# Study of bacterial communities in two step treatment of post methanated distillery effluent by bacteria and constructed wetland plant treatment system

**THESIS**

SUBMITTED TO

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# CERTIFICATE

This is to certify that the thesis entitled **"Study of bacterial communities in two step treatment of post methanated distillery effluent by bacteria and constructed wetland plant treatment system"** submitted by **Mr. Vineet Kumar** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulation-1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of **Doctor of Philosophy** of the University

Date: April 2, 2018

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# DECLARATION

I, **Vineet Kumar** hereby declare that the work which is being presented in the thesis entitled **"Study of bacterial communities in two step treatment of post methanated distillery effluent by bacteria and constructed wetland plant treatment system"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh is an authentic record of my own work carried out during the period from October 2012 to April 2018 under the supervision of **PROF. RAM CHANDRA**, *Professor and Dean*, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.

The matter presented in this thesis has not been submitted by me for the award of any other degree in any other University/Deemed University without proper citation.

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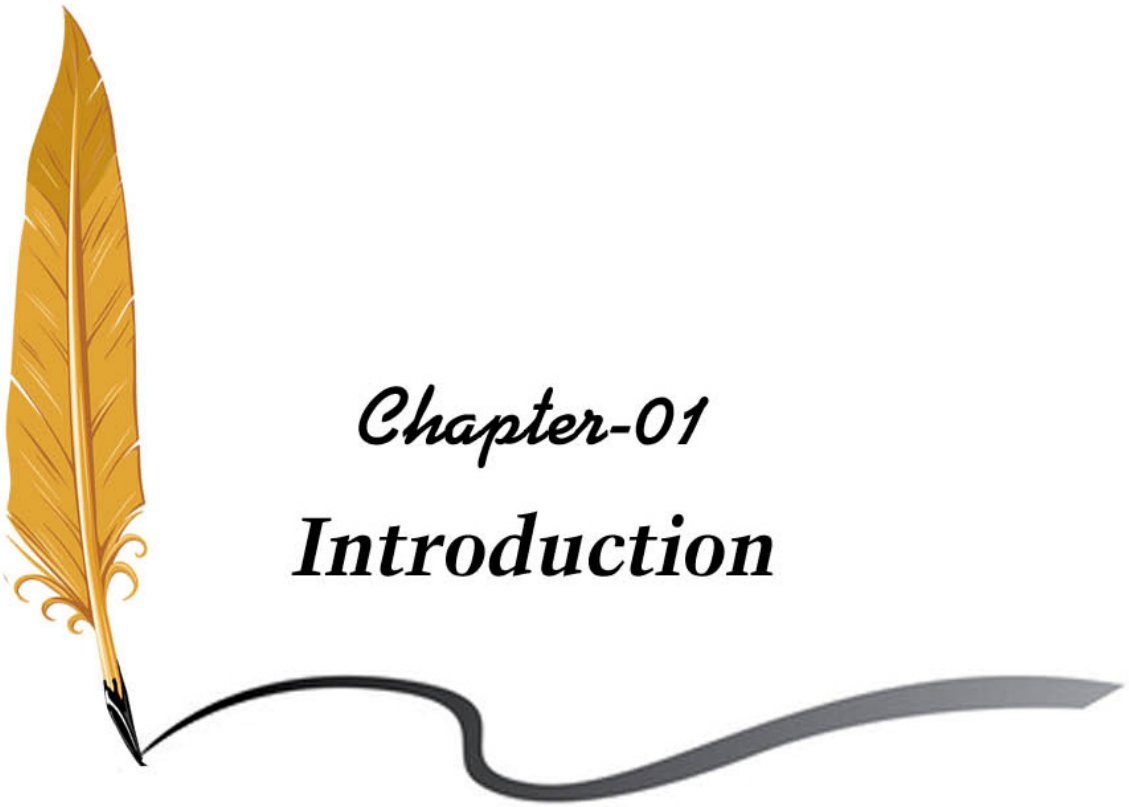
# ABBREVIATIONS AND SYMBOLS

| | | |
|---|---|---|
| **α** | **:** | Alpha |
| **Al** | **:** | Aluminium |
| **$AlCl_3$** | | Aluminium chloride |
| **amu** | **:** | Atomic mass unit |
| **ANOVA** | | Analysis of variance |
| **AAS** | **:** | Atomic absorption spectrophotometer |
| **ATP** | **:** | Adenosine triphosphate |
| **APS** | **:** | Ammonium per sulfate |
| **ASS** | **:** | Ammonium salt sugars |
| **~** | **:** | Approx |
| **β** | **:** | Beta |
| **BD** | **:** | Bacterial decolourised |
| **$BaSO_4$** | **:** | Barium sulfate |
| **BLAST** | **:** | Basic local alignment search tool |
| **BOD** | **:** | Biological oxygen demand |
| **BSA** | **:** | Bovine serum albumin |
| **BSTFA** | **:** | N,O-bis(trimethylsilyl)trifluoroacetamide |
| **C** | **:** | Carbon |
| **$CaCl_2$** | **:** | Calcium chloride |
| **CaO** | **:** | Calcium oxide |
| **Cd** | **:** | Cadmium |
| **CEC** | **:** | Cation exchange capacity |
| **CFU** | **:** | Colony forming unit |
| **cm** | **:** | Centimeter |
| **COD** | **:** | Chemical oxygen demand |
| **Cr** | **:** | Chromium |
| **Cu** | **:** | Copper |
| **$CuSO_4$** | **:** | Copper sulfate |
| **CW** | **:** | Constructed wetland |
| **°C** | **:** | Degree centigrade |
| **DO** | **:** | Dissolved oxygen |
| **dNTP** | **:** | Deoxynucleotide triphosphate |
| **dATP** | **:** | Deoxyadenosine triphosphate |
| **dGTP** | **:** | Deoxyguanosine triphosphate |
| **dTTP** | **:** | Deoxythymidine triphosphate |
| **dCTP** | **:** | Deoxycytidine triphosphate |
| **DNA** | **:** | Deoxyribo nucleic acid |
| **DAD** | **:** | Diode array detector |
| **DSW** | **:** | Distillery spent wash |
| **eV** | **:** | Electron volt |
| **EC** | **:** | Electrical conductivity |
| **EDCs** | **:** | Endocrine disrupting chemicals |
| **EDTA** | **:** | Ethylenediamine tetra acetic acid |
| **EtBr** | **:** | Ethidium bromide |
| **F** | **:** | Forward |
| **Fe** | **:** | Iron |
| **$FeCl_3$** | **:** | Ferric chloride |
| **$FeSO_4$** | **:** | Ferrous sulfate |
| **Fig.** | **:** | Figure |
| **FT-IR** | **:** | Fourier transform-infrared spectroscopy |
| **λ** | **:** | Wavelength |

| | | |
|---|---|---|
| **g** | **:** | Grams |
| **g $L^{-1}$** | **:** | Gram per liter |
| **GC-MS** | **:** | Gas chromatography-mass spectrometery |
| **GPM** | **:** | Glucose peptone melanoidins |
| **h** | **:** | Hours |
| **$H_2S$** | **:** | Hydrogen sulfide |
| **$H_2O_2$** | **:** | Hydrogen peroxide |
| **HCl** | **:** | Hydrochloric acid |
| **$HgCl_2$** | **:** | Mercuric chloride |
| **$HNO_3$** | **:** | Nitric acid |
| **HPLC** | **:** | High performance liquid chromatography |
| **HRT** | **:** | Hydraulic retention time |
| **IU** | **:** | International unit |
| **kV** | **:** | Kilovolt |
| **Kb** | **:** | Kilo base |
| **Kbp** | **:** | Kilo base pair |
| **kDa** | **:** | Kilo Dalton |
| **$K_2HPO_4$** | **:** | Dipotassium hydrogen orthophosphate |
| **L** | **:** | Liter |
| **LiP** | **:** | Lignin peroxidase |
| **Ltd.** | **:** | Limited |
| **M** | **:** | Mol |
| **mm** | **:** | Millimeter |
| **mM** | **:** | Milimolar |
| ***m/z*** | **:** | Mass-to-charge ratio |
| **µg** | **:** | Microgram |
| **µl** | **:** | Microlitre |
| **µm** | **:** | Micrometer |
| **mg** | **:** | Milligrams |
| **mL** | **:** | Milliliter |
| **mmol $L^{-1}$** | **:** | Milimolar per liter |
| **$m^2/g$** | | Metre square per gram |
| **$m^3d^{-1}$** | **:** | Cubic metre per days |
| **mg $L^{-1}$** | **:** | Milligram per liter |
| **mg $kg^{-1}$** | **:** | Milligram per kilogram |
| **MEGA** | **:** | Molecular evolutionary genetics analysis |
| **$MgSO_4$** | **:** | Magnesium sulfate |
| **$MgCl_2$** | **:** | Magnesium chloride |
| **Mn** | **:** | Manganese |
| **MnP** | **:** | Manganese dependent peroxidase/Manganese peroxidase |
| **$MnCl_2$** | **:** | Manganese chloride |
| **MRPs** | **:** | Maillard reaction products |
| **MIP** | **:** | Manganese independent peroxidase |
| **MR** | **:** | Methyl red |
| **Min** | **:** | Minutes |
| **MW** | **:** | Molecular weight |
| **N** | **:** | Normality |
| **NaOH** | **:** | Sodium hydroxide |
| **$Na_2SO_4$** | **:** | Sodium sulfate |
| **ng·µ$L^{-1}$** | **:** | Nanogram per microliter |
| **ng** | **:** | Nanogram |
| **nm** | **:** | Nanometer |
| **Ni** | **:** | Nickel |

| | | |
|---|---|---|
| **NaCl** | **:** | Sodium chloride |
| **NaOH** | **:** | Sodium hydroxide |
| **NIST** | **:** | National Institute of Standard and Technology |
| **NCBI** | **:** | National Center for Biotechnology Information |
| **O** | **:** | Oxygen |
| **OTU** | **:** | Operational taxonomic unit |
| **OD** | **:** | Optical density |
| **(O-F) test** | **:** | Oxidative-fermentative |
| **OsO4** | **:** | Osmium tetroxide |
| **%** | **:** | Percent |
| ***p*** | **:** | Para |
| **pmol** | **:** | Picomole |
| **pUC** | **:** | Plasmid vector UC |
| **Pvt.** | **:** | Private |
| **pH** | **:** | Potential of hydrogen |
| **Pb** | **:** | Lead |
| **PAGE** | **:** | Polyacrylamide gel electrophoresis |
| **PCR** | **:** | Polymerase chain reaction |
| **PMDE** | **:** | Post methanated distillery effluent |
| **PMDS** | **:** | Post methanated distillery sludge |
| **QIIME** | | Quantitative Insights Into Microbial Ecology |
| **$R_f$** | **:** | Retardation factor |
| **R** | **:** | Reverse |
| **RFLP** | **:** | Restriction fragment length polymorphism |
| **rRNA** | **:** | Ribosomal ribonucleic acid |
| **rDNA** | **:** | Ribosomal deoxyribonucleic acid |
| **RDP** | **:** | Ribosomal database project |
| **rpm** | **:** | Revoultion per minute |
| **RT** | **:** | Retention time |
| **SEM** | **:** | Scanning electron microscope |
| **EDS** | **:** | Energy dispersive X-ray spectrometer |
| ***Sau*3A** | **:** | *Staphylococcus aurous* restriction enzyme 3A |
| **Sp.** | **:** | Species |
| **Sec** | **:** | Seconds |
| **SDS** | **:** | Sodium dodecyl sulfate |
| **SD** | **:** | Standard deviation |
| **SGA-MRPS** | **:** | Sucrose-glutamic acid Maillard reaction Product |
| **SCB** | **:** | Sodium cacodylate buffer |
| **SPSS** | **:** | Statistical package for Social Sciences |
| **T4** | **:** | Bacteriophage T4 |
| ***Taq*1** | **:** | *Thermus aquaticus* type I |
| **TMCS** | **:** | Trimethylchlorosilane |
| **TLC** | **:** | Thin layer chromatography |
| **TOC** | **:** | Total organic carbon |
| **TDS** | **:** | Total dissolved solids |
| **TS** | **:** | Total solids |
| **TSS** | **:** | Total suspended solids |
| **TN** | **:** | Total nitrogen |
| **TEM** | **:** | Transmission electron microscope |
| **TMS** | **:** | Trimethylsilyl |
| **TAE** | **:** | Tri-acetate-EDTA |
| **TEMED** | **:** | N, N,N’N’-tetramethylethylenediamine |
| **U** | **:** | Unit |

| | | |
|---|---|---|
| **U $mL^{-1}$ $min^{-1}$** | **:** | Unit per milliliter per minute |
| **UV** | **:** | Ultraviolet |
| **V** | **:** | Volt |
| **VS** | **:** | Volatile solid |
| **V3** | **:** | Variable region third |
| **V4** | **:** | Variable region four |
| **v/v** | **:** | Volume/ volume |
| **w/v** | **:** | Weight/volume |
| **X-Gal** | **:** | 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranosides |
| **Zn** | **:** | Zinc |
| **$ZnSO_4$** | **:** | Zinc sulfate |
| **3′** | **:** | Three prime |
| **5′** | **:** | Five prime |
| < | **:** | Less than |
| > | **:** | Greater than |



# Chapter-01

# Introduction

# Chapter-1

## 1.1 Introduction

The discharge of pollutants into the environment from distilleries is a threat to ecosystem due to big environmental hassle. In India, alcohol distilleries are one of the most agro-based polluting agro-based industries; in addition, they are high consumer of raw water. Most of the distilleries co-exist with sugar mills and utilize the molasses from cane sugar manufacturing as the starting material for alcohol production. India being the largest rice producing country has a tremendous amount of rice grain as the raw material after sugarcane molasses. The process of alcohol production and effluent generation has been illustrated in Fig.1.1. The main source of effluent generation is the distillation process. During the distillation process, an average 12-15 L effluent is generated for each litre of ethanol produced. In India, there are more than 319 sugarcane molasses based distilleries releasing approximately $3.5\times10^{13}$ kL spent wash annually (Bharagava et al. 2009). Spent wash is generally characterized with unpleasant odour, deep brown colour, high level of biological oxygen demand (BOD; 42,000-46,666 mg $L^{-1}$), chemical oxygen demand (COD; 90,000-104,130 mg $L^{-1}$), total dissolved solids (TDS; 77,000-81,733 mg $L^{-1}$), total nitrogen (TN; 1635-2,800 mg $L^{-1}$) sulphate (1738-5760 mg $L^{-1}$) and the presence of heavy metals (i.e. Fe, Zn, Cu, Ni, Mn), and nitrogen containing complex organic pollutants with low pH (Chandra et al. 2004a; Tiwari et al. 2013). Spent wash characteristics are dependent on the variety of substrates for alcohol fermentation, such as cereal malt (rice, barely, wheat, and maize) sugarcane molasses and grapes. Due to high pollution nature of distillery spent wash, Ministry of Environment and Forest (MoEF), Government of India listed alcohol industries at the top among the 'Red category' industries (Tewari et al. 2007). Regarding water pollution, the Indian government made rules and regulation in 1976 and again revised it in 1983 (Suneeth Kumar 1998). Bureau of Indian Standard (BIS) provides guidelines to the authorities of state and central government which would help to decide on suitable restrictions on effluent disposal and to the industry for selecting an appropriate technology, suitable site, and the degree of treatment required for effluents before their disposal. In 2003, the Central Pollution Control Board (CPCB), India stipulated that distilleries should achieve zero discharge in inland surface water courses by the end of 2005. Consequently, distillery effluent must be properly treated before it is discharged into the environment (Tiwari et al. 2007). Distillery spent wash treatment is being carried out generally by three routes in the industry; (i) concentration followed by incineration; (ii) direct oxidation by air at a high temperature followed by aerobic treatment, and (iii) anaerobic digestion with biogas recovery followed by aerobic polishing. Out of three routes, anaerobic digestion has been considered as the most attractive first step technique for the treatment of distillery effluent due to its reputation as a low-cost and eco-friendly technique besides its biomethane generation potential (Satyawali and Balakrishnan 2008a,b; Oller et al. 2011). The effluent received after anaerobic treatment (methanogensis) is called post-methanated distillery effluent (PMDE).

PMDE is characterised by extremely higher level of BOD (4316-9800 mg $L^{-1}$), COD (18,700-34366 mg $L^{-1}$), TDS (17612-32266 mg $L^{-1}$) sulphate (97-2786 mg $L^{-1}$), phenol (6893 mg $L^{-1}$) with high pH (7.6-8.5), strong odour and dark brown colour. Besides organic content, PMDE also contains nitrogen (1313-4096 mg $L^{-1}$), potassium (537-1370 mg $L^{-1}$) and phosphorous (111-1625 mg $L^{-1}$) which can lead to eutrophication of waster bodies (Chandra et al. 2012; Sowmeyan and Swaminathan 2008). In addition, PMDE retains high amount of Fe (0.75-42.00 mg $L^{-1}$), Zn (1.00-1.24 mg $L^{-1}$), Cu (0.55-0.75 mg $L^{-1}$), Mn (1.20-43.60 mg $L^{-1}$), Ni (0.31 mg $L^{-1}$), Pb (0.22-0.23 mg $L^{-1}$) and Cd (1.30 mg $L^{-1}$) (Bharagava and Chandra 2010a; Chandra et al. 2012). In addition, Indian distilleries produce ~1500 tonnes of post methanated distillery sludge (PMDS) per day during anaerobic digestion of spent wash (Kansal et al. 1998) which is characterized by high organic matter, TDS, phenol, sulphate and heavy metals along with melanoidins. Anaerobic digestion is preferred due to fact that a significant component of spent wash is biodegradable. Since, the anaerobic digestion is reported to remove about 40-50% COD and 60-65% BOD (Arimi et al. 2015). This means that spent wash after anaerobic digestion still contains some organic load and is not safe for discharge in environment. The color of spent wash converts darker with higher TDS after anaerobic treatment due to complexation of organic and inorganic pollutants present in spent wash. Therefore, extended aeration of distillery effluent does not change its physico-chemical properties even after aeration.

The conventional anaerobic-aerobic treatment processes can accomplish the degradation of melanoidins only upto 6 % or 7% (Ojijo et al. 2010; Blonskaja and Zub 2009). Satyawali and Balakrishnan (2008a) reported that anaerobic digestion treatment does not result in effective color removal. When the untreated or partially treated spent wash gets released into surface water resources, the dark coloration of PMDE hinders the penetration of sunlight into the water, thereby decreasing the photosynthetic activity and eventually affecting the life of aquatic microbiome (Fig. 1.2). Therefore, it is necessary to study additional pre or post treatment methods to remove color from molasses-based distillery effluent. The complexity of wastewater makes difficult the development of effective technologies for tertiary treatment and color removal, before its disposal into the environment. For instance, Indian distilleries have been directed by MoEF to achieve zero liquid discharge into the surface water body from December 2005. In many instances, industries dilute the PMDE by mixing with raw water before discharge in order to meet the set waste disposal standard. This dilution even though accepted in some regions, is of great environmental concern as it does not reduce the absolute pollution load of the PMDE (Chaudhari et al. 2007). Various physico-chemical methods such as column filtration (Satyawali and Balakrishnan 2008b), coagulation/flocculation (Fan et al. 2011; Liang et al. 2009a), adsorption (Onyango et al. 2011), chemical precipitation/coagulation (Chandra and Singh 1999; Liang et al. 2009b), UV/$H_2O_2$ treatment (Dwyer and Lant 2008) and ozone oxidation (Kim et al. 1985; Pena et al. 2003) has been reported for removal of COD and decolourisation of PMDE, but these techniques are not feasible at large scale due to high cost, blockage of filtration devices and sensitivity to variable water input. Moreover,

due to high TDS it generates huge amount of toxic sludge and other secondary pollutants (Liang et al. 2009b; Chandra et al. 2008).

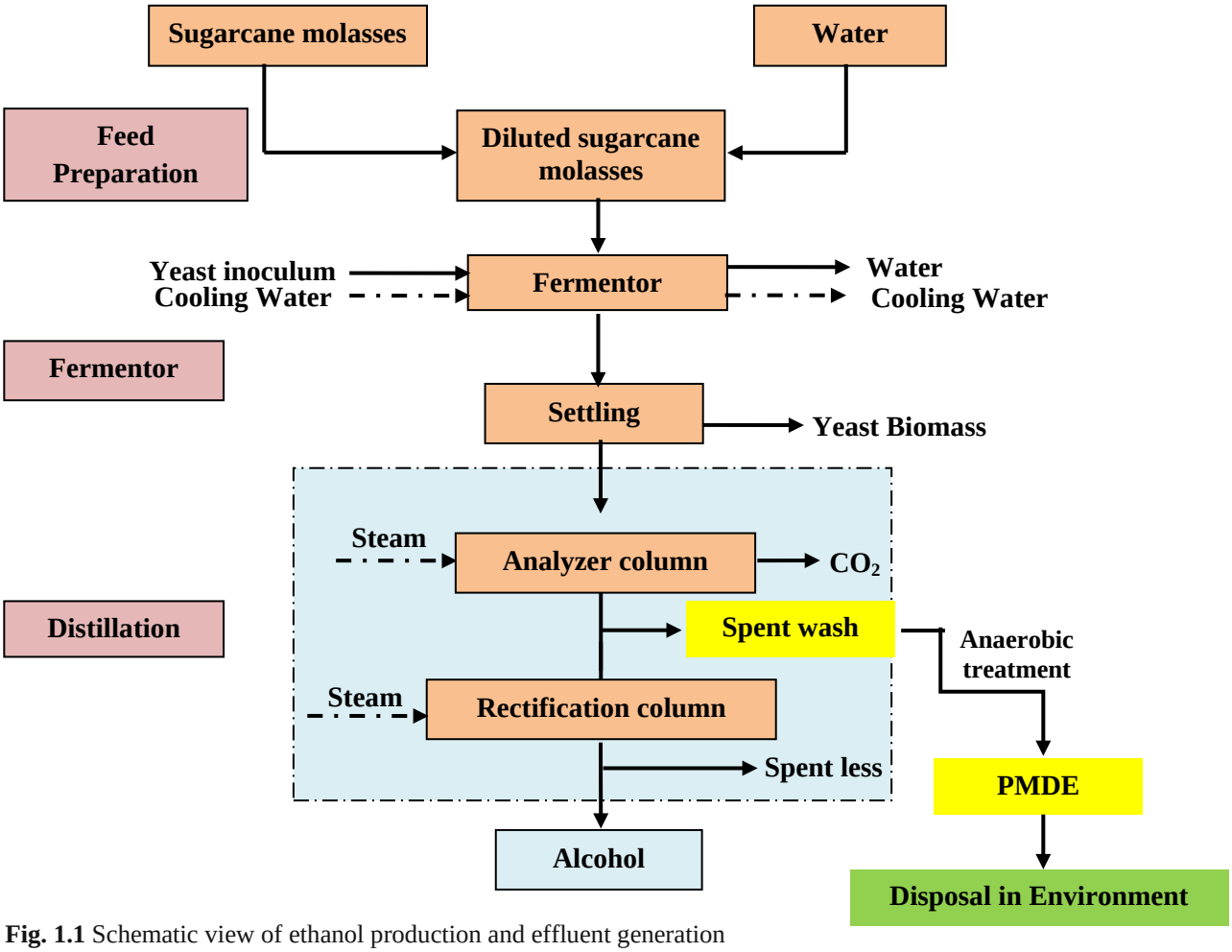



**Fig. 1.1** Schematic view of ethanol production and effluent generation


**Fig. 1.2** Distillery wastes and their environmental impacts (a) PMDE (b) aquatic pollution (c) leaching of PMDE in soil (d) underground waster pollution

In recent years, the independent role of microorganisms in the degradation and decolourisation of PMDE has been proposed by using a number of microorganisms such as bacteria, fungi, yeast and cynobacteria. The growing acceptance of microorganisms for decolourisation and detoxification of melanoidins containing distillery effluent are due to facts that some microorganisms have a specific extracellular ligninolytic enzymatic system known as manganese peroxidase (MnP), laccase and lignin peroxidase (LiP) capable of breaking a large number of C═C, C=O and C≡N bonds present in melanoidins (Miyata et al. 2000). The degradation and decolourisation of melanoidins by fungus has been reported more effective due to prevalence of ligninolytic enzymes, which metabolizes melanoidins as a sole carbon and nitrogen sources (Miyata et al. 2000; González et al. 2008). But, due to growth limitation of fungus in aquatic conditions at low pH restricted its large scale application for development of effluent treatment process. In contrast, the bacterial consortium has been reported more promising due to their immense environmental adaptability and versatile metabolic pathway for catabolism of complex recalcitrant compounds. Some worker have reported the bacterial decolourisation and degradation of sugarcane molasses melanoidins as well as model melanoidins both (Kumar and Chandra 2006; Bharagava and Chandra 2010a,b; Bharagava et al. 2009) but the decolourisation was achieved only up to certain limit. Moreover, most of decolourisation and degradation of molasses-melanoidins is reported at 475 nm only as purified melanoidins through dialysis process with specific molecular weight, while the molasses-melanoidins contains mixture of maillard reaction products (MRPs) (i.e. initial, intermediate and advanced stages with variable molecular weight). However, due to complex nature of molasses-melanoidins at variable absorption spectrum at different wavelength is essential to understand the mechanism of melanoidins decolourisation and degradation and its metabolic products. But, no study has been conducted for assessment of molasses-melanoidins degradation present in its original form for the feasibility of any potential bacterial consortium. Therefore, prior to attempting the degradation and decolourisation of distillery effluent, the degradation of molasses-melanoidins with mixture of complex MRPs should be evaluated for its degradability. In addition, the optimisation of PMDE degradation process by potential bacterial consortium at various concentration and TDS will be essential prior to development of two step treatment process.

Constructed wetland (CW) treatment systems are engineered systems that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and their associated microbial assemblages to assist in treating wastewater. These systems are low cost self sustaining, easily operated and have been successfully employed worldwide, over the last six decades, to remove a diverse array of contaminants from municipal, agricultural and industrial wastewater (Vymazal 2014; 2011; Zhang et al. 2015a,b). Thus, CW system planted with potential wetland plant would be an effective device as biofilter for the pilot or large scale treatment system. Different species of wetland plants, including *Phragmites karka* and *Phragmites australis* have been used in constructed wetland treatment system in tropical areas (Vymazal et al. 2009; Sochacki et al. 2015; Badejo et al. 2015). Plants can provide favorable condition for

microbial colonization of the rhizosphere for symbiotic degradation and detoxification of pollutants (Stottmeister et al. 2003; Doran 2009; Kadlec and Wallance 2009). On the other hand, plants possess very complex, efficient, pronounced and versatile modes of metabolism that could degrade and even mineralize highly toxic xenobiotic compounds. The complex microbial communities which are created by the interaction with wastewater, play a key role in wetland biogeochemical cycling and are mainly responsible for degradation of the target organic pollutants under aerobic and anaerobic conditions (Stottmeister et al. 2003; Ibekwe et al. 2007; Ligi et al. 2014). Combination of wetland treatment technology after bacterial degradation offers an advanced and innovative treatment system for removal of color from PMDE and further reduction of BOD, COD and organic pollutants for safe disposal. The microbial community structure and diversity of wetland plant is very important for the treatment efficiency of pollutants and ecosystem stability (Hallberg and Johnsonb, 2005; Sirivedhim and Gray 2006). However, to date, only a limited numbers of studies have focused on microbial community investigation in laboratory scale units and full scale and CW under specification conditions during PMDE treatment (Chandra et al. 2012; Chaturvedi et al. 2006). But no study has examined the rhizosphere bacteria associated with wetland plants and their possible role in phytoremediation mediated by wetland plant. The combined effect of plant associated bacterial species or that of rhizosphere bacterial species and biotransformation of effluent by aerobic bacteria for the decolorization is yet not reported in wetland ecosystem. Recent developments have indicated that hybrid methods that combine partial treatment with two or more steps favour more complete treatment of this effluent (Thompi 2000). Considering the advantages and the disadvantages of different treatment technologies, no single technology can be used for complete treatment of distillery effluent. Hence, there is a need to establish a comprehensive treatment approach involving sequential treatment technologies. Therefore, integrated biological treatment methods have been practised by coupling or integrating two or more biological processes for the treatment of complex industrial wastewaters. Two step treatment by using integrated bacteria and constructed wetland treatment process to be a novel and promising approach for bioremediation of industrial wastewater. Integrated biological systems have been characterized by their low operating costs and efficiency in pollutant removal, they may be widely applied for the treatment of highly polluted complex industrial effluents. Therefore, integrated use of bacteria and constructed wetland plant treatment system in sequential manner has been proposed for degradation and detoxification of PMDE. In first step bacterial treatment, the complexes pollutants are reduced or biotransform to another form which are much helpful or bioavailable for the second step treatment. In fact, the real idea of phytoremediation using CW is to achieve the enhanced degradation of pollutants by using plants and their root associate microorganisms.

### 1.2 Colourants of post methanated distillery effluent

That dark brown color of PMDE is generally attributed to the existence of wide variety naturally polymeric colorants such as carotenoids, chlorophyll, resins, fatty acids, heme

pigment, anthocyanins, tannins, riboflavin, betalains, quinone pigments, polyphenols melanoidins, caramels, alkaline degradation product of hexose (ADPH) melanin, and metal sulfides (Pant and Adholeya 2007a). Among these colourants, melanoidins, plant derived polyphenols, ADPH, and caramels have a high potential of occurrence of colour in PMDE.

### 1.2.1 Melanoidins

The origin of brown colour in PMDE is primarily associated with the dark brown melanoidins pigments and a class of compounds known as MRPs (Fan et al. 2011; Arimi et al. 2015). Melanoidins are one of the final products of the non-enzymatic maillard reaction between sugars and amino acids, peptides or proteins sugars at high temperature >50 $^{o}$C and pH 4-7. The Maillard reaction (MR) is actually a complex series of reactions; the first stage involves the initial condensation of the sugar with protein and the isomerization of the resulting products to form Amadori or Heyns products. Amadori product and its dicarbonyl derivates can undergo concurrently retro-aldol reactions producing more reactive $C_2$, $C_3$, $C_4$ and $C_5$ sugar fragments, such as hydroxyacetone derivatives, glyceraldehydes and diketones. This reaction is called as Strecker degradation and it is characterized by the production of $CO_2$ (Arimi et al. 2015). The aldehydes formed may be important as auxiliary flavour compounds and they also contribute to melanoidin formation. The formation of melanoidins from sugar and amino acids reactions is shown in Fig. 1.3a.

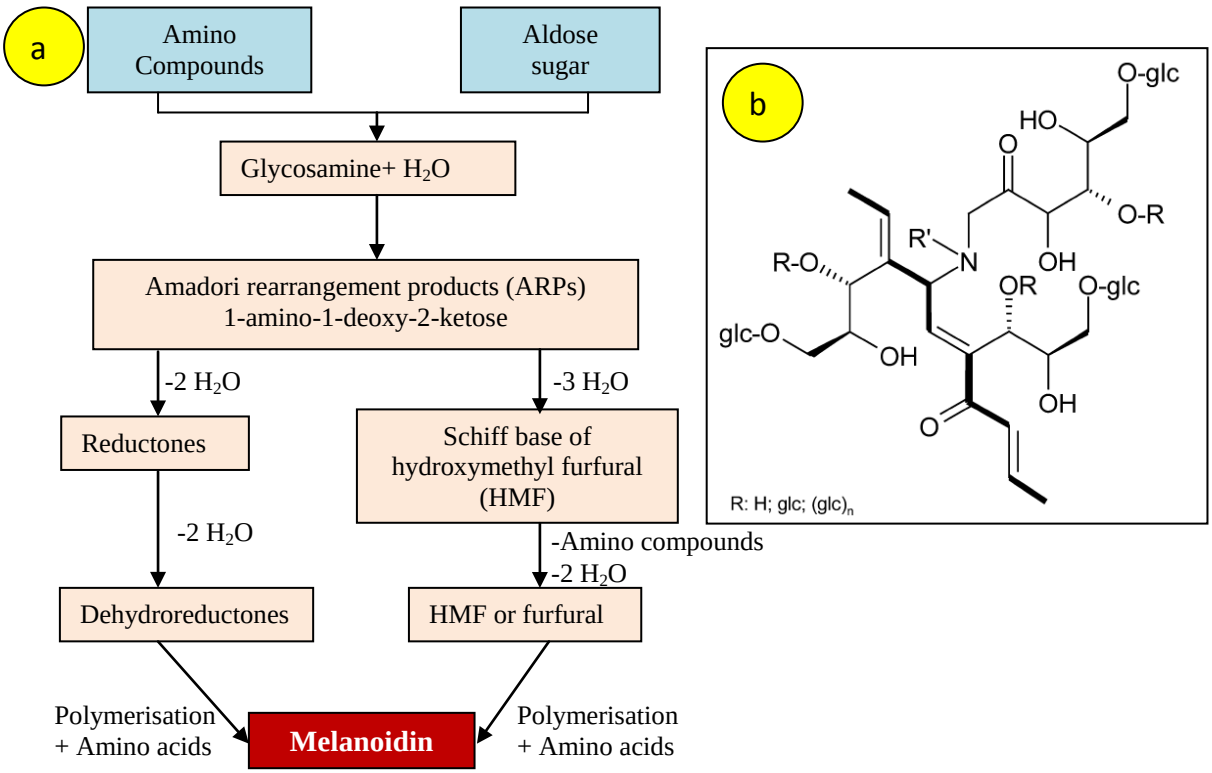



**Fig. 1.3** The formation of advanced MRPs (Melanoidin) **(a)** the basic structure of melanoidin proposed by Cammerer et al. (2002)

Melanoidins have conjugated carbon-carbon double bonds –C=C- in their structure that are responsible for their brown color (Kim et al. 1985). Melanoidins are generally regarded to be heterogenous, high molecular weight negatively charged, acidic and highly dispersed polymer due to dissociation of carboxylic acid and phenolic groups with similar chemical properties to humic substances (Migo et al. 1993). The empirical formula of melanoidins has been suggested as $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ and molecular weight between 5kDa and 40kDa. In sugar processing, melanoidins are formed during purification and evaporation steps. The elemental and chemical structure of melanoidins depends heavily on the nature and molecular concentration of reactant and reaction conditions (pH, temperature, heating time and solvent). Cammerer et al. (2002) have proposed the basic structure of melanoidins pigment (Fig. 1.3b) formed from 3-deoxyhexosuloses and Amadori reaction products.

### 1.2.2 Caramels

Caramel is a brownish black viscous liquid formed by caramelisation process. The caramelisation reaction occurs when sugar heated in the absence of nitrogen compounds and the process can be catalysed by acid and bases. During a caramelization reaction, the sugar initially undergoes dehydration and then condensation or polymerisation into complex molecules. The generation of colour in caramelisation requires that sugars, normally monosaccharides structure, should first undergo intramolecular rearrangement. Depending on the time and temperature, yellow or brown solution are obtained. In the sugar degradation reaction, osuloses are formed, which are considered to be intermediate of caramelisation. Osuloses lead to the formation of typical components of caramel colour and caramel flavour.

### 1.2.3 Polyphenols

The alcohol distilleries produce large volume of waste- water having phenolic compounds, which give a high inhibitory and antibacterial activity to this wastewater, thus slowing down the anaerobic digestion process. Polyphenols are naturally occurring antioxidant compounds found largely in the fruits, vegetables, cereals and beverages. They are categorised into three broad classes: phenolic acid, flavonoids and tannins. Phenolic acids have been detected in PMDE, including benzoic acid and its derivatives (e.g. gentisic acid, gallic acid), cinnamic acid and its derivatives (*p*-coumaric acid, caffeic acid, chlorogenic acid, and ferulic acid) (Payer et al. 2005, 2006; Incedayi et al. 2010), which impart high antibacterial activity (Borja et al. 1993; Keyser et al. 2003). In PMDE, a number of compounds containing this highly reactive phenol group are produced and extracted into the PMDE and responsible its colour. Phenolic acids are initially clear but can be oxidised to a brownish-yellow colour with the exposure to oxygen.

### 1.2.4 Alkaline degradation products of hexose

The ADPH, together with the melanoidins are responsible for up to 80% of colour in PMDE. ADPH are formed when hexoses (glucose or fructose) are degraded in alkaline

media at high temperature (>80 $^0$C). Sucrose is a non-reducing sugar that is only degraded by alkali at high temperatures (Yang and Montgomery 1996). Sugarcane molasses have a high content of sucrose and some glucose and fructose undergoes both reversible and irreversible reaction in aqueous solution and produced ADPH.

### 1.3 Environmental impacts and health hazard from post methanated distillery effluent

The disposing of untreated and partially treated distillery effluent into the environment is unsafe to the ecosystem due to high pollution potential and presence of several unknown recalcitrant pollutants. PMDE is slightly alkaline in nature their high organic content can cause considerable aquatic and soil pollution. Molasses based distillery effluent contains intense quantities of recalcitrant organic compounds and metal sulfides in the form of dark colored organic pollutants. The intense color of PMDE is due to the presence of a dark brown, acidic melanoidins pigment. These antioxidant and recalcitrant polymers (melanoidins) cannot be easily degraded by conventional biological treatment methods, namely, anaerobic digestion (biomethanation), anaerobic lagoons, and activated sludge process due to their complex structure and xenobiotic nature and in some cases are both mutagenic and carcinogenic. PMDE have also been characterised for heavy metals, viz.Fe, Cu, Zn, Ni, Mn, and Pb, and inorganic ions such as $Ca^{2+}$, $K^{+}$ and $Na^{+}$ (Harada et al. 1996; Ramana et al. 2002; Natarajan et al. 2006) and sulphates (Harada et al., 1996; Ramana et al., 2002). High concentrations of these constituents (Ramana et al. 2002; Eusébio et al. 2004), plus other nutrients such as nitrate and phosphate, make the possible discharge of PMDE into water bodies problematic, as they cause eutrophication and other adverse environmental effects (Borja et al. 1993; Collins et al. 2005). It has been reported that melanoidins have net negative charges, hence, different heavy metals ($Cu^{2+}$, $Cr^{3+}$, $Fe^{3+}$, $Zn2^{+}$ and $Pb^{2+}$ etc.) present in distillery effluent strongly bind to form organo-metallic complex with melanoidins (Migo et al. 1997). The high metal binding tendency of melanoidins also enhances vulnerability of organo-metallic complex towards its toxicity in environment. In soil, it inhibits seed germination by reducing alkalinity as well as manganese accessibility and ultimately effects agricultural crop production. Jagdale and Savant (1979) indicated that non-judicious use of spent wash might adversely affect the crop growth and soil properties by increasing soil salinity. Jadhav and Savant (1975) also noted adverse effect on water retention, hydraulic conductivity and water stable aggregates of soil with the application of spent wash in high amount. Constant disposal/irrigation of soil with distillery effluent adversely affects the soil microorganisms that are involved in regulating the various process of nutrient recycling in soil. It alters soil and groundwater quality (Juwarkar and Dutta 1989). Jain et al. (2005) indicated that long-term indiscriminate use of PMDE could lead to significant leaching of inorganic salts. Although leaching of salts has the potential to affect the quality of groundwater. Bharagava and Chandra (2010a) have also reported that PMDE have deleterious effects on seed germination and seedling growth parameters in *Phaseolus mungo* (L). The inhibition in seed germination at higher PMDE content might be attributed to high salt concentration

and TDS, which increases high osmotic pressure (OP) and anaerobic conditions, respectively. These conditions affect various biochemical and physiological activities such as movement of solute, respiration and enzymatic process of seed germination. It has been also reported that high PMDE concentration also acts as an inhibitor for plant growth hormone(s) (auxin and gibberline), which play an important role in plant growth and development (Subramani et al. 1997). Moreover, Bharagava et al. (2008a) have reported that at higher PMDE concentration, the entrance of potentially toxic trace elements into the protoplasm may result in the reduction of intermediate metabolites, which are responsible for the reduction in plant growth parameters. Moreover, the sulfide has strong tendency of metal binding, which contributes more toxicity and colour to the effluent. Discharge of partially treated or untreated distillery effluent also affects the aquatic life (Kumar and Gopal, 2001; Saxena and Chauhan, 2003; Matkar and Gangotri, 2003; Ramakritinan et al. 2005). It reduces penetration of sunlight in water bodies decreased photosynthetic activity, and dissolved oxygen concentration causing death of aquatic organisms due to oxygen deficiency (Raghukumar et al. 2004; Kumar and Chandra 2006). Undiluted effluent has toxic effect on fishes and other aquatic organisms. The $LC_{50}$ for distillery effluent has been estimated at 0.5% using a bio-toxicity study on fresh water fish *Cyprinus carpio* var. communis. Impact of distillery effluent on carbohydrate metabolism of freshwater fish, *C. carpio* has also been studied (Ramakritinan et al. 2005). The respiratory process in *C. carpio* under distillery effluent stress is affected resulting in a shift towards anaerobiosis at organ level during sublethal intoxication. Leaching of ions affects groundwater quality by altering physicochemical properties such as color, pH, and electrical conductivity (Jain et al. 2005). In addition to COD and BOD pollution, PMDE contain phenolic compounds, mainly gallic acid, *p*-coumaric acid and gentisic acid, which impart high antibacterial activity (Borja et al., 1993; Seghezzo et al., 1998; Keyser et al. 2003). Phenolic acid form a relatively small portion of PMDE, but have great negative effects upon treatment systems as well as environmental damage if released untreated. These compounds have been implicated in the inhibition of biological treatment system for distillery wastes. Many phenolic compounds are not readily biodegradable and are toxic to microorganisms, even at low concentrations. Phenol can even inhibit microbial growth that are capable of using aromatic compounds as their sole source of carbon and energy. Several problems have been encountered during the biological treatment of wine distillery wastewater because of high toxicity and the inhibition of biodegradation due to the presence of polyphenolic compounds (Goodwin et al. 2001), which demonstrated the antibacterial activity reported in the earlier literature (Borja et al. 1993). Polyphenol concentrations in some distillery wastewaters vary considerably and can range from 29 to 474 mg $L^{-1}$ (Bustamante et al. 2005). Polyphenols are responsible for strong inhibitory effects on microbial activity and must be removed during wastewater treatment, owing to the environmental and public health risks they pose. Humans exposed to phenol at 1300 mg $L^{-1}$ of concentration exhibit significant increases in diarrhoea, dark urine, mouth sores and burning of the mouth (Collins et al. 2005).

## 1.4 Challenges for the biodegradation and bioremediation of post methanated distillery effluent

The major challenges of PMDE are the removal of TDS and degradation of color contributing compounds like malanoidins, polyphenols, caramel, ADPH, and their metabolic products as well as inorganic compounds before its safe disposal into the environment. Different feedstocks, fermentation processes and biological treatments may bring about inconsistent structures of individual colorant (Arimi et al. 2014). Different polysaccharide, amino acids and other organic compounds react at various proportions; the polymerization probably occurs in complex ways and extends to different levels. Accordingly, it is not easy to characterize those pigments. It should also be highlighted that multiple colorants instead of one type of pigment alone may result in the undesirable color, which will then increase the difficulty of decolorization and organic elimination of PMDE. It is reported that caramels and ADPH can be reduced by 70% in the aerobic stage. However, melanoidins and plant polyphenols exhibit strong antimicrobial and antioxidant properties (Arimi et al. 2014). Conventionally biological processes are capable of accomplishing the degradation of the melanoidins up to merely 67% (Mohana et al. 2009); however, the small size pigments probably repolymerize during biological processes. In addition to be colored components, melanoidins and polyphenols may also cause the formation of aromatic halogenated disinfection by-products (DBPs) during chlorine disinfection of wastewater effluents. Recent studies have shown that aromatic halogenated DBPs generally presented significantly higher developmental toxicity and growth inhibition than aliphatic halogenated DBPs (Jiang et al. 2017; Liu and Zhang 2014; Meng et al. 2016). Given that the chlorine disinfection has so far, the molasses based distillery effluent has been mostly studied in the related research, where melanoidins were considered as the leading or even unique type of pigments (Hatano et al. 2008; Liang et al. 2009a,b; Liakos and Lazaridis 2014). Thus, advanced techniques are strongly required to eliminate the organic matter and colorants from the biologically treated distillery wastewater, which has been increasingly recognized as a tough challenge (Arimi et al. 2014; Prajapati and Chaudhari 2015; Tsioptsias et al. 2015). The characterisation of organic pollutants present in spent wash and PMDE is essential prior to its treatment by bacteria and constructed wetland plant treatment system. The bacterial communities grown in such adverse environment is very much important to understand the microbiology of distillery waste. Further, the assessment of molasses-melanoidins by potential bacterial consortium will be a primary step for development of two step treatment process for the PMDE treatment. However, the decolourisation of PMDE is a critical concern and understanding of the structure and characteristics of the melanoidins is required to develop an appropriate process scheme for their removal. In addition, the detail of enzyme and its mode of action also need to be revealed. Moreover, the effect of sulfides and metals along with phenolic compounds required systematic studies to establish the mechanism of biological decolourisation prior to its scope to develop an industrial scale decolourisation technique for safe disposal. The sugarcane molasses, which is used as raw material for alcohol production in distilleries contain high amount of sulfate and aromatic alcoholic

moieties as by products from sugar industries. Further, sulfate and heavy metals present in distillery effluent get reduced into black coloured precipitate of metal sulfides which act as competitive inhibitor for sulfate reducing bacteria and non-sulfate reducing bacteria (McCartney and Oleszkiewicz 1991) leading to inhibition of methanogenesis or sulphate reduction and giving toxicity to PMDE. Recently, the two step/sequential applications of bacteria and wetland plants have been reported to be very promising for detoxification but this has to be optimized yet with detailed microbiology of wetland plants, plant rhizospheres and detoxification mechanism. The interaction of melanoidins with other toxic compounds present in PMDE under the influence of different environmental condition is not known. Chemical structure of different types of melanoidins in PMDE is complicated and yet to be explored. Moreover, the nature of recalcitrant toxic compounds and extent of toxicity added in environment by melanoidins, phenolics, sulphates, phosphates and heavy metals is to be understood.

Keeping in view of all facts, my topic of study is degradation and decolourisation of PMDE in two step treatment system of bacteria and wetland plant for environmental safety. The study has been focused on characterisation of persistent organic pollutants of spent and PMDE by GC-MS analysis to reveal the nature of pollutants present in distillery waste. Prior to develop the two step treatment system for PMDE degradation and decolourisation, assessment of natural and synthetic melanoidins degradability and the effect of distillery effluent co-pollutants are warranted. Therefore, optimisation of its colourant reduction by potential bacterial consortium in presence of heavy metals and other co-pollutants has been studied. Further, before the construction of wetland plant treatment system, the phytoextraction potential of several wetland plants has also been studied. Finally, the study has investigated the degradation and decolourisation of PMDE by bacteria and pilot scale constructed wetland plant treatment system using two steps. The bacterial communities which grows around the rhizospheric zone and degrades the organic pollutants of PMDE is not much known from wetland plant *Phragmites communis* because they indirectly support for plant growth.

The thesis of study has been compiled into following chapters;

Chapter first has introduced the basic information on the topic of thesis. The information related with number of sugarcane molasses based distilleries installed in India and their wastewater generated in the environment. Further, this chapter also provide comprehensive information on the distillery wastewater colorants, as well as its toxicological effects on environment, human and animal health.

Chapter two of this thesis has described the objectives of this study, keeping in view exploring the novel methodology and scientific findings for scale up to develop a new method for industrial scale application has also been described.

Chapter three of this thesis has described the comprehensive existing information on various physico-chemicals, biological (i.e. aerobic and anaerobic), enzymatic treatment as well as emerging techniques for the treatment of PMDE.

Chapter four has described the physico-chemical characteristics of spent wash and PMDE as per approved standard methods. In addition, this section has also described the

characerisation of various persistent organic pollutants of spent wash and PMDE after liquid-liquid extraction and analysed by GC-MS to reveal the nature of pollutants present in this waste. Further, the environmental impact assessment of spent wash and PMDE was done by using seed (*Phaseolus mungo* L. and *Triticum aestivum*) germination test.

Chapter five of this study comprised the decolourization and degradation of sucrose glutamic acid-Maillard reaction products (SGA-MRPs) by developed bacterial consortium. Further, the decolourization of SGA-MRPs by the developed MnP and laccase producting bacterial culture was also studied by using different carbon and nitrogen sources at different environmental conditions. Further, the change in absorption peaks (200-700 nm) by UV-Vis spectrophotometric analysis has been correlated for their structural changes and reduction in color by investigation through FT-IR, HPLC, and GC-MS analysis. Furthermore, to evaluate the stability of potential bacterial consortium at variable nutritional and environmental conditions was assessed for optimum degradation and decolourisation of SGA-MRPs. The reduction of toxicity after bacterial degradation was also evaluated by using seed germination test of *P. mungo* L.

Chapter six of this study comprised the degradation and decolourization of molasses-melanoidins extracted from PMDE by potential bacterial consortium, and optimization of nutritional and environmental parameters for the maximum degradation and decolourisation of melanoidins by bacterial consortium. This chapter also deals with the complete physico-chemical analysis of molasses-melanoidins before and after bacterial decolourisation. Further, the change in absorption peaks (200-700 nm) by UV-Vis spectrophotometric analysis has been correlated for their structural changes and reduction in color by investigation through FT-IR, HPLC, and GC-MS analysis. Furthermore, tThe toxicity assessment has been done in pre and post degradation of molasses-melanoidins by using *P. mungo* L. seeds.

Chapter seven of this study deals with the complete physico-chemical analysis of fresh post methanated distillery sludge (PMDS) and distillery spent wash as well as distillery sludge leachate prepared from in-situ degraded distillery sludge. To accomplish the identification of chemical nature of distillery waste, we employed several organic solvents to extract the broad range organic pollutants under acidic conditions using the liquid–liquid extraction method. Further, the extracted organic compounds present in this waste has been detected and characterised by GC-MS analysis. Simultaneously, the dominant autochthonous bacterial communities were investigated by the RFLP method using a metagenomics approach to reveal the microbial niche in this polluted environment.

Chapter eight has described physico-chemical characteristics of distillery sludge before and after *in*-situ phytoremediation. In addition, the complex organic compounds present in distillery sludge has been detected and characterised by GC-MS analysis. Further, to assess the phytoextraction potential of collected native plants (i.e., *Dhatura stramonium, Achyranthes* sp., *Kalanchoe pinnata, Trichosanthes dioica, Trichosanthes dioica, Cannabis sativa, Amaranthus spinosus* L., *Croton bonplandianum, Solanum nigrum, Ricinus communis, Setaria viridis, Blumea lacera, Argemone mexicana,*

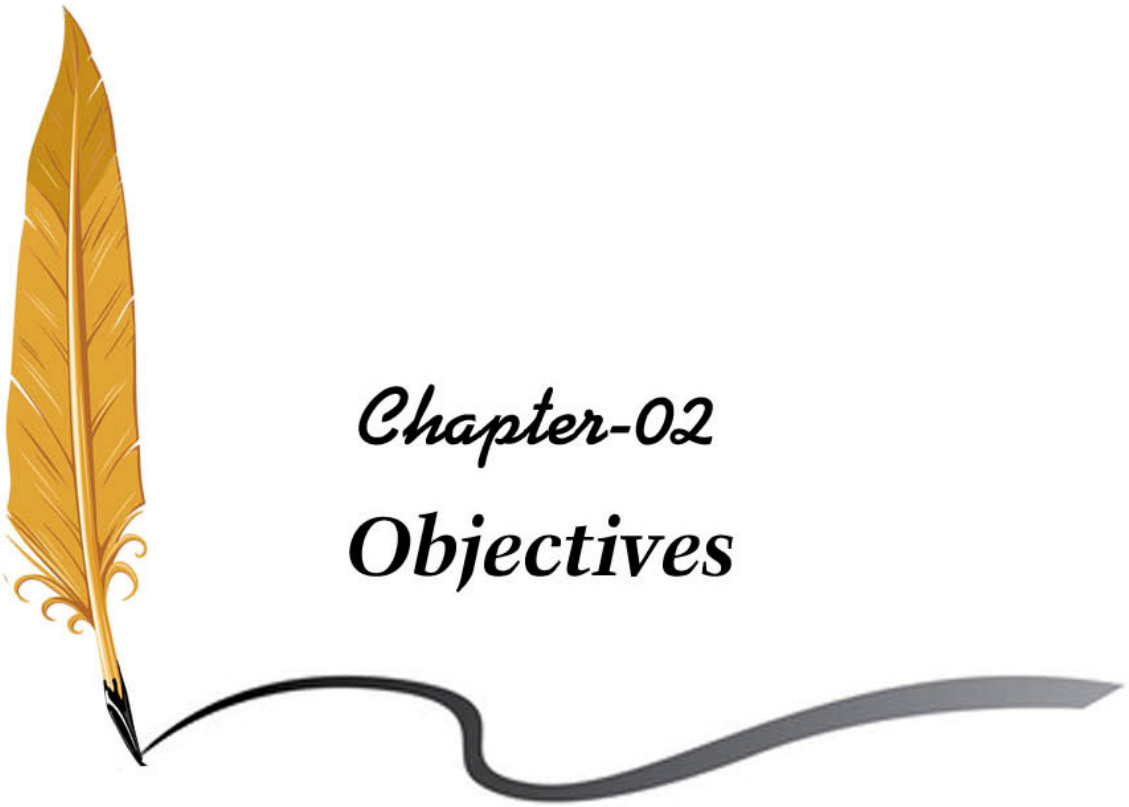
*Saccharum munja, Cynodon dactylon, Pennisetum purpureum, Chenopodium album, Rumex dentatus, Tinospora cordifolia, Calotropis procera,* and *Basella alba*) growing on stabilised PMDS in natural condition, the heavy metals analysis was done. In addition, the bioaccumulation coefficient factor (BCF) and translocation factor (TF) for various heavy metals in different parts of native plants was evaluated. Further, the histological changes in root of native plants at cellular level in all native plants after accumulation of heavy metals have also been investigated by TEM analysis.

Chapter nine of this study deal with designing detail of pilot scale constructed wetland plant treatment system and the optimization for enhancement of PMDE decolourisation by *P. communis* after bacterial treatment in constructed wetland treatment system. The detail physico-chemical characteristics of PMDE collected from the effluent treatment plant of M/s Unnao distillery and Breweries, Unnao (U.P.), India before and after bacteria and wetland plants rhizosphere treatment has also described. Further, this chapter has also shown the complete emphasis of bacterial communities' analysis growing in *P. communis* rhizosphere through next generation sequence (NGS) technology during PMDE degradation and decolourisation. The environmental safety of biphasic treated PMDE has also been shown with seed germination test using with *P. mungo* L.

Chapter ten has summarized the findings of thesis. This section has mentioned the brief findings of each chapter.

Chapter eleven described the concerned references cited in the whole thesis. The reference section has been written in a standard format and all the important references related with topic have been included.

Chapter twelve has been annexed with the title page of published papers and other scientific output of work. The cover page of each published original research papers in National and International journal has also been attached.



# Chapter-02

# Objectives

# Chapter-2

**Objectives**

The objectives of my study were as below;

**1. Investigation of persistent organic pollutants (POPs) of post methanated distillery effluent (PMDE) causing environmental pollution**

- Physico-chemical analysis of spent wash and PMDE
- Characterisation of organic compounds of spent wash by GC-MS analysis
- Characterisation of organic compounds of PMDE by GC-MS analysis persist in PMDE
- Toxicity assessment of spent wash and PMDE by seed germination test
  (a) By using seeds of *Phaseolus mungo* L.
  (b) By using seeds of *Triticum aestivum* L.

**2. Isolation and characterisation of manganese peroxidase and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard (SGA-MRPs) reaction products at different nutritional and environmental conditions**

- Isolation and purification of bacterial strains.
- Screening of MnP and laccase producing and melanoidin degrading bacterial strains
- Characterization of potential MnP and laccase producing bacterial strains.
  (a) Biochemical characterization.
  (b) Molecular characterization
- Decolourization and degradation of SGA-MRPs by developed bacterial culture.
- Optimization of nutritional and environmental parameters.
- Evaluation of bacterial growth, biomass, and scanning electron microscopic (SEM) of bacterial consortium
- Characterization of organic compounds of SGA-MRPs
- Toxicity assessment of Maillard reaction products before and after bacterial treatment

**3. Evaluation of molasses-melanoidins degradation and decolourisation by potential bacterial consortium at different nutritional and environmental conditions**

- Decolourization and degradation of molasses-melanoidins by bacterial consortium.
- Optimization of nutritional and environmental parameters.
- Evaluation of bacterial growth, biomass, and scanning electron microscopic (SEM) of bacterial consortium during molasses-melanoidins degradation
- Characterization of organic compounds of molasses-melanoidins
- Toxicity assessment of molasses-melanoidins before and after bacterial treatment

**4. Profiling of dominant bacterial communities and metabolic products of spent wash and post methanated distillery sludge during *in*-situ bioremediation of distillery waste**

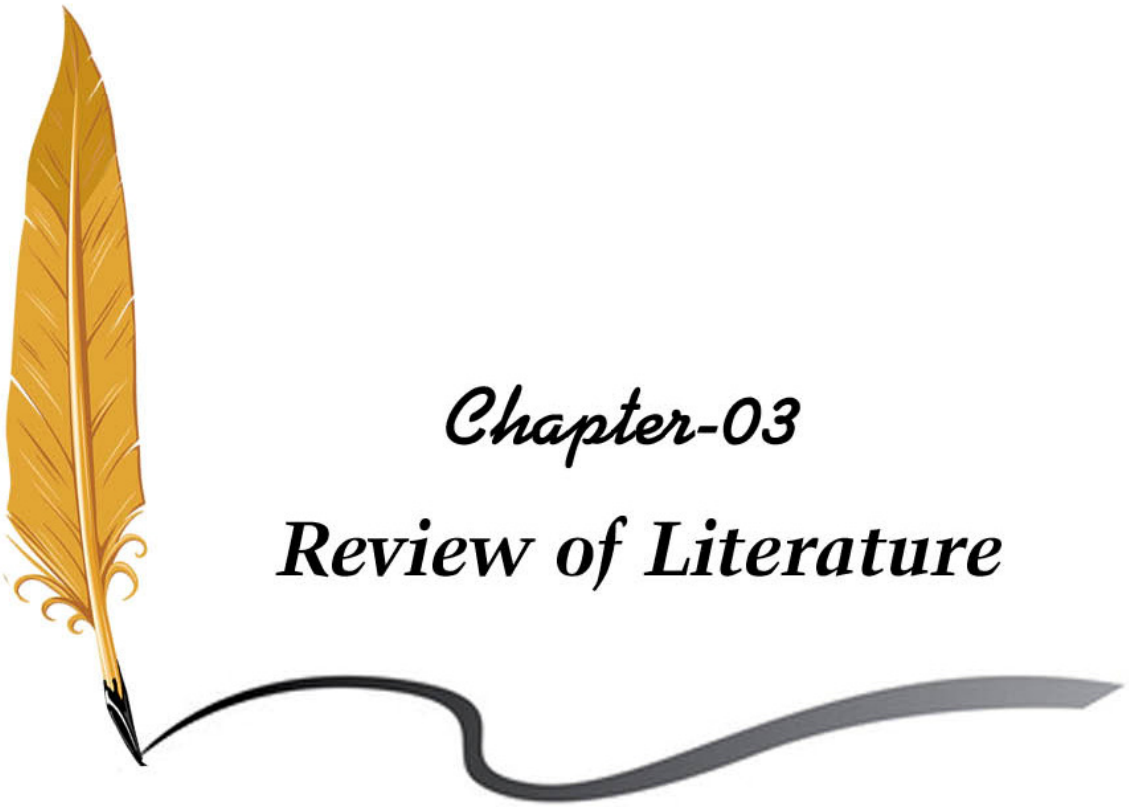
- Physico-chemical analysis of spent wash, post methanated distillery sludge (PMDS) and leachate.
- Characterization of organic compounds of spent wash, PMDS and leachate.
- Analysis of uncultured bacterial communities grown in spent wash and PMDS
- Phytotoxicity assessment of spent wash
- Phytotoxicity and genotoxicity evaluation of sludge leachate

**5. Assessment of phytoextraction capacity of potential native plants grown on endocrine disrupting chemicals rich complex distillery waste**

- Physico-chemical analysis of distillery sludge before and after *in*-situ phytoremediation
- Detection of EDCs and other organic pollutants from distillery sludge
- Heavy metal accumulation in different part of native plants
- Cellular observation of plant root tissues by TEM after accumulation of heavy metals

**6. Analysis of dominant rhizospheric bacterial communities from *Phragmites communis* growing in constructed wetland plant treatment system for decolourisation and detoxification of bacterial treated post methanated distillery effluent (PMDE)**

- Two step treatment of PMDE by bacteria and constructed wetland plant treatment system.
- Physico-chemical analysis of distillery effluent before and after bacteria and constructed wetland plant treatment system.
- Detection and characterization of various metabolites produced during the degradation and decolourisation of PMDE in two step treatment
- Characterisation of rhizospheric bacterial communities of *P. communis*
- Effect of bacteria and CW plant treated PMDE on seed germination and seedling growth of *Phaseolus mungo* L.



# Chapter-03

# Review of Literature

Anaerobic treatments remove most of its biodegradable organic material as reflected in the reduced BOD and COD values of the PMDE. Aerobic treatment using activated sludge process break down these polymeric materials into low-molecular weight compounds without removing colour and hence colouring material still remain in the PMDE in the form of high level of COD. However, colorants of PMDE such as lignin, caramel, ADPH, and melanoidins persist, as these bodies of bio-refractory. Current treatment technologies used to treat PMDE includes physical, chemical, and biological methods before its disposal. The selection of treatment methods depends on various factors viz. treatment efficiency, treatment cost, local geography, climate, land use, regulatory constraints, and public acceptance of the treatment.

### 3.1. Physico-chemical treatment

Physico-chemical treatment methods are combination of physical and chemical technologies. Removal of suspended solids from the PMDE is physical operation while reduction of the dissolved solid is a chemical process. Both operations are done on PMDE by adding chemicals. Several physico-chemical methods have been shown to decrease the pollutants load of distillery effluent. These include coagulation/flocculation, electrocoagulation, thermolysis, membrane filtration, oxidation by ozone, chlorine dioxide, hydrogen peroxide and radiation, and adsorption to material such as chitosan and activated carbon.

#### 3.1.1 Adsorption

Activated carbon is the most widely studied adsorbent, and it has been found to adsorb a variety of materials such as metals, phenols, dyes, and other organic compounds and bio-organisms, and is therefore used for the removal of pollutants from wastewaters by adsorption. The activated carbons were prepared from agro-residues such as sugarcane bagasse, fly ash, rice husk ash, wood ash, and wood saw dust. In distillery wastewater treatment, the interface is between the liquid and solid surface that is artificially provided. The material removed from the liquid phase is called the adsorbate and the material providing the solid surface is called the adsorbent. This process has been also found successful in removing harmful parameters like COD and color from distillery effluent (Swamy et al. 1997). Decolourisation of distillery effluent include adsorption on commercial as well as indigenously prepared activated carbons has been reported by various researchers (Satyawali and Balakrishnan 2009; Chandra and Pandey 2000). Satyawali and Balakrishnan (2007) investigated the decolourisation of anaerbically treated distillery effluent using different agro residue based activated carbon and commercial prepared activated carbons (ACs). Colour removal was 50% among all the agro-residue based carbons at the highest investigated dosages of 5g/100 mL, whereas commercial ACs performed better with 80% colour removal at the same highest dosage. Since, then, commercial ACs surface area (1520 $m^2/g$) was three fold higher than for bagasse (492

$m^{2/}g$). The use of chemically modified bagasse using 2-diethylaminoethyl (DEAE) chloride hydrochloride and 3-chloro-2-hydroxypropyltrimethyl ammonium chloride (CHPTAC) was found to be capable of decolorizing diluted spent wash (Mane et al. 2009). The 0.6 g of chemically modified bagasse in contact with 100 mL spent wash reduced 50% color in 4 h. The decolonization of biomethanated distillery effluent in packed bed using commercial activated charcoal reduces 99% color and 90% of BOD. Comparative studies of color removal have been performed using commercial activated carbon and bagasse fly ash. The 58% color removal with 30 $g/dm^3$ of bagasse flyash and 80.7% color removal with 20 $g/dm^3$ of commercial ACs are reported. Few studies have been done using natural carbohydrate polymer chitosan. The study (Lalov et al. 2000) on the diluted distillery wastewater using chitosan as an anion exchanger gave 98% color and 99% COD removal of 10 $g/dm^3$ and 30 min contact time. Ramteke et al. (1989) reported color removal up to 98% with pyrochar. They reported to achieve the same level of color removal with larger doses of the indigenously prepared powdered and granular pyrochar in comparison to commercial ACs. ACs are not low-cost materials; hence, in spite of their good efficiency and applicability for adsorbing a wide variety of materials, their use can sometimes be restricted due to economic considerations

### 3.1.2 Membrane filtration

Membrane filtration (MF) is a term used to describe the removal of particulates from a feed stream. There are two embodiments of the membrane filtration process, namely ultrafiltration (UF) and microfiltration (MiF). Two other important MF processes are used in wastewater treatment, namely reverse osmosis (RO) and nanofiltration (NF). RO and NF processes are designed to remove dissolved species from the feed stream, and are not intended to be filtration processes. MF technology cannot be directly applied to treat distillery effluent due to high TDS. de Wilde (1987) carried out electrodialysis for distillery effluent after the removal of suspended and undissolved matter. It was found that between 50% and 60% of $K^+$ could be removed from the distillery effluent at a current efficiency of about 50-55% and a DC power consumption of 0.75-0.85 kWh per kilogram of potassium removed. Pre-treatment of spent wash with ceramic membranes was performed prior to anaerobic digestion is reported to halve the COD from 36,000 to 18,000 mg $L^{-1}$ (Chang et al. 1994). Ceramic membranes with 0.05 mm pore size were chosen based on the particle size distribution in raw wastes. In this pre-treatment, COD was reduced from 36000 to 18000 mg $L^{-1}$ and suspended solids were almost completely removed. Tertiary treatment of PMDE by NF was carried out in a spiral wound NF membrane module, which was done by Rai et al. (2008) under different operating conditions. They obtained COD and TDS in the range of 96–99.5% and 85–95%, respectively. The color removal was in the range of 98–99.5%. Nataraj et al. (2006) reported the membrane based NF and RO processes can be used to reduce the TDS, COD, and $K^+$ content of distillery effluent by 99.80, 99.90, and 99.99%, respectively. Nakhla et al. (2006) studied the applicability of a submerged vacuum UF membrane technology in combination with the biological treatment system. The system achieved 95-96.5% BOD

removal and 99% COD removal. The reactor achieved a superior quality that may meet reuse criteria at reasonable cost. Submerged nanofiltration for removal of melanoidins from the biologically treated molasses fermentation wastewater was evaluated by Liu et al (2013). The melanoidins could be effectively removed from the biologically treated molasses fermentation wastewater by SNF and the electrolytes presented in the effluent were nearly not retained by the membrane.

### 3.1.3 Coagulation and flocculation

Coagulation is the use of chemicals to cause pollutants to agglomerate and subsequently settle out during sedimentation. The removal of colour containing compounds and COD from distillery effluent was reported using inorganic coagulants-$FeCl_3$, $AlCl_3$ and poly-aluminium chloride (PAC) (Chaudhari et al. 2007). Migo et al. (1993) used a commercial inorganic flocculent [Fe(OH)n(SO4)3-n/2]m, a polymer of ferric hydroxide for the emoval of molasses-derived melanoidin in alcohol distillery wastes. Decolorization yields of 32, 87 and 94% were obtained for fresh slops and for biodigester and lagooned effluents, respectively, at a flocculant dosage of 4% v/v. The reduction in total organic carbon (TOC) was 21% for fresh slops and averaged to more than 73% for the biodigester and lagooned effluents. Pikaev et al. (2001) reported that treatment of distillery wastewater using iron sulfate [$Fe_2(SO_4)_3$] as a coagulant results, 40% removal of wastewater pollutants. Beltrain de Heredia et al. (2005) also achieved 55% reduction in COD by using integrated Fenton-coagulant/flocculation process in distillery wastewater treatment. In another report, addition of $FeSO_4$, $AlCl_3$, CaO, $FeCl_3$ along with coagulants reduced the colour to 95%, 74.4%, 80.2%, and 83%, respectively, while COD was reduced as 78%, 61.3%, 39.8%, and 55%, respectively (Chaudhari et al. 2007). *Moringa oleifera* seeds were also used as coagulant for removal of color from distillery spent wash. During the color removal, the dosage (20 and 60 mL), pH (7 and 8.5) and concentration of 0.25 M had been found to be the optimum conditions for maximum 56% and 67% color removal using NaCl and KCl salts, respectively (Krishna Prasad 2009). Fan et al. (2011) reported that anaerobic treatment of distillery effluent enhanced the coagulation efficiency markedly, with $FeCl_3$, achieving 94% colour removal and 96% COD removal, while ACH/polyDAMAC gave 70% and 56 % removal respectively. Armini et al. (2015) used manganese oxides for the color removal from melanoidins rich distillery effluent. Manganse oxides can oxidize aromatic amine to quinones and dimer products. It can oxidise the long chain amines to nitrone, which may further dimerize to bigger compounds and precipitate out of the solution by MnOx. Liako and Lazaridis (2014) studied the effect of $FeCl_3.6H_2O$ on the removal of melanoidins from simulated and real wastewater by coagulation and electro-flotation. They showed that coagulation experiment could achieve 90% color removal from all wastewater at pH 5.0. However, recent studies have pointed out several serious drawbacks of using aluminum salts, such as Alzheimer's disease (Miller et al. 1984; Letterman and Driscoll 1988; Qureshi and Malmberg 1985). There is also the problem of reaction of alum with natural alkalinity present in the water leading to

a reduction of pH and a low efficiency in coagulation of cold waters (Haarhoff and Cleasby 1988; Morris and Knocke 1984).

### 3.1.4 Electrochemical/Electrocoagulation

The electrochemical treatment is widely used to remove the color from distillery effluent. These processes use electron as main reagent, but also require the presence of supporting electrolytes. Electrocoagulation involves the consumption of metals from anode, with simultaneous formation of hydroxyl ions and hydrogen gas occurring at the cathode. Therefore, the electrochemical treatment methods such as electrocoagulation (EC) may be used effectively for the removal of organic pollutants in terms of lower operating time and costs. In the electrochemical process, the pollutants are destroyed by either the direct or indirect oxidation process. In a direct anodic oxidation process, the pollutants are first adsorbed on the anode surface and then destroyed by the anodic electron transfer reaction. In an indirect oxidation process, strong oxidants such as hypochlorite/chlorine, ozone, hydrogen peroxide are electrochemically generated. The pollutants are then destroyed in the bulk solution by oxidation reaction of the generated oxidant. All the oxidants are generated in-situ and are utilized immediately. Among the oxidants hypochlorite is cheaper and most of the effluents have a certain amount of chloride. The electrochemical method involves the application of electrical current to the effluent to convert chloride to chlorine/hypochlorite. The chlorine and hypochlorite oxidize the pollutants and are then reduced to chloride ions. Thakur et al. (2009) studied the effect of pH 3.5‑9.0 on EC treatment of anaerobically treated distillery effluent. The 43.71, 48.9, and 31.5% COD reduction was obtained at pH 3.5, 6.5, and 9.0, respectively, with a current density of 117 $A/m^2$ in 60 min. Similarly, electrochemical degradation to treat spent wash with ruthenium oxide-coated titanium mesh acting as anode and SS as cathode was performed between pH 4.0 and 9.0 (Prasad and Srivastava 2009). Increase in initial pH was found to decrease the decolorization efficiency. Kumar et al. (2009) also studied the effect of pH for treatment of BDE. The 32.66%, 39.95%, and 44% COD reduction was obtained at pH 2, 5, and 8, respectively, with a current density of 133.94 $A/m^2$ in 90 min. Parajapati and Chaudhari (2014) reported 93% COD reduction and 87% color reduction using iron electrode at their optimum condition. Parajapati and Chaudhari (2014) found 93% COD and 76% color reduction using aluminum electrode. Kobya and Gengec (2012) also reported that EC process using aluminium electrode was able to decolourise melanoidins upto 98% at pH 4.2. The decolourisation performance was dependent on pH value since the lower pH values led to faster reaction and higher decolourisation.

### 3.1.5 Thermolysis

The treatment of distillery effluent by thermolysis is occurs due to the presence of various components like reduced carbohydrates, proteins, lignin, and minerals (Chaudhari et al. 2008). The functional groups are responsible for the reaction and formation of solid residues. The thermolysis process for anaerobically treated distillery effluent has also been reported by Dhale and Mahajani (2000). The effluent was heated in the absence of air at

150 $^{o}$C resulting in 35% COD reduction in t=0.6 h. Chaudhari et al. (2005) reported COD reduction of anaerobically treated distillery effluent at 100 $^{o}$C and atmospheric pressure. Catalysts used were CuO, $CuSO_4$, Mn/Ce oxide, and Mn/Cu oxide. About 70%, 65%, 35%, 38%, 40%, and 36% COD reduction was obtained at pH 2, 2, 4, 6, 8, and 10, respectively, using CuO catalyst. These data show acidic condition to be best for treatment process. Similar studies were performed for treatment of spent wash (Chaudhari et al. 2008). The COD reduction of 58%, 60%, 51%, 36%, 30%, and 32% was obtained at pH 1, 2, 4, 6, 8, and 10, respectively, at 140 $^{o}$C and autogenous pressure. Thermolysis process has been also performed by Lele et al. (1989) for treatment of spent wash at temperatures in the range of 160‑250 $^{o}$C and autogenous pressures. They observed that there was no further COD reduction after a treatment time of 2 h. The authors reported zero-order COD reduction kinetics. The zero-order rate was evaluated as 6.67, 10.40, 10.8, and 14.40 kg/m$^3$h at 160, 200, 230, and 250 $^{o}$C, respectively. Their work supported the work of Daga et al. (1986), where an increase in temperature results in an increase in COD reduction.

### 3.1.6 Oxidation process

Oxidation processes are a set of chemical treatment procedures designed to remove organic (and sometimes inorganic) materials in wastewater by oxidation through reactions with hydroxyl (OH) radicals.

#### 3.1.6.1 Ozone oxidation

Ozonation process seems to be a promising technology for the treatment of melanoidins containing distillery effluent. Ozone is a powerful oxidant for water and wastewater treatment. When ozone comes in contact with effluent, ozone reacts with organic compounds in two different ways: by direct oxidation as molecular ozone or by indirect reaction through the formation of secondary oxidants like free radical species, in particular the OH radicals. Both ozone and OH radicals are strong oxidants and are capable of oxidizing a number of compounds and finally COD value is reduced. Melanoidins have conjugated –C=C- bond in their structure that are responsible for the brown colour (Kim et al. 1985). The colour removal from distillery effluent was probably due to the fact that ozone is able to break down the conjugated –C=C- bonds, thus breaking the chromophore of the pigment. Carboxylic acids have been indicated as products of melanoidins ozonation (Kim et al. 1985). Carboxylic acids hardly react with ozone but they contribute to COD. Ozone only transforms the dark collared polymeric compounds in the effluent (Pena et al. 2003). Benitez et al. (2003) study the oxidation of the organic substrate present in wastewaters generated in wine distilleries effluent by an ozonation process. The ozonation process is conducted in a subsequent first discontinuous and a second continuous periods. It is reported that the maximum COD is reduced by 5‑25.2% and the total aromatic compound content is reduced by 16.8‑51.4% at the optimum condition. A catalyst has also enhanced the efficiency of ozonation process. Recent works have demonstrated the combined use of ozone and aerobic oxidation for the treatment of synthetic and distillery wastewater (Beltran et al. 2001; Pena et al. 2003). Further,

Sangave et al. (2007) has also used combination of ultrasound (US) and ozone treatment process to treat distillery effluent pre-treated with thermal pre-treatment and anaerobic treatment process. For US-treated sample, a maximum of 13% COD reduction was attained at the end of 48 h of aerobic oxidation, while for the ozone-treated effluent a maximum of 45.6% COD reduction was obtained. Asaithambi et al. (2012) has reported a hybrid technique of ozone-assisted electrocoagulation for the removal of color and COD in the industrial effluent and they achieved a maximum removal of COD 83% at a current density of 3 $Adm^{-2}$, initial COD concentration 2500 ppm, initial pH 6.0, and an $O_3$ mixture flow rate 15 L $min^{-1}$, while complete color removal was observed within 2 h of process time. Sreethawong and Chavadej (2008) have used iron oxide as a heterogeneous catalyst to enhance the ozone oxidation process. They achieved a maximum 80% COD and 50% color reduction during process. Oxidation by ozone could achieve 80% decolourisation of anaerobically treated distillery effluent with simultaneous 15-25% COD reduction (Pena et al. 2003). Recently, Kumar et al. (2006) has reported that COD was removed upto 95% from treated anaerobically distillery spent wash through ozone treatment.

### 3.1.6.2 Hydrogen peroxide treatment

The chemical decolourisation of model melanoidins prepared from glucose-glycine system by $H_2O_2$ treatment studied by Hayase et al. (1984) reported about 64% and 97% decolourisation of melanoidin using $H_2O_2$ in neutral (pH 7.0) and alkaline (pH 10.0) medium, respectively. He suggested that the degradation of melanoidins by active oxygen species such as $H_2O_2$, which is secondarily produced by the enzymatic oxidation of glucose into gluconic acid by glucose-oxidase enzyme. They proposed that hydrogen peroxide reacts with hydroxyl anion to give perhydroxyl anion ($HOO^-$), which has a strong nucleophilic activity and per hydroxyl anion was considered to attack the nucleophilically carbonyl groups of melanoidins.

### 3.1.6.3 Advanced oxidation treatment/photocatalytic treatment

In recent years, advanced oxidation processes (AOPs) have been intensively studied due to their efficiency to oxidize variety of organic contaminants. Among these AOPs, heterogeneous photocatalysis employing semiconductor catalysts ($TiO_2$, ZnO, $Fe_2O_3$, CdS, GaP, and ZnS) have demonstrated their efficiency in degrading a wide range of ambiguous refractory organics into readily biodegradable compounds, and eventually mineralized them to innocuous carbon dioxide and water.

## 3.2 Biological processes

Biological treatments have been recognized as effective methods of treatment for complex industrial wastewaters. In biological treatment, both anaerobic and aerobic systems are commonly used to treat the wastewaters from agro-industrial plants including distilleries. In the recent years, increasing attention is also being directed towards utilizing microbial activity (pure bacteria and fungi) for the decolourization and mineralization of distillery effluent. Moreover, the biologically treated effluent could be used safely and effectively to

increase the soil productivity. This section is discussed in detail as anaerobic and aerobic treatments.

### 3.2.1 Anaerobic treatment

Anaerobic digestion has been considered as an attractive biotechnological method for degrading a variety of polluting organic wastes. However, it is a complex process requiring the concerted activity of multiple microbial populations interacting in a trophic web. The breakdown of organic chemicals in an anaerobic reactor usually involves several consequent degradation phases such as hydrolysis, acidogenesis, and then methanogenesis. In the first two phases, organic pollutants are hydrolyzed and/or fermented into intermediate short-chain fatty acids (e.g., lactate, butyrate and propionate), which are further degraded to acetate and $H_2/CO_2$. In the last phase, acetate and $H_2/CO_2$ are converted into methane. Anaerobic digestion is widely accepted first step of distillery spent wash treatment. Anaerobic biological treatment effectively removes 90% of the COD (70% for the industrial scale), 80–90% of the BOD along with the recovery of 85–90% of biogas generated (Sankaran et al. 2014). A typical BOD/COD ratio of 0.8-0.9 indicates suitability of distillery wastewaters for biological treatment (Mohana et al. 2007). Up flow UASB reactor is the most popular high rate digester that has been utilized for anaerobic treatment of various types of industrial wastewaters. Treatment by a UASB reactor resulted in 75% COD removal in spent wash. Wolmarans and De Villiers (2002) have also reported COD removal efficiency of greater than 90% over three seasons in a UASB plant treating distillery wastewater. In another anaerobic treatment method, fluidized bed reactors contain an appropriate media such as sand, gravel, or plastics for bacterial attachment and growth. A two stage process with an anaerobic filter followed by a UASB reactor was investigated by Blonskaja et al. (2003). The acidogenic and methanogenic phases were clearly separated ensuring better conditions for the methanogens. COD reduction was 54% and 93% in the first and second stage, respectively. The highest BOD removal is possible in open lagoon, whereas highest biomethane produced is in up flow UASB bioreactor. Compared to aerobic system, it has slow growth rate, mainly associated with methanogenic bacteria. Therefore, it requires a long retention time, and also only a small portion of the degradable organic waste is being synthesized to new cells (Pant and Adholeya 2007b). Goodwin and Stuart (1994) studied two identical UASB reactors operated in parallel as duplicates for 327 days for the treatment of malt whisky pot ale and achieved COD reductions of up to 90% for influent concentrations of 3526–52126 mg $L^{-1}$. When the OLRs of 15 kg $m^3$ day and above were used, the COD removal efficiency dropped to less than 20%, in one of the duplicate reactors. A mesophilic two-stage system consisting of an anaerobic filter (AF) and an UASB reactor was found suitable for anaerobic digestion of distillery waste, enabling better conditions for the methanogenic phase (Blonskaja et al. 2003). The optimum conditions for the stable work of reactor are: for the acidogenic stage, organic loading of 2–4 kg COD $m^{-3}$ $day^{-1}$ at pH 6.0 and for the methanogenic stage, organic loading of 1–2 kg COD $m^{-3}$ $day^{-1}$ at pH 7.6. An advanced version of UASB system was reported by

Driessen and Yspeert (1999), wherein they used an internal circulation reactor characterized by biogas separation in two stages within a reactor with a high weight/diameter ratio and the gas driven internal effluent circulation. This system could handle high upflow liquid and gas velocities making possible treatment of low strength effluents at short hydraulic retention times as well as treating high strength effluents such as from brewery at very high volumetric loading rates up to 35 kg COD $m^{-3}$. Effect of addition of macronutrients and micronutrients in the distillery effluent was investigated on the performance of simulated UASB system by Sharma and Singh (2001). Calcium and phosphate were found to be detrimental to treatment efficiency. Uzal et al. (2003) investigated the biochemical methane potential of malt whisky distillery wastewater both with and without basal medium to observe the effect of nutrient supplementation. When a COD concentration of 20920 mg $L^{-1}$ was maintained in the influent to the first stage, the effluent quality of the first stage began to deteriorate. A significant colour change was observed from black to brownish black and then to brown, and this was thought to be due to reduced metabolic activity owing to the toxic effect of the wastewater on granular biomass, thus increasing oxidation-reduction potential. It was concluded that two stage UASB reactor configuration is an efficient system for malt whisky wastewater treatment until up to 33866 mg $L^{-1}$ influent COD concentration. For the overall sequential system (anaerobic/aerobic) treatment COD and BOD removal efficiencies were 99.5% and 98.1%, respectively, for the treatment of malt whisky wastewater. In aerobic phase, the effluent of anaerobic bioreactor is exposed to atmospheric oxygen in a tank with homogenizers for proper mixing of the effluents. BOD is reduced to 200 and effluent diluted with wastewater from bottling and washer sections and disposed of after clarification (Ramendra and Awasthi 1992). The stabilized sludge serves as a soil conditioner and plant nutrient.

### 3.2.2 Aerobic treatment

Aerobic processes are biological treatment processes that occur in the presence of oxygen. The aerobic treatment of industrial wastewaters usually depends on the oxidative activities of microorganisms. Although, a large number of microorganisms, such as bacteria, cynobacteria, yeast, fungi, etc. have been used for treatment of PMDE. Filamentous fungi can be of important sources of phenolic-degrading organisms, as they frequently grow on wood, utilizing lignin as a carbon source. Aerobic processes are usually applied as post anaerobic treatment steps, based on pollutant degradation by the utilization of specific microorganisms, either as pure strains or as a bioconsortium, with air supply.

#### 3.2.2.1 Fungal treatment

In recent years, several basidiomycetes and ascomycetes type fungi have been used in the decolourisation of natural and synthetic melanoidins in connection with colour reduction of effluent from distilleries. The aim of fungal treatment is to purify the effluent by consumption of organic substances, thus, reducing its COD and BOD, and at the same time to obtain some valuable product, such as fungal biomass for protein-rich animal feed,

extracellular organic acids or some specific fungal metabolites. There were several reports that indicated that some fungi in particular have such a potential (Pant and Adholeya 2007a, 2009a). *Phanerochaete chrysosporium* and *Trametes versicolor* are the most widely studied for the decolourisation and degradation of distillery effluent. *P. chrysosporium* JAG 40 resulted in 80% decolourization of diluted synthetic melanoidin (absorbance unit of 3.5 at 475 nm), as well as with 6.25% anaerobically digested spent wash (Kumar et al. 1998; Dahiya et al. 2001a). Thakkar et al. (2006) studied the biocatalytic decolorization of molasses by *P. chrysosporium.* Bioremediation potential of *P. chrysosporium* strains NCIM 1073, NCIM 1106 and NCIM1197 to decolorise molasses in solid and liquid molasses media was studied. Strains varied in the pattern of molasses decolorization on solid medium by giant colony method. Under submerged cultivation conditions, strain NCIM 1073 did not decolorise molasses while, strains NCIM 1106 and NCIM 1197 could decolorise molasses up to 82% and 76%, respectively. Under stationary cultivation conditions, none of the strains could decolorise molasses. *Coriolus versicolor* immobilized in a packed-bed reactor reduced the COD of spent wash by a further 50.3%, giving an overall reduction in COD of 77% to 15,780 mg $L^{-1}$ (Fitzgibbon et al. 2007). One of the most studied fungus having the ability to degrade and decolorize distillery effluent is *Aspergillus* sp. *A. fumigatus* G-2-6, *A. niger, A. niveus,* and *A. fumigates* UB260 brought about an average of 60‑85% COD reduction along with 65‑78% decolorization (Miranda et al. 1996; Shayegan et al. 2005). *A. fumigatus* has been found to be effective for decolourization of anaerobically treated distillery wastewater (Mohammad et al. 2006). Fungal consortium was employed in fluidized film aerobic system (FFAS). The analyzed effluent at the end of FFAS treatment showed a reduction of 70% in BOD and 63% in COD without causing any colour change (Ravikumar et al. 2007). The fungi *Geotrichum candidum*, *Coriolus versicolor*, *P. chrysosporium*, and *Mycelia sterilia* were screened for their ability to decolourize spent wash and to reduce the COD level. A 10 day pre-treatment with *Geotrichum candidum* at 30 0C resulted in reducing the COD by 53.17% and total phenols by 47.82%, enabling other bioremediating organisms to grow. *Flavodon flavus* (Klotzsch) Ryvarden, a basidiomycete (NIOCC strain 312) isolated from decomposing leaves of a sea grass, decolourized pigments in distillery effluent by 80% after 8 days of incubation, when used at concentrations of 10% and 50% (Raghukumar and Rivonkar 2001). Treatment of distillery spent wash with ascomycetes group of fungi such as *Penicillium* spp., *P. decumbens,* and *P. lignorum* resulted in about 50% reduction in color and COD and 70% phenol removal (Jimnez et al. 2003). Kahraman and Yesilada (2003) reported molasses decolourisation in semi solid state cultivation by fungi *C. versicolour, Funalia trogii, P. chrysosporium* and *Pleurotus pulmonarius* with cotton stalks being used as additional source of carbon. *C. versicolour* decolourized 48% of 30% diluted vinasse without any additional carbon source which increased to 71% on addition of cotton stalks. *A. niveus,* a litter degrading fungi was used by Angayarkanni et al. (2003) for the treatment of distillery effluent using paddy straw, sugarcane bagasse, molasses and sucrose as carbon source for growth of fungus in the effluent. Sugarcane bagasse at 1% (w/v) concentration resulted in maximum removal of colour (37%) and COD (91.68%). The decrease in colour removal in this study might be due to the fact that the effluent

taken for study was alkaline (pH 9.0) and the melanoidins responsible for colour were more soluble in the alkaline pH. In the acidic pH, the melanoidins might be precipitated and removed easily. Raghukumar and Rivonkar (2001) isolated a marine fungus, *Flavodon flavus*, which was more effective in decolorizing raw molasses spentwash than was the molasses wastewater collected either after anaerobic treatment or after aerobic treatment. Tondee and Sirianutapiboon (2006) isolated *Issatchenkia orientalis* yeast from fruit sample which showed 60% melanoidin decolorization at 30 °C in 7 days under aerobic condition. Kaushik and Thakur (2009) isolated five different fungi from distillery mill contaminated site and the two isolates having higher capabilities to remove colour were identified as *Emericella nidulans* var. lata and *Neurospora intermedia*, respectively. Maximum color was removed at pH 3, temperature 30 °C, stirring 125 rpm, dextrose (0.05%) and sodium nitrate (0.025%) by both fungi. Ravikumar (2015) demonstrated the ability of *Cladosporium cladosporioides* in biodegradation and decolorization of distillery spent wash. They observed that during the initial period of 2 days, there is no significant change in the color removal of the spent wash; and maximum decolorization of 72.3 % was achieved with COD reduction of 74.8 % on 5th day. COD reduction and decolorization percentage increased till 5th day and then remained constant. The thermotolerant *Candida tropicalis* decolorized complex melanoidin compounds at a wide range of temperature and pH conditions, in the presence of small amounts of carbon and nitrogen sources, within a short incubation period of 24 h. This strain could reduce environmental pollution problems due to molasses wastewater discharge by decolorizing melanoidin pigment under a cost-effective and eco-friendly process (Tiwari et al. 2012). But, the large scale applications of these techniques have own constraint due to slow growth cycle, huge spore formation, low pH range (3.0–5.0), and adverse submerged aquatic environment for growth of fungus (Arimi et al. 2014).

### 3.3.2.2 Bacterial treatment

Bacterial cultures have a very high potential for both bioremediation and decolorization of anaerobically treated distillery spent wash. Pioneering work on spent wash decolourizarion by bacteria was done by Kumar et al. (1997). They observed that two aerobic bacterial isolates LA-1 and D-2 brought about maximum decolourization (36.5% and 32.5%) and COD reduction (41% and 39%) under optimized conditions in eight days. Kambe et al. (1999) screened out various microorganisms for their ability to decolorised molasses wastewater under thermophilic and anaerobic conditions. Strain MD-32, which was newly isolated from a soil sample, was selected as the best strain. From taxonomical studies, the strain was concluded to belong to the genus *Bacillus*, most closely resembling *B. smithii*. The strain decolorized 35.5% of molasses pigment within 20 days at 55 $^{o}$C under anaerobic conditions but no decolorization activity was observed when it was cultivated aerobically. Cibis et al. (2002) achieved biodegradation of potato slops (distillation residue) by a mixed population of bacteria under thermophilic conditions up to 60 $^{0}$C. A COD removal of 77% was achieved under non-optimal conditions. Petruccioli et al. (2000) used an air bubble column reactor with activated sludge carrying self adapted

microbial population in both free and immobilized on polyurethane particles for treating aerobic winery wastewater. The highest COD removal rate was with free activated sludge in the bubble column reactor. The most prominent bacterial species isolated from the reactor liquid belonged to *Pseudomonas* while *Bacillus* was isolated mostly from colonized carriers. *P. fluorescens*, decolourized melanoidin wastewater (MWW) up to 76% under non-sterile conditions and up to 90% in sterile samples (Dahiya et al. 2001b). Sirianuntapiboon and Phothilangka (2004) used an acetogenic bacterium to obtain a decolourization yield of 76.4% under optimal nutrient conditions. However, this value was only 7.3%, by using anaerobic pond. Also, it required sugar, especially glucose and fructose for decolourization of MWWs. The decolourization activity might be due to a sugar oxidase. Acetogenic bacteria are capable of oxidative decomposition of melanoidins. Lactic acid bacteria (*Lactobacillus coryniformis, L. sakei, L. plantarum, Weisella soli, Pediococcus parvulus, P. pentosaceus*) were used to decolorise the melanoidins. The isolate *L. plantarum* exhibit 44% decourisation of melanoidins (Krzywonos and Seruga 2012). *Lactobacillus plantarum* No.PV71-1861, showed the potential for decolorization of molasses wastewater under both anaerobic and facultative conditions. The highest melanoidins pigment removal efficiencies and growth yield of 76.6% and 2.6 mg $mL^{-1}$, respectively were observed within 7 days of culture (Tondee and Sirianuntapiboon 2008). Santal (2016) reported a novel bacterium *Paracoccus pantotrophus* strain SAG1 to decolourise melanoidins of distillery effluent up to 81.2% in the presence of glucose and $NH_4NO_3$. Many attempts for decolourisation and detoxification of synthetic melanoidins were carried out with bacterial culture such as by individual and mixed cultures of *Bacillus* sp. for biodegradation of organic compounds of molasses melanoidins from biomethnated distillery spent wash during the decolourisation by potential bacterial consortium (Yadav and Chandra 2012; Jiranuntipon et al. 2008). An effort has also been made by the Kryzwonos (2012) using the consortia of genus Bacilli to decolorize the melanoidins. Two mixed bacterial cultures of the genus *Bacillus* (C1 and C2) were tested for color removal ability. Kumar and Chandra (2006) studied on the decolorization of synthetic melanoidins (i.e., GGA, GAA, SGA and SAA) by three *Bacillus* isolates *B. thuringiensis* (MTCC 4714), Bacillus brevis (MTCC 4716) and *Bacillus* sp. (MTCC 6506). Significant reduction in the values of physicochemical parameters was noticed along with the decolorization of all four melanoidins (10%, v/v). *B. thuringiensis* (MTCC 4714) caused maximum decolorization followed by *B. brevis* (MTCC4716) and *Bacillus* sp. (MTCC6506). A mixed culture comprised of these three strains was capable of decolorizing all four melanoidins. The medium that contained glucose as sole carbon source showed 15% more decolorization than that containing both carbon and nitrogen sources. Melanoidin SGA was maximally decolorized (50%) while melanoidins GAA was decolorized least in the presence of glucose as a sole energy source. On the other hand, *B. megaterium* SW3 and *B. subtilis* SW8 were used for decolorization and bioremediation of distillery spent wash. Both bacterial isolates were grown well in 7.5% (v/v) diluted digested spent wash supplemented with glucose and urea as a readily available carbon and nitrogen source. Optimum condition for growth and distillery effluent decolorization were at pH 7.2, temperature 37 °C, glucose 5% (w/v), and urea 2% (w/v) in a minimal salt

medium. The maximum decolorization (51%, 52.5% and 57%) and COD reduction (53%, 54.5% and 59%) was found after 5 days of incubation under the optimized condition were achieved for cultures SW3, SW8 and consortia (SW3 + SW8) respectively (Sivakumar et al. 2006). Similarly, Yadav and Chandra (2012) developed the consortia *Proteus mirabilis* (IITRM5), *Bacillus* sp. (IITRM7), *Raoultella planticola* (IITRM15) and *Enterobacter sakazakii* (IITRM16) in the ratio of 4:3:2:1. This consortia was responsible for the 75% decolorization of melanoidins within 10 days. The degradation of synthetic and natural melanoidins was studied by using the axenic and mixed bacterial consortium (*B. licheniformis* (RNBS1), *Bacillus* sp. (RNBS3) and *Alcaligenes* sp. (RNBS4). Results have revealed that the mixed consortium was more effective compared to axenic culture decolorising 73.7% and 69.8%% synthetic and natural melanoidins whereas axenic cultures RNBS1, RNBS3 and RNBS4 decolorized 65.88%, 62.5% and 66.1% synthetic and 52.6%, 48.9% and 59.6% natural melanoidins, respectively. Consortia of *Pseudomonas, Stenotrophomonas* and *Proteus* species were also viable microorganisms to biodegrade and decolorize anaerobically treated spent wash. The treatment results showed that67±2% decolorization within 24h and 51±2% COD reduction within 72h when incu bated at 37 $^{0}$C under static condition in biomethanated spent wash supplementedwith0.5% glucose, 0.1% $KH_2PO_4$, 0.05%KCl and 0.05% $MgSO_4.7H_2O$ (Mohana et al. 2007). Chaturvedi et al. (2006) isolated and characterized root associated rhizospheric bacterial communities of *Phragmites australis* grown on distillery waste contaminated site. Fifteen culturable bacterial sp. were identified based on the 16S rRNA sequencing from the rhizosphere soil; they are *Microbacterium hydrocarbonoxydans* (AJ880397), *Achromobacter xylosoxidans* (AJ880764), *B. subtilis* (AJ880760), *B. megaterium* (AJ880767), *B. anthracis* (AJ880766) from upper zone; *B. licheniformis* (AJ880762), *A. xylosoxidans* (AJ880763), *Achromobacter* sp. (AJ880396), *B. thuringiensis* (AJ868359), *B. licheniformis* (AJ880758), *B. subtilis* (AJ880761) frommiddle zone and *Staphylococcus epidermidis* (AJ880759), *P. migulae* (AJ887999), *Alcaligens faecalis* (AJ880765) *B. cereus* (AJ853737) from lower zone of rhizosphere. All the fifteen isolates grew on effluent supplemented medium as a sole carbon source resulting in the reduction of the levels of distillery pollutants and their color by 75.5%. Concomitantly there is a reduction in biological oxygen demand (BOD), chemical oxygen demand (COD), phenol, sulfate, heavy metals. Practical application of this study is the decolorization of the dark distillery effluents.

### 3.3.2.3 Cynobacterial/Algal treatment

Microalgae treatment is of superior importance in today's era. It attracts scientist not only by treating waste but also by its products/by products which are in high demand. Microalgae treatment is commonly suggested after the anaerobic digestion of spent wash since the process is energy efficient and also micro- algae have the ability to take up its nutrients (majorly inorganic compounds) requirement from biomethanated spent wash and energy requirement from the sun. Additionally, it has the mechanism of taking carbon dioxide and converting them into oxygen as electron donor thereby reducing the energy

need of the aerobic treatment. Kalavathi et al. (2001) reported degradation and metabolization of the melanoidin in distillery effluent by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. The organisms degrade melanoidins due to production of $H_2O_2$, hydroxyl, perhydroxyl and active oxygen radicals, resulting in the 60 % decolourization of the effluent. They examined that enzyme such as glucose oxidase present in microalgae cleave the ns for maximum $H_2O_2$ production from microalgae namely glucose oxidase, manganese dependent peroxidase and at least two manganese independent peroxidase. $MnSo_4$, methylviologen, reduced glutathionine, riboflavin, ascorbic acid could be used for improving the degradation rate of melanoidins by microalgae. Cynobacteria growing in media containing both pigment and effluent have been demonstrated to possess higher glutamine synthetase activity. Another study conducted by Patel et al. (2001) examined the 96%, 81% and 26% decolorisation of distillery effluent through bioflocculation by *Oscillatoria* sp., *Lyngbya* sp. and *Synechocystis* sp., Respectively. Marine cynobacteria excretes colloidal substances like lipopolysaccharides, poly hydroxybutyrate, poly hydroxyalconate, protein etc. during stationary phase, which has either OO- of half ester sulfate ($OSO^{3-)}$ reactive group and get electrostatically complexed with active cationic site of organic matter and result in flocculation. In a other study, Valderrama et al. (2002) reported 52% colour removal from distillery effluent when using a combined treatment of with *Lemna minuscule* and *Chlorella vulgaris*. The microalgal treatment removed nutrients and organic matter from wastewater and produced oxygen for other organisms. The macrophyte removed organic matter and eliminated the microalgae form treated wastewater. However, despite the potential of aquatic macrophytes in cleaning wastewaters the use of these plants in designing a low cost treatment system is still at experimental stage and is considered to be a potentially important area of environmental management. Recently, Krishnamoorthy et al. (2017) have showed the treatment of anaerobically digested distillery wastewater with Oscillatoria sp.. This oraganisms reduced COD up to 55% of anaerobically digested distillery wastewater. Solovchenko et al. (2014) has investigated the possibilities of distillery effluent bioremediation along with a new *Chlorella sorokiniana* sp. cultivated in a semi-batch mode in a high-density photobioreactor. A decrease in COD of the PMDE from 20000 to ca. 1500 mg $L^{-1}$ was achieved over four days with a decline in nitrate (>95%), phosphate (77%) and sulfate (35%).

### 3.3 Recent approaches for degradation and decolurisation of PMDE

#### 3.3.1 Phytoremediation approach and constructed wetland

Phytoremediation with wetland plants is an eco-friendly, aesthetically pleasing, cost-effective, solar-driven, passive technique that is useful for cleaning up environmental pollutants with low to moderate levels of contamination (USEPA 2000). Phytoremediation of melanoidins and heavy metals containing distillery effluent is comparatively a new approach, and to date, only a limited number of plant species have been explored for the degradation of melanoidins; for instance, *Phragmites karka, P. australis, P. communis, Typha angustifolia, Cyperus esculentus* (Chandra and Yadav 2010, 2011; Chaturvedi et al.

2006). Recently, aquatic macrophyte *Potamogeton pectinatus* was used for bioaccumulate heavy metals (Fe, Cu, Zn and Mn) and efficiently cleanup the distillery effluent (Singh et al. 2005). For the removal of industrial contamination there are some significant work has been done by Billore et al. 1999 for horizontal subsurface flow gravel bed constructed wetland with *P. karka* in Central India. In another study, Billore et al. (2001) reported on the use of an horizontal flow constructed wetland to treat the secondary treated distillery effluent from a private distillery, Associated Alcohols and Breweries, Ltd. at Khodigram village in the outskirts of Baraha town in Central India. The $BOD_5$ and COD concentrations in the distillery effluent even after the conventional secondary treatment amounted to 2540 and 13,866 mg $L^{-1}$, respectively and, therefore additional treatment was necessary. The system achieved COD, $BOD_5$, TKN and TP reductions of 64%, 84%, 59% and 79%. The study indicated that constructed wetlands may be a suitable tertiary treatment option. In another study, Trivedy and Nakate (2000) employed *T. latipholia* for distillery effluent treatment in a constructed wetland. The system resulted in 78% and 47% reduction in COD and BOD respectively in a period of 10 days. Increasing concentration of the effluent greatly reduced the biomass of the plant with maximum accumulation of Fe being recorded in plants growing in 100% effluent. Recently, Chaturvedi et al. (2006) reported the phytoremediation potential of *P. australis* grown on distillery effluent contaminated site. She also characterised the diverse bacterial species from rhizospheric zone of *P. australis.* The culturable bacterial were beneficial for the degradation of toxic constituents present in the distillery waste. They observed that 75.5% reduction of color by the same bacteria along with concomitant reduction in COD, BOD, phenol, sulphate and heavy metals values. Hatano et al. (2016) examine the chelating property of melanoidin like product (MLP) and to evaluate the facilitatory influence on the phytoextraction efficiency of Japanese radish *Raphanus sativus* var. *longipinnatus*. They showed that MLP binds to all the metal ions examined and the binding capacity of MLP toward Cu2+ seems to be the highest among them. The metal detoxification by MLP followed the order of $Pb^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+} > Fe^{2+} > Cd^{2+} > Co^{2+}$. Furthermore, in the phytoextraction experiment using copper sulfate, the application of MLP accelerated the detoxification of copper and the bioavailability in radish sprouts. In another study, Hatano and Yamatsu (2018) evaluated the facilitatory effect of MLP on phytoextraction in a medium including cadmium or lead, the concentrations of which were adjusted around the regulation values of the Soil Contamination Countermeasures Act in Japan. Three Brassica species were tested based on their fast growth, high biomass productivity, and heavy metal absorption. Both biomass and lead uptake in the nutrient medium with 1 mM lead nitrate were significantly increased by the addition of MLP, and almost all of the lead was accumulated in the root tissue. Therefore, MLP were able both to detoxify lead ions and to improve their bioavailability in Brassica species.

### 3.3.2 PMDE treatment and decolourisation by phase separation and hybrid technology

Two-stage processes and hybrid reactors a two stage process with an anaerobic filter followed by a UASB reactor was investigated by Blonskaja et al. (2003). The acidogenic and methanogenic phases were clearly separated ensuring better conditions for the methanogens. COD reduction was 54% and 93% in the first and second stage, respectively. In another study on a two-phase thermophilic system, 65% COD reduction combined with a three-fold increase in biogas yield over a single phase system was observed (Yeoh 1997). Boopathy and Tilche (1991) studied the anaerobic digestion of 3–12 times diluted beet molasses wastewater, without pH adjustment, in a hybrid anaerobic baffled reactor (HABR). Additional nitrogen and phosphorus were provided in the form of urea (0.007 g/g of COD) and diammonium hydrogen phosphate (0.0006 g/g of COD). The reactor consisted of three chambers and a final settler. 77% COD removal at a loading rate of 20 kg COD/$m^3$ d was obtained. Several variations of the UASB reactor have been investigated for distillery wastewater treatment. In large scale operations, highly variable process wastewater flows makes it difficult to maintain suitable inlet UASB flow rate; further, prevention of the loss of low density granules is also important. To overcome these problems, Akunna and Clark (2000) used granular-bed anaerobic baffled reactor (GRABBR). The reactor consisted of 10 equal compartments, each of which was further divided into two with suitable baffles. Acidogenesis was found to be predominant in the compartments near the inlet and methanogenesis in those located near the outlet. 82–90% COD reduction was observed at a HRT of 4 d. Yet another modification is upflow blanket filter (UBF) in which the packing is limited to 5–10% height of the reactor. This configuration resulted in 70% COD removal in sugarcane molasses distillery spent wash (Bardiya et al. 1995). Vlissidis and Zouboulis (1993) have investigated the thermophilic anaerobic treatment of wastewater from the processing of beet molasses. The process consisted of two stages: anaerobic digestion in up flow sludge bed reactor followed by coagulation-flocculation with lime. The HRT was 11 d in the bioreactor and 2.5 h in the flocculator-precipitator tank. On an average, the overall treatment scheme resulted in 86% BOD and 71% COD removal. Biogas produced in the anaerobic reactor had a methane content of 76%. This configuration was reported to be efficient in treating undiluted wastewaters.

### 3.3.3 Microbial enzymes in degradation and decolourisation of PMDE

Enzymatic degradation of synthetic melanoidins and anaerobically digested spent wash is facilitated by non-specific ligninolytic enzymes like MnP, laccase and LiP. Although, the enzymatic system related with decolourisation of melanoidins is yet to be completely understood, it seems greatly connected with fungal ligninolytic mechanisms. One of the enzymatic studies regarding melanoidins decolourisation was reported by Ohmomo et al. (1985). They used *C. versicolour* Ps4a, which decolourized molasses wastewater 80% in darkness under optimum conditions. Decolourization activity involved two types of intracellular enzymes, sugar-dependent and sugar-independent. One of these enzymes

required no sugar and oxygen for appearance of the activity and could decolourize MWW up to 20% in darkness and 11–17% of synthetic melanoidins. Thus, the participation of these $H_2O_2$ producing enzymes as a part of the complex enzymatic system for melanoidins degradation by fungi should be taken into account while designing any treatment strategy.

Colour removal of synthetic melanoidins by *C. hirsutus* involved the participation of MIP and MnP and the extracellular $H_2O_2$ produced by glucose-oxidase, without disregard of a partial participation of fungal laccase Miyata et al. (1998). The authors used *C. hirsutus* pellets to decolorize a melanoidins containing medium. It was elucidated that extracellular $H_2O_2$ and two extracellular peroxidase, MIP and MnP were involved in decolourisation activity. Mansur et al. (1997) obtained a maximum decolourization of around 60% on day 8 after inoculating with fungus *Trametes* sp. I-62. Here effluent was added at a final concentration of 20% (v/v) after 5 days of fungal growth, the time at which high levels of laccase activity were detected in the extracellular mycelium. A melanoidin mineralizing 47 kDa extracellular protein corresponding to the major mineralizing enzyme system from *T. versicolour* was isolated by Dehorter and Blondeau (1993). This $Mn^{2+}$ dependent enzyme system required oxygen and was described to be as peroxidase. D'souza et al. (2006) reported 100% decolourization of 10% spent wash by a marine fungal isolate whose laccase production increased several folds in the presence of phenolic and non-phenolic inducers. Boer et al. (2006) had isolated and purified the melanoidin decolourising enzymes (MnP) from *L. edodes*. Two peaks of MnP as MnP1 and MnP2 were obtained by gel filtration. They revealed that the purified enzyme yielded a single band after denaturing SDS-PAGE. A molecular mass of 46 kDa was estimated after SDSPAGE, and this molecular mass was confirmed by Sephadex G-100 gel filtration. Gonzalez et al. (2007) report the induction of laccases by molasses wastewaters and molasses melanoidins in the *Trametes* sp. I-62. The time course of effluent decolorization and laccase activity in the culture supernatant of the fungus were correlated. The expression of laccase genes lcc1 and lcc2 increased as a result of the addition of complete molasses wastewater and its high molecular weight fraction to fungal cultures. Tapia-Tussell et al. (2015) also reported the expression of laccase genes in the *T. hirsuta* strain Bm-2, in the presence of phenolic compounds, as well as its effectiveness in removing colorants from vinasse. In the presence of all phenolic compounds, increased levels of laccase-encoding mRNA were observed. Transcript levels in the presence of guaiacol were 40 times higher than those in the control. The lcc1 and lcc2 genes of *T. hirsuta* were differentially expressed; guaiacol and vanillin induced the expression of both genes, whereas ferulic acid only induced the expression of lcc2. The discoloration of vinasse was concomitant with the increase in laccase activity. The highest value of enzyme activity (2543.7 U $mL^{-1}$) was obtained in 10% (v/v) vinasse, which corresponded to a 69.2% increase in discoloration. Pant and Adholeya (2007a) isolated two fungal strains from the soil of a distillery effluent contaminated site producing ligninolytic enzymes and having the potential to decolourize distillery effluent. These two isolates along with one isolate of *Pleurotus florida* EM 1303 were assessed for their ligninolytic enzyme activity in culture filtrate as well as after solid state fermentation on two substrates wheat straw and corncob

powder. Both *P. pinophilum* TERI DB1 and *A. gaisen* TERI DB6 were found to produce laccase, MnP and LiP. The immobilized fungal biomass was then used for decolourization of the biomethanated distillery wastewater and observed the reduction in colour up to the magnitude of 86, 50, and 47% with *P. florida, P. pinophilum*, and *A. gaisen* respectively. Singh et al. (2015) reported the degradation of glucose-glycine Maillard products using immobilised laccase lead to 47% decolourisation in 6 h at pH 4.5 and 28 °C. A combined treatment technique consisting of enzymatic hydrolysis, followed by aerobic biological oxidation was investigated for the treatment of alcohol distillery spent wash. Sangave and Pandit (2006a) used cellulase enzyme for the pre-treatment step with an intention of transforming the complex and large pollutant molecules into smaller molecules. It was suggested that enzymatic pre-treatment of the distillery effluent leads to in situ formation of the hydrolysis products, which have different physical properties and are easier to assimilate than the parent pollutant molecules by the microorganisms, leading to faster initial rates of aerobic oxidation even at lower biomass levels. Sangave and Pandit (2006b) used irradiation and ultrasound combined with the use of an enzyme as pretreatment technique for treatment of distillery wastewater. The combination of the ultrasound and enzyme yielded the best COD removal efficiencies as compared to the processes when they were used as stand-alone treatment techniques. The white-rot basidiomycete *T. versicolour* is an active degrader of humic acids as well as of melanoidins. The MnP and laccase activity in the culture supernatant decreases with the increase in the time duration and further bacterial incubation showed gradual decrease of both enzyme activities. The enzyme activity has direct co-relation with the melanoidin decolorization. The initiation of MnP activity in culture supernatant starts at 48 h of bacterial growth and remains active up to 192 h, whereas, the laccase induction starts at 96 h and its activity decreased after 192 h. This resulted in the initial role of MnP in melanoidins degradation (Yadav and Chandra 2012; Bharagava et al. 2009). The melanoidin decolorization has also been reported by MnP activity in fungus (Pant and Adholeya 2007a; Raghukumar et al. 2004). Recently, Mahgoub et al. (2016) reported the biodegradation and decolorization of melanoidin solutions by manganese peroxidase yeasts. He isolated yeast cultures showed a noticeable organic matter reduction and decolorization capacity reaching up to 70% within 2–5 days. However, the corresponding yeast cultures grown in glucose peptone yeast extract medium using real melanoidin wastewater at 30 W C showed lower organic matter and color removal capacity, reaching about 60% within 2–5 days.

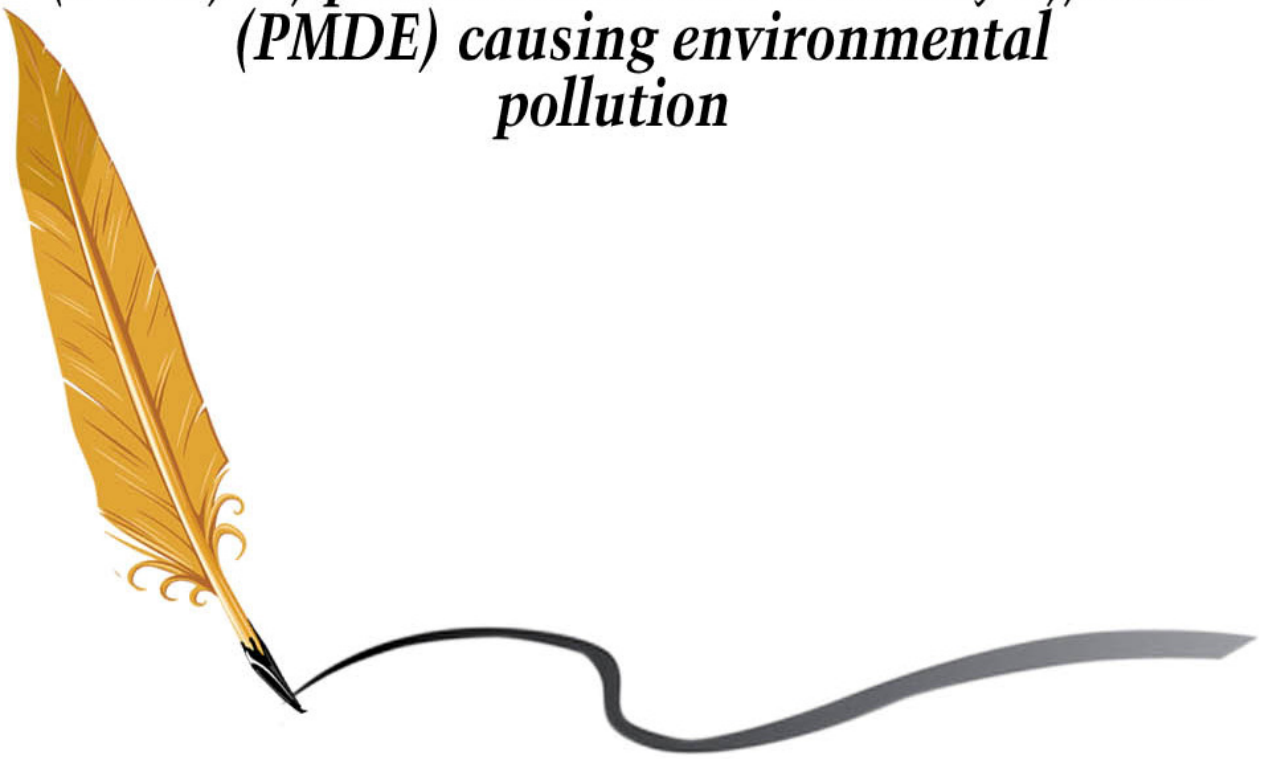
### 3.4 Recent approaches for bioremediation of PMDE

Biological methods provide an eco-friendly approach for wastewater treatment, but these methods also have some technical difficulties as far as in situ administration of pollutant is concerned. The complex industrial wastewater containing different types of pollutants do not easily decolorized by single step treatment process. Therefore, treatment of high-strength complex wastewater by a novel two-step treatment/phase separation method using bacteria and constructed wetland plant treatment system might be a novel and more promising approach for bioremediation of industrial wastewater. Two step treatment found to be more efficient than individual bacteria and plant because integration of bacterial

treated effluent (biodegradative bacteria) with constructed wetland treatment system is commonly effective at facilitating the breakdown of contaminants of wastewater and increase the bioavailability of contaminants to plant. Modern approaches like use of bacteria, fungi, yeast and their combinatorial systems have proven to be effective for decolorization of distillery effluent. Combination of wetland treatment technology after bacterial degradation offers an excellent system for removal of color from industrial wastewater and further reduction of BOD, COD, TDS and heavy metals for safe disposal. For instance, Ghosh et al. (2002) investigate the treatment of anerobically digested distillery effluent by a two stage bioreactor by using *P. putida* U followed by *Aeromoans* sp. strain EMa in a two stage bioreactor. In the first stage, *P. putida* reduced the COD and colour of distillery effluent up to 44.4 and 60%, respectively, while in the second stage, *Aeromonas* sp. strain Ema, reduced the effluent COD up to about 44.4%. Kumar and Chandra (2004) successfully treated distillery effluent in two stage treatment process involving transformation of recalcitrant coloring components of the effluent by aerobic bacterium *B. thuringiensis* followed by subsequent reduction of remaining load of pollutants by a macrophyte *Spirodela polyrrhiza* Schliden. A similar biphasic treatment of the effluent was carried out in a constructed wetland with *B. thuringiensis* and *T. angustata* L. by Chandra et al. (2008c) which resulted in 98-99% BOD, COD and colour reduction after 7 days. The results suggested that the bacterial pre-treatment of PMDE, integrated with phytoremediation will improve the treatment process of PMDE and promote safer disposal of this waste. Kaushik et al. (2010) investigated treatment of distillery spent wash in three stage bioreactor by using fungus followed by bacteria. Treatment was first carried out by *Emiricella nidulans* var lata. *Neurospora intermedia* followed by *Bacillus* sp.. The treated spent wash showed significant reduction in color (82%) and COD (93%) after 30 h. Chandra et al. (2012) reported 94.5 % and 96.0% reduction in BOD and COD values in two stage sequential treatment of PMDE by bacteria and *Phragmites communis*. He also characterised *P. communis* rhizosphere bacterial communities and metabolic products during the two stage sequential treatment of PMDE by bacteria and wetlands plant. A two-stage sequential treatment for anaerobically digested wastewater from a cane molasses-based distillery was reported by Pant and Adholeya (2009a). In the first step, the effluent was treated in a hydroponic based system using two plant species (*Vetiveria zizanioides* and *Phragmites karka*) to reduce the high nitrogen content of the effluent. Roots of these plants showed profuse growth on effluent. Nitrogen removal to the tune of 84% was achieved. When this hydroponically treated effluent was subjected for treatment by fungal isolates, 86.33% decolorization was obtained with *Pleurotus florida* Eger EM1303 followed by *Aspergillus flavus* TERIDB9 (74.67%), with significant reduction in COD as well. The suggested method is thus important from the point of treating distillery wastewaters without the need of high dilutions and addition of supplementary carbon sources. The efficacy of two step treatment approach has been demonstrated under pilot scale. For organic in-contaminants present in wastewater, this approach found to be effective in the field scale and it is likely during the next five to ten years its use will become widespread.

# Chapter-04

# *Investigation of persistent organic pollutants (POPs) of post methanated distillery effluent (PMDE) causing environmental pollution*



# Chapter-4

## Investigation of persistent organic pollutants (POPs) of post methanated distillery effluent (PMDE) causing environmental pollution

### 4.1 Introduction

Distillery is one of the most highly polluting and growth-oriented industries in India with reference to the extent of water and soil pollution and the quantity of wastewater generated. Sugarcane molasses-based distillery spent wash (DSW) is characterized by a high BOD, COD, TDS, and the presence of heavy metals, sulfur and nitrogen containing complex organic pollutants with low pH (Chandra et al. 2004; Saliha 2005). Because of the presence of unknown recalcitrant organic compounds in this waste, it is difficult to identify methods for its safe disposal and management. In organic-rich environments, heavy metal toxicity is aggravated by its high tendency to bind with organic compounds and its solubility under acidic conditions (Rosselli et al. 2003). Therefore, an adequate treatment is required before the effluent discharged into the environment. Among the different process available for treatment of DSW, biomethanation is a popular anaerobic convention process which produces methane to meet a part of the power requirement in distilleries. After conventional anaerobic treatment the DSW become biologically inert because accumulation of non-biodegradable/recalcitrant compounds particularly melanoidins, which contribute to latent BOD and TDS (Fitzgibbon et al. 1995). However, the detailed chemical properties of various pollutants present in PMDE before and after anaerobic treatment have yet to be elucidated. The toxicity of these unknown compounds present in PMDE is suspected to occur via complexation of several organic pollutants with various heavy metals. The nature of complex pollutants of distillery waste contaminated sites is still unknown. Therefore, the present study was conducted to characterize the chemical nature of unknown pollutants present in DSW and PMDE collected from a sugarcane molasses-based distillery plant. To accomplish this, we employed several organic solvents to extract the broad range organic pollutants under acidic conditions using the liquid–liquid extraction method. Subsequently, extracted pollutants were identified by GC-MS. The toxicity assessment of DSW and PMDE was also evaluated by using seed germination test of *Phaseolus mungo* L and *Triticum aestivum***.**

### 4.2 Material and Methods

#### 4.2.1 Sample collection

DSW and PMDE was collected aseptically in presterilized plastic containers (capacity 20 L; Tarson Production Pvt. Ltd., USA) from the DSW and PMDE collection tank of M/s Unnao Distillers, Unnao, located in Uttar Pradesh, India (26°320″N,80°30′0″E) (Fig. 4.1). The distillery plant has an annual installed capacity of $9\times10^6$ L for alcohol production and generates approximately $12.6\times10^7$ L of effluent annually.


**Fig. 4.1** View of distillery effluent and their impact in environmental (a) spent wash generated after alcohol production (b) post methanated distillery effluent discharged after anaerobic treatment (c,d) soil pollution due to PMDE

### 4.2.2 Physico-chemical analysis of DSW and PMDE

The DSW and PMDE was processed within 24 h for analysis of different physicochemical parameters including color, odor, pH, BOD, COD, TS, TDS, phenol, sulfate, phosphate, and heavy metals based on the standard methods for examination of water and wastewater (APHA 2012).

#### 4.2.2.1 Color (Visual Comparison Method)

Color in water may be resulted due to the presence of natural metallic ions (Fe and Mn), humus and peat materials, plankton, weeds and industrial wastes. Color is removed to make water suitable for general and industrial application. Colored industrial waste water may require color removal before discharge into water courses. In some highly colored industrial wastewaters like distillery effluent, color is principally contributed by melanoidins.

**(a) Principle**

Color was determined by the visual comparison method of the sample with known concentration of colored samples. Comparison may also be made with special, properly calibrated, colored glass disks. The platinum-cobalt method of color measurement is the

standard method, the unit of color being that produced by 1.0 mg platinum/l in the form of chloroplatinate ion. The ratio of cobalt to platinum may be varied to match the hue in special cases.

**(b) Apparatus**

a. Nessler tubes: Matched, 50 mL, tall form.
b. pH meter: for determining sample pH

**(c) Reagents**

a. Potassium chloroplatinate ($K_2PtCl_6$)
b. Cobaltous chloride ($CoCl_6.6H_2O$)
c. Hydrochloric acid (HCl)

**(d) Procedure**

a. Preparation of standards: Dissolved 1.246 g potassium chloroplatinate, $K_2PtCl_6$ (equivalent to 500 mg metallic Pt) and 1.0 g crystallized cobaltous chloride, $CoCl_2.6H_2O$ (equivalent to about 250 mg metallic Co) in distilled water containing 100 ml of concentrated HCl and diluted to 1000 ml with distilled water. This stock standard has a colour of 500 units. Then, prepared standards having colour of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, and 70 by diluting 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, and 7.0 mL stock standard with distilled water to 50 ml in Nessler tubes and protected these standards against evaporation and contamination when not in use.

**(e) Calculation**

**Calculated the color units by the following equation**

$$\text{Colour units} = \frac{A \times 50}{B}$$

Where

A = Estimation colour of a diluted sample
B = mL sample taken for dilution.

**4.2.2.2 Biological oxygen demand (5-Day BOD Test)**

**(a) Principle**

The method consisted of filling with samples to overflowing an airtight bottle of the specified size and incubating it at the specified size and incubating it at the specified temperature for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, and BOD is computed from the difference between initial and final D.O. Because the initial D.O. is determined immediately after the dilution is made, all oxygen uptake, including that occurred during the first 15 min is included in BOD measurement.

**(b) Apparatus**

a. Incubation bottles: 300 mL capacity.

b. Air incubator or water bath: Thermostatically controlled at 20±1 ºC exclude all light to prevent the possibility of photosynthetic production of DO.

**(c) Reagents**

a. Phosphate buffer solution: Dissolved 8.5 g $KH_2PO_4$, 21.75 g $K_2HPO_4$, 33.4 g $Na_2HPO_4.7H_2O$ and 1.7 g $NH_4Cl$ in about 500 mL distilled water and diluted to 1000 mL. The pH should be 7.2 without further adjustment.

b. Magnesium sulfate solution: Dissolved 22.5 g $MgSO_4.7H_2O$ in distilled water and diluted to 1000 mL.

c. Calcium chloride solution: Dissolved 27.5 g $CaCl_2$ in distilled water and diluted to 1000 ml.

d. Ferric chloride solution: Dissolved 0.25 g $FeCl_3.6H_2O$ in distilled water and diluted to 1000 mL.

e. Sodium sulfite solution: Dissolved 1.575 g $Na_2SO_3$ in 1000 mL distilled water.

f. Manganous sulphate solution: Dissolved 480 g $MnSO_4.4H_2O$ in 1000 mL distilled water.

g. Alkai-iodide azide reagent: Dissolved 500 g NaOH and 135 g NaI in 1000 mL distilled water.

h. Starch: Dissolved 2 g starch in 1000 mL distilled water.

i. Standard sodium thiosulphate: Dissolved 6.025 g $Na_2S_2O_3.5H_2O$ and 0.4 g NaOH in 1000 mL distilled water.

**(d) Procedure**

a. Preparation of dilution water: Placed 300 mL sample water in a suitable bottle and added 1ml each of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions/l of water, seeded dilution water 1mL $L^{-1}$. Temperature was maintained at 20±1 ºC.

b. Determination of initial D.O.: To the sample collected in a 300 mL bottle, added 1.0 mL $MnSO_4$ solution followed by addition of 1.0 mL alkaline-iodide-azide reagent, mixed by inverting the bottle a few times. Let the precipitate to settle sufficiently and added 1.0 mL concentrated $H_2SO_4$ and mixed again by inverting bottle several times until dissolution was completed, titrated with 0.025 M $Na_2S_2O_3$ solution to a pale straw colour. Added few drops of starch solution and continued titration till disappearance of blue colour.

c. Incubation: Incubated at 20±1 ºC BOD bottles containing 1000 × dilution, seed controls and dilution water blanks.

d. Determination of final D.O.: After 5 day incubation period determined dissolved oxygen in sample dilution as described above.

**(e) Calculation**

$$BOD_5 \text{ (mg } L^{-1}) = \frac{(D_1-D_2)- (B_1-B_2)\, f}{P}$$

Where,

$D_1$ = D.O. of diluted sample immediately after preparation in mg $L^{-1}$

$D_2$ = D.O. of diluted sample after 5-day incubation at 20 ºC in mg $L^{-1}$

P = Decimal volumetric fraction of sample used
$B_1$ = D.O. of seed control before incubation in mg $L^{-1}$
$B_2$ = DO of seed control after incubation in mg $L^{-1}$
f = Ratio of seed in diluted sample to seed in seed control

### 4.2.2.3 Chemical oxygen demand (Open Reflux Method) (COD)

**(a) Principle**

Majority of organic matter are oxidized by a boiling mixture of chromic and sulfuric acids. A sample is refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced ($K_2Cr_2O_7$) is titrated with ferrous ammonium sulfate to determine the amount of ($K_2Cr_2O_7$) consumed and the oxidizable organic matter is calculated in terms of oxygen equivalent.

**(b) Apparatus**

a. Reflux apparatus: Consisting of 250 mL Erlenmeyer flasks with ground-glass 24/40 neck and 300 mm jacket Liebig, west or equivalent condenser with 24/40 ground-glass joint.
b. Hot plate: Having sufficient power to produce at least 1.4 w/$cm^2$ of heating surface.

**(c) Reagents**

a. Standard potassium dichromate solution, 0.0417 M: Dissolved 12.259 g $K_2Cr_2O_7$, previously dried at 103 $^oC$ for 2 h, in distilled water and diluted to1000 mL.
b. Sulphuric acid reagent: Added 5.5 g $Ag_2SO_4$ in 1000 g sulphuric acid ($H_2SO_4$). Let stood for 1 to 2 days to dissolve $Ag_2SO_4$.
c. Ferroin indicator solution: Dissolved 1.484 g phenonthroline monohydrate and 695 mg $FeSO_4.7H_2O$ in distilled water and diluted to 100 mL.
d. Standard ferrous ammonium sulphate (FAS), titrant, 0.25N: Dissolved 98 g ferrous ammonium sulphate [$Fe(NH_4)_2(SO_4)_2.6H_2O$] in distilled water. Added 20 mL of concentrated sulphuric acid ($H_2SO_4$), and diluted to 100 mL. Standardized the solution against standard $K_2Cr_2O_7$ solution as follows: Diluted 10 mL standard $K_2Cr_2O_7$ up to 100 ml. Added 30 mL concentrated hydrogen sulfides and cooled and titrated with FAS titrant using 0.10 to 0.15 mL (2 to 3 drops) ferroin indicator.

$$\text{Molarity of FAS solution} = \frac{\text{Volume of 0.0417 M } K_2Cr_2O_7 \text{ solution titrated (mL)} \times 0.25}{\text{Volume of FAS used in titration (mL)}}$$

e. Mercuric sulphate: Powdered mercuric sulphate ($HgSO_4$).

**(d) Procedure**

Taken 1.0 mL of sample and diluted to 50 mL in a 250 mL refluxing flask, added 1.0 g $HgSO_4$, some glass beads and 5 mL sulphuric acid reagent, (added very slowly), with mixing to dissolve $HgSO_4$. Cooled while mixing to avoid the loss of volatile materials and added 25 mL of 0.0417 M $K_2Cr_2O_7$ solution and mixed. Attached the flask to a condenser and turned on cooling water. Remaining sulphuric acid reagent (70 mL) was added

through open end of condenser, continued swirling and mixing while adding sulphuric acid reagent. Open end of condenser was covered with a small beaker to prevent foreign material from entering refluxing mixture and refluxed for 2 h. Cooled and washed down condenser with distilled water. Disconnected reflux condenser and diluted mixture to about twice its volume with distilled water. Cooled to room temperature and titrated excess $K_2Cr_2O_7$ with FAS using 2-3 drops (0.10 to 0.15 mL) ferroin indicator. The first sharp change from blue-green to reddish brown was taken as end point of the titration. In same manner, refluxed and titrated a blank containing the reagents and a volume of distilled water equal to that of sample.

**(e) Calculation**

$$\text{COD (mg } O_2\text{ /l)} = \frac{(A-B) \times M \times 8000}{\text{mL of sample}}$$

Where,

A = mL FAS used for blank

B = mL FAS used for sample

M = Molarity of FAS

### 4.2.2.4 Total Nitrogen (Macro-Kjeldhal Method)

The Kjeldhal method determines nitrogen in the trinegative state. It fails to account nitrogen in the form of azide, azime, azo, hydrazone, nitrate, nitroso, oxime and semi-carbazone. This method is used for samples having $NH_3$-N concentration greater than 5 mg $L^{-1}$. Since, distillery effluent is waste water and contains large quantities of nitrogen, hence we employed this method. This method also gives least relative error as compared to other methods.

**(a) Principle**

In presence of sulphuric acid ($H_2SO_4$), potassium sulphate ($K_2SO_4$) and mercuric sulphate ($HgSO_4$) as catalyst, amino nitrogen of many organic materials are get converted into ammonium sulphate [$(NH_4)_2SO_4$]. Free ammonia and ammonium-nitrogen also are converted to $NH_4)_2SO_4$. During sample digestion, a mercury ammonium complex is formed and then decomposed by sodium thiosulphate ($Na_2S_2O_3$). After decomposition, ammonia is distilled from an alkaline medium and absorbed in boric or sulphuric acid. The ammonia is determined colorimetrically or titration with a standard mineral acid.

**(b) Apparatus**

a. Digestion apparatus: Kjeldhal flask with a total capacity of 800 ml, a heating device that can provide the temperature range of 365 to 370 ºC for effective digestion.

b. Distillation apparatus: A borosilicate glass of 800 to 2000 ml capacity attached to a vertical condenser so that the outlet tip may be submerged below the surface of the receiving acid solution.

**(c) Reagents**

Ammonia free water was used in making all of reagents.

a. Digestion reagent: Dissolved 134 g $K_2SO_4$ in 650 mL water containing 200 ml of concentrated $H_2SO_4$. Added with stirring 25 mL $HgSO_4$ solution and diluted the combined solution to 1000 ml with distilled water. Temperature was kept close to 20 $^oC$ to prevent crystallization.

b. Sodium hydroxide-sodium thiosulphate reagent: Dissolved 500 g NaOH and 25 g $Na_2S_2O_3.5H_2O$ in distilled water and diluted to 1000 mL.

c. Mixed indicator solution: Dissolved 200 mg methyl red indicator in 100 ml 95% ethyl alcohol. Combined the solution and mixed well.

d. Boric acid solution: Dissolved 20 g $H_3BO_4$ in distilled water, added 10 ml mixed indicator solution and diluted to 1000 mL.

e. Standard sulfuric acid titrant, 0.02 N: Diluted 200 ml 0.1 N standard acid to 1000 ml with distilled water and standardized by potentiometric titration of 15 mL 0.05 N $Na_2CO_3$.

**(d) Procedure**

A measured volume of sample (25 mL of distillery effluent) was taken in 800 mL Kjeldhal flask to which 50 mL digestion mixture was added carefully. After mixing, the contents were heated gently under a hood to remove acid fumes. Then, it was boiled briskly until the volume was greatly reduced (to about 25 mL) and copious white fumes emanated. The digestion was continued for an additional 30 min or up to charred ash. After digestion, flask and contents were allowed to cool and diluted up to 300 mL with distilled water and mixed well. Tilted the flask and added carefully 35 mL sodium hydroxide-thiosulphate reagent to form an alkaline layer at the flask bottom. Connected flask to steamed-out distillation apparatus and shaken the flasks to insure complete mixing. A black precipitate, HgS was formed. The contents were then distilled and 300 mL distillate was collected in other flask containing 50 mL boric acid (absorbent) solution. This purple coloured indicator solution turns green, indicating the presence of nitrogen. Now titrated the solution against 0.02 N $H_2SO_4$ as titrant. The end point was indicated by the reappearance of purple color. A blank was run simultaneously throughout the process.

**(e) Calculation**

$$N_{org}\ (mg\ L^{-1}) = \frac{(A-B) \times 280}{mL\ of\ sample}$$

Where,

A = Volume of $H_2SO_4$ titrated for sample (mL)

B = Volume of $H_2SO_4$ titrated for blank (mL)

**4.2.2.5 Total dissolved solids (dried at 180 °C)**

**(a) Principle**

A well mixed sample is filtered through a standard glass fiber filter, and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180 °C. The

increase in dish weight represents the total dissolved solids. This procedure may be used for drying at other temperatures.

**(b) Apparatus**

a. Evaporating dishes: Dishes of 100 mL capacity made of one of the following materials:
1) Porcelain, 90 mm diam.
2) Platinum-Generally satisfactory for all purposes.
3) High-silica glass.
b. Muffle furnance for operation at 550 °C.
c. Steam bath
d. Desiccator, provided with a desiccant containing a color indicator of moisture concentration or an instrument indicator.
e. Drying oven, for operating at 103 to 105 °C.
f. Analytical balance, capable of weighing to 0.1 mg.
g. Magnetic stirrer with TFE stirring bar.
h. Wide-bore pipets
i. Graduated cylinder
j. Low form beaker
k. Glass-fiber filter disks without organic binder
l. Filtration apparatus with reservoir and coarse (40 to 60 µm) fritted disk as filter support. Suction flask of sufficient capacity for sample size selected.
4) Drying oven, for operation at 180±2 °C.

**(c) Procedure**

Heated clean dish to 103 to 105 ºC for 1 h. Cooled in desiccator and weighed immediately. Now 50 ml of well mixed sample was taken in a pre-weighed dish and evaporated to dryness in a drying oven at approximately 2 ºC below boiling to prevent splattering. Dried evaporated sample for at least 1 h in an oven at 103 to 105 ºC, cooled dish in desiccators to balance temperature and weighed. Repeated cycles of drying, cooling, desiccating and weighing till a constant weight was obtained, or until weight change was less than 4% of previous weight or 0.5 mg.

**(e) Calculation**

$$\text{mg total dissolved solids/L} = \frac{(A-B) \times 1000}{\text{sample volume, mL}}$$

Where,

A= weight of dried residue + dish, mg, and

B= weight of dish, mg

**4.2.2.6 Total solids (Dried at 103-105 °C)**

**(a) Principle**

A well mixed sample is evaporated in a weighed dish and dried to constant weight in an oven at 103 to 105 °C. The increase in weight over that of the empty dish represents the total solids.

**(b) Apparatus**

a. Evaporating dishes: Dishes of 100 mL capacity made of one of the following materials:
1) Porcelain, 90 mm diam
2) Platinum-Generally satisfactory for all purposes.
3) High-silica glass.
b. Muffle furnace for operation at 550 °C.
c. Steam bath.
d. Desiccator, provided with a desiccant containing a color indicator of moisture concentration or an instrumental indicator.
e. Drying oven, for operating at 103 to 105 °C.
f. Analytical balance, capable of weighing to 0.1 mg.
g. Magnetic stirrer with TFE stirring bar.
h. Wide-bore pipets.
i. Graduated cylinder
j. Low-form beaker

**(c) Procedure**

Heated clean dish to 103 to 105 ºC for 1 h. Cooled in desiccator and weighed immediately. Now 50 ml of well mixed sample was taken in a pre-weighed dish and evaporated to dryness in a drying oven at approximately 2 ºC below boiling to prevent splattering. Dried evaporated sample for at least 1 h in an oven at 103 to 105 ºC, cooled dish in desiccator to balance temperature and weighed. Repeated cycles of drying, cooling, desiccating and weighing till a constant weight was obtained, or until weight change was less than 4% of previous weight or 0.5 mg.

**(e) Calculation**

$$\text{mg total solids/L} = \frac{(A-B) \times 1000}{\text{Sample volume, mL}}$$

Where,

A= weight of dried residue + dish, mg, and

B= weight of dish, mg

**4.2.2.7 Estimation of phenolics**

**(a) Principle**

Phenols, defined as hydroxy derivatives of benzene and its condensed nuclei may occur in domestic and industrial waste waters. Steam-distillable phenols react with 4-amino antipyrine at pH 7.9±0.1 in presence of potassium ferricyanide to form a coloured antipyrine dye. This dye is extracted from aqueous solution with $CHCl_3$ and the

absorbance is measured at 460 nm. This method covers the phenol concentration ranging from 1.0 µg $L^{-1}$ to over 250 µg $L^{-1}$ with a sensitivity of 1 µg $L^{-1}$.

**(b) Apparatus**

a. Photometric equipment: A spectrophotometer.
b. Filter funnels: Buchner type with fritted disk.
c. Filter paper: alternatively, an appropriate 11 cm filter paper for filtering chloroform ($CHCl_3$) extracts instead of the buchner-type funnels and anhydrous $Na_2SO_4$ can be used.
d. pH meter.
e. Separatory funnels: 1000 mL Squibb form, without ground-glass stoppers and TFE stopcocks.

**(c) Reagents**

Prepared all the reagents in distilled water, it should be free of phenols and chlorines.
a. Stock phenol solution: Dissolved 100 mg phenol in freshly boiled and cooled distilled water and diluted to 100 mL.
b. Intermediate phenol solution: Diluted stock phenol solution (1 mL) in freshly boiled and cooled distilled water to 100 ml; [1 mL = 10 µg phenol, Prepared daily].
c. Standard phenol solution: Diluted 50 ml intermediate phenol solution to 500 ml with freshly boiled and cooled distilled water. [1 mL = 1 µg phenol, Prepared with in 2 h of use].
d. Bromate-bromide solution: Dissolved 2.784 g anhydrous potassium bromo-oxide ($KBrO_3$) in distilled water. Then, added 10 g potassium bromide (KBr) crystals, dissolved and diluted to 1000 mL.
e. Hydrochloric acid: Concentrated hydrochloric acid (HCl)
f. Ammonium hydroxide ($NH_4OH$), 0.5 N: Diluted 35 mL fresh concentrated $NH_4OH$ to 1000 ml with distilled water.
g. Standard sodium thiosulphate titrant, 0.025 M: Dissolved 6.025 g $Na_2S_2O_3.5H_2O$ and 0.4 g NaOH in 1000 mL distilled water.
h. Starch solution: Dissolved 2.0 g laboratory-grade soluble starch and 0.2 g salicylic acid, as a preservative, in 1000 ml hot distilled water.
i. Phosphate buffer solution: Dissolved 104.5 g $K_2HPO_4$ and 72.3 g $KH_2PO_4$ in water and dilute to 1000 mL distilled water (pH=6.8).
j. 4-aminoantipyrine solution: Dissolved 2.0.g 4-aminoantipyrine in water and diluted to 100 mL. Prepared daily.
k. Potassium ferricyanide solution: Dissolved 8.0 g $K_3Fe(CN)_6$ in water and diluted to 100 ml. Filtered, if necessary, it was stored in brown glass bottle or prepared freshly.
l. Chloroform: Laboratory grade chloroform ($CHCl_3$).
m. Sodium sulphate: Anhydrous sodium sulphate ($Na_2SO_4$).
n. Potassium iodide: Laboratory grade potassium iodide (KI) crystals.

**(d) Procedure**

A measured volume (25 mL) of sample was taken in a distillation flask and its pH was adjusted to 2-3 using 1N hydrochloric acid and removed oil and grease from the sample by transferring it in a separatory funnel and extracted oil and grease with 25 mL of chloroform. Repeated this process twice to ensure complete removal of oil and grease, then added four drops of orthophosphoric acid and three drops methyl orange indicator to the sample. Distilled the solution and placed 500 mL distillate in a 1000 mL flask. Prepared a 500 ml blank and a series of 500 mL phenol standards 5, 10, 20, 30, 40, and 50 µg phenol. The treated samples, blank and standards as follows: added 12.0 mL 0.5 N $NH_4OH$ and immediately adjusted pH to 7.9±0.1 with phosphate buffer (10 mL) and transferred it to a 1000 mL separating funnel. Added 3 mL of amino antipyrine solution, mixed well followed by addition of 3 ml $K_3Fe(CN)_6$ solution. Mixed the solution well and let colour to develop for 15 min. Extracted immediately with 50 ml of chloroform each time. Shaken the separating funnel many times (10 times), let $CHCl_3$ to settle, shaken again and let the $CHCl_3$ to settle again. Filtered each $CHCl_3$ extract through filter paper or fritted glass funnels containing a 5 g layer of anhydrous $Na_2SO_4$. The dried extract was collected in clean test tubes or cells for absorbance measurements. Read absorbance of samples and standard against the blank at 460 nm. Constructed calibration curve by plotting absorbance against the micrograms of phenol concentration and calculated the amount of phenol in samples using this curve.

**(e) Calculation**

$$\text{Phenol } (\mu g\ L^{-1}) = \frac{A \times 100}{B}$$

Where,

A = µg phenol in sample, from calibration curve

B = mL original sample

**4.2.2.8 Total organic carbon (High temperature combustion method)**

**(a) Principle**

The sample is homogenized and diluted as necessary and microportion is injected into a heated reaction chamber packed with an oxidative catalyst such as cobalt oxide, platinum group metals, or barium chromate. The water is vaporized and the organic carbon is oxidized to $CO_2$ and $H_2O$. The $CO_2$ from oxidation or organic and inorganic carbon is transported in the carrier gas streams and is measured by means of a nondispersive infrared analyzer, or titrated coulometrically. Because total organic carbon is measured, inorganic carbon must be removed by acidification and sparging or measured separately and TOC obtained by difference.

**(b) Apparatus**

a. Total organic carbon analyser

b. Sampling, injection, and sample preparation accessories, as prescribed by instrument manufacture.

c. Sample blender or homogenizer.
d. Magnetic stirrer and TFE-coated stirring bars.
e. Filtering apparatus and 0.45 µm filter.
(c) Reagents
a. Reagent water: Prepare reagents, blanks, and standard solution from reagent water with TOC value less than 2× the MDL.
b. Acid: Phosphoric acid, $H_3PO_4$
c. Organic carbon stock solution: Dissolve 2.1254 g anhydrous primary standard grade potassium biphthalate, $C_8H_5KO_4$, in carbon free water and dilute to 1000 mL; 1.0 mL=1.00 mg carbon. Prepare laboratory control standards using any other appropriate organic-carbon containing compound adequate purity, stability, and water solubility. Preserve by acidifying with $H_3PO_4$ or $H_2SO_4$ to pH≤2, and store at 4 $^oC$.
d. Inorganic carbon stock solution: Dissolve 4.4122 g anhydrous sodium carbonate in water, add 3.497 g anhydrous sodium bicarbonate and dilute to 1000 mL; 1.00 mL=1.00 mg carbon.
Carrier gas: Purified oxygen or air, $CO_2$ free and containing less than 1 ppm hydrocarbons (as methane)
f. Purging gas: Any gas free of $CO_2$ and hydrocarbons.

**(d) Procedure**
a. Instrument operation: Follow manufacturer's instructions for analyzer assembly, testing, calibration, and operation. Adjust to optimum combustion temperature before using instrument; monitor temperature to insure stability.
b. Sample treatment: If a sample contains gross solids or insoluble matter, homogenize until satisfactory replication is obtained. Analyze a homogenizing blank consisting of reagent water carried through the homogenizing treatment.
If inorganic carbon must be removed before analysis, transfer a representative portion (10 to 15 mL) to a 30-mL beaker, add acid to reduce pH to 2 or less, and purge with gas for 10 min. Inorganic carbon also may be removed by stirring the acidified sample in a beaker while directing a stream of purified gas into the beaker. Because volatile organic carbon will be lost during purging of the acidified solution, report organic carbon as total nonpurgeable organic carbon. Check efficiency of inorganic carbon removal for each sample matrix by splitting a sample into two portions and adding to one portion an inorganic carbon level similar to that of the sample. The TOC values should agree; if they do not, adjust sample container, sample volume, pH, purge gas flow rate, and purge time to obtain complete removal of inorganic carbon. If the available instrument provides for a separate determination of inorganic carbon (carbonate, bicarbonate, free $CO_2$) and total carbon, omit decarbonation and determine TOC by difference between TC and inorganic carbon. If dissolved organic carbon is to be determined, filter sample through 0.45 µm pore-diam filter; analyze a filtering blank.
c. Sample injection: Withdraw a portion of prepared sample using a syringe fitted with a blunt-tipped needle. Select sample volume according to manufacturer's direction. Stir samples containing particulates with a magnetic stirrer. Select needle size consistent with

sample particulate size. Repeat injection until consecutive measurements are obtained that are reproducible to within ±10%

d. Preparation of standard curve: Prepare standard organic and inorganic carbon series by diluting stock solutions to cover the expected range in samples within the linear range of the instrument. Dilute samples higher than the linear range of the instrument in reagent water. Inject and record peak height or area of these standards and a dilution water blank. Plot carbon concentration in mg $L^{-1}$ against corrected peak height or area on rectangular coordinate paper.

**(e) Calculation**

Calculate corrected instrument response of standards and samples by subtracting the reagent-water blank instrument response from that of the standard and sample. Prepare a standard curve of corrected instrument response vs. TOC concentration. Subtract procedural blank from each sample instrument response and compare to standard curve to determine carbon content. Apply appropriate dilution factor when necessary. Subtract inorganic carbon from total carbon when TOC is determined by difference.

### 4.2.2.9 Chloride ($Cl^-$) (Argentometric Method)

**(a) Principle**

In a neutral or slightly alkaline solution, potassium chromate can indicate the end point of the silver nitrate titration of chloride. Silver chloride is precipitated quantitatively before red silver chromate is formed.

**(b) Apparatus**

a. Erlenmeyer flask, 250 mL.

b. Buret, 50 mL.

**(c) Reagents**

a. Potassium chromate indicator solution: Dissolve 50 g $K_2CrO_4$ in a little distilled water. Add $AgNO_3$ solution until a definite red precipitate is formed. Let stand 12 h, filter, and dilute to 1 L with distilled water.

b. Standard silver nitrate titrant, 0.0141M (0.0141N): Dissolve 2.395 g $AgNO_3$ in distilled water and dilute to 1000 ml. Standardize against NaCl by the procedure described in 4b below; 1.00 mL = 500 µg $Cl^-$. Store in a brown bottle.

c. Standard sodium chloride, 0.0141M (0.0141N): Dissolve 824.0 mg NaCl (dried at 140°C) in distilled water and dilute to 1000 mL; 1.00 mL = 500 µg $Cl^-$.

d. Special reagents for removal of interference:

1) Aluminum hydroxide suspension: Dissolve 125 g aluminum potassium sulfate or aluminum ammonium sulfate, $AlK(SO_4)_2 \cdot 12H_2O$ or $AlNH_4(SO_4)_2.12H_2O$, in 1 L distilled water. Warm to 60 °C and add 55 mL conc. ammonium hydroxide ($NH_4OH$) slowly with stirring. Let stand about 1 h, transfer to a large bottle, and wash precipitate by successive additions, with thorough mixing and decanting with distilled water, until free from chloride. When freshly prepared, the suspension occupies a volume of approximately 1 L.

2) Phenolphthalein indicator solution.
3) Sodium hydroxide, NaOH, 1N.
4) Sulfuric acid, $H_2SO_4$, 1N.
5) Hydrogen peroxide, $H_2O_2$, 30%.

**(d) Procedure**

a. Sample preparation: Use a 100-mL sample or a suitable portion diluted to 100 mL. If the sample is highly colored, add 3 mL $Al(OH)_3$ suspension, mix, let settle, and filter. If sulfide, sulfite, or thiosulfate is present, add 1 mL $H_2O_2$ and stir for 1 min.
b. Titration: Directly titrate samples in the pH range 7 to 10. Adjust sample pH to 7 to 10 with $H_2SO_4$ or NaOH if it is not in this range. For adjustment, preferably use a pH meter with a non-chloride-type reference electrode. (If only a chloride-type electrode is available, determine amount of acid or alkali needed for adjustment and discard this sample portion. Treat a separate portion with required acid or alkali and continue analysis.) Add 1.0 mL $K_2CrO_4$ indicator solution. Titrate with standard $AgNO_3$ titrant to a pinkish yellow end point. Be consistent in end-point recognition. Standardize $AgNO_3$ titrant and establish reagent blank value by the titration method outlined above. A blank of 0.2 to 0.3 mL is usual.

**(e) Calculation**

$$\text{mg } Cl^-/L = \frac{(A-B) \times N \times 35450}{\text{sample volume, mL}}$$

Where,

A= mL titration for sample
B= mL titration for blank, and
N= normality of AgNO3
mg NaCl/L = (mg $Cl^-$/L) × 1.65

**4.2.2.10 Sulfate ($SO_4^{2-}$) (Gravimetric Method with Drying of Residue)**

**(a) Principle**

Sulfate is precipitated in a hydrochloric acid (HCl) solution as barium sulfate ($BaSO_4$) by the addition of barium chloride ($BaCl_2$). The precipitation is carried out near the boiling temperature, and after a period of digestion the precipitate is filtered, washed with water until free of $Cl^-$, ignited or dried, and weighed as $BaSO_4$.

**(b) Apparatus**

a. Steam bath.
b. Drying oven, equipped with thermostatic control.
c. Muffle furnace, with temperature indicator.
d. Desiccator.
e. Analytical balance, capable of weighing to 0.1 mg.
f. Filter: Use one of the following:

1) Fritted-glass filter, fine (‘‘F’’) porosity, with a maximum pore size of 5 µm.
2) Membrane filter, with a pore size of about 0.45 µm.
b. Vacuum oven.

**(c) Reagents**

a. Methyl red indicator solution: Dissolve 100 mg methyl red sodium salt in distilled water and dilute to 100 mL.
b. Hydrochloric acid, HCl, 1 + 1.
c. Barium chloride solution: Dissolve 100 g BaCl2·2H2O in 1 L distilled water. Filter through a membrane filter or hard- finish filter paper before use; 1 mL is capable of precipitating approximately 40 mg $SO_4^{2-}$.
d. Silver nitrate-nitric acid reagent: Dissolve 8.5 g AgNO3 and 0.5 mL conc. $HNO_3$ in 500 mL distilled water.

**(c) Procedure**

a. Precipitation of barium sulfate: A measured volume of sample (50 mL) was taken in a flask and pH was adjusted to 4.5 to 5.0 with HCl using a pH meter or the orange color of methyl red indicator. Added 1 to 2 mL HCl and heated to boiling and while stirring gently, slowly added warm $BaCl_2$ solution until precipitation appeared to be completed, then added about 2 mL in excess, digested precipitate overnight at 80 to 90 ºC.
b. Filtration and Weighing: Filtered $BaSO_4$ through a pre-weighed membrane filter at room temperature. Washed precipitate with several small portions distilled water until washings are free of $Cl^-$ as indicated by testing with $AgNO_3$-$HNO_3$ reagent. Added a few drops of silicone fluid to the suspension before filtering, to prevent adherence of precipitate to holder. Dried filter and precipitated in a conventional oven at a temperature of 103 to 105 ºC. Cooled in desiccator and weighed.

**(e) Calculation**

$$\text{mg } SO_4^{2-}/L = \frac{\text{mg } BaSO_4 \times 411.5}{\text{mL sample}}$$

**4.2.2.11 Phosphate**

**(a) Principle**

Organic phosphates are formed primarily by biological processes. They are contributed to sewage by body wastes and food residues and also may be formed from orthophosphates in biological treatment processes or by receiving water biota. Molybdophosphoric acid is formed and reduced by stannous chloride to intensely coloured molybdenum blue. The minimum detectable concentration is about 3 µg phosphate/l. The sensitivity at 0.3% absorbance is about 10 µg P/l for an absorbance change of 0.009.

**(b) Apparatus**

a. Colorimetric equipment: Spectrophotometer (400-490 nm).
b. Acid washed glassware: Cleaned all glassware with hot diluted HCl and rinsed well with distilled water,

c. Filtration apparatus and filter paper: Whatman No. 42 or equivalent.

**(c) Reagents**

a. Phenolphthalein indicator aqueous solution.

b. Strong-acid solution: Slowly added 300 ml of concentrated $H_2SO_4$ to about 600 mL distilled water. When cold, added 4.0 ml concentrated $HNO_3$ and diluted to 1000 mL.

c. Ammonium molybdate reagent I: Dissolved 25 g $(NH_4)_6MO_7O_{24}.4H_2O$ in 175 ml distilled water. Cautiously added 280 ml concentrated $H_2SO_4$ to 400 ml distilled water. Cooled, added molybdate solution and diluted to 1000 mL.

d. Stannous chloride reagent I: Dissolved 2.5 g fresh $SnCl_2.2H_2O$ in 100 mL glycerol. Heated in a water bath and stirred with a glass-rod to hasten dissolution. The reagent was stable and required neither preservative nor special storage.

e. Standard phosphate solution: Dissolved 219.5 mg anhydrous $KH_2PO_4$ in distilled water and diluted to 1000 mL; 1.00 mL= 50.0 μg $PO_4^{3-}$- P.

**f. Reagents for extraction:**

1. Benzene-isobutanol solvent: Mixed equal volumes of benzene and isobutyl alcohol.

2.Ammonium molybdate reagent II: Dissolved 40.1g $(NH_4)_6MO_7O_{24}.4H_2O$ in approximately 500 ml distilled water. Slowly added 396 mL ammonium molybdate reagent I. Cooled and diluted to 1000 mL

3. Alcoholic sulphuric acid solution: Cautiously added 20 mL conc. $H_2SO_4$ to 980 ml methyl alcohol with continuous mixing.

4. Diluted stannous chloride reagent II: Mixed 8 mL stannous chloride reagent I with 50 ml glycerol. The reagent was stable for at least 6 months.

**(d) Procedure**

A measured volume (20 mL) of sample was taken and diluted to 100 ml with distilled water and added 4.0 mL molybdate reagent I and 0.5 mL (10 drops) stannous chloride reagent I. After 10 min, but before 12 min, measured colour photometrically at 690 nm and compared with a calibration curve using distilled water blank.

**(e) Calculation**

$$P\ (mg\ L^{-1}) = \frac{mg\ P\ (in\ approximately\ 104.5\ ml\ final\ volume)}{mL\ of\ sample} \times 1000$$

**4.2.2.12 Metals [Inductively Coupled Plasma (ICP) Emission Spectroscopy Method]**

**(a) Principle**

To reduce interference by organic matter and to convert metals associated with particulates to form (usually the free metal ions) that can be determined by electrothermal atomic absorption spectrometry or inductively coupled plasma spectroscopy. This method is designed to determine trace metals and metalloids in surface, ground and drinking waters also.

**(b) Apparatus**

a. Hot plate

b. Conical (Erlenmeyer) flask: 125 mL, or griffin beakers, 150 mL, acid washed and rinsed with water

c. Volumetric flask, 100 mL

d. Watch glass, ribbed and unribbed

e. Safety shield

f. Safety goggles

**(c) Reagents**

a. Concentrated Nitric acid ($HNO_3$) and Perchloric acid ($HClO_4$)

b. Acid water solution containing HCl and water in a 1:1 ratio.

**(c) Procedure**

1. 1g of soil/water sample was placed in a 250 mL beaker
2. HNO3 and $HClO_4$ in 3:1 ratio were added in the sample.
3. The sample was digested on a hot plat at 100 0C for 4-5 hrs until a whitish brown dry mass was obtained.
4. The sample after digestion was treated with acid water mixture and filtered through Whatman No. 42 filter paper.
5. The filtrate was analyse for total Fe, Mn, Ni, Zn, Cu, Pb and Cd by using atomic absorption spectrophotometer.

**(e) Calculations**

Concentration of element (mg $L^{-1}$) = (Observed concentration – Blank) × Dilution factor
Results were calculated up to three significant figures.

### 4.2.3 Extraction and identification of various persistent organic pollutants from spent wash and PMDE

#### 4.2.3.1 Liquid-liquid extraction

The organic pollutants present in DSW were extracted by acetone, *n*-hexane, ethyl acetate, methanol, and isopropanol under acidic conditions (pH <2.0) as previously described (Bharagava and Chandra 2009). To extract the organic pollutants, a fixed volume (10 mL) of spent wash/PMDE was acidified with 35% hydrochloric acid and then placed in a separating funnel (100 mL), after which an equal volume of ethyl acetate was added and the mixture was shaken continuously for 5 h with intermittent rests for liquid–liquid extraction (Fig. 4.2). The extraction was repeated successively three times to complete extraction of organic pollutants. The organic layers obtained from spent wash were kept together and concentrated on a rotatory evaporator until complete solvent evaporation (Rotavapor RE 120, Buchi, Flawil, Sweden) at ≤40 °C. A similar process was followed for the extraction of various organic compounds products from PMDE. An aliquot of the

concentrate was dissolved in 3.0 mL of methanol, filtered through 0.22 µm syringe filters (Millipore Ltd., Bedford, MA, USA), and used for further GC-MS analysis.

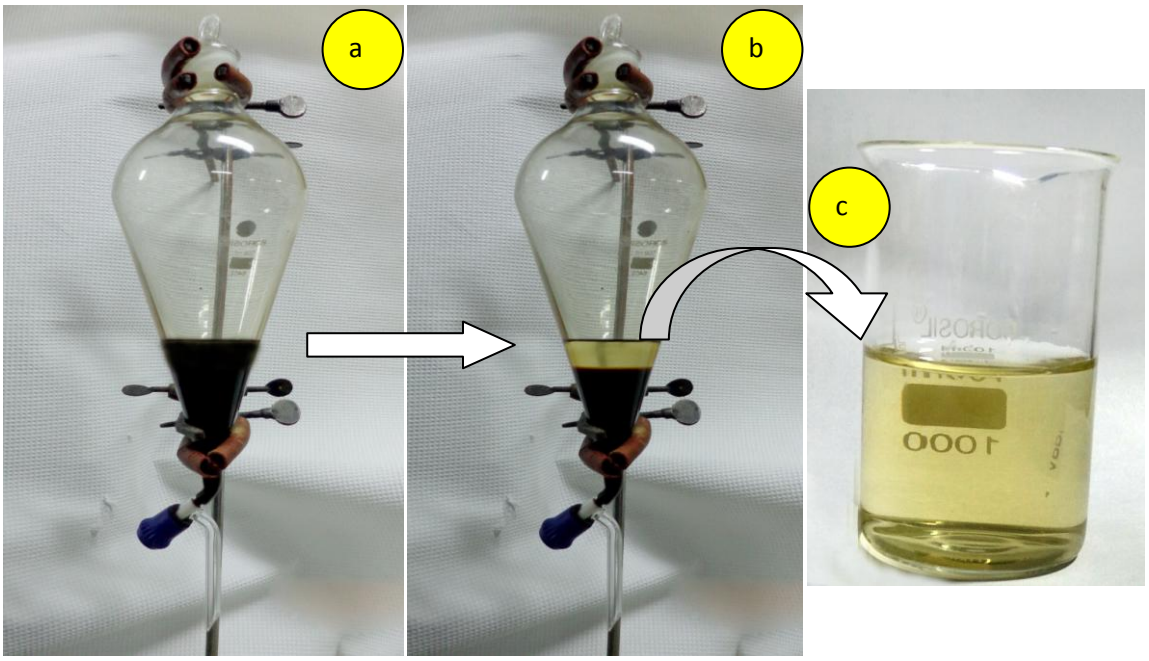



**Fig. 4.2** Extraction of organic pollutants by using liquid-liquid extraction procedure (a) spent wash mixed with organic solvent (b) after intermittent rests for liquid–liquid extraction organic layer was formed (c) organic layer obtained from spent wash+solvent mixture

### 4.2.3.2 GC-MS analysis

In GC-MS analysis, the extracted samples were derivatised with trimethylsilyl (TMS) as described by Minuti et al. (2006). In this method, the extracted samples (300 µl) were transferred into GC vials and evaporated to dryness with nitrogen gas. Derivatization was performed by adding 50 µl of pyridine to the sample and then subjecting it to silylation with 80 µl trimethylsilyl BSTFA and TMCS. Next, the mixture was heated at 70 °C for 30 min with periodic shaking to dissolve the residues, after which it was subjected to GC-MS analysis. The system consisted of a Thermo Scientific Trace GC Ultra Gas Chromatograph equipped with a TriPlus auto sampler coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific, FL, USA). An aliquot (2.0 µl) of silylated sample was automatically injected into DB-5MS capillary column [30 m length × 0.25 µm I.D. × 0.25 mm film thickness of 5% phenyl and 95% methylpolysiloxane (v/v)] with helium as the carrier gas at a flow rate of 1.1 mL $min^{-1}$. The initial oven temperature was 65 °C (hold for 2 min), after which it was increased to 230 °C at 6 °C min and then to 290 °C (hold for 20 min) at a rate of 10 °C min . The transfer line temperature and ion source temperature were kept at 290 and 220 °C, respectively. The mass spectrometer was operated in positive electron ionization (+EI) mode at an electron energy of 70 eV with a solvent delay of 7 min. Initially, derivatization of organic compounds was conducted in full scan mode using a mass range of 45–800 amu. The NIST mass spectral database library (version 1.0.0.12) available with the instrument was used to identify selected peaks.

### 4.2.4 Phytotoxicity assay of DSW and PMDE

The phytotoxicity of any environmental sample using higher plants is a basic and authentic tool for toxicity evaluation of any environmental sample. Therefore, the toxicity of DSW against *P. mungo* L. and *T. aestivum* seed germination was studied as previously described (OECD 2003; Sharma et al. 2002). Prior to preparation of various concentrations of DSW and PMDE, the pH was adjusted to 7.0 with 1N NaOH solution. For the seed germination experiment, DSW was applied at 1, 2, 4, and 5% (*v*/*v*). The surfaces of the seeds were sterilized with 0.1% HgCl for 2 min to avoid any fungal contamination, after which they were subjected to repeated washings with sterilized distilled water (Santal et al. 2011). Subsequently, 10 seeds of *P. mungo* L. and *T. aestivum* were placed separately in sterilized glass petri dishes of uniform size lined with two Whatman no. 1 filter paper disks. These filter discs were then moistened with 10 mL of tap water for control and with the same volume of DSW, after which they were incubated at room temperature for a period of three consecutive days. A similar process was followed for the phototoxicity assessment of PMDE. Germination, which was considered the visible protrusion of the radical from the seed coat, was expressed as a percentage as previously described (Bharagava and Chandra 2010a). Different seed germination parameters like germination percentage, relative toxicity percentage, phytotoxicity percentage, and stress tolerance index of seedling were also studied sing the following formula as described by David Noel and Rajan (2015).

$$\text{Germination (\%)} = \frac{\text{No. of seed planted}}{\text{No. of seed germinated}}$$

$$\text{Relative toxicity (\%)} = \{(x-y)/x\} \times 100$$

Where,

x= Germination percentage in control at particular hours of incubation

y= Germination percentage in presence of effluent at the same hours of incubation

$$\text{Phtotoxicity (\%)} = \frac{\text{Radical length of control} - \text{radical length of test of tested}}{\text{Radical length of control}} \times 100$$

$$\text{Stress tolerance index} = \frac{\text{Mean length of longest root in treatment}}{\text{Mean length of longest root in control}}$$

## 4.3 Results and Discussion

### 4.3.1 Physico-chemical analysis of DSW and PMDE

The physico-chemical characteristics of DSW showed high BOD (42,000), COD (90,000), TDS (77,776), TS (83,084), chloride (2200), phenol (4.20), sulfate (5760), and phosphate (5.36 mg $L^{-1}$) levels with acidic pH (4.07), molasses like odor, and dark brown color. In addition, the DSW showed high levels of different heavy metals (Fe, Mn, and Zn) as shown in Table 4.1. These values are far greater than the permissible limit of industrial discharge (USEPA 2002). These results were in accordance with those of previous studies (Chandra et al. 2004). The pungent smell of the mixture of boiled sugar and alcohol from spent wash was caused by residual organic products and fermented reducing sugar. In contrast to DSW, the physicochemical characteristics of PMDE also showed high BOD (6,500), COD (10,864), TDS (10,764), TS (12,248), chloride (19,993.8), phenol (3.98), sulfate (4850.45), and phosphate (5.12 mg $L^{-1}$) levels with alkaline pH (8.17), sulphur like odor, and dark brown color. In addition, the PMDE showed high levels of various heavy metals as shown in Table 4.1.

**Table 4.1** Physico-chemical characteristics of spent wash and post methanated distiller effluent

| **Parameter** | **Spent wash** | **Post methanated distillery effluent** | **Discharge Permissible limit (USEPA, 2002)** |
|---|---|---|---|
| Color appearance | Dark Brown | Dark Brown | Not clear |
| Color Intensity | 1,50,000 | 1,98,000 | Not clear |
| pH | 4.07±0.12 | 8.17±0.30 | 5-9 |
| Temperature | 90±2.0 | 35±1.1 | 40 |
| BOD | 42,000±1848 | 6500±266.5 | 40.00 |
| COD | 90,000±3600 | 10,864±391.10 | 120.00 |
| TS | 83,084±2907 | 12,248.0±502.16 | 2200 |
| TDS | 77,776±3768 | 10,764±409.03 | 2100 |
| Chloride | 2200±105 | 19,993.8±599.75 | 750.00 |
| Phenol | 4.20±1.8 | 3.98±1.22 | 0.50 |
| Sulphate | 5760±260 | 4850.45±120.14 | 750.00 |
| Phosphate | 5.36±0.168 | 5.12±0.001 | Not clear |
| Total nitrogen | 2800±130 | 2650±121.74 | 25 |
| Total organic carbon | 25368±1.060 | 2085.6±10.38 | Not clear |
| Trace Elements | | | |
| Mn | 4.556±0.159 | 4.978±0.01 | 0.20 |
| Cr | 1.05±0.031 | 1.257±001 | 0.05 |
| Zn | 2.487±0.094 | 2.678±0.04 | 2.00 |
| Cu | 0.337±0.016 | 0.878±0.00 | 0.50 |
| Fe | 163.947±6.52 | 128.64±1.57 | 2.00 |
| Pb | BDL | BDL | 0.05 |
| Cd | BDL | BDL | 0.01 |
| Ni | 1.175±0.047 | 1.425±0.00 | 0.10 |

All values are mean (n=3)±SD in mg $l^{-1}$ except color intentity (Co-Pt unit), pH, and temperature ($^0$C)

The dark color of DSW and PMDE is known to be generated by the formation of Maillard product due to complexation of amino-carbonyl compounds during the condensation of sugarcane juice for the sugar manufacturing process. Subsequently, the Maillard product is

precipitated out as waste containing poor quality sugar and particulate matter known as molasses. Further, during the fermentation process, available sugar is fermented while other constituents remain unchanged. Hence, after ethanol recovery during the distillation process, the residual material showed high BOD, COD, TDS, and TS owing to the presence of several unknown residual organic pollutants generated during the sugar manufacturing and distillation process of fermented sugarcane molasses slurry. Some of these organic pollutants might have originated from the sugarcane plant during the extraction of sugarcane juice as plant phenolics or plant steroids. Similarly, the high concentration of Fe, Mn, Zn, Cr and Ni might be due to the corrosion of pipes, fermentor vessels, and the plant extract. The physicochemical properties of distillery effluent observed herein were in accordance with those of previous studies (Chandra et al. 2004; Tiwari et al. 2013).

### 4.3.2 Characterization of organic pollutants

#### 4.3.2.1 Acetone extract of DSW

GC-MS analysis of acetone extracted DSW samples revealed several peaks at different RTs (Fig. 4.3), with the major peaks noted at RT 8.66, 13.78, 16.01, 22.18, and 22.81, which corresponded to 1,3-propanediol, TMS ether; butanedioic acid, bis(TMS) ester; 2,3-butandiol, bis-O-TMS; cyclooctene, 1,2-bis(trimethylsiloxy) and Dribohexanoic acid, 3-deoxy 2,56,tris-O-(TMS); and lactone, respectively, based on comparison with the NIST library. Several minor peaks were also noted at RT 9.88, 10.27, 11.54, 11.87, 15.52, 17.34, 17.54, 19.60,20.19, 22.15, 23.54, 24.19, 24.87, 26.48, and 30.61. This corresponded to propanoic acid, 3-[(TMS)oxy), TMS ether; butanoic acid, 3-methyl-2-[(TMS)oxy], TMS ether; D-erythrotetrofuranose, tris-O-(TMS); pentanoic acid; resorcinol, O-bis(TMS); malic acid (O-(TMS)bis(TMS ester); 2-methyl1,3-butanediol 2-TMS; 2-furancarboxylic acid, 5[[(TMS) oxy] methyl], TMS ester; 2,3,5-Tri-O-TMS-arabino1,5-lactone, tricarballylic acid TMS; benzoic acid,3,4-bis [(TMS)oxy], TMS ester; tertbutylhydroquinone, bis (TMS) ether; TMS 3,5-dimethoxy-4-9 TMS oxy)benzoate, vanillylpropionic acid, bis (TMS); and benzene acetic acid, α,4bis[(TMS) oxy]methyl ester, respectively. The majority of these compounds were characterized as organic acids generated from fermented sugarcane molasses slurry but discharged as waste in spent wash after ethanol recovery as shown in Table 4.2. Hence, the production of these compounds supported the low pH (2.0) of DSW. However, the detected compounds (pentanoic acid, butanedioic acid bis(TMS) ester, 2-furancarboxylic acid, 5[[(TMS) oxy] methyl], TMS ester, tricarballylic acid, and benzoic acid) have been listed as potential endocrine disrupting chemicals (EDCs) by the USEPA (2012). Moreover, 1,3-propanediol has been reported as a product of glycerol fermentation by *Clostridium* sp. (Kubik et al. 2012). *Clostridium* sp. has been reported in sugarcane processing plant as well (Sharmin et al. 2013); hence, these products might have originated during fermentation of sugarcane molasses. However, their fate in the environment along with the detected compounds is unknown. Tricarballylic acid has also been reported as a bacterial fermentation product,

but its accumulation in mammalian tissue inhibits the metabolic process (Russell and Forsberg 1986). Moreover, benzoic acid is known to have harmful effects, including the ability to alter hormone secretion in animals (Mineo et al. 1995). Thus, the majority of these detected compounds indicated that the waste poses an environmental risk to aquatic flora and fauna at distillery waste disposal sites.

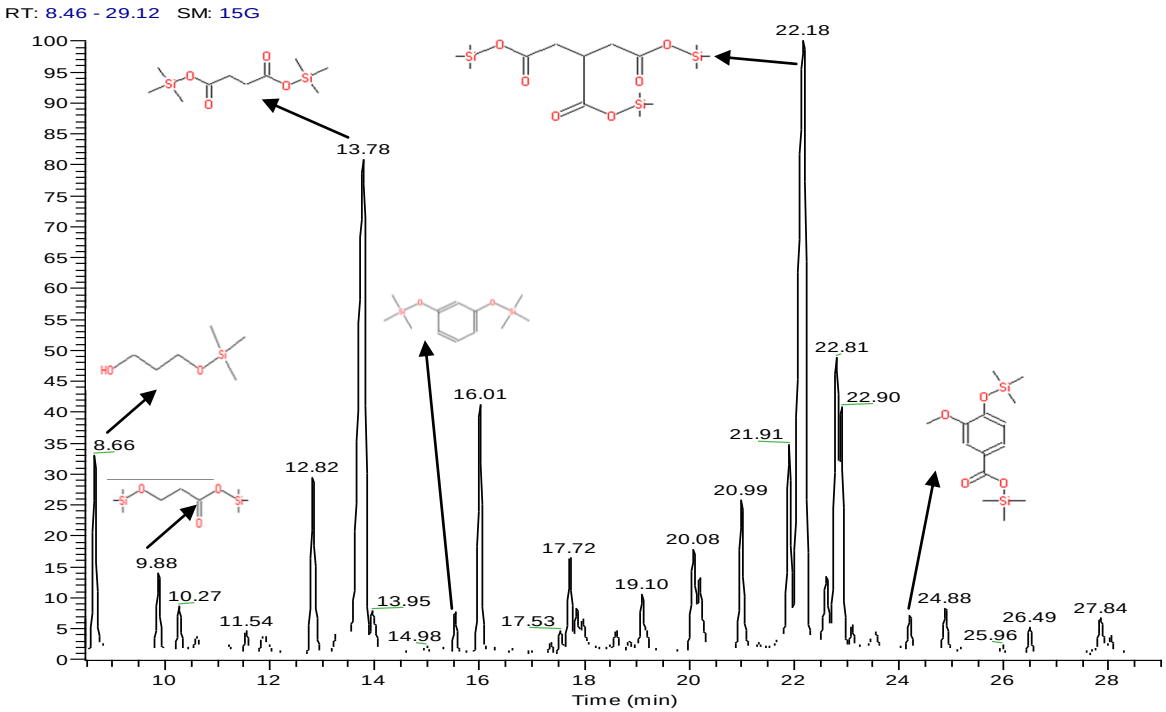



**Fig. 4.3** TIC chromatogram obtained during GC-MS analysis of acetone extract of spent wash

**Table 4.2** Organic compounds identified by GC-MS in acetone extract of DSW

| SI. No. | RT | Identified Compounds |
|---|---|---|
| 1. | 8.66 | 1,3-Propanediol, TMS ether |
| 2. | 9.88 | Propanoic acid, 3-[(TMS)oxy)-TMS ether |
| 3. | 10.27 | Butanoic acid, 3-methyl-2-[(TMS)oxy]-TMS ether |
| 5. | 11.54 | D-Erythrotetrofuranose, tris–O-(TMS) |
| 6. | 11.87 | Pentanoic acid |
| 7. | 13.78 | Butanedioic acid, bis(TMS) ester |
| 8. | 15.52 | Resorcinol, O-bis(TMS) |
| 9. | 16.01 | 2,3-Butandiol, bis-O-(TMS) |
| 10. | 17.34 | Malic acid (O-(TMS)-bis(TMS ester) |
| 11. | 17.54 | 2-Methyl-1,3-Butanediol 2TMS |
| 12. | 19.60 | 2-Furancarboxylic acid, 5-[[( TMS) oxy] methyl], TMS ester |
| 13. | 20.19 | 2,3,5-Tri-O- TMS-arabino-1,5-lactone |
| 14. | 22.18 | Cyclooctene, 1,2-bis(trimthylsiloxy) |
| 15. | 22.15 | Tricarballylic Acid TMS |
| 16. | 22.81 | D-ribo-Hexanoic acid, 3-deoxy-2,56, tris-O-(TMS), lactone |
| 17. | 23.54 | Benzoic acid, 3,4-bis [(TMS)oxy], TMS ester |
| 18. | 24.19 | Tert-butylhydroquinone, bis (TMS) ether |
| 19. | 24.87 | TMS 3,5-dimethoxy-4-9 TMS oxy)benzoate |
| 20. | 26.48 | Vanillypropionic acid, bis (TMS) |
| 22. | 30.61 | Benzeneacetic acid, α,4-bis[(TMS) oxy]-methyl ester |

RT: Retention Time (Min); TMS: Trimethylsilyl

### 4.3.2.2 Ethyl acetate extract of DSW

The GC-MS total ion current (TIC) chromatogram of ethyl acetate extract of DSW showed several major peaks at RT 8.23, 12.90, 13.78, 16.00, 20.09, 22.23, 22.63, 24.89, and 27.83 (Fig. 4.4). These compounds were characterized as L-lactic acid, TMS ether, TMS ester; 1,3-propanediol, TMS ether; butanedioic acid, bis(TMS)ester; silane, [1,4-phenylenebis(oxy)]bis(trimethyl); benzoic acid, 4[(TMS)oxy], TMS ester; tricarballylic acid 3-TMS; benzoic acid, 3-methoxy4[(TMS)oxy], TMS ester; trimethylsilyl 3,5 dimethoxy-4-(TMSoxy)benzoate; and silane, (preg-5ene-3β,11β,17,20β-tetraylteraoxy) tetrabis (trimethyl), respectively. Other minor peaks were also observed at RT values of 8.48, 10.33, 10.70, 11.86, 12.24, 13.94, 15.22, 16.56, 18.59, 19.18, 20.25, 20.62, 20.98, 21.32, 25.96, and 27.01, which corresponded to acetic acid, [(TMS)oxy], TMS ester; butanoic acid, 3-methyl-2-[(TMS), TMS ester; valeric acid, 5-methoxy, TMS ester; 213 hydroxysocaproic acid, TMS ether; ethyl(TMS) succinate; diethyl methyl succinate; lactic acid dimer, bis(TMS); 2-methyl-1,3-propanediol-2-TMS; 2-furancarboxylic acid, 5[[(TMS)oxy]methyl], TMS ester; benzenepropanoic acid, α[(TMS)oxy], TMS ester; ethyl-TMS dipropylmalonate; bicyclo[4.3.0]nonane-2-one, [Z]cis-8-(phenyl1trimethylsilylmethylene), 2-hydroxyheptanoic acid 2-TMS; Derythrohex-2-enoic acid, 2,3, di-O-methyl5,6-bis-O-(TMS)-γ-lactone; 3-vanil-1,2-propanediol 3-TMS; and β-D-galactopyranoside, methyl 2,6-bis-O-(TMS)cyclic butylboronate, respectively.

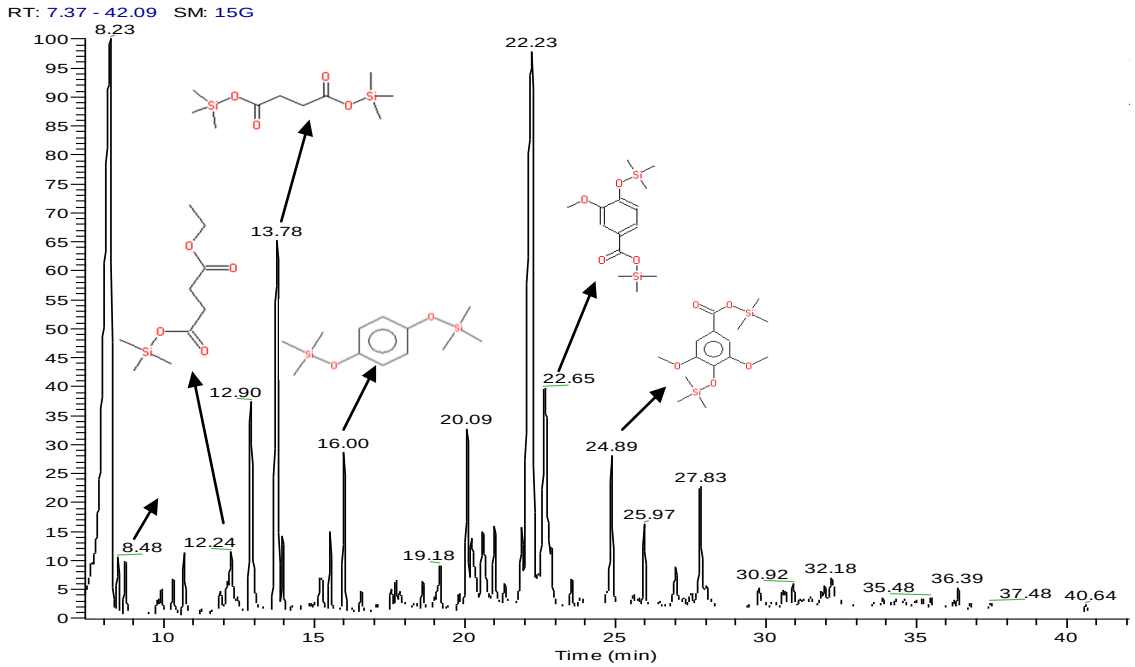



**Fig. 4.4** TIC chromatogram obtained during GC-MS analysis of ethyl acetate extract of DSW

The maximum numbers of identified compounds were observed in the ethyl acetate extract of DSW (Table 4.3). These findings demonstrated that ethyl acetate was the best solvent for the extraction of organic compounds present in sugarcane molasses based DSW. However, L-lactic acid, TMS ether, TMS ester; acetic acid, [(TMS)oxy], TMS ester; butanoic acid, 3methyl2[(TMS), TMS ester; valeric acid, 5-methoxy, TMS ester;

butanedioic acid, bis(TMS)ester; and tricarballylic acid 3-TMS and benzoic acid, 3-methoxy-4-[(TMS)oxy] TMS ester have been listed as potential EDCs by the USEPA (2012). In addition, benzenepropanoic acid has been reported to have toxic effects in the environment, while 2-hydroxysocaproic acid has been reported to be an antiinflammatory and antimicrobial agent (Nieminen et al. 2014).

**Table 4.3** Organic compounds identified by GCMS in ethyl acetate extract of DSW

| SI. No. | RT | Identified Compounds |
|---|---|---|
| 1. | 8.23 | L-Lactic acid, TMS ether, TMS ester |
| 2. | 8.48 | Acetic acid, [(TMS)oxy], TMS ester |
| 3. | 10.33 | Butanoic acid, 3-methyl-2-[(TMS), TMS ester |
| 4. | 10.70 | Valeric acid, 5-methoxy, TMS ester |
| 5. | 11.86 | 2-Hydroxysocaproic acid, TMS ether |
| 6. | 12.24 | Ethyl(TMS) succinate |
| 7. | 12.90 | 1,3-Propanediol, TMS ether |
| 8. | 13.78 | Butanedioic acid, bis(TMS)ester |
| 9. | 13.94 | Diethyl methylsuccinate |
| 10. | 15.22 | Lactic acid dimer, bis(TMS) |
| 11. | 16.00 | Silane, [1,4-phenylenebis(oxy)]bis(trimethyl) |
| 12. | 16.56 | 2-Methyl-1,3-Propanediol 2TMS |
| 13. | 18.59 | 2-Furancarboxylic acid, 5 -[[(trimethylsilyl)oxy]methyl], TMS ester |
| 14. | 19.18 | Benzenepropanoic acid, α-[(TMS)oxy], TMS ester |
| 15. | 20.09 | Benzoic acid, 4-[(TMS)oxy], TMS ester |
| 16. | 20.25 | Ethyl-TMS dipropylmalonate |
| 17. | 20.62 | Bicyclo[4.3.0]nonane -2-one,[Z]-cis-8-(phenyl-1-trimethylsilylmethylene) |
| 18. | 20.98 | 2-Hydroxyheptanoic acid 2TMS |
| 19. | 21.32 | D-erythro-Hex-2-enoic acid, 2,3,-di-O-methyl-5,6-bisO-(TMS)-Ɣ-lactone |
| 20. | 22.23 | Tricarballylic acid 3TMS |
| 21. | 22.65 | Benzoic acid, 3-methoxy-4-[(TMS)oxy], TMS ester |
| 22. | 24.89 | TMS 3,5 dimethoxy-4-(TMS oxy)benzoate |
| 23. | 25.96 | 3-Vanil-1,2-Propanediol 3-TMS |
| 24. | 27.01 | B-D-Galactopyranoside, methyl 2,6-bis-O-(TMS)- cyclic butyboronate |
| 25. | 27.83 | Silane, (preg-5-ene-3β,11β,17,20β-tetraylteraoxy)tetrabis (trimethyl) |

RT: Retention Time (Min); TMS: Trimethylsilyl

### 4.3.2.3 Isopropanol and methanol extract of DSW

The GC-MS TIC chromatogram of DSW extracted by isopropanol and methanol showed relatively fewer peaks than the acetone and ethyl acetate extracts of DSW (Fig. 4.5a,b). However, major peaks were noted in the isopropanol extracts at RT 8.49, 13.75, and 16.01 corresponding to propanoic acid, 29[(TMS)oxy], TMS ester; butanedioic acid, bis(TMS)ester; and 2-methyl-1,3-propanediol2TMS, respectively, while a minor peak was noted at RT 15.32 and characterized as butane, 1,2,4-tris (trimethylsilyloxy) (Table 4 (a)). Moreover, very minor peaks were observed at RT 8.85, 9.14, 10.53, 12.87, 13.11, 13.99, 16.01, 17.80, 18.59, 20.21, 21.54, 21.90, and 22.79; however, these could not be identified because they were not available in the NIST library.

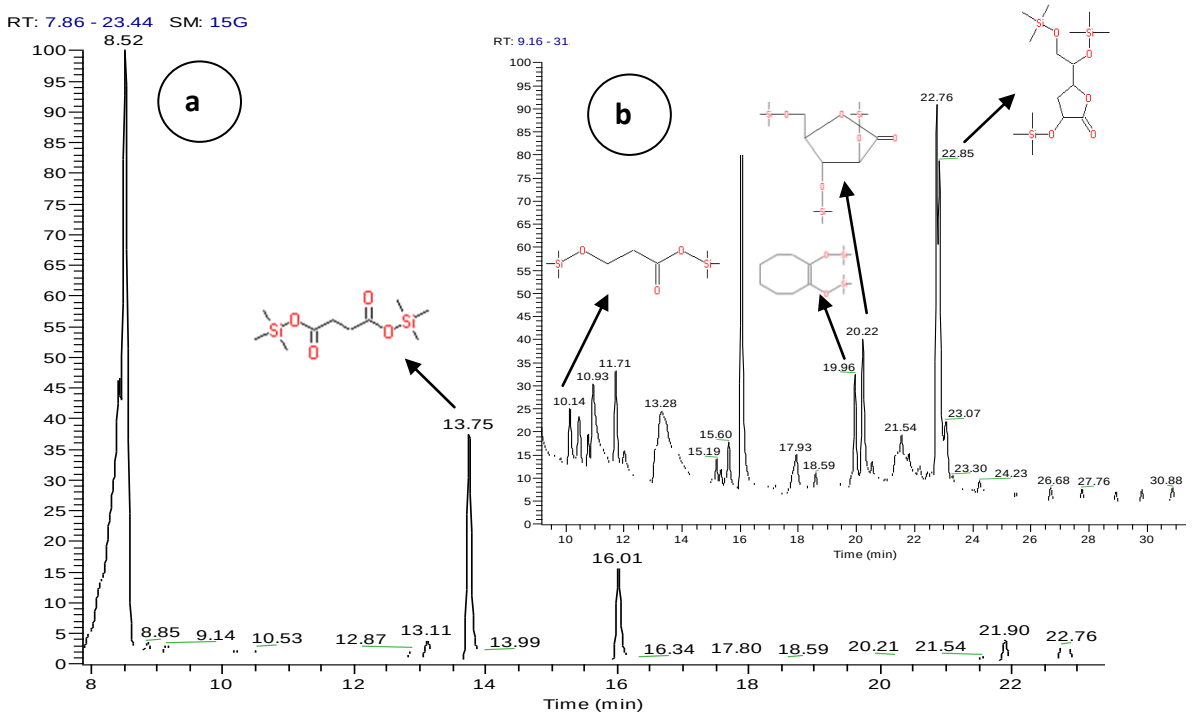



**Fig. 4.5** TIC chromatogram obtained during GC-MS analysis of (a) isopropanol (b) methanol extract of DSW

Simultaneously, the GC-MS TIC chromatogram of methanol extract of DSW showed major peaks at RT 16.04, 20.22, and 22.85, which reflected 3,6-didoxa-2,7disilaoctane,2,2,4,5,7,7-hexamethyl, 2,3,5,tri-O-trimethylsilyarabino-1,5-lactone, and D-ribohexanoic acid, 3-deoxy-2,5,6 tris-O-(TMS)lactone, respectively (Fig. 3b and Table 4b). Moreover, minor peaks were observed at RT 10.14, 10.44, 10.93, 11.71, and 19.95, which corresponded to propanoic acid, 3[(TMS)oxy], TMS ester, butanoic acid, 3-methyl-2[(TMS)oxy], TMS ester, 2-methylbutanoic acid, 3-(t-butyldimethylsilyloxy) methyl ester, erythro-pentitol 2-dedoxy1,3,4,5-tetrakis-O-(TMS), and cyclooctene, and 1,2-bis(trimethylsilyloxy), respectively (Table 4.4b). However, the roles of several other detected organic compounds in the environment have yet to be elucidated.

**Table 4.4** Organic compounds identified by GC-MS in (a) isopropanol extract and (b) methanol extract of DSW

| **(a)** | | |
|---|---|---|
| **SI. No.** | **RT** | **Identified Compounds** |
| 1. | 8.49 | Propanoic acid, 2-9 (TMS)oxy],- TMS ester |
| 2. | 13.75 | Butanedioic acid, bis(TMS)ester |
| 3. | 15.32 | Butane, 1,2,4-tris (trimethylsilyloxy) |
| 4. | 16.01 | 2-Methyl-1,3-Propanediol-2-TMS |
| **(b)** | | |
| 1. | 10.14 | Propanoic acid, 3-[(TMS)oxy], TMS ester |
| 2. | 10.44 | Butanoic acid, 3-methyl-2-[(TMS)oxy], TMS ester |
| 3. | 10.93 | 2-Methylbutanoic acid, 3-(t-butlydimethylsilyloxy),-methyl ester |
| 4. | 11.71 | Erythro-pentitol, 2-dedoxy-1,3,4,5-tetrakis-O-(TMS) |
| 5. | 16.04 | 3,6-Didoxa-2,7-Disilaoctane, 2,2,4,5,7,7-Hexamethyl |
| 6. | 19.95 | Cyclooctene, 1,2-bis-(trimethylsilyloxy) |

| | | |
|---|---|---|
| 7. | 20.22 | 2,3,5,-tri-O-trimethylsily-arabino-1,5-lactone |
| 8. | 22.85 | D-ribo-hexanoic acid, 3-deoxy-2,5,6 tris-O-(TMS)-lactone |

RT: Retention Time (Min); TMS: Trimethylsilyl

### 4.3.2.4 Ethanol and *n*-hexane extract of DSW

The GC-MS TIC chromatogram of DSW extracted by ethanol and n-hexane showed few major peaks (Fig. 4.6). However, major peaks were noted in ethanol extracts at RT 8.48, 13.76, and 16.03, corresponding to D-lactic acid-DiTMS; butanedioic acid, bis(TMS)ester; and 3,6-dioxa-2,7-disilaoctane, 2,2,4,7,7 pentamethyl, respectively. Moreover, minor peaks were noted at RT 12.39, 17.77, 20.21, 21.07, 22.76, and 23.12 corresponding to ethyl (trimethyl)succinate, erythritol per TMS, 2,3,4,5-tetrahydroxypentanoic acid1,4-lactone, tris; cyclooctene, 1,2 bis(trimethylsiloxy); D-ribohexanoic acid, 3-deoxy2,5,6-tris-O-(TMS)lactone; and α-D-galactopyranose,1,2,3-tris-O-(TMS), cyclic methylboronate, respectively (Fig. 4.6a and Table 4.5a). Simultaneously, the GC-MS TIC chromatogram of methanol extract of DSW showed major peaks at RT 35.69, 36.56, and 37.57, corresponding to 3,4-dihyroxymandelic acid, ethyl ester, tri-TMS, octadecane, 3-ethyl-5-(2-ethylbutyl) and celidonioll, deoxy, respectively (Fig. 4.6b and Table 4.5b). Some other minor peaks were also observed at different RT that could not be identified. These compounds may adversely affect aquatic organisms at the receiving ecosystem because of their toxic nature (USEPA 2012). Hence, this is strong evidence of the toxic nature of DSW. However, their fate in the environment and biogeochemical cycle has yet to be revealed.

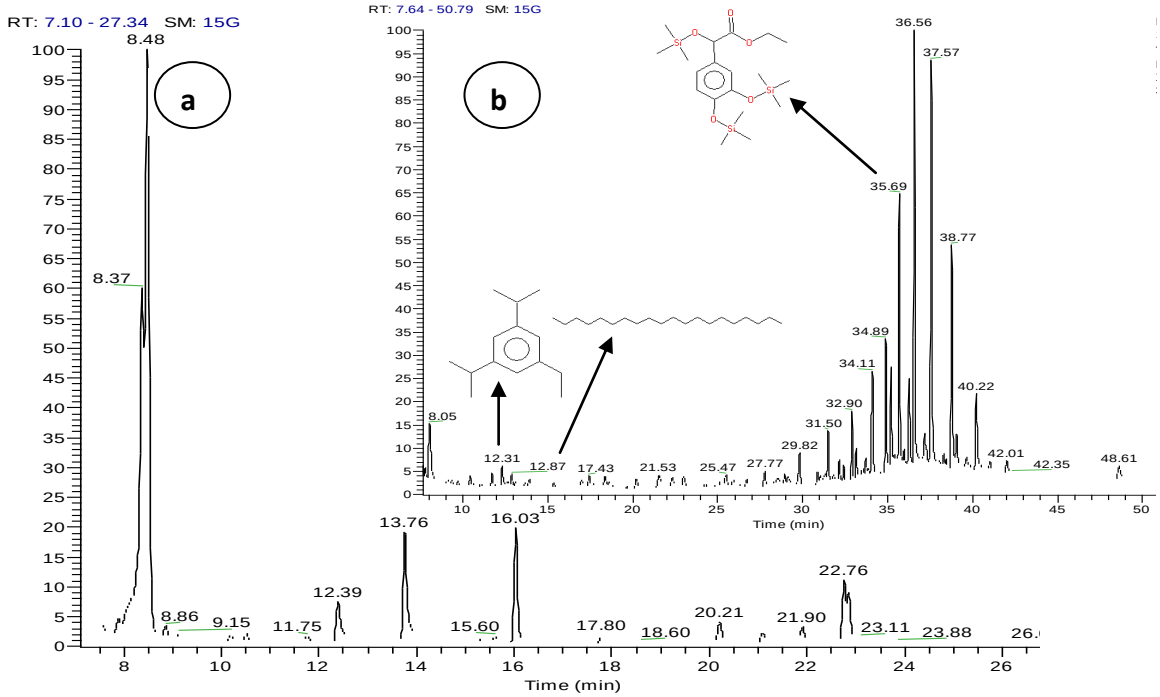



**Fig. 4.6** TIC chromatogram obtained during GC-MS analysis of DSW (a) ethanol extract and (b) *n*-hexane extract

**Table 4.5** Organic compounds identified by GC-MS in (a) ethanol extract and (b) *n*-hexane extract of DSW

| **(a)** | | |
|---|---|---|
| **SI. No.** | **RT** | **Identified Compounds** |
| 1. | 8.48 | D-Lactic acid-Di TMS |
| 2. | 12.39 | Ethyl (trimethyl)succinate |
| 3. | 13.76 | Butanedioic acid, bis (TMS)ester |
| 4. | 16.03 | 3,6-Dioxa-2,7-disilaoctane,2,2,4,7,7-pentamethyl |
| 5. | 17.77 | Erytritol per-TMS |
| 6. | 20.21 | 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone, tris (TMS) |
| 7. | 21.07 | Cyclooctene, 1,2-bis(trimethylsiloxy) |
| 8. | 22.76 | D-Ribo-Hexanoic acid, 3-deoxy-2,5,6-tris-O-(TMS)lactone |
| 9. | 23.12 | α-D-Galactopyranose, 1,2,3-tris-O-(TMS), cyclic methylboronate |
| **(b)** | | |
| 1. | 12.31 | Benzene, 1-ethyl-3,5-disopropyl |
| 2. | 12.87 | Eicosane |
| 3. | 35.69 | 3,4-Dihyroxymandelic acid, ethyl ester, tri-TMS |
| 4. | 36.56 | Octadecane,3-ethyl-5(2-ethylbutyl) |
| 5. | 37.57 | Celidonil, Deoxy |

RT: Retention Time (min); TMS: Trimethylsilyl

### 4.3.3 Characterization of organic pollutants

#### 4.3.3.1 Acetone extract of PMDE

GC-MS analysis of acetone extracted PMDE samples revealed several peaks at different RTs (Fig. 4.7), with the major peaks noted at RT 27.09, 27.93, and 36.22, which corresponded to 2,3-bis[(TMS)oxy]propyl esterate; silane, [[(3b)-cholest-5-en-3-yl]oxy]trimethyl and stimgmasterol TMS ether, respectively, based on comparison with the NIST library. Several minor peaks were also noted at 8.39, 8.98, 9.02, 11.90, 14.76, 18.09, 21.87, 22.14, 23.84, 36.06 and 42.51. This corresponded to silane,[(1-ethylpentyl]oxy) trimethyl; D-lactic acid-DITMS; 3-methyl-1,3 bis(TMS) butane; trisiloxane, 1,1,1,5,5,5,-hexamethyl-3,3-bis[(TMS)oxy]; 3,7-Dioxa-2,8-dislanonane, 2,2,8,8-tetramethyl-5- [(TMS); erythritolper-TMS; hexadecanoic acid, TMS ester; octadecanoic acid, TMS ester; O-TMS-cannabinol; campesterol TMS; and β-sitosterol TMS ester (Table 4.6). However, the detected compounds (hexadecanoic acid, octadecanoic acid, stimgmasterol and β–sitosterol) have been listed as potential EDCs by the USEPA (2012).

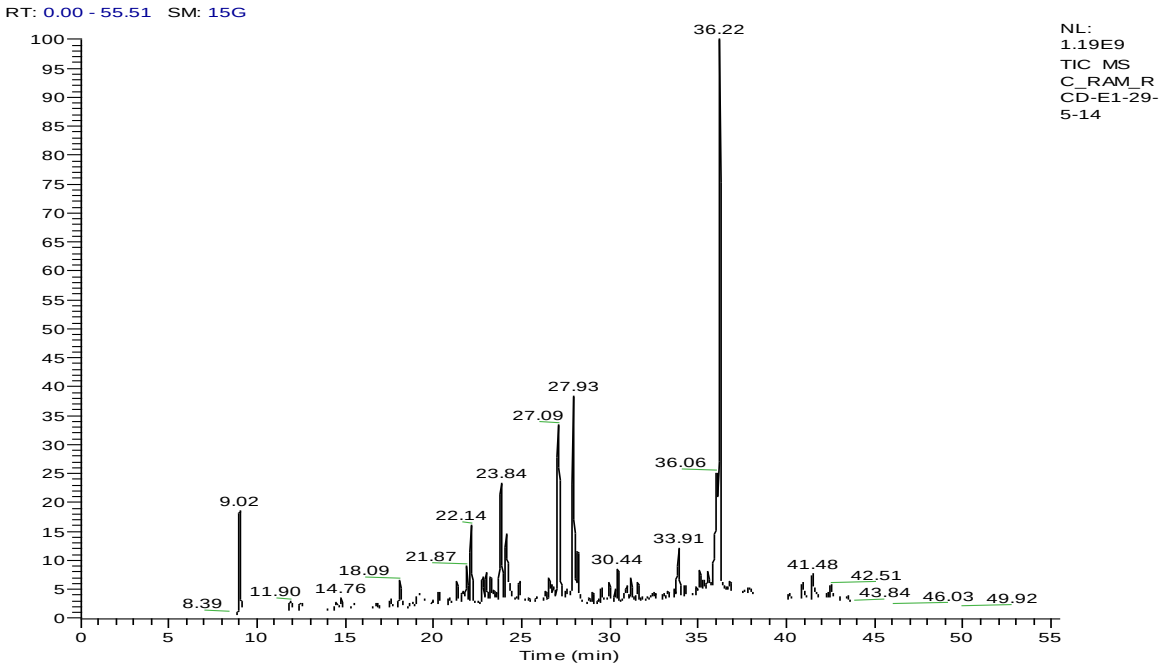



**Fig. 4.7** TIC chromatogram obtained during GC-MS analysis of acetone extract of PMDE

**Table 4.6** Organic compounds identified by GC-MS in acetone extract of PMDE

| SI. No. | RT | Name of Compound |
|---|---|---|
| 1. | 8.39 | Silane,[(1-ethylpentyl]oxy) trimethyl |
| 2. | 8.98 | D-Lactic Acid-DITMS |
| 3. | 9.02 | 3-Methyl-1,3 bis(TMS) butane |
| 4. | 11.90 | Trisiloxane, 1,1,1,5,5,5,-hexamethyl-3,3-bis[(TMS)oxy] |
| 5. | 14.76 | 3,7-Dioxa-2,8-dislanonane, 2,2,8,8-tetramethyl-5-[(TMS) |
| 6. | 18.09 | Erythritolper-TMS |
| 7. | 21.87 | Hexadecanoic acid, TMS ester |
| 8. | 22.14 | Octadecanoic acid, TMS ester |
| 9. | 23.84 | O-TMS-Cannabinol |
| 10. | 27.09 | 2,3 Bis[(TMS)oxy]propyl esterate |
| 11. | 27.93 | Silane, [[(3b)-cholest-5-en-3-yl]oxy]trimethyl |
| 12. | 36.06 | Campesterol TMS |
| 13. | 36.22 | Stimgmasterol TMS ether |
| 14. | 42.51 | β –Sitosterol TMS ester |

RT: Retention Time (min); TMS: Trimethylsilyl

### 4.3.3.2 Ethyl acetate extract of PMDE

The GC-MS TIC chromatogram of ethyl acetate extract of PMDE showed several major peaks at RT 18.92, 21.27, 24.01, 24.99, 27.23, 30.15, 35.11, 39.72, and 40.59 (Fig. 4.8). These compounds were characterized as 2,4-Imidazolidinedione, 1-[[(5-nitro-2-firnayl)methylene]amino; 2-propenoic acid, oxybis(methyl-2,1-ethanediyl)ester; 2,5-cyclohexade-1,4-dione,2,6-bis(1,1-dimethylethyl); 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; hexadecanoic acid, methyl ester; octadecanoic acid, methyl ester; butyl-11-eicosenoate; cholest-8(14)-en-3-one, (5α); and 3β, 5α-ergosta-8,24(28)-dien-3-ol,

respectively. Other minor peaks were also observed at RT values of 8.07, 8.25, 9.03, 9.73, 11.92, 14.53, 15.45, 15.67, 15.75, 16.64, 17.49, 19.32, 19.55, 19.71, 20.50, 21.93, 22.20, 22.59, 22.93, 23.89, 25.15, 29.47, 29.48, 29.85, 31.70, 42.10, 47.10, and 48.14, which corresponded to acetamide, 2,2,2-trifluoro-N-methyl-(TMS); 2-butanol, tert-butyldimethylsilyl ether; ethanedioic acid, bis(TMS)ester; pyridine, 3-trimethylsiloxy; 3-hydroxy-6-methypyridine-1-TMS; 1,4-Di-methylpyrrolo(1,2-A)pyrazine; docosane; 1-galactopyranone, 6-deoxy-1,2-bis-O-(TMS)-cyclic; benzenepropanoic acid, TMS ester; decanoic acid, TMS ester; benzeneacetic acid, α,4-bis[(TMS)oxy], TMS ester; 1-hexadecanol, 2-methyl; hexadecane; 5-hydroxy-2,2-dimethyl-5,6-bis(2-oxo-1-propyl)-1-cyclohexanone; dodecenoic acid, TMS ester; benzoic acid, 2,5-bis(TMSoxy)-TMS ester; 1-tetradecene, 2-decyl; 1,2-propanediol, 3-(octadecycloxy)-diacetate; anthacene; β-D-glucopyranoside, methyl 2,3-bis-O-(TMS)-, cyclic methylboronate; 2-butanone, 4-[2-isopropyl-5-methyl; octatriacontyl pentafluoropropionate; 17-pentatriacontene; 1,4-ethano-1,2,3,4-tetrahydroanthracen-3-ol-benzylidene; pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl); 24-ethyl-delta(22) coprostenol; stigmasta-5,22-Dien-3-ol (3β, 22E); and β-sitosterol, respectively (Table 4.7). These compounds may adversely affect aquatic organisms at the receiving ecosystem because of their toxic nature (USEPA 2012). The maximum numbers of identified compounds were observed in the ethyl acetate extract of PMDE (Table 4.7). These findings demonstrated that ethyl acetate was the best solvent for the extraction of organic compounds present in sugarcane molasses based PMDE.

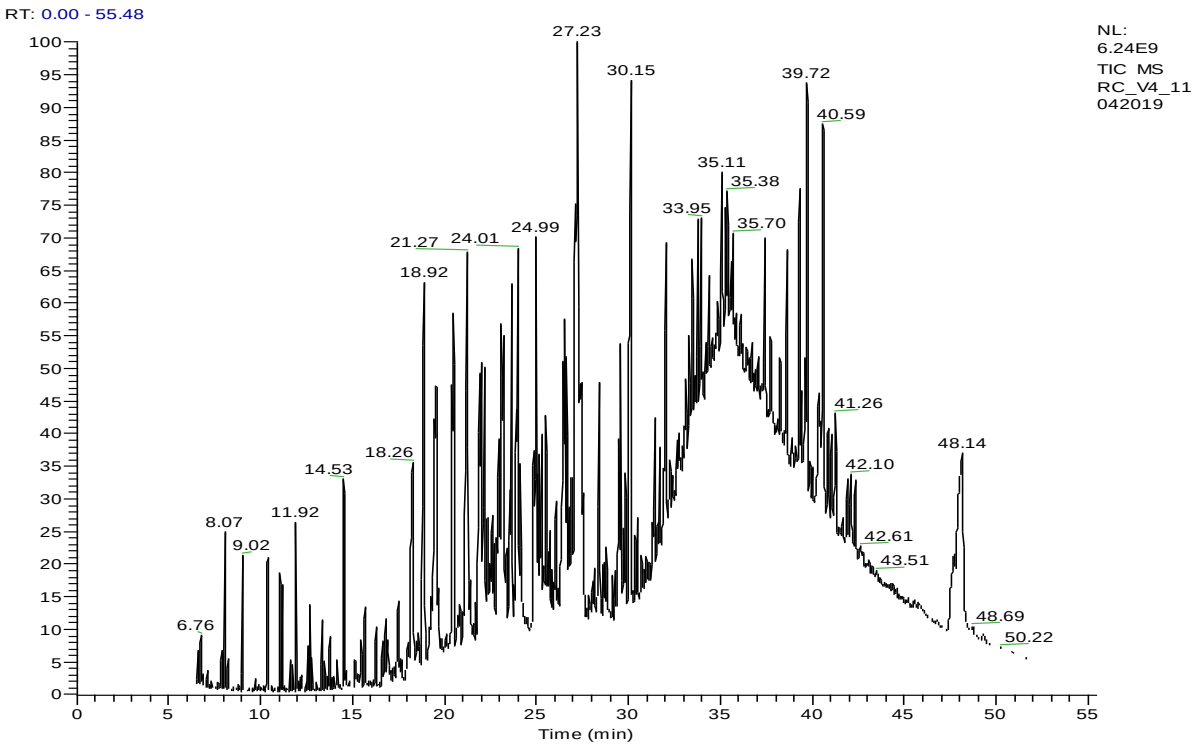



**Fig. 4.8** TIC chromatogram obtained during GC-MS analysis of ethyl acetate extract of PMDE

**Table 4.7** Organic compounds identified by GC-MS in ethyl acetate extract of PMDE

| SI. No. | RT | Name of Compound |
|---|---|---|
| 1. | 8.07 | Acetamide, 2,2,2-trifluoro-N-methyl-(TMS) |
| 2. | 8.25 | 2-Butanol, tert-butyldimethylsilyl ether |
| 3. | 9.03 | Ethanedioic acid, bis(TMS)ester |
| 4. | 9.73 | Pyridine, 3-trimethylsiloxy |
| 5. | 11.92 | 3-Hydroxy-6-methypyridine-1-TMS |
| 6. | 14.53 | 1,4-Dimethylpyrrolo(1,2-A)pyrazine |
| 7. | 15.45 | Docosane |
| 8. | 15.67 | 1-Galactopyranone, 6-deoxy-1,2-bis-O-(TMS)-cyclic methylboronate |
| 9. | 15.75 | Benzenepropanoic acid, TMS ester |
| 10. | 16.64 | Decanoic acid, TMS ester |
| 11. | 17.49 | Benzeneacetic acid, α,4-bis[(TMS)oxy], TMS ester |
| 12. | 18.92 | 2,4-Imidazolidinedione, 1-[[(5-nitro-2-firnayl)methylene]amino |
| 13. | 19.32 | 1-Hexadecanol, 2-methyl |
| 14. | 19.55 | Hexadecane |
| 15. | 19.71 | 5-Hydroxy-2,2-dimethyl-5,6-bis(2-oxo-1-propyl)-1-cyclohexanone |
| 16. | 20.50 | Dodecenoic acid, TMS ester |
| 17. | 21.27 | 2-Propenoic acid, oxybis(methyl-2,1-ethanediyl)ester |
| 18. | 21.93 | Benzoic acid, 2,5-bis(TMSoxy)-TMS ester |
| 19. | 22.20 | 1-Tetradecene, 2-decyl |
| 20. | 22.59 | 1,2-Propanediol, 3-(octadecycloxy)-diacetate |
| 21. | 22.93 | Anthacene |
| 22. | 23.89 | β-D-Glucopyranoside, methyl 2,3-bis-O-(TMS)-, cyclic methylboronate |
| 23. | 24.01 | 2,5-Cyclohexade-1,4-dione,2,6-bis(1,1-dimethylethyl) |
| 24. | 24.99 | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione |
| 25. | 25.15 | 2-Butanone, 4-[2-isopropyl-5-methyl |
| 26. | 27.23 | Hexadecanoic acid, methyl ester |
| 27. | 30.15 | Octadecanoic acid, methyl ester |
| 28. | 29.47 | Octatriacontyl pentafluoropropionate |
| 29. | 29.48 | 17-Pentatriacontene |
| 30. | 29.85 | 1,4-Ethano-1,2,3,4-tetrahydroanthracen-3-ol-benzylidene |
| 31. | 31.70 | Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) |
| 32. | 35.11 | Butyl-11-eicosenoate |
| 33. | 39.72 | Cholest-8(14)-en-3-one, (5α) |
| 34. | 40.59 | 3β, 5α-ergosta-8,24(28)-dien-3-ol |
| 35. | 42.10 | 24-Ethyl-delta(22) coprostenol |
| 36. | 47.10 | Stigmasta-5,22-Dien-3-ol (3β, 22E) |
| 37. | 48.14 | β-sitosterol |

RT: Retention Time (min); TMS: Trimethylsilyl

### 4.3.3.3 Isopropanol and methanol extract of PMDE

The GC-MS TIC chromatogram of PMDE extracted by isopropanol and methanol showed relatively fewer peaks than the acetone and ethyl acetate extracts of PMDE (Fig. 3a,b). However, one major peak was noted in the isopropanol extracts at RT 27.53

corresponding to acetophenone, 2-(trimethylsiloxy) (Table 4.8). Moreover, several minor peaks were observed at RT 8.54, 11.71, 12.08, 14.07, 15.35, 18.60, 22.32, 22.98, 25.88, 26.75, 29.84, 31.53, 32.93, 34.15, 35.24, 36.30, 37.54, and 39.08; however, these could not be identified because they were not available in the NIST library (Fig. 4.9a). Simultaneously, the GC-MS TIC chromatogram of methanol extract of PMDE showed major peaks at RT 9.68, 27.71, and 35.92, which corresponded to 5-methyl-2-(1-methylethyl)cycohexanol; phenol,4,4'-thiobis[2-(1,1dimethylethyl)-6-methyl; and 2,6,10,14,18,22-tetracohexaene, 2,6,10,19,23 hexamethyl, respectively (Table 4.8). Some other minor peaks were also observed at different RT that could not be identified (Fig. 4.9b) However, the roles of several other detected organic compounds in the environment have yet to be elucidated.

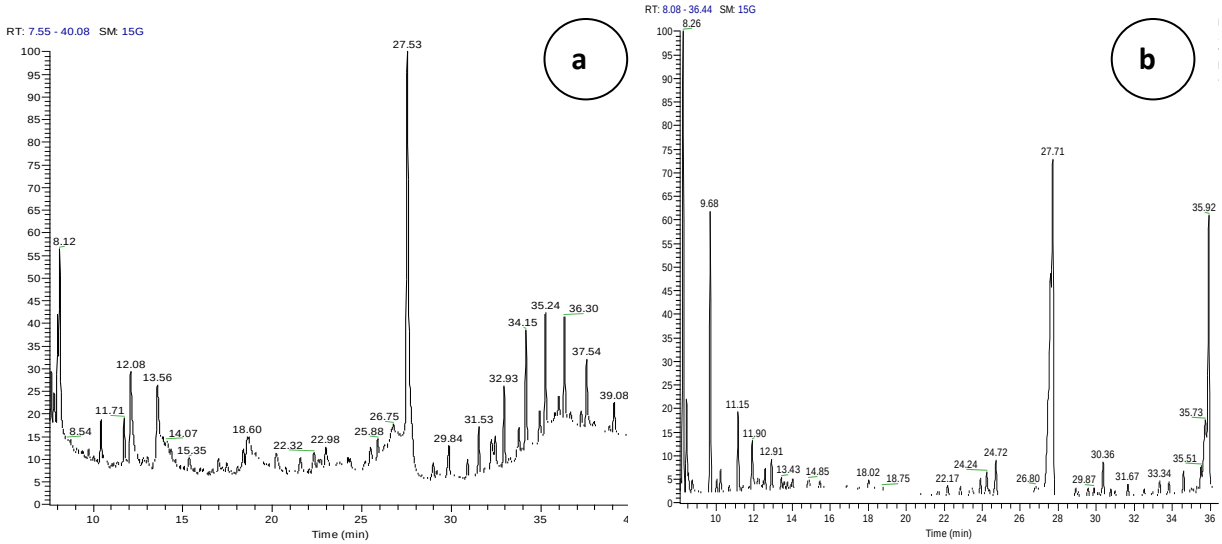



**Fig. 4.9** TIC chromatogram obtained during GC-MS analysis of PMDE; (a) isopropanol extract and (b) methanol extract

**Table 4.8** Organic compounds identified by GC-MS in (a) isopropanol extract and (b) methanol extract of PMDE

| **(a)** | | |
|---|---|---|
| **SI. No.** | **RT** | **Name of Compound** |
| 1. | 8.11 | D-Lactic acid-DITMS |
| 2. | 12.09 | 3-Ethylphenol, trimethylsilyl ether |
| 4. | 27.53 | Acetophenone, 2-(trimethylsiloxy) |
| **(b)** | | |
| 1. | 9.68 | 5-methyl-2-(1-methylethyl)cycohexanol |
| 2. | 27.71 | Phenol,4,4'-thiobis[2-(1,1dimethylethyl)-6-methyl |
| 3 | 30.35 | Octadecanoic acid |
| 4. | 33.33 | Cholest-5-en-7-one,3-ol |
| 5. | 35.92 | 2,6,10,14,18,22-tetracohexaene, 2,6,10,19,23 hexamethyl |
| 6. | 38.96 | 3β, 5α-ergosta-8,24(28)-dien-3-ol |
| 7. | 40.09 | 24-Ethyl-delta(22) coprostenol |
| 8. | 41.34 | Stigmasta-5,22-Dien-3-ol (3β, 22E) |
| 9. | 41.55 | β-sitosterol |

### 4.3.3.4 Ethanol and *n*-hexane extract of PMDE

The GC-MS TIC chromatogram of PMDE extracted by ethanol and *n*-hexane showed few major peaks (Fig. 4.10). However, major peaks were noted in ethanol extracts at RT 13.71, 27.99, 34.21, 34.52, and 36.14, corresponding to ethanedioic acid, bis(TMS)ester; tentradecanoic acid, TMS ester; hexadecanoic acid, 2-[(TMS)oxy]-1-[[(TMS)oxy]methyl)ethyl ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; and octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester, respectively. Simultaneously, the GC-MS TIC chromatogram of *n*-hexane extract of PMDE showed major peaks at RT 27.94, 34.51, and 36.12, corresponding to benzoic acid, 3,4,5-tris(TMSoxy)-TMS ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; and octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester, respectively. Some other minor peaks were also observed at different RT that could not be identified. However, the detected compounds (hexadecanoic acid, octadecanoic acid, stimgmasterol, benzoic acid, and β–sitosterol) have been listed as potential EDCs by the USEPA (2012).

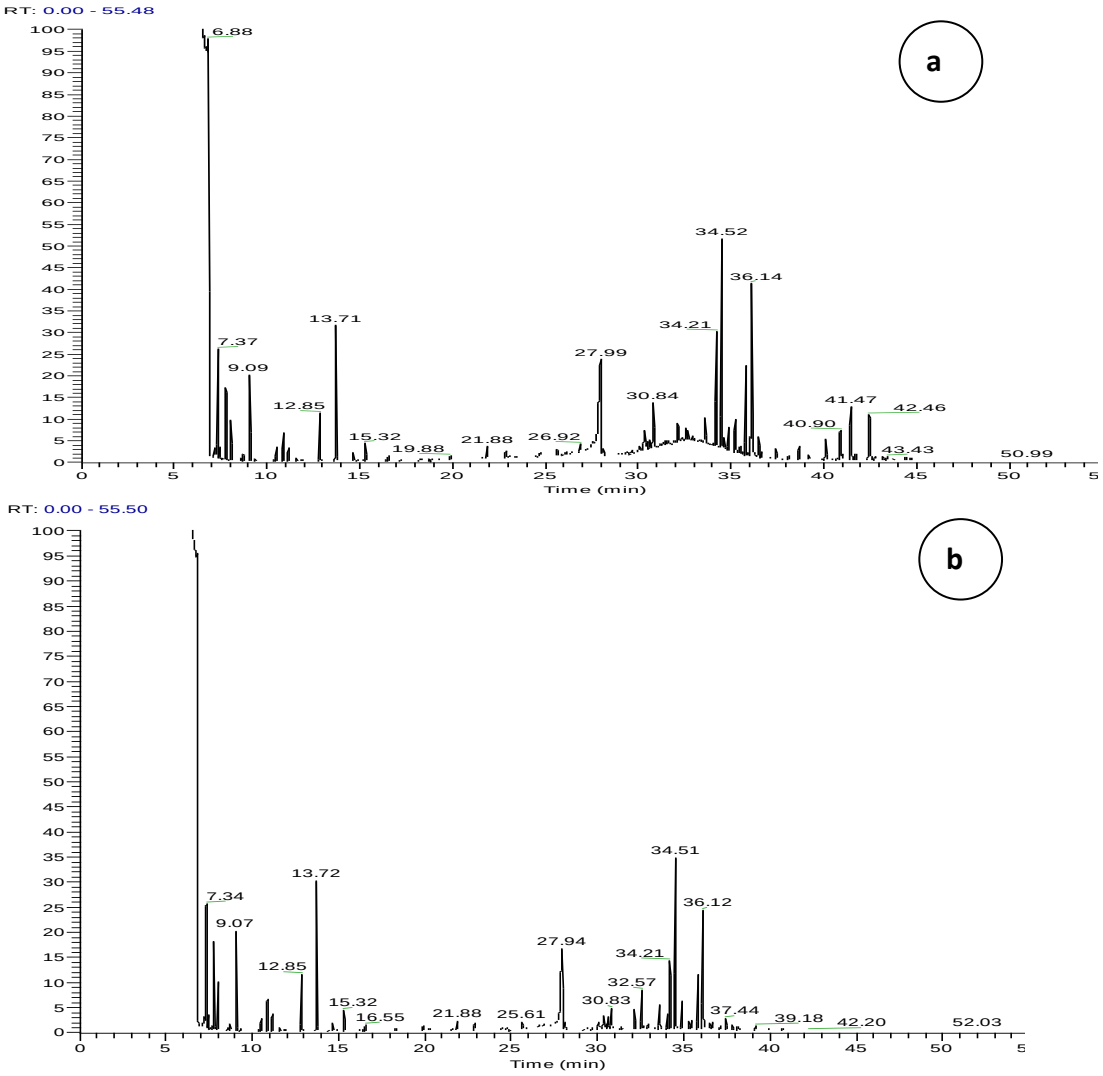



**Fig. 4.10** TIC chromatogram obtained during GC-MS analysis of PMDE; (a) ethanol and (b) *n*-hexane extract

**Table 4.9** Organic compounds identified by GC-MS in (a) ethanol (b) *n*-hexane extract of PMDE

| **(a)** | | |
|---|---|---|
| **S. No.** | **RT** | **Name of Compound** |
| 1. | 13.71 | Ethanedioic acid, bis(TMS)ester |
| 2. | 27.99 | Tetradecanoic acid, TMS ester |
| 3. | 30.32 | Hexadecanoic acid, 3,7,11,15-tetramethyl-,trimethyl ester |
| 4. | 30.84 | Octadecanoic acid, TMS ester |
| 5. | 34.21 | Hexadecanoic acid, 2-[(TMS)oxy]-1-[[(TMS)oxy]methyl)ethyl ester |
| 6. | 34.52 | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 7. | 35.82 | 2-Monostearin TMS ether |
| 8. | 36.14 | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 9. | 36.48 | 2,6,10,14,18,22-Tetracosahexane,2,6,10,15,19,23-hexamethyl |
| 10. | 38.68 | Hexacosanoic acid |
| 11. | 40.11 | Silane,[[(3β,22E)-ergosta-7,22-dien-3-yl]trimethyl |
| 12. | 42.46 | Silane, trimethyl[[(3β,5α)-stigmastan-3-yl]oxy]- |
| 13. | 43.19 | Stigmasterol TMS ether |
| 14. | 44.41 | β-sitosterol TMS ether |
| **(b)** | | |
| 1. | 13.72 | Ethanedioic acid, bis(TMS)ester |
| 2. | 27.94 | Benzoic acid, 3,4,5-tris(TMSoxy)-TMS ester |
| 3. | 32.57 | Hexanedioic acid, dicotyl ester |
| 4. | 34.51 | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 5. | 35.31 | Heptacosane |
| 6. | 35.81 | 2-Monostearin TMS ether |
| 7. | 36.12 | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 8. | 37.81 | Benzenamine, 4-octyl-N-(4-Octylphenyl) |
| 9. | 40.70 | Silane, [[(3β)-cholest-5-en-3-yl |

RT: Retention Time (min); TMS: Trimethylsilyl

### 4.3.4 Toxicity of DSW and PMDE

The seed germination test is a common and basic tool for toxicity evaluation of the environmental safety of industrial waste (OECD 2003). The comparative seed germination test of green gram (*P. mungo* L.) and wheat *(T. aestivum*) showed that DSW was more toxic to *T. aestivum* than *P. mungo* L. (Fig. 4.11a and Table 4.10). Consequently, no seed germination was observed in *T. aestivum* at 4% DSW, while 20% seed germination was noted in *P. mungo* L. at the same concentration (Fig. 4.11b). In contrast to DSW, the comparative seed germination test of *P. mungo* L. and *T. aestivum* showed that 85% seed germination was observed in *P. mungo* L. at 5%, while 26% seed germination was noted in *T. aestivum* at the same concentration (Fig. 4.12a and Table 4.11). No seed germination was observed in *P. mungo* L. and *T. aestivum* at 10% concentration of PMDE (Fig. 4.12b). *P. mungo* L. has a seed coat around the embryo, while *T. aestivum* does not. Therefore, pollutants in DSW and PMDE might impact the embryo of *T. aestivum*, while merely penetrating the seed coat of *P. mungo* L., which would explain the differences in its effects. Similar patterns have been reported in previous studies of *T. aestivum* by Pandey et al. (2007, 2008). Moreover, radical growth inhibition showed a similar pattern (Fig.

4.11 and 4.12). Further, a study conducted by Chandra et al. (2008a) revealed that distillery sludge amended soil showed delayed flowering and reduced pod formation in *P. mungo* L., which apparently indicated suppression of reproductive hormones. These findings supported the presence of high levels of EDCs in DSW and PMDE along with other toxic compounds.

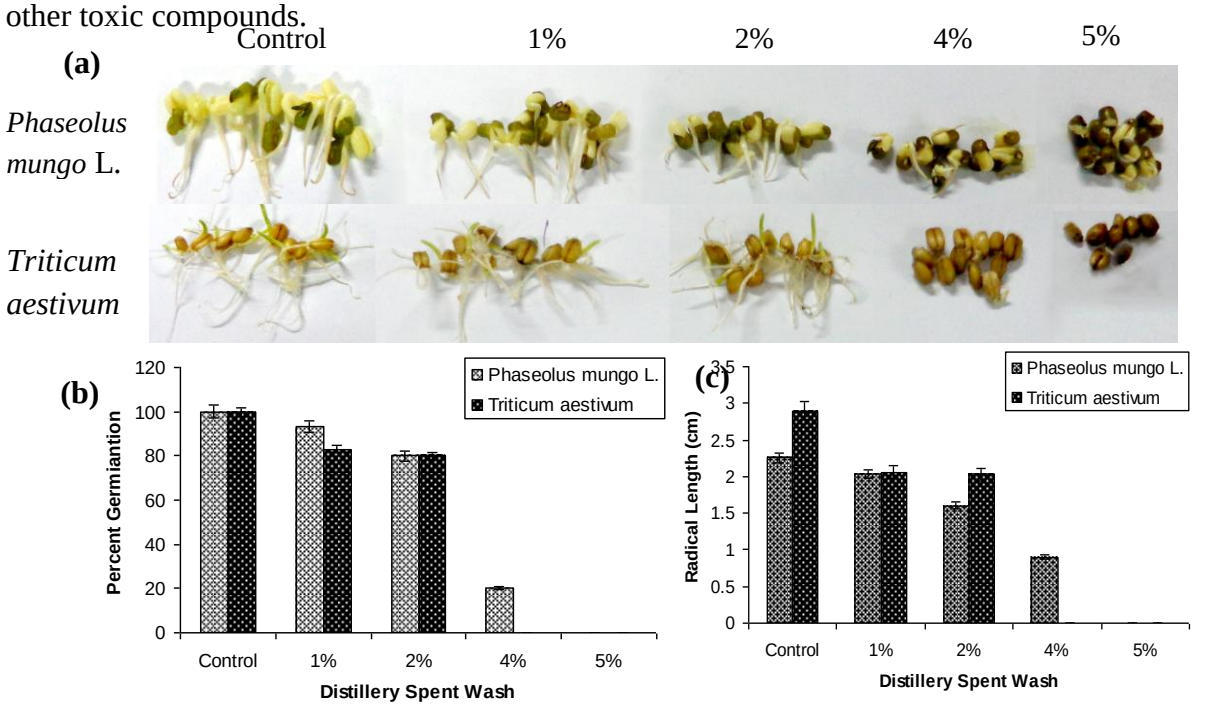



**Fig. 4.11** Effects of various concentrations of DSW on *Phaseolus mungo* L. and *Triticum aestivum* seeds vs. control (tap water): (a) morphological effect, (b) percent germination, and (c) radical length

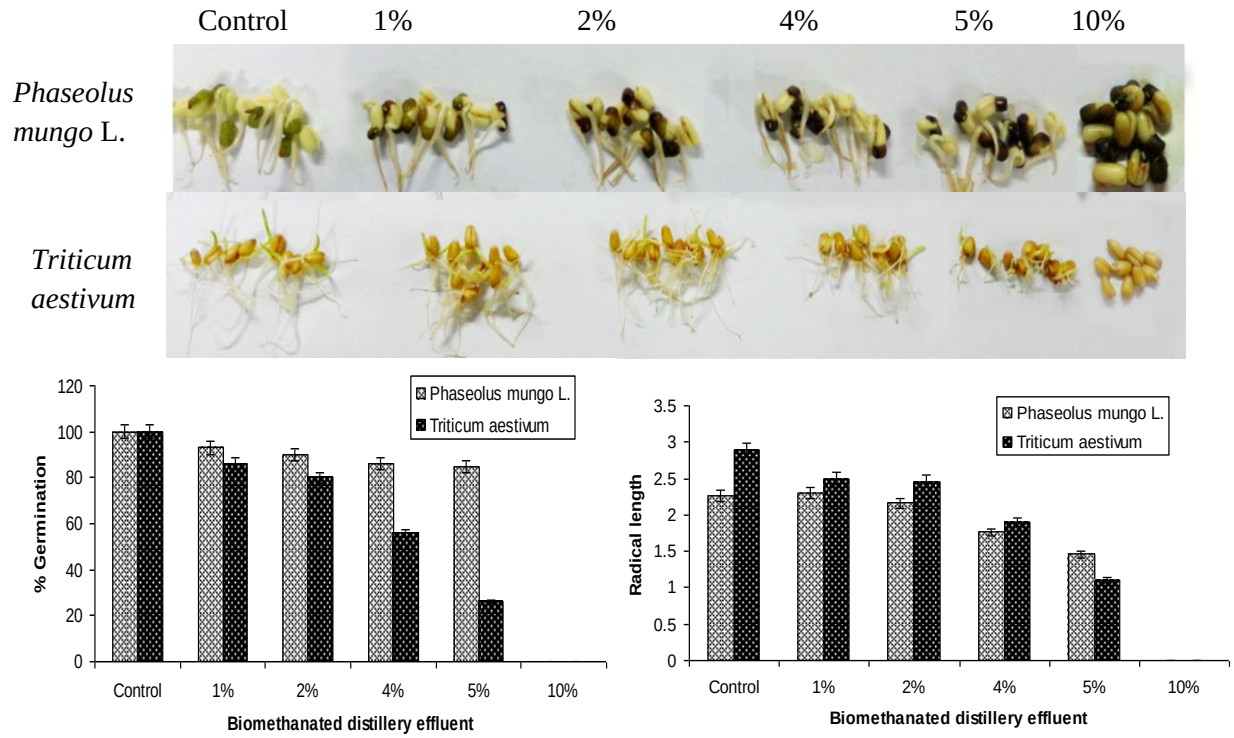



**Fig. 4.12** Effects of various concentrations of PMDE on *Phaseolus mungo* L. and *Triticum aestivum* seeds vs. control (tap water): (a) morphological effect (b) percent germination, and (c) radical length

**Table 4.10** Effects of various concentrations of DSW on the growth of *Phaseolus mungo* L. and *Triticum aestivum*

| Plant | Treatment | No. of seed sown | Germination (%) | Relative toxicity | Radical length (cm) | Phytotoxicity (%) | Radical growth inhibition (cm) | Stress tolerance index |
|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 100±0.00 | 00±00 | 2.26±0.01 | 00±00 | NI | 00 |
| *Phaseolus mungo* L. | 1% | 10 | 93.30±2.89 | 6.70±0.35 | 2.03±0.06 | 10.17±0.01 | 0.23±0.01 | 0.89±0.01 |
| | 2% | 10 | 80±2.40 | 20±0.71 | 1.60±0.04 | 29.20±0.02 | 0.66±0.04 | 0.70±0.02 |
| | 4% | 10 | 20±0.84 | 80±2.40 | 0.90±0.02 | 60.17±0.01 | 1.36±0.03 | 0.39±0.00 |
| | 5% | 10 | NG | NG | NG | NG | NG | NG |
| *Triticum aestivum* | Control | 10 | 100±0.00 | NI | 2.90±0.09 | - | NI | - |
| | 1% | 10 | 83±4.50 | 17±±0.68 | 2.06±0.06 | 28.96±0.01 | 0.84±0.01 | 0.71±0.01 |
| | 2% | 10 | 80±3.88 | 20±±0.60 | 2.03±0.08 | 30.00±0.02 | 0.87±0.02 | 0.70±0.00 |
| | 4% | 10 | NG | NG | NG | NG | NG | NG |
| | 5% | 10 | NG | NG | NG | NG | NG | NG |

All value are mean (n=3) ± S.D., NG= No growth or germination was observed, NI= No inhibition was observed, Control=tape water

**Table 4.11** Effects of various concentrations of PMDE on the growth of *Phaseolus mungo* L. and *Triticum aestivum*

| | **Treatment** | **No. of seed sown** | **Germination (%)** | **Relative toxicity** | **Radical Length (cm)** | **Phytotoxicity (%)** | **Radical growth inhibition (cm)** | **Stress tolerance index** |
|---|---|---|---|---|---|---|---|---|
| | Control (tap water) | 10 | 100±0.00 | 00±00 | 2.26±0.07 | NI | NI | 00 |
| *Phaseolus mungo* L. | 1% | 10 | 93±0.00 | 07±0.01 | 2.03±0.01 | 7.0±0.00 | 0.23±0.01 | 0.89±0.01 |
| | 2% | 10 | 90±0.00 | 10±0.02 | 2.16±0.01 | 10±0.00 | 0.1±0.00 | 0.95±0.01 |
| | 4% | 10 | 86±0.00 | 14±0.00 | 1.76±0.01 | 14±0.00 | 0.5±0.00 | 0.77±0.01 |
| | 5% | 10 | 85±0.00 | 15±0.01 | 1.46±0.02 | 15±0.00 | 0.8±0.00 | 0.64±0.00 |
| | 10% | 10 | NG | | NG | - | -- | -- |
| *Triticum aestivum* | Control (tap water) | 10 | 100±0.00 | 00 | 2.90±0.09 | NI | NI | -- |
| | 1% | 10 | 86±0.00 | 14±0.01 | 2.50±0.01 | 14±0.00 | 0.4±0.00 | 0.86±0.01 |
| | 2% | 10 | 80±0.00 | 20±0.10 | 2.46±0.02 | 20±0.00 | 0.44±0.01 | 0.84±0.01 |
| | 4% | 10 | 56±0.00 | 44±0.02 | 1.90±0.01 | 44±0.00 | 1.09±0.01 | 0.65±0.02 |
| | 5% | 10 | 26±0.00 | 74±1.01 | 1.10±0.01 | 74±0.00 | 1.8±0.02 | 0.37±0.01 |
| | 10% | 10 | NG | | NG | - | | |

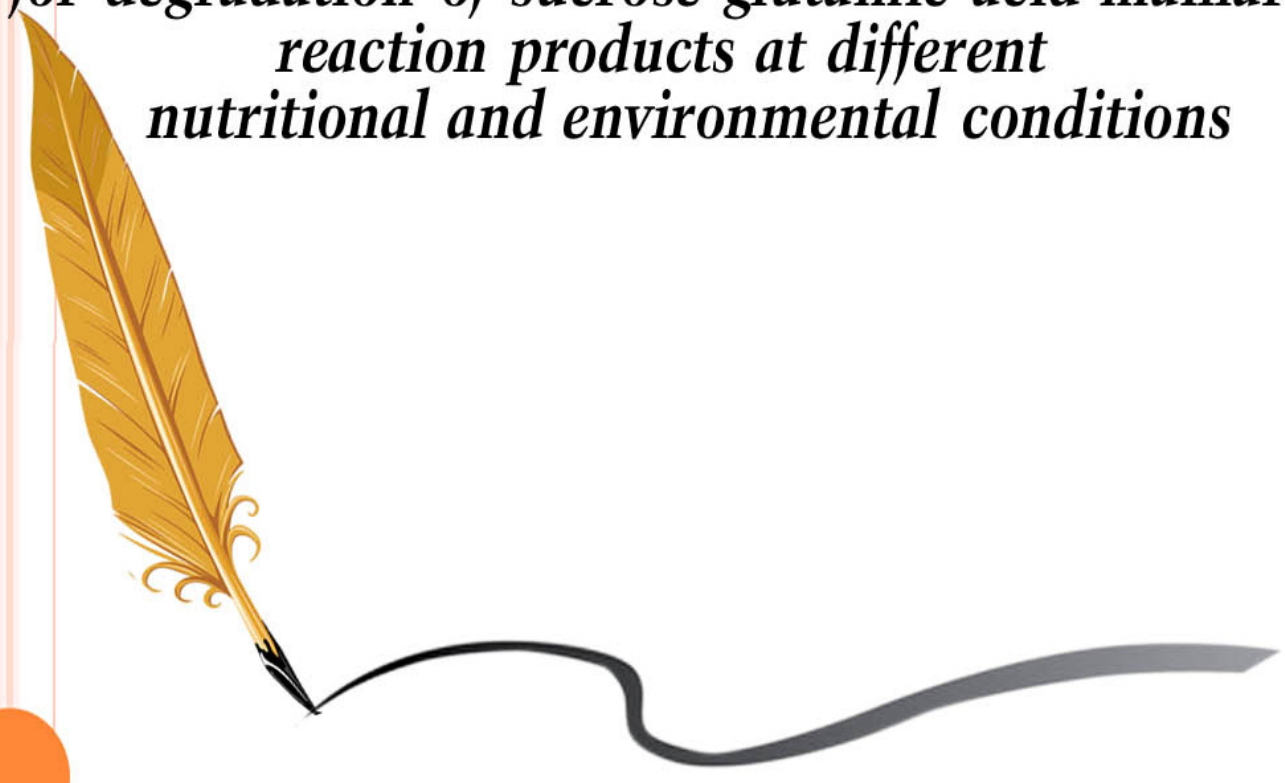
All value are mean (n=3) ± S.D., NG= No growth or germination was observed, NI= No inhibition was observed

## Conclusion

This study revealed that sugarcane molasses-based DSW contains several EDCs, as well as other complex organic pollutants that are generated during the fermentation process or inherited from molasses. PMDE also contain several residual recalcitrant organic pollutants plus heavy metals, the majority of which are environmental toxicants, endocrine disrupting chemicals (i.e., β-sitosterol) and also DNA fragmentation inducers (i.e., tetradecanoic acid), which are still fairly unknown. Moreover, a phytotoxicity assay with *P. mungo* and *T. aestivum* revealed the presence of highly toxic compounds in the DSW and PMDE. As a result, DSW poses a strong environmental risk to flora and fauna in the ecosystem; nevertheless, they are discharged into systems where they may impact biota and benthic organisms.

# Chapter-05

# *Isolation and characterisation of manganese peroxidase and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard reaction products at different nutritional and environmental conditions*



# Chapter-5

## Isolation and characterisation of manganese peroxidase and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard reaction products at different nutritional and environmental conditions

### 5.1 Introduction

In recent years, the biological approaches with microbial decolourisation process of effluent for optimisation of various parameters at laboratory conditions have drawn attention of various workers world over to explore the feasible technology because it may lead eco-sustainable and cost effective alternative to chemical methods (Kaushik et al. 2010). Some worker have reported the bacterial decolourisation and degradation of sugarcane molasses-melanoidins as well as model melanoidins both (Kumar and Chandra 2006; Chandra et al. 2009; Bharagava and Chandra 2009). But, due to complex nature of melanoidins in sugarcane molasses with mixture of various other organic compounds it shows variable absorption range which makes more difficult to understand the mechanism of melanoidins decolourisation and characterization of its metabolic products. Therefore, prior to attempting the degradation and decolourisation of distillery effluent, the degradation of model melanoidins with mixture of complex MRPs should be evaluated for its degradability. But, no any such study has been reported so far for assessment of melanoidins degradation as whole to assess the capability of bacterial consortium. Therefore, the main objective of this study was to investigate the MnP and laccase producing bacterial consortium capability for the degradation and decolourisation of complex sucrose glutamic acid-maillard reaction products (SGA-MRPs), which are predominantly present in sugarcane juice composition which generates the complex of molasses melanoidins due to thermal degradation reaction at subsequent stage (Walford 1996; Eggleston and Vercellotti 2000). Further, the change in absorption peaks (200-700 nm) by UV-Vis spectrophotometric analysis has been correlated for their structural changes and reduction in color by investigation through FT-IR, HPLC, and GC-MS analysis. Furthermore, to evaluate the stability of potential bacterial consortium at variable nutritional and environmental conditions was assessed for optimum decolourisation of SGA-MRPs. The reduction of toxicity after bacterial degradation was also evaluated by using seed germination test of *Phaseolus mungo* L.

### 5.2 Material and Methods

#### 5.2.1 Collection of distillery sludge sample

The sludge sample was collected from the dumping site of M/s Unnao Distilleries and Breweries, located in Unnao, Uttar Pradesh (26°320″N,80°30′0″E), India for isolation of potential bacterial strains (Fig. 5.1).


**Fig. 5.1** Distillery sludge discharged from anaerobic reactor after methanogenesis of spent wash (a) fresh distillery sludge (b) degraded distillery sludge at dumping site of M/s Unnao Distilleries and Breweries Ltd.

### 5.2.2 Isolation and screening of melanoidins tolerant, MnP, and laccase producing bacterial strains

For isolation of bacteria strains, 10 g of sludge sample was transferred to a 250 mL Erlenmeyer flask containing 100 mL sterile GPM broth (g $L^{-1}$; glucose 1%, peptone 0.1%, $MgSO_4.7H_2O$ 0.05 %, $K_2HPO_4$ 0.1%). The flasks were incubated at 37±1 °C in a refrigerated incubator shaker (Orbitek, Scigenic Biotech, India) at 120 rpm for 7 days. The flask containing the sample showing the decolourisation was selected (Fig. 5.2). Subsequently, an aliquot (1.0 mL) was taken serially diluted in order of $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$, then after diluted sample (0.1 mL) was spread on GPM agar medium plates. Further, the bacterial colonies showing clear zone on agar plates were purified on GPM agar plate by streak plate method. To investigate, the bacterial tolerance on melanoidins purified bacterial strains were spread on pre-sterilised GPM agar medium plate amended with different concentration of molasses melanoidins (1000, 1500, 2000, 2500, 3000, and 3500 mg $L^{-1}$ w/v). Each plate was incubated at 37±1 °C for 48 h and observed for growth of tolerant bacterial colonies. The bacterial strains tolerant to high concentration of melanoidins were selected and screened for MnP (EC 1.11.1.13) and laccase (EC 1.10.3.2) activity by plate assay method. For enzyme screening, the substrate used for MnP was phenol red (Lobachemie, India) (Pangallo et al. 2007), while laccase activity was detected in presence of guaiacol (Sigma, USA) as a substrate in B&K agar medium containing (g $L^{-1}$) dextrose 1%, peptone 0.5%, beef extract 0.3%, NaCl 0.5%, and $CuSO_4$ (1 mM) (D'Souza et al. 2006). The conversion of dark pink to yellow colour indicated the presence of MnP activity and reddish brown colour zones in the medium indicated the presence of laccase activity.

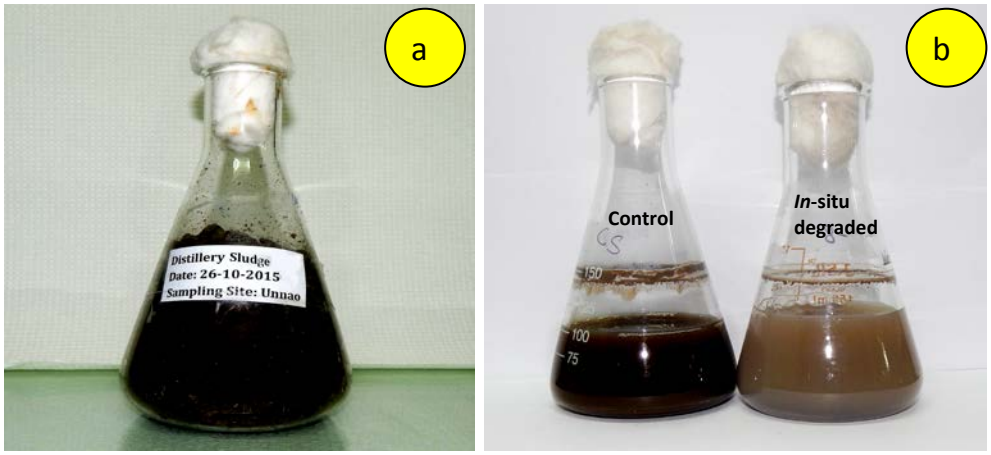



**Fig. 5.2** Isolation of melanoidin degrading bacteria from distillery sludge (a) collected distillery sludge sample (b) Distillery sludge sample for enrichment of bacteria (a) flask showing decolourisation of sludge leachate after 7 days of incubation

### 5.2.3 Identification of bacterial strains

#### 5.2.3.1 Morphological and biochemical characterisation

The isolated bacterial strains were identified based on morphological and biochemical characteristics by using the standard procedure described by Barrow and Feltham (2003).

##### 5.2.3.1.1 Gram Staining

**(a) Principle**

Christian Gram discovered the gram staining, a differential staining method which differentiates bacteria either into Gram positive or Gram negative. Staining is based on the principle of component of cell wall of bacterial cell. The Gram-negative bacterial cell wall is thin, complex, multilayered structure and contains relatively high lipid contents in addition to protein and mucopeptide. The high lipid content is readily dissolved by alcohol resulting in the formation of large pores in the cell wall, which does not close appreciably on dehydration of cell wall protein. Thus, facilitating the leakage of crystal violet iodine complex and resulting in the decolourization of the bacterium that later take the counter strain and appears pink. In contrast, the gram positive bacterial cell wall is thick and chemically simple, mainly composed of protein and cross linked by mucopeptide. This cell wall, when treated with alcohol causes dehydration and closure of cell wall pores thereby not allowing the loss of crystal- iodine complex and cells remains violet.

**(b) Reagents**

**Ammonium oxalate-crystal violet stain**

**Solution A**

| | | |
|---|---|---|
| Crystal violet | : | 10 g |
| Ethanol (95%) | : | 100 mL |

Mixed and dissolved

**Solution B**

| | | |
|---|---|---|
| Ammonium oxalate | : | 1 g |

Distilled water : 100 mL
For use, mixed 2 mL of solution A and 80 mL of solution B
**Solution A + Solution B = Crystal violet**
**Lugol's iodine**
Iodine : 5 g
Potassium iodide (KI) : 10 g
Distilled water : 100 mL
Dissolved the iodide and iodine in some of the water and adjusted to 100 mL with distilled water.
**Ethyl alcohol 95 %**
95 ml ethyl alcohol + 5 ml distilled water
**Safranine**
Safranine : 2.5 g
Ethyl alcohol 95% : 10 mL
These were added in 100 mL distilled water

**(d) Procedure**
Bacterial cells were grown on nutrient agar plates to mid log phase and a smear of cells was prepared on a clean microscopic slide. The slides were flooded with ammonium oxalate-crystal violet stain for one min and then wash with distilled water. Now, apply Lugol's iodine solution for half min and after then the iodine solution was drain off but do not wash. The smear was decolourized with a few drops of acetone and washed thoroughly with water. Counter stain the slides with 0.5% safranin for half min, washed again and stand slide on end to drain or blot dry and then observed microscopically.

**(e) Interpretation**
**Violet colour:** Gram positive; **Pink colour:** Gram negative

**5.2.3.1.2 Motility**

**(a) Principle**
Bacterial strain may be flagellated or non-flagellated. When bacterial strains are flagellated then it shows motility or movement.

**(b) Composition of Motility Media (g $L^{-1}$)**
Peptone : 10 g
Mean extract : 3 g
NaCl : 5 g
Agar : 4 g
Gelatin : 80 g
Distilled water : 1000 mL
Soaked the gelatin in water for 30 min, added other ingredients, heated to dissolve and sterilized at 115 ºC for 20 min.

**(c) Procedure**

The motility medium was stab-inoculated with a straight needle to a depth of about 5 mm. The tube was left overnight incubation (at or below the optimum growth temperature).

**(d) Interpretation**

Motile organisms migrate throughout the medium, which becomes turbid in semi-solid media was scored positive for motility. Growth of non-motile organisms is confined to the stab inoculum was showing negative.

**5.2.3.1.3 Catalase**

**(a) Principle**

Catalase acts as a catalyst to breakdown the hydrogen peroxide into oxygen and water. An organism is tested for catalase activity by bringing it into contact with hydrogen peroxide. Oxygen bubbles are released which is the gaseous product of enzymatic activity, which indicated the liberation of oxygen and presence of bacterial catalase enzyme. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. $H_2O_2$ is lethal to bacterial cells.

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O+O_2$$

**(b) Procedure**

The test was done by placing a drop of 3-6% $H_2O_2$ on a microscope slide. Then, by using an applicator stick, touch the colony and formed a smear on slide. Slide was observed for the formation of bubbles.

**(c) Interpretation**

Rapid effervescence or production of gas bubbles (molecular oxygen) has indicated the positive test.

**5.2.3.1.4 Oxidative-fermentative (O-F) test**

**(a) Principle**

O.F. medium contains low concentration of peptone, high concentration of carbohydrate and low concentration of agar as comparison to carbohydrate fermentative medium. The lower protein to carbohydrate ratio reduces the formation of alkaline amines that can neutralize the small quantities of weak acid that may form oxidative metabolism. The relatively large amount of carbohydrate serves to increase the amount of acid that can potentially be formed. The semi solid consistency of the agar permits acids that form on the surface of agar to permeate through the medium, making interpretation of pH shift of the indicator easier to visualize.

**(b) Composition of O.F. medium (g $L^{-1}$)**

Peptone : 2 g

| | | |
|---|---|---|
| NaCl | : | 5 g |
| $K_2HPO_4$ | : | 0.3 g |
| Agar | : | 3 g |
| Distilled water | : | 1000 mL |
| Glucose | : | 1% |
| BCP | : | 0.2% aqueous solution (15 mL) |

**(c) Procedure**

O.F. medium constituents were dissolved in 1000 mL double distilled water, heated to dissolved the solids and pH was adjusted to 7.1, filtered and added the indicator, sterilized at 115 ºC for 20 min. Added a sterile solution of the appropriate carbohydrate aseptically to give a final concentration of 1%. Mixed and distributed aseptically into sterile tubes of not more than 16 mm diameter.

Glucose was autoclaved separately to low temperature after that these were mixed aseptically to medium, inoculated the duplicate tubes by stabbing with a sterilized straight wire. To one of the tubes added a layer of melted soft liquid paraffin to a depth of about 1 cm. Incubated and examined daily for up to 14 days.

**(d) Interpretation**

| **Open Tube** | **Covered tube** | |
|---|---|---|
| Oxidation | Acid (yellow) | Alkaline (green) |
| Fermentation | Acid (yellow) | Acid (yellow) |
| Non-Sacchrolytic | blue or green | Alkaline (green) |

**5.2.3.1.5 Indole Production**

**(a) Principle**

The test organisms were grown in a media containing tryptophan. Indole is a volatile substance (easily vaporized) and its production was detected by Kovac's reagent, which contained (*p*)-dimethylamino benzaldehyde. This reacted with indole producing red coloured compound.

**(b) Kovac's reagent for indole (g $L^{-1}$)**

| | | |
|---|---|---|
| p-dimethylamino benzaldehyde: | | 5 g |
| Amyl alcohol | : | 75 mL |
| Conc. HCl | : | 25 mL |

**(c) Procedure**

Dissolved *p*-dimethylamino benzaldehyde in alcohol by gently warming in a water bath at 50-55 ºC, cooled and added the acid with care. Protected from light and stored at 4 ºC.
Inoculated peptone water or nutrient broth and incubated for 48 h. Added 0.5 mL Kovac's reagent for indole production, shaken well and examined after about 1 min.

**(d) Interpretation**

A red colour in the reagent layer indicates indole production.

#### 5.2.3.1.6 Citrate utilization test

**(a) Principle**

Carbon source utilization and their application to identification are limited mainly to tests for the utilization of citrate. Other citrate media, such as Christensen's contain additional nutrients.

**(b) Reagents and media**

The test was performed by using HiMedia Rapid Biochemical Identification kit, [Enterobacteriaceae Identification Kit (KB002 HiAssorted®)] for gram negative rod. KB002 is the comprehensive test system used for identification of gram negative Enterobacteriaceae species and other non-fastidious, Gram-negative rods.

**(c) Procedure**

The biochemical strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24 h. The indices obtained after reading and results were interpreted using the HiMedia result interpretation chart supplied with Biochemical Identification kit. The organisms were identified to species level. The bromothymol blue pH indicator is a deep forest green at neutral pH. If citrate is present, a degradation product is produced which increase in medium pH to above 7.6, bromothymol blue changes to blue. The blue color indicates the positive result.

**(c) Interpretation**

**Blue color and streak of growth:** Citrate utilized
**Original green color of medium:** Citrate not utilized

#### 5.2.3.1.7 Lysine utilisation

**(a) Principle**

The purpose is to see if the microbe can use the amino acid lysine as a source of carbon and energy for growth. Use of lysine is accomplished by the enzyme lysine decarboxylase. This enzyme attack the carboxylic group of amino acid lysine, with formation of amine cadaverine. These by-products are sufficient to raise the pH of the media so that the broth turns purple.

**(b) Reagents and media**

The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**

The strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24 h. The indices obtained after reading the results were interpreted using the HiMedia result interpretation chart supplied with Biochemical Identification kit.

**(d) Interpretation**

If the inoculated medium is light purple, or if there is no color change, the organism is decarboxylase-negative for that amino acid. If the medium turns dark purple, the organism is decarboxylase-positive for that amino acid.

**5.2.3.1.8 Ornithine utilisation**

**(a) Principle**

Ornithine Decarboxylase is used for detection of the ability of microorganisms to decarboxylate ornithine. Decarboxylation is the process in which bacteria that possess specific decarboxylase enzyme attack amino acids at their carboxyl end (-COOH) to yield an amine or a diamine and carbon dioxide. The amino acid L-ornithine is decarboxylated by the enzyme ornithine decarboxylase to yield the diamine putrescine and carbon dioxide.

**(b) Reagents and media**

The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**

The procedure of this test was same as described in section 5.2.3.1.7.

**(d) Interpretation**

The production of this amine elevates the pH of the medium towards alkalinity, changing the color of the indicator from light purple to dark purple. If the organism does not produce the appropriate enzyme, the medium remains light purple.

**5.2.3.1.9 Urease activity**

**(a) Principle**

The test organisms were cultured in a medium containing urea and indicator phenol red. If the stain is urease producing, the enzyme will break down the urea by hydrolysis to give ammonia and $CO_2$ with the release of ammonia. The medium becomes alkaline as shown by a change in colour of the indicator to red-pink.

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$$

**(b) Reagents and media**

The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**

The procedure of this test was same as described in section 5.2.3.1.7.

**(c) Interpretation**

**Pink color and streak of growth:** Urea Utilised

**Organish yellow color of medium:** Urea not utilized

### 5.2.3.1.10 Phenylalanine deaminase

**(a) Principle**

This test determines whether the microbe produces the enzyme phenylalanine deaminase, which is needed for it to use the amino acid phenylalanine as a carbon and energy source for growth.

**(b) Reagents and media**

The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**

The procedure of this test was same as described in section 5.2.3.1.7.

**(d) Interpretation**

If phenylalanine deaminase is present, a degradation product is produced from phenylalanine. The product combines with iron compounds in an acidic environment to produce a green color. The green color indicates the positive result.

### 5.2.3.1.11 Nitrate reduction

**(a) Principle**

Nitrate reduction may be shown either by detecting the presence of one of the breakdown products or by showing the disappearance of nitrate from the medium. The products of reduction may include nitrite, hyponitrite, hydroxylamine, ammonia, nitrous oxide or gaseous nitrogen. The first test to be applied aims at showing the presence of nitrite. When this test is negative (i.e. nitrite is not detected) the medium is tested to see whether there is residual nitrate, if this test also is negative it confirms that the first stage of the breakdown has been completed and the nitrite further broken down. In uninoculated nitrate broth and with cultures of organisms that do not reduce nitrate, the test for nitrite is negative until zinc dust or other reducing agent is added to the culture medium to reduce the nitrate contained in it. To detect small amounts of residua nitrate the amount of zinc added may be critical. The tests are very sensitive and it is important to check the uninoculated medium for nitrite, which should not be present.

**(b) Reagents and media**

The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**

The procedure of this test was same as described in section 5.2.3.1.7.

**(d) Interpretation**

Appearance of pinkish red color, which showed the presence of nitrite and thus shows that nitrate has been reduced, indicates a positive reaction.
Tubes not showing red colour within 5 min, added powdered zinc and allowed to stand.
**Pinkish Red color:** Nitrate present in medium (i.e. not reduced by the organism).
**Absence of red color (colorless):** Nitrate absent in medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced).

#### 5.2.3.1.12 Hydrogen sulfide ($H_2S$) production test

**(a) Principle**
The $H_2S$ test is one that can be made as sensitive as required with an adequate sulfur source (cysteine) and a delicate indicator (lead acetate papers) almost all the enteric bacteria can be shown to be able to produce $H_2S$. Tested in this way an accurate estimation can be obtained of an organism's catabolic power in relation to sulfur compounds, but it is not possible to distinguish readily between those organisms with much and those with little ability to produce $H_2S$. With a poor medium or a less sensitive (ferrous chloride or lead acetate in the medium) only the strong $H_2S$ producer are detected.

**(b) Reagents and media**
The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(c) Procedure**
The procedure of this test was same as described in section 5.2.3.1.7.

**(d) Interpretation**
**Black colour:** $H_2S$ produced by organism
**Organish Yellow color (colorless):** $H_2S$ not produced by organism

#### 5.2.3.1.13 Sugar fermentation test

**(a) Principle**
The sugar fermentation tests were performed for the detection of acid and gas production by isolated bacterium. The test was performed in a fermentation tube as well as kit that contained Durham tube (a small tube placed in inverted position in the fermentation tube) for the detection of gas production, as an end product of metabolism.

**(b) Reagents and media**
The reagent and media used in this test was same as described in section 5.2.3.1.6.

**(b) Procedure**
To perform sugar fermentation test (i.e. Glucose, adonitol, lactose, arabinose, sorbitol) the biochemical strips were inoculated with isolated bacterium suspension and incubated at 37 °C for 24-48 h. The indices obtained after reading the results were interpreted using the

HiMedia result interpretation chart supplied with Biochemical Identification kit. In addition, to perform sugar fermentation test total seven fermentation medium were prepared that contain different different sugar (Glucose, Sucrose, Maltose, Lactose, Cellobiose, Mannitol, Raffinose). Inoculation the isolated bacteria in to each fermentation tube and keep one a inoculated tube of each fermentation broth as a comparative control. After inoculation the fermentation tubes were incubated at 35 °C for 24-48 hours.

**(d) Interpretation**

**Yellow color:** Glucose, adonitol, lactose, arabinose, and sorbitol was fermented by bacteria

**Pinkish Red/Red:** Glucose, adonitol, lactose, arabinose, and sorbitol was not fermented by bacteria

### 5.2.3.2 Molecular characterisation

The isolated potential MnP and laccase producing bacterial strains were characterized by 16S rRNA gene sequence analysis. The total genomic DNA from the overnight growth culture of bacterial consortium was extracted using the method described by Kapley et al. (2001). Briefly, about 5 µl DNA sample was used to amplify the 16S rDNA gene using universal eubacterial 27F and 1492R primers (Eden et al. 1991) in a reaction mixture which contained 1×PCR buffer, 3.0 mM $MgCI_2$, 200 µM of each of dNTPs, and 2.5U of *Taq* polymerase in a final volume of 50 µl. The thermocycling program was as follows: initial denaturation at 94 °C for 1 min, primer annealing at 55 ºC for 1 min, and a final extension at 72 °C for 3 min for a total of 35 cycles. The PCR amplicon were electrophoresed through 1.2% (w/v) agarose gel in 1× TAE buffer using 1 Kb DNA ladder (M/s Merck Biosciences, India), then visualized by staining with ethidium bromide. Finally, the amplified 16S rDNA gene amplicon were purified from gel using a PCR cleanup kit (Merck Biosciences, India) and sequenced using 1492R and 27F eubacterial primer in an automated DNA sequencer. A phylogenetic tree was generated using the MEGA software (version 6.0) by the Neighbor-Joining method (Tamura et al. 2013).

### 5.2.4 Maillard products decolurisation and degradation studies

### 5.2.4.1 Preparation and physico-chemical analysis of maillard products

SGA-MRPs were prepared following the method described by Bharagava et al. (2009). Briefly, 1.0M sucrose, 1.0M glutamic acid, and 0.5M sodium carbonate were dissolved in 1.0 L of doubled distilled water and the pH of the reaction mixture was adjusted to 8.0 with 1N NaOH. After this, the solution was refluxed at 110 °C for 8 h (Fig. 5.3). The prepared SGA-MRPs were diluted to 10% of its original concentration using distilled water to obtain the OD 3.5±0.02 at 250 nm. This prepared SGA-MRPs solution was used for physico-chemical and decolourisation studies. The physico-chemical parameters (i.e. color, pH, BOD, COD, TDS, etc.) of SGA-MRPs were analysed as per standard methods (APHA 2012).

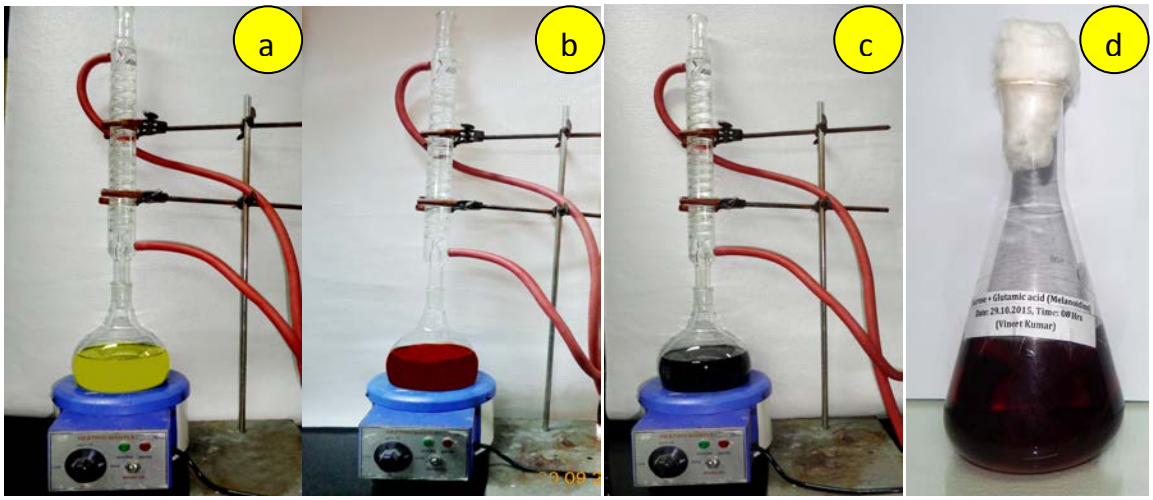



**Fig. 5.3** Synthesis of maillard reaction products in laboratory at different time interval (a) after 3 h (b) 6 h (c) 8 h (d) and synthesized sucrose-glutamic acid maillard reaction products

### 5.2.4.2 Construction of bacterial consortium for decolourisation studies

A loopful pure culture of isolated bacteria strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 was transferred to a 50 mL modified GPM broth medium supplemented with $K_2HPO_4$ (0.1%), $MgSO_4.7H_2O$ (0.05%) and molasses melanoidins (1000 mg $L^{-1}$) and incubated for 24 hrs in continuous shaking condition (125 rpm) at 37±1 °C. After 24 hrs, the equal volume of each bacterial strains containing cell density $3.1 \times 10^4$ cells $mL^{-1}$ was used for preparation of different bacterial consortium. The bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 were taken in the various combinations i.e. in the ratio of 1:1:1:1, 1:2:1:1, 1:2:2:1, 1:2:2:2, 2:1:1:2; 2:2:1:1, 2:2:2:1 and 2:2:2:2 for optimal decolourisation of SGA-MRPs.

### 5.2.4.3 Decolourisation assay

The decolourisation experiment was carried out in Erlenmeyer flasks (250 mL) containing 100 ml of previously prepared (as mentioned in section 2.3) sterile SGA-MRPs solution supplemented with (glucose 1%, peptone 0.1%, $K_2HPO_4$ (0.1%) and $MgSO_4.7H_2O$ (0.05% g $L^{-1}$) and 1.0 mL of trace elements solution containing $CuSO_4.5H_2O$ (0.003%), $MnCl_2.4H_2O$ (0.001%), $ZnSO_4 \cdot 7H_2O$ (0.001%), and $CaCl_2.2H_2O$ (0.002%), and pH was adjusted to 7.0 with 1N NaOH (Bharagava et al. 2009). The flasks were inoculated with 1% (v/v) over night grown culture of bacterial consortium (OD 0.24) and incubated at 37±1 °C under shaking flask condition (125 rpm) for 192 h. The decolourisation of SGA-MRPs was monitored by measuring the change in absorbance maxima of the melanoidins at 250 nm after scanning of absorbance maxima by using a UV-vis spectrophotometer (Evoluation-201, Thermo Scientific, USA). Simultaneously, the un-inoculated flask contain same medium and condition was used as control. The % decolorization was calculated as

$$\text{Decolourisation (\%)} = \frac{\text{Initial absorbance } (A_0) - \text{final absorbance } (A_1)}{\text{Initial absorbance } (A_0)} \times 100$$

#### 5.2.4.4 Optimisation of culture conditions for decolourisation and degradation of SGA-MRPs

##### 5.2.4.4.1 Optimisation of nutritional parameters

The effect of different carbon source viz. sucrose, fructose, glucose, xylose, lactose, and starch at 1.0% (w/v) were evaluated for the SGA-MRPs decolourisation and degradation. In another experiment, different organic and inorganic nitrogen sources viz. peptone, beef extract, yeast extract, sodium sulphate, ammonium chloride and urea were added into the GPM medium at 0.5% (w/v) concentration. The effect of carbon and nitrogen sources were optimised for decolourisation and degradation SGA-MRPs at different concentration viz. 0.1 to 1.0 (w/v)

##### 5.2.4.4.2 Optimisation of environmental conditions

To evaluate the effect of different environmental conditions the same experiment was carried out at different temperature (25-50 °C), pH (4-12), and shaking speed (100—220 rpm).

### 5.2.5 Evaluation of bacterial growth, biomass, and SEM observation of bacterial consortium

During decolourisation experiment, the optical density was continuously measured till 192 h at 12 h periodic interval by taking absorbance at 620 nm by UV-Vis spectrophotometer as the measurement of the bacterial cell growth. While the bacterial sample for biomass determination and SEM micrograph was collected from the flask after 144 h of incubation. First, the bacterial cells were centrifuged (6500 ×g) for 10 min at 4 $^{o}$C, the pellets were washed thrice with distilled water to remove the attached medium contents. For bacterial biomass determination, the pellet was dried in an hot air oven at 80 °C until getting a constant dried weight reported in the form of dry cell mass (g $L^{-1}$). For SEM micrograph, the bacterial cells were fixed with 1% (w/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 2 h and washed again with distilled water. The fixed samples were then dehydrated through 25%, 50%, 75%, 95% and 100% ethanol solutions in increasing concentration for 5 min, at each step. The samples were then dried in critical point drier and coated with a thin conductive film of gold in a sputtering coater and observed under SEM (FEI Quanta 450, Hillsboro, USA).

### 5.2.6 Estimation of MnP and lacasse activity

To measure the ligninolytic activity during degradation, the bacterial degraded supernatant was obtained by centrifugation at 6,500 ×g for 10 min at 4 $^{o}$C. The MnP and lacasse activity was determined using the phenol red (Lobachemie, Mumbai) and guaiacol (HiMedia, Mumbai), respectively as described earlier (Arora et al. 2002). For MnP activity, 5.0 mL of reaction mixture composed by 1.0 mL sodium lactate (50 mM, pH 5.0), 1.0 mL sodium succinate buffer (50 mM, pH 4.5), 0.4 mL manganese sulphate (0.1 mM),

0.7 mL phenol red (0.1 mM), 0.4 mL $H_2O_2$ (50 mM), 1.0 mg $mL^{-1}$ gelatin and 0.5 mL of enzyme extract. Blanks contained the same mixture solution without enzyme. The reaction was initiated at 25 $^oC$ by the addition of $H_2O_2$ and the rate of $Mn^{3+}$-malonate complex formation was monitored at every minute by measuring the increase in absorbance at 610 nm and the reaction was interrupted by the addition of 40 µl of 5 N NaOH. But, laccase reaction mixture containing 3.8 mL acetate buffer (10 mM, pH 5.0), 1.0 mL guaiacol (2 mM) and 0.2 mL of enzyme extract was incubated at 25 °C for 2 h. The absorbance was read at 450 nm using UV-Vis spectrophotometer after 2 h when reaction time completed. The enzyme activity was expressed as international unit (IU), where 1 IU represents the amount of enzyme that forms 1.0 µmol of product per minute under standard assay conditions.

### 5.2.7 UV-Vis and FT-IR spectroscopic analysis

UV-visible absorption spectra of the treated and untreated SGA-MRPs solution were recorded using a UV-Vis spectrophotometer in the wavelength range between 200-700 nm at room temperature. Further, FT-IR analysis of the sample was performed in range of 400 to 4000 $cm^{-1}$ using a spectrophotometer (Nicolet$^{TM}$ 6700, Thermo Scientific, USA) in order to reveal the chemical nature of the SGA-MRPs. The separated samples of SGA-MPs were mixed with potassium bromide (KBr) to prepare the pellet for FT-IR analysis.

### 5.2.8 Extraction and identification of various organic compounds

The organic compounds present in treated and untreated SGA-MRPs were extracted by ethyl acetate under acidic condition (pH <2.0) as described in chapter no. 04 (Bharagava and Chandra 2009)

#### 5.2.8.1 HPLC analysis

HPLC was carried out using waters, 515 HPLC instrument equipped with a diode array detector system (1100 series, Agilent Technologies, USA) and reverse phase $C_{18}$ column (250 × 4.6 mm, 5 µm particle size) by using the gradient of solvent A (Milli-Q water) and solvent B (acetonitrile with 0.1% TFA) (Merck, Germany) at a flow rate 0.4 mL $min^{-1}$ for 60. The detection was monitored at wavelength 240 nm (absorption maxima) to assess the decolourisation and degradation of maillard products. 50 µl of methanol extract was injected into the column by using a glass microsyringe (Agilent Technologies, USA)

#### 5.2.8.2 GC-MS analysis

In GC-MS analysis, the extracted samples were derivatised with trimethylsilyl (TMS) as described in chapter no. 04 (Minuti et al. 2006). After extraction, the extracted samples were analysed by Thermo Scientific Trace GC Ultra Gas Chromatograph equipped with a TriPlus auto sampler coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific, FL, USA). The operation conditions were same as described in chapter no. 04.

### 5.2.9 Toxicity assessment of maillard reaction products

The toxicity effect of untreated and treated SGA-MRPs was studied on *P. mungo* L. seed germination using Petri dish method as described in chapter no. 04 (OECD 2003; Sharma et al. 2002). The seed germination parameters like germination percentage, germination index, relative toxicity percentage, phytotoxicity percentage and stress tolerance index were calculated using the formula described earlier (David Noel and Rajan 2015).

### 5.2.10 Statistical data analysis

All the experiment was carried out in triplicate and the results were presented as the mean of three independent observations. To confirm the variability of data obtained and validity of results, the mean concentration of various physico-chemical parameters of untreated and treated SGA-MRPs was calculated by Student's '*t*' test by using SPSS (version 17.0, Chicago, USA).

## 5.3 Results and discussion

### 5.3.1 Isolation and screening of bacterial strains

Twenty seven morphologically distinct bacterial strains were isolated from distillery sludge. Only four bacterial isolates namely IITRCS01, IITRCS06, IITRCS07, and IITRCS11 could show the MnP activity by changing the deep orange to light yellow color at melanoidins (800 mg $L^{-1}$) containing phenol red amended modified GPM agar plates, as well as laccase activity by developing a reddish brown color zone on B&K agar plate during the screening of potential bacterial strains capable for growth on SGA-MRPs amended media (Fig. 5.4, 5.5 and Table 5.1). The screened bacterial strains also showed tolerance for growth on melanoidins amended GPM agar plate at 3500 mg $L^{-1}$ concentration as shown in Fig. 5.6. The phenol red changed from deep orange to light yellow color during screening of peroxidase activity in bacteria has been used as an indicator of MnP activity shown in Fig. 5.4. This change in colour of phenol red occurred due to oxidation of glucose by sugar oxidase enzyme, resulting in the production of $H_2O_2$ and media acidification (i.e. lowering of pH) which is required for the melanoidins degradation (Aoshima et al. 1985; Hwang et al. 2011). The guaiacol is also a chromogenic substrate that is used for quick screening of microbial strains producing laccases by means of a color reaction (Okino et al. 2000). The selected four bacterial strains produced the laccase which catalyzed the oxidation of guaiacol to form reddish brown halo zones in the medium (Fig. 5.5). This observation corroborated with earlier finding for screening of MnP and laccase producing bacterial strains by Chandra and Singh (2012).

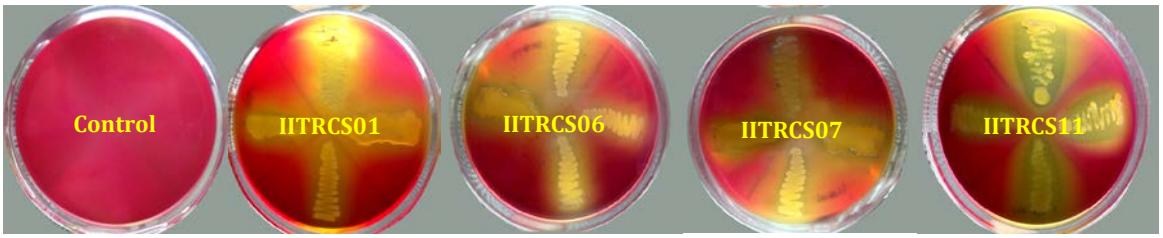



**Fig. 5.4** Potential bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 showing MnP activity phenol red amended modified GPM agar plates

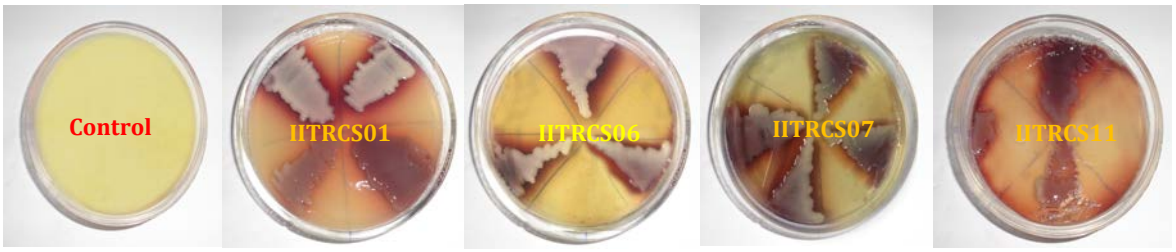



**Fig. 5.5** Potential bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 showing laccase activity on guaiacol amended B&K agar plates

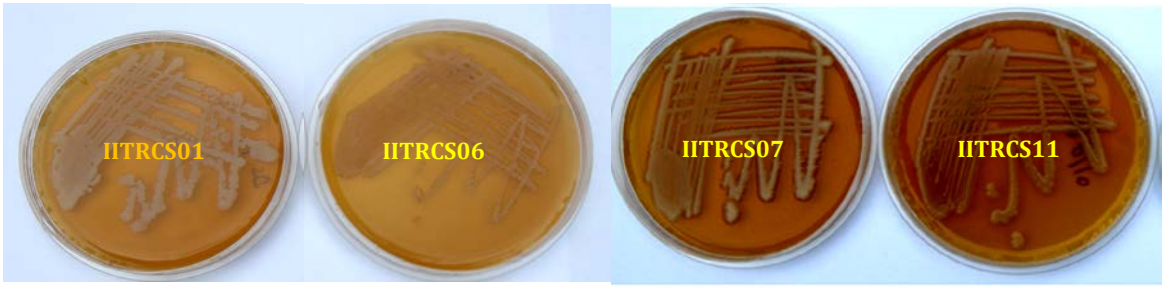



**Fig. 5.6** Potential bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 showing melanoidins tolerance activity on melanoidins amended GPM agar plates

**Table 5.1** Screening of bacterial strains on the basis of growth tolerance and enzyme activity

| Bacterial Strains | Molasses concentration (w/v) | | | | Enzyme Screening | |
|---|---|---|---|---|---|---|
| | 1000 | 2000 | 3000 | 3500 | MnP | Laccase |
| IITRCS 01 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| IITRCS 02 | ++ | ++ | + | + | + | - |
| IITRCS 03 | ++ | + | - | - | + | - |
| IITRCS 04 | ++ | ++++ | +++ | +++ | + | ++ |
| IITRCS 05 | ++ | ++ | ++ | ++ | - | + |
| IITRCS 06 | ++++ | ++++ | ++++ | ++++ | +++ | +++ |
| IITRCS 07 | ++++ | ++++ | ++++ | ++++ | +++ | +++ |
| IITRCS 08 | ++ | ++ | ++ | - | + | ++ |
| IITRCS 09 | +++ | + | - | | - | + |
| IITRCS 10 | ++ | ++ | + | + | + | + |
| IITRCS 11 | ++++ | ++++ | ++++ | ++++ | +++ | +++ |
| IITRCS 12 | ++ | - | - | - | + | + |
| IITRCS 13 | + | + | - | - | - | + |
| IITRCS 14 | ++ | ++ | - | - | - | - |
| IITRCS 15 | + | + | - | - | + | + |

| | | | | | | |
|---|---|---|---|---|---|---|
| IITRCS 16 | ++ | - | - | - | + | - |
| IITRCS 17 | ++ | + | - | - | - | - |
| IITRCS 18 | ++ | +++ | ++++ | ++ | ++ | +++ |
| IITRCS 19 | ++ | + | - | - | + | + |
| IITRCS 20 | + | + | - | - | + | + |
| IITRCS 21 | ++ | ++ | + | + | - | + |
| IITRCS 22 | +++ | ++ | + | - | - | + |
| IITRCS 23 | + | + | - | - | + | - |
| IITRCS 24 | ++ | ++ | + | + | + | - |
| IITRCS 25 | +++ | +++ | ++ | + | - | + |
| IITRCS 26 | ++ | + | + | - | + | - |
| IITRCS 27 | + | + | + | - | - | + |

++++= luxuriant growth; +++=moderate growth; ++=slower growth; +=very slow growth; -=no growth; +++=high enzyme activity

### 5.3.2 Characterisation and identification of bacterial strains

On account of morphological and biochemical test, bacterial strain IITRCS01 was characterized as Gram negative, rod shaped, non-motile, non-spore forming bacterium and given positive test for catalase, O-F, citrate, lysine, urease, nitrate, and produced acid from fermentation of glucose, adonitol, lactose, arabinose, and sorbitol. But, this strain showed negative test for indole, ornithine decarboxylase, phenylalanine deaminase, and $H_2S$. The bacterial strains IITRCS06 was characterised as Gram negative, rod shaped, motile bacilli given positive tests for catalase, O-F, citrate, lysine, ornithine decarboxylase and H2S production and given acid after fermentation of glucose. While this bacterium strain showed negative test for indole, urease, phenylalanine, nitrate reduction and not produced acid ferment the sugar adonitol, lactose, arabinose, and sorbitol. The bacterial strains IITRCS11 and IITRCS07 were characterised as Gram negative, rod shaped, motile, non-spore forming bacteria given positive tests for catalase, O-F, citrate, ornithine decarboxylase, nitrate, , and produced acid from fermentation of glucose, lactose, sucrose, maltose, arabinose. But, both the strains showed negative test for indole, lysine decarboxylase, phenylalanine deamination, urease, phenylalanine deaminase, and $H_2S$ production. The morphological, physiological, and biochemical characteristics of bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 as shown in Fig. 5.7, 5.8, and 5.9 and Table 5.2.

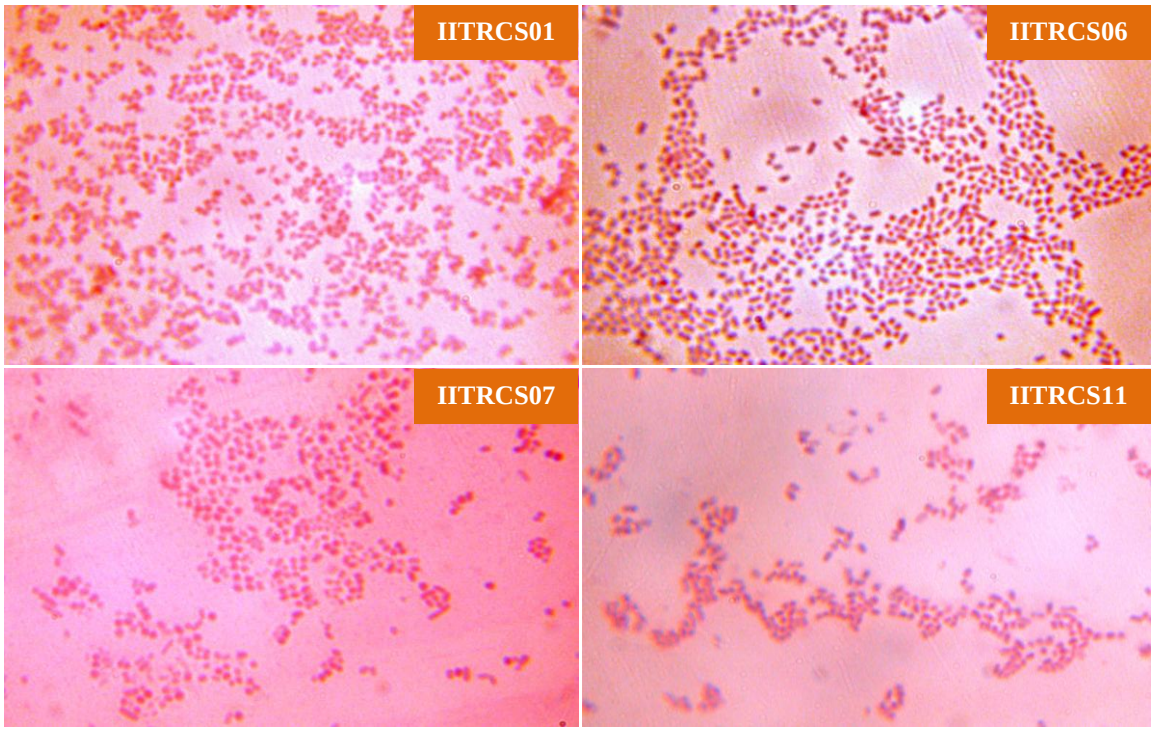



**Fig. 5.7** Morphological characteristics of isolated bacterial strains IITRCS01, IITRCS06, IITRCS07 and IITRCS11

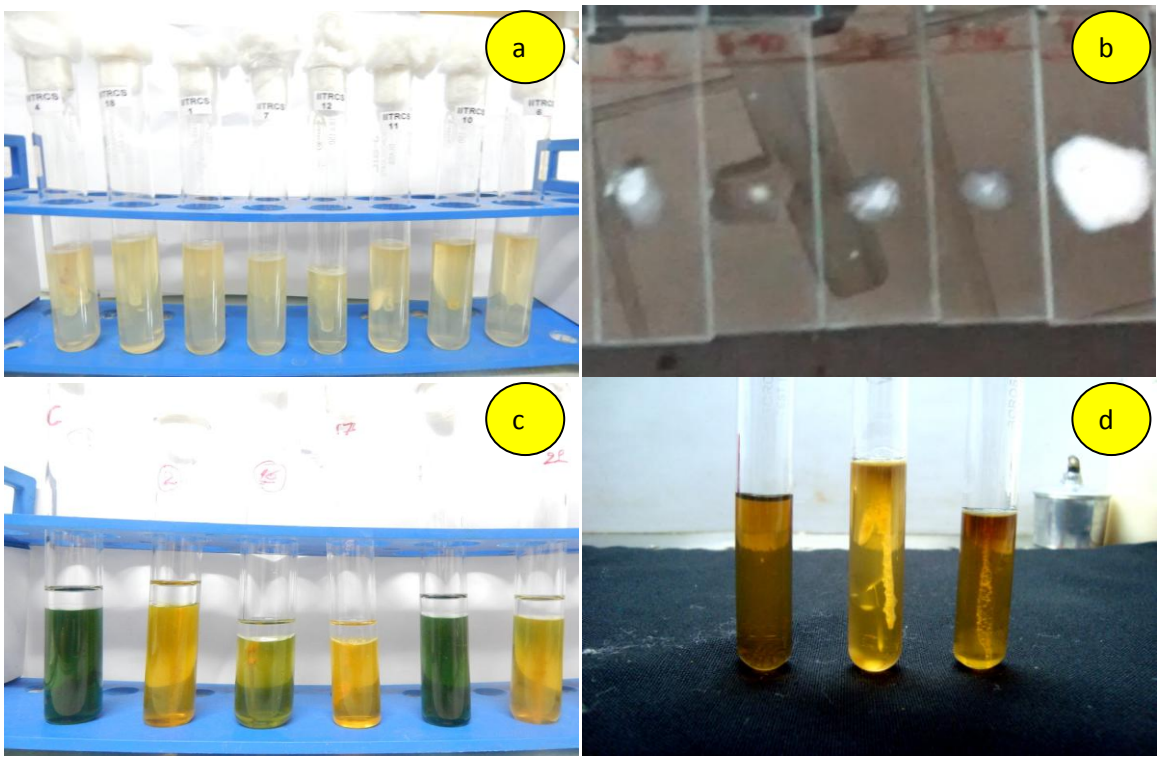



**Fig. 5.8** Various morphological and biochemical reactions showed by isolated bacterial strains IITRCS01, IITRCS06, IITRCS07 and IITRCS11 (a) motility test (b) catalase test (c) oxidative-fermentative test (d) $H_2S$ production test

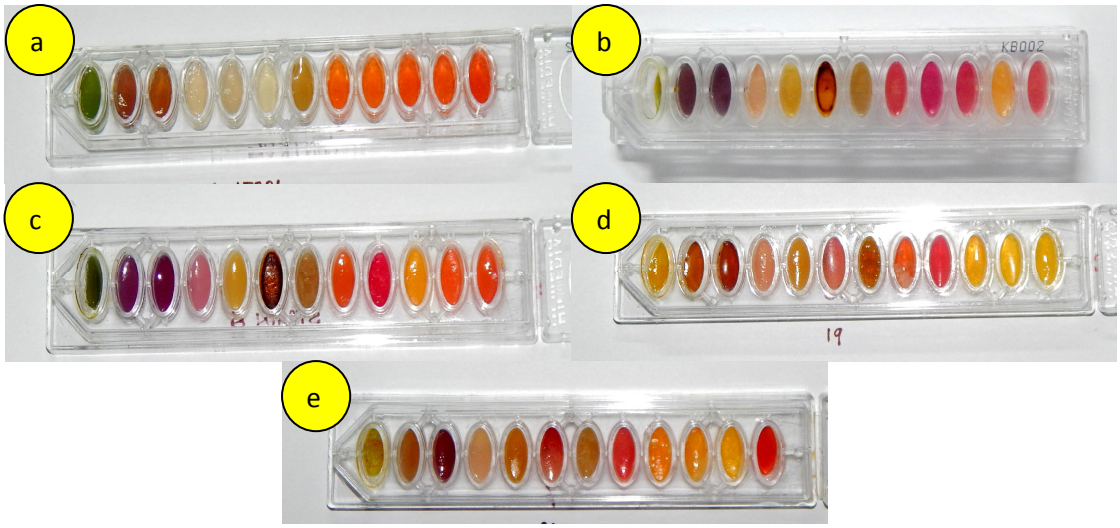



**Fig. 5.9** Biochemical characterization of isolated bacterial strains using HiMedia Rapid Biochemical Identification kit (a) control (b) IITRCS01 (c) IITRCS06 (d) IITRCS07 (e) IITRCS11

**Table 5.2** Morphological and biochemical characteristics of isolated bacterial strains

| **Characteristics** | **Bacterial Isolates** | | | |
|---|---|---|---|---|
| | **IITRCS01** | **IITRCS06** | **IITRCS07** | **IITRCS11** |
| **Morphological test** | | | | |
| Gram stain | - ve | -ve | - ve | - ve |
| Shape | R | R | R | R |
| Arrangement | Single/in chains | Single/in chains | Single/in chains | Single/in chains |
| Motility | Non-motile | Motile | Motile | Motile |
| Capsule | + | - | - | - |
| Spore staining | - | - | - | - |
| **Biochemical test** | | | | |
| Catalase | + | + | + | + |
| Oxidative Fermentation (O-F) | + | + | + | + |
| Indole production | - | - | - | - |
| Citrate utilisation | + | + | + | + |
| Lysine utilisation | + | + | - | - |
| Ornithine utilisation | - | + | + | + |
| Urease | + | - | - | - |
| Phenylalanine deaminase | - | - | - | - |
| Nitrate reduction | + | - | + | + |
| $H_2S$ production | - | + | - | - |
| Sugar fermentation test | | | | |
| Glucose | + | + | + | + |
| Adonitol | + | - | + | + |
| Lactose | + | - | + | + |
| Arabinose | + | - | + | + |
| Sorbitol | + | - | + | + |

+ve: Gram positive; -ve: Gram negative +: Positive; -: Negative; O: Oxidative; F: Fermentative; R: Rod shape

Further, the molecular identification of screened bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 showed specific amplification of a single band of around 1500 bp of 16S rRNA gene as shown in Fig. 5.10.

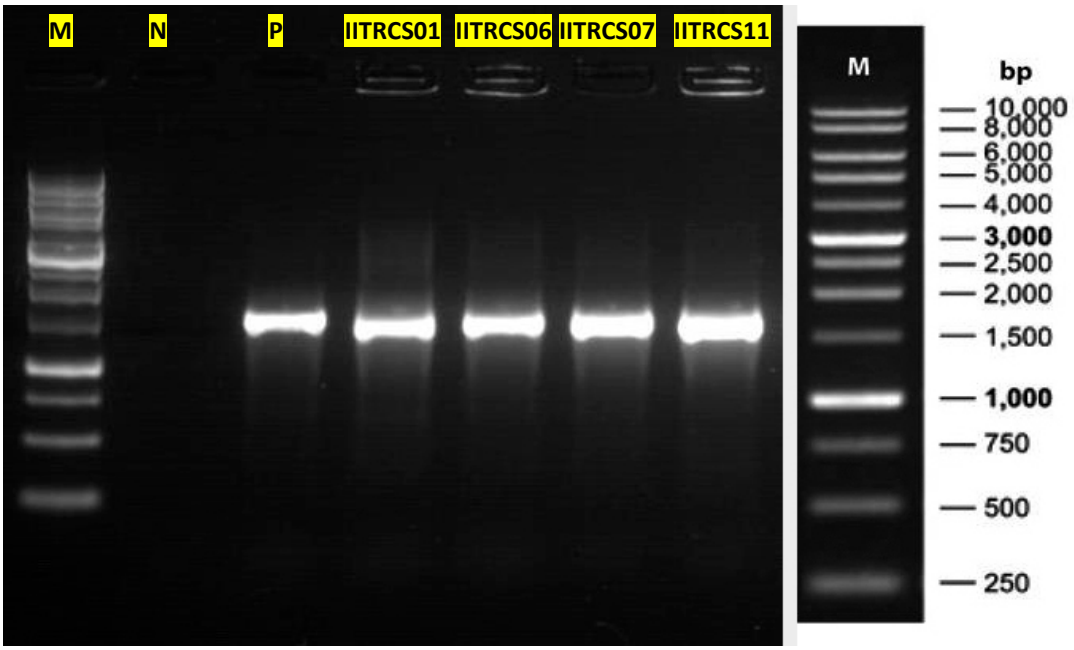



**Fig. 5.10** PCR amplification of 16S rRNA gene of isolated bacterial strains; Lane M 10,000 bp DNA ladder; N: PCR positive non-template control (water only); P: positive control (DNA extracted from *E. coli* used as template)

The BLAST analysis of nucleotide sequence data of representative isolates was showed 100% sequence homology with *Klebsiella pneumoniae* (KU726952), *Salmonella enterica* (NR044372), *Enterobacter aerogenes* (NR024643), and *Enterobacter cloaceae* (KJ437492) found in the GenBank database. Hence, based on the sequence homology, the bacterial strain IITRCS01, IITRCS06, IITRCS07, and IITRCS11 were identified as *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), *E. cloaceae* (KU726957), respectively. These bacterial species belonged to group *Gammaproteobacteria*. The class *Gammaproteobacteria* constitutes a very large and diverse group of bacteria that exhibits enormous variety in terms of their phenotype and metabolic capabilities (Woese et al. 1985; Brenner et al. 2005; Kersters et al. 2006). Members of this group exhibit broad ranges of aerobicity, of trophism, including chemoautotrophism and photoautotrophism, and of temperature adaptation. The class *Gammaproteobacteria* also includes enteric bacteria and it is well known for harbouring large numbers of human, animal and plant pathogens (Brenner et al. 2005; Kersters et al. 2006). Although, *Gammaproteobacteria* has only the taxonomic rank of class within the phylum *Proteobacteria*. Further, the phylogenetic tree was showed the interrelationship of bacterial species with the most closely related genera inferred from sequences of the 16S rRNA gene as shown in Fig. 5.11.

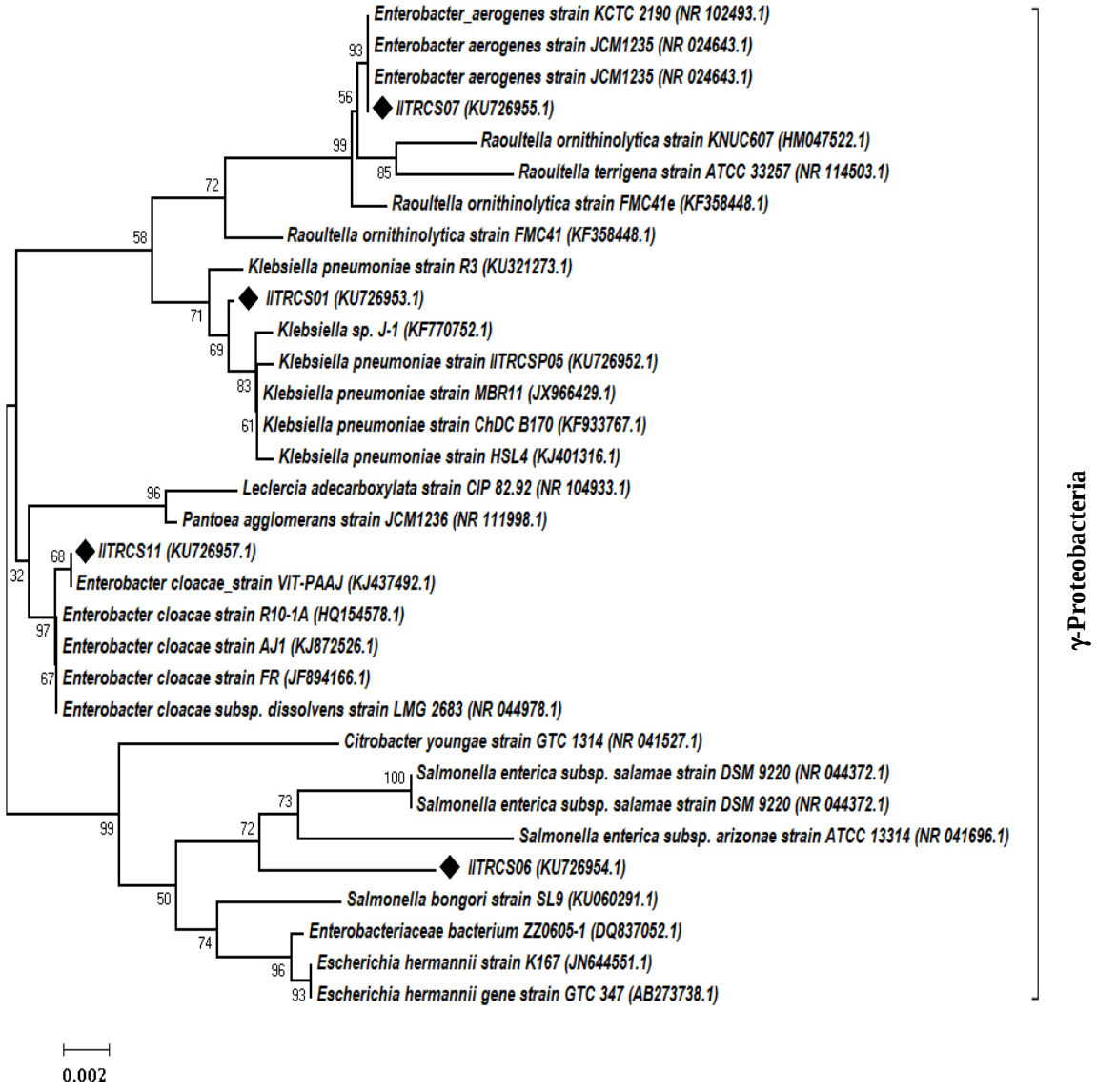



**Fig. 5.11** Phylogenetic tree showing the inter-relationship of isolated bacterial strains with the most closely related species inferred from sequences of 16S rRNA gene. The tree was generated using Neighbour-Joining method with a bootstrap value of 1,000 replicates. The scale bar shows 0.05% sequence divergence.

### 5.3.3 Physico-chemical analysis

The physico-chemical characteristics of untreated SGA-MRPs showed dark brown in color at pH 6.51. In addition, the concentration of BOD (8641), COD (11,586), TDS (1050.77), VS (457.83), and phenolics (110.56 mg $L^{-1}$) were found higher as shown in Table 4.3. Thus, the high BOD and COD values of the MRPs solution are due to lack of oxygen molecule in MRPs during the synthesis of MRPs after dehydration process. Since, some caramelized sugar may be also generated during the MRPs synthesis. Therefore, high TDS of solution is found in this study. In contrast to above, the bacterial treated SGA-MRPs showed reduction in all physico-chemical parameters as shown in Table 5.3. The colour of SGA-MRPs is dark brown which turned light brown after bacteria treatment as shown in Fig. 5.12. The reduction in colour was noted due to depolymerisation of

melanoidins as reported earlier (Verma et al. 1974). At the initial stage of bacterial incubation during the decolourisation process the pH of medium was noted 4.1. This might be due to generation of some organic acids with bacterial reaction in medium composition (Davídek et al. 2006). But, it gradually moves towards alkaline condition (pH 8.1) after 192 h of bacterial incubation. The increased pH of media might be due to depolymerisation of MRPs by enzyme activity of bacterial consortium. This induced the solubility and utilisation of melanoidins which favoured the mineralisation and co-metabolism of MRPs (Bharagava et al. 2009). Therefore, a sharp reduction in BOD (2568), COD (4201), TDS (687.92), VS (113.41) and phenolics (55.77 mg $L^{-1}$) was noted as shown in Table 5.3. This indicated the degradation of MRPs by inoculated bacterial consortium due to presence of ligninolytic enzyme activities. All the physico-chemical parameters of treated SGA-MRPs were found significantly higher ($p<0.001$) that the untreated SGA-MRPs except for pH ($p<0.05$). These results also corroborated to the previous findings regarding the degradation of natural and synthetic melanoidins (Bharagava et al. 2009; Kumar and Chandra 2006; Yadav and Chandra 2012).

**Table 5.3** Physico-chemical analysis of sucrose glutamic acid-Maillard reaction products

| **Parameter** | **Untreated (control)** | **Bacterial Treated** | | **Percent Reduction** | **Discharge Permissible limit (USEPA, 2002)** |
|---|---|---|---|---|---|
| | | **After 96 h** | **After 192 h** | | |
| Color appearance | Dark Brown | Dark Brown | Light brown | - | - |
| pH | 6.9±0.0 | 4.1±0.10[b] | 8.1±0.20[c] | -14.28 | - |
| BOD | 8641±160.33 | 5472±1.04[a] | 2568±2.01[a] | 70.28 | 40.00 |
| COD | 11586±92.82 | 4867±1.29[a] | 4201±3.11[a] | 63.74 | 120.00 |
| TDS | 1050.77±2.39 | 997.1±1.01[b] | 687.92±2.90[a] | 34.53 | - |
| VS | 457.83±9.39 | 254.24±1.11[a] | 113.41±1.12[a] | 75.22 | - |
| Phenolics | 110.56±1.40 | 89.01±0.01[b] | 55.77±1.43[a] | 49.55 | 750.00 |

All values are mean (n=3)±SD in mg $L^{-1}$ except pH; Student's t-test (two tailed as compared with untreated sample); a- Highly significant at $p < 0.001$, b- significant at $p<0.05$


**Fig. 5.12** Decolourisation of SGA-MRPs (a) untreated (b) bacterial treated after 192 h incubation

### 5.3.4 Effect of different carbon and nitrogen sources

SGA-MRPs decolourisation was monitored by using different carbon sources at 1% (w/v) concentration for 192 h of incubation, and the results are illustrated in Fig. 5.13a. It was observed that glucose was the best carbon source, allowing maximum decolourisation (65.8%) at 1% (w/v) concentration. The decolourizing ability of bacterial consortium was tested at different concentration showed that the efficiency of consortium increase with increase in glucose concentration from 0.1% to 1% reaching maximum decolourisation (65.8%) at 1.0%. While in absence of glucose slow growth of bacterial consortium was noted. However, sucrose, xylose and lactose were found less effective than glucose showed decolourisation up to 63-61.2%. Fructose was relatively poor co-substrate, aiding only 40% decolourisation. Fructose was relatively poor co-substrate, aiding only 40% decolourisation. This revealed that the carbon source present in MRPs is not readily being utilised by bacterial consortium. But, the supplementation of glucose in media was readily available for bacterial growth. This indicated strong evidence that incubated bacterial consortium is not able to utilise the SGA-MRPs for its growth and metabolism. Similar observation has also been reported by previous worker in case of anaerobially treated distillery spent wash (Ghosh et al. 2004). This indicated that our bacterial consortium was more effective for decolourisation and degradation of MRPs. This might be due to induction of hight amount of ligninolytic enzyme which facilitated for the decolourisation processes (Kumar and Chandra 2006).

Simultaneously, the effect of different organic and inorganic nitrogen sources at a concentration of 0.5% (w/v) along with glucose (1.0 % w/v) showed that peptone was most effective organic nitrogen source which enhanced up to 72% decolourisation of maillard products at 0.1% (w/v) concentration (Fig. 5.13b). Because, peptone are rich in free amino acids and short peptides, which support cell growth with a low consumption rate of the carbon source and minimize the accumulation of by-products (Lau et al. 2004). Further, increase in concentration of peptone resulted in continuous reduction in degradation capability of the developed bacterial consortium inhibited the decolourisation process. Moreover, presence of other organic nitrogen sources i.e. beef extract, and yeast extract could show the SGA-MRPs decolorization up to 55-58% only, while urea could shown the decolourisation up to 15%, whereas inorganic nitrogen sources such as sodium sulphate and ammonium chloride could show the decolourisation up to 32%, and 25%, respectively. Similar, effect has also been reported by previous workers for melanoidins decolourisation (Tiwari et al. 2013; Tiwari et al. 2012; Yadav and Chandra 2012; Sirianuntapiboon et al. 2004). Hence, the developed bacterial consortium utilised tiny amount of peptone for higher decolourisation, BOD, and COD reduction compared to other researchers ever reported.

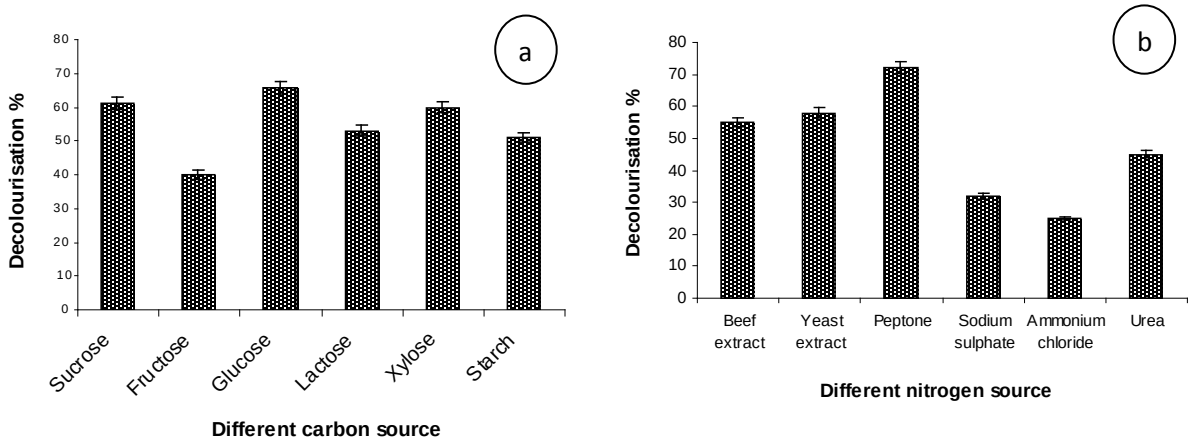



**Fig. 5.13** Effect of different nutrient on SGA-MRPs decolourisation (a) carbon source (b) nitrogen source

### 5.3.5 Effect of pH, temperature and shaking speed

The variation in SGA-MRPs decolourisation process was directly affected with change in pH and temperature of culture conditions. The optimum SGA-MRPs decolourisation (72%) was noted at pH 8.1 by developed bacterial consortium after 192 h of incubation. Further, increase or decrease in pH inhibited the decolourisation process. This might be due to inhibition of the enzyme activity (Fig. 5.14a). For developed aerobic bacterial consortium comprising *Bacillus* sp., *Bacillus licheniformis*, and *Alcaligenes* sp. was showed highest decolourisation activity at pH 7.0 (Bharagava and Chandra 2010b). In another study, the optimum pH for melanoidins decolourisation by bacterial strains *Alcaligenes faecalis* $SAG_5$ was note at pH 7.5 (Santal et al. 2011). Similarly, the developed bacterial consortium showed maximum decolourisation (70%) at 37 °C. While further, increase in temperature up to 45 °C adversely affected the growth, metabolism and MRPs decolourisation ability of bacterial consortium (Fig. 5.14b). Similar pattern is also reported in other studies also (Mohana et al. 2007; Santal et al. 2011). However, the maillard products were significantly affected by various shaking speed also. It was observed that the optimum decolourisation (70%) by potential bacterial consortium was noted at 180 rpm. However, increase in shaking speed resulted decrease in decolourisation process. This might be due to mechanical injury of bacterial cell wall at a higher shaking speed (Tiwari et al. 2014; Yadav and Chandra 2012).

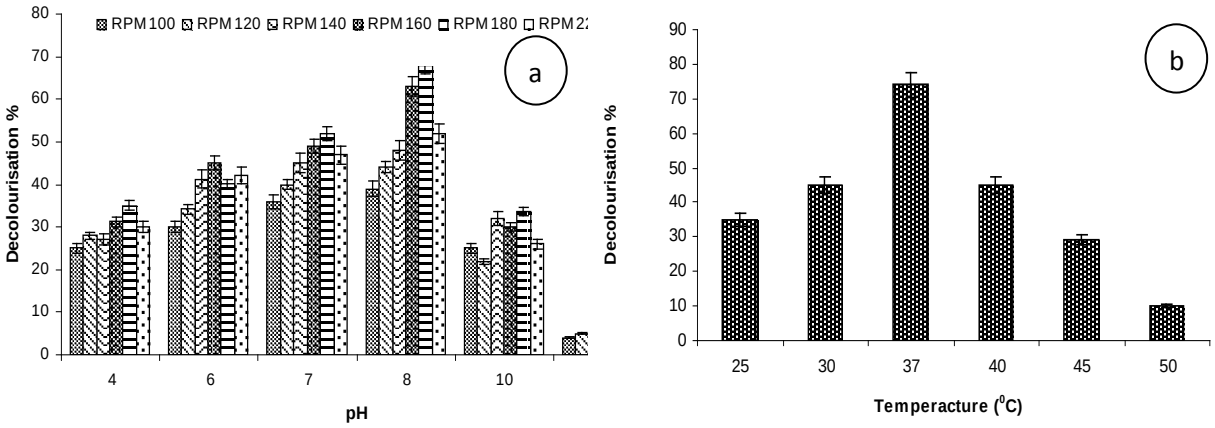



**Fig. 5.14** Effect of different environmental parameters on SGA-MRPs decolourisation (a) temperature (b) pH and shaking speed at 37 °C for 24-192 h incubation

### 5.3.6 Bacterial growth and biomass

For efficient and potential consortium, different inoculums ratio of bacterial strains were also optimized. For efficient and potential consortium, different inoculums ratio of bacterial strains were also optimized. Consortium having 1:1:1:1 inoculums ratio of bacteria showed maximum (74%) decolourisation of MRPs within biomass (5.15 $gL^{-1}$; CFU $mL^{-1}$ $28\times10^6$) $OD_{620}$ (2.49) at 144 h of incubation (Fig. 5.15a) Further, incubation did not increase the rate of decolourisation. The direct correlation of $OD_{620}$ with high bacterial biomass indicated the direct involvement in decolourisation and degradation process of MRPs. This indicted high bacterial growth was due to utilisation of MRPs as a carbon source for its growth. Beside, the SEM analysis of bacterial biomass showed thick biomass under SEM study also supported the high biomass of bacteria (Fig. 5.15b).

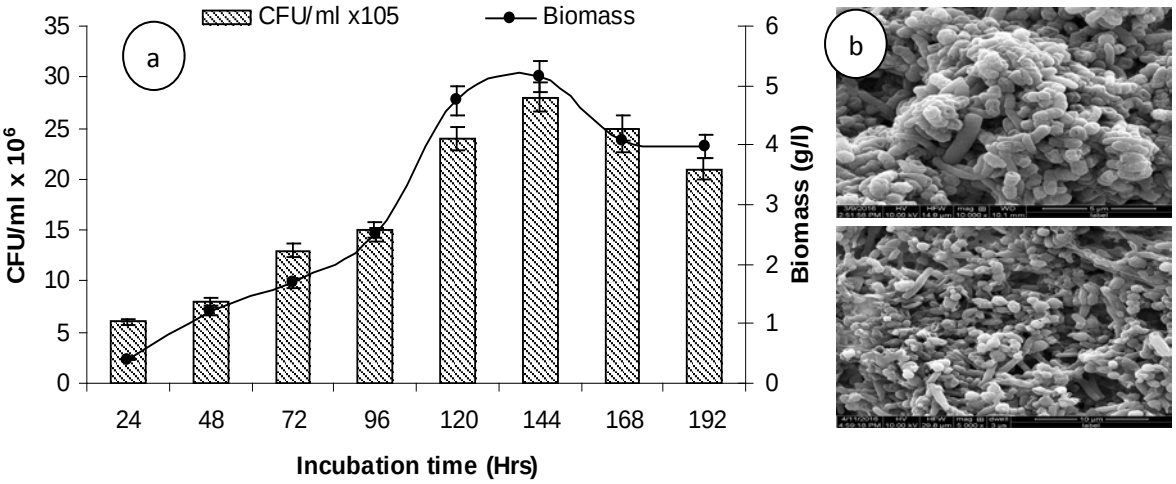



**Fig. 5.15** Growth pattern and (b) scanning electron micrograph of developed bacterial consortium during degradation and decolourisation of SGA-MRPs

### 5.3.7 Enzyme activity

To observe the function of ligninolytic enzymes in the decolourisation and degradation of SGA-MRPs, enzymes were measured and MnP was found to be the dominating enzymes at the initial stage of decolourisation. MnP activity was noted maximum at 120 h (2.8 U $mL^{-1}$ $min^{-1}$), and laccase activity was found maximum (1.91 U $mL^{-1}$ $min^{-1}$) at 144 h of incubation (Fig. 5.16). But, after 120 h and 144 h incubation showed gradual decrease of MnP and laccase enzyme activities of bacterial consortium, respectively. The colour reduction of MRPs was significantly initiated at 72 h and achieve maximum decolourisation at 144 h incubation. The MnP and laccase have a broad substrate oxidising enzymes reported for detoxification and degradation of phenolic and non-phenolic polymeric compounds (Rodriguez et al. 1999; Gonzalaez et al. 2008). The presence of melanoidins and other similar compounds can induce the expression of MnP and laccase protein both are having synergistic action in the degradation and depolymerisation of melanoidins and other industrial pollutants (Miyata et al. 2000; Gonzalaez et al. 2008). In our study, MnP was profusely present at the initial phase of bacterial growth, while laccase was produced in later phase of growth during MRPs degradation. The findings of this study are well supported by earlier results reported by various workers (Pant and Adholeya 2009b; Pant and Adholeya 2007a; Yadav and Chandra 2012).)

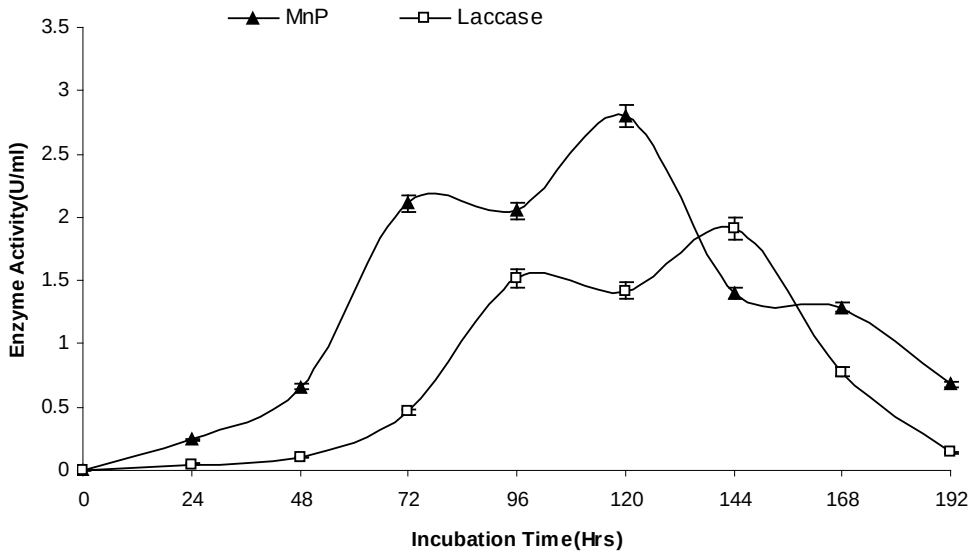



**Fig. 5.16** MnP and laccase activity in broth assay during decolourisation and degradation of SGA-MRPs

### 5.3.8 UV-Vis and FT-IR spectroscopy

UV-Vis spectrophotometric analysis is the most common used method of the MR (Martins et al. 2003; Echvarria et al. 2014). Therefore, The UV-Vis spectroscopy analysis in range between 200-700 nm for the change of maillard products was done at different time intervals during the optimum decolourisation of SGA-MRPs. The result revealed that maillard products showed different absorption peaks in the UV region and their maximum absorbance was noted at 250 nm (Fig. 5.17). In addition, MRPs showed different stable

peaks in a range of between 200-410 nm. The peaks were decreases as the day progresses, which showed the decolourization of MRPs by bacterial consortium at the end of the 192 h of incubation. The stable peaks absorbance in UV region indicated the presence of several organic compounds formed during the early stages of the MR (Echavarria et al. 2013a,b). While, the absorbance of either 280 nm, 320-350 nm or 420-450 nm corresponding to early MRPs for pyrazine compounds (at 280 nm) (Gu et al. 2010), advanced stage for soluble pre-melanoidins formation (at 320-350 nm) and final MRPs (at 420-450 nm) corresponding to polymerised MRPs in medium (Billaud et al. 2004). On the basis of our findings, it can be stated that the formation of various peaks in UV and visible region indicated the presence of complex mixture of early, advanced and final MRPs. Our findings corroborated with the earlier results reported by Echvarria et al. (2013a,b).

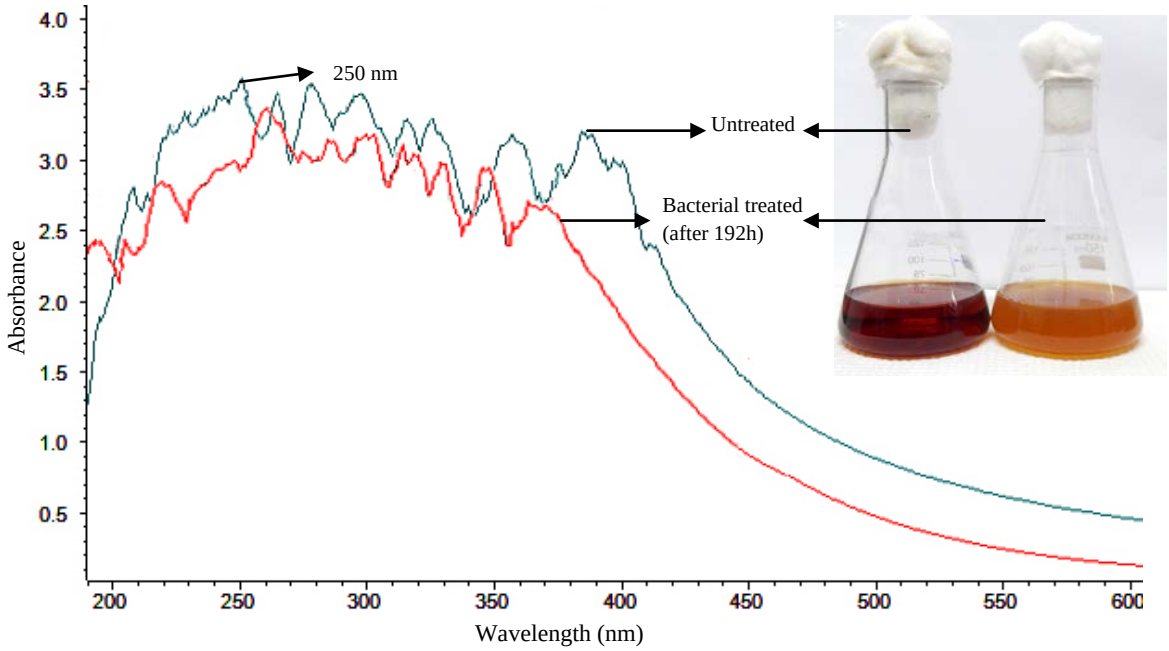



**Fig. 5.17** Analysis of untreated and bacterial treated SGA-MRPs at different time interval by UV-visible spectrum analysis

FT-IR analysis supported the depolymerisation of SGA-MRP after bacterial treatment by shifting the various functional groups as shown in Fig. 5.18. The FT-IR spectrum of the SGA-MRPs is characterized by an intense absorption between 3200 to 3600 $cm^{-1}$ represented the broad peaks of stretching vibration of O-H of COOH and the N-H stretching of amides. The region between 2800 to 3000 $cm^{-1}$ exhibited the stretching of C-H from $-CH_3$ present in hydrocarbons chain (Kadam et al. 2013; de Oliveira Silva et al. 2012). The control sample showed the peaks at 2976.8, and 2930.3 $cm^{-1}$ of C-H asymmetric stretching vibration of –CH, and $-CH_3$ functional groups. The peak value 2930.3, and 2946.8 was represented the C-H vibrational stretching from $CH_3$ present in hydrocarbon chain. An intense band in the 1500-1750 $cm^{-1}$ range (1596.0 and 1739.5) may correspond to carbonyl groups in primary amide functions, or it could appear because of the existence of C-O stretching of $COO^-$ ketonic C-O and aromatic C-C conjugated with

$COO^-$. The absorption band at 1411.1 and 1457.8 $cm^{-1}$ might appear due to aliphatic C-H bending and $COO^-$ asymmetric stretching in spectra. The untreated sample showed absorptions peak between 1000 and 1200 $cm^{-1}$ in untreated and simulated spectra could appear due to C-O bonds (ethers and alcohols) and/or minerals. While a band at 922.8, and 978.0 $cm^{-1}$ indicated the presence of C–O stretching of polysaccharides or Si–O-asymmetric stretch. The untreated sample also showed an intense peak at 847.7, 778.5, 629.6 and 535.4 $cm^{-1}$ represent the sulfate, carbohydrate, alkyl halides, nitro group, and C-S bond (Kadam et al. 2013). The treated sample showed a narrow peak at 3414.0 $cm^{-1}$ represented the stretching vibration of O-H group. The spectra corresponding to treated sample was displays bands at 2855.4 and 2924.8 $cm^{-1}$, probably due to stretching vibrations of $CH_3$ and $CH_2$ groups, respectively. The broad stretching adsorption band peak at 2160.0 $cm^{-1}$ could be assigned to C=C stretching vibrations. The absorption band at 1444.9 $cm^{-1}$ might appear due to aliphatic C-H bending. The occurrence of aromatic groups is also suggested by absorptions at around 700 $cm^{-1}$. Moreover, most of the bands were similar between untreated and simulated samples. These results are corroborated by previous finding (Ramezani and Mohammadi 2011). The extra peaks in the treated effluent are due to degradation of SGA-MPs by developed bacterial consortium.

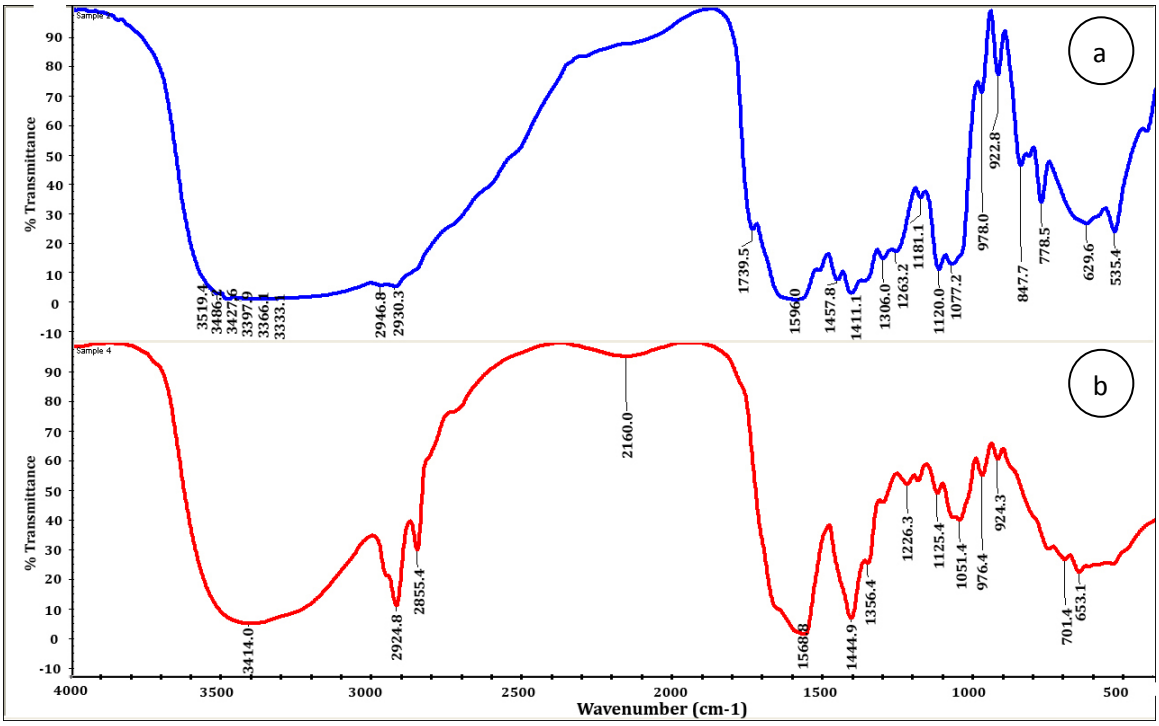



**Fig. 5.18** FT-IR spectra of SGA-MRPs (a) untreated (b) bacterial treated after 192h

### 5.3.9 HPLC analysis

The HPLC analysis report demonstrating the area, height, and retention time, before and after bacterial treatment of SGA-MRPs which confirms the degradation of melanoidins (Fig. 5.19). The HPLC analysis of untreated samples extracted with ethyl acetate showed a single major peak at RT of 2.40 min, while in 96 h treated sample was less compared to untreated sample clearly indicates the capability of the bacterial consortium to decolorize

and degrade the SGA-MRPs by their enzymatic action. In addition, there were some extra peaks appeared in bacterial treated sample suggesting the formation of several new metabolites. Further, after 192 h treatment of SGA-MRPs, the HPLC chromatogram also showed shifting of peak, decrease in peak area height, and generation of some new peaks compared to untreated sample. This suggested the biotransformation and biodegradation of SGA-MRPs in various metabolites by developed bacterial consortium.

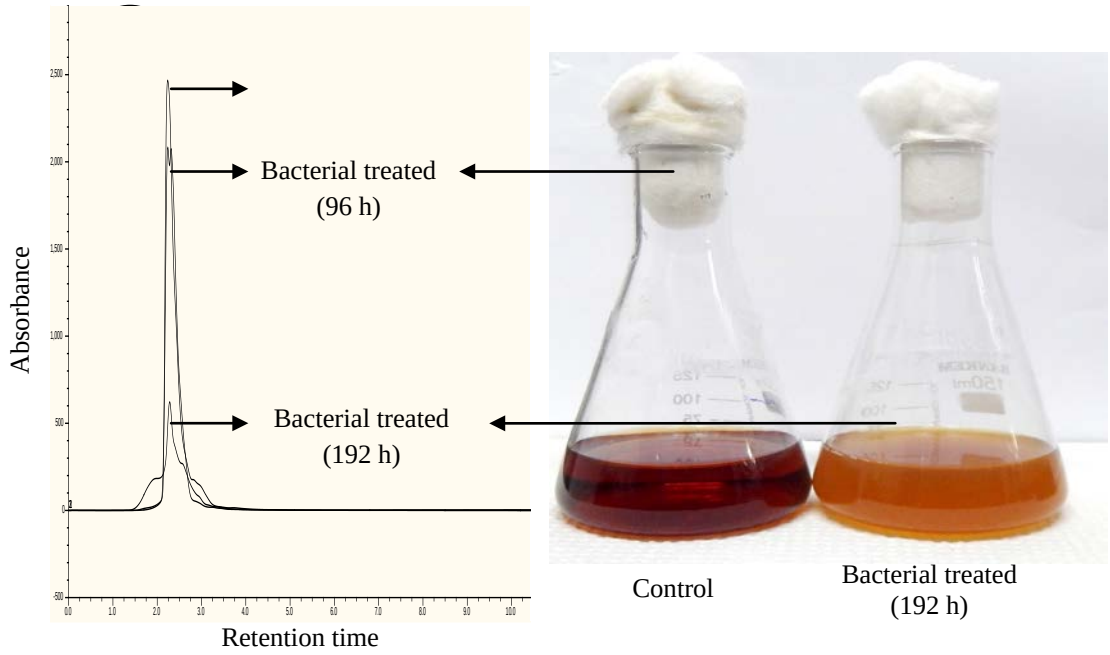



**Fig. 5.19** HPLC analysis (at 250 nm) of control and bacterial treated SGA-MRPs at different time interval

### 5.3.10 Identification of organic pollutants and their metabolites

In the present study, the GC–MS analysis of ethyl acetate extracted untreated SGA-MRPs showed the existence of different types of organic pollutants, most of which were biotransformed and biodegraded during bacterial treatment (Fig. 5.20 & Table 5.4). The major organic compounds detected in untreated sample at different RT were nonane, 3,7-dimethyl (RT:7.76), 1-octanol,2,2-dimethyl (RT:7.85), dodecane, 2,6, 10-trimethyl (RT:8.05), D-lactic acid-DiTMS (RT:8.34), 1-dodecene (RT:8.41), undecane, 2-methyl (RT: 8.80), butanoic acid, TMS ester (RT:9.12), dodecane (RT:9.28), silanol, trimethyl, benzoate (RT:12.36), heptadecane, 2,6,10,15-tetramethyl (RT: 13.22), benzeneacetic acid, TMS ester (RT:13.44), 1-tetradecanol (RT:13.60), 2-Bromo dodecane (RT:13.93), pentadecane (RT:14.03), dodecane, 2,6,11-trimethyl (RT:14.07), heptadecane (RT: 14.47), tetradecane (15.77), hexadecane, 2,6,10,14-tetramethyl (RT:17.60), phenol,2,4-bis (1,1dimethylethyl) (RT:17.95), 1-tetradecanol (RT:18.17), tetradecane, 2,6,10-trimethyl (RT:18.30), hexadecane (RT:19.82), dodecanoic acid, TMS ester (RT:20.77), docosane (RT:21.68), pyrazine, 2,5-dimethyl-3-propyl (RT:21.56), 2,6-diisopropylnaphthalene (RT:22.19), 3,4-tetramethylene-5,5-pentamethyle-2-nepyrazoline (RT:22.66), hexadecane, 2,6,11,15-tetramethyl (RT:25.64), 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-

dione (RT:25.21), heptacosane (RT:29.40), hexadecanoic acid, TMS ester (RT:27.55), 1H-purin-6-amine,[(2-fluorophenyl)methyl (RT:27.90), pentacosane (RT:29.29), 2,2-bis [(4-trimethylsiloxy)phenyl]propane (RT:30.00), nonacosane (RT:32.30), 1,2-benzenedicarboxylic acid, bis (2-ethylhexyl)ester (RT:33.51), hexadecanoic acid (RT:33.67), octadecane, 3-ethyl-5-(2-ethylbutyl) (RT:33.97), and methylenebis (2,4,6-triisopropylphenylphosphine) (RT:43.94). The major organic compounds detected in untreated sample were organic acids, pyrazine and phenolic compounds. Lactic acid, benzeneacetic acid, and benzenedicarboxylic acid are the alkaline degradation products of hexose. Pyrazines are the amdori products play a key role during the maillard product synthesis. Phenolic compounds are the fragmentary product of amadori compound. The presence of organic acids, phenolics and long chain aliphatic compounds in wastewater was reported previously by various workers (Gonzalez et al. 2000).

However, the analysis of bacteria treated SGA-MPs sample has showed the existence of various organic compounds such as, 3-hydroxy-2-butanone, TMS ester (RT:9.41), ethanolamine, TBS (RT:9.65), butane, 2,3-bis (TMSoxy) (RT:10.52), silanol, trimethyl, pentadecane (RT:14.03), undecenoic aicd, TMS ester (RT:18.51), cis-5-dodecanoic acid, TMS ester (RT:22.71), tetradecanoic acid, ethyl ester (RT:24.28), 2-hexadecanol acetate (RT:25.52), trans-9-octadecenoic acid 1TMS (RT:25.60), cis-10-heptadecenoic acid, TMS ester (RT:28.79), octadecanoic acid, ethyl ester (RT:29.72), 11-trans-octadecenoic acid, TMS ester (RT:30.10), methyl 19-methyl-eicosanoate (RT:32.16), 1-docosanol, acetate (RT:33.40), docosanoic acid, methyl ester (34.08), 17-pentatriacontene (RT:35.13), 2-monostearin, TMS ether (RT:35.30), octadecanoic acid, 2,3-bis [(TMS)oxy]propyl ester (RT:35.58), and ethyl tetracosanoate (RT:35.72) at different RT. These compound were completely absent in untreated sample, they are the degraded products of SGA-MRPs and few as new metabolites. Similar, compounds have been identified in distillery effluent after treatment with *Emericella nidulans* var. lata, *Neurospora intermedia* followed by *Bacillus* sp. in three stage bioreactor (Kaushik et al. 2010). However, several organic compounds detected in untreated sample were diminished after bacterial treatment. This suggested that complex and high molecular weight compounds were degraded by bacterial consortium with the help of extracellular ligninolytic enzymes. These organic compounds were utilized as sole carbon, nitrogen and energy source and played a key role in the decolourisation and degradation of SGA-MRPs.

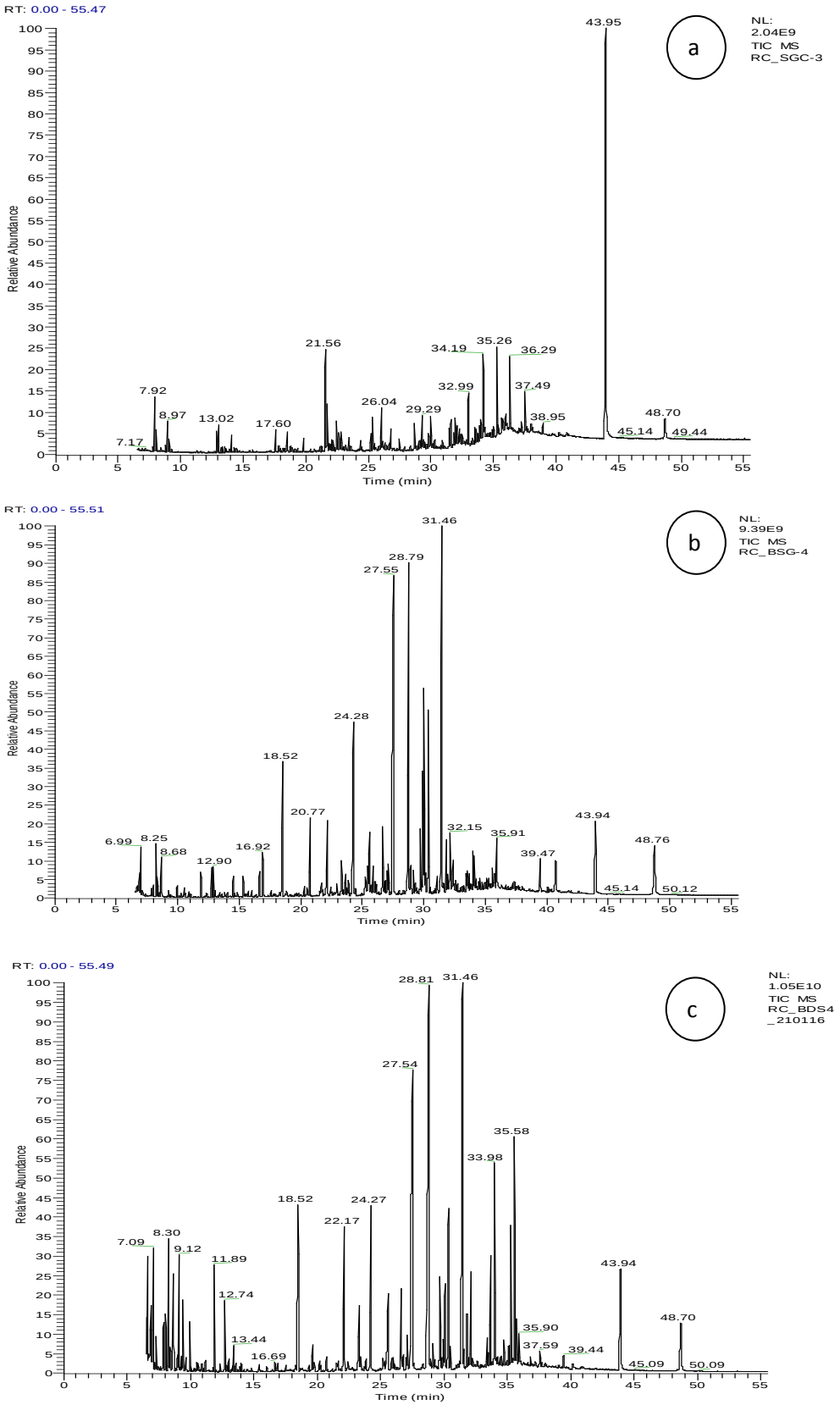



**Fig. 5.20** GC-MS chromatogram of organic compounds extracted with ethyl acetate from SGA-MRPs (a) control (b) bacterial degraded after 96 h incubation (c) bacterial degraded after 192 h incubation

**Table 5.4** Organic compounds identified by GC–MS analysis extracted with ethyl acetate from untreated and bacterial treated SGA-MRPs

| SI. No. | Name of Compound | RT | | BT | |
|---|---|---|---|---|---|
| | | | C | 96 h | 192 h |
| 1. | Nonane, 3,7-dimethyl | 7.76 | + | - | - |
| 2. | Butane, 2,3-bis(trimethylsiloxy) | 7.82 | - | + | - |
| 3. | 1-Octanol,2,2-dimethyl | 7.85 | + | - | - |
| 4. | 3,6-Dioxa-2,7-disilaoctane,2,2,4,5,7,7-hexamethyl | 8.00 | - | + | - |
| 5. | Dodecane, 2,6, 10-trimethyl | 8.05 | + | - | - |
| 6. | D-Lactic acid-DiTMS | 8.34 | + | - | - |
| 7. | 1-Dodecene | 8.41 | + | - | - |
| 8. | Piperidine, 1,4-dimethyl | 8.68 | - | + | - |
| 9. | Undecane, 2-methyl | 8.80 | + | - | - |
| 10. | Butanoic acid, TMS ester | 9.12 | + | - | - |
| 11. | Dodecane | 9.28 | + | + | - |
| 12. | 3-Hydroxy-2-butanone. TMS ester | 9.41 | - | - | + |
| 13. | Ethanolamine, TBS | 9.65 | - | - | + |
| 14. | Silane, trimethyl(4-methylphenoxy) | 10.31 | - | + | - |
| 15. | Butane, 2,3-bis(TMSoxy) | 10.52 | - | - | + |
| 16. | α-D-Mannopyranoside, methyl, cyclic 2,3:4,6-bis(butyloboronate) | 10.53 | - | + | - |
| 17. | 2-Hexenoic acid, 5-(1-ethoxyethoxy), 2-(TMS)ethyl ester | 10.57 | - | + | - |
| 18. | Silanol, trimethyl, benzoate | 12.36 | + | - | - |
| 19. | Octanoic acid, TMS ester | 12.75 | - | + | - |
| 20. | Silanol, trimethyl-phosphate | 12.90 | + | - | - |
| 21. | Heptadecane, 2,6,10,15-tetramethyl | 13.22 | + | - | - |
| 22. | Benzeneacetic acid, TMS ester | 13.44 | + | - | - |
| 23. | 1-Tetradecanol | 13.60 | + | - | - |
| 24. | Butanedioic acid, bis(TMS)ester | 13.82 | - | + | - |
| 25. | 2-Bromo dodecane | 13.93 | + | - | - |
| 26. | Pentadecane | 14.03 | + | - | + |
| 27. | Dodecane, 2,6,11-trimethyl | 14.07 | + | - | - |
| 28. | Vanillin, tert-butyldimethylsilyl ether | 14.45 | - | + | - |
| 29. | Heptadecane | 14.47 | + | - | - |
| 30. | 4-Methoxy-α-(TMS)benzenemethanol | 14.51 | - | + | - |
| 31. | Nonanoic acid, TMS ester | 14.85 | - | + | - |
| 32. | 1H-Purine-2,6-dione, 3,7-dihydro-1,3-dimethyl-7-(TMS) | 15.34 | - | + | - |
| 33. | Tetradecane | 15.77 | + | - | - |
| 34. | Decanoic acid, TMS ester | 16.92 | - | + | - |
| 35. | Hexadecane, 2,6,10,14-tetramethyl | 17.60 | + | - | - |
| 36. | 2-(2-(2-Butoxyethoxy)ethoxy)ethoxy-trimethylsilane | 17.84 | + | - | - |
| 37. | Phenol,2,4-bis(1,1dimethylethyl) | 17.95 | + | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 38. | 1-Tetradecanol | 18.17 | + | - | - |
| 39. | Tetradecane, 2,6,10-trimethyl | 18.30 | + | - | - |
| 40. | Undecenoic aicd, TMS ester | 18.51 | - | - | + |
| 41. | Undecenoic acid, TMS ester | 18.52 | - | + | - |
| 42. | Hexadecane | 19.82 | + | - | - |
| 43. | Pumiliotoxin | 20.52 | - | + | - |
| 44. | Dodecanoic acid, TMS ester | 20.77 | + | + | + |
| 45. | Pyrazine, 2,5-dimethyl-3-propyl | 21.56 | + | - | - |
| 46. | Docosane | 21.68 | + | + | + |
| 47. | Glucopyranose-1,2,3,5-Di-methylboronate-TMS | 22.11 | - | + | - |
| 48. | 2,6-Diisopropylnaphthalene | 22.19 | + | - | - |
| 49. | 3,4-tetramethylene-5,5-pentamethyle-2-nepyrazoline | 22.66 | + | - | - |
| 50. | cis-5-Dodecanoic acid, TMS ester | 22.71 | - | + | + |
| 51. | Leucine | 22.82 | + | - | - |
| 52. | Tetradecanoic acid, ethyl ester | 23.32 | - | + | - |
| 53. | Myristoleic acid ITMS | 23.87 | - | + | - |
| 54. | Tetradecanoic acid, TMS ester | 24.20 | - | + | - |
| 55. | Tetradecanoic acid, ethyl ester | 24.28 | - | - | + |
| 56. | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | 25.21 | + | - | - |
| 57. | Hexadecane, 2,6,11,15-tetramethyl | 25.34 | + | - | - |
| 58. | *n*-Pentadecanoic acid, TMS ester | 25.43 | - | + | |
| 59. | 2-Hexadecanol acetate | 25.52 | - | - | + |
| 60. | 1,4-Diaza-2,5-dioxa-3-isobutyl bicycle nonane | 25.60 | - | + | - |
| 61. | Trans-9-Octadecenoic acid 1TMS | 25.60 | - | - | + |
| 62. | cis-9-Hexadecenoic acid, TMS ester | 27.14 | - | + | - |
| 63. | Hexadecanoic acid, TMS ester | 27.55 | + | + | + |
| 64. | 1H-Purin-6-amine,[(2-fluorophenyl)methyl | 27.90 | + | - | - |
| 65. | cis-10-Heptadecenoic acid, TMS ester | 28.79 | - | + | + |
| 66. | Pentacosane | 29.29 | + | - | - |
| 67. | Heptacosane | 29.40 | + | - | - |
| 68. | Octadecanoic acid, ethyl ester | 29.72 | - | + | + |
| 69. | 9,12-Octadecadienoic acid, TMS ester | 29.93 | - | + | - |
| 70. | 2,2-Bis[(4-trimethylsiloxy)phenyl]propane | 30.00 | + | - | - |
| 71. | 11-Trans-Octadecenoic acid, TMS ester | 30.10 | - | + | + |
| 72. | Octadecanoic acid, TMS ester | 30.38 | - | + | - |
| 73. | cis-10-Nonadecenoic acid, TMS ester | 31.46 | - | + | + |
| 74. | Methyl 19-methyl-eicosanoate | 32.16 | - | + | + |
| 75. | Nonacosane | 32.30 | + | - | - |
| 76. | 2-Acetyl-3-(2-cinnamido)ethyl-7-methoxyindole | 32.38 | - | + | - |
| 77. | 17-Pentatriacontane | 33.41 | - | + | - |

| | | | | | |
|---|---|---|---|---|---|
| 78. | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester | 33.51 | + | - | - |
| 79. | 1-Docosanol, acetate | 33.40 | - | - | + |
| 80. | Hexadecanoic acid | 33.67 | + | - | - |
| 81. | Dotriacontane | 33.90 | - | + | - |
| 82. | Octadecane, 3-ethyl-5-(2-ethylbutyl) | 33.97 | + | - | - |
| 83. | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester | 33.98 | - | - | + |
| 84. | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester | 33.99 | - | + | - |
| 85. | Docosanoic acid, methyl ester | 34.08 | - | - | + |
| 86. | Ethyl docosanoate | 34.09 | - | + | - |
| 87. | Cis-13-Docodenoic acid, TMS ester | 34.24 | - | + | - |
| 88. | Docosanoic acid, TMS ester | 34.45 | - | + | - |
| 89. | Quercetin 7,3',4',-trimethoxy | 34.72 | - | + | - |
| 90. | Heptacosane | 34.99 | - | + | - |
| 91. | 17-Pentatriacontene | 35.13 | - | - | + |
| 92. | 2-Monostearin, TMS ether | 35.30 | - | + | + |
| 93. | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester | 35.58 | - | - | + |
| 94. | Ethyl tetacosanoate | 35.72 | - | - | + |
| 95. | 2,6,10,14,18,22-Tetracohexane, 2,6,10,15,19,23-hexamethyl | 35.91 | - | + | - |
| 96. | Silane, [[(3β)-cholest-5-en-3-yl]oxy]TM | 39.47 | - | + | - |
| 97. | Silane, (Ergosta-5,7,22-trien-3β-yl)TM | 40.71 | - | + | - |
| 98. | Methylenebis (2,4,6-triisopropylphenylphosphine) | 43.94 | + | - | - |

TMS: Trimethylsilyl; C: Control; BT: Bacterial treated; RT: Retention time

### 5.3.11 Effect of SGA-MRPs on seed germination and seedling growth

The phytotoxicity of untreated and treated sample was assessed by observing the germination percentage and measuring the length of the radical of *P. mungo* L. seeds. It was observed that untreated sample was highly toxic in nature and showed only 75, 70, 60, 15% germination at 2.5, 5, 10 and 15 % concentration of SGA-MRPs. But, after treatment with bacteria, the toxicity of SGA-MRPs was reduced extensively and showed 90, 90, 80, 25, and 20% germination at 2.5, 5, 10, 15, and 20% concentration of SGA-MRPs (Table 5.5 and Fig. 5.21). Suppression of germination at high concentration of SGA-MRPs might be due to the occurrence of highly toxic organic pollutants and dissolve solid which absorbed by the seed before germination and affecting different biochemical and physiological process of seed germination (Bharagava and Chandra 2010a). Increased in percent germination in treated SGA-MRPs might be due to decrease of organic compounds that has created favorable environment for germination and utilisation of nutrient present in maillard products. The percent germination and radical length were combined for a comprehensive interpretation of maillard products toxicity in term of germination index (GI). The GI of untreated and treated SGA-MRPs values ranged

between 0.05-0.61 and 0.10-0.85, respectively. The GI was decreased with increased concentration of treated sample compared to untreated sample. The percent phytotoxicity analysis of untreated sample revealed that the phytotoxicity increased with increased in SGA-MRPs concentration but in degraded SGA-MRPs it gradually decreased. Various parameters were studies showed seedling vigor index (16.5-180 and 30-252), stress tolerance index (37.28-81.35 and 50.84-94.91), relative toxicity (85-25 and 80-10) with untreated and treated SGA-MPs, respectively. These results clearly indicated that the toxicity of SGA-MRPs was reduced appreciably after bacterial treatment.

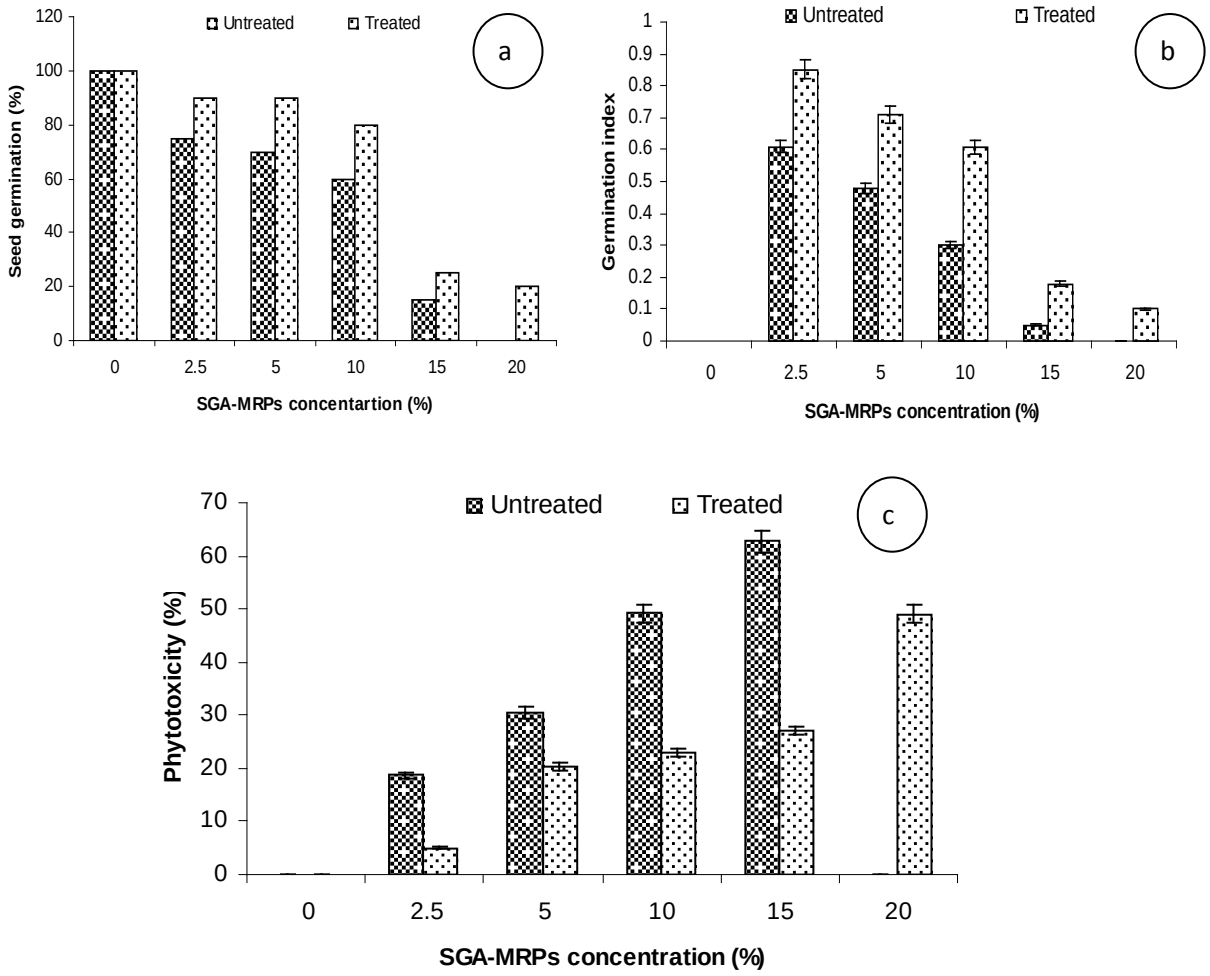



**Fig. 5.21** Effects of various concentrations of untreated and bacterial treated SGA-MRPs on the seed germination and seedling growth of *Phaseolus mungo* L. (a) percent germination (b) germination index (c) phytotoxicity percent (0%: tap water used as a control)

**Table 5.5** Effects of various concentrations of untreated and bacterial treated SGA-MRPs on the seed germination and seedling growth of *Phaseolus mungo* L.

| Samples | Treatment | Germination (%) | Germination index | Phytotoxicity (%) | Seedling vigor index | Stress tolerance index (%) | Relative toxicity (%) | Radical length (cm) |
|---|---|---|---|---|---|---|---|---|
| Untreated | 2.5% | 75±0.00 | 0.61±0.00 | 18.64±0.09 | 180±8.01 | 81.35±0.02 | 25±0.01 | 2.40±0.01 |
| | 5% | 70±0.00 | 0.48±0.00 | 30.50±0.06 | 143.50±4.21 | 69.49±0.02 | 30±0.03 | 2.05±0.01 |
| | 10% | 60±0.00 | 0.30±0.00 | 49.15±0.07 | 90±0.08 | 50.84±0.01 | 40±0.02 | 1.50±0.00 |
| | 15% | 15±0.00 | 0.05±0.00 | 62.71±0.04 | 16.5±0.01 | 37.28±0.01 | 85±0.11 | 1.10±0.00 |
| | 20% | NG | - | - | - | - | - | - |
| Treated | 2.5% | 90±0.00 | 0.85±0.00 | 5.08±0.01 | 252±14.01 | 94.91±0.03 | 10±0.00 | 2.80±0.01 |
| | 5% | 90±0.00 | 0.71±0.00 | 20.33±0.01 | 211.5±9.14 | 79.66±0.02 | 10±0.00 | 2.35±0.01 |
| | 10% | 80±0.00 | 0.61±0.00 | 23.05±0.03 | 181.6±4.21 | 76.94±0.02 | 20±0.01 | 2.27±0.02 |
| | 15% | 25±0.00 | 0.18±0.00 | 27.11±0.02 | 53.75±0.04 | 72.88±0.01 | 75±0.09 | 2.15±0.01 |
| | 20% | 20±0.00 | 0.10±0.00 | 49.15±0.01 | 30±0.00 | 50.84±0.01 | 80±0.17 | 1.50±0.00 |
| Control | 0% | 100±0.00 | - | - | 295±24.01 | - | - | 2.95±0.01 |

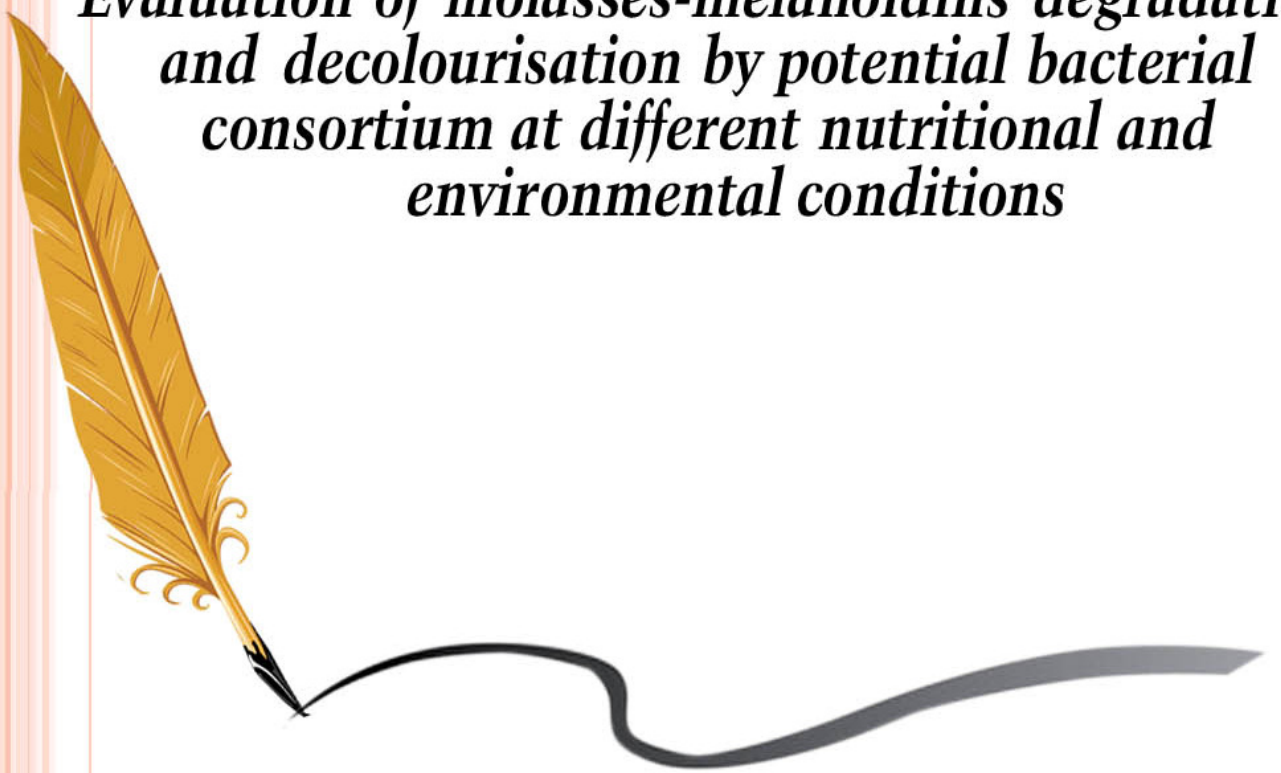
All values are mean of three replicates ± S.D. NG: No germination; Control: tap water

## Conclusion

This study has revealed that SGA-MRPs showed mixture of variable molecular weight melanoidins. Therefore, several peaks were noted in rage of 200-450 nm. Further, this study also showed that the developed bacterial consortium has ability to degrade and decolourise recalcitrant complex SGA-MRPs polymer up to 70% in existence of adequate carbon and nitrogen source at broad range of temperature, pH, and shaking speed within a incubation period of 192 h. GC-MS and other spectrophotometric analysis of untreated and bacterial treated sample of SGA-MRPs have shown that the majority of the colours containing compounds (melanoidins) detected in untreated sample were diminished from the treated sample by MnP and laccase enzyme activity of bacteria. Hence, the optimized conditions for developed bacterial consortium can be used for decolourisation of MRPs containing industrial wastewater at large scale.

# Chapter-06

# Evaluation of molasses-melanoidins degradation and decolourisation by potential bacterial consortium at different nutritional and environmental conditions



# Chapter-6

## Evaluation of molasses-melanoidin degradation and decolourisation by potential bacterial consortium at different nutritional and environmental conditions

### 6.1 Introduction

Melanoidins is a major colouring constituents of dark-brown effluent released from ethanol producing distillery industries. Most of decolourisation and degradation of molasses-melanoidins is reported at 475 nm only as purified melanoidins through dialysis process with specific molecular weight, while the molasses-melanoidins contains mixture of maillard reaction products (MRPs) (i.e. initial, intermediate and advanced stages with variable molecular weight). However, due to complex nature of molasses-melanoidins at variable absorption spectrum at different wavelength is essential to understand the mechanism of melanoidins decolourisation and degradation and its metabolic products. But, no study has been conducted for assessment of molasses-melanoidins degradation present in its original form for the feasibility of any potential bacterial consortium. Hence, the prime objectives of this study were to optimize the environmental conditions for the decolourisation and degradation of molasses-melanoidins extracted from PMDE. Further, the analysis of absorption spectrum between 200-700 nm by UV-Vis spectrophotometer has been correlated for their structural changes and reduction in color by investigation through TLC, HPLC, FT-IR and GC-MS analysis. Furthermore, the stability of developed potential bacterial consortium at variable nutritional and environmental conditions was evaluated for its effective degradation and degradation of molasses-melanoidins containing distillery effluent for development of feasible technology. The toxicity evaluation of molasses-melanoidins prior and after bacterial degradation was also done by using seed germination test of *Phaseolus mungo* L.

### 6.2 Material and Methods

#### 6.2.1 Collection of distillery effluent

PMDE after secondary treatment (final treatment) was collected aseptically in pre-sterilised plastic container (capacity 20 litre; Tarson Production Pvt. Ltd., USA) from the effluent collection tank of M/s Unnao Distilleries, located in Unnao, Uttar Pradesh ($26^{o}320''N,80^{o}30'0''E$), India (Fig. 6.1). All the collected samples were transported to laboratory and kept at $4^{0}C$ for further analysis.


**Fig. 6.1** Post methanated distillery effluent (PMDE) discharged after anaerobic digestion

### 6.2.2 Extraction of molasses melanoidins

Molasses melanoidins were extracted from PMDE by following the isopropanol method described by Kalavathi et al. (2001). In this method, the equal volume of PMDE and isopropanol (v/v) was taken in separating funnel and mixed vigorously. Subsequently, precipitate at bottom of the separating funnel was separated and dried in a hot air oven at 80 $^{o}$C, powdered and used in this study.

### 6.2.3 Medium to study decolourisation

The medium used for melanoidins decolourisation study containing (g $L^{-1}$) glucose 1%, peptone 0.5%, $K_2HPO_4$ 0.1%, and $MgSO_4.7H_2O$ 0.05% w/v). This medium was supplemented with different concentration of molasses melanoidins viz. 1000-3500 mg $L^{-1}$ (w/v). The final pH of medium was adjusted to 7.0 using 0.1N HCl or 0.1N NaOH.

### 6.2.4 Preparation of bacterial culture

An aerobic bacterial consortium consisting four bacterial strains *Klebsiella pneumoniae* (KU726953), *Salmonella enterica* (KU726954), *Enterobacter aerogenes* (KU726955), *Enterobacter cloaceae* (KU726957) isolated from distillery sludge sample to degrade synthetic MRPs as described in chapter 05 was used in this study. The seed culture was prepared for degradation by inoculating the pure bacterial colonies in broth medium and incubated for 24 h in continuous shaking condition (120 rpm) in 250 mL Erlenmeyer flask containing sterilised 100 mL mineral salt medium supplemented with $K_2HPO_4$ (0.1%), $MgSO_4.7H_2O$ (0.05), and molasses melanoidins (1000 mg $L^{-1}$) and incubated at 35±1 °C and 130 rpm.

### 6.2.5 Physico-chemical analysis of molasses melanoidins

The physico-chemical properties such as colour, pH, BOD, COD, TDS, TS, VS, chloride, total nitrogen, ammonical nitrogen, phenol, sulphate, phosphate and heavy metals of

molasses-melanoidins before and after bacterial decolourisation were carried out using standard methods (APHA 2012).

### 6.2.6 Measurement of decolourisation efficiency

The decolourisation experiment was carried out in Erlenmeyer flasks (250 mL) containing 100 mL of previously prepared sterile melanoidins containing medium. The flasks were inoculated with overnight grown fresh culture of four identified bacterial strains i.e. *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), and *E. cloaceae* (KU726957). The each bacterial culture was inoculated for their exponential phase with highest optical density (O.D.) such as *K. pneumoniae* ($OD_{620}$ 0.24), *S. enterica* ($OD_{620}$ 0.24), *E. aerogenes* ($OD_{620}$ 0.24), and *E. cloaceae* ($OD_{620}$ 0.24). The different bacteria were inoculated in flask in equal ratio i.e. 1:1:1:1 and incubated at 37±1 °C under shaking flask condition (120 rpm) for 192 h. The sample were collected at every 24 h interval during incubation and centrifuged at 10,000 ×g for 10 min at 4 °C for measurement of decolourisation efficiency. Melanoidins decolourisation efficiency was monitored by measuring the change in absorbance maxima of the melanoidins at 295 nm after scanning of absorbance maxima by using a UV-vis spectrophotometer (Evoluation-201, Thermo Scientific, USA). Simultaneously, the control flasks without any bacterial inoculation were also incubated in shaker with same temperature and shaking speed. Finally, the ratio of bacterial cell in flask was found in ratio of 2:1:2:2. This indicated the optimum ratio of different bacteria in consortium for maximum decolourisation. Therefore, the ratio of different bacteria in consortium was selected in ratio of 2:1:2:2 for optimisation of decolourisation process at different nutritional and environmental parameters. The decolourisation efficiency was expressed as percent (%) of decolourisation.

$$\text{Decolourisation (\%)} = \frac{(A_i)-(A_t)}{(A_i)} \quad 100$$

Where, $A_i$ is the initial absorbance and $A_f$ is the absorbance of medium after decolourisation at the $\lambda_{max}$ 295 nm

### 6.2.7 Optimisation of culture conditions for decolourisation and degradation of melanoidins

#### 6.2.7.1 Effect of carbon and nitrogen source

To find the most appropriate carbon source viz. sucrose, fructose, glucose, xylose, lactose, and starch at 1.0% (w/v) were evaluated for the melanoidins decolourisation and degradation. In another experiment, different organic and inorganic nitrogen sources viz. peptone, beef extract, yeast extract, sodium sulphate, ammonium chloride and urea were added into the GPM medium at 0.5% (w/v) concentration to evaluate the best nitrogen source for melanoidins decolourisation and bacterial growth.

#### 6.2.7.2 Effect of environmental parameters

To evaluate the effect of different environmental parameters on melanoidins decolourisation, the same experiment was carried out at varying temperature (25-50 °C), pH (4-12), and shaking speed (100—220 rpm).

### 6.2.8 Evaluation of bacterial growth, biomass, and SEM observation of bacterial consortium

During decolourisation experiment, bacterial cell growth was monitored by measuring the optical density (O.D.) at 620 nm at 12 h periodic interval. While the bacterial sample for biomass determination and SEM micrograph was collected from the flask after 144 hrs of incubation. First, the bacterial cells were centrifuged (6500 $\times$g) for 10 min at 4 $^{o}$C, the pellets were washed thrice with distilled water to remove the attached medium contents. For bacterial biomass determination, the pellets was dried in an hot air oven at 80 °C until getting a constant dried weight reported in the form of dry cell mass (g $L^{-1}$). For SEM micrograph, the bacterial cells were fixed with 1% (w/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 2 h and washed again with distilled water. The fixed samples were then dehydrated through 25%, 50%, 75%, 95% and 100% ethanol solutions in increasing concentration for 5 min, at each step. The samples were then dried in critical point drier and coated with a thin conductive film of gold in a sputtering coater and observed under SEM (FEI Quanta 450, Hillsboro, USA).

### 6.2.9 Enzyme assay

To measure the MnP and laccase activity during melanoidins degradation, the bacterial degraded supernatant was obtained by centrifugation at 6,500 ×g for 10 min at 4 $^{o}$C. The MnP and laccase activity was determined using the phenol red (Lobachemie, Mumbai) and guaiacol (HiMedia, Mumbai), respectively as described in chapter no. 04 (Arora et al. 2002). One international unit (IU) of enzyme activity was defined as 1.0 µM product formed per minute under assay conditions.

### 6.2.10 Decolourisation and biodegradation analysis

#### 6.2.10.1 UV-Vis and FT-IR spectroscopic analysis

Melanoidins decolourisation was quantitatively analysed using a UV-Vis spectrophotometer in the wavelength range between 200-700 nm at 25 °C, whereas biodegradation was monitored by FT-IR analysis of the sample was performed in range of 400 to 4000 $cm^{-1}$ using a spectrophotometer (Nicolet$^{TM}$ 6700, Thermo Scientific, USA) in order to reveal the chemical nature of the melanoidins. The separated samples of melanoidins were mixed with potassium bromide (KBr) to prepare the pellet for FT-IR analysis.

#### 6.2.10.2 Detection of EDCs and other organic compounds

The EDCs and other organic compounds present in treated and untreated melanoidins were extracted by ethyl acetate under acidic condition (pH <2.0) as described in chapter no. 04 (Bharagava and Chandra 2009) The organic phase was dried over anhydrous $Na_2SO_4$, and the solvent was removed using a stream of nitrogen at room temperature. The residues were dissolved in 2.0 mL methanol. The sample in methanol was filtered through 0.22-μm syringe filters and used for TLC, HPLC and GC-MS analysis.

#### 6.2.10.3 TLC and HPLC analysis

The concentrated sample resolved on a pre-coated silica-gel TLC plate (silica G 20×20 cm, 0.25 mm thickness). This plate was developed in an organic solvent system toluene: chloroform: methanol: water (10:10:9:9 v/v). After development, the bands of decolourisation were observed under UV light through gel documentation system (Syngene, 230V-2A, UK). HPLC analysis was performed on 515 HPLC instrument equipped with a diode array detector system (1100 series, Agilent Technologies, USA) and reverse phase $C_{18}$ column (250 × 4.6 mm, 5 μm particle size) by using the gradient of solvent A (Milli-Q water) and solvent B (acetonitrile with 0.1% TFA) (Merck, Germany) at a flow rate 0.4 mL $min^{-1}$ for 60. The detection was monitored at wavelength 240 nm (absorption maxima) to assess the decolourisation and degradation of melanoidins.

#### 6.2.10.4 GC-MS analysis

Identification of EDCs and other organic compounds before and after bacterial treatment was carried out by GC–MS analysis. In GC-MS analysis, the extracted samples were derivatised with trimethylsilyl (TMS) as described by chapter no. 04 (Bharagava and Chandra 2009). Further, the organic compounds were identified by matching with the mass spectrum library NIST v. 1.0.0.12 available with instrument.

### 6.2.11 Phytotoxicity assessment

The toxicity assessment of untreated and treated melanoidins was studied on *P. mungo* L. seed germination using petri dish method as described in chapter no. 04 (OECD 2003; Sharma et al. 2002).

### 6.2.12 Statistical analysis

Each experiment was performed in triplicate. The standard deviation (SD) was calculated using Microsoft Excel, and results presented as mean ± SD value. The mean value of various physico-chemical parameters of untreated and treated melanoidins was assessed Student's '*t*' test by using SPSS software package program version 22.0.

## 6.3 Results and discussion

### 6.3.1 Physico-chemical analysis

The physico-chemical properties of control and bacterial decolourised melanoidins containing samples are presented in Table 6.1. The value of colour intensity and pH was higher in the control sample. In addition, the concentration of BOD, COD, TS, TDS, VS, chloride, total nitrogen, ammonical nitrogen, phenol, sulphate, phosphate and various heavy metals such as Mn, Cr, Zn, Ni, Cu, Fe, Pb, and Cd was found higher as shown in Table 6.1. The high pH of control sample might be due to the binding of high concentrations of soluble salts and heavy metals with melanoidins. The high BOD and COD values of the prepared melanoidins solution suggested the presence of organic and inorganic compounds in higher quantities. Since, some caramelized sugar might be also generated during the MR. Therefore, high TDS of solution is found in this study. In addition, the high content of heavy metals in distillery effluent could be due to the corrosive effect of sugarcane juice during the sugar manufacturing process. Besides, metals might be also added during the fermentation and distillation processes of fermented sugarcane molasses in distilleries, which is finally discharged as PMDE with alkaline pH. Our findings also corroborated well with earlier observation (Bharagava and Chandra 2009, 2010). Phenols are fragmentary product of amadori compound which play a key role during the melanoidins formation. The high levels of organic and inorganic parameters in distillery effluent are also corroborated with previous observations (Yadav and Chandra 2012, 13; Bharagava and Chandra 2010a).

In contrast to above, the bacterial decolourised sample obtained after 168 h incubation showed reduction in all physico-chemical parameters as shown in Table 6.1. This indicated the biodegradation and biotransformation of various organic and inorganic contents by potential bacterial consortium. Initially, during degradation of melanoidins the pH of medium was decreased to 4.47, but after 168 h of bacterial growth the pH was gradually enhanced. The reduction of pH at initial stage of bacterial growth might be due to production of organic acid such as ethanedioic acid, acetic acid, phosphoric acid, and octadecenoic acid (Davídek et al. 2006). The increased pH of media might be due to depolymerisation of MRPs and degradation of organic compounds by enzymatic activity of bacterial consortium during degradation of melanoidins. This induced the solubility and utilisation of melanoidins which favoured the mineralisation and co-metabolism of melanoidins (Bharagava et al. 2009). However, the evaluation of BOD and COD values at various time intervals showed that, there was gradual decrease in BOD and COD values with increase in percent decolorization (76%) as shown in Fig. 6.2. BOD/COD ratio is typically a measurement used to describe the organic composition of wastewater. This BOD/COD ratio has been commonly used as an indicator for biodegradability index (Metcalf and Eddy 2003). In our study, the BOD/COD ratio of bacterial decolourised melanoidins was found 0.46. This indicates the utilisation of organic and inorganic chemical compounds present in melanoidins containing sample by bacterial consortium. It appears that bacterial consortium carried out biodegradation of some organic components

of MRPs. Statistical analyses revealed that there was statistically significant difference ($p<0.001$) between all physic-chemical parameters of control and bacterial decolourised melanoidins except pH.

**Table 6.1** Physico-chemical characteristics of molasses-melanoidins before and after bacteria decolourisation

| S.No. | Parameter | Values | Bacterial degraded | | % Reduction |
|---|---|---|---|---|---|
| | | | After 72 h | After 168 h | |
| 1. | Color Intensity | 150000±2.64 | 90002±2.64 | 15244±3.60[a] | 89.83 |
| 2. | pH | 8.53±0.01 | 4.47±0.00 | 8.76±0.02[a] | -2.69 |
| 3. | BOD | 6296.66±6.65 | 4252.66±1.52 | 2932±51.15[a] | 53.43 |
| 4. | COD | 2487.66±110.12 | 2235±1.00 | 1352.33±1.52[a] | 25.51 |
| 5. | TS | 6940±5.29 | 5525±2.64 | 4772±3.60[a] | 45.6 |
| 6. | TDS | 10527.66±6.65 | 6424±3.51 | 4534.33±5.50[a] | 56.92 |
| 7. | V S | 1059.33±2.08 | 753.01±2.64 | 242±5.13[a] | 77.15 |
| 8. | Chloride | 2746±4.35 | 1525.66±1.52 | 805±8.38[a] | 70.68 |
| 9. | Total nitrogen | 195.33±4.06 | 184.12±1.48 | 164.66±2.08[a] | 15.70 |
| 10. | Phenol | 6892±7.54 | 4520.53±3.27 | 1997±1.00[a] | 71.02 |
| 11. | Sulphate | 13463.33±3.78 | 5247.19±0.83 | 3756.66±6.80[a] | 72.09 |
| 12. | Phosphate | 41.52±1.60 | 31.60±0.47 | 17.28±0.06[a] | 58.38 |
| 13. | Trace Elements | | | | |
| a | Mn | 8.20±0.09 | 6.21±0.01 | 4.26±0.01[a] | 48.04 |
| b | Cr | 2.97±0.026 | 2.02±0.02 | 1.64±0.02[a] | 44.78 |
| c | Zn | 16.61±0.00 | 8.52±0.00 | 3.89±0.01[a] | 76.58 |
| d | Cu | 2.55±0.01 | 1.232±0.00 | 0.22±0.02[a] | 91.37 |
| e | Fe | 373.95±0.36 | 150.725±0.66 | 17.06±0.02[a] | 95.43 |
| f | Pb | 2.59±0.00 | 1.92±0.00 | 1.44±0.02[c] | 44.40 |
| g | Cd | BDL | BDL | BDL | - |
| h | Ni | 4.18±0.00 | 1.25±0.01 | 1.05±0.00[a] | 74.88 |

All values are mean (n=3)±SD in mg $l^{-1}$ except color intensity (Pt-Co unit), pH, and temperature (°C), Student's t-test (two tailed as compared to untreated sample); a- Highly significant at $p < 0.001$; c- Less significant at $p<0.05$

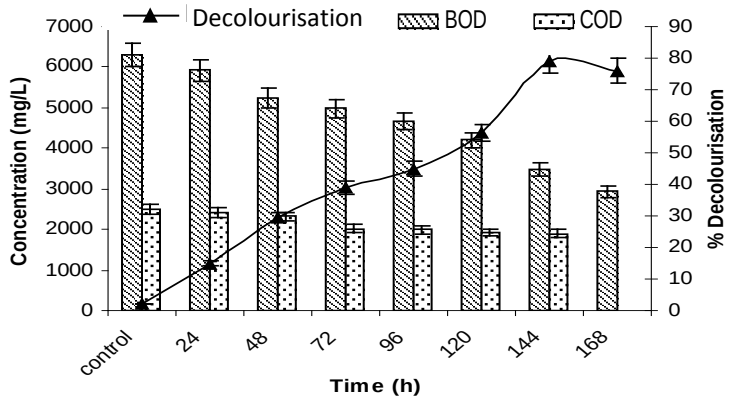



**Fig. 6.2** Decolourisation of molasses-melanoidins, BOD and COD reduction at various time intervals

### 6.3.2 Effect of different carbon and nitrogen sources

Melanoidins decolourisation was evaluated by using different carbon sources at 1% (w/v) concentration for 168 h of bacterial incubation. The results showed that the efficiency of consortium increase with increase in glucose concentration from 0.1% to 1% achieved maximum decolourisation (76%) at 1.0% concentration of glucose (Fig. 6.3a). This indicated that decolourisation of melanoidins was result of co-metabolism of MRPs polymer in presence of glucose. While, further increase in glucose concentration does not support the bacterial growth and decolourisation of melanoidins. However, sucrose, starch, lactose, xylose and fructose were found less effective compared to glucose, showed decolourisation of melanoidins up to 42-58%. This indicated that carbon bounds with melanoidins are not bioavailable to growing bacterial consortium. Therefore, the decolourisation of melanoidins without supplementary of carbon source is very not observed. But, the presence of glucose in media was readily available to bacterial consortium for their growth. Carbon source provided energy for survival and growth of microorganisms, which are essential for the cleavage of conjugated C═C, C=O and C≡N bonds of melanoidins by electron donation from hydrogen peroxide ($H_2O_2$) generated by glucose by the action of bacterial glucose oxidase in growth media (Miyata et al. 1998, 2000; Kalavathi et al. 2001). Similar observation has also been reported by previous workers during bacterial treatment of anaerobically treated distillery spent wash (Santal et al. 2011; Ghosh et al. 2004). This indicated that our bacterial consortium was more effective for decolourisation and degradation of melanoidins containing effluent. This might be due to presence of hight amount of enzyme which facilitates the decolourisation and degradation processes of complex MRPs (Kumar and Chandra 2006).

The effect of different organic and inorganic nitrogen sources at a concentration of 1.0 % (w/v) along with glucose (1.0% w/v) showed that peptone at 0.2% (w/v) concentration was found most effective organic nitrogen source which enhanced decolourisation of melanoidins upto 78% (Fig. 6.3b). Further, increase in concentration of peptone resulted in gradual decline in degradation capability of the developed bacterial consortium inhibited the decolourisation process. This might be due to allosteric inhibition of bacterial enzymes. However, presence of other organic nitrogen sources i.e. beef extract, yeast extract, and urea could show the melanoidins decolorization upto 34-78% only, whereas inorganic nitrogen sources such as sodium sulphate, and ammonium chloride could show the decolourisation upto 21% and 25%, respectively. This finding showed the inhibitory effect of sodium sulphate and ammonium chloride on bacterial growth. Kumar and Chandra (2006) and Miyata et al. (2000) have also studied the inhibitory effect of inorganic nitrogen sources for melanoidins decolourisation. Similarly, various nitrogen sources were optimized by various workers for decolorization of melanoidins, but peptone was reported most effective for color reduction (Sirianuntapiboon and Phothilangka 2004; Tiwari et al. 2013; Ravikumar et al. 2011). Kirk et al. (1978) reported that in presence of peptone microorganisms catalyse enzymatic degradation of lignin and lignin-like compounds during the secondary phase of metabolic growth. Induction and secretion of MnP is triggered by nutrient limitations such as carbon

and nitrogen sources (Wong 2009). At high concentration, there was no significant melanoidins decolourisation was noted due to surplus supplementation of nitrogen which inhibited the bacterial growth. Hence, bacterial consortium utilised tiny amount of peptone for highest decolourisation, BOD, and COD reduction.

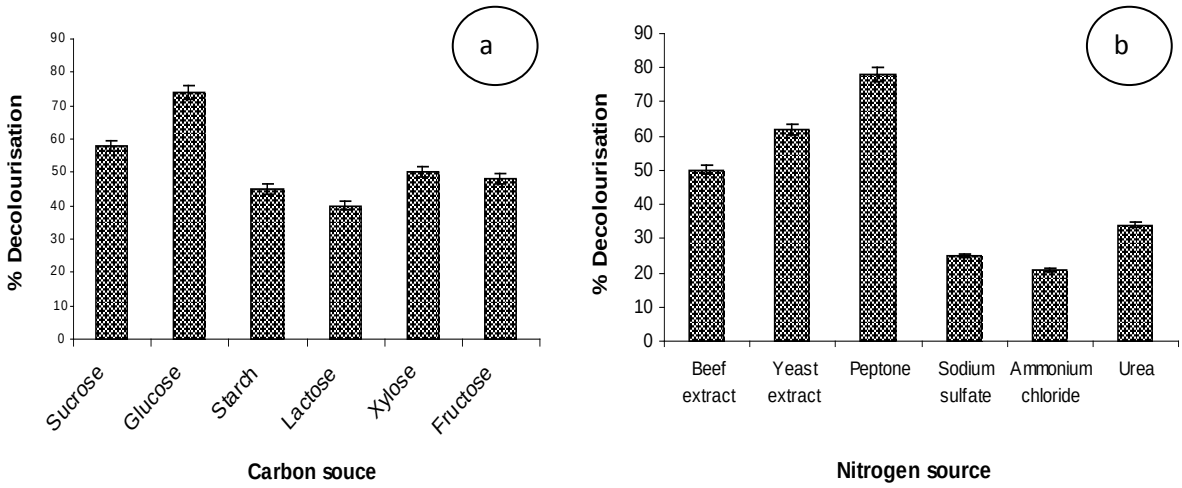



**Fig. 6.3** Effect of different nutritional parameters on degradation and decolourisation of molasses-melanoidins (a) effect of carbon sources (b) effect of nitrogen sources

### 6.3.3 Effect of pH, temperature and shaking speed

The pH and temperature are important factors for the optimal decolourisation of melanoidins and physiological performance of bacterial culture. The maximum decolourisation (79%) of melanoidins by bacterial consortium was found at pH 8.1. This showed the optimum pH of growing bacterial consortium for decolourisation of melanoidins. At alkaline pH (8.1), the higher decolourisation of melanoidins might be due to combined action of bacterial enzyme and loosen of conjugated C═C, C=O and C≡N bonds of melanoidins. Further, increase in pH inhibited the decolourisation process of melanoidins (Fig. 6.4a). This might be due to either toxicity of variable pH or low bio-availability of melanoidins. At high pH decrease in color was due to depolymerization of melanoidins by bacterial consortium. Our results are supported the previous observation of Hayase et al. (1984), who achieve maximum decolourisation of melanoidins by $H_2O_2$ treatment at alkaline pH. The fluctuation in the optimum pH (8.1), melanoidins decolourisation was reduced might be due to inhibition of the enzyme activities responsible for decolourisation of melanoidins, or less of cell viability. Because, all enzymes are made up of proteins, hence, some proteins are denatured at high or low pH range. Similarly, it was also observed that increase in temperature (25–37 °C) enhanced decolourisation of melanoidins from 35 to 79% (Fig. 6.4b). The developed bacterial consortium also showed maximum decolourisation (81%) of melanoidins at 37 °C. While further, the increase in temperature up to 45 °C adversely affected the growth and decolourisation ability of the bacterial consortium. Similar pattern of melanoidins decolourisation at variable temperature is also reported by other researchers (Santal et al.

2011; Tiwari et al. 2013; Mohana et al. 2007). Further, the effect of shaking speed showed the optimum decolourisation of melanoidins by potential bacterial consortium was observed at 180 rpm as shown in Fig. 6.4a. However, increase in shaking speed resulted decrease in decolourisation process of melanoidins. This might be due to breakage of bacterial cell wall at a higher shaking speed. In addition, bacterial cells might be stressed and could not grow well at very high shaking speeds. These results were in accordance with those of previous studies (Yadav and Chandra 2012; Tiwari et al. 2014). The decolourisation and degradation of melanoidins was greatly influenced by concentration of melanoidins. The decolourisation rate of melanoidins by bacterial strains was decreased with an increase concentration of melanoidins. Maximum decolourisation (79%) of melanoidins was observed at 3200 mg $L^{-1}$ concentration. Further increase in melanoidins concentration (3200-3500 mg $L^{-1}$) inhibited the melanoidins decolourisation process.

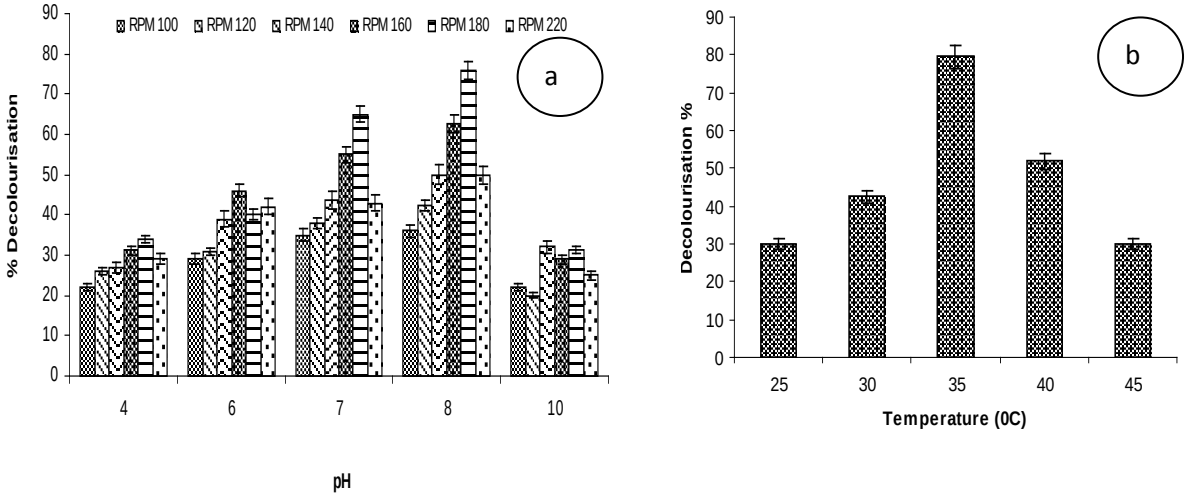



**Fig. 6.4** Effect of different environmental parameters on degradation and decolourisation of molasses-melanoidins (c) effect of pH and shaking speed (effect of temperature)

### 6.3.4 Bacterial growth and biomass

The periodic monitoring of bacterial growth curve and biomass showed maximum $OD_{620}$ (2.49) and biomass (5.22 $gL^{-1}$; CFU/ml $27.5\times10^{6}$) at 144 h incubation period during decolourisation process (Fig. 6.5 and 6.6). This indicated the most optimum condition for highest bacterial growth and biomass production to achieve maximum decolourisation (79%) of melanoidins. In addition, the SEM analysis of growing bacterial biomass showed dense bacterial cell. This indicated rapid bacterial growth in melanoidins containing medium this resulted for degradation and decolourisation of melanoidins. Further, incubation of bacterial consortium did not increase the rate of melanoidins decolourisation. The direct correlation of $OD_{620}$ with high bacterial biomass also indicated the direct involvement of bacterial consortium in decolourisation and degradation process of melanoidins.

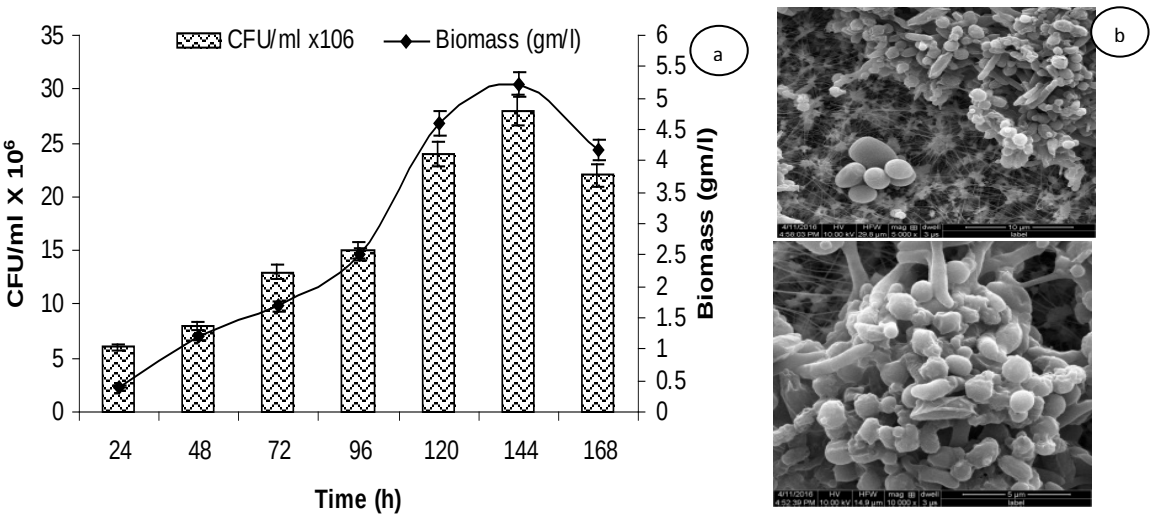



**Fig. 6.5 (a)** Growth pattern and (b) scanning electron micrograph of developed bacterial consortium during degradation and decolourisation of molasses-melanoidins

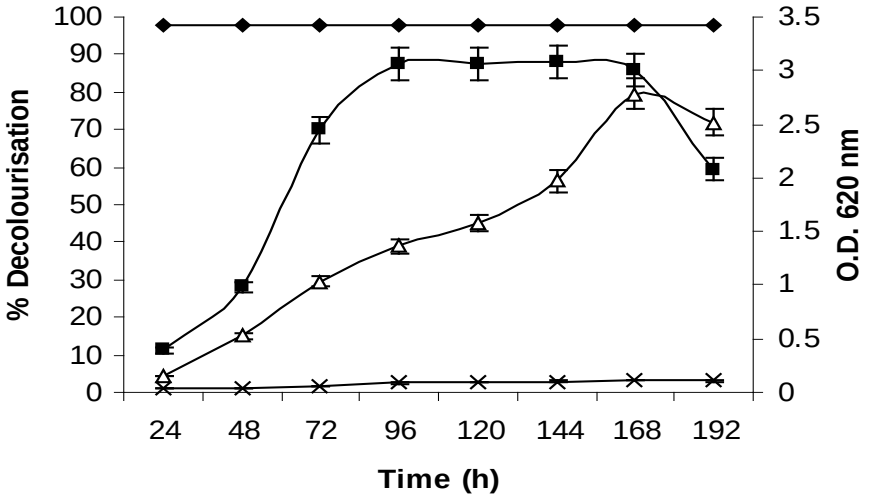



**Fig. 6.6** Bacterial growth and decolorization of molasses-melanoidins

### 6.3.5 Enzymatic mechanism of melanoidins degradation

Ligninolytic enzymes play a key role in decolourisation and degradation of colored effluents and support the growth of microorganisms for utilization of complex recalcitrant compounds (Bonugli-Santos et al. 2012; Pant and Adholeya 2007a). During melanoidins degradation, the MnP was found to be the dominating enzyme at the initial stage of melanoidins degradation. The activity of MnP reached maximum at 168 h (3.8 U $mL^{-1}$ $min^{-1}$), while the laccase activity was found maximum (2.39 U $mL^{-1}$ $min^{-1}$) at 144 h of incubation (Fig. 6.7) and showed maximum decolourisation (79%) of melanoidins. This might be due to direct involvement of MnP for the cleavage of conjugated C═C, C=O and C≡N bonds of melanoidins for degradation, while further incubation due to generation of phenolic compounds induce the laccase production from bacterial cells. Therefore, laccase

was noted higher concentration at the later stage of growing bacterial consortium during the decolourisation evaluation.

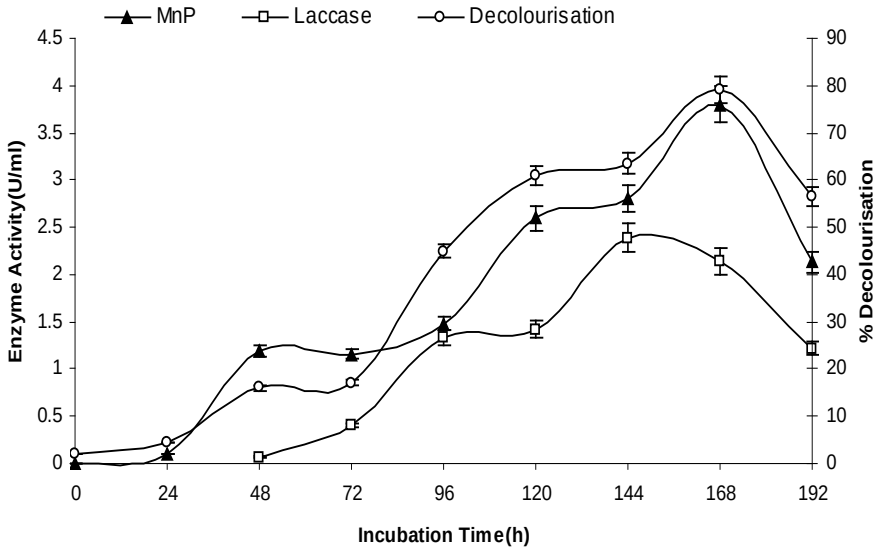



**Fig. 6.7** Manganese peroxidase (MnP) and laccase activity during decolurisation and degradation of molasses-melanoidins

The MnP and laccase have a broad substrate oxidising enzymes capable for breaking a large quantity of different chemical bonds present in phenolic and non-phenolic recalcitrant compounds (Bonugli-Santos et al. 2012; Wong 2009). But, after 168 and 144 h incubation, it showed gradual decrease of MnP and laccase enzyme activities of potential bacterial consortium, respectively. Miyata et al. (1998) verified that synthetic melanoidins are decolorized by the participation of MnP and manganese independent peroxidase (MIP) of *Trametes hirsutus* pellets, and the extracellular $H_2O_2$ produced by glucose oxidase along with partial participation of laccase. The presence of melanoidins and other similar compounds can induce the expression of MnP and laccase genes, both described as having synergistically action in the degradation and depolymerisation of melanoidins and other industrial pollutants (González et al. 2008; Miyata et al. 2000). The depolymerisation of melanoidins can be ascribed to the action of MnP and laccase activity. The findings of our study are well supported by earlier observations reported by various researchers (Pant and Adholeya 2007a; Bharagava et al. 2009; Yadav and Chandra 2012).

### 6.3.6 UV-Vis and FT-IR spectroscopy

UV-Vis spectrophotometric analysis is the most commonly used methods of the MR (Martins and Van Boekel 2003; Echavarría et al. 2014). The result revealed that melanoidins showed different absorption peaks in the UV region and their maximum absorbance was noted at $\lambda_{max}$ 295 nm. In addition, melanoidins also showed different stable peaks in a range of between 200-400 nm (Fig. 6.8). The comparative UV-Vis absorption spectrum of control and bacterial decolourised melanoidins sample was showed the reduction of absorption spectrum in bacterial degraded sample at the end of the 168 h

of incubation as shown in Fig. 6.8. Several organic compounds such as hydroxymethyl furfural, reductones, and furanones formed during the early stages of the MR is related to absorbance in UV region (Echavarría et al. 2013a,b). The expansion of the MR is generally monitored by the enhanced in absorbance of either 280 nm, 320-350 nm or 420-450 nm corresponding to early MR products for pyrazine compounds (at 280 nm) (Gu et al. 2010), advanced stage for soluble pre-melanoidins formation (at 320-350 nm) and final MRPs (at 420-450 nm) corresponding to development of in the medium and formation of specific compounds (Billaud et al. 2004). On the basis of our findings, it can be stated that the formation of various peaks in UV and visible region indicated the presence of complex mixture of early, advanced and final MRPs. Our findings are well corroborated with the earlier results reported by Echvarria et al. (2013a,b). UV-visible spectroscopic analysis indicates loss of melanoidins peaks and a significant spectral shift indicating biotransformation of melanoidins to unrelated metabolites. The shifting of peaks indicated the biotransformation of melanoidins molecule to some other metabolites.

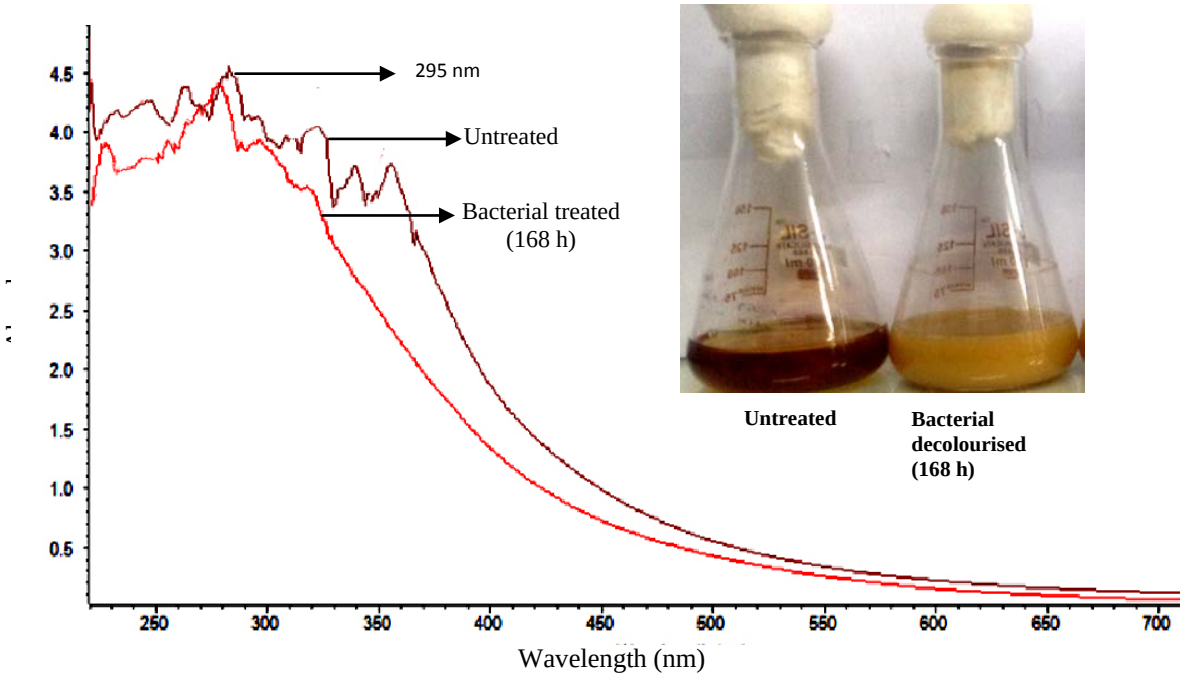



**Fig. 6.8** UV–Vis spectral analysis of the control and bacterial decolourised molasses-melanoidins

Further, the FT-IR spectra analysis also revealed significant absorbance intensities between control and bacterial treatment sample as shown in Fig. 6.9. The absorption band and their tentative assignment are given in Table 6.2. The appearance and disappearance of some peaks as well as shifting in peaks are indicated the conversion of complex toxic compounds into simpler metabolites.

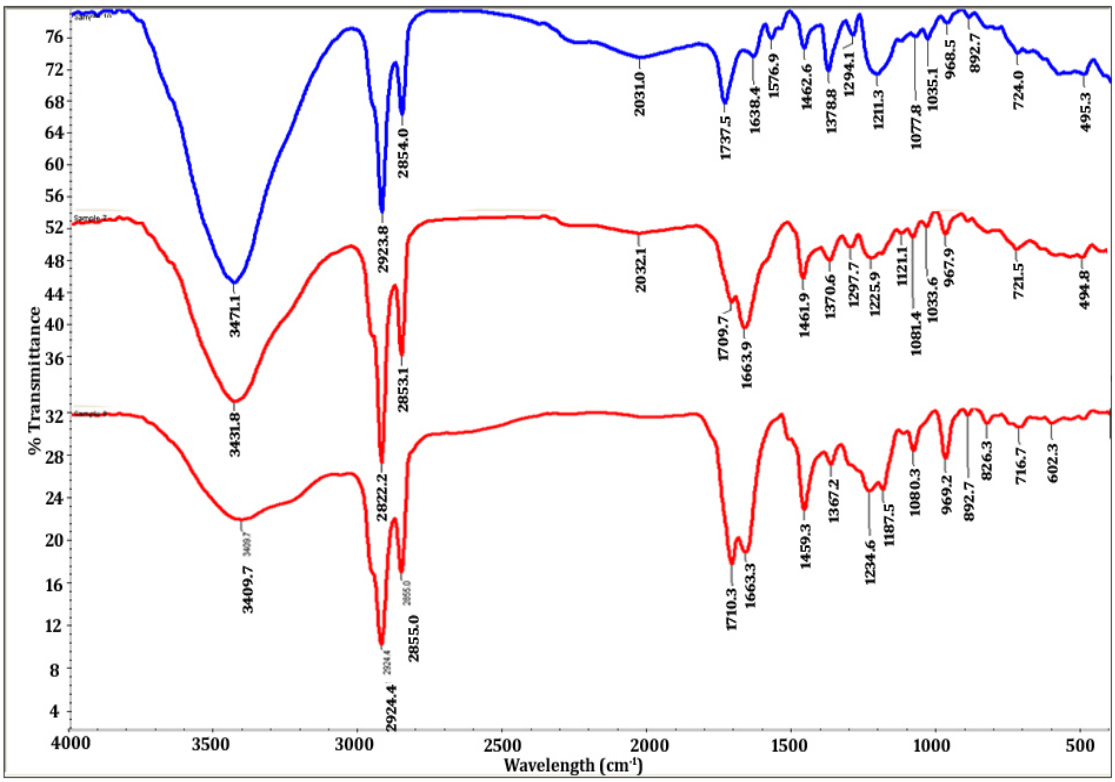



**Fig. 6.9** FT-IR spectra of molasses-melanoidins and its biodegradation metabolites (a) untreated (b) bacterial treated after 72 h (c) bacterial treated after 168 h

**Table 6.2** Assignment of IR absorption bands in the spectra of molasses-melanoidins (a) control (b) bacterial decolourised after 72 h and 168 h

| Untreated | | Bacterial Treated | | | |
|---|---|---|---|---|---|
| | | After 72 h | | After 168 h | |
| **Wave number ($cm^{-1}$)** | **Tentative assignments/interpretation** | **Wave number ($cm^{-1}$)** | **Tentative interpretation** | **Wave number ($cm^{-1}$)** | **Tentative assignments/interpretation** |
| 3910.8 | OH stretching of phenols | 3431.8 | OH and/or NH stretching of COOH and NH groups | 3409.7 | O-H stretching of acids, alcohols and phenols |
| 3434.7 | OH and/or NH stretching of COOH and NH groups | 2922.2 | C–H stretching of aliphatic compounds | 2924.4 | C-H stretching of –CH, $-CH_2$, and $-CH_3$ |
| 2923.8 | C-H stretching of –CH, $-CH_2$, and $-CH_3$ | 2853.1 | C-H stretching of $-CH_3$ in hydrocarbon chains | 2855.0 | C-H stretching of –CH, $-CH_2$, and $-CH_3$ |
| 2854.0 | C-H stretching of CH, $-CH_2$, and $-CH_3$ functional groups | 2032.1 | C=C stretching vibrations | 1710.3 | C=O of COOH, C=O or ketonic carbonyl |
| 2031.0 | C=C stretching vibrations | 1709.7 | C=O of COOH, C=O or ketonic carbonyl | 1663.3 | C=O stretching of secondary amide groups/C=O stretching of H-bonded conjugated ketons |
| 1737.5 | C=O of COOH, C=O or ketonic carbonyl | 1663.9 | C=O carbonyl stretching of amides-II | 1459.3 | aliphatic C-H bending and $COO^-$ asymmetric stretching |
| 1638.4 | C-O stretching of $COO^-$ ketonic C-O and aromatic C-C conjugated with $COO^-$ | 1461.9 | C-H bending and $COO^-$ asymmetric stretching of aliphatic compounds | 1367.2 | Aromatic ring stretch |
| 1576.9 | ketonic C-O and aromatic C-C conjugated with $COO^-$ | 1370.6 | C-H bending and $COO^-$ asymmetric stretching of aliphatic compounds | 1234.6 | C–O stretch of OH-deformation of COOH |
| 1462.6 | aliphatic C-H bending and $COO^-$ asymmetric stretching | 1297.7 | C-N bonds in aromatic amine functions | 1187.5 | S=O stretching of sulfone groups |
| 1378.8 | Aromatic ring stretch and $COO^-$ antisymmetric stretching | 1225.9 | C–O stretch of OH-deformation of COOH | 1080.3 | Carboxylic acids, esters, ethers, alcohols and anhydrides |
| 1294.1 | C-N stretch of aromatic amine functions | 1121.1 | S=O stretching in sulfone groups | 969.2 | C–O stretching of polysaccharides |
| 1077.8 | C–O stretching of $COO^-$ group | 1081.4 | Si–O-asymmetric stretch | 892.7 | OH- deformation in carboxyl groups |
| 1035.1 | C-O bonds stretching of ethers and alcohols | 1033.6 | C-O bonds (ethers and alcohols) | 826.3 | Aromatic out-of-plane C-H bending |

### 6.3.7 TLC and HPLC analysis

The TLC chromatogram under UV light showed dark spot in control while decolourised sample after 168 h bacterial incubation had three bands corresponding to retardation factor ($R_f$) value of 0.64, 0.71, and 0.78 as shown in Fig. 6.10a. This clearly indicated the degradation of melanoidins into intermediate metabolic products with $R_f$ value 0.64, 0.71, and 0.78. Further, the HPLC analysis of control sample also showed a single major peak at RT 2.0 min, while in 72 h bacterial decolourised sample was less compared to untreated sample. This indicated the capability of the bacterial consortium to decolorize and degrade melanoidins by their enzymatic action. Further, after 168 h treatment of melanoidins, the HPLC chromatogram also showed shifting of peak, decrease in peak area height (Fig. 6.10b). The differences in the peak pattern confirm biodegradation of melanoidins. This study revealed that potential bacterial consortium effectively reduced pollutants level of melanoidins containing medium. These findings are well supported by earlier results reported by various workers (Tiwari et al. 2012; Bharagava et al. 2009; Yadav and Chandra 2012).

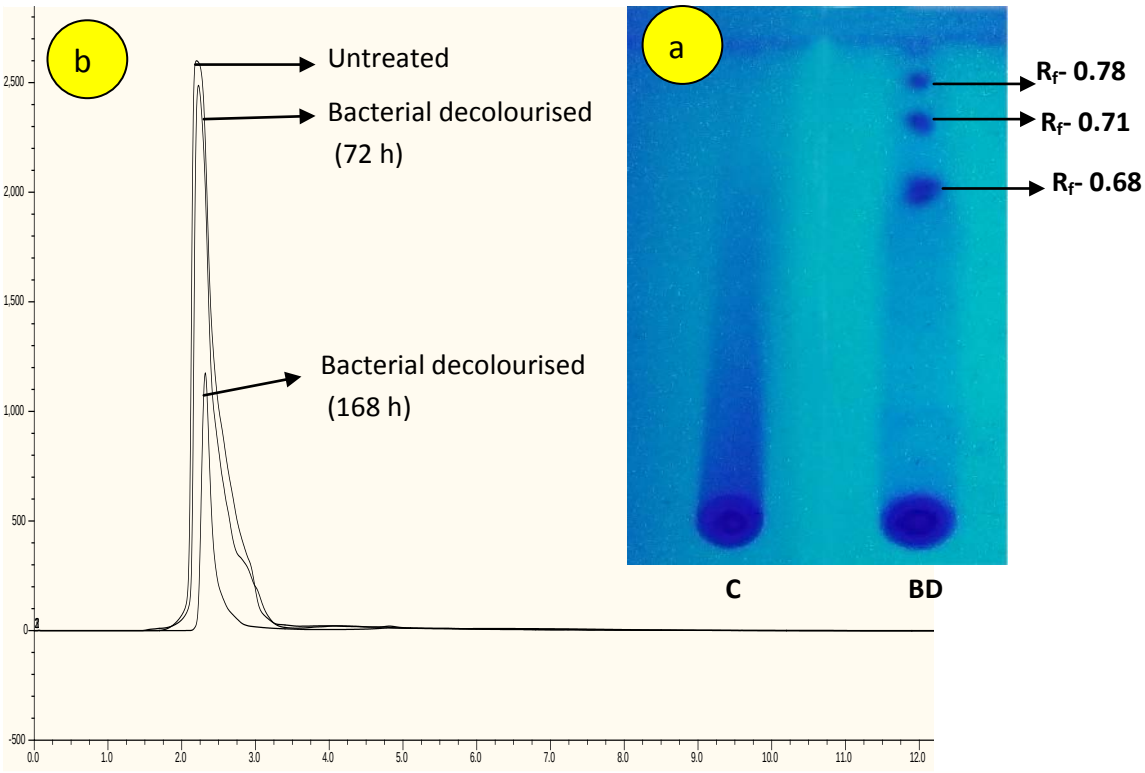



**Fig. 6.10** Chromatograph of melanoidins before and after bacterial decolourised during (a) TLC (b) and HPLC analysis. C=control (untreated); D=bacterial decolourised sample after 168 h incubation

### 6.3.8 GC-MS analysis

The GC-MS chromatogram of organic compounds extracted with ethyl acetate from untreated and bacterial treated sample is shown in Fig. 6.11 and organic compounds have been identified in detail at various RT based upon mass to charge ratios (m/z). The major

peaks detected in the control samples at different RT were identified as silanol, trimethyl-,trimester with boric acid (RT:7.06), D-(-)lactic acid, trimethyl ether, TMS ester (RT:9.08), butyldimethyl(2-styry[1,3]dithian-2-yl) silane (RT:10.88), propane, 1,2,3-tris[(tert-butyldimethylsilyl)oxy] (RT:1285), acetic acid, [bis[(TMS)oxy]phosphinyl]-TMS ester (RT:13.71), hexadecanoic acid, 2-[(TMS)oxy]-1,3-propenediyl ester (RT:27.99), 9,12-octadecanoci acid(Z,Z)-, TMS ester (29.98), octadecanoic acid, ethyl ester (RT:30.84), cis-10-nonadecenoic acid (RT:31.88), hexadecanoic acid (RT:34.50), and octadecanoic acid, ethyl ester (RT:36.11). Several minor peaks were also noted at RT: 7.06; 7.76; 10.51; 11.07; 12.35; 12.85; 17.35; 19.09; 21.25; 23.08; 23.70; 29.95; 29.98; 30.84; 33.49; 34.50; 35.11; 35.50; 35.75; 35.82; 35.83; 36.10; 37.43; 39.17; and 39.40. This corresponded to silanol, trimethyl-trimester with boric acid; bis(dimethyl-t-butylsilyl)oxalate; D-lactic acid; bis(dimethyl-t-butylsilyl)oxalate; ethanedioic acid; butyldimethyl(2-styry[1,3]dithian-2-yl)silane; silanol, trimethyl-,benzoate; propane, 1,2,3-tris[(tert-butyldimethylsilyl)oxy]; pyrrolozine1-one,7-propyl; undecenoic acid; dodecanoic acid; dotriacontate; pyrrolo(1,2-a)pyrazine-1,4-dione; 11-cis-octadecenoic acid, TMS ester; 9,12-octadecanoci acid(Z,Z)-,TMS ester; octadecenoic acid; 1,2-benzenedicarboxylic acid, bis (2-ethylhexyl)ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; β-sitosterol; querecetin 7, 3',4' trimethoxy; 1,7-pentatriacontene; 2-monostearin; 1-monolinoleoylglycerol TMS ester; octadecanoic acid, 2,3 bis[(TMS)oxy]propyl ester; stigmasta-5, 22-dien-3-ol (3 β,22E), stigmasterol; and silane[[(3β)-cholesta-5-en-3-yl]trimethyl, respectively. Moreover, some minor peaks were observed at different RT, but these peaks could not be identified because they were not available in the NIST library.

D(-)lactic acid, trimethyl ether, TMS ester and acetic acid, [bis[(TMS)oxy]phosphinyl]-TMS ester are the major volatile alkaline degradation products of hexose (ADPH) sugar and discharged in effluent after anaerobic treatment. These detected compounds have been listed as EDCs by the USEPA (2012). The octadecanoic and hexadecanoic acid were also detected in our study; they are plant origin fatty acid which have been reported to be antiquoroum sensing molecules of bacterial product (Singh et al. 2013). Moreover, in some studies octadecanoic and hexadecanoic acid has also been reported to be a toxic compound in aquatic systems and DNA fragmentation inducer in a human melanoma cell line, respectively (Kamaya et al. 2003; de Sousa Andrade et al. 2005). However, the toxicity of octadecanoic and hexadecanoic acid is well known on metabolic activity of bacteria (Koster and Cramert 1987). But, these compounds are also listed under EDCs as per USEPA (2012). Similar compounds have been reported by various researchers (Gonzalez et al. 2000; Quinn et al. 2007). Similarly, ethanedioic acid,pyrrolizidine, and benzenedicarboxylic acid have been detected in control sample. Ethanedioic acid is a toxic colourless crystalline organic acid found in some plants while pyrrolizidine and benzenedicarboxylic acid are the fragmentary ADPH. These compounds might occur as a residual fraction of the distillation process during ethanol production and remain after anaerobic treatment of spent wash. However, dotriacontane, a phyohydrocarbons, have also been identified in untreated sample. These compounds have been as key constituents of environmental pollutants responsible for dermal irritation

(Muhammad et al. 2005). Apart from this, other compounds were also identified as stigmasta-5, 22-dien-3-ol (3 β,22E), stigmasterol and β-sitosterol. These are the major phytosterols with a chemical structure similar to cholesterol, soluble in water at all pH values and previously reported to be present in *Saccharum officinarum* L. and effluent discharged from sugarcane molasses based distilleries. These compounds are screened under the EDCs list of USEPA (2012). In a previous study it has been reported that in environment, aerobic and/or anaerobic microorganisms may transform β-sitosterol and other sterols into androgenic hormones i.e., 5-β-androstan 3,17-dione and androstan 4-en-3,17-dion (Taylor et al. 1981). Such androstane derivatives of sterols may ultimately interfere with endocrine system of fish and produce hermaphroditism or other morphological defects. A role of these compounds in masculinising the fish population and reducing fish numbers has been suggested (Jenkins et al. 2003). We have also detected several other organic compounds in control and bacterial decolourised sample. These organic compounds constituent the main components of spent wash and discharge in PMDE from yeast or original sugarcane molasses, which remains after secondary treatment of spent wash. The presence of organic acids, phenolics and long chain aliphatic compounds in distillery effluent was reported previously by various workers (Gonzalez et al. 2000; Yadav and Chandra 2012). The maximum numbers of organic compounds present in untreated sample are well-known toxicants; complex and high molecular weight compounds pose an environmental risk to plant and animal including human. However, the major peaks correspond to toxic organic compounds detected in the control sample diminished after bacterial treatment and appearance of few new peak indicated the generation of new metabolic products as shown in Fig. 6.11 and Table 6.3. This suggested that complex and high molecular weight organic compounds were degraded by bacterial consortiums with the help of extracellular ligninolytic enzymes utilize its as sole source of carbon and nitrogen and play a major role in the decolourisation and degradation of melanoidins. The new metabolic products extracted with ethyl acetate from bacterial decolourised sample were identified as bis(dimethyl-t-butylsilyl)oxalate (RT: 7.77), butane, 2,3-bis(trimethylsiloxy) (RT:7.87), 3-benyl-1,4-diaza-2,5-dioxobicyclo (RT:4.3.0), nonane (RT:31.87); 2-amino-1-phenylethanol, TMS ether (RT:35.24), and 1-monolinoleoylglycerol TMS ether (RT:40.66).

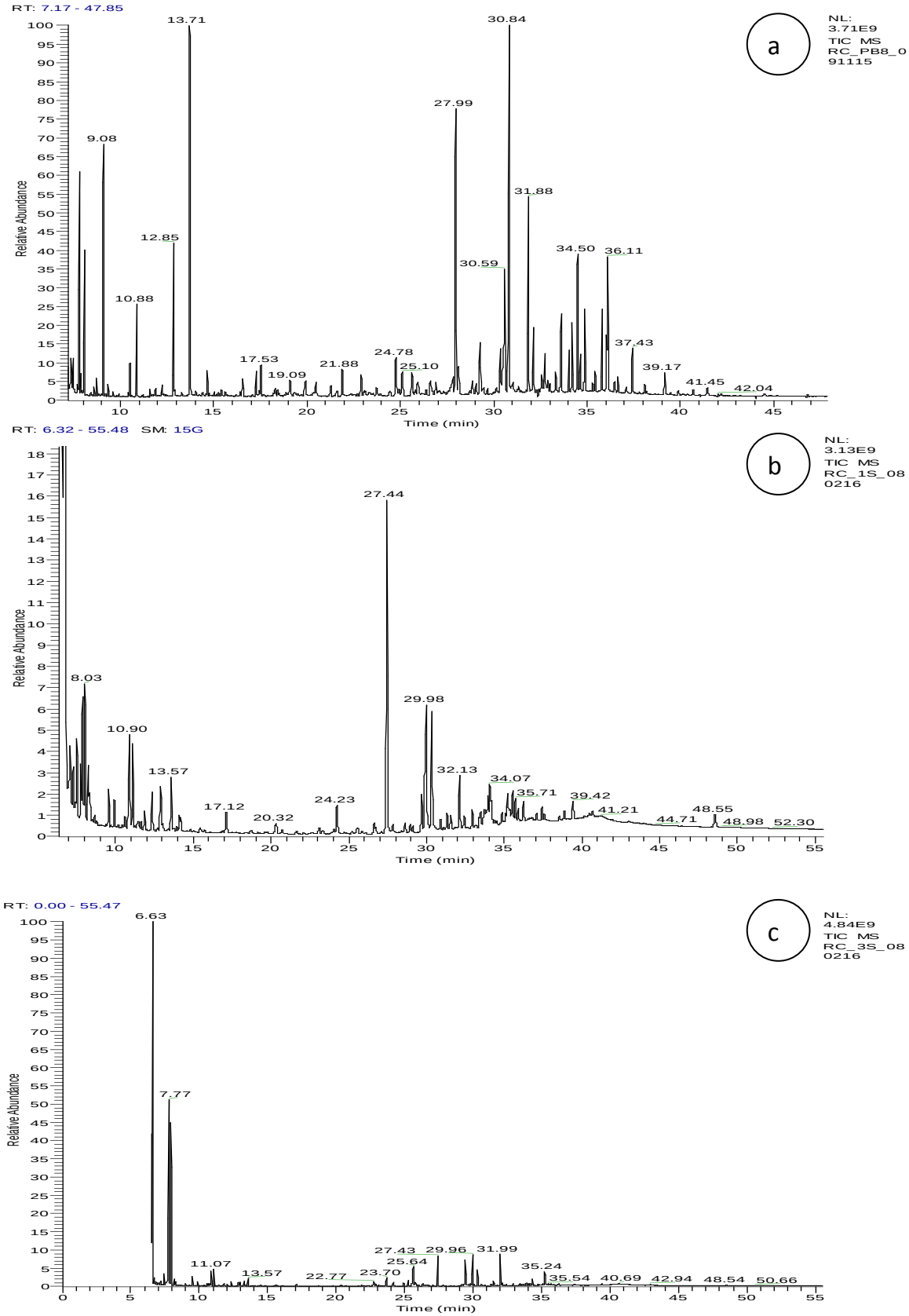



**Fig. 6.11** GC-MS chromatogram of TMS derivatized organic compounds extracted from molasses-melanoidins (a) control (b) bacterial degraded after 72 h (c) bacterial degraded after 168 h

**Table 6.3** Organic compound identified by GC-MS analysis extracted with ethyl acetate extract from untreated and bacterial decolourised molasses-melanoidins

| S.No. | RT | Compound Name | C | BD | |
|---|---|---|---|---|---|
| | | | | 72 h | 168 h |
| 1. | 6.63 | Propanoic acid, 2-methyl-2-[(TMS)oxy], TMS ether | - | - | + |
| 2. | 7.06 | Silanol, trimethyl-,trimester with boric acid | + | - | - |
| 3. | 7.28 | L-Lactic acid | + | - | - |
| 4. | 7.76 | Bis(dimethyl-t-butylsilyl)oxalate | + | - | - |
| 5. | 7.77 | 2-Methyl-1,3-Propanediol 2TMS | - | - | + |
| 6. | 7.87 | Cyclohexanol, 4-[(TMS)oxy]-cis | + | - | - |
| 7. | 7.95 | Butane, 2,3-bis(Trimethylsiloxy) | - | - | + |
| 8. | 9.08 | D-(-)Lactic acid, trimethyl ether, TMS ester | + | - | - |
| 9. | 10.51 | Ethanedioic acid | + | + | - |
| 10. | 10.88 | t-Butyldimethyl(2-styry[1,3]dithian-2-yl) silane | + | + | + |
| 11. | 11.07 | Butane-1,3-diol, 1-methylene-3-methyl, bis(TMS)ether | + | + | + |
| 12. | 12.35 | Silanol, trimethyl-,benzoate | + | - | - |
| 13. | 12.85 | Propane, 1,2,3-tris[(tert-butyldimethylsilyl)oxy] | + | - | - |
| 14. | 13.71 | Acetic acid, [bis[(TMS)oxy]phosphinyl]-TMS ester | + | + | - |
| 15. | 17.27 | 5-Methyl-1,3-diazaadamantan-6-one | - | + | - |
| 16. | 17.53 | Pyrrolozine1-one,7-propyl | + | - | - |
| 17. | 19.09 | Undecenoic acid | + | - | - |
| 18. | 20.49 | Acetic acid, [[bis[(TMS)oxy]phosphinyl]oxy]-TMS ester | - | + | - |
| 19. | 21.88 | Silanol, trimethyl phosphate | - | + | - |
| 20. | 21.25 | Dodecanoic acid | + | - | - |
| 21. | 23.08 | Dotriacontate | + | - | - |
| 22. | 23.70, | Pyrrolo(1,2-a)pyrazine-1,4-dione, | + | - | - |
| 23. | 23.73 | Phosphoric acid, bis(TMS)2,3-bis[(TMS)oxy]propyl ester | - | + | - |
| 24. | 27.98 | Hexadecanoic acid | + | + | - |
| 25. | 29.28 | cis-10-Heptadecenoci acid | - | + | - |
| 26. | 29.95 | 11-cis-Octadecenoic acid, TMS ester | + | + | - |
| 27. | 29.98 | 9,12-Octadecanoci acid(Z,Z)-, TMS ester | + | - | - |
| 28. | 30.59 | 11-trans-Octadecenoic acid | - | + | - |
| 29. | 30.84 | Octadecanoic acid | + | + | - |
| 30. | 31.88 | 1-Eicosanol | + | - | - |
| 31. | 31.88 | cis-10-Nonadecenoic acid | - | + | - |
| 32. | 31.97 | 3-Benyl-1,4-diaza-2,5-dioxobicyclo(4.3.0)nonane | - | - | + |
| 33. | 32.77 | β-D-Galactopyranoside, methyl 2,3-bis-O-(TMS), cyclic methylboronate | - | + | - |
| 34. | 33.49 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester | + | - | - |
| 35. | 34.50 | Hexadecanoic acid | + | - | - |
| 36. | 34.05 | 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester | + | + | - |

| | | | | | |
|---|---|---|---|---|---|
| 37. | 34.50 | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester | + | + | - |
| 38. | 34.63 | cis-13-Docosenoic acid | - | + | - |
| 39. | 35.11 | 1,7-Pentatriacontene | + | - | - |
| 40. | 35.24 | 2-Amino-1-phenylethanol, TMS ether | - | - | + |
| 41. | 35.50 | β-Sitosterol | + | - | - |
| 42. | 35.75 | Querecetin 7, 3',4' Trimethoxy | + | - | - |
| 43. | 35.82 | 2-Monostearin | + | + | - |
| 44. | 35.83 | 1-Monolinoleoylglycerol TMS ester | + | - | - |
| 45. | 36.10 | Octadecanoic acid, 2,3 bis[(TMS)oxy]propyl ester | + | + | - |
| 46. | 36.11 | Octadecanoic acid, ethyl ester | + | - | - |
| 47. | 37.43 | Stigmasta-5,22-dien-3-ol (3 β,22E) | + | - | - |
| 48. | 39.17 | Stigmasterol | | | |
| 49. | 39.40 | Silane[[(3β)-cholesta-5-en-3-yl]trimethyl | + | - | |
| 50. | 40.66 | 1-Monolinoleoylglycerol TMS ether | - | - | + |

RT: Retention time (in minutes); C: Control; BD: Bacterial decolourised; +: Present; -: Absent; TMS: Trimethylsilyl

### 6.3.9 Phytotoxicity evaluation of untreated and bacterial treated sample

The phytotoxicity assessment using higher plants is a fundamental and reliable tool for toxicity evaluation of any environmental sample (OECD 2003). The seed germination test of green gram (*P. mungo* L.) showed inhibitory effects of untreated and bacterial decolurised mellanoidins sample at different concentrations in terms of seed germination and growth parameters of seedlings (Table 6.3 and Fig. 6.12). Seeds germinated at rate of 98% with 2% (v/v) concentration of melanoidins containing solution, as the concentration increased the percent germination decrease, with 80% at 5% concentration. There was no seed germination at a concentration 10% after 24 h. However, in bacterial treated sample 80% germination was recorded in up to a 10% (v/v) concentration, which was higher than with untreated sample.

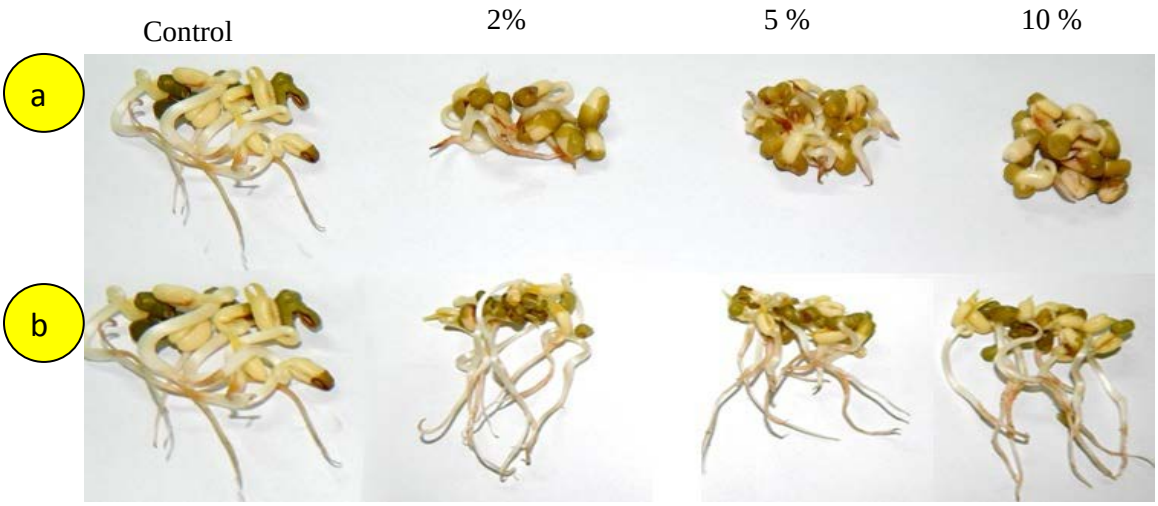



**Fig. 6.12** Toxicity effect of molasses-melanoidins on seedling growth of *Phaseolus mungo* L. seeds cultivated with (a) untreated (b) treated sample; Control; tap water

According to Bharagava and Chandra (2010a), suppression of seed germination at high concentration of melanoidins might be due to the occurrence of toxic organic compounds and dissolve solid which absorbed by the seed prior to germination and affecting different biochemical and physiological process of seed germination. Increased in percent germination in bacterial decolourised melanoidins might be due to lower presence of organic compounds that has created favorable environmental conditions for seed germination and utilization of nutrient present in melanoidins. With respect to the seedling growth (radical length), the radical length of seeds exposed to untreated sample varies from 18.12 to 2.21 cm at different concentration of melanoidins containing wastewater. When the seeds had been exposed to 10% (v/v) untreated sample they showed no radical development, but after bacterial treatment the seeds showed development of a radical (15.11cm) at 10% concentration of melanoidins. The various parameters like germination index, % phytotoxicity and stress tolerance index was also observed in untreated and treated melanoidins as shown in Table 6.4. These results clearly indicated that the toxicity of melanoidins before bacterial treatment was very high which inhibited the growth of seeds but after treatment the toxicity of melanoidins decrease.

**Table 6.4** Effect of different concentration of molasses-melanoidins (treated and untreated) on seedling growth of *Phaseolus mungo* L.

| Sample | No. of seed plated | Concentrat-ion (%) | Germination (%) | Germination index | Reduction in germination (%) | Radical length (cm) | Phytotoxicity (%) | Stress tolerance Index |
|---|---|---|---|---|---|---|---|---|
| Control | 10 | 0 | 100±0.00 | 00±0.00 | 00±0.00 | 18.12±0.82 | 00±0.00 | 00±0.00 |
| Untreated | 10 | 2 | 90±0.00 | 0.17±0.01 | 10±0.00 | 03.50±0.17 | 80.68±3.64 | 0.19±0.00 |
| | 10 | 5 | 80±0.00 | 0.09±0.00 | 20±0.00 | 02.21±0.07 | 87.80±3.99 | 0.12±0.00 |
| | 10 | 10 | NG | -- | -- | -- | -- | -- |
| Treated | 10 | 02 | 90±0.00 | 0.84±0.04 | 10±0.00 | 17.10±0.77 | 05.62±0.23 | 0.94±0.04 |
| | 10 | 05 | 85±0.00 | 0.71±0.02 | 15±0.00 | 15.21±0.65 | 16.05±0.64 | 0.83±0.04 |
| | 10 | 10 | 80±0.00 | 0.66±0.00 | 20±0.00 | 15.11±0.54 | 16.61±0.52 | 0.83±0.01 |

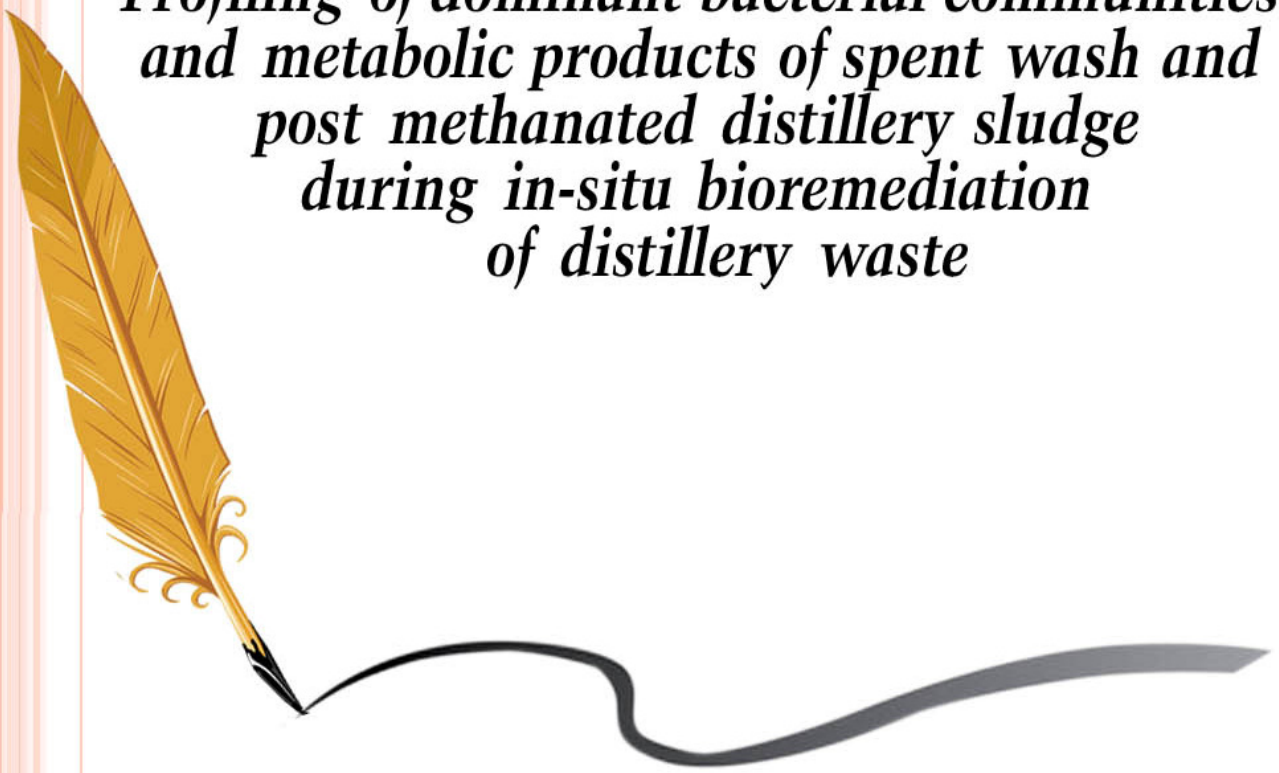
All values are mean of three replicate ± S.D. NG: No germination; Control: Tap water

## Conclusion

The study revealed that the waste discharged from sugarcane molasses based distilleries have EDCs like containing organo-metallic complex which can be degraded by potential bacterial consortium with supplementary source of glucose (1.0%) and peptone (0.2%) at optimum temperature (35 °C), pH (8.1) and shaking speed (180 rpm) through MnP and laccase enzyme activities as bio-tools for degradation and decolourisation of industrial effluent. The metabolic products analysis by TLC, HPLC, and GC-MS analysis confirmed the biodegradation of molasses-melanoidins by potential bacterial consortium consisting of *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), and *E. cloaceae* (KU726957). The toxicity reduction after decolourisation confirmed the reduction of toxic compounds from the molasses-melanoidins organo-metallic complex

# Chapter-07

# Profiling of dominant bacterial communities and metabolic products of spent wash and post methanated distillery sludge during in-situ bioremediation of distillery waste



# Chapter-7

## Profiling of dominant bacterial communities and metabolic products of spent wash and post methanated distillery sludge during *in*-situ bioremediation of distillery waste

### 7.1 Introduction

Microbial communities are fundamental components of any ecosystem, playing a primary and critical role in the metabolism of organic matter to maintain biogeochemical cycles in various critical environments (Fuhrman 2009). They are predominantly involved in the bioremediation of contaminated sites, and several microorganisms, which degrade a wide range of pollutants have been described (Loviso et al. 2015). The diversity and activity of microbial communities are important indices of soil quality because soil microbes play significant roles in recycling of plant nutrients, maintenance of soil structure, fertility, and detoxification of noxious chemicals, as well as control of plant pests and plant growth (Elsgaard et al. 2001; Filip 2002). The nature of complex pollutants at distillery waste contaminated sites and the potential for microbial strains to grow in such environments are still unknown. Several microorganisms degrading a wide range of pollutants have been described by various researchers under different environmental conditions (Loviso et al. 2015; Chandra et al. 2011; Płaza et al. 2006). Hence, not only detailed information regarding microbial communities at any polluted site reflects the relationship with pollutants but also information regarding the bioremediation potential of microbes toward specific pollutants for its management. Moreover, some studies have indicated a relationship between soil microbial communities and environmental pollutants, including heavy metals (Muller et al. 2001; Pennanen et al. 1996; Perkiomaki et al. 2003). The bacterial diversity and community structure in an aerated lagoon were also revealed by ribosomal intergenic spacer analysis and 16S rDNA sequencing (Yu and Mohn 2001). Therefore, the present study was conducted to characterize the chemical nature of unknown pollutants present in distillery spent wash (DSW) and post methanated distillery sludge (PMDS) collected from a sugarcane molasses-based distillery plant. Simultaneously, a study was conducted to reveal the endemic bacterial communities growing in specific environments that might be responsible for amelioration of these pollutants, eventually leading to biological succession and bioremediation for eco-restoration of polluted sites. To accomplish this, we employed several organic solvents to extract the broad range organic pollutants under acidic conditions using the liquid–liquid extraction method. Subsequently, extracted pollutants were identified by GC-MS analysis. Simultaneously, the dominant autochthonous bacterial communities were investigated by the RFLP method using a metagenomics approach to reveal the microbial niche in this polluted environment. The results presented herein provide useful information that will help understand the mechanism *of in*-situ or *ex*-situ bioremediation for safe disposal of such waste.

## 7.2 Material and methods

### 7.2.1 Sample collection (spent wash, distillery sludge)

The spent wash was collected aseptically in presterilized plastic containers (capacity 20 L; Tarson Production Pvt. Ltd., USA) from the spent wash collection tank of M/s Unnao Distillers, Unnao, located in Uttar Pradesh, India (26° 320″ N, 80° 30′ 0″ E) (Fig. 7.1). While, the fresh PMDS sample was collected in clean pre-sterilised polythene bags from the effluent treatment plant (ETP) for the same distillery (Fig. 7.1). The plant has a capacity for 9000 kL of alcohol production and generates ~800 tonnes of sludge annually (AIDA 2004). After 90 days of *in*-situ bioremediation the degraded sludge sample was collected from the sludge dumping site of the distillery plant, which is located inside the premises. All the sludge samples collected were transported to the laboratory and used for the analysis of physico-chemical parameters, detection of organic pollutants, detection of bacterial communities and preparation of leachate.


**Fig. 7.1** Distillery waste (a) spent wash in collection tank (b) methane reactor for treatment of spent wash (c) post methanated distillery sludge discharged from methane reactor after anaerobic treatment of spent wash (d) collection of degraded post methanated distillery sludge

### 7.2.2 Preparation of sludge leachate

The sludge leachate was prepared by stirring it in distilled water (1:1 w/v) for approximately 48 h and allowing the sludge suspension to stand still for 6 hrs in an Erlenmeyer flask (250 mL). Clear supernatant was pumped out and filtered by passing

through Whatman filter Paper. Newly prepared sludge leachate was taken as 100%, with different concentrations prepared from it by adding distilled water to a final concentration of 1.0%, 2.5%, 5% and 10% for the analysis of various physicochemical parameters, detection of organic pollutants, phytotoxicty, and genotoxicity assays (Fig. 7.2).

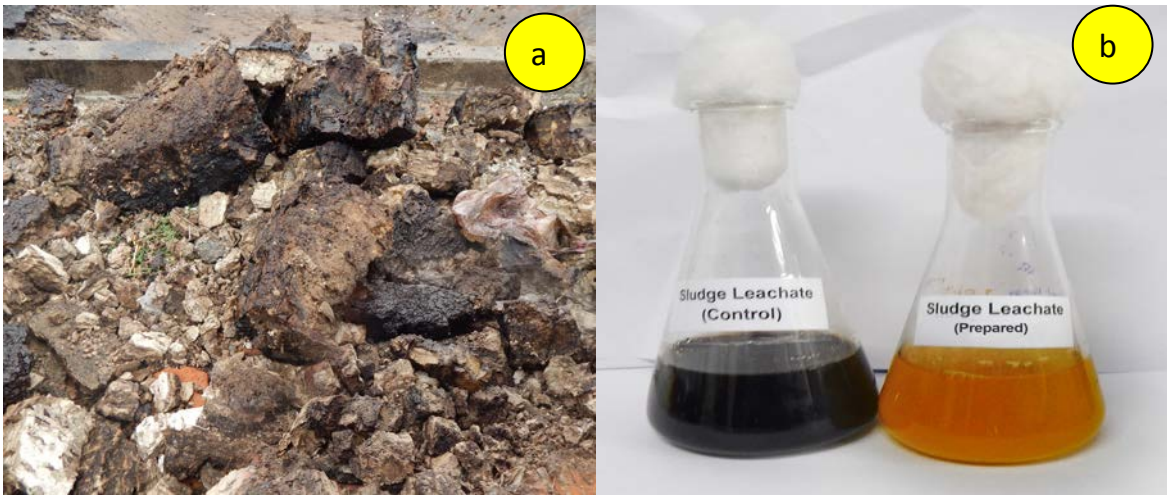



**Fig. 7.2** Distillery waste (a) post methanated distillery sludge (PMDS) after *in*-situ bioremediation (b) prepared distillery sludge leachate from fresh as well as degraded post methanated distillery sludge

### 7.2.3 Physicochemical analysis of spent wash, distillery sludge and sludge leachate

The physicochemical parameters of fresh distillery sludge and sludge leachate samples i.e. pH, EC, salinity, chloride, sodium, nitrate, ammonical nitrogen were estimated according to the method of Kalra and Maynard (1991). The pH and EC of sludge samples (1:1 sludge:water) and leachate were measured using an Orion meter (Model-960, Thermo Scientific, USA) and an Orion conductivity meter (Model-150, Thermo Scientific, USA), respectively. The heavy metals in the sludge and leachate samples were analysed with standard methods (APHA 2012) by atomic absorption spectrophotometry (ZEEnit 700, Analytic Jena, Germany) after nitric acid-perchloric acid digestion method no. 3030H. Elemental analysis was performed for total C, $N_2$, $H_2$ and $O_2$ by using an elemental analyser (EuroVector EA 3000, University of AL-al-Bayt, Jordan). In addition, the SEM-EDS analysis of PMDS was performed using a scanning electron microscope (JEOL JSM-6490LV, Massachuselts, USA). Operating conditions were: accelerating voltage 20 kV, probe current 45 nA and counting time 60 sec. The physicochemical parameters were also analysed in the degraded sample after 90 days of bioremediation of sludge *in*-situ.

### 7.2.4 Extraction and identification of various organic compounds from fresh and degraded sludge

#### 7.2.4.1 Solid-liquid extraction of distillery sludge and liquid–liquid extraction of spent wash and distillery leachate

The fresh PMDS sample (5.0 g) was weighed and put into an Erlenmeyer flask (250 mL); 5 ml of *n*-hexane was added and it was mixed vigorously. After 30 min, a glass magnetic stirring bar was added to the inoculated sample. The samples were processed by vortex agitation (2 min), sonication (2 min on and 30 sec off, ×3) and centrifuged (15 min 10,000 ×g). In order to extract organic pollutants from distillery leachate, a fixed volume (10 mL) of sludge leachate was acidified with 35% (v/v) hydrochloric acid and placed in a separating funnel (100 mL), after which an equal volume of *n*-hexane was added and the mixture was shaken continuously for 5 h with intermittent rests for liquid–liquid extraction. The extraction was repeated successively three times to complete the extraction of organic pollutants. Subsequently, the organic layer was collected from the sludge and leachate, dehydrated over anhydrous $Na_2SO_4$ and dried under a stream of $N_2$ gas. A similar process was followed for the extraction of metabolic products from a degraded sludge sample after bioremediation *in*-situ. The dry residue obtained was dissolved in 1.0 mL ethyl acetate and filtered through 0.22 µm syringe filters (Millipore Ltd., Bedford, Massachusetts, USA) and used for further FT-IR and GC-MS analysis.

#### 7.2.4.2 GC-MS analysis

In GC-MS analysis, the extracted PMDS and leachate samples were derivatised with trimethylsilyl (TMS) as described in chapter 04 (Minuti et al. 2006). The derivatised sample was subjected to GC-MS analysis. The operational condition of GC-MS for characterisation of organic compounds is described in chapter no. 04.

### 7.2.5 Analysis of uncultured bacterial communities growing in spent wash and distillery sludge

#### 7.2.5.1 Genomic DNA extraction and purification

The total genomic DNA from the collected DSW sample was extracted using the method described by Zhou et al. (1996). Briefly, aliquots (1.0 mL) of DSW were mixed with 2.0 ml of extraction buffer [100 mM TrisHCl (pH 8.0), 1.5 m NaCl, 100 mM sodium phosphate (pH 8.0), and 1% hexadecyl trimethyl ammonium bromide] in 10 mL screw caped polypropylene microcentrifuge tubes. After proper mixing, 13 µl of proteinase K (10 mg $mL^{-1}$ ) was added to the tube. All tubes were incubated at 37 °C with shaking for 45 min, after which 160 µl of 20% sodium dodecyl sulfate was added and vortexed for 30 s. The samples were then incubated at 60 °C for 1.5 h, during which time the samples were mixed thoroughly every 15 min. Next, the tubes were shaken for 5 min at high speed on a MiniBead Beater to lyse the cells. After lysis, the lysate was centrifuged at 15,000×g for 10 min at 4 °C in a microfuge. The supernatant obtained was then mixed with equal quantities of chloroform and isoamyl alcohol (24:1) and extracted two to three times. The aqueous layer was separated and precipitated with 0.6 volume of isopropanol, after which the samples were centrifuged at 5000 ×g for 10 min at 4 °C in a microfuge and aliquots of 300–500 µl of the supernatants were purified by passing through a spin column that had previously been equilibrated and slurried with 20 mM potassium phosphate buffer (pH

7.4) by centrifugation at 1000 ×g for 2 min (Merck Biosciences, Mumbai, Maharashtra, India).

While the total genomic DNA from degraded PMDS samples after 90 days of *in*-situ bioremediation was extracted following the protocol of Berthelet et al. (1996). An aliquot (2.0 g) of sludge sample was mixed with 5.0 ml of 0.1 M sodium phosphate buffer (pH 8.0) in screw-capped polypropylene micro-centrifuge tubes (10 mL capacity) containing 5.0 g silica beads (0.1 mm diameter) followed by addition of 500 mM Tris-HCl (pH 8.0) and 10% SDS. The tubes were shaken for 5 min at high speed on a Mini-Bead beater to lyse the cells. After lyses, the lysate was centrifuged (15,000 ×g; 10 min; 4 °C) in a microfuge and the supernatant obtained was mixed with double the volume of 7.5 M ammonium acetate and incubated on ice for 10 min. The samples were then centrifuged (15,000 ×g; 10 min; 4 °C) in a microfuge and aliquots of 300-500 µl supernatant were purified by centrifugation (1,000 ×g; 2 min) through a spin column (Bangalore Genei, India), which was previously equilibrated and slurried with 20 mM potassium phosphate buffer (pH 7.4).

### 7.2.5.2 PCR amplification of 16S ribosomal RNA genes

The PCR amplification of 16S rDNA genes derived from DSW and degraded PMDS was performed with universal eubacterial primers (27F) 5′ AGAGTTTGATCMTGGCTCAG 3′ and (1492R) 5′ TACGGYTACCTTGTTACGACTT 3′ using a Thermocycler (Lane 1991). The reaction mixture contained 5 µl of template DNA, 1× PCR buffer, 10 mM of each dNTP, 3.0 mM MgCl, 10 pmol of primer, and 2.5 U of *Taq* DNA polymerase (Bangalore Genei, India) in a final volume of 50 µl. Reactions without template DNA were conducted as a negative control. The complete reaction mixture was overlaid with mineral oil and incubated in a thermal cycler (Agilent Technologies, Sure Cycler8800). The cycling program consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min, followed by final extension at 72 °C for 10 min. The PCR products were then electrophoresed through 1.2% (*w*/*v*) agarose gel in 1× TAE buffer using 1 Kb DNA ladder (M/s Merck Biosciences, India) as a molecular weight marker, then visualized by staining with EtBr. Finally, the amplified 16S rDNA gene products were gel purified using a PCR cleanup kit (Merck Biosciences, Mumbai, Maharashtra, India) and used for clone library preparation.

### 7.2.5.3 Cloning of 16S rDNA PCR products

For cloning, 10 µl purified PCR products were made blunt-ended by treatment with 10 U of the large (Klenow) fragment of DNA polymerase I, after which the 5′ end was phosphorylated with 10 U of T4 polynucleotide kinase (Promega, Madison, WI, USA). The reaction mixture, also contained 10 µl 10× Klenow buffer [0.5 M Tris HCl (pH 7.5 at 25°C), with 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mg BSA per mL, 1 mM ATP, 200 µM dATP, 200 µM dCTP, 200 µM dGTP and 200 µM dTTP also present]; the total volume was 100 µl and the prepared solution was incubated at 37 °C for 1.0 h (Moyer

1994). The blunt-ended PCR-amplified 10S rDNA gene products were again purified with Quiaex and ligated into a *Sma*I-digested dephosphorylated pUC18 vector (Promega, Madison, Wisconsin, USA). Competent *Escherichia coli* XL1 blue MRF′ cells (Stratagene) were transformed using the method described by Morission (1977). The positive recombinants were screened for α-complementation with X-Gal as a substrate on solid agar medium plates supplemented with ampicillin (150 µg/mL).

#### 7.2.5.4 16S rDNA RFLP analysis

Recombinant plasmids were isolated from overnight cultures by alkaline lysis (Sambrook and Russell 2001). The cloned 16S rDNA gene fragments were then digested with the restriction enzymes *Taq*I and *Sau*3AI, either separately or together (Merck Biosciences, Mumbai, Mahararashtra, India). The restriction digestion was performed at 37 °C for 2 h in a 50 µl reaction mixture containing 12 µl (~200 ng) of PCR amplified 16S ribosomal RNA (rRNA) gene product, 0.5 µl (5U) of restriction endonucleases (*Taq*I/*Sau*3AI) (Merck Biosciences, Mumbai, Maharashtra, India), 5 µl of reaction buffer, and 32.5 µl of autoclaved MilliQ water. The restriction digestion process was repeated twice to establish the reproducibility of results. The resulting RFLP products were separated by gel electrophoresis in 1.8% (*w*/*v*) agarose gel and Tris-borate-EDTA buffer at 75V for 5 h. Next, gels were stained with EtBr (1.0 µg mL), and DNA bands were visualized using the Gel Documentation System (Syngene, USA). The DNA fragments were compared with a 100 and 500bp DNA ladder (Merck Biosciences, Mumbai, Maharashtra, India) to determine the molecular weight and size.

#### 7.2.5.5 Sequencing of cloned 16S rDNA PCR fragments

Based on differences in the RFLP profiles generated, 10 bands generated by digestion of DSW by *Taq*I and *Sau*3AI were selected for sequence analysis. Based on differences in the RFLP profiles generated (spent wash), 29 bands generated by digestion of DSW by *Taq*I and *Sau*3AI were selected for sequence analysis. Selected bands were gel purified using gel extraction kits (Merck Biosciences, Mumbai, Maharashtra, India) and sequenced using the M13 forward (5′-GTAAAACGACGGCCAGT 3′) and reverse universal primers (5′-CAGGAAACAGCTATGAC-3′) (Shi-Yan et al. 2007) and an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, MA, USA). The samples were then sequenced using an automatic DNA sequencer (ABI PRISM® 310 Genetic Analyzer, USA). The partial sequences obtained were subjected to BLAST analysis using the online option available at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al. 1997).

#### 7.2.5.6 Phylogenetic analysis and nucleotide sequence accession number

A phylogenetic tree was generated using MEGA-6.0 software (Tamura et al. 2013). All query sequences and other homologous sequences available online in the NCBI (http://ncbi.nlm.nih.gov/) nucleotide database were saved in a single FASTA file format

after retrieval. Furthermore, all sequences were saved in one FASTA format file and then subjected to multiple sequence alignment using MEGA-6.0, which was subsequently used to reconstruct phylogenetic trees by the Neighbor-Joining method using the MEGA-6.0 Draw Tree tool (Larkin et al. 2007) with a bootstrap value of 1,000 replicates. The nucleotide sequences of identified bacterial communities were also deposited in GenBank under accession numbers FJ227502–FJ227522 and FJ227533–FJ227540 (for DSW) and FJ227502–FJ227522 and FJ227533–FJ227540 (for PMDS).

### 7.2.6 Toxicity assessment

#### 7.2.6.1 Phytotoxicity assay of spent wash

The toxicity assessment of distillery spent wash was studied on *Phaseolus mungo* L. seed germination using Petri dish method as previously described in chapter no. 04 (OECD 2003; Sharma et al. 2002).

#### 7.2.6.2 Phytotoxicity assay of PMDS

For the seed germination experiment, distillery leachate was applied at 1.0%, 2.5%, 5% and 10% (v/v). The surfaces of the seeds were sterilized with 0.1% $HgCl_2$ for 2 min to remove any fungal contamination, after which they were subjected to repeated washings with sterilized distilled water. Subsequently, 10 seeds of *P. mungo* L. were placed separately in sterilized glass Petri dishes of uniform size lined with two Whatman No. 1 filter paper discs. Discs were then moistened with 10 mL tap water for controls and with the same volume of distillery leachate, after which they were incubated at 25 $^0$C for a period of 2 consecutive days. The bioassay was performed on three replicate samples. The percentage seed germination (SG) and the percentage phytotoxicity (RI) was calculated with the formula previously described (Oleszczuk et al. 2012):

$$SG/RI = \frac{(A-B)}{A} \times 100$$

Where,

A is mean seed germination and root length in controls,

B is mean seed germination and root length in test experiments.

Other SG parameters such as the germination index (GI), relative percentage toxicity, percentage phytotoxicity, and the stress tolerance index were calculated using the formula described by David Noel and Rajan (2015).

#### 7.2.6.3 Genotoxicity assay of PMDS

The root tip cells of onion, *Allium cepa*, were used to test the genotoxic effects of fresh and degraded sludge leachate. The test was carried out as described by Fiskesjo (1985). The onion bulbs were previously germinated in tap water at room temperature. When the seeds reached about 2.0 cm in length, they were transferred to test tubes containing

different concentrations of distillery sludge leachate (1%, 2.5%, 5% and 10%), while one tube with distilled water was used as a control. After 24 hrs of treatment, the root tips were collected and fixed in Carnoy's fluid (ethanol-glacial acetic acid 3:1; v/v) for 24 h at 4 °C, in order to arrest mitosis (Fiskesjo 1993, 1997). Afterwards, they were washed with distilled water and placed in 70% (v/v) ethanol for 24 hrs at room temperature. The root tips of bulbs were hydrolysed with 1M HCl at 60 °C for 4–5 minutes to dissolve cell walls. After hydrolysis, the roots were washed with distilled water and approximately 1–2 mm of root tips were cut off and processed for staining with hematoxylin. Five slides were prepared for each concentration and for the control, out of which five (at 500 cells per slide) were analysed with a light microscope (Leica Microsystem, Germany; x1000). A total of 2500 cells were evaluated for each concentration. In the analysis, the following chromosomal and nuclear aberrations were considered: chromosome adherence, c-mitosis, chromosome bridges, vagrant chromosomes, disordered metaphase and anaphase, lagging chromosomes, multipolarity and polyploidy. Mitotic index (MI) and mitotic inhibition were also an indication of cytotoxicity. In the MI study, MI % was determined with the following formula, as described by Fiskesjo (1997).

$$\text{Mitotic index (\%)} = \frac{\text{Total number of cells in division}}{\text{Total cell number of cells observed}} \times 100$$

$$\text{Mitotic inhibition (\%)} = \frac{\text{Mitotic index in control group - Mitotic index in test group}}{\text{Mitotic index in control group}} \times 100$$

### 7.3 Results and discussion

#### 7.3.1 Physicochemical analysis of the spent wash, distillery sludge and leachate

The physicochemical properties of PMDS and its leachate are shown in Table 7.1. The value of pH and EC were higher in the sludge. In addition, the sludge showed high concentrations of ions eg. $Na^+$, $Cl^-$, $NO_3^-$ and $NH_4^+$ (Table 7.1). It also had a high content of various heavy metals, where the highest content was noted for Fe followed by Mn, Zn, Cu, Cr, Pb, Ni, and Cd. Leachate analysis also demonstrated high values for pH and EC, BOD, COD, TDS and various heavy metals as shown in Table 7.1. This indicated the high leaching properties of the pollutants present in PMDS. The high pH of sludge and leachate might be the presence of large amounts of salt. The alkalinity of the waste arises from the combined residual effect of carbonates, bicarbonates, and hydroxides, which are used in a distillery for pH adjustment during the fermentation process and the washing of fermentation products (Tiwari et al. 2013). The high values of EC of indicated the role of various cations and anions, such as sodium, chloride, nitrate, and ammonium ions, which are present in both fresh distillery sludge and leachate. The high pH and EC of sludge could be due to the presence of high concentrations of soluble salts. Several authors have also reported higher values of these cations and anions in distillery waste (Chandra et al. 2008a). The high content of heavy metals in distillery sludge could be due to the corrosive effect of sugarcane juice during the sugar manufacturing process. Metals might be added further during the fermentation and distillation processes of sugarcane molasses in

distilleries, as it is finally discharged as a spent wash under highly acidic conditions and again, undergoes treatment in an anaerobic reactor and all of these processes potentially induce metal corrosion of metal pipes. This may be the main source of the heavy metal content of distillery sludge. The high levels of organic and inorganic parameters in PMDS are also concordant with previous reports (Chandra et al. 2008).

**Table 7.1** Physico-chemical analysis of disposed distillery sludge and leachate

| S. No. | Physico-chemical parameters | Distillery sludge | Distillery sludge leachate | Sludge leachate after *in*-situ remediation | % Reduction of sludge leachate |
|---|---|---|---|---|---|
| 1. | pH | 8.00±0.21 | 7.99±0.10 | 7.114±0.21[a] | 10.96 |
| 2. | EC | 4.1± 2.11 | 3.9±1.14 | 2.4±1.34[a] | 38.46 |
| 3. | Sodium ($Na^+$) | 56±1.31 | 55.108±0.97 | 13.352±0.80[a] | 75.77 |
| 4. | Chloride ($Cl^-$) | 1825±0.10 | 7034.6.±0.89 | 6878.674±129.45[ns] | 83.14 |
| 5. | Nitrate ($NO_3^-$) | 110±3.14 | 109.916±1.30 | 46.295±0.90[a] | 57.88 |
| 6. | Ammonical Nitrogen ($NH_4^+$) | 190±1.24 | 187.794±0.94 | 85.594±0.71[a] | 54.42 |
| 7. | TDS | - | 16550.4±2.07 | 2406±89.19[a] | 85.56 |
| 8. | BOD | - | 11050.2±2.28 | 2555.698±1.78[a] | 76.87 |
| 9. | COD | - | 22421±1.58 | 4512.934±1.90[a] | 79.87 |
| 10. | Total Organic Carbon | 17.318±0.21 | 16.404±0.28 | 7.407±1.09[a] | 66.48 |
| 11. | Total Nitrogen | 2.463±0.01 | 2.084±0.04 | 1.982±0.05[c] | 57.29 |
| 12. | Total Hydrogen | 4.013±0.04 | 3.668±0.38 | 2.93±0.05[c] | 69.41 |
| 13. | Total oxygen | 36.251±1.11 | 35.408±0.58 | 26.039±3.52[b] | 9.42 |
| 14. | Trace Elements | | | | |
| a | Iron (Fe) | 2403±3.11 | 1401.22±1.86 | 852.528±2.05[a] | 39.15 |
| b | Zinc (Zn) | 210.15±2.14 | 95.273±0.68 | 21.463±1.67[a] | 77.47 |
| c | Copper (Cu) | 73.62±1.14 | 62.928±1.20 | 11.526±1.02[a] | 81.68 |
| d | Chromium (Cr) | 21.825±0.41 | 18.447±0.60 | 9.140±0.49[a] | 50.45 |
| e | Cadmium (Cd) | 1.440±0.12 | 1.166±0.15 | 0.94±0.05[c] | 19.38 |
| f | Manganese (Mn) | 126.30±0.94 | 94.602±1.13 | 45.105±0.72[a] | 52.32 |
| g | Nickel (Ni) | 13.425±0.21 | 8.302±0.31 | 2.250±0.20[a] | 72.89 |
| h | Lead (Pb) | 16.33±1.11 | 14.311±1.65 | 7.222±0.38[a] | 49.53 |

All values are mean of three replicate ± SD and presented in mg $kg^{-1}$ and ml $L^{-1}$ for distillery sludge and distillery leachate, respectively except electrical conductivity (µS $cm^{-1}$), t-test (two tailed as compared to fresh distillery leachate); a-Highly Significant at $p<0.001$; b-Significant at $p< 0.01$; c-Less significant at $P<0.05$; ns- Non significant at $p>0.05$

The leachate obtained after 90 days of *in*-situ bioremediation of PMDS had lower values of various physiochemical parameters (Table 7.1). This indicates that the biodegradation of various organic and inorganic content by autochthonous bacterial communities growing in PMDS had occurred. The majority of bacteria, actinomycetes and fungi are saprophytes and so they decompose organic matter. They hydrolyse and oxidize various complex organic and inorganic compounds through enzymic processes, resulting in the reduction in some physicochemical parameters following *in*-situ bioremediation. The SEM-EDS

analysis of PMDS also showed heterogenous morphology (Fig. 7.3), and its elemental composition included C (24.10%), O (50.08), Si (8.67%), Al (5.22%) and Fe (4.27%) as the main elements (Table 7.2).

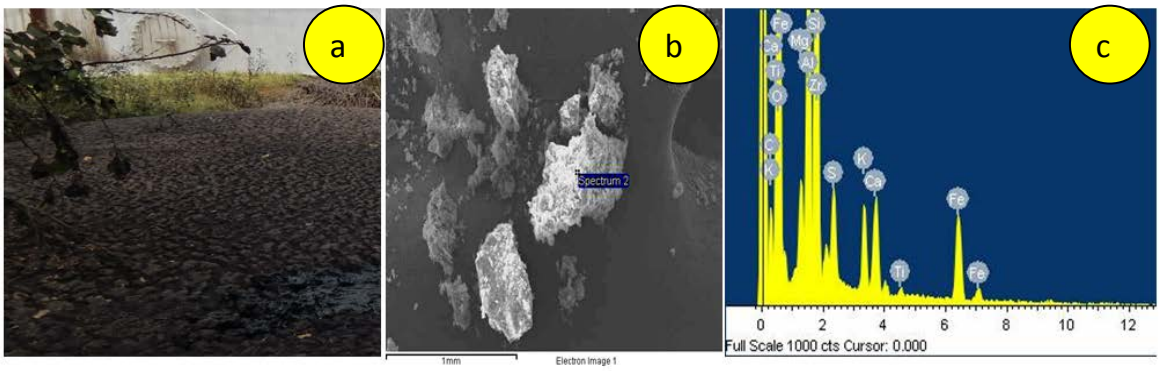



**Fig. 7.3** Post methanated distillery sludge; (a) distillery sludge dumped after methanogenesis of spent wash (b) SEM image of the morphology of sludge (100×) (c) elemental analysis image (EDS) of sludge

**Table 7.2** Elemental composition (EDS) of PMDS

| Element | Weight % |
|---|---|
| C-K | 24.10 |
| O-K | 50.08 |
| Mg-K | 1.49 |
| Al-K | 5.22 |
| Si-K | 8.67 |
| S-K | 1.41 |
| K-K | 1.55 |
| Ca-K | 1.81 |
| Ti-K | 0.20 |
| Fe-K | 4.27 |
| Zr-K | 1.19 |
| Total | 100.00 |

The K following every element indicated the K-shell of the specific atom

### 7.3.2 Characterization of organic compounds

#### 7.3.2.1 Spent wash

The detail of identified compound in distillery spent wash is described in chapter no. 04.

#### 7.3.2.2 PMDS and leachate

The GC-MS chromatogram of compounds extracted from PMDS and leachate with *n*-hexane is shown in Fig. 7.4 and organic compounds have been identified in detail at various retention times based upon mass to charge ratios (m/z). The results show that fresh PMDS and leachate contained large numbers of organic compounds as identified by GC-MS (Table 7.3). The major compounds were identified as saturated fatty acids (propanoic acid, dodecanoic acid, tetradecanoic acid, n-pentadecanoic acid, octadecanoic acid and hexadecanoic acid). Other compounds were also identified as stigmasta-5, 22-dien-3-ol(3 β,22E), stigmasterol and β-sitosterol. Other compounds such as 1-propanol, 3-

(octadecycloxy), D- lactic acid, TMS ether, TMS ester, 2-methyl-4-keto-pentan-2-ol, 1 methylene-3-methyl–butanol, benzene, 1,3-bis (1,1-dimethylethyl), phosphoric acid, 1-phenyl 1-propanol, 2-isoropyl-5-methyl-1-heptanol, 5-methyl-2-(1-methylethyl) cycohexanol, 2-ethylthio-10-hydroxy-9-methoxy-1,4 anthraquinone, tert-hexadecanethiol and 2,6,10,14,18,22-tetracosahexane 2,6,10,18,19,23 hexamethyl were detected in sludge as well as sludge leachate samples. GC-MS analysis of organic extracts in the sample identified more than 35 organic compounds some of which were mutagens, carcinogens, and environmental endocrine disrupters. Octadecanoic acid and hexadecanoic were detected in our study, which have been reported to be antiquorum sensing molecules in bacterial products (Singh et al. 2013). However, in some studies octadecanoic acid has also been reported to be a toxic compound in aquatic systems (Kamaya et al. 2003), while hexadecanoic acid has been reported to be a DNA fragmentation inducer in a human melanoma cell line (de Sousa Andrade et al. 2005). Similar compounds have been reported by various researchers, and so our data support results reported previously (González et al. 2000; Quinn et al. 2007). This finding also corroborated previous studies (Kaushik et al. 2010). These organic compounds constitute the main components of wastewater and discharge in sludge from yeast or original sugarcane molasses, which remains after the secondary treatment of distillery effluent. Apart from this, other compounds were also identified as stigmasta-5, 22-dien-3-ol(3β,22E), stigmasterol and β-sitosterol. These are major phytosterols with a chemical structure similar to cholesterol, soluble in water at all pH values and previously reported to be present in sugarcane (*Saccharum officinarum* L.) and the wastewater from sugarcane molasses. These compounds are screened under the list of environmental endocrine disrupting chemicals (EDCs; as in USEPA 2012). In a previous study, it was reported that in nature, aerobic, and/or anaerobic microorganisms may transform b-sitosterol and other sterols into androgenic hormones such as 5-β-androstan 3,17-dione and androstan 4-en-3,17-dion (Taylor et al. 1981). Such androstane derivatives of sterols may ultimately interfere with endocrine systems and produce hermaphroditism or other morphological defects. A role of these compounds in masculinizing the fish population and reducing fish numbers has been suggested (Jenkins et al. 2003). However, tetradecane and other similar phyo hydrocarbons (heptacosane dotriacontane, lanosta-8,24-dien-3-one) have also been identified, and reported as key constituents of environmental pollutants responsible for dermal irritation (Muhammad et al. 2005).

However, in the GC-MS chromatogram of the *n*-hexane extract obtained from the degraded sludge sample after 90 days of *in*-situ bioremediation the disappearance of several peaks and generation of some new peaks was clear. This indicated the degradation of major toxic organic compounds had occurred (mutagens, carcinogens and environmental endocrine disrupters) along with the simultaneous biotransformation of some new compounds (Fig. 7.4 and Table 7.3). However, the role of several other organic compounds detected in the environment is still of interest and requires detailed investigation for environmental safety.

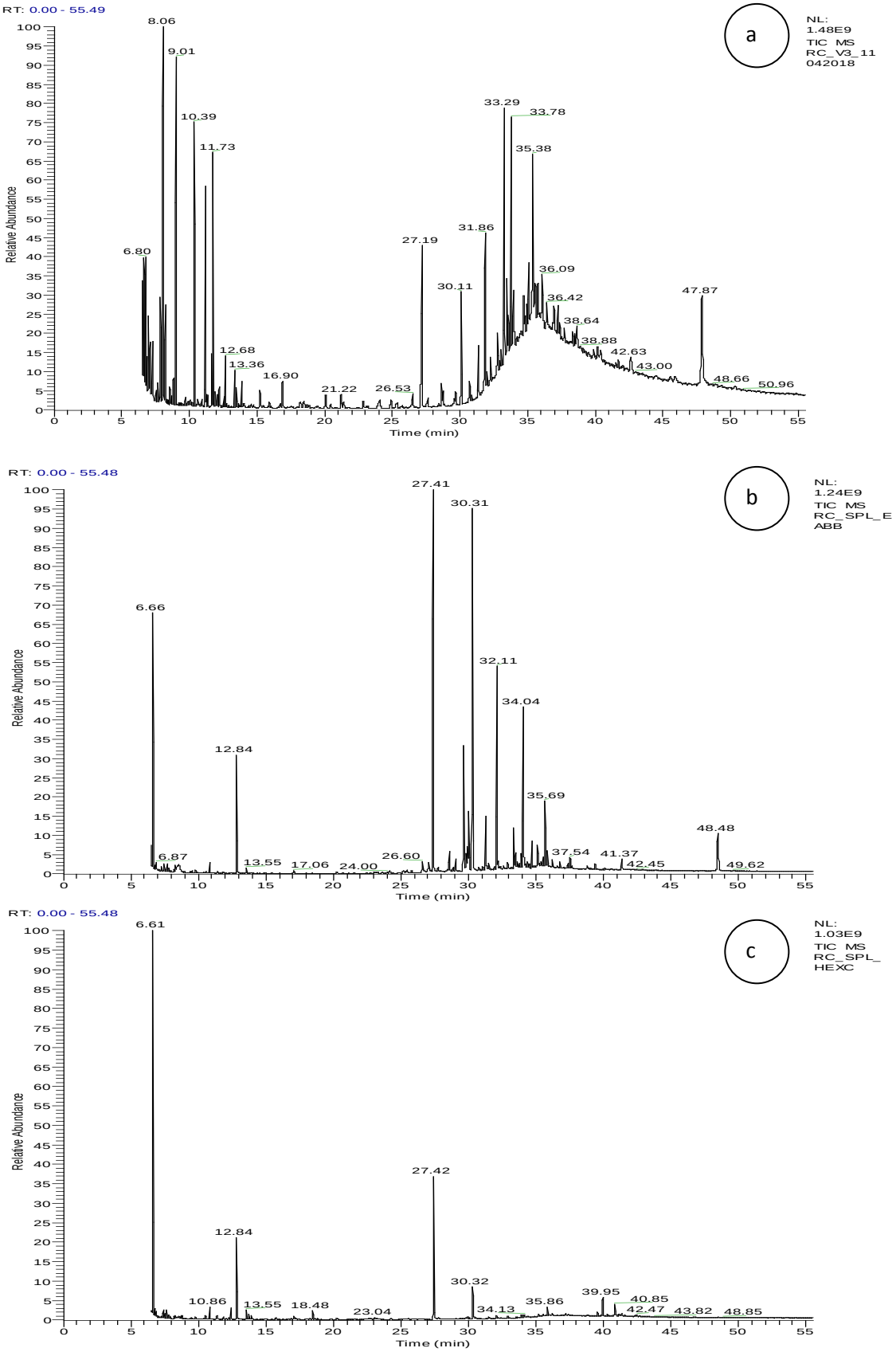



**Fig. 7.4** GC-MS chromatogram of organic compounds extracted with n-hexane from; (a) fresh distillery sludge, (b) fresh distillery leachate, (c) distillery leachate after 90 days of *in*-situ bioremediation.

**Table 7.3** Identified organic compounds by GC-MS present in *n*-hexane extract of distillery sludge and leachate

| S.No. | RT | Name of Compound | DS | DSLC | DSLD |
|---|---|---|---|---|---|
| 1. | 7.33 | 1-Propanol, 3-(octadecycloxy) | - | + | + |
| 2. | 8.15 | 2-methyl-4-keto pentan-2-OL | + | + | - |
| 3. | 8.16 | D-Lactic acid, TMS esther, TMS ester | + | + | + |
| 5 | 10.41 | Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-(CAS) | - | + | - |
| 6. | 11.13 | 1 methylene-3-methyl-Butanol | + | - | - |
| 7. | 12.46 | Benzene, 1,3-bis(1,1-dimethylethyl) | + | + | - |
| 8. | 12.86 | Phosphoric acid | + | + | - |
| 9. | 12.68 | 3,7-Dioxa-2,8-disilanonane, 2,2,8,8-tetramethyl-5-[(TMS)oxy | - | - | + |
| 10. | 13.55 | Docosanoic acid, docosyl ester | - | + | + |
| 11. | 13.74 | 2-isoropyl-5-methyl-1-heptanol | + | - | - |
| 12. | 14.84 | 1-phenyl 1-propanol | + | + | - |
| 13. | 15.70 | Tetradecane | + | - | - |
| 14. | 16.69 | Tert-Hexadecanethiol | - | - | + |
| 15. | 17.23 | Decane, 2,3,5,8 tetramethyl | + | + | - |
| 16. | 17.50 | Propanoic acid | + | + | - |
| 17. | 18.47 | Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy ethyl ester | - | - | + |
| 18 | 19.12 | 1-Dodecanol | + | - | - |
| 19. | 19.73 | Docosane | + | + | - |
| 20. | 20.73 | Dodecanoic acid | + | - | - |
| 21. | 21.62 | Heptacosane | + | + | - |
| 22. | 22.13 | Dotriacontane | + | + | - |
| 23. | 22.63 | 1-Hexadecanol, 2 methyl | - | - | + |
| 24. | 23.01 | Tert-Hexadecanethiol | + | - | - |
| 25. | 24.24 | Tetradecanoic acid | + | + | - |
| 26. | 25.28 | n-Pentadecanoic acid | + | + | - |
| 27. | 27.15 | Hexadecanoic acid, TMS ester | + | + | - |
| 28. | 28.63 | Palmidrol | - | - | + |
| 29. | 30.34 | Octadecanoic acid | + | + | - |
| 30. | 31.85 | Hexanedioic acid, bis(2-ethylhexyl ester) | - | + | + |
| 31. | 34.71 | Querecetin 7,3',4'-Trimethoxy | - | | + |
| 32. | 35.08 | 1H-Purin-6-amine, (fluorophenyl)methyl | - | + | + |
| 33. | 35.09 | 2-monostearin TMS ether | - | - | + |
| 34. | 35.91 | 2,6,10,14,18,22-Tetracosahexane 2,6,10,18,19,23 hexamethyl | + | + | - |
| 35. | 37.41 | Butyl 11-eicosenoate | - | - | + |
| 36. | 41.33 | Stigmasta-5,22-dien-3-ol(3β,22E) | + | + | - |
| 37. | 41.55 | Stigmasterol | + | + | - |
| 38. | 42.14 | Lanosta-8, 24 dien-3-one | + | + | - |
| 39. | 42.61 | 1-Monolinoleoylglycerol TMS ether | - | - | + |
| 40. | 42.40 | Spirostan-3-one (5α, 20β, 25R) | + | - | + |
| 41. | 42.66 | β-Sitosterol trimethyl ether | + | + | - |

DS: Distillery sludge; DSLC: Distillery sludge leachate control; DSLD: Distillery sludge leachate degraded after 90 days in-situ bioremediation

### 7.3.3 Characterisation of bacterial communities using 16S rRNA gene analysis

#### 7.3.3.1 Bacterial communities of spent wash

Digestion of the 16S rDNA genes derived from the bulk DNA of uncultured bacterial communities in DSW with *Taq*1 and S*au3*A1 revealed the presence of 29 uncultured bacterial species clones (Fig. 7.5). Specifically, fragments of 1519, 1517, 1513, 1516, 1519, 1515, 1515, 1514, 1449, 1516, 1566, 1452, and 1518 bp (Fig. 6.5a), 1467, 1515, 1496, 1507, 1449, 1466, 1467, and 1472 bp (Fig. 7.5b), and 1514, 1515, 1519, 1515, 1518, 1513, 1524, and 1512 bp (Fig. 7.5c) were observed. The results showed the dominance of *Bacillus* sp. as well as evidence of hydrolysis of some complex organic compounds present in the DSW. Phylogenetic analysis revealed that the dominant bacterial species belonged to Firmicutes (Fig. 7.6). The presence of *Firmicutes* as a dominant group is quite rare in natural samples. However, lignocellulosic materials such as sugarcane molasses/sugarcane bagasse represent an abundant, inexpensive source of organic material that can be used as a carbon source for growing bacteria (Womersley 2006). Further, disposed DSW might be a major source of microbial nutrients (nitrogen, phosphate, and carbon source). Thus, these results indicate that *Firmicutes* might have wide ranging metabolic capabilities, as well as the ability to use various sugar and organic compounds as a sole carbon source. Therefore, this ability creates a specific niche of these bacterial communities that may lead to in situ bioremediation of these pollutants. Moreover, they are endospore formers that grow aerobically; thus, in the presence of organic compounds under anaerobic conditions, they might be able to grow slowly and become viable after discharge of sludge (Whitman 2009; Priest 1993). Park et al. (2007) also reported the dominance of *Bacillus* sp. growing on a rotating activated bacillus contactor biofilm for advanced wastewater treatment. PhyloChip analysis has also revealed the dominance of *Firmicutes* growing within sugarcane processing plants (Sharmin et al. 2013).

The results also showed the dominance of *Stenotrophomonas* sp. in the DSW discharging collection pond, which is characterized by high temperature, low pH, and high BOD and COD. These findings reflect the thermotolerant, versatile, and adaptive nature of *Stenotrophomonas* sp. The 16S rRNA gene sequence of *Stenotrophomonas* sp. was shown to belong to *γ-Proteobacteria*, a class of several medically, ecologically, and scientifically important groups of bacteria (Fig. 7.7). Moreover, the bacteria were shown to be adaptable in acidrich environments containing plant phenolics and heavy metals with very low dissolved oxygen. The versatility and adaptability of *Stenotrophomonas* sp. have been reported from the diverse environmental conditions (Ryan et al. 2009; Assih et al. 2002). Additionally, some species of such as *Stenotrophomonas maltophilia* have been reported for the degradation of *p*-nitrophenol and polycyclic aromatic hydrocarbons (Zhang et al. 2007; Juhasz et al. 2000; Dungan et al. 2003). This bacterium has also been reported to have intrinsic resistance to various heavy metals (Alonso et al. 2000). Taken together, these properties of *Stenotrophomonas* sp. support our findings and provide strong evidence of the survival and specialized adaptation to oxygen limiting environments of these autochthonous bacteria in this specific ecological niche.

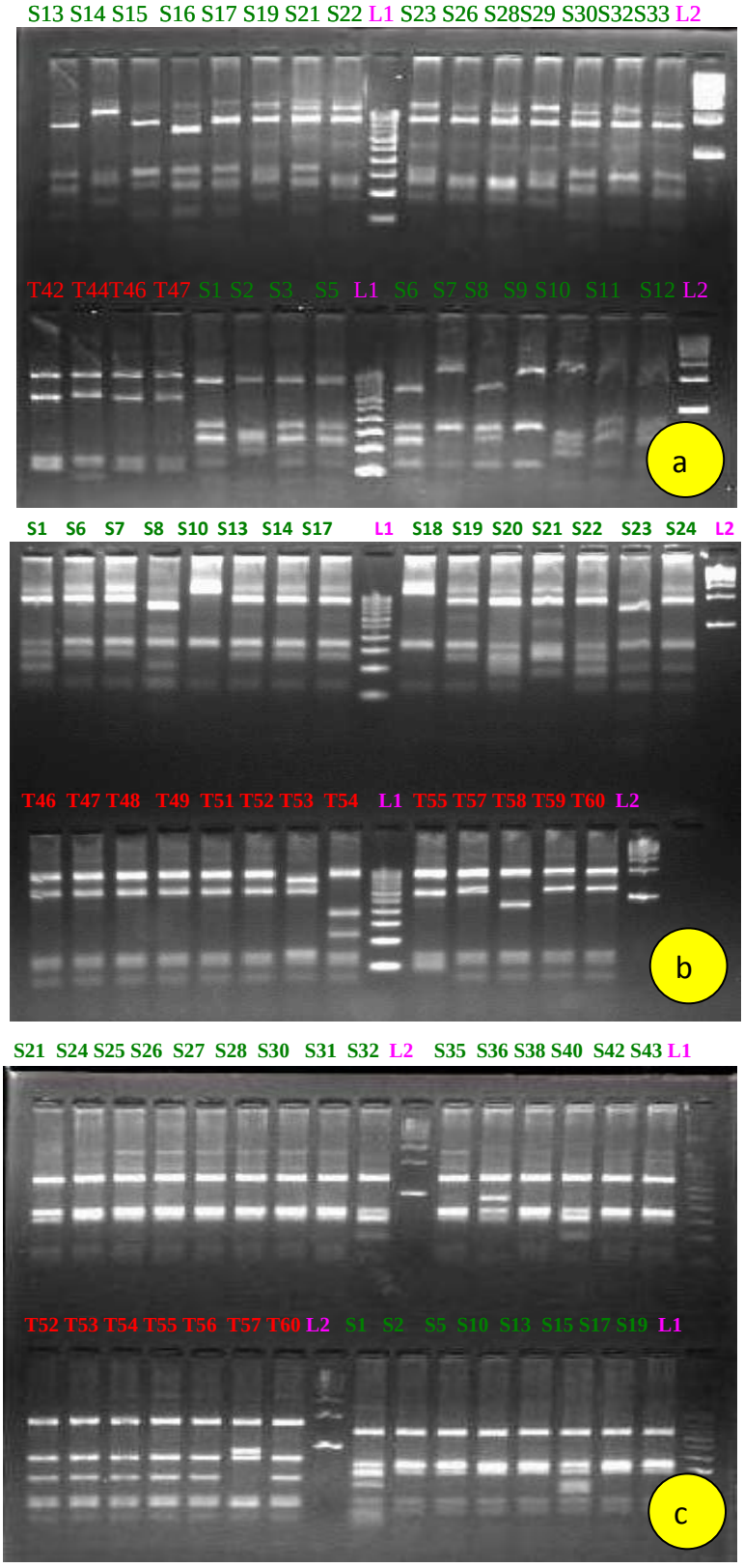



**Fig. 7.5** Restriction pattern using *Taq*I and *Sau*3AI endonucleases. The picture shows that the clones T42–T47 (labeled in red) and S1–S33 (labeled in green) (a), T46–T60 (labeled in red) and S1–S24 (labeled in green) (b), and T52–T60 (labeled in red) and S1–S43 (labeled in green) (c) are PCR positive products, which were subjected to RFLP using *Taq*I and *Sau*3AI, respectively. L1, 100 bp ladder; L2, 500 bp ladder

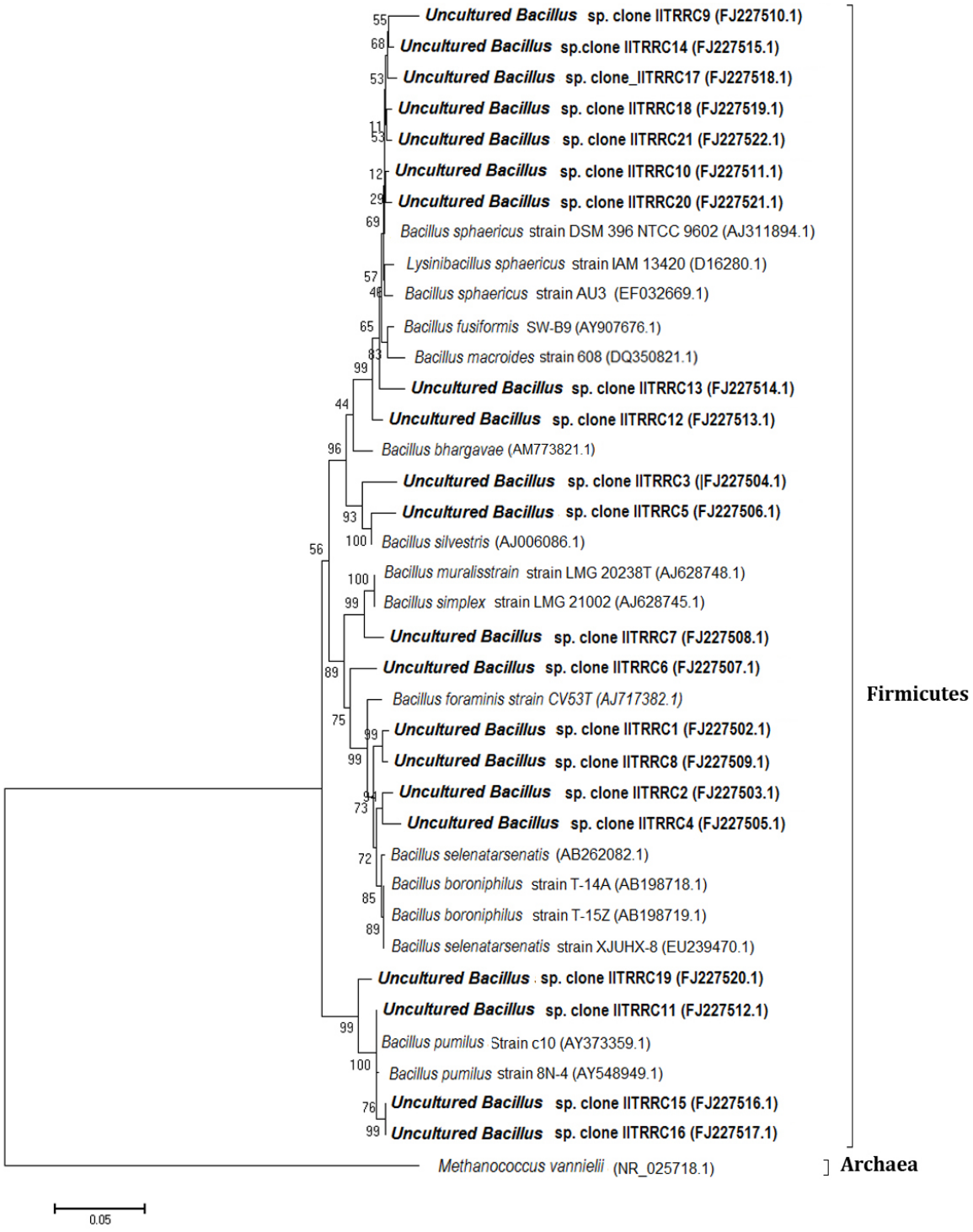



**Fig. 7.6** Phylogenetic tree showing the interrelationship of uncultured bacterial community with the most closely related species inferred from sequences of the 16S rRNA gene. The tree was generated using the neighbour-joining method. The bootstrap values based on 1000 replicates are shown at the branching points. The query sequences are *boldface*. All the query sequences fell in the phylum *Firmicutes.* Accession numbers of sequences are given in square brackets at the end of the sequence. *Methanococcus vannielii* strain SB in the Archaea domain was utilized as the out group. The scale bar shows 0.05% sequence divergence.

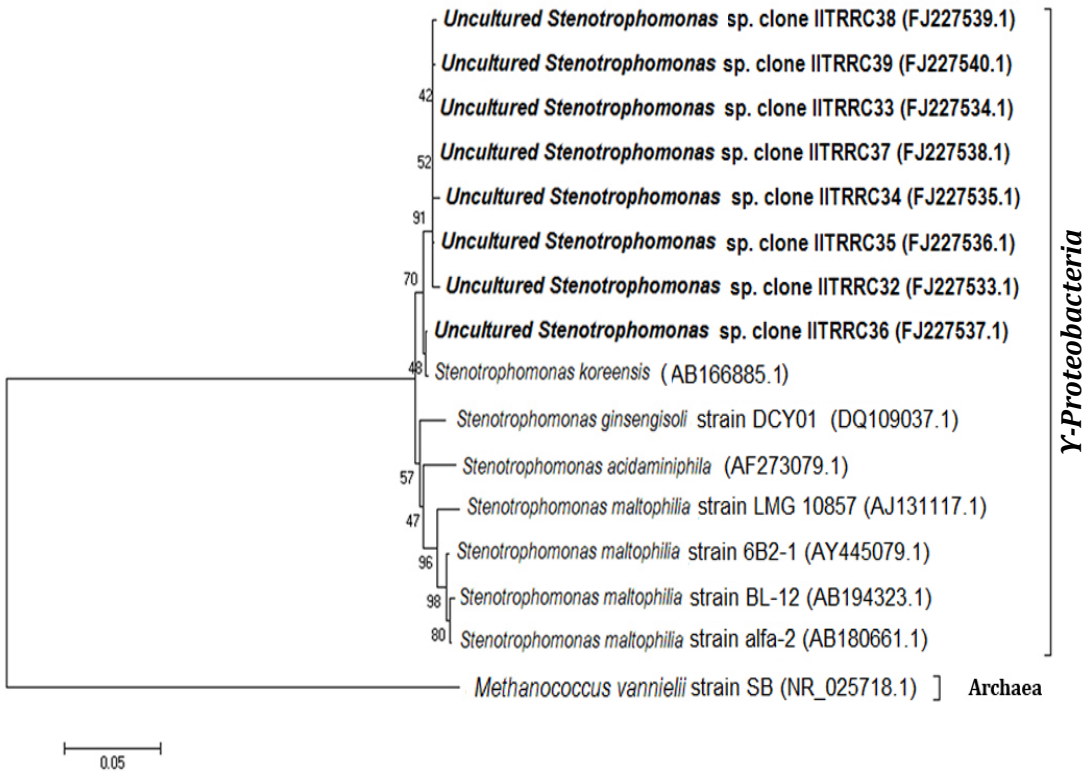



**Fig. 7.7** Phylogenetic tree showing the interrelationship of uncultured bacterial community with the most closely related species inferred from sequences of the 16S rRNA gene. The tree was generated using the neighbour-joining method. The bootstrap values based on 1000 replicates are shown at the branching points. The query sequences are *boldface*. All query sequences fell in the phylum *γ-Proteobacteria*. Accession numbers of sequences are given in square brackets at the end of the sequence. *Methanococcus vannielii* strain SB in the Archaea domain was utilized as the outgroup. The *scale bar* shows 0.05% sequence divergence

### 7.3.3.2 Bacterial communities of PMDS

The restriction digestion of the 16S rDNA genes derived from the bulk DNA from the uncultured bacterial community growing in PMDS with *Taq*1 and *Sau*3A1 restriction enzymes demonstrated the presence of 10 clones of uncultured bacterial species (Fig. 7.8) Specifically, fragments of 1503, 1496, 1515, 1519, 1515, 1521, 1515, 1500 and 1513 bp were observed. RFLP analysis indicated that clones of uncultured *Bacillus* sp. (9) were dominant in degraded PMDS followed by clones of *Enterococcus* sp. (1). The genus *Bacillus* is also characterized by its cellular properties, which are Gram-positive, have a low G+C content, are mostly straight rod shaped, and occur singly, in pairs, or chains. They also form endospores and grow aerobically. So, in the presence of organic compounds under anaerobic conditions they could grow slowly or are in the inactive phase and become viable after the discharge of sludge (Whitman 2009). The heat resistant nature of the endospores accounts for the presence of *Bacillus* sp. in cooling tower water, as the heat-exchange process selects for heat resistant microorganisms that become even more dominant as water is recycled (Sharmin et al. 2013). Also, Park et al. (2007) reported the dominance of *Bacillus* sp. growing on a Rotating Activated Bacillus Contactor Biofilm (RABCS) as used for advanced wastewater treatment by culture-dependent methods. The

second member of the phylum *Firmicutes* noted in this study was *Enterococcus* sp. These bacteria are characterized as being Gram-positive, cocci-shaped, often occurring in pairs (diplococci) or short chains, non-endospore forming, aerobic or facultative anaerobes, capable of the fermentation of sugars and of producing lactic acid or other acidic products. One study has also revealed the presence of *Bacillus* and *Enterococcus* sp. (España-Gamboa et al. 2012) in the anaerobic sludge of an upflow anaerobic sludge blanket (UASB) reactor for the treatment of distillery spent wash. This indicated that bacteria are able to exploit carbon compounds from spent wash as a necessary source of energy, and can adapt to this environment by using their endogenous enzymatic systems. This corroborates our results.

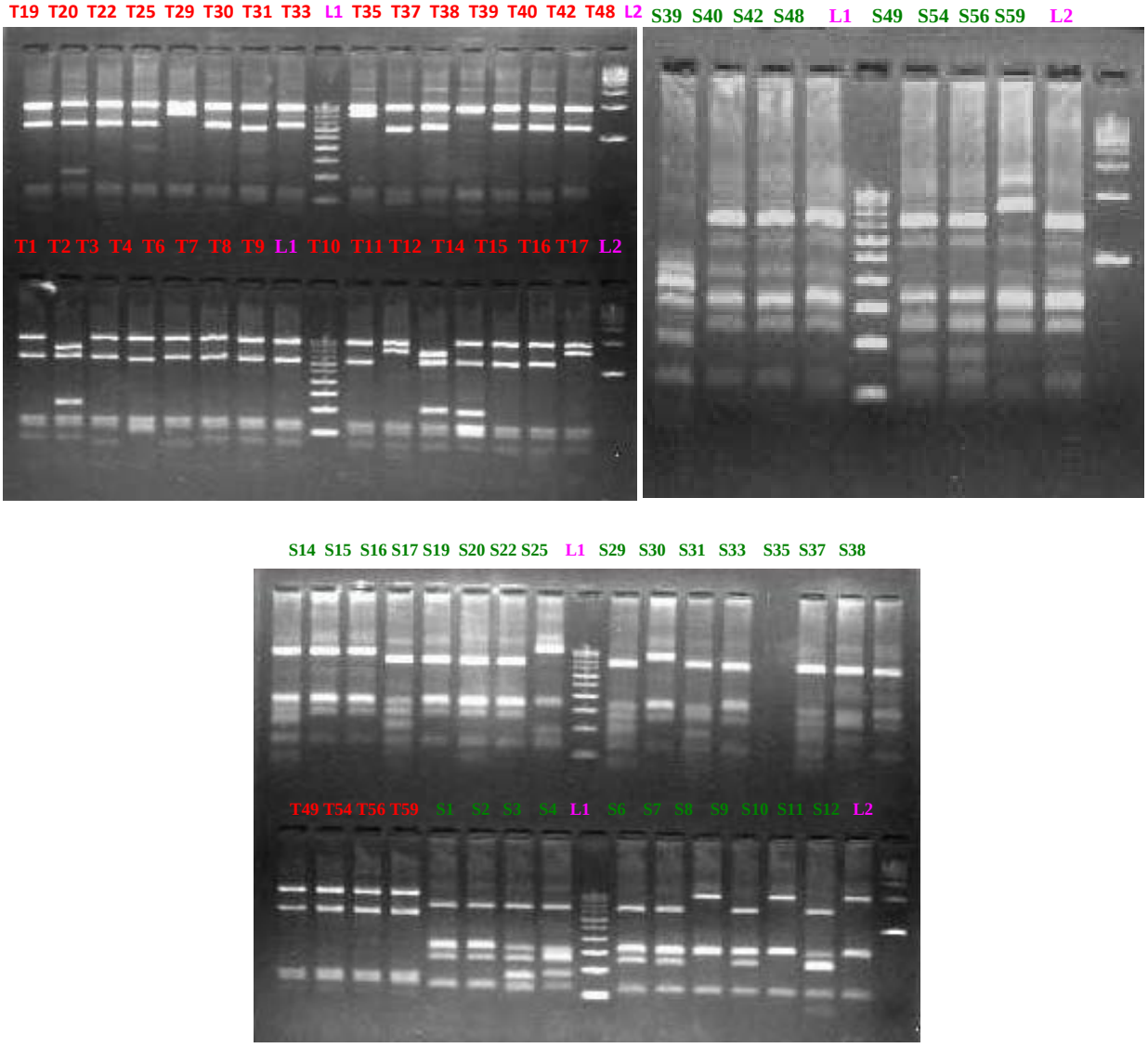



**Fig. 7.8** RFLP profile generated by the restriction digestion of 16S rRNA gene derived from bacterial community growing on distillery sludge using *Taq*1 (a) *Sau*3AI (b) and *Taq*1 and *Sau*3AI restriction endonuclease together (c). The above picture shows the clones, labeled from T1-T48 (A) S39-S59 (B) T49-T59 are PCR positive products. L1-100 bp ladder; L2-500 bp ladder.

On the basis of phylogenetic relationships, the dominant bacterial species identified belong to the phylum *Firmicutes* (Fig. 7.9). Disposed PMDS might be a major source of microbial nutrients (nitrogen, phosphate and carbon sources). PhyloChip analysis has also been used to assess the dominance of *Firmicutes* growing within sugarcane processing plants (Sharmin et al. 2013). Acharya et al. (2011) also studied the dynamics of the microbial communities within anaerobic biphasic fixed film bioreactor treatment distillery spent wash, showing the clear dominance by *Firmicutes* in a methanogenic bioreactor, which indicates the high degree of diversity of this phylum. The presence of *Firmicutes* as a dominant group is quite rare for natural samples. However, lignocellulosic material, such as sugarcane molasses/sugarcane bagasse represents an abundant, inexpensive source of organic material, which can be a carbon source for a growing bacterial population. Hence, the results of the current study agree with previously reported data from autochthonous bacterial communities in similar environments (Chandra et al. 2012). PMDS contains much more various organic and inorganic complex pollutants at much greater concentrations, but the autochthonous bacterial communities identified were specific and capable of growing in this environment, which may allow *in*-situ bioremediation of pollutants.

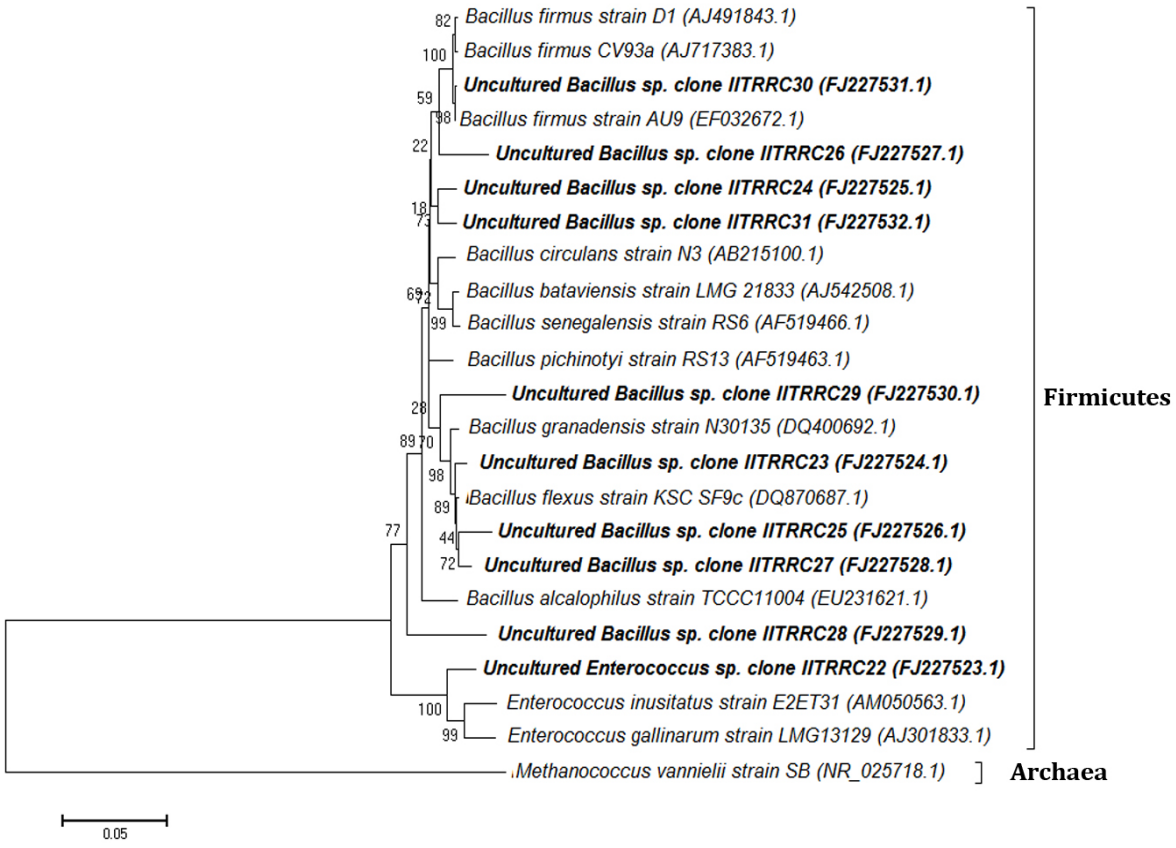



**Fig. 7.9** Phylogenetic tree generated via Neighbour-Joining method showing the relationship of uncultured bacterial community growing in PMDS with their closest homologues based on 16S rRNA gene sequences. The query sequences were denoted in boldface, after tree reconstruction it showed, that all the query sequences were lying in phylum *Firmicutes* of bacteria. All the accession numbers of sequences were written in square bracket at the end of sequence name. *Methanococcus vannielii* strain SB in the Archaea domain was utilised as the out group. The scale bar represents 0.05 changes per nucleotide.

### 7.3.4 Phytotoxicity assay

#### 7.3.4.1 Phytotoxicity of DSW

The comparative seed germination test of green gram (*P. mungo* L.) and wheat *(T. aestivum*) showed that DSW was more toxic to *T. aestivum* than *P. mungo* L. The detail result of phytotoxicity assessment of DSW is mentioned in chapter no. 04.

#### 7.3.4.1 Phytotoxicity of PMDS

The seed germination test of green gram (*P. mungo* L.) showed inhibitory effects of fresh and degraded sludge leachate at different concentrations in terms of seed germination and growth parameters of seedlings (Fig. 7.10 and Table 7.4). Seeds germinated at rate of 100% with a 1% (v/v) concentration of fresh leachate, as the concentration increased the percent germination decreased, with 2.5% and 5% (v/v) resulting in 90% and 78% germination, respectively. There was no seed germination at a concentration of 10% (v/v) fresh sludge leachate after 24 h. However, in degraded sludge leachate 85% germination was recorded in up to a 10% (v/v) concentration, which was higher than with untreated leachate. With respect to the seedling growth (radical length), the radical length of seeds exposed to fresh (control) and degraded sludge leachate varied from 1.9–0.50 cm and 2.0–1.1 cm, respectively. When the seeds had been exposed to 10% (v/v) untreated leachate they showed no root development, but after treatment seeds showed development of a radical (1.1 cm). Inhibition of radical length was considered to be the first evidence of an effect of organic pollutants and of metal toxicity in plants. The promotion of seedling growth by lower concentrations of leachate might be due to the presence of lower concentrations of toxicant in samples tested. While the suppression of germination at high concentrations of leachate might be due to the presence of highly toxic organic compounds and dissolved solid, which were absorbed by the seeds before germination and affected various physiological and biochemical processes of SG (Bharagava and Chandra 2010).

The germination percentage and the radical length were combined to give a comprehensive interpretation of leachate toxicity in terms of the germination index. The germination index (GI) values of untreated and treated leachate ranged between 0.19–0.95 and 1.0–0.46, respectively. The GI decreased with increasing concentrations of leachate. The maximum relative toxicity of control and degraded sludge leachate was noted as 22% at a concentration of 5% (v/v) and 15% at an 85% (v/v) concentration, respectively. The control sludge leachate showed greater relative toxicity. The percentage phytotoxicity analysis of the leachate obtained from controls revealed that phytotoxicity increased with increases in leachate concentrations, but in degraded leachate it gradually decreased. However, the extent of toxicity was found to be higher [75% at a 5% (v/v) concentration] of fresh sludge (control) leachate, while at the same concentration only 5% toxicity was noted in the degraded sludge leachate. The stress tolerance index of seedlings was at a minimum at a 5% (v/v) concentration of treated leachate. These results clearly indicate that the toxicity of sludge leachate was reduced significantly after *in*-situ bioremediation.

The inhibitory effect of the fresh sludge leachate on seed germination and seedling growth might be attributed to the high salt load and metal content, which induces both a high osmotic pressure and anaerobic conditions. It has also been reported that a high salt load and metal content acts as an inhibitor of plant hormones (amylases, auxins, gibberellins, and cytokinins), which are required mainly for SG, seedling growth and plant development, respectively (Ahsan et al. 2007). One study showed that distillery-sludge-amended soil delayed flowering and reduced pod formation in *P. mungo* L., which apparently provides evidence for the suppression of reproductive hormones by the toxicants present in distillery sludge (Chandra et al. 2008a). These findings support the presence of high levels of EDCs plus other toxic compounds in distillery sludge.

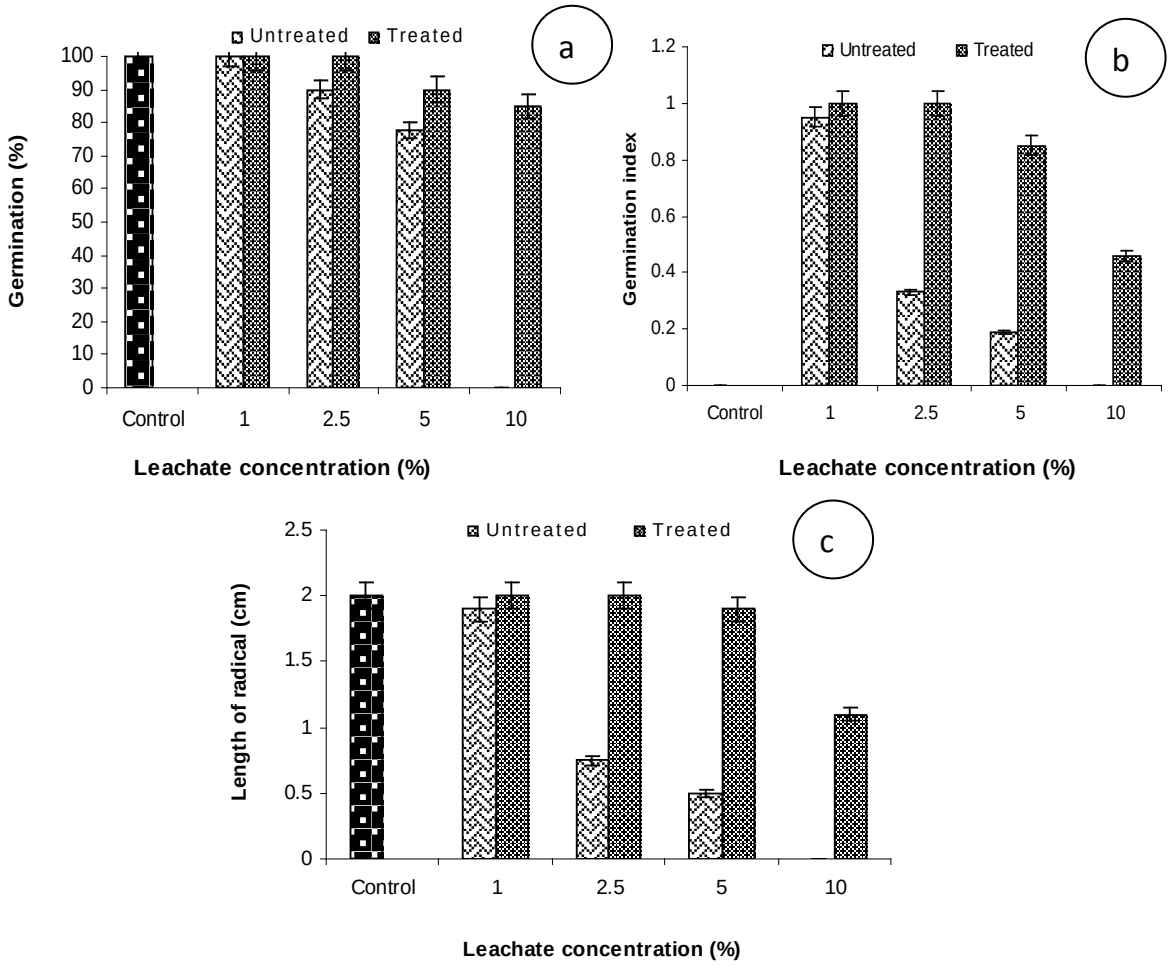



**Fig. 7.10** Effects of various concentrations of sludge leachate on *Phaseolus mungo* L. vs. control (tap water); (a) percent germination, (b) germination index, (c) radical length.

**Table 7.4** Effect of different concentration of distillery sludge leachate on seedling growth of *Phaseolus mungo* L.

| Concentration (%) | Germination (%) | | Relative toxicity (%) | | Radical length (cm) | | Germination index | | Phytotoxicity (%) | | Stress tolerance Index | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Untreated | Treated | Untreated | Treated | Untreated | Treated | Untreated | Treated | Untreated | Treated | Untreated | Treated |
| 1 | 100±0.00 | 100±0.00 | 00±00 | 00±00 | 1.9±0.04 | 2.00±0.01[a] | 0.95±0.10 | 1.0±0.08[a] | 5.0±0.01 | 00±00[ns] | 0.95±0.02 | 1.00±0.01[ns] |
| 2.5 | 90±0.00 | 100±0.00 | 10±0.00 | 00±00 | 0.75±0.02 | 2.00±0.02[a] | 0.33±0.02 | 1.0±0.01[a] | 62.50±2.77 | 00±00[a] | 0.37±0.01 | 1.00±0.04[a] |
| 5.0 | 78±0.00 | 90±0.00 | 22±0.00 | 10±0.00 | 0.50±0.04 | 1.9±0.02[a] | 0.19±0.00 | 0.85±0.00[a] | 75±2.98 | 5.0±1.4[a] | 0.25±0.03 | 0.95±0.00[a] |
| 10 | NG | 85±0.00 | NG | 15±0.00 | NG | 1.1±0.01[a] | NG | 0.46±0.00[a] | NG | 45±1.2[a] | NG | 0.55±0.00[a] |
| Control | 100±00 | 100±00 | 00±00 | 00±00 | 2.00±0.10 | 2.00±0.18[ns] | 00±00 | 00±00 | 00±00 | 00±00 | 00±00 | 00±00 |

NG: No germination was observed; All the values are means of triplicate (n=3)±SD. The statistical significance between the values of control to their respective treated samples was evaluated by one way ANOVA.

a- significant level $p<0.001$

ns-non significant level $p>0.05$

### 7.3.5 Genotoxicity assay of PMDS

The results of genotoxic studies showed a concentration dependent reduction of the MI of root tip meristem cells of *Allium cepa*, after 24 h of treatment with different concentrations of distillery sludge leachate (Table 7.5). It was found that MI decreased with increasing concentrations (as percentages) of fresh sludge leachate compared to degraded sludge leachate and the decrease in MI was in the order 1%>2.5%>5%>10%, which produced MI values of 16.6, 10.32, 6.68, 3.0, respectively. Trace metals and other organic pollutants have been considered responsible for diminishing the MI of *A. cepa* exposed to industrial wastewater (Chandra et al. 2005; Carita and Marin-Morales 2008). All tested samples induced chromosome abnormalities at all concentrations of fresh sludge leachate. Maximal chromosomal aberrations were recorded at a 10% concentration, showing 165% aberration and a total of 124 aberrant cells. The chromosomal aberrations observed included morphologically altered cells with losses of genetic material, disturbed metaphase, c-mitosis, chromosome bridges, sticky chromosomes, laggard chromosomes, polyploidy cells and apoptotic bodies (Fig. 7.11 and Table 7.6). The reason for such effects could be due to the presence of toxic substances in the liquid medium, which may disturb division, causing a relatively high number of aberrations. c-Metaphase may result from the action of aneurogenic agents on the cell; compounds that promote complete inactivation of the mitotic spindle (Fiskesjo 1993; Fernandes et al. 2007). Such alterations may generate other types of cell abnormalities, such as polyploid cells (Odeigah et al. 1997).

Exposure to distillery sludge leachate adversely affected the shape of cells of *A. cepa*. The percentage of morphologically altered cells was higher during treatment with different concentrations of leachate. Our study showed the presence of both c-mitosis (c-metaphase) and polyploid cells after treatment with distillery leachate exposure for 24 hrs. The frequency of cells with laggard and sticky chromosomes significantly increased with increasing distillery leachate concentrations (Table 7.6). These were seen mostly in anaphase-telophase aberration tests. The presence of chromosomal adherence reinforces the evidence of the aneugenic action of organic pollutants present in distillery leachate. However, organic pollutants disturb the balance in the quantity of histones or other proteins responsible for controlling the proper structure of nuclear chromatin. Chromosome stickiness reflects a highly toxic effect, which probably leads to cell death (Fiskesjo 1997). Similar observations have been reported in *A. cepa* root after treatment with distillery effluent (Hemavanthi et al. 2015). This increased stickiness also leads to the formation of chromosome bridges. Our findings are well corroborated with earlier studies (Hemavanthi et al. 2015). In our study, the significant decrease in the MI and chromosome abnormalities observed after *in*-situ bioremediation, indicated the detoxification of distillery leachate (Table 7.5). The most likely reason for the high genotoxicity and cytotoxicity of these industrial water samples is the complex assortment of organic pollutants produced during the sugarcane molasses distillation process. In summary, genotoxicity results indicate that distillery sludge and leachate produce genotoxicity and

may exert potentially harmful effects on flora and fauna, however, this toxicity was reduced after 90 days of *in-situ* bioremediation.

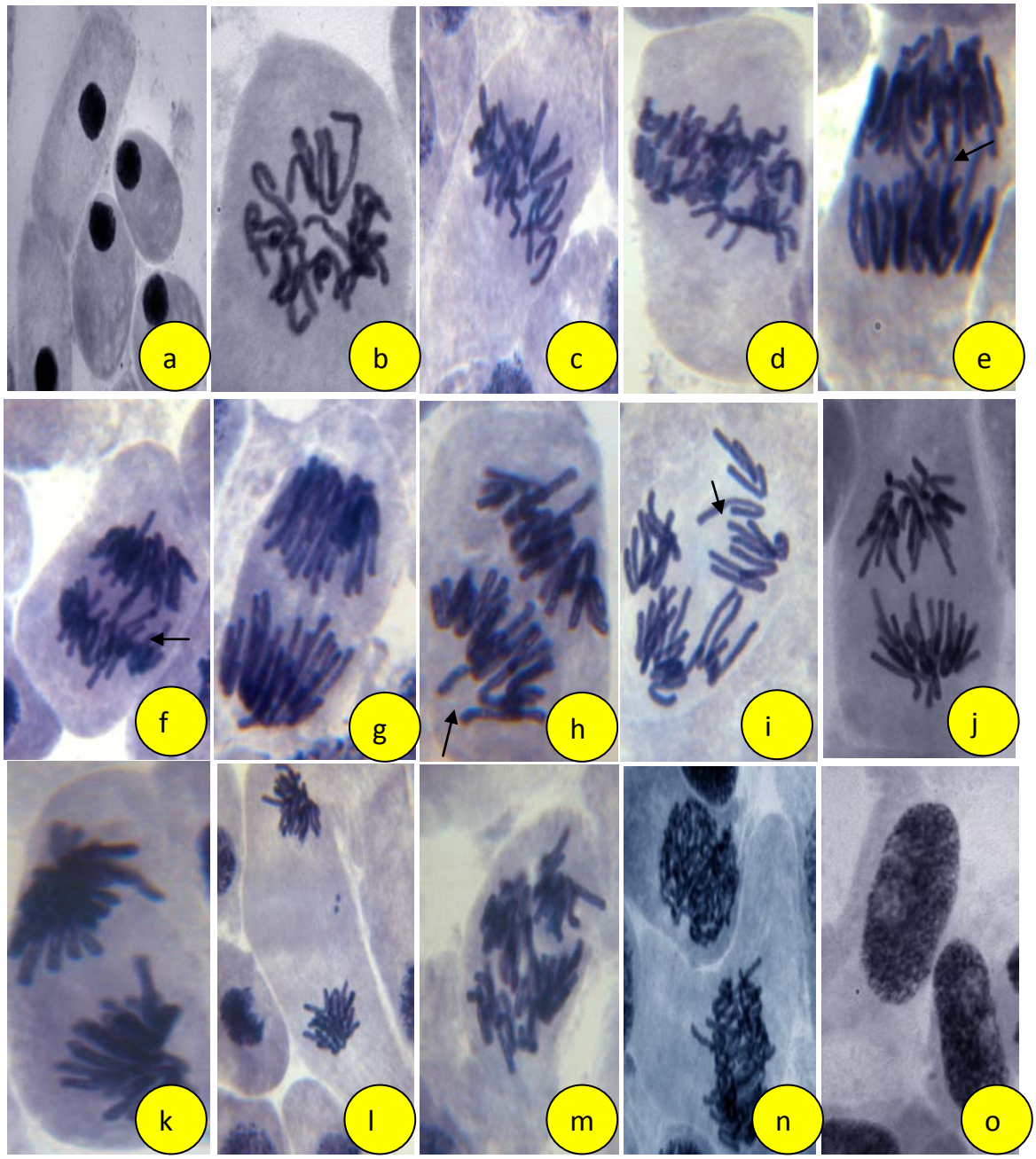



**Fig. 7.11** Different chromosome aberration observed in meristematic cells of *Allium cepa* (2n = 16) treated with distillery sludge leachate; (a) change in nucleus position in morphological altered cell, (b) prophase with genetic material loss, (c,d) disturb metaphase, (e) disturb anaphase, (c-mitosis) (e,f) chromosome bridge in anaphase, (g) sticky chromosome in anaphase, (h,i) laggard chromosome with diagonal anaphase, (j) disturb chromosome in anaphase, (k) sticky chromosome in anaphase, (l,m) sticky chromosome in telophase (chromosome adherence), (n) polyploidy cell, (o) apoptotic bodies (Magnification:1000X).

**Table 7.5** Mitotic index and Mitotic inhibition at different concentration of distillery sludge leachate

| PMDS leachate Concentration (%) | Total no. of cells | | Total no. of dividing cell | | Mitotic index (%) | | Mitotic inhibition (%) | |
|---|---|---|---|---|---|---|---|---|
| | Untreated | Treated | Untreated | Treated | Untreated | Treated | Untreated | Untreated |
| 1.0 | 2500 | 2500 | 415±20.75 | 551±19.28[a] | 16.6±0.74 | 22.04±0.90[a] | 28.93±1.27 | 5.65±0.19[a] |
| 2.5 | 2500 | 2500 | 258±12.38 | 475±18.05[a] | 10.32±0.36 | 19.00±0.86[a] | 55.82±1.95 | 18.66±0.55[a] |
| 5.0 | 2500 | 2500 | 167±8.14 | 315±35.12[a] | 6.68±0.24 | 12.6±0.44[a] | 71.40±3.28 | 46.06±1.42[a] |
| 10 | 2500 | 2500 | 75±2.25 | 269±9.14[a] | 3.0±0.12 | 10.76±0.40[a] | 87.15±4.00 | 53.93±1.94[a] |
| Control | 2500 | 2500 | 584±28.61 | 584±27.51[ns] | 23.36±0.93 | 23.36±1.14[ns] | - | - |

All the values are means of triplicate (n=5)±SD. The statistical significance between the values of control to their respective treated samples was evaluated by one way ANOVA. a- significant level $p<0.001$; ns-non significant level $p>0.05$

**Table 7.6** Cytological effect of distiller leachate on the *Allium cepa* root meristem cell after 24 h

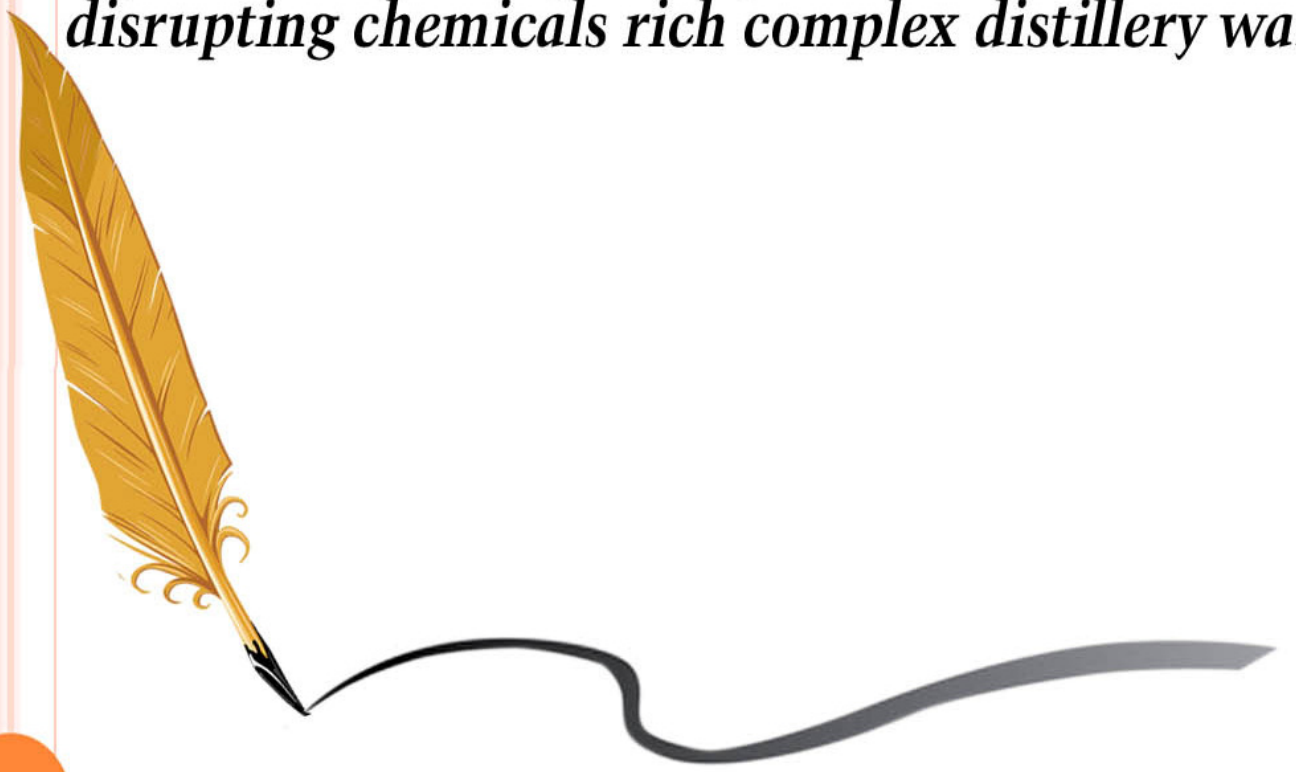
| Aberrant / Leachate concentration | Morphological alerted cell | Prolonged prophase | Disturb metaphase | Multipolar anaphase | C-mitosis (vagrant chromosome) | Chromosome bridge | Spindle disturbance cell at anaphase | Laggard chromosome multipolarity cell | Sticky chromosome | Polyploid cell | Apoptotic bodies | % Aberrant cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Untreated | | | | | | | |
| 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0.51 |
| 1 | 0 | 1 | 2 | 0 | 2 | 1 | 2 | 1 | 3 | 2 | 1 | 3.61 |
| 2.5 | 20 | 10 | 8 | 3 | 8 | 6 | 4 | 2 | 5 | 10 | 1 | 29.84 |
| 5.0 | 50 | 17 | 12 | 5 | 10 | 9 | 7 | 4 | 11 | 12 | 2 | 85.62 |
| 10 | 42 | 11 | 15 | 2 | 12 | 11 | 9 | 7 | 12 | 2 | 1 | 165 |
| | | | | | Treated | | | | | | | |
| 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0.51 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| 2.5 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.42 |
| 5.0 | 3 | 3 | 3 | 1 | 0 | 1 | 0 | 1 | 2 | 1 | 1 | 5.07 |
| 10 | 5 | 4 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 7.06 |

## Conclusion

This study has revealed that sugarcane molasses-based DSW, PMDS and its leachate contain several residual recalcitrant organic pollutants plus heavy metals, the majority of which are environmental toxicants (i.e. octadecanoic acid), endocrine disrupting chemicals (i.e. β-sitosterol) and also DNA fragmentation inducers (i.e. tetradecanoic acid), which are still fairly unknown. Moreover, a phytotoxicity assay with *P. mungo* and *T. aestivum* revealed the presence of highly toxic compounds in the DSW, PMDS and leachate. As a result, this waste poses a strong environmental risk to flora and fauna in the ecosystem; nevertheless, they are discharged into systems where they may impact biota and benthic organisms. However, DSW also contained high levels of *Bacillus* sp. and *Stenotrophomonas* sp., which might contribute to *in*-situ bioremediation of sites that have been impacted by DSW. Moreover, during bioremediation of PMDS *in*-situ, *Bacillus* sp. and *Enterococcus* sp. were noted growing dominantly as autochthonous bacterial communities of the phylum *Firmicutes.* This showed a capability to degrade the toxic pollutants present in PMDS. This bacterial community also demonstrated a special niche in high concentrations of Fe, Zn, Cu, Mn and Pb, and an environment rich in complex organic compounds containing mutagenic molecules and EDCs. The findings of the present study will be useful for monitoring and managing distillery waste environmentally and for the eco-restoration of polluted sites.

# Chapter-08

# Assessment of phytoextraction capacity of potential native plants grown on endocrine disrupting chemicals rich complex distillery waste



# Chapter-8

## Assessment of phytoextraction capacity of potential native plants grown on endocrine disrupting chemicals rich complex distillery waste

### 8.1 Introduction

Distilleries are major source of heavy metal pollution in environment due to due to discharge of their huge amount of complex waste (Chandra et al. 2004). The phytoremediation of heavy metals contamination is reported as a cost-effective 'green' technology based on the use of metal-accumulating plants to remove toxic metals and other complex organic pollutants including phenolic compounds from effluent contaminated sludge (Rosselli et al. 2003; McGrath and Zhao 2003; Smith et al. 2007). One of the effective strategies of phytoremediation for metals contaminated sludge may be phytoextraction through uptake and accumulation of metals into plants-shoot which subsequently can be harvested and removed from the polluted site through phytomining. The majority of phytoextraction studies have been conducted on pot experiment and laboratory hydroponic solution (Solhi et al. 2005; Wenzel et al. 2003) whereas very few studies have been attempted to evaluate the potential of natural hyperaccumulators of high biomass crop plants for phytoextraction under field condition (Yoon et al. 2006; Shu et al. 2002; McGrath and Zhao 2003; McGrath et al. 2006; Hammer and Keller 2003). Therefore, it is important to examine the native plants as natural source with less cost evolvement for phytoremediation because these plants have better potential in terms of its survival, growth and reproduction under the environmental stress than plant introduced from other environment. However, the detail knowledge regarding the magnitude and heavy metal accumulation pattern in different plants is unknown. Furthermore, the distribution and translocation of different heavy metal in their various parts is also unknown. But, these potential growing native plants are commonly used by animal and humans as food material. Thus, prior to application of these plants for phytoremediation of distillery waste contaminated site there is an urgent need to reveal the phytoextraction potential of these native plants for heavy metals accumulation and translocation in their various parts. Moreover, it is also important to investigate the threshold limit for accumulation of these potential growing native plants without indicating any adverse effect on plant for sustainable application. Recently the accumulation of various heavy metals by wetland plant in constructed wetland treatment system from metal contaminated waste has been reported an effective tools for treatment and management of industrial waste (Liu et al. 2007; Deng et al. 2004; Yoon et al. 2006). Moreover, the microscopic observation on cellular organelles of root of hyperaccumulator plants after phytoextraction of heavy metals from the distillery sludge which is a mixture of very complex organic is still completely unknown. Therefore, the present study has been focused on detail investigation of complex organic pollutants present in distillery sludge by GC-MS analysis and microscopic histological observations of root of growing weeds and grasses by TEM

for heavy metal accumulation in their parts to reveal the hyperaccumulation mechanism of these potential plant species in presence of complex pollutants.

## 8.2 Materials and methods

### 8.2.1 Site description

The test site of the experiments selected for soil and plant sampling was located in Unnao, Uttar Pradesh, India as shown in Fig. 8.1. The samples were taken from the sludge dumping site of M/S Unnao Distilleries & Breweries.

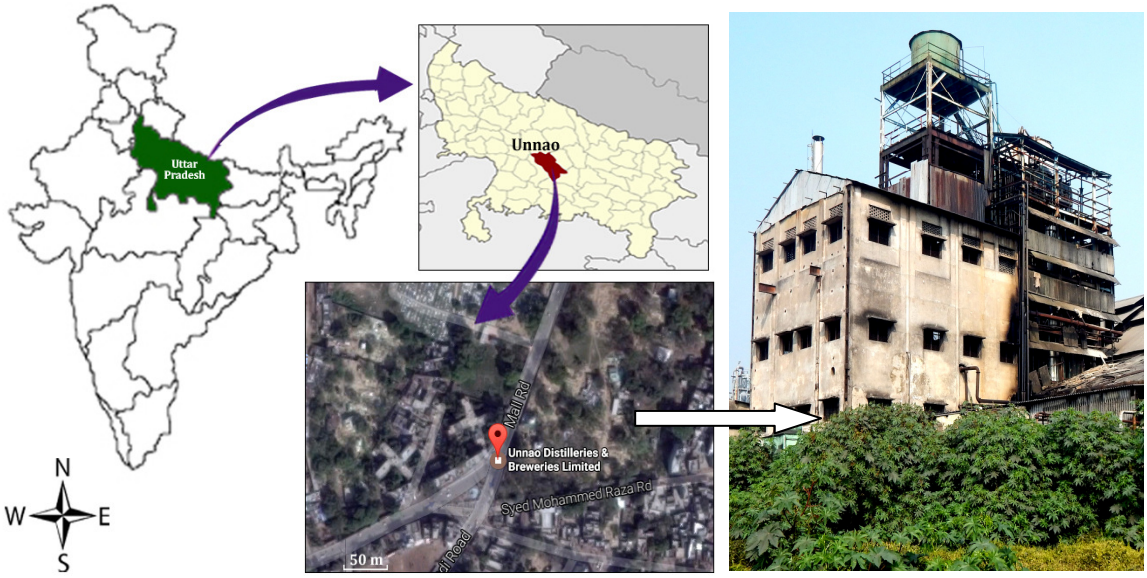



**Fig. 8.1** Location map of study site Unnao Distilleries and Breweries Limited (26°320″N, 80°30′0″E)

### 8.2.2 Collection of plant and distillery sludge sample

Twenty one native plants species (weeds and grasses) were collected based on dominant species luxuriantly growing on disposed distillery sludge. These twenty one plants species were identified from different genera and nine families where three genera belong to Poaceae family indicated the most dominant taxa growing on metal containing distillery sludge (Table 8.2). These native plants species were uprooted with associated sludge samples and carried in pre-sterilized polythene bags for the analysis of accumulated heavy metal in different parts of growing plants (Fig. 8.2). Besides, the fresh disposed dried distillery sludge cakes were collected in clean pre-sterilized polythene bags from sludge dumping site of distillery plant located inside the premises of industry. The fresh as well as ameliorated distillery sludge after plant growth was collected randomly in triplicate from three different points of same location. This process was repeated three times in different seasons from same place which was protected from any outside interference of human and animals.


**Fig. 8.2** Collection of native plants luxuriantly growing on stabilised post methanated distillery sludge (a-b); *Ricinus communis* (c) *Cannabis sativa* (d) *Parthenium hysterophorous* (e) *Achyranthes* sp. (f) *Rumex dentatus* (g) *Saccharum munja* (h) *Argemone mexicana (i) Cynodon dactylon* (j) *Blumea lacera (k) Solanum nigrum* (l) *Chenopodium album* (m) *Achyranthes sp.* (n) *Croton bonplandianum* (o) collection of fresh distillery sludge

**Table 8.1** Native plants growing on stabilised post methanated distillery sludge

| S. No. | Plant Species | Common Name | Family |
|---|---|---|---|
| 1. | *Dhatura stramonium* | Dhatura | Solanaceae |
| 2. | *Achyranthes* sp. | Chaff flower | Amaranthaceae |
| 3. | *Kalanchoe pinnata* | Life Plant | Crassulaceae |
| 4. | *Trichosanthes dioica* | Parval | Cucurbitaceae |
| 5. | *Parthenium hysterophorous* | Congress grass | Asteraceae |
| 6. | *Cannabis sativa* | Bhang | Cannabaceae |
| 7. | *Amaranthus spinosus* L. | Amarant | Amaranthaceae |
| 8. | *Croton bonplandianum* | Kala Bhanga | Euphorbiaceae |
| 9. | *Solanum nigrum* | Kali Makoy | Solanaceae |
| 10. | *Ricinus communis* | Castor oil plant | Euphorbiaceae |
| 11. | *Setaria viridis* | Green foxtail | Poaceae |
| 12. | *Blumea lacera* | Janglimuli | Asteraceae |
| 13. | *Argemone mexicana* | Mexican poppy | Papaveraceae |
| 14. | *Saccharum munja* | Munja | Poaceae |
| 15. | *Cynodon dactylon* | Bermuda grass | Poaceae |
| 16. | *Pennisetum purpureum* | Elephant grass | Poaceae |
| 17. | *Chenopodium album* | Goosefoots | Amaranthaceae |
| 18. | *Rumex dentatus* | Toothed dock | Polygonaceae |
| 19. | *Tinospora cordifolia* | Giloy | Menispermaceae |
| 20. | *Calotropis procera* | Madhaar | Asclepiadaceae |
| 21. | *Basella alba* | Pui | Basellaceae |

### 8.2.3 Physico-chemical analysis of distillery sludge

The physico-chemical parameters of distillery sludge sample i.e. pH, electrical conductivity (EC), chloride ($Cl^-$), sodium ($Na^+$), and nitrate were estimated according to the method described by Kalra and Maynard (1991). The phenol contents in sludge were analyzed as per standard methods described in APHA (2012). The pH and EC values (sludge:water =1:2.5w/v) of sludge samples were measured by using Orion meter (Model-960, Thermo Scientific, FL, USA) and Orion conductivity meter, respectively (Chandra et al. 2008). The total content of Fe, Zn, Cu, Mn, Ni, and Pb in dry weight sample of sludge was measured by atomic absorption spectrophotometer (ZEEnit 700, Analytic Jena, Germany) after acid digestion using method no. 3030H of the standard methods for examination of water and wastewater as described in chapter no. 04 (APHA 2012).

### 8.2.4 Detection of EDCs and other organic pollutants from distillery sludge

#### 8.2.4.1 Extraction of organic compounds from distillery sludge by solid-liquid extraction method

The EDCs and other organic compounds were extracted from distillery sludge by using two different solvent to detect broad range of organic compounds at pH <2.0 as described in chapter no. 04 ((Bharagava and Chandra 2009). To extract the organic compounds, the fresh distillery sludge sample (5.0 g) was weigh and put into an Erlenmeyer flask (250

mL), added 5 mL of *n*-hexane and ethyl acetate in each flask separately and mix vigorously (Fig. 8.3). The sample were processed via vortex agitation (for 2 min), sonication (2 min on and 30 sec off, repeated three times) and centrifuged for 15 min at 10,000 ×g. The extraction was repeated successively three times to complete extraction of organic compounds from distillery sludge. The organic layer was obtained from sludge, dehydrated over $Na_2SO_4$ and dried under a stream of nitrogen gas. The dry residue obtained was dissolved in 1.0 mL ethyl acetate and filtered through 0.22 µm syringe filters (Millipore Ltd., Bedford, Massachusetts, USA) and used for further GC-MS analysis.


**Fig. 8.3** Extraction of organic pollutants from distillery sludge by using liquid-liquid extraction procedure (a) collected fresh distillery sludge (b) extraction of organic compounds from distillery sludge by liquid-liquid extraction procedure

### 8.2.4.2 GC-MS analysis

In GC-MS analysis, the extracted samples were derivatised with trimethylsilyl (TMS) as described in chapter no. 03 (Minuti et al. 2006). After extraction, the extracted samples were analysed by Thermo Scientific Trace GC Ultra Gas Chromatograph equipped with a TriPlus auto sampler coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific, FL, USA). The operation conditions were same as described in chapter no. 04.

### 8.2.5 Digestion of plant samples for heavy metals estimation

In order to estimate the metal content of native plants, the uprooted plants were washed thoroughly with deionized water to remove sludge particles from the roots, followed by rinsing with a 10 mmol $L^{-1}$ solution of calcium chloride. Subsequently, the plants root, shoot and leaves were separated and chopped into small pieces and the resulting biomass was oven-dried at 70 $^0$C for 5 days till constant weight. The dried plant parts were ashed in a muffle furnace at 460 °C for 6 h. Further, the weighed ash from these samples was digested in 2% $HNO_3$ and filtered through a 0.45 µm glass fibre filter (AOAC 2002). One gram (1.0 g) of dried and sieved sediments was digested with 10 mL of $HNO_3$. If brown

fumes appeared, 5 mL of $HNO_3$ was added and digestion continued till no generation of brown fumes. Subsequently, the sample was cooled, 2 mL water and 3 mL of water was added and digestion continued followed by USEPA method 3050-B (EPA 1996). The concentrations of Cr, Zn, Mn, Ni, Cu, Fe, Cd, and Pb were determined by AAS.


**Fig. 8.4** Plant sample for heavy metal estimation (a) dried plant sample after grinding (b) prepared plant sample after digestion with nitric:perchloric acid

### 8.2.6 Metal accumulation efficiency

To evaluate the metal accumulation efficiency in plants, we have calculated the bioaccumulation coefficient factor (BCF) and translocation factor (TF). BCF is defined as the ratio of metal concentration in the root to soil, and TF is the ratio of metal concentration in shoot to the root. BCF and TF were calculated as below mentioned formula which has also been reported earlier by Yoon et al. (2006).

$$BCF = \frac{\text{Concentration of metal in plant root (mg kg}^{-1})}{\text{Concentration of metal in distillery sludge (mg kg}^{-1})}$$

$$TF = \frac{\text{Concentration of metal in plant shoot (mg kg}^{-1})}{\text{Concentration of metal in plant root (mg kg}^{-1})}$$

Both BCF and TF have to be considered for evaluating whether a plant is a metal hyperaccumulator. A hyperaccumulator plant should have BCF >1 or TF >1, as well as total accumulation >1000 mg $kg^{-1}$ of Cu, Co, Cr, Ni or Pb, or >10,000 mg $kg^{-1}$ of Mn or Zn (Baker and Brooks 1989; Kabata-Pendias, 2011).

### 8.2.7 Cellular observation of plant root tissues by Transmission electron microscopy

Root segments (~2.0 mm in length) of selected plants were quickly immersed in $H_2S$ saturated water as pre-treatment for 30 min at room temperature to precipitate trace elements (Khan et al. 1984). Further, the root sample was washed with 0.1M SCB (sodium cacodylate buffer, pH 7.2) and fixed in 2.5 % glutaraldehyde (v/v) prepared in sodium cacodylate (Ladd Research Industries, Williston, USA) buffer (pH 7.2) for 2h at 4 °C. Root tissue was washed three times with 0.1M SCB with 10 min interval between each washing and post fixed in 1% $OsO_4$ for overnight. Fixed tissue were washed with SCB,

dehydrated in graded acetone series (50, 60, 70, 80, 90, 95, and 100%) and emended in araldite-DDSA mixture (Ladd Research Industries, Williston, USA). After backing at 60 °C, blocks were cut (60-80nm thick) by an ultramicrotome (Cryo Leice EM UC7, Leica Microsystem, India) and sections were stained by uranyl acetate and lead citrate. Analysis of section was done under TEM (FEI Tecnai$^{TM}$ G$^2$ Spirit Twin, Hillsboro, USA) at accelerating voltage of 80 KV.

### 8.2.8 Statistical data analysis

*In*-situ phytoremediation compared with the original distillery sludge (Pre-treatment) was calculated using Student's *t*-test using the SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL, USA). Significant and non-significant data has been shown in Table 8.2. Further, the reduction/change in pre and post treatment of distillery sludge due to phytoremediation after six months also listed in the Table 8.2. The mean metal concentrations of plant material were reported in mg kg$^{-1}$ based on dry weight.

## 8.3 Results and discussion

### 8.3.1 Changes in physico-chemical parameters of distillery sludge

The physico-chemical properties of distillery sludge are shown in Table 8.2. The value of pH and EC, $Na^+$, $Cl^-$, $NO_3^-$, phosphate, sulfate and phenol were higher in the sludge. In addition, the sludge showed high content of various heavy metals Fe, Zn, Cu, Ni, Mn, Pb and Cd which are beyond the permissible limit (Table 8.2) and toxic to human and animal health due to their long-term persistence and accumulation from contaminated sites of environment (Barakat 2011). The alkalinity (pH 8.2) of the distillery sludge arises from the combined residual effect of bicarbonates, carbonates, and hydroxides, which are used in a distillery for pH adjustment during the fermentation process (Tiwari et al. 2013). The high values of EC indicated the role of various cations and anions, such as $Na^+$, $Cl^-$, $NO_3^-$, which are present in distillery effluent. The high content of heavy metals in distillery sludge could be due to the corrosive effect of sugarcane juice during the sugar manufacturing process in sugar industries and further concentrations during the fermentation and distillation process of sugarcane-molasses in distilleries which is finally discharged as spent wash. This might be the main source of the heavy metal content in distillery sludge. Several authors have also reported higher values of pH, EC, salts and heavy metals in biomethanated distillery sludge (Chandra et al. 2008a). But, the sludge obtained after 90 days of *in*-situ phytoremediation had lower values of various physio-chemical parameters as shown in Table 8.2. The result revealed that initial pH of distillery sludge was found to be alkaline in nature (pH 8.1) which gradually changed into acidic (pH 6.76) might be due to rhizospheric interaction of microbial population with organo-metallic complex, where several root exudates are released in form of organic acids and amino acids as metal chelators. This decreases the pH of sludge and facilitates the microbial growth. This resulted enhanced the phytoextraction process due to bioavailability of metals and organic nutrient from sludge at acidic pH. This study

collaborates with previous observations (Sessitsch et al. 2013; Rajkumar et al. 2012). Consequently, the plant accumulated significant amount of heavy metals from distillery sludge. EC of post-treated distillery sludge was also increased this indicated the function of cations/anions present in sludge.

**Table 8.2** Physico-chemical characteristics of distillery sludge before and after phytoremediation

| S. No. | Parameter | Fresh distillery sludge | Distillery sludge after *in*-situ phytoremediation | % Change | Permissible limit (USEPA 2002) |
|---|---|---|---|---|---|
| 1. | pH | 8.1±0.00 | 6.76±0.23* | 16.54 | -- |
| 2. | EC | 4.12±0.01 | 5.44±0.46*** | -24.26 | -- |
| 3. | Sodium | 42.13±1.00 | 28.36±0.53* | 32.68 | 200 |
| 4. | Chloride | 1272.74±5.13 | 646.22±24.69* | 49.22 | 1500 |
| 5. | Nitrate | 85.89±0.76 | 43.69±1.16* | 49.13 | 10 |
| 6. | Phosphate | 2268.83±1.70 | 1589.63±6.55* | 29.93 | -- |
| 7. | Sulfate | 145.07±0.68 | 121.78±0.96* | 16.05 | -- |
| 8. | Phenol | 501.34±1.22 | 421.76±0.98* | 15.87 | -- |
| 9. | Heavy metals | | | | |
| a | Fe | 5264.49±59.64 | 2372.45±21.58* | 54.93 | 2.0 |
| b | Zn | 43.47±1.31 | 28.21±0.40* | 35.10 | 2.0 |
| c | Cu | 847.46±1.00 | 141.45±1.00* | 83.30 | 0.5 |
| e | Mn | 238.47±0.83 | 63.17±0.26* | 73.51 | 0.20 |
| f | Ni | 15.60±0.54 | 4.02±0.01* | 74.23 | 0.1 |
| g | Pb | 31.22±1.14 | 12.22±0.12* | 60.85 | 0.05 |
| h | Cd | 1.466±0.011 | 1.29±0.018* | 10.78 | 0.01 |

All values are mean three replicate (n=3)±standard deviation and presented in mg $kg^{-1}$ except pH and electrical conductivity (μS $cm^{-1}$). Student's *t*-test (two tailed as compared to pre-treated sludge), where *Highly significant at $p < 0.001$, *** Less significant at $p<0.05$

### 8.3.2 Characterization of EDCs and other organic pollutants

#### 8.3.2.1 Ethyl acetate extract of distillery sludge

The GC-MS analysis of ethyl acetate extracted samples from distillery sludge revealed several major peaks at RT 13.72, 28.08, 30.90, 34.21, 34.52, 36.14, 37.11, 40.96, 41.57, 42.57, 43.28 as shown in Fig. 8.5. These compounds were characterized as ethanedioic acid, bis(TMS)ester; benzoic acid, 3,4,5 tris(TMS oxy)-TMS ester; 11-trans-octadecenoic acid, TMS ester; hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; dotriacontane; 5α-cholestane,4-methylene; 24-ethyl-δ-(22)-coprostenol, TMS; campesterol TMS; and β-sitosterol, respectively. Other minor peaks were also observed at RT values of 7.42, 7.54, 8.62, 12.85, 15.45, 18.48, 19.31, 21.88, 24.74, 31.87, 34.84, 35.83, 38.68, 40.12, 40.19, 40.71, 41.98, 44.73, and 45.37which corresponded to silane, (4-ethylphenyl)trimethyl; benzene, 1-ethyl-2-methyl; 2,3-D2-methylsuccinic acid 2TMS; β-eudesmol, TMS ether; benzoic acid, 2-methyl-,

trimethylsilyl ester; phenol, 2,4-bis(1,1-dimethylethyl); phenol, 2, 6-bis(1,1-dimethylethyl); tentradecanoic acid, TMS ester; 9,12-octadecadienoic acid (Z,Z)- TMS ester; cis-10-nonadecenoic acid, TMS ester; docosanoic acid, TMS ester; 2-monostearin TMS ether; hexacosanoic acid; silane,[[(3β,22E)-ergosta-7,22-dien-3-yl]trimethyl; octacosanol; stigmasterol TMS ether; ergosten-3β-ol; lanosterol; 9,19-cyclocholestan-3-one, 4,14-dimethyl (Table 8.3).

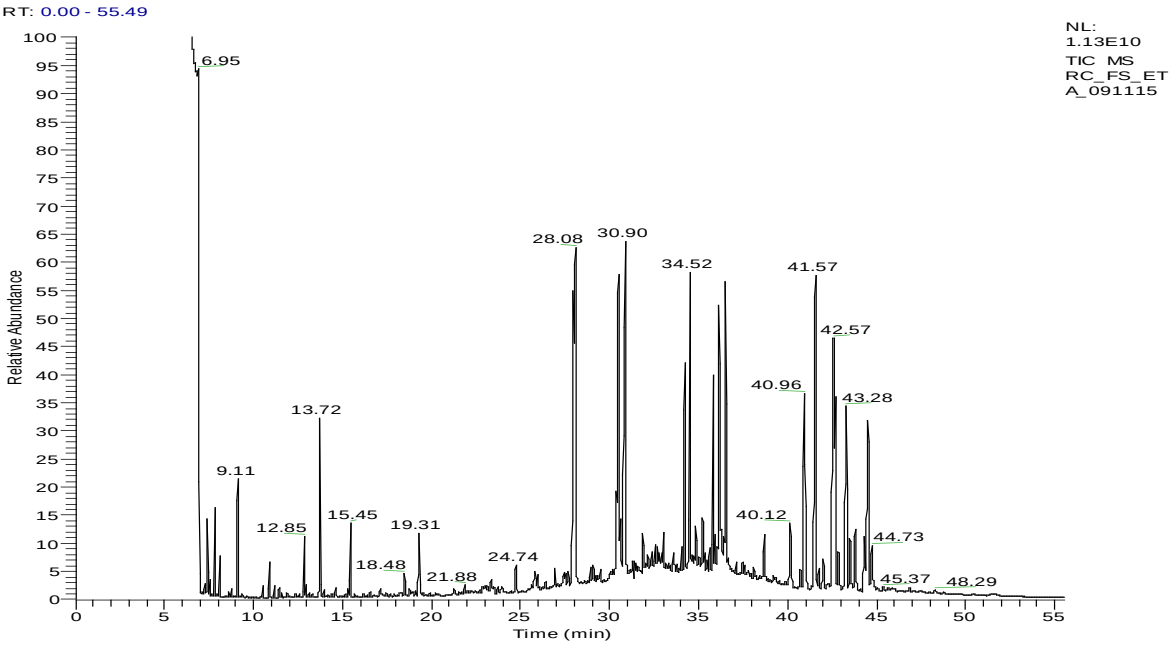



**Fig. 8.5** GC-MS Chromatogram of organic compounds extracted from distillery sludge with ethyl acetate

**Table 8.3** Identified EDCs and other organic pollutants by GC-MS present in ethyl acetate extract of distillery sludge

| S. No. | RT | Identified compounds |
|---|---|---|
| 1. | 7.42 | Silane, (4-ethylphenyl)trimethyl |
| 2. | 7.54 | Benzene, 1-ethyl-2-methyl |
| 3. | 8.62 | 2,3-D-2-methylsuccinic acid 2TMS |
| 4. | 13.72 | Ethanedioic acid, bis(TMS)ester |
| 5. | 12.85 | β-Eudesmol, TMS ether |
| 6. | 15.45 | Benzoic acid, 2-methyl-, trimethylsilyl ester |
| 7. | 18.48 | Phenol, 2,4-bis(1,1-dimethylethyl) |
| 8. | 19.31 | Phenol, 2, 6-bis(1,1-dimethylethyl) |
| 9. | 21.88 | Tetradecanoic acid, TMS ester |
| 11. | 24.74 | 9,12-Octadecadienoic acid (Z,Z)- TMS ester |
| 10. | 28.08 | Benzoic acid, 3,4,5 tris(TMS oxy)-TMS ester |
| 12 | 30.90 | 11-trans-Octadecenoic acid, TMS ester |
| 13. | 31.87 | cis-10-Nonadecenoic acid, TMS ester |
| 14. | 34.21 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester |
| 15. | 34.52 | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 16. | 34.84 | Docosanoic acid, TMS ester |
| 17. | 35.83 | 2-Monostearin TMS ether |

| | | |
|---|---|---|
| 18. | 36.14 | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 19. | 37.11 | Dotriacontane |
| 20. | 38.68 | Hexacosanoic acid |
| 21. | 40.12 | Silane,[[(3β,22E)-ergosta-7,22-dien-3-yl]trimethyl |
| 22. | 40.19 | Octacosanol |
| 23. | 40.71 | Stigmasterol TMS ether |
| 24. | 40.96 | 5α-Cholestane,4-methylene |
| 25. | 41.57 | 24-Ethyl-δ-(22)-coprostenol, TMS |
| 26. | 41.98 | Ergosten-3β-ol |
| 27. | 42.57 | Campesterol TMS |
| 28. | 43.28 | β-Sitosterol |
| 29. | 44.73 | Lanosterol |
| 30. | 45.37 | 9,19-Cyclocholestan-3-one, 4,14-dimethyl |

TMS: Trimethylsilyl; RT: Retention Time (Min)

The primary sources of phenolic compounds in sludge are sugarcane extracted during production which remains as complex in cane molasses. In addition, some other forms of phenolic compounds are also derived from the aromatic amino and or fragmentary unit of melanoidins present in the original molasses or from the rest of the yeast cells that remain in the distillery. The presence of phenolic compounds in distillery sludge would impose the toxic effect to the soil microbial communities and plants (Zhou and Wu 2012; Wu et al. 2015). Similarly, the benzoic acid would impose the harmful effect on the plant root and sludge microbial communities which will inhibits the metal accumulation and biotransformation of organo-metallic complex. Besides, the octadecanoic acid and hexadecanoic acid have detected in sludge also. They are plant origin fatty acids which have been reported for its toxicity to aquatic ecosystem and genetic material also (de Sousa Andrade et al. 2005; Kamaya et al. 2003). These compounds are also reported in the humic substances, which support its origin from the plant sources (Roselli et al. 2003). Moreover, benzene, phenol, hexadecanoic acid, octadecenoic acid, benzoic acid, nonadecenoic acid have been listed as potential EDCs by the USEPA (2012), which confirm for presence of EDCs rich organo-metallic compounds in distillery sludge. Besides, the study also showed that these organic compounds constituted the main components of wastewater and discharge in sludge from yeast or original sugarcane molasses, which remains after the secondary treatment of spent wash. Apart from this, other compounds were also identified as stigmasterol, l, 5α-cholestane, 24-ethyl-δ-(22)-coprostenol, campesterol and β-sitosterol in distillery sludge. They are the major plant sterols (phytosterols) and stanols (phytostanols) and very similar in structure to cholesterol. These compounds are also listed under EDCs as per USEPA (2012). But, smooth growth of native plants indicates the capabilities of microbial communities which might be detoxifying the complex compounds which are subsequently accumulated by weeds and grasses from its habitat. In a previous study, it has been reported that in nature, microorganisms may transform β-sitosterol and other sterol derivatives into androgenic hormones such as 5-β-androstan 3, 17-dione and androstan 4-en-3, 17-dion (Taylor et al. 1981). This also supported to create the EDCs rich environment in distillery sludge. The

effect of androstan is to interfere with endocrine system of fish to produce hermaphroditism or other morphological defect and reducing fish number (Jenkins et al. 2003). Moreover, silane,[[(3β,22E)-ergosta-7,22-dien-3-yl]trimethyl and ergosten-3β-ol are also detected in sludge sample. These are the major fungal and yeast sterol present in plasma membrane and might be generated in sludge from yeast or original sugarcane molasses. The majority of these detected compounds indicated that the waste poses an environmental risk to aquatic flora and fauna at distillery waste disposal sites. These compounds are either generated during the fermentation of sugarcane molasses or the biological treatment of spent wash during methanogenesis.

### 8.3.2.2 *n*-Hexane extract of distillery sludge

GC-MS analysis of *n*-hexane extracted sludge samples revealed several peaks at different RT (Fig. 7.5), with the major peaks noted at RT: 7.34, 9.07, 13.71, 34.50, 36.11, which corresponded to acetic acid, [(TMS)oxy],-TMS ester; benzene, 1-ethyl-4-methyl; 2-Butenedioic acid, bis (TMS)ester; hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester; and octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester , respectively (table 3). Several minor peaks were also noted at RT: 14.64, 16.40, 16.85, 19.11, 21.88, 27.97, 29.34, 30.82, 31.34, 32.56, 33.61, 35.80, 39.17 and 44.37 as shown in Fig. 8.6. This corresponded to docosane; n-pentadioic acid, bis(TMS) ester; decanedioc acid; ethanol, 2(octadecyoxy)-; benzoic acid, 3,4,5-tris(TMS oxy), TMS ester; hexadecanoic acid, TMS ester; quercetin 7,3',4'-trimethoxy; octadecanoic acid, TMS ester; octadecanoic acid, TMS ester; 1H-purin-6-amine,[(2-fluorophenyl)methyl]; hexanedioic acid, dioctyl ester; tentradecanoic acid, TMS ester; 2-Monostearin TMS ether; 9,12-octadecadienoic acid (Z,Z)-2,3-bis[(TMS)oxy] propyl ester; glycocholic acid methyl ester TMS (Table 8.4). These complex pollutants in distillery sludge are generated during the process of the distillation of fermented molasses slurry and the subsequent methanogenesis of the spent wash.

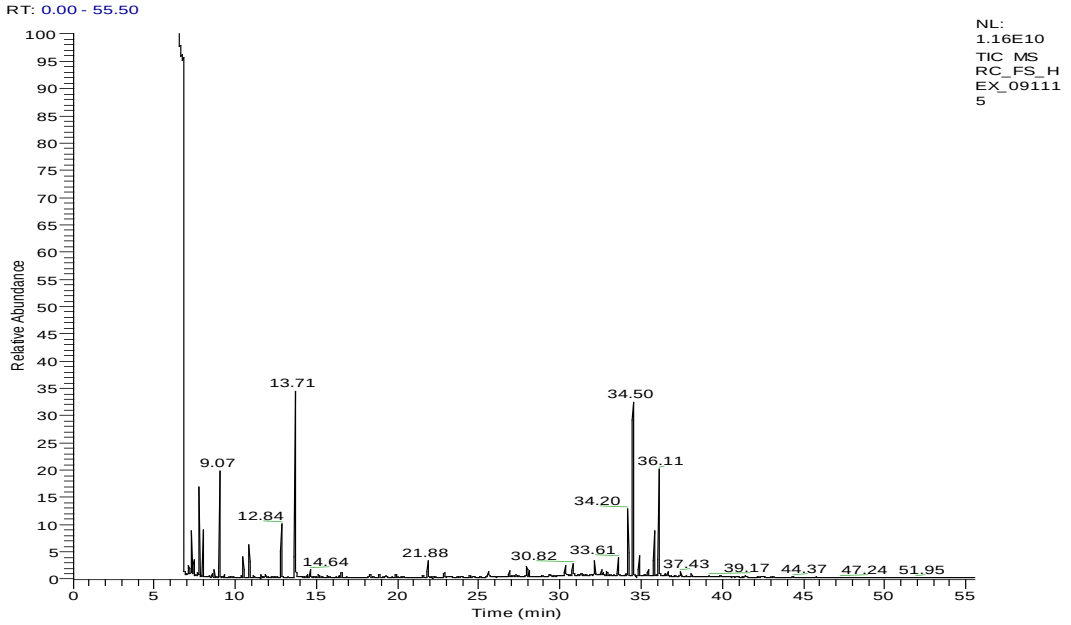



**Fig. 8.6** GC-MS chromatogram of organic compounds extracted from distillery sludge with *n*-hexane

**Table 8.4** Identified EDCs and other organic pollutants by GC-MS present in *n*-hexane extract of distillery sludge

| S. No. | RT | Identified compounds |
|---|---|---|
| 1. | 7.34 | Acetic acid, [(TMS)oxy],-TMS ester |
| 2. | 9.07 | Benzene, 1-ethyl-4-methyl |
| 3. | 13.71 | 2-Butenedioic acid, bis (TMS)ester |
| 4. | 14.64 | Docosane |
| 5. | 16.40 | n-Pentadioic acid, bis(TMS) ester |
| 6. | 16.85 | Decanedioc acid |
| 7. | 19.11 | Ethanol, 2(octadecyoxy)- |
| 8. | 21.88 | Benzoic acid, 3,4,5-tris(TMS oxy), TMS ester |
| 9. | 27.97 | Hexadecanoic acid, TMS ester |
| 10. | 29.34 | Quercetin 7,3',4'-trimethoxy |
| 11. | 30.82 | Octadecanoic acid, TMS ester |
| 12. | 31.34 | 1H-Purin-6-amine,[(2-fluorophenyl)methyl] |
| 13. | 32.56 | Hexanedioic acid, dioctyl ester |
| 14. | 33.61 | Tentradecanoic acid, TMS ester |
| 15. | 34.50 | Hexadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 16. | 35.80 | 2-Monostearin TMS ether |
| 17. | 36.11 | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester |
| 18. | 39.17 | 9,12-Octadecadienoic acid (Z,Z)-2,3-bis[(TMS)oxy] propyl ester |
| 19. | 44.37 | Glycocholic acid methyl ester TMS |

TMS: Trimethylsilyl; RT: Retention Time (Min)

### 8.3.3 Heavy metal accumulation in different part of plants

The concentration of Fe, Zn, Cu, Mn, Ni, Pb and Cd accumulation in the plants tissues collected from distillery sludge contaminated site showed variable pattern of metal accumulation and distribution in their various parts as shown in Table 8.5 and Fig. 8.7. The data had shown the metal content in the plant tissues variable among species at the polluted sites indicating their different capacities for metal uptake. In our study, total Fe concentration in plant samples ranged from 14.955 mg $kg^{-1}$ (*R. communis*) to 1196.626 mg $kg^{-1}$ (*S. munja*), with the maximum level in root of *S. munja* (1004.538 mg $kg^{-1}$), shoot of *R. dentatus* (855.024 mg $kg^{-1}$) and leaves of *T. cordifolia* (214.48 mg $kg^{-1}$) (Table 8.5). Similar pattern of Fe accumulation in root of plants have been reported by other researchers also from polluted site containing the complex organo-metallic compounds (Mazumdar and Das 2015; Gupta and Sinha 2007). Since, Fe is an essential micronutrient that is present in distillery sludge in high amount, it act an important role in influencing the plant's capacity to withstand metal stress and positively affect its phytoextraction potential (Qureshi et al. 2010). From a toxicological perspective, excess Fe often precipitates on the roots which causes phytotoxicity as this might interfere with other plant processes (Wheeler et al. 1993). In our study, high Fe concentration was determined in all plant samples as shown in Table 8.5.

The total Zn concentration in plant was found in variable ranged from non-detectable (*C. procera*) to 263.223 mg $kg^{-1}$ (*R. dentatus*) and maximum concentration was found in root (64.500 mg $kg^{-1}$), shoot (137.463 mg $kg^{-1}$) and leaves (86.096 mg $kg^{-1}$) of *B. lacera*, *R. dentatus*, and *C. album*, respectively (Fig. 8.7). The zinc could not detected heavy metals in their different part of *C. procera*. Since, Zn is an essential micronutrient to plants and means concentration in normal plants (above ground tissues) is 66 mg $kg^{-1}$ (Outridge and Noller, 1991) but at higher concentration of Zn would be toxic to plant. Mazumdar and Das (2015) reported a range of 13.3 to 393.84 mg $kg^{-1}$ Zn concentration accumulated in root of 25 wetland plant species grown on paper mill contaminated site.

Cu concentration in the plants found as between 2.807 mg $kg^{-1}$ (*R. communis*) to 696.366 mg $kg^{-1}$ (*R. dentatus*). The maximum concentration of Cu was observed in root (253.95 mg $kg^{-1}$) and shoot (506.829 mg $kg^{-1}$) of *C. dactylon* and *R. dentatus*, respectively which reflects its high tolerance towards Cu, while the leaves of *A. mexicana* accumulated significant concentration (38.785 mg $kg^{-1}$) of Cu (Table 8.5 and Fig. 8.7). Like Fe, Cu is also essential micronutrient to plant growth, but cause toxic effects when root and shoot accumulate Cu levels exceeding 20 mg $kg^{-1}$ (Borkert et al. 1998). But, our results showed that nine plant species collected from the distillery sludge dumping site contained high concentration of Cu than this threshold. This indicated the hyperaccumulation characteristics of plants. The similar observations have been reported in root and shoot of native plants growing on urban waste contaminated site from Florida (Yoon et al. 2006).

The total Mn content in plants varied between 4.171 mg $kg^{-1}$ *R. communis* and 178.406 mg $kg^{-1}$ in *R. dentatus*. Among plant species with higher Mn concentration, *R. dentatus* showed highest accumulation of Mn (113.56 mg $kg^{-1}$) in their shoot followed by root 45.585 mg $kg^{-1}$, and 59.769 mg $kg^{-1}$ concentration in leaves of *C. album* as shown in Table 8.5 and Fig. 8.7. Mn is also an essential micronutrient for plants and used in photosynthesis and many redox enzymatic processes (Memon et al. 2001; Carranza-Alvarez et al. 2008). In many cases, it was predicted that Mn might improve heavy metal toxicity, reducing root growth inhibition effect or alteration of mitotic activity (Gabbrielli and Pandolfini 1984; Roberston 1985). Our result indicated that plants usually contained higher concentration of metals in order of shoot > root > leaves. Similar observations have also been reported in previous studies (Chandra and Yadav 2011a,b)

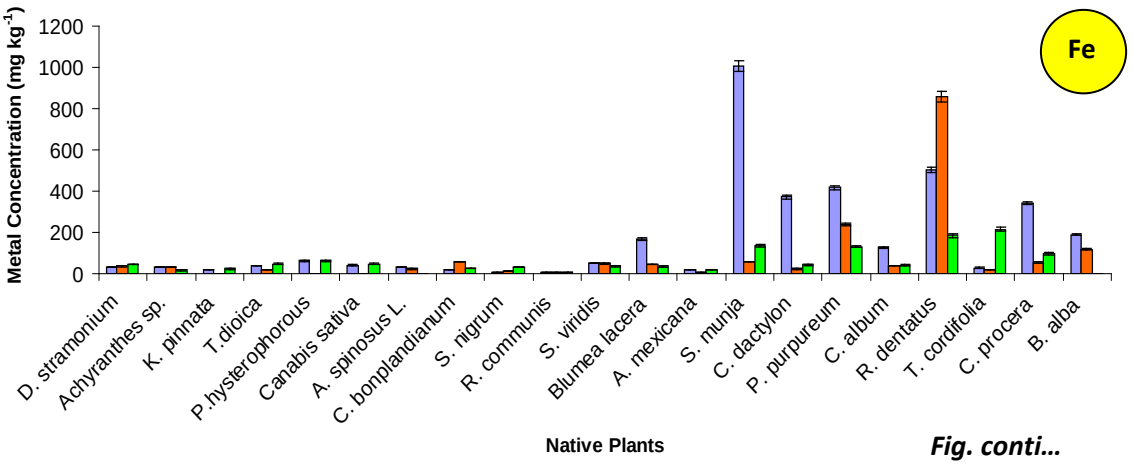



***Fig. conti…***

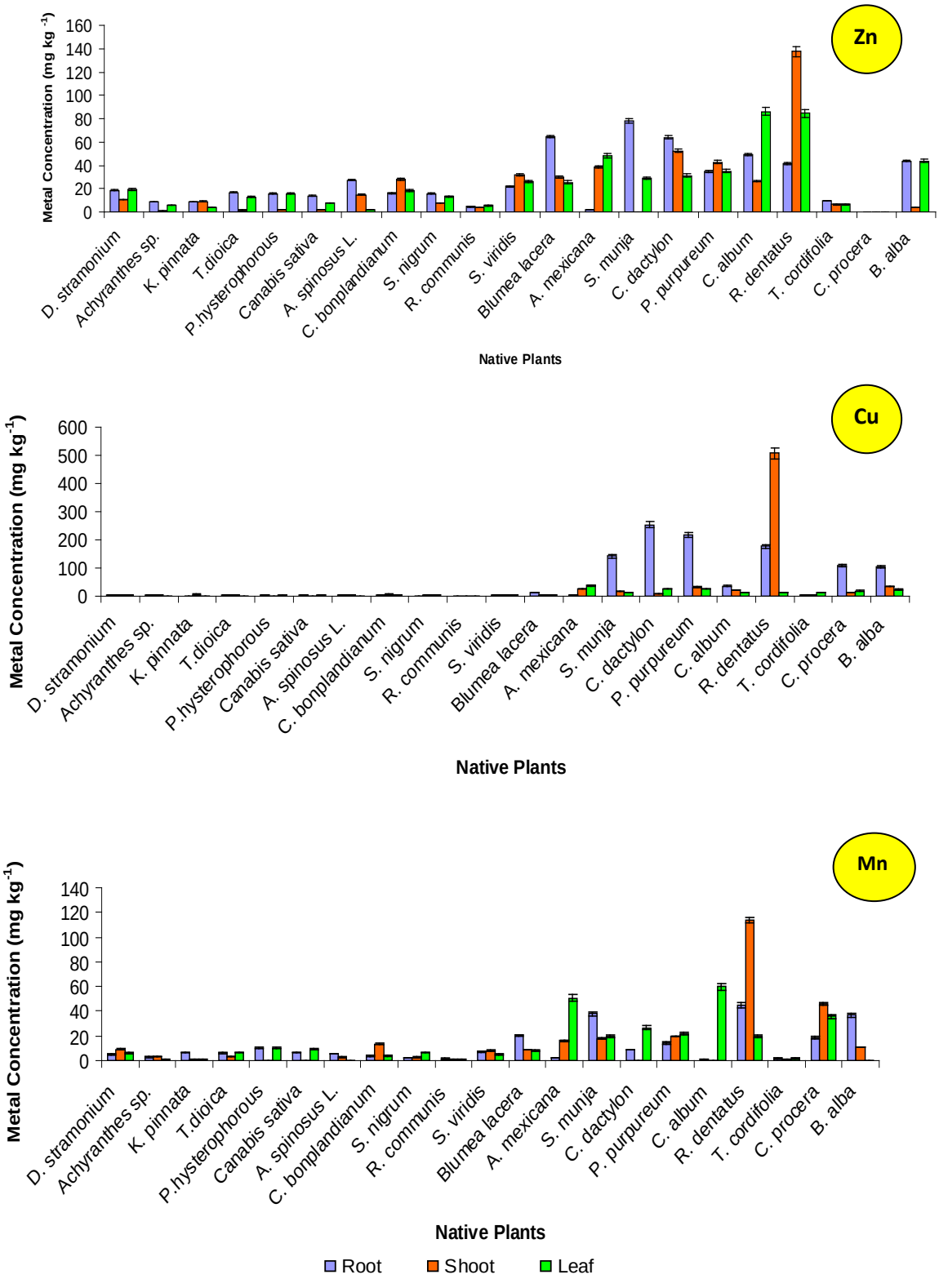



**Fig**. **8.7** Accumulation of heavy metals in different parts of native plants collected from distillery sludge dumping site

In the present study, different plants have exhibited varying degree of Ni accumulation and it has ranged between non-detactable (*C. dactylon*, *C. procera*) to 8.399 mg $kg^{-1}$ (*R. dentatus*) as shown in Table 8.5. Metal accumulation pattern from distillery sludge showed that Ni accumulation was maximum in root of *R. dentatus* (6.997 mg $kg^{-1}$), shoot of (3.51 mg $kg^{-1}$), and leaves of *T. cordifolia* (2.124 mg $kg^{-1}$) (Table 8.6 and Fig. 8.8). Ni is reported to improve the growth and development, being an important component of some enzymes but at higher concentration, it causes phytotoxicity by inhibiting seed germination, plant growth and productivity (Rao and Sresty 2000; Sengar et al. 2008). Normal Ni concentration in plants ranges from 0.5 to 5.0 mg $kg^{-1}$; the values 5.0 mg $kg^{-1}$ are toxic to plant (Allen 1989). In our study, most of the plant accumulated the Ni in their different parts beyond the permissible limit except *C. dactylon* and *C. procera* as shown in Tables 8.5. Result conducted by Kumar et al. (2013a) showed that Ni accumulation was higher in ranged between 2.52 mg $kg^{-1}$ to 14.19 mg $kg^{-1}$ and 1.81 mg $kg^{-1}$ to 15.39 mg $kg^{-1}$ in root and shoot of weed plants grown on industrial waste contaminated soil.

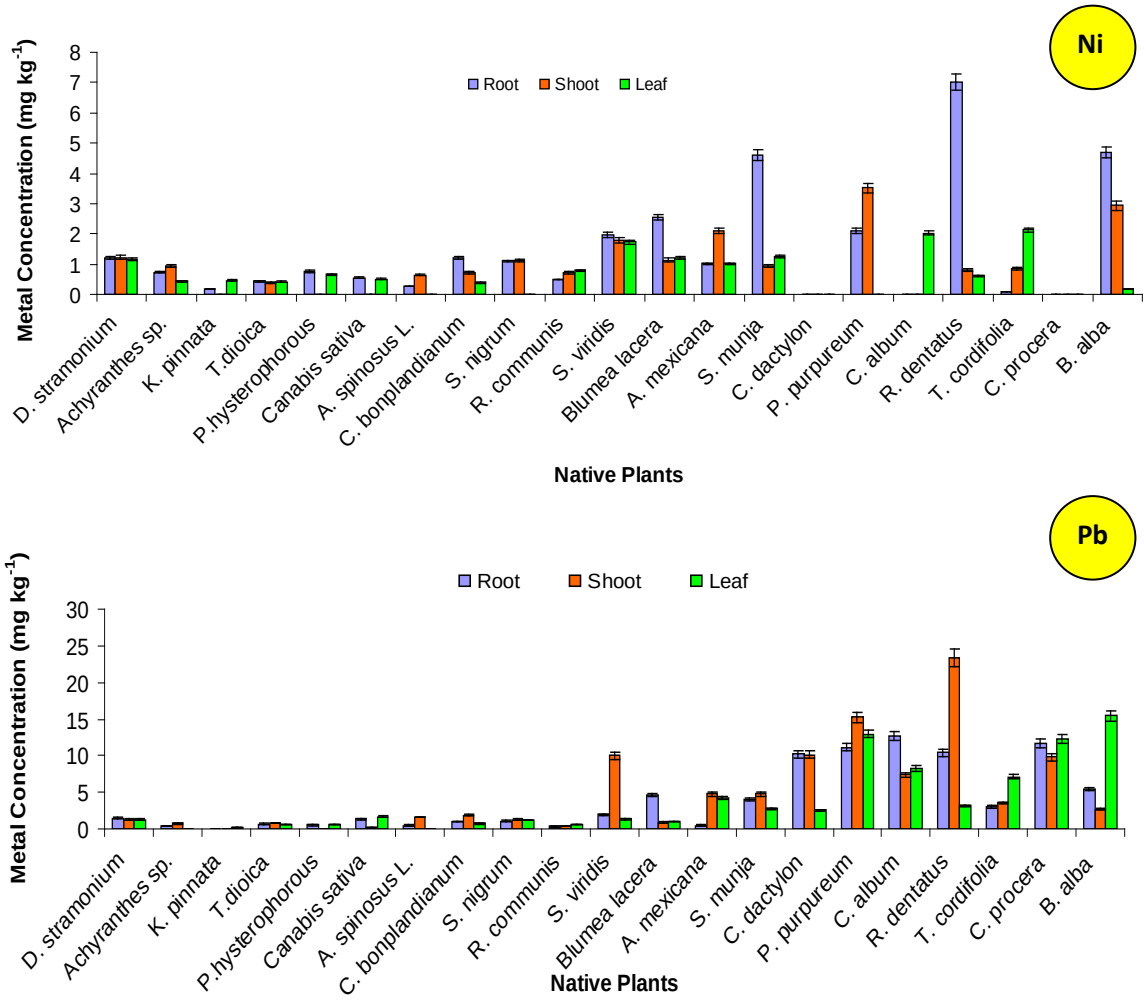



**Fig. 8.8** Accumulation of heavy metals in different parts of native plants collected from distillery sludge dumping site

The total Pb concentration in the plant ranged from 9429 mg $kg^{-1}$ (*A. mexicana*) to 36.996 mg $kg^{-1}$ (*R. dentatus*) as shown in Table 8.5 and Fig. 8.8. The highest Pb accumulation was observed in shoot of *R. dentatus* (23.414 mg $kg^{-1}$), root of *C. album* (12.748 mg $kg^{-1}$) and leaves of *P. purpureum* (12.978 mg $kg^{-1}$). Pb is not an essential element for plant; some plant species proliferated in Pb-contaminated area and accumulated it in their different parts but they might be toxic in high concentration (i.e. 27 mg $kg^{-1}$) (Levy et al. 1999). Our results indicated that plants grown in Pb contaminated sludge are usually contained higher concentration than this threshold which supported the tolerance capability of plants. Kumar et al. (2013a) also found the Pb concentration in plant root and shoots ranged between 1.14 mg $kg^{-1}$ to 58.25 mg $kg^{-1}$ and 5.17 mg $kg^{-1}$ to 44.28 mg $kg^{-1}$, respectively.

The total Cd concentration in the plant ranged from non-detectable to 0.435 mg $kg^{-1}$ (*D. stamonium* and *A. spinosus*) (Table 8.5 and Fig. 8.9) The highest Cd accumulation was observed in root of *B. lacera* (0.315 mg $kg^{-1}$), shoot of *C. bonplandianum* (0.270 mg $kg^{-1}$) and leaves of *A. spinosus* (0.210 mg $kg^{-1}$). Cd is a toxic element for plant damage photosystems I and II, alter the synthesis of RNA, inhibit chlorophyll synthesis and transport of Zn, Fe and Cu, and few species can tolerate their high concentrations in soil (Galfati et al. 2011; Sanita di Toppi and Gabbrielli 1999). The permissible limit of Cd in plant recommended by WHO is 0.02 mg $kg^{-1}$ (Nazir et al. 2015). But, in our study, most of the plant accumulated the Cd beyond the permissible limit except *R. communis, A. mexicana, S. munja, C. dactylon, P. purpureum, C. album, R. dentatus, T. cordifolia, C. procera,* and *B. alba* as shown in Tables 8.5. The distillery sludge also showed the Cd concentration beyond the permissible limit. The higher than toxic level of metal concentrations in root of some native plant species indicated that in addition to internal metal detoxification, tolerance mechanism in the form of exclusion strategies might be operating exists (Baker 1981).

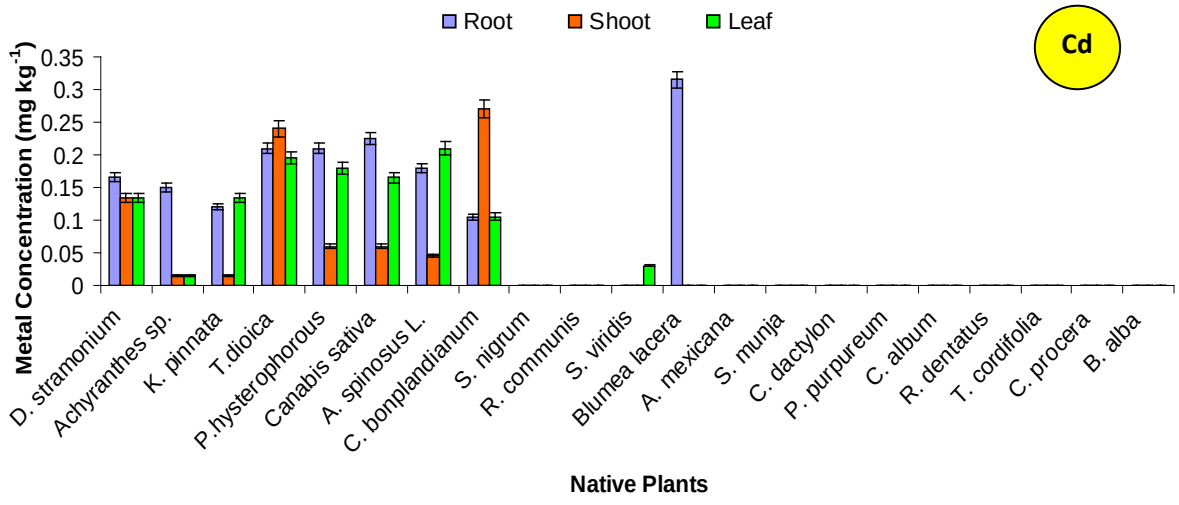



**Fig. 8.9** Accumulation of heavy metals in different parts of native plants collected from distillery sludge dumping site

Overall, the total metal accumulation was in the order Fe > Cu > Zn > Mn > Pb > Ni > Cd which varied from one part to another and highest metal accumulation was found in the order; root > leaves > shoot. The accumulation of heavy metals varied greatly among plant species and uptake of heavy metals by any other plant species depends on the metal availability, pH of substrate, and chemical nature of other organic co-pollutants (Laghlimi et al. 2015; Grisey et al. 2012). In addition, the other factors regulate the metal accumulation in plants pattern associated with environmental factors, plant genotype, type of plant root system, redox states of metals, and the response of plants to elements in relational to seasonal cycles (Rosselli et al. 2003; McGrath and Zhao 2003). pH play an important role in availability of metals to plant, because the alkaline pH of soil or sludge restricted metal mobility for accumulation in plants. The metal mobilisation and bioavailability was higher reported in acidic environment which favour the phytoextraction process of plant (Laghlimi et al. 2015). Our results indicated that distillery sludge generated after methanogenesis of spent wash initially alkaline in nature with mixture of several organo-metallic compounds along with melanoidins. This complex organic waste having strong binding tendency with various trace elements. However, due to specific plant-microbes interaction loosen the organo-metallic bond which make metal availability to plant (Sessitsch et al. 2013; Rajkumar et al. 2012). In the present study, the accumulation of tested metals in most of the plants was found higher in root tissues of the plants. This indicated the concentration dependent mobility of metals in plant tissues. It is also possible that metal accumulation in root iron oxyhydrate plaque layer on the root surface of wetland plants (Deng et al. 2004; Guo and Cutright, 2014). In comparison with the ranges of metal concentrations in the distillery sludge and in the underground tissues, the concentrations of Fe, Zn, Cu, Mn, Ni, Pb and Cd in shoots and leaves were maintained at low levels (Table 8.5). The results presented here suggest that this metal-tolerating strategy is widely evolved and exists in plant species when they grow in metal-contaminated areas. Similar indication has also been reported in previous studies regarding the heavy metals accumulation (Gupta and Sinha 2006; Nouri et al. 2009).

**Table 8.5** Heavy metal accumulation (mg $kg^{-1}$ DW) in root, shoot and leaves of various plant species

| Plant | Plant parts | Heavy metal | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Fe** | **Zn** | **Cu** | **Mn** | **Ni** | **Pb** | **Cd** |
| *D. stramonium* | Leaves | 44.160±0.020 | 19.050±0.019 | 3.735±0.018 | 6.090±0.007 | 1.170±0.015 | 1.275±0.089 | 0.135±0.009 |
| | Shoot | 36.300±0.126 | 10.500±0.035 | 2.670±0.004 | 9.150±0.012 | 1.215±0.016 | 1.305±0.014 | 0.135±0.009 |
| | Root | 31.320±0.003 | 18.600±0.002 | 3.855±0.003 | 5.010±0.01 | 1.200±0.01 | 1.500±0.003 | 0.165±1.32 |
| | Total | **111.78±0.149** | **48.15±0.056** | **10.26±0.025** | **20.25±0.029** | **3.585±0.41** | **4.08±0.106** | **0.435±1.338** |
| **Accumulation pattern** | | L>S>R | L>R>S | R>L>S | S>L>R | L>S>R | R>S>L | R>S>L |
| *Achyranthes* sp. | Leaves | 16.230±0.080 | 5.550±0.034 | 1.320±0.024 | 1.320±0.033 | 0.435±0.016 | BDL | 0.015±0.000 |
| | Shoot | 33.765±0.042 | 1.305±0.007 | 3.115±0.012 | 3.495±0.015 | 0.945±0.010 | 0.735±0.034 | 0.015±0.00 |
| | Root | 30.990±0.003 | 8.700±0.005 | 2.685±0.002 | 2.835±0.012 | 0.735±0.004 | 0.480±0.000 | 0.150±0.001 |
| | Total | **80.985±0.125** | **15.555±0.016** | **7.12±0.038** | **7.65±0.060** | **2.115±0.030** | **1.215±0.034** | **0.18±0.001** |
| **Accumulation pattern** | | S>R>L | R>L>S | S>R>L | S>R>L | S>R>L | S>R | R>S>L |
| *K. pinnata* | Leaves | 22.951±0.063 | 3.750±0.099 | 1.832±0.026 | 1.515±0.026 | 0.465±0.020 | 0.225±0.004 | 0.135±0.025 |
| | Shoot | 2.372±0.042 | 9.150±0.035 | 6.375±0.012 | 0.615±0.015 | BDL | BDL | 0.015±0.00 |
| | Root | 21.705±0.004 | 8.550±0.001 | 1.531±0.002 | 6.675±0.004 | 0.165±0.003 | BDL | 0.120±0.001 |
| | Total | **47.028±0.109** | **21.45±0.135** | **9.738±0.040** | **8.805±0.045** | **0.63±0.023** | **0.225±0.004** | **0.27±0.026** |
| **Accumulation pattern** | | L>R>S | S>R>L | S>L>R | R>L>S | L>R | -- | L>R>S |
| *T. dioica* | Leaves | 47.011±0.018 | 12.600±0.054 | 2.055±0.039 | 6.661±0.011 | 0.420±0.016 | 0.660±0.010 | 0.195±0.003 |
| | Shoot | 17.175±0.023 | 1.455±0.041 | 3.000±0.004 | 3.345±0.036 | 0.390±0.018 | 0.825±0.019 | 0.240±0.021 |
| | Root | 37.381±0.001 | 16.800±0.003 | 2.715±0.005 | 6.181±0.002 | 0.435±0.001 | 0.690±0.002 | 0.210±0.001 |
| | Total | **101.567±0.042** | **30.855±0.098** | **7.77±0.048** | **16.187±0.049** | **1.245±0.035** | **2.175±0.031** | **0.645±0.025** |
| **Accumulation pattern** | | L>R>S | R>L>S | S>R>L | L>R>S | R>L>S | S>R>L | S>R>L |
| *P. hysterophorous* | Leaves | 61.590±0.011 | 15.90±0.081 | 2.265±0.017 | 10.170±0.005 | 0.660±0.018 | 0.585±0.023 | 0.180±0.005 |
| | Shoot | BDL | 1.650±0.049 | BDL | BDL | BDL | BDL | 0.060±0.002 |
| | Root | 61.815±0.004 | 15.90±0.02 | 2.280±0.002 | 10.185±0.003 | 0.765±0.006 | 0.510±0.002 | 0.210±0.002 |
| | Total | **123.405±0.015** | **33.45±0.150** | **4.545±0.019** | **20.355±0.008** | **1.425±0.024** | **1.095±0.025** | **0.45±0.009** |
| **Accumulation pattern** | | R>L | R>L>S | R>L | R>L | R>L | L>R | R>L>S |
| *C. sativa* | Leaves | 48.250±0.007 | 7.800±0.001 | 2.700±0.008 | 9.135±0.033 | 0.495±0.046 | 1.710±0.008 | 0.165±0.012 |
| | Shoot | BDL | 1.650±0.010 | BDL | BDL | BDL | 0.270±0.020 | 0.060±0.002 |
| | Root | 41.385±0.01 | 13.800±0.005 | 2.595±0.006 | 6.660±0.004 | 0.555±0.001 | 1.365±0.006 | 0.225±0.005 |
| | **Total** | **89.635±0.017** | **23.25±0.016** | **5.295±0.012** | **15.795±0.037** | **1.05±0.047** | **3.345±0.034** | **0.45±0.019** |
| **Accumulation pattern** | | L>R | R>L>S | L>R | L>R | R>L | L>R>S | R>L>S |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *A. spinosus* L. | Leaves | BDL | 1.650±0.018 | BDL | BDL | BDL | 0.022±0.005 | 0.210±0.002 |
| | Shoot | 22.640±0.010 | 14.550±0.042 | 2.385±0.022 | 2.730±0.017 | 0.645±0.017 | 1.590±0.022 | 0.045±0.00 |
| | Root | 31.785±0.02 | 27.150±0.008 | 3.220±0.004 | 5.655±0.008 | 0.270±0.003 | 0.495±0.01 | 0.180±0.004 |
| | Total | **54.425±0.030** | **43.35±0.068** | **5.605±0.026** | **8.385±0.025** | **0.915±0.020** | **2.107±0.037** | **0.435±0.006** |
| **Accumulation pattern** | | R>S | R>S>L | R>S | R>S | S>R | S>R>L | L>R>S |
| *C. bonplandianum* | Leaves | 24.615±0.202 | 18.450±0.034 | 3.000±0.025 | 4.005±0.004 | 0.390±0.007 | 0.735±0.020 | 0.105±0.004 |
| | Shoot | 59.475±0.041 | 28.200±0.006 | 6.360±0.034 | 13.41±0.037 | 0.705±0.004 | 1.830±0.016 | 0.270±0.004 |
| | Root | 20.640±0.001 | 16.050±0.002 | 2.265±0.002 | 3.765±0.004 | 1.185±0.004 | 0.960±0.00 | 0.105±0.001 |
| | Total | **104.73±0.244** | **62.7±0.032** | **11.625±0.061** | **21.18±0.045** | **2.28±0.015** | **3.525±0.036** | **0.48±0.009** |
| **Accumulation pattern** | | S>L>R | S>L>R | S>R>L | S>L>R | R>S>L | S>R>L | S>R>L |
| *S. nigrum* | Leaves | 30.495±0.005 | 13.200±0.090 | 2.895±0.024 | 6.435±0.019 | BDL | 1.185±0.015 | 0.03±0.002 |
| | Shoot | 11.295±0.008 | 7.950±0.034 | 2.640±0.042 | 2.700±0.004 | 1.125±0.022 | 1.275±0.040 | BDL |
| | Root | 5.175±0.003 | 15.900±0.001 | 0.810±0.001 | 1.785±0.001 | 1.095±0.002 | 1.080±0.001 | BDL |
| | Total | **46.965±0.016** | **37.05±0.125** | **6.345±0.067** | **10.92±0.024** | **2.22±0.024** | **3.54±0.056** | **0.03±0.002** |
| **Accumulation pattern** | | L>S>R | R>L>S | L>S>R | L>S>R | S>R | S>L>R | - |
| *R. communis* | Leaves | 4.515±0.042 | 5.401±0.012 | 0.885±0.020 | 1.261±0.008 | 0.795±0.018 | 0.585±0.025 | BDL |
| | Shoot | 4.785±0.013 | 3.901±0.012 | 1.051±0.036 | 1.335±0.026 | 0.721±0.012 | 0.375±0.014 | BDL |
| | Root | 5.655±0.003 | 4.351±0.001 | 0.871±0.002 | 1.575±0.000 | 0.495±0.003 | 0.315±0.001 | BDL |
| | Total | **14.955±0.058** | **13.653±0.025** | **2.807±0.058** | **4.171±±0.034** | **2.011±0.033** | **1.275±0.040** | -- |
| **Accumulation pattern** | | R>S>L | L>R>S | S>L>R | R>S>L | L>S>R | L>S>R | - |
| *S. viridis* | Leaves | 36.060±0.008 | 25.650±0.189 | 4.040±0.024 | 5.150±0.012 | 1.730±0.010 | 1.320±0.015 | 0.030±0.003 |
| | Shoot | 49.740±0.025 | 31.500±0.017 | 4.930±0.049 | 8.160±0.018 | 1.790±0.037 | 9.990±0.017 | BDL |
| | Root | 52.860±0.017 | 21.750±0.023 | 4.070±0.007 | 7.290±0.017 | 1.960±0.019 | 1.935±0.026 | BDL |
| | Total | **138.66±0.050** | **78.9±0.229** | **13.04±0.080** | **20.6±0.047** | **5.48±0.066** | **4.245±0.058** | **0.030±0.003** |
| **Accumulation pattern** | | R>S>L | S>L>R | S>R>L | S>R>L | R>S>L | S>R>L | - |
| *B. lacera* | Leaves | 34.200±0.014 | 25.500±0.049 | 4.290±0.014 | 7.820±0.004 | 1.190±0.008 | 1.010±0.003 | BDL |
| | Shoot | 43.950±0.050 | 30.300±0.020 | 4.590±0.020 | 8.600±0.007 | 1.130±0.013 | 0.890±0.006 | BDL |
| | Root | 167.85±0.238 | 64.500±0.009 | 13.980±0.01 | 20.270±0.07 | 2.550±0.081 | 4.590±0.153 | 0.315±0.002 |
| | Total | **246±0.302** | **120.3±0.078** | **22.86±0.045** | **36.69±0.018** | **4.87±0.102** | **6.49±0.162** | **0.315±0.002** |
| **Accumulation pattern** | | R>S>L | R>S>L | R>S>L | R>S>L | R>L>S | R>L>S | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *A. mexicana* | Leaves | 77.78±1.581 | 48.364±0.152 | 38.785±0.623 | 50.7±0.1 | 1.425±0.00 | 4.158±0.00 | BDL |
| | Shoot | 6.418±0.130 | 38.783±0.015 | 25.202±0.1 | 16.148±0.001 | 2.094±0.001 | 4.763±0.045 | BDL |
| | Root | 18.34±0.078 | 1.768±0.046 | 2.423±0.005 | 2.469±0.018 | 1.007±0.001 | 0.503±0.00 | BDL |
| | **Total** | **102.538±1.789** | **88.915±0.213** | **63.988±0.728** | **69.317±0.119** | **4.526±0.002** | **9.429±0.045** | -- |
| **Accumulation pattern** | | L>R>S | L>S>R | L>S>R | L>S>R | S>L>R | S>L>R | - |
| *S. munja* | Leaves | 135.058±0.037 | 28.651±0.04 | 12.812±0.003 | 19.311±0.09 | 1.265±0.001 | 2.79±0.00 | BDL |
| | Shoot | 57.44±0.618 | BDL | 18.081±0.011 | 18.023±0.00 | 0.93±0.00 | 4.744±0.028 | BDL |
| | Root | 1004.128±3.140 | 78.151±0.01 | 142.184±0.985 | 37.983±0.136 | 4.621±0.349 | 3.949±0.005 | BDL |
| | **Total** | **1196.626±3.795** | **106.802±0.05** | **173.077±0.999** | **75.317±0.226** | **6.816±0.350** | **11.483±0.033** | -- |
| **Accumulation pattern** | | R>L>S | R>L | R>S>L | R>L>S | R>L>S | S>R>L | |
| *C. dactylon* | Leaves | 42.734±0.516 | 31.207±0.007 | 26.695±0.198 | 26.666±0.215 | BDL | 2.530±0.02 | BDL |
| | Shoot | 22.639±0.217 | 52.330±0.122 | 8.721±0.007 | BDL | BDL | 10.15±0.00 | BDL |
| | Root | 372.548±0.628 | 64.031±0.032 | 253.95±1.01 | 8.845±0.055 | BDL | 10.232±0.020 | BDL |
| | **Total** | **437.921±1.361** | **147.568±0.161** | **289.366±1.215** | **35.511±0.270** | **--** | **22.912±0.04** | -- |
| **Accumulation pattern** | | R>L>S | R>S>L | R>L>S | L>R | -- | R>S>L | -- |
| *P. purpureum* | Leaves | 131.908±1.731 | 35.065±0.044 | 25.197±0.045 | 22.189±0.030 | BDL | 12.978±0.025 | BDL |
| | Shoot | 239.042±1.997 | 42.872±0.101 | 31.808±0.00 | 19.787±0.005 | 3.51±0.060 | 15.212±0.086 | BDL |
| | Root | 418. 01±1.732 | 34.900±0.085 | 218.696±0.899 | 14.334±0.031 | 2.096±0.052 | 11.161±0.004 | BDL |
| **Total** | | **789.960±5.46** | **112.837±0.230** | **275.701±0.944** | **56.31±0.066** | **5.606±0.112** | **39.351±0.115** | -- |
| **Accumulation pattern** | | R>S>L | S>L>R | R>S>L | L>S>R | S>R | S>L>R | -- |
| *C. album* | Leaves | 41.764±1.053 | 86.096±0.095 | 13.949±0.187 | 59.769±0.168 | 2.032±0.00 | 8.267±0.072 | BDL |
| | Shoot | 39.099±1.00 | 26.466±0.472 | 22.18±0.02 | BDL | BDL | 7.368±0.035 | BDL |
| | Root | 126.783±2.016 | 48.888±0.00 | 36.955±1.005 | 1.286±0.002 | BDL | 12.748±0.075 | BDL |
| | **Total** | **207.646±4.069** | **161.45±0.567** | **73.084±1.212** | **61.055±0.170** | **2.032±** | **28.383±0.257** | -- |
| **Accumulation pattern** | | R>L>S | L>R>S | R>S>L | L>R | - | R>L>S | -- |
| *R. dentatus* | Leaves | 184.505±0.583 | 84.466±0.141 | 13.201±0.003 | 19.807±0.199 | 0.612±0.00 | 3.142±0.044 | BDL |
| | Shoot | 855.024±0.00 | 137.463±0.039 | 506.829±1.538 | 113.56±0.415 | 0.79±0.00 | 23.414±0.102 | BDL |
| | Root | 501.597±2.460 | 41.294±0.084 | 176.336±0.017 | 45.039±0.007 | 6.997±0.006 | 10.440±0.02 | BDL |
| | **Total** | **1541.126±3.043** | **263.223±0.264** | **696.366±1.558** | **178.406±0.62** | **8.399±0.006** | **36.996±0.074** | -- |
| **Accumulation pattern** | | S>R>L | S>L>R | S>R>L | S>R>L | R>S>L | S>R>L | -- |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *T. cordifolia* | Leaves | 214.48±3.875 | 6.321±0.023 | 12.979±0.026 | 10.984±0.006 | 2.124±0.005 | 7.124±0.114 | BDL |
| | Shoot | 21.119±0.007 | 6.321±0.079 | 3.770±0.00 | 0.722±0.00 | 0.836±0.00 | 3.543±0.002 | BDL |
| | Root | 28.477±0.602 | 9.679±0.048 | 5.847±0.010 | 1.642±0.098 | 0.08±0.00 | 3.030±0.01 | BDL |
| | **Total** | **264.076±4.484** | **22.321±0.15** | **22.596±0.036** | **13.348±** | **3.04±0.005** | **13.697±0.126** | -- |
| **Accumulation pattern** | | L>R>S | R>S>L | L>R>S | L>R>S | L>S>R | L>S>R | -- |
| *C. procera* | Leaves | 96.468±1.253 | BDL | 19.047±0.005 | 35.634±0.012 | BDL | 12.301±0.011 | BDL |
| | Shoot | 53.430±0.999 | BDL | 14.416±0.102 | 45.585±0.089 | BDL | 9.854±0.022 | BDL |
| | Root | 342.407±2.960 | BDL | 110.06±0.05 | 18.311±0.007 | BDL | 11.688±0.299 | BDL |
| | **Total** | **492.8±5.212** | **--** | **143.523±0.157** | **99.530±** | **--** | **33.843±0.332** | -- |
| **Accumulation pattern** | | R>L>S | - | R>L>S | S>L>R | -- | L>R>S | -- |
| *B. alba* | Leaves | BDL | 62.352±0.038 | 22.857±0.071 | BDL | 0.168±0.00 | 15.462±0.218 | BDL |
| | Shoot | 118±1.00 | 3.902±0.00 | 35.422±0.051 | 11.044±0.023 | 2.935±0.069 | 2.684±0.103 | BDL |
| | Root | 189.06±6.970 | 43.851±0.044 | 105.910±0.027 | 36.912±0.165 | 4.699±0.472 | 5.41±0.045 | BDL |
| | **Total** | **307.06±7.97** | **110.105±0.082** | **164.189±0.149** | **47.596±0.188** | **7.802±0.541** | **23.556±0.366** | -- |
| **Accumulation pattern** | | **R>S** | **L>R>S** | **R>S>L** | **R>S** | **R>S>L** | **L>R>S** | |

All the values are mean of three replicates (n=3) ± standard deviation (SD), BDL: Below detection limit, R: Root, S: Shoot, L: Leaves

### 8.3.4 Bioaccumulation coefficient factor and translocation factor

The BCF and TF values have been used to evaluate the phytoextraction and phytostabilisation potential of plant species as per reported by previous worker (MacFarlane et al. 2007; Yoon et al. 2006). The BCF refers to the most important plant feature in phytoremediation; the uptake of metals, their mobilization into plant tissues, and storage in the aerial plant biomass (McGrath and Zhao 2003). The results of BCF for each heavy metal in all tested plant samples are shown in Table 8.6. The result revealed that among the nine plant species, *S. munja*, *C. dactylon*, *P. purpureum*, *R. dentatus*, and *B. alba* has a high BCF values (>1) for Zn, Cu, and Ni while the plant samples all tested plants had a low BCF values (<1) for Fe, Mn, Pb and Cd as shown in Table 8.6. The high BCF>1 of *S. munja*, *C. dactylon*, *P. purpureum*, *R. dentatus*, and *B. alba* indicated that these plants have potential for phytoextraction of Zn, Cu and Ni from contaminated site. All tested plants species showed lowest values of BCF (<1) for Fe, Mn, Pb and Cd indicated that plants had difficulties in mobilizing these metals in root zone (Table 8.6). The process of metal uptake and accumulation from soil to root by different plants depends on the concentration of available metals in soils, solubility sequences and evapo-transpiration rate, hydropotential, photosynthetic activity of plant (Rajkumar et al. 2012; Ma et al. 2011).

**Table 8.6** BCF of different heavy metals in various plants growing on distillery sludge

| **Plants Code** | **Bioconcentration factor (BCF)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Zn** | **Mn** | **Ni** | **Cu** | **Fe** | **Pb** | **Cd** |
| *D. stramonium* | 0.088 | 0.039 | 0.089 | 0.052 | 0.013 | 0.091 | 0.114 |
| *Achyranthes* sp. | 0.041 | 0.022 | 0.054 | 0.036 | 0.012 | 0.029 | 0.104 |
| *K. pinnata* | 0.040 | 0.052 | 0.012 | 0.020 | 0.009 | - | 0.083 |
| *T. dioica* | 0.079 | 0.048 | 0.032 | 0.036 | 0.015 | 0.042 | 0.145 |
| *P. hysterophorous* | 0.075 | 0.080 | 0.056 | 0.030 | 0.025 | 0.031 | 0.145 |
| *C. sativa* | 0.065 | 0.052 | 0.041 | 0.035 | 0.017 | 0.083 | 0.156 |
| *A. spinosus* L. | 0.129 | 0.044 | 0.020 | 0.043 | 0.013 | 0.030 | 0.125 |
| *C. bonplandianum* | 0.076 | 0.029 | **1.185** | 0.030 | 0.008 | 0.058 | 0.072 |
| *S. nigrum* | 0.075 | 0.014 | 0.081 | 0.011 | 0.002 | 0.066 | - |
| *R. communis* | 0.020 | 0.012 | 0.036 | 0.011 | 0.002 | 0.019 | - |
| *S. viridis* | 0.103 | 0.057 | 0.145 | 0.055 | 0.021 | 0.118 | - |
| *B. lacera* | 0.306 | 0.160 | 0.189 | 0.189 | 0.069 | 0.281 | 0.218 |
| *A. mexicana* | 0.062 | 0.039 | 0.250 | 0.017 | 0.007 | 0.041 | -- |
| *S. munja* | **2.770** | 0.601 | **1.149** | **1.005** | 0.423 | 0.323 | -- |
| *C. dactylon* | **2.269** | 0.140 | -- | **1.795** | 0.157 | 0.837 | -- |
| *P. purpureum* | **1.237** | 0.226 | 0.521 | **1.546** | 0.176 | 0.913 | -- |
| *C. album* | **1.733** | 0.020 | -- | 0.261 | 0.053 | **1.043** | -- |
| *R. dentatus* | **1.463** | 0.712 | **1.740** | **1.246** | 0.211 | 0.854 | -- |
| *T. cordifolia* | 0.343 | 0.025 | 0.019 | 0.041 | 0.012 | 0.247 | -- |
| *C. procera* | -- | 0.289 | -- | 0.778 | 0.144 | 0.956 | -- |
| *B. alba* | **1.554** | 0.584 | **1.168** | 0.748 | 0.796 | 0.442 | -- |

All values are mean of three replicates (n=3), Values >1 are indicated in *boldfaces*

TF, the ratio of shoot to root metals, indicates internal metal transportation from root to shoot. For different plant species, the TF of Fe, Zn, Cu, Mn, Ni, Pb and Cd were variable as shown in Table 8.7. The order of TF values for various metals were as Zn > Ni > Cu > Pb > Mn > Cd > Fe. The maximum TF was found for Zn (TF: 21.936) by *A. maxicana* and the minimum was 0.031 for Fe by *B. alba*. However, the maximum TF was also obtained for Ni and Pb by *T. cordifolia* and *A. maxicana*, respectively as shown in Table 7.7. While, the plant species such as *A. mexicana*, *P. purpureum*, and *R. dentatus* had showed TF>1 for Zn (TF: 21.936, 1.228, 3.328), Cu (TF: 10.401, 2.874), Mn (TF: 6.540, 1.380, 2.512 ) Ni (TF: 2.079, 1.674, 10.45), and Pb (TF: 9.469, 1.362, 2.242). *T. cordifolia,* and *S. munja* also showed BCF>1 for Ni, Pb and Cd (Table 8.7). The high TF values of these metals might be due to high bioavailability of heavy metals, hydropotential, evapo-transpiration rate and photosynthetic activity of plant, and higher biomass production to fast growth; the higher transpirational pull out, higher biomass production due to fast growth; these plants have also vital characteristics to be used in phytoextraction of these metals as indicated by Yoon et al. (2006). The high metal accumulation might be attributed to well develop detoxification mechanism based on sequestration of heavy metal ions in vacuoles by binding them on appropriate ligands such as organic acid, protein and peptides in presence of enzymes that can function at high level of metalicilous conditions (Yang et al. 2005).

However, the TF value of Fe, Zn, Cu, Mn, and Ni in *S. munja, C. dactylon, P. purpureum, T. cordifolia, C. procera,* and *B. alba* was found <1 as shown in Table 7.7, while TF value of Cd in *D. stramonium, Achyranthes* sp. *K. pinnata, P. hysterophorous, C. sativa,* and *A. spinosus* L. was found <1, which indicates that these plant was not an accumulator and there was a tolerance mechanism through which maximum Fe, Zn, Cu, Mn, Ni, and Cd accumulated only in plant root not in shoot (Baker and Brooks 1989). The transport of metals from roots to shoot includes long distance translocation in the xylem and storage in the vacuole of leaf cells and these processes affected by element speciation, soil pH, and many other factors (McGrath and Zhao 2003; Liu et al. 2007). The heavy metal tolerance plants species with BCF >1 and TF <1 could be good candidates for phytostabilization of contaminated sites (Fitz and Wenzel 2002; Yoon et al. 2006). Example of such plants in our study included S. *munja, C. dactylon, C. album*, for Zn and Cu, *P. purpureum* for Cu, *S. munja, R. dentatus, B. alba* for Zn, and Ni, and *C. album* for Pb (Table 8.7). Phytostabilisation is a strategy of plant, in which plant species reduce the mobility and biovailability of heavy metals in environment. Plant can immobilise heavy metals through adsorption to roots surface, precipitation, and accumulation by roots or within rhizosphere soil. This process reduces the metal mobility and leaching into ground water, and also reduces the metal bioavailability entry into the food chain.

**Table 8.7** TF of different heavy metals in various plants growing on distillery sludge

| Plants Name | Translocation factor (TF) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Zn** | **Mn** | **Ni** | **Cu** | **Fe** | **Pb** | **Cd** |
| *D. stramonium* | 0.564 | **1.826** | **1.025** | 0.692 | **1.159** | 0.87 | 0.818 |
| *Achyranthes* sp. | 0.15 | **1.232** | **1.285** | **1.160** | **1.089** | **1.531** | 0.100 |
| *K. pinnata* | **1.070** | 0.092 | - | **4.163** | 0.109 | - | 0.125 |
| *T. dioica* | 0.086 | 0.541 | 0.896 | **1.104** | 0.459 | **1.195** | **1.142** |
| *P. hysterophorous* | 0.103 | - | - | - | - | - | 0.285 |
| *C. sativa* | 0.119 | - | - | - | - | 0.197 | 0.266 |
| *A. spinosus* L. | 0.535 | 0.482 | 2.388 | 0.740 | 0.712 | **3.212** | 0.250 |
| *C. bonplandianum* | **1.757** | **3.561** | 0.594 | **2.807** | **2.881** | **1.906** | **2.571** |
| *S. nigrum* | 0.5 | **1.512** | **1.027** | **3.259** | **2.182** | **1.180** | - |
| *R. communis* | 0.896 | 0.847 | **1.456** | **1.206** | 0.846 | **1.190** | - |
| *S. viridis* | **1.448** | **1.119** | 0.913 | **1.211** | 0.940 | 0.511 | - |
| *B. lacera* | 0.469 | 0.424 | 0.443 | 0.328 | 0.261 | 0.193 | - |
| *A. mexicana* | **21.936** | **6.540** | **2.079** | **10.401** | 0.034 | **9.469** | -- |
| *S. munja* | -- | 0.474 | 0.201 | 0.127 | 0.057 | **1.201** | -- |
| *C. dactylon* | 0.817 | -- | --- | 0.034 | 0.060 | 0.991 | -- |
| *P. purpureum* | **1.228** | **1.380** | **1.674** | 0.145 | 0.571 | **1.362** | -- |
| *C. album* | 0.541 | -- | -- | 0.600 | 0.308 | 0.577 | -- |
| *R. dentatus* | **3.328** | **2.512** | 0.112 | **2.874** | **1.704** | 2.242 | -- |
| *T. cordifolia* | 0.653 | 0.439 | **10.45** | 0.644 | 0.741 | **1.169** | -- |
| *C. procera* | -- | **2.489** | -- | 0.130 | 0.156 | 0.843 | -- |
| *B. alba* | 0.088 | 0.299 | 0.624 | 0.334 | 0.031 | 0.496 | -- |

All values are mean of three replicates (n=3), Values >1 are indicated in boldfaces

### 8.3.5 Ultrastructural observations of root tissues of potential native plants after phytoextraction of heavy metals

The effect of heavy metals on cellular organization is an important factor in understanding the morphological and physiological alterations induced by heavy metals due to complementarity of structure and function (Bini et al. 2012). The anatomical observations through TEM in the root of various potential native plants showed apparent formation of multi-nucleolus, multi-vacuoles and deposited metal granules in the cellular components of plants. The *P. hysterophorous* which was growing luxuriantly on the distillery sludge showed formation of multi-nucleolus, multi-vacuoles, and dense deposition of heavy metal granules towards the cell wall region and cytoplasm both as shown in Fig. 8.10(a-c). Similarly, *C. sativa* a native plant was growing dominantly showed very frequent formation of glomerated multi-vacuoles near the cell wall and deposition of metals granules in the intercellular spaces as characteristics features of *C. sativa* root TEM structure. Besides, the deformities of cell wall and creation of intercellular spaces and deposition of metals granules at the surface of cell wall was also observed as specific features of this plant but the plants were found growing

very healthy throughout the year even after variation in weather as TEM observations have shown in Fig. 8.10(d-f). *S. nigrum* also showed the similar properties with dominant features of metal deposition in the root cell wall [Fig. 8.10(g-i)]. The metal analysis of different parts of *S. nigrum* revealed that Zn was accumulated maximum in root while Fe was transported maximum in leaves of plants. The *R. Communis* was found very potential for the heavy metal phytoextraction and phytoremediation of distillery sludge. The cellular compartment of root showed a characteristic feature for formation of vacuoles in a chain. This may be as an excretory property of accumulated metals through vacuoles which appeared very clearly in TEM observation as shown in Fig. 8.8(j-l). The metal granules were observed abundantly in the cell cytoplasm also along with cell wall deposition. The metal granules and multi-nucleolus formation in nucleus along with projecting out structure from the nuclear membrane with metal granules were observed clearly in the TEM structure [Fig. 8.10(j-l)].

The TEM micrograph of *A. mexicana* and *S. munja* root showed formation of multi-vacuoles, multi-nucleolus and changed of nuclear shape as shown in Fig. 8.11(a-h). This indicated the synthesis of protein for their tolerance mechanism in process of accumulation of heavy metals in high concentration in their cellular compartment. In addition, the apparent deposition of metals in form of electron dense granules in vacuoles, cell cytoplasm, and cell wall also indicated heavy metal hyperaccumulation tendency of plant. The heavy metal deposition in periphery of the cell wall of *A. mexicana* and *S. munja* were also observed as shown in Fig. 8.11(a-h). The heavy metal depositions near the cell wall play a significant role in heavy metals tolerance by preventing the circulation of free metals ions in the cytosol. This observation established a new findings on the heavy metals detoxification mechanism of plants based on sequestration of heavy metals ions in vacuoles and deposition on the cell wall as shown in Fig. 8.11.

Similarly, the root tissue of *C. dactylon* also showed formation of multi-nucleolus, multi-vacuoles, thicking of middle lamella, and round shaped mitochondria with metal deposition in cell wall, cytoplasm, and middle lamella as shown in Fig. 8.11(i-l). The multi-vacuoles formation and deposition of metal granules in cell wall indicated a plant tolerance mechanism for metal accumulation and detoxification at higher quantity in their cellular tissues (Tong et al. 2004). The round shaped mitochondria were some of the prominent feature observed in root of *C. dactylon.* The multi-vacuoles formation and depositions of metal granules in cell wall, and cytoplasm in root of *P. hysterophorous, C. album*, *S. nigrum*, and *R. communis* growing under heavy metal stress as adaptive mechanism at high concentration of metals in presence of organic pollutants.

The ultrastructural observation of root tissue of *P. purpureum* and *C. album* at low and high magnification showed deposition of metal granules inside the cell wall, cell membrane, cytoplasm, and nucleoplasm as shown in Fig. 8.11(m-t). The accumulation of heavy metals inside the cell wall and cell membrane might occurred due to apoplastic and symplastic binding of heavy metals which indicated a tolerance mechanism of plant preventing the circulation of free metals in the cytosol (Kupper et al. 2000; Najeeb et al. 2011; Kramer et al.

2000). This findings are corroborated with the earlier observation reported by various workers (Alkatib et al. 2013; Daud et al. 2013).

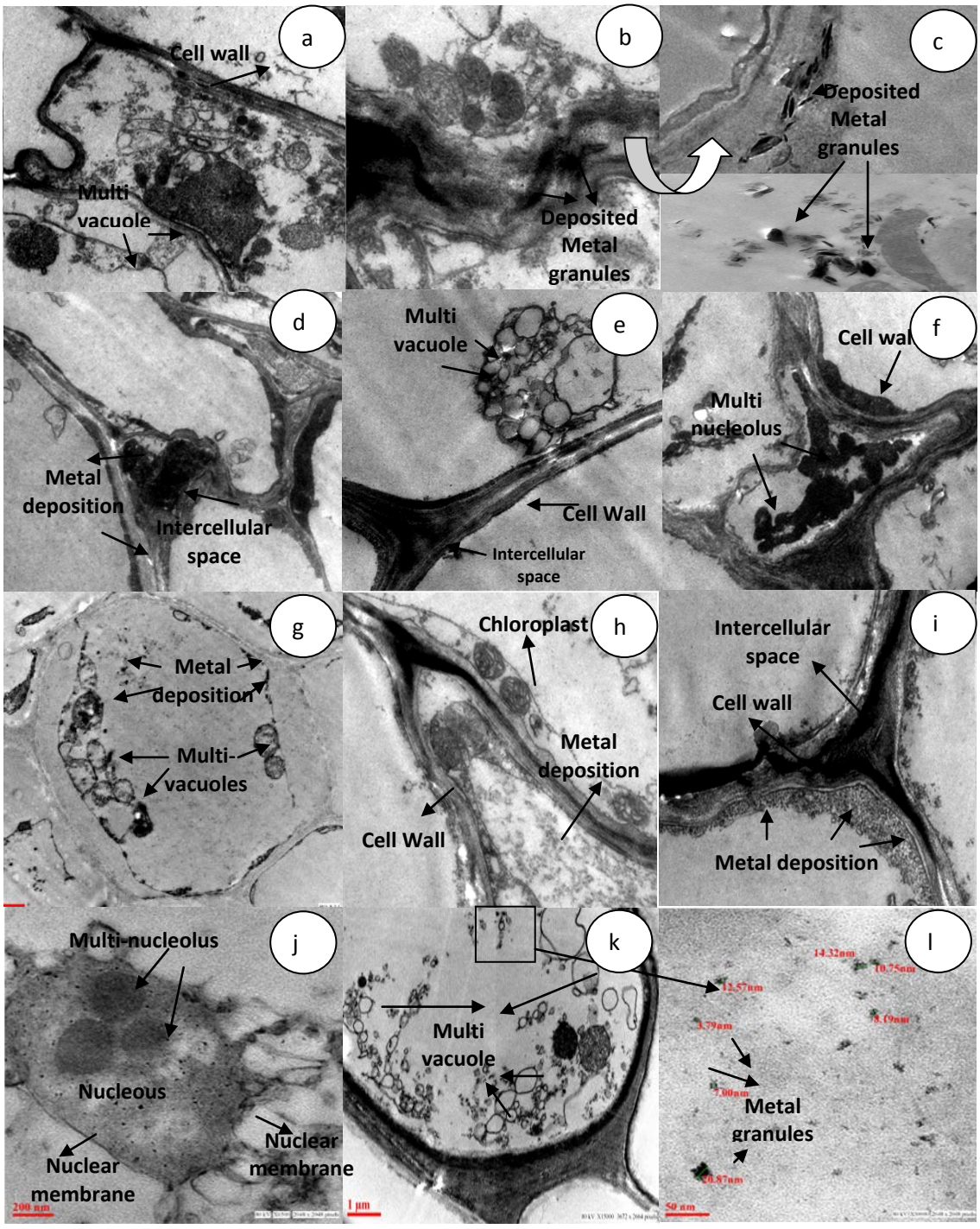



**Fig. 8.10** TEM images of native plants root after phytoextraction of heavy metals (a-c) *Parthenium hysterophorous* (d-f) *Cannabis sativa* (g-i) *Solanum nigrum* (j-l) *Ricinus communis*

The TEM micrograph of root tissue of *R. dentatus* and *T. cordifolia* showed plasmolytic shrinkage, round shaped mitochondria formation and deposition of heavy metal granules on periphery and inside the cell wall as shown in Fig. 8.12(a-g). In addition, the root observation of *R. dentatus* and *T. cordifolia* also showed an increased number of round shaped mitochondria which are become enlarged in size. The greater number of mitochondria formation indicated the production of larger amount of energy in form of ATP inside the cell, which is needed in order to combat the heavy metal toxicity. Similar pattern of mitochondria formation have been reported by Jin et al. (2008) and Alkhatib et al. (2013) in presence of high concentration of Cd and Pb in hydroponic conditions.

The root cells of *C. procera* showed deposition of electron dense granules of metals in cell cytoplasm as shown in Fig. 8.12(h-k). However, the multi-vacuoles and multi-nucleolus formation could be observed in root cells of *B. alba* as shown in Fig. 8.12(l-o). The heavy metal deposition inside the vacuoles, middle lamella, and cell wall could be seen in this plant root. In addition, the entire plant root also showed thickening of cell wall. The production of greater number of nucleolus and vacuoles at elevated level of heavy metals increase the production of ribosome and mRNA, which ultimately enhance the production of new proteins being involved in the heavy metals tolerance in plant (Najeeb et al. 2011). Our results corroborated with the observations reported by earlier study for the formation of multi-vacuoles and multi-nucleolus by Zn and Cd along with metal deposition in the cell wall of *Brassica juncea* (Maruthi Sridhar et al. 2005). All the plant species in our study were luxuriantly growing on disposed distillery sludge without showing any morphological deformities in their aerial parts. This indicated that these plant species have strong potential and adaptation for survival and growth on organo-metallic and EDCs containing distillery waste for *in*-situ phytoremediation. These growing plants have also given a strong evidence for heavy metals monitoring and phytoremediation of hazardous complex industrial waste for eco-restoration of polluted sites.

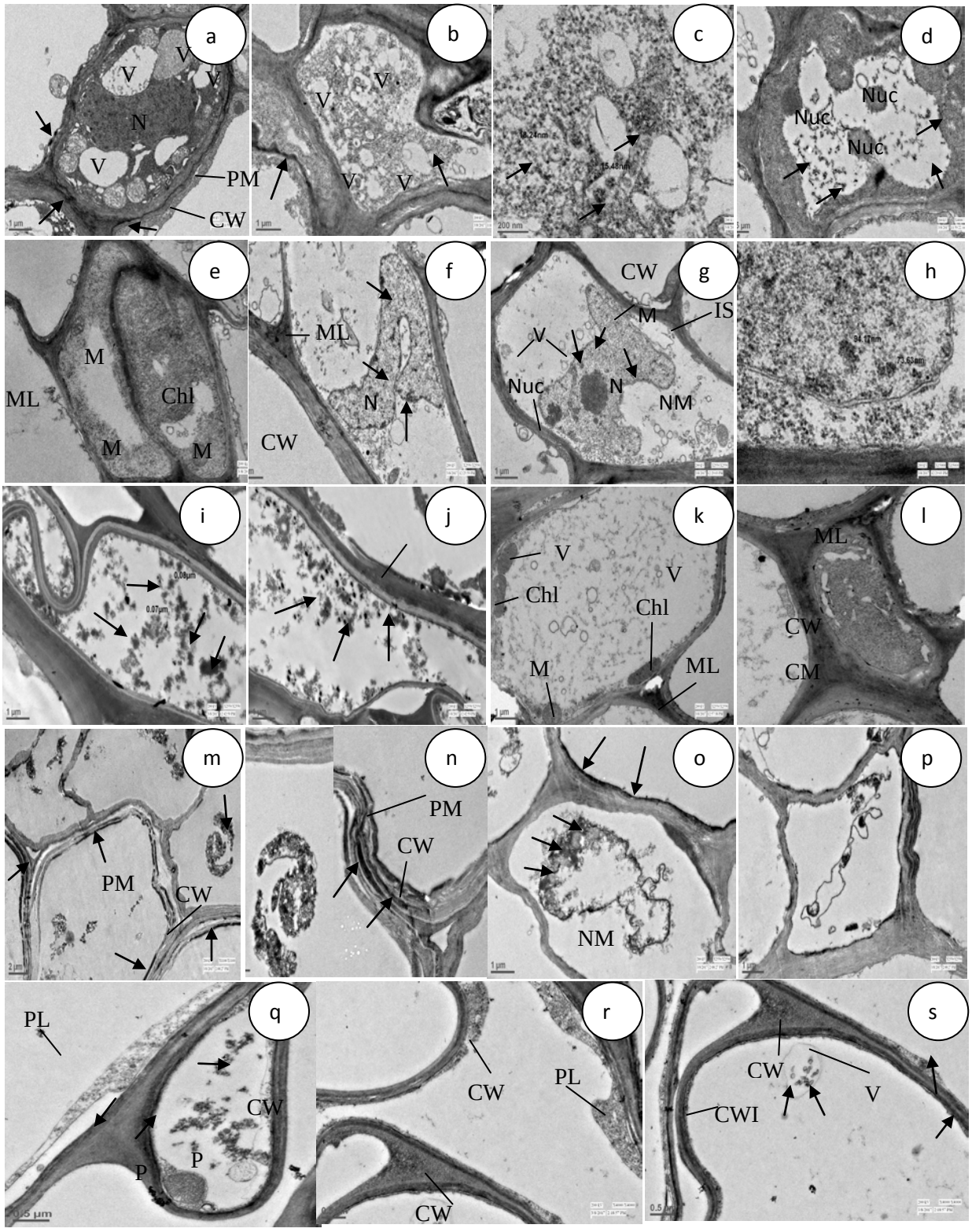



**Fig. 8.11** Electron micrographs of transverse section of plants root after phytoextraction of heavy metals (a-d) *Argemone mexicana* (e-h) *Saccharum munja* (i-l) *Cynodon dactylon* (m-q) *Pennisetum purpureum* (r-t) *Chenopodium album.* V: Vacuole; PM: Plasma membrane; P: Peroxisome; CW: Cell wall; CWI: Cell wall invegination CM: Cell membrane; Nuc; Nucleolus Chl: Chloroplast; ML: Middle lamella; N: Nucleus; Arrow (⟷) indicated metals deposition; IS: Intercellular space

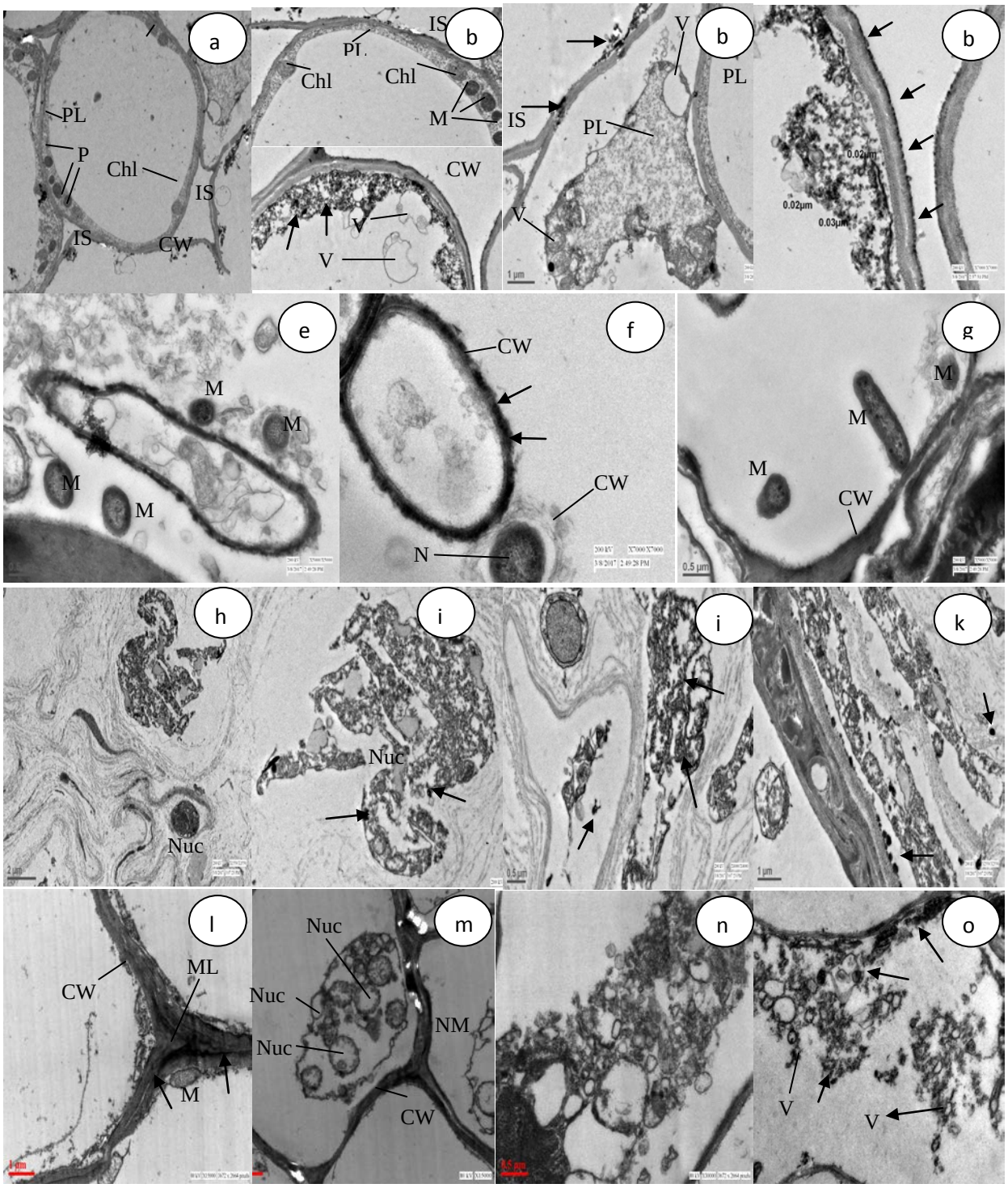



**Fig. 8.12** Electron micrographs of transverse section of plants root after phytoextraction of heavy metals (a-d) *Rumex dentatus* (e-g) *Tinospora cordifolia* (h-k) *Calotropis procera* (l-o) *Basella alba.* V: Vacuole; PM: Plasma membrane; P: Peroxisome; PL: plasmolysis CW: Cell wall; CM: Cell membrane; Chl: Chloroplast; N: Nucleus; Nuc: Nucleolus; Arrow (⟶) indicated metal depositions

## Conclusion

The study concluded that the distillery sludge discharged from sugarcane-molasses based distillery contained several heavy metals Fe (5264.49), Zn (43.47), Cu (847.46), Mn (238.47), Ni (15.60), and Pb (31.22 mg $kg^{-1}$) and EDCs as well as other toxic organic pollutants which has strong binding tendency with metal ions. The comparative extraction process for various organic pollutants showed that ethyl acetate compared to *n*-hexane was the best solvent for the extraction of EDCs and other organic compounds present in distillery sludge. The metal accumulation pattern of weeds and grasses growing naturally in the contaminated environment revealed that all the plant species accumulated high amount of metals in root, shoot and leaves. Further, BCF and TF of *A. mexicana, S. munja, C. dactylon, P. purpureum, C. album, R. dentatus, T. cordifolia, C. procera,* and *B. alba* for Zn, Cu and Ni were found >1 while the TF of most of the growing plant for Zn, Cu, Mn, Ni and Pb was noted >1. Thus, it was observed that all the growing plants have been found for hyperaccumulation properties of heavy metals from organo-metallic polluted site mixed with androgenic and mutagenic compounds. The TEM observation in root tissues of all plant species showed apparent formation of multi-nucleus, multi-nucleolus, multi-vacuoles, mitochondria and dense deposition of metal granules in the cellular organelle of plant which supported the plant tolerance mechanism at higher concentration of heavy metals in presence of other complex organic pollutants. Thus, the study recommended that these plants can be used for *in*-situ monitoring and phytoextraction of heavy metal from organo-metallic waste contaminated sites. Further, it is also recommended that these plants should be prohibited for the use of food and fodder due to health hazards.

# Chapter-09

# Analysis of dominant rhizospheric bacterial communities from Phragmites cummunis growing in constructed wetland plant treatment system for decolourisation and detoxification of bacterial treated post methanated distillery effluent

# Chapter-9

## Analysis of dominant rhizospheric bacterial communities from *Phragmites communis* growing in constructed wetland plant treatment system for decolourisation and detoxification of bacterial treated post methanated distillery effluent

### 9.1 Introduction

Constructed wetland treatment system are engineered and design to exploit natural processes, including those of microbial communities associated with wetland plants and soils, for cleanup of wastewater (Kadlec and Wallaces 2009; Stromeister et al. 2003). It is an eco-friendly, cost-effective, reliable technology that is useful for cleaning up nutrient overloads, toxic metals, and organic pollutants present in municipal solid waste leachates, industrial wastewaters, agricultural, storm runoff, sludges, pharmaceutical waste, and acid mine drainage, etc. (Vymazal 2014; Wu et al. 2015; Zhang et al. 2015). CWs are generally categorized into two basic types: free water surface system (FWS-CWs) (which includes the free water table on top of the soil) and subsurface flow system (SSF-CWs) (which includes vertical and horizontal subsurface flow CWs, depending on the direction of the flow). In recent years, SSF-CWs have attracted more and more studies and applications in China and Europe, due to its decreased demand for land and high efficiency for various pollutants. Macrophytes are the main biological component of wetland ecosystems; they contribute directly to pollution reduction through uptake and assimilation and indirectly by facilitating the growth of important pollutant-reducing microorganisms through complex interactions in the rhizosphere (Pang et al. 2016). Plant act as catalysts for purification reactions by increasing the microbial diversity in the root zone through the release of oxygen and exudates and promotion of a variety of chemical and biochemical reactions that enhance purification (Stottmeister et al. 2003). The complex microbial community associated with filter material roots, created by interactions with wastewater, is mainly responsible for the degradation efficiency of pollutants and ecosystem stability (Stottmeister et al. 2003, Pang et al. 2016). In addition, wetland plants have high remediation potentials for macronutrients due to their generally fast growth and high biomass production *Phragmites sp.* is a cosmopolitan highly resilient and very productive emergent microphyte, which fulfils diverse role in freshwater environment and is widely used in constructed wetland for wastewater treatment (Vymazal 2014). These plants can withstand extreme environmental conditions, including the presence of toxic contaminants such as heavy metals. A detailed knowledge of microbial communities at any polluted site not only reflects their relationships with pollutants, but also reflects information with respect to the bioremediation potential of microbes on specific pollutants. This may indicate a direction of the bio-stimulation or bio-augmentation for the restoration of any polluted ecosystem. High-throughput sequencing technology is a highly efficient too for identifying the profile of complicated microbial communities (Wang et al. 2015a,b; Yang et al. 2015). Due to its relatively low cost and great throughput, 16S rDNA Illumina Miseq

has been applied to study the microbial diversity in various environments (Moreau et al. 2014; Zhang et al. 2015; Walujkar et al. 2014; Glenn 2011; Liu et al. 2012). The objective of the present study was to investigate the bacterial community in the substrate, the V3-V4 regions of the bacteria 16S rDNA were sequenced via an Illumina MiSeq 2500 platform.

## 9.2. Material and Methods

### 9.2.1 Construction of wetland plant treatment system and acclimatization of *Phragmites communis* plants

The pilot scale horizontal subsurface flow-constructed wetland (HSSF-CW) plant treatment system was constructed in 7 × 20 m area located at the Babasaheb Bhimrao Ambedkar University, where in 19 × 4 m area; a zig-zag channel was constructed using cement to prevent the seepage. Upstream channel depth and width were kept at 50 and 40 cm, respectively, while the downstream dept and width were kept at 58 and 40 cm, respectively, thereby maintaining a 0.88% slope that promoted hydraulic flow. The horizontal bed was filled with pebbles, coarse gravel and sand particles, each layer being 8 cm deep, as in the design of Swindell and Jackson (1990) and the EPA design manual (EPA 1998). A view of two step treatment process for PMDE degradation and decolourisation is illustrated in Fig. 9.1 and 9.2.

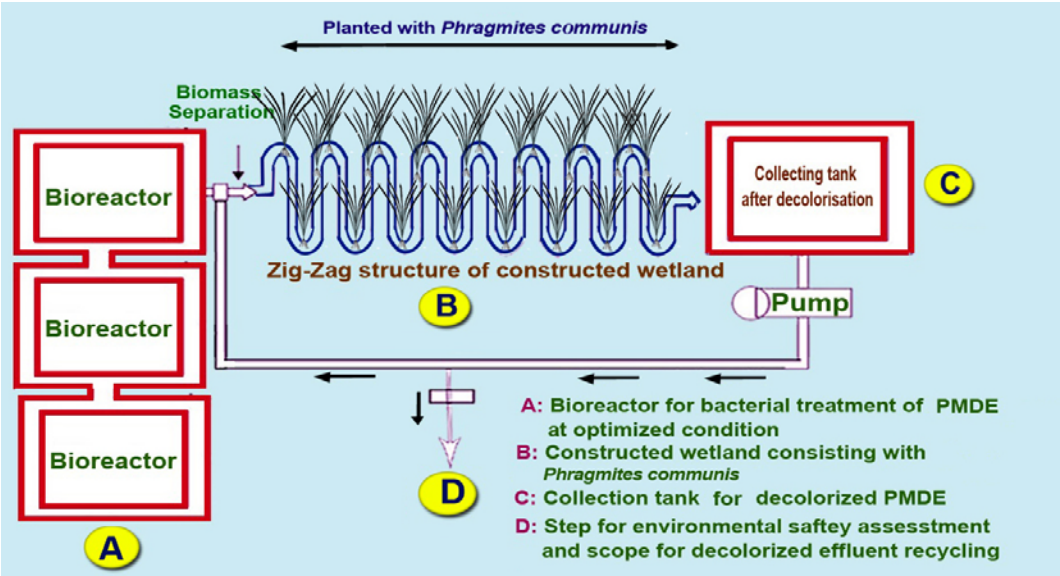



**Fig. 9.1** Schematic view of two step treatment system for PMDE decolourisation and degradation for environmental safety

Naturally growing wetland plant *P. communis* were collected from a wetland area polluted with PMDE. The young buds (plantlets) of the subterranean rhizomes of this species were acclimatized for more than 60 days in HSSF-CW. During this period, the plants were fed with 5% (v/v) Hoagland's solution (Hoagland and Arnon 1938) for 30 days; the solution contained (in g $L^{-1}$); $Ca(NO_3)_2 \cdot 4H_2O$ (1.18), $KNO_3$ (0.51), $K_2HPO_4$ (0.14), $MgSO_4 \cdot 7H_2O$ (0.49), $H_3BO_3$ (0.0029), $MnCl_2 \cdot 4H_2O$ (0.0018), $FeCuH_4O_6$ (0.005), $CuSO_4 \cdot 5H_2O$ (0.00008), $ZnSO_4$ (0.0022), and $H_2MoO_4$ (0.00002). Subsequently, the plants were further

acclimatized for 30 days by means of subsurface flow circulation containing PMDE mixed with the above mentioned Hoagland's solution (30:70 v/v). The diluted effluent was re-circulated using a pump to maintain natural wetland conditions. The upstream flow rate was 2 L $min^{-1}$ versus 1.5 L $min^{-1}$ downstream. The layout of the two step plant treatment system for PMDE decolourisation and degradation is shown in Fig. 9.2.

### 9.2.2 Bacterial treatment of PMDE at optimized conditions in bioreactor

The bacterial treatment of PMDE was carried out in an open bioreactor containing 350 lit of diluted PMDE with color range 1,40,000 co.pt amended with modified GPM medium containing (in %) glucose (1%), peptone (0.1%), $K_2HPO_4$ (0.1%) and $MgSO_4.7H_2O$ (0.05%). The bioreactor was inoculated with four bacterial strains [*Klebsiella pneumoniae* (KU726953), *Salmonella enterica* (KU726954), *Enterobacter aerogenes* (KU726955), *Enterobacter cloaceae* (KU726957) with highest $OD_{620}$ 0.24 for each bacterial strains, with synthetic and molasses-melanoidins degrading ability previously isolated from distillery sludge and treated up to 144 h of incubation period. One bioreactor without bacterial inoculums was served as the control. The cultures were incubated at 180 rpm for 168 h with continuous aeration. After 168 h, aeration was stopped and the reactors were left for 24 h to settle down the bacterial biomass. Two parallel sets of bioreactors were operated for the control and experimental treatments to maintain continuous flow with and without bacterial pretreatment of the PMDE in the HSSF-CW plant treatment system (Fig. 9.1). Another set of effluent with the same concentration but without bacterial pretreatment was also circulated through a separate wetland system to serve as a control. The decolourisation of PMDE was monitored spectrophotometrically in terms of bacterial growth and decrease in color intensity at 620 and 250 nm (absorption maxima), respectively and the percent decolourisation was calculated by using the formula as described in chapter no. 05.

### 9.2.3 Integration of bacterial pretreated PMDE with HSSF-CW plant treatment system

The bacterial treated PMDE was fed into zigzag shaped HSSF-CW plant treatment system with 50 × 40 cm upstream depth and width and 58 × 40 cm down stream depth and upstream flow rate of 2 L $min^{-1}$ and a downstream flow rate of 1.5 L $min^{-1}$. The bacterial treated distillery effluent was circulated up to 168 h until the BOD and COD of the PMDE reached a value of 100 mg $L^{-1}$. The decolourization of distillery effluent was monitored periodically at the regular interval of 24 h and after 168 h of treatment, the treated PMDE was collected in a collection tank and used for the analysis of various physico-chemical parameters.

### 9.2.4 Optimization of flow rate (FR) and hydraulic retention time (HRT) for PMDE decolourisation

The proper functioning of wetland treatment system depends on the interaction between the plants, microbes, wastewater characteristics and operational condition. The FR and

HRT determine the interaction of wastewater with plant and microbes. In our study decolourisation was optimized at different flow rate from 10-30 $m^3d^{-1}$ by putting the controlling knob at connecting points of bioreactor (bacterial treatment) and upstream part of constructed wetland system.

### 9.2.5 Physico-chemical analysis of the PMDE before and after treatment

The physico-chemical parameters of the PMDE were periodically analyzed to assess the performance of the CW plant treatment system. All the physico-chemical parameters in pre and post treated PMDE prior to its final discharge was periodically monitored by analyzing colour, pH, BOD, COD, TDS, TD, etc. as per standard methods (APHA 2012).

### 9.2.6 Detection and characterization of various metabolites produced during the degradation and decolourisation of PMDE in two step treatment

#### 9.2.6.1 Liquid-liquid extraction

A portion (150 mL) of untreated, bacteria as well as CW treated PMDE samples was centrifuged at 6000 ×g for 10 min at 4 °C in a microfuge to separate suspended particles and bacterial biomass from control and treated samples, respectively. The supernatant obtained was vacuum evaporated at 40 °C to concentrate metabolites and reduce volume up to 50%. Each 100 mL aliquot of the concentrated sample was then mixed with equal volume of ethyl acetate and the mixture was shaken continuously for 5 h with intermittent rests for liquid–liquid extraction. The organic layers obtained from each sample were kept together and concentrated on a rotatory evaporator until complete solvent evaporation at ≤40 °C. An aliquot of the concentrate was dissolved in 3.0 mL of methanol, filtered through 0.22 μm syringe filters (Millipore Ltd., Bedford, MA, USA), and used for metabolites analysis.

#### 9.2.6.2 UV-Vis, HPLC, and GC-MS analysis

The PMDE was analysed using a UV-Vis spectrophotometer in the wavelength range between 200-700 nm at 25 °C, in order to reveal the chemical nature of the metabolites. The HPLC analysis of decolourised PMDE as well as metabolites generation was monitored by 515 HPLC instrument equipped with a diode array detector system (1100 series, Agilent Technologies, USA) and reverse phase $C_{18}$ column by using the gradient of solvent A (Milli-Q water) and solvent B (acetonitrile with 0.1% TFA) at a flow rate 0.4 mL $min^{-1}$ for 60 min. The detection was monitored at wavelength 250 nm (absorption maxima) to assess the decolourisation and degradation of melanoidins. For GC-MS analysis, the extracted samples were derivatised with TMS as described by Minuti et al. (2006). After derivatisation sample was subjected to GC-MS analysis. The operational conditions for GC-MS analysis of organic compounds is described in chapter no. 04 (Bharagava and Chandra 2009).

### 9.2.7 Characterisation of rhizospheric bacterial communities of *Phragmites communis*

#### 9.2.7.1 Collection of sample

The plant was selected at the growing stage as excretes more volume of root exudates and can bear more diversified rhizosphere microbes in its roots. To collect the rhizospheric sludge sample, roots with sludge were transferred into polyethylene bags and vigorously shaken by hand for 10 min until sludge samples non-adhering to roots were completely removed. The roots with adhering sludge were subsequently shaken sterile saline solution (NaCl 0.009 g $kg^{-1}$) to remove the sludge and centrifuged twice (1700 ×g, 10 min) to concentrate sludge particles in the pellet. The pellet was collected for subsequent DNA extraction, 16S rDNA PCR amplification and Illumina MiSeq sequencing. Bacterial communities in the rhizosphere of this plant are of great interest because of their potential for bioremediation of industrial effluent (Chaturvedi et al. 2006).


**Fig. 9.2** Two step treatment of PMDE by bacteria and constructed wetland plant treatment system (a-c) view of pilot scale HSSF-CW plant treatment system planted with *P. communis* (d) uprooted *P. communis* (e) rhizosphere of *P. communis*

### 9.2.7.2 Community DNA Extraction and PCR amplification

0.25 g of the sludge pellet was prepared for DNA extraction. The total microbial community DNA was extracted using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. DNA yields were verified by the 260/280 nm and 260/230 nm absorption rations, measured using Nano-DropND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) as described by Kumbhare et al. (2015). The integrity of genomic DNA was evaluated via 1 % agarose gel electrophores and purified by the gel extraction kit (Omega) is as shown in Fig. 9.3. DNA products were then stored at -20 °C before analysis. Then, DNA was diluted to 1 $ng \cdot \mu L^{-1}$ in sterile water. The universal primer sets 341F (5`-CCTAYGGGRBGCASCAG-3`) and 785R (5`-GACTACHVGGGTATCTAATCC-3`) were used for amplification of the V3-V4 hypervariable regions of 16S rDNA (Wang et al. 2015). A 10 ng template, 0.5 µL of forward primers and 0.5 µl of reverse primers were added into a 25 µl reaction system for the PCR reaction. The amplification of the 16S rRNA gene was performed using the denaturation at 94 °C for 5 min, which was followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 7 min. The PCR products were purified using AMPure XP beads (Agencourt Bioscience, USA). The concentrations of the PCR products were fluorometrically quantified by the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) before being sequenced on the Miseq platform (Illumina, San Diego, CA, USA),

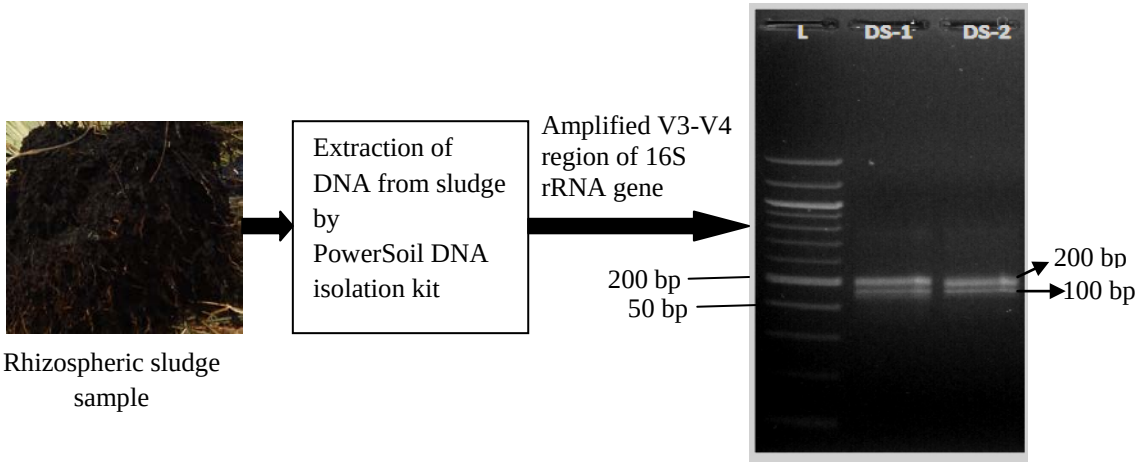



**Fig. 9.3** Gel electrophoresis of amplified V3-V4 region of 16S rRNA genes of rhizospheric sludge sample of *P. communis*; L: DNA ladder; DSI: Control; DS2: Constructed wetland treated

### 9.2.7.3 16S rRNA Illumina Miseq sequencing

The MiSeq sequencing procedures were conducted on an Illumina MiSeq platform at the M/s AgriGenome Labs Pvt Ltd., India. The library preparation of 16S rRNA gene amplicons was in accordance with the protocols of Illumina (USA). The sequencing libraries were constructed with an NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs Inc., Boston, MA, USA), and then a Qubit 2.0 Fluorometer (Life Invitrogen, Inc., Carlsbad, CA, USA) was used to assess the libraries quality. Then, the

libraries were sequenced on an Illumina MiSeq 2500 platform. These amplicon libraries were further processed for sequencing using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA). The resultant product was screened with the LabChip GX (Perkin Elmer, Waltham, MA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Subsequently, the 16S rRNA gene library was sequenced on the Illumina MiSeq platform using 2× 250 bp chemistry.

#### 9.2.7.4 16S rDNA sequence processing and bacterial diversity, richness and taxonomic distribution of taxa analysis

The diversity and composition of bacterial communities of the paddy soil samples were analysed based on the raw sequencing data obtained from the Illumina MiSeq platform. Raw sequences through paired end sequencing were merged based on the method described by Magoc and Salzberg (2011). Raw sequences were processed using the QIIME software package (version 1.7.0 (Caporaso et al. 2010a). Sequences were denoised, and trimmed to remove bar codes and primers and clustered into operational taxonomic units (OTUs) based on their sequence similarity at 97% using ULUST programme (UCLUST version 1.2.22q). In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), the representative sequence identified from each OTUs was then multiple aligned against the ‘‘Core Set” dataset in the Green-Gene database using PyNAST software (version 1.2) (Caporaso et al., 2010b). Further, these representative sequences aligned against reference chimeric data sets. The taxonomic assignment was carried out with the Ribosomal database project (RDP) Classifier (version 2.2) (Cole et al. 2009) against SILVA OTUs database (version 123) using a confidence threshold of 70% (Quast et al. 2013). Krona charts were calculated using the Krona Tools available from http://krona.sourceforge.net (Ondov et al. 2011). Alpha diversity is applied in analysing complexity of species diversity for a sample through Shannon, Chao1, and Observed-species indices. The chao1 metric estimates the species richness while Shannon metric is the measure to estimate observed OUT abundances, and accounts for both richness and evenness. Rarefaction curves were generated based on these three metrics by QIIME (Version 1.7.0).

### 9.2.8 Phytoxicity assessment of PMDE for environmental safety

The toxicity assessment of untreated and treated melanoidins was studied on *Phaseolus mungo* L. seed germination using Petri dish method as described in chapter no. 04 (OECD 2003; Sharma et al. 2002).

## 9.3 Results and discussion

### 9.3.1 Bacterial decolourisation of PMDE in bioreactor

Bacterial consortium comprising decolourized 10% PMDE up to 71% at optimized condition of nutrient and different parameters after 168 h of incubation. After 168 h of incubation, bacterial consortium was unable to decolorized PMDE higher than 71 %. This

might be due to higher concentration of toxic organic and inorganic compounds which inhibit the bacterial growth. During bacterial treatment along with colour other pollution parameter i.e. COD, BOD, TDS, and TS were also reduced after 168 h of incubation (Fig. 9.4 and Table 9.1).

### 9.3.2 Optimization of FR and HRT in CW plant treatment system

Supernatant of bacterial treated PMDE was integrated in to HSSF-CW plant treatment system with different FR and HRT to obtain the more decolourisation (Fig. 9.4). FR from 10 to 28.8 $m^3d^{-1}$ showed very slight difference in PMDE decolourisation. However, FR at 30 $m^3d^{-1}$ showed non-significant decolourisation as compared to 28.8 $m^3d^{-1}$ flow rate. In contrast, PMDE decolourisation was greatly affected by HRT. Gradual increases in HRT increases the decolourisation process. The optimum decolourisation was found at 7 days HRT. Beyond this HRT (7 days) decolourisation was constant.

### 9.3.3 Physico-chemical analysis of PMDE

The maximum decolourisation of PMDE was observed (98%) after bacterial and CW treatment as shown in Fig. 9.4. Further, the physico-chemical properties of PMDE prior to bacterial and CW rhizospheric treatment samples are presented in Table 9.1. The value of colour intensity and pH was founder higher in the untreated PMDE. In addition, the concentration of BOD (6500), COD (10864), TS (12248.0), TDS (10764), SS (754.0), VS (9014.0), TN (2085.6) chloride (19993.8), sulphate (15247.5), phenol (2040.0 mg $L^{-1}$), and various heavy metals such as Fe (41.50), Zn (1.12), Ni (0.25), Mn (23.54), Pb (0.18), Cu (1.24) and Cd (0.08 mg $L^{-1}$) was found higher. But, bacterial treated sample of PMDE obtained after 168 h incubation showed reduction in all physico-chemical parameters as shown in Table 9.1. This indicated the biodegradation and biotransformation of various organic and inorganic contents by potential bacterial consortium. Moreover, the constructed wetland plant rhizosphere treated PMDE sample has also shown reduction of BOD COD, TDS, SS, VS, TN, chloride, sulphate, phenol (Fig. 9.5) and various heavy metals such as Fe, Zn, Ni, MN, Pb, Cu, and Cd as shown in Table 9.1. This indicated that during the bacterial treatment of PMDE, organic compounds and metal complexes get biotransformed into such forms that become easily bioavailable to wetland plants rhizosphere microflora and plant root. The degradation and metabolization of recalcitrant organic and inorganic compounds by bacterial consortium and their rhizospheric assisted utilization by wetland plants has also been reported by various authors (Stottmeister et al. 2003). The decolorization of PMDE is a combined phenomenon of bacterial transformations followed by the bacterial assisted wetland plants rhizospheric treatment, which grows in the vicinity of plant roots and supported the bioaccumulation of toxic metals by wetland plants. The growing bacterial communities helped in degradation of toxic wastes and plant accumulates the heavy metals in its tissues. Both of them played a major role in the bioremediation of PMDE. Therefore, all the values of BOD, COD, phenol, sulfate, and heavy metals were reduced (Table 9.1 and Fig. 9.5). The phytoremediation of heavy metals by *Phragmites* and other wetland plants from aqueous solution has been also reported in previous studies (Chandra and Yadav 2011). Moreover,

the removal of toxic metals from industrial wastewaters by wetland plants can also takes place through the process of filtration, sedimentation of suspended particles, adsorption phenomenon, uptake into plant materials and precipitation of metals by biogeochemical process. As in case of the surface flow of aqueous phase through constructed wetland plants, $Fe^{2+}$ get oxidized into $Fe^{3+}$ by abiotic and microbial oxidation processes while other metals get immobilized by the microbial dissimilatory sulfate reducing mechanisms (Stottmeister et al. 2003). This finding is in good agreement with Chandra et al. (2012) who found that the plants and their rhizospheric bacterial communities had a significant impact on the removal efficiency of all physic-chemical parameters of PMDE.

**Table 9.1** Physico-chemical analysis of PMDE

| S.No. | Parameters | Untreated (control) | Bacterial Treated (168 h) | Constructed wetland treated (168 h) | % Reduction |
|---|---|---|---|---|---|
| 1. | Colour | Dark Brown | Light brown | Light yellow | - |
| 2. | C.I. | 140000 | 85040 | 22350[a] | 84.03 |
| 3. | pH | 7.1±0.30 | 7.4±0.28[a] | 7.1±0.12[a] | 4.04 |
| 4. | BOD | 5500±266.5 | 2345±96.14[a] | 450±78.45[a] | 91.81 |
| 5. | COD | 10864±391.10 | 7760±372.48[a] | 555±11.45[a] | 92.84 |
| 6. | TS | 12248.0±502.16 | 6956±208.68[a] | 411±102.22[a] | 94.09 |
| 7. | TDS | 10764±409.03 | 6232.0±255.12[a] | 315.85±13.12[a] | 94.93 |
| 8. | SS | 754.0±27.14 | 198.0±72.71[a] | 29.41±1.25[a] | 85.14 |
| 9. | VS | 9014.0±270.42 | 3526±148.09[a] | 241.64±91.45[a] | 93.14 |
| 10. | TN | 2085.6±10.38 | 1801.11±1.21[a] | 150.14±12.01[a] | 91.66 |
| 11. | Chloride | 19993.8±599.75 | 3098.7±127.95[a] | 241.74±54.47[a] | 92.19 |
| 12. | Sulfate | 15247.5±609.9 | 6792.0±474.84[a] | 758.54±123.74[a] | 88.83 |
| 13. | Phenol | 2040.0±295.68 | 940.0±6.4[a] | 108.47±5.21[a] | 88.51 |
| 14. | Phosphate | 5.36±0.168 | 2.45±0.01[a] | 0.46±0.01a | 91.41 |
| 15 | Heavy Metals | | | | |
| A | Fe | 41.50±1.57 | 29.88±3.52[a] | 0.84±0.001[a] | 97.16 |
| B | Zn | 1.12±0.00 | 0.91±0.00[a] | 0.10±0.00[a] | 89.01 |
| C | Ni | 0.25±0.00 | 0.15±0.00[a] | 0.02±0.00[a] | 86.66 |
| D | Mn | 23.54±0.70 | 14.28±0.00$_a$ | 2.45±0.00[a] | 82.84 |
| E | Pb | 0.18±0.00 | 0.07±0.00[a] | 0.001±0.00[a] | 98.57 |
| F | Cu | 1.24±0.00 | 0.80±0.00[a] | 0.002±0.00[a] | 99.75 |
| G | Cd | 0.08±0.00 | 0.03±0.00[b] | BDL | - |

All the values are in (mg $L^{-1}$) and means of three replicate (n=3)±SD except pH and C.I.: colour intensity (Pt-Co), Student's *t*-test (two tailed as compared to untreated sample), where *Highly significant at $p < 0.001$, [b] Less significant at $p < 0.05$

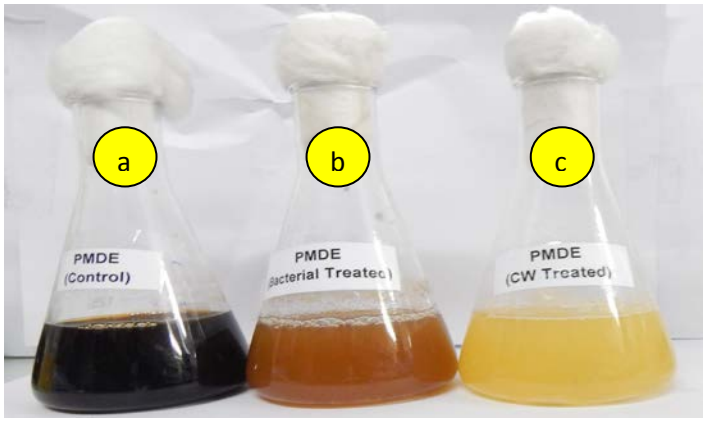



**Fig**. **9.4** Erlenmeyer flask showing decolourisation of PMDE (a) untreated (b) after bacterial treatment (c) after pilot scale HSSF-CW plant treatment system

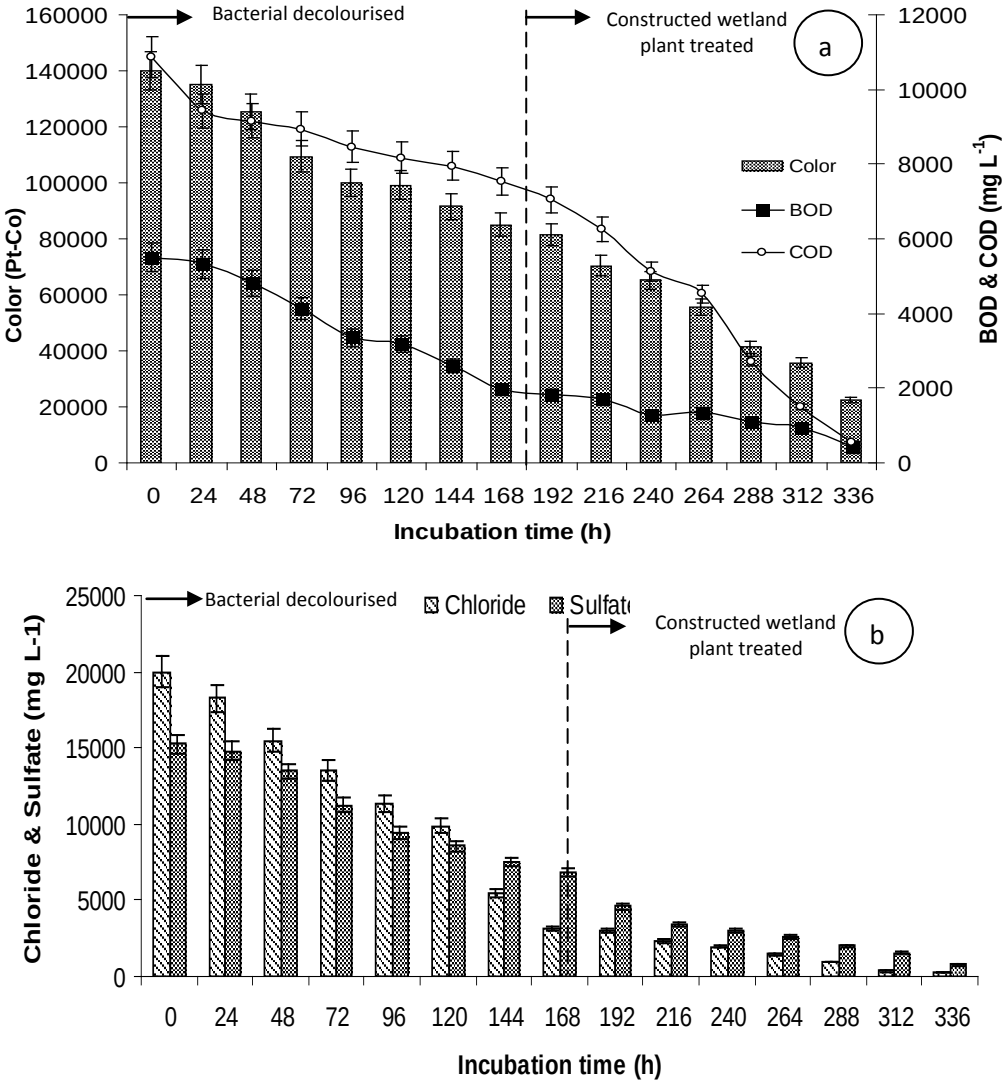



**Fig. 9.5** Reduction in pollution levels of PMDE as a result of two step treatment of bacteria and constructed wetland plant treatment systems (a) reduction of BOD, COD and color (b) chloride and sulfate

### 9.3.4 Characterization of various metabolites

The UV-Vis spectrophotometry analysis revealed that melanoidins containing PMDE showed different absorption peaks in the UV region and their maximum absorbance was noted at $\lambda_{max}$ 250 nm. In addition, PMDE also showed different stable peaks in a range of between 200-400 nm (Fig. 9.6a). The peaks were decreases as the day progresses, which showed the decolourization of PMDE by bacterial consortium at the end of the 168 h of incubation. UV-visible spectroscopic analysis indicates loss of melanoidins and other compounds peaks and a significant spectral shift indicating biotransformation of melanoidins to some other metabolites. The formation of various peaks in UV and visible region indicated the presence of complex mixture of early, advanced and final MRPs (Echvarria et al. 2013a,b).

HPLC analysis of untreated samples showed a single major peak at RT of 3.40 min, while in bacteria as well as CW treated sample was less compared to untreated sample clearly indicates the capability of the bacterial consortium to decolorize and degrade the PMDE (Fig. 9.6b). In addition, there were some extra peaks appeared in bacterial and CW treated sample suggesting the formation of several new metabolites. The HPLC chromatogram also showed shifting of peak, decrease in peak area height, and generation of some new peaks compared to untreated sample. This suggested the biotransformation and biodegradation of melanoidins in various metabolites by developed bacterial consortium. Our findings are well corroborated with the earlier results reported by

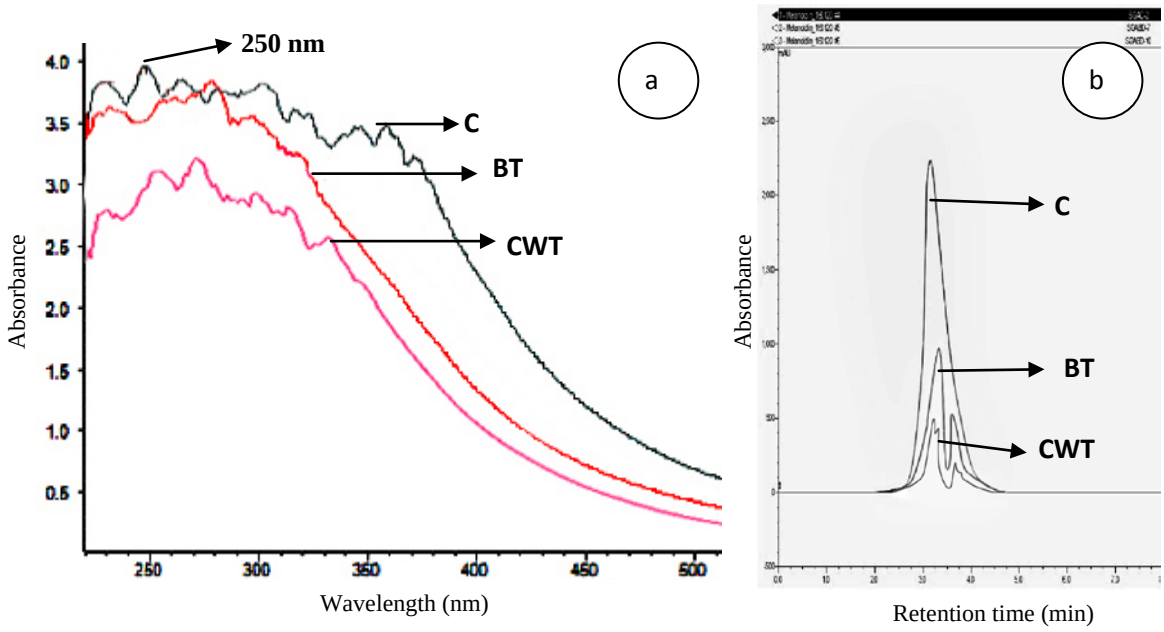



**Fig. 9.6** Analysis of control and treated PMDE at different time interval by (a) UV-visible spectrum analysis (b) HPLC analysis (at 250 nm). BT: bacterial treated; CWT: constructed wetland treated

In the present study, the GC–MS analysis of ethyl acetate extracted untreated PMDE showed the existence of different types of organic pollutants, most of which were biotransformed and biodegraded during bacterial and CW plant treatment (Fig. 9.7 &

Table 9.2). The details of identified compound in untreated PMDE has been described in chapter no. 03. However, the analysis of bacteria treated PMDE has showed the existence of various organic compounds such as, acetamide, 2,2,2-trifluoro-N-methyl-(TMS) (RT: 8.07), β-eudesmol, TMS ether (RT: 8.25), propanoic acid, 3-[(TMS)oxy]- TMS ester (RT: 9.87), phenylethanolamine TMS (RT: 11.72), octanoic acid, TMS ester (RT: 12.46), benzeneacetic acid, TMS ester (RT: 13.15), pyrrole-2-carboxylic acid, N-TMS-, TMS ester (RT: 14.27), nonanoic acid, TMS ester (RT: 14.57), tetradecane (RT: 15.44), decanoic acid, TMS ester (RT: 16.64), silane, trimethyl(undecycloxy) (RT: 16.88), benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester (RT: 18.17), palimitic acid, 2(tetradecycloxy)ethyl ester (RT:19.31), hexadecen, 1-ol, trans-9 (RT: 19.38), hexadecane (RT: 19.55), dodecenoic acid, TMS ester (RT: 20.50), dotriacontane (RT: 22.32), 7-ethyl-3-methyl-8-(propylsulfany)-3,7-dihydro-1H-purine-2,6-dione (RT: 22.63), pentatriacontane (RT: 23.08), eicosane (RT:23.22), β-D-glucopyranoside, methyl 2,3-bis-O-(TMS)-, cyclic methylboronate (RT:23.89), 2,5-cyclohexade-1,4-dione,2,6-bis(1,1-dimethylethyl) (RT: 24.10), ethanol, 2-(octadecycloxy) (RT: 26.41), eicosane (RT: 26.53), octatriacontyl pentafluoropropionate (RT: 29.47), docosane (RT: 29.56), cis-10-nonadecenoic acid, TMS ester (RT: 31.16), tetrapentacontane, 1,54-dibromo (RT: 33.94), 2-monostearin TMS ether (RT: 35.09), and octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester (RT:35.37). These compounds were completely absent in untreated sample, they are the degraded products of melanoidins and few as new metabolites. Similar, compounds have been identified in distillery effluent after treatment with *Emericella nidulans* var. lata, *Neurospora intermedia* followed by *Bacillus* sp. in three stage bioreactor (Kaushik et al. 2010). Most of the metabolic products have been also reported from distillery effluent by fungal treatment (Gonzalez et al. 2000) and also as degradation products of synthetic and natural melanoidin after bacterial degradation as published in earlier studies (Bharagava and Chandra 2010; Chandra et al. 2009). However, several organic compounds detected in untreated sample were diminished after bacterial treatment. This suggested that complex and high molecular weight compounds were degraded by bacterial consortium with the help of extracellular ligninolytic enzymes.

The GC-MS analysis of wetland plant rhizosphere treated PMDE revealed one major peaks at different RT: 27.32 (Fig. 9.7), which corresponded to o-trimethylsilyl-cannabinol. Several minor peaks were also noted at RT: 8.12, 9.87, 15.44, 20.50, 22.63, 26.41, 30.26, 35.09, and 37.11, corresponding to D-lactic acid-DITMS; propanoic acid, 3-[(TMS)oxy]- TMS ester; tetradecane; dodecenoic acid, TMS ester; 7-ethyl-3-methyl-8-(propylsulfany)-3,7-dihydro-$^{1}$H-purine-2,6-dione; ethanol, 2-(octadecycloxy); 1-monolinoleoyglycerol TMS ester; 2-monostearin TMS ether; and dotriacontane, respectively. All these observations have revealed that the developed bacterial consortium and unculturable bacterial communities growing in wetland plant rhizosphere exposed to PMDE are capable for efficient degradation and metabolization of organic and inorganic pollutants present in PMDE. The disappearance of most of organic compounds from bacteria and wetland plant rhizosphere treated PMDE could be related with color removal associated with bacterial degradation of organic and inorganic pollutants.

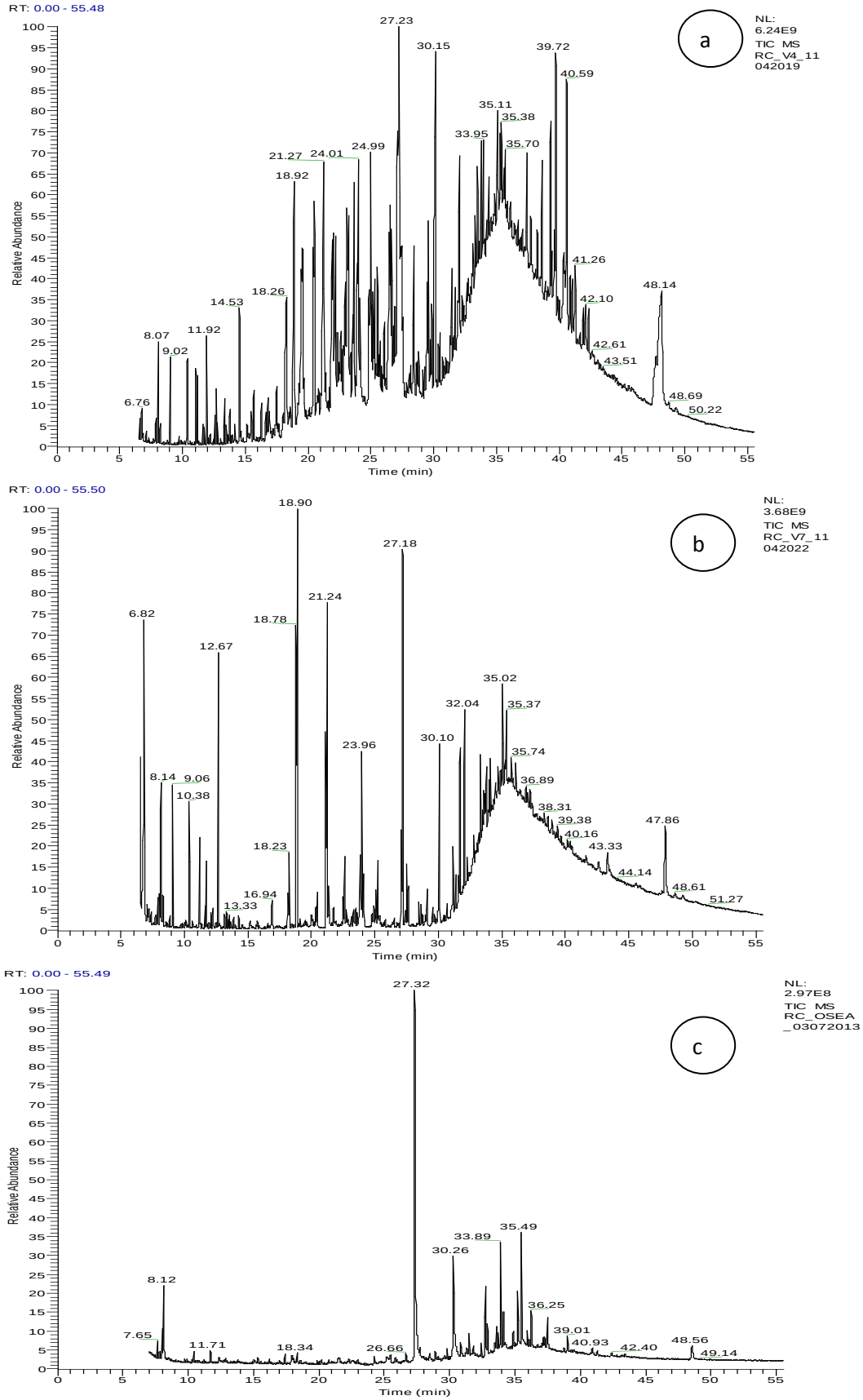



**Fig. 9.7** GC-MS analysis of untreated and treated PMDE samples extracted in ethyl acetate; (a) control (b) bacterial treated (c) constructed wetland treated

**Table 9.2** Compounds identified by GC–MS analysis extracted with ethyl acetate from PMDE

| S. No. | Name of Compound | RT | C | BT | CWT |
|---|---|---|---|---|---|
| 1. | Acetamide, 2,2,2-trifluoro-N-methyl-(TMS) | 8.07 | + | + | - |
| 2. | D-Lactic acid-DITMS | 8.12 | - | - | + |
| 3. | 2-Butanol, tert-butyldimethylsilyl ether | 8.25 | + | - | - |
| 4. | β-Eudesmol, TMS ether | 8.25 | - | + | - |
| 5. | Ethanedioic acid, bis(TMS)ester | 9.03 | + | - | - |
| 6. | Pyridine, 3-trimethylsiloxy | 9.73 | + | - | - |
| 7. | Propanoic acid, 3-[(TMS)oxy]- TMS ester | 9.87 | - | + | + |
| 8. | Phenylethanolamine TMS | 11.72 | - | + | - |
| 9. | Silanamine, 1,1,1-trimethyl-N-(TMS)-N-[2(TMS)oxy]ethyl | 11.73 | + | - | - |
| 10. | 3-Hydroxy-6-methypyridine 1TMS | 11.92 | + | - | - |
| 11. | Octanoic acid, TMS ester | 12.46 | - | + | - |
| 12. | Benzeneacetic acid, TMS ester | 13.15 | - | + | - |
| 13. | Pyrrole-2-carboxylic acid, N-TMS-, TMS ester | 14.27 | - | + | - |
| 14. | Nonanoic acid, TMS ester | 14.57 | - | + | - |
| 15. | 1,4-Dimethylpyrrolo(1,2-A)pyrazine | 15.12 | + | - | - |
| 16. | Tetradecane | 15.44 | - | + | + |
| 17. | Docosane | 15.45 | + | - | - |
| 18. | 1-Galactopyranone, 6-deoxy-1,2-bis-O-(TMS)-cyclic methylboronate | 15.67 | + | - | - |
| 19. | Benzenepropanoic acid, TMS ester | 15.75 | + | - | - |
| 20. | Decanoic acid, TMS ester | 16.64 | + | + | - |
| 21. | Silane, trimethyl(undecycloxy) | 16.88 | + | + | - |
| 22. | Benzeneacetic acid, α,4-bis[(TMS)oxy], TMS ester | 17.49 | + | - | - |
| 23. | Emetan, 1’,2-didehydro-6’,7’,10,11-tetramethoxy | 18.02 | + | - | - |
| 24. | Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester | 18.17 | - | + | - |
| 25. | 2,4-Imidazolidinedione, 1-[[(5-nitro-2-firnayl)methylene]amino | 19.26 | + | - | - |
| 26. | Palimitic acid, 2(tetradecycloxy)ethyl ester | 19.31 | - | + | - |
| 27. | 1-Hexadecanol, 2-methyl | 19.32 | + | - | - |
| 28. | Hexadecen, 1-ol, trans-9 | 19.38 | - | + | - |
| 29. | Hexadecane | 19.55 | + | + | - |
| 30. | 5-Hydroxy-2,2-dimethyl-5,6-bis(2-oxo-1-propyl)-1-cyclohexanone | 19.71 | + | - | - |
| 31. | Dodecenoic acid, TMS ester | 20.50 | + | + | + |
| 32. | 2-Propenoic acid, oxybis(methyl-2,1-ethanediyl)ester | 21.24 | + | - | - |
| 33. | Benzoic acid, 2,5-bis(TM Soxy)-TMS ester | 21.93 | + | - | - |
| 34. | 1-Tetradecene, 2-decyl | 22.20 | + | - | - |
| 35. | Dotriacontane | 22.32 | - | + | - |
| 36. | 1,2-Propanediol, 3-(octadecycloxy)-diacetate | 22.59 | + | - | - |
| 37. | 7-Ethyl-3-methyl-8-(propylsulfany)-3,7-dihydro-1H-purine-2,6-dione | 22.63 | - | + | + |
| 38. | Anthacene | 22.93 | + | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 39. | Pentatriacontane | 23.08 | - | + | - |
| 40. | Eicosane | 23.22 | - | + | - |
| 41. | β-D-Glucopyranoside, methyl 2,3-bis-O-(TMS)-, cyclic methylboronate | 23.89 | + | + | - |
| 42. | 2,5-Cyclohexade-1,4-dione,2,6-bis(1,1-dimethylethyl) | 24.10 | - | + | - |
| 43. | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | 24.84 | + | - | - |
| 44. | 2-Butanone, 4-[2-isopropyl-5-methyl | 25.15 | + | - | - |
| 45. | Hexadecanoic acid, methyl ester | 27.14 | + | - | - |
| 46. | O-Trimethylsilyl-Cannabinol | 27.32 | - | - | + |
| 47. | Ethanol, 2-(octadecycloxy) | 26.41 | - | + | + |
| 48. | Eicosane | 26.53 | - | + | - |
| 49. | 2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl) | 26.63 | + | - | - |
| 50. | Octadecanoic acid, methyl ester | 30.14 | + | - | - |
| 51. | Octatriacontyl pentafluoropropionate | 29.47 | - | + | - |
| 52. | 17-Pentatriacontene | 29.48 | + | - | - |
| 53. | 1,4-Ethano-1,2,3,4-tetrahydroanthracen-3-ol-benzylidene | 29.85 | + | - | - |
| 54. | Docosane | 29.56 | - | + | - |
| 55. | 1-Monolinoleoyglycerol TMS ester | 30.26 | - | - | + |
| 56. | cis-10-Nonadecenoic acid, TMS ester | 31.16 | - | + | - |
| 57. | Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) | 31.70 | + | - | - |
| 58. | Tetrapentacontane, 1,54-dibromo | 33.94 | - | + | - |
| 59. | 2-monostearin TMS ether | 35.09 | - | + | + |
| 60. | Octadecanoic acid, 2,3-bis[(TMS)oxy]propyl ester | 35.37 | - | + | - |
| 61. | Dotriacontane | 37.11 | - | - | + |
| 62. | Butyl 11-eicosenoate | 37.41 | + | - | - |
| 63. | Cholest-8(14)-en-3-one, (5α) | 39.31 | + | - | - |
| 64. | α-Homocholest-4a-en-3-one | 40.58 | + | - | - |

+: present; −: absent; C: control; BT: bacterial treated; CWT: constructed wetland treated; RT: retention time (in min)

### 9.3.5 Bacterial communities analysis

#### 9.3.5.1 Analysis of sequencing data/Diversity analysis and richness of OTUs

When compared to other molecular methods, 16S rDNA gene sequencing technologies provide a more complete, efficient and effective DNA amplification of bacterial population in compost samples. 16S rRNA gene sequencing of the V3-V4 hypervariable regions is a powerful tool to estimate total bacterial abundance and diversity (Rosselli et al. 2016). In this study, a total of 1191014 and 901757 high quality reads with an average length of 250 bases were obtained from **DS1** (Control), and **DS2** (treated), respectively using Illumina MiSeq sequencing analysis (Table 9.3). After quality filtering, the MiSeq sequencing yielded 1189451 and 900797 reads of DS1 and DS2, respectively. A total of 621897 OTUs were identified from 1904491 reads. From 621897 total OTUs, 539635 singletons were removed and 82262 OTUs were selected for further analysis (Table 9.4). Overall, a total of 82262 OTU were generated after clustering at 97% similarity level from

1904491 reads. The graphical representation of reads and OTU proportion is shown in Fig. 9.8.

**Table 9.3** Illumina MiSeq sequencing raw read summary

| Sample | Total reads | Passed conserved region filter | Passed mismatch filter |
|---|---|---|---|
| DS1 | 1191014 | 1191014 | 1189451 |
| DS2 | 901757 | 901757 | 900797 |

**Table 9.4** Pre-processing of Illumina MiSeq sequencing sequence read data

| Sample | Consensus Read | Chimeric Sequences | Pre-processed reads |
|---|---|---|---|
| DS1 | 1189451 | 113453 | **1075931** |
| DS2 | 900797 | 72237 | **828560** |
| | | **Total** | **1904491** |

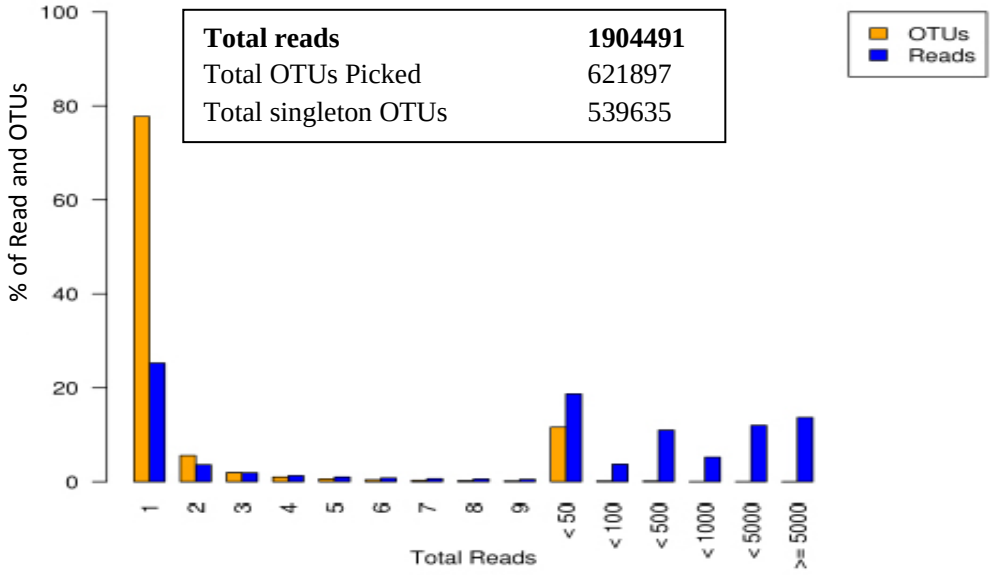



**Fig. 9.8** Graphical representation of reads and OTU distribution from DS1 and DS2. The blue bar represents percentage of total OTUs in the read-count groups. The red bar represents percentage of total read contributed by the OTUs in the read-count group.

The average GC content distribution of the sequenced read of the DS1 and DS2 is shown in Fig. 9.9. The x-axis represents average GC content in the sequence and y-axis represents percentage of sequences. The reads have GC content in the range 30-60%.

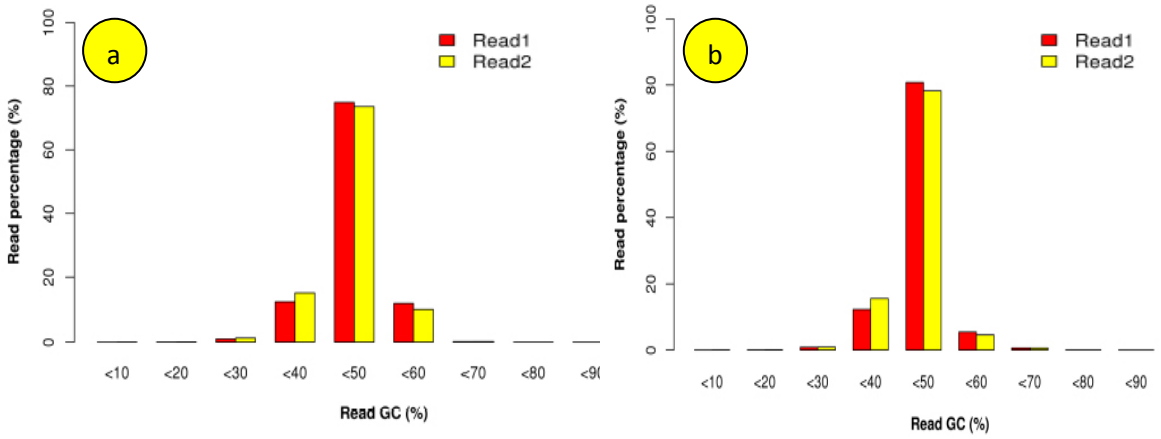



**Fig.9.9** Read GC distribution of (a) DS1 (b) DS2

The rarefaction curves tended to approach the saturation plateau in DS1 and DS2 samples (Fig. 9.10). Rarefaction curves demonstrated that OTU abundance were diverse between DS1 and DS2. The alpha diversity estimation demonstrated that the diversity of bacterial communities is abundant in DS1. Higher Shannon and Chao 1 indices indicated the rhizospheric bacterial community diversity of DSI and DS2. The larger Shannon index values, the higher alpha diversity. The results suggested that these libraries detected a large majority of the rhizospheric bacterial diversity in the samples used in our study.

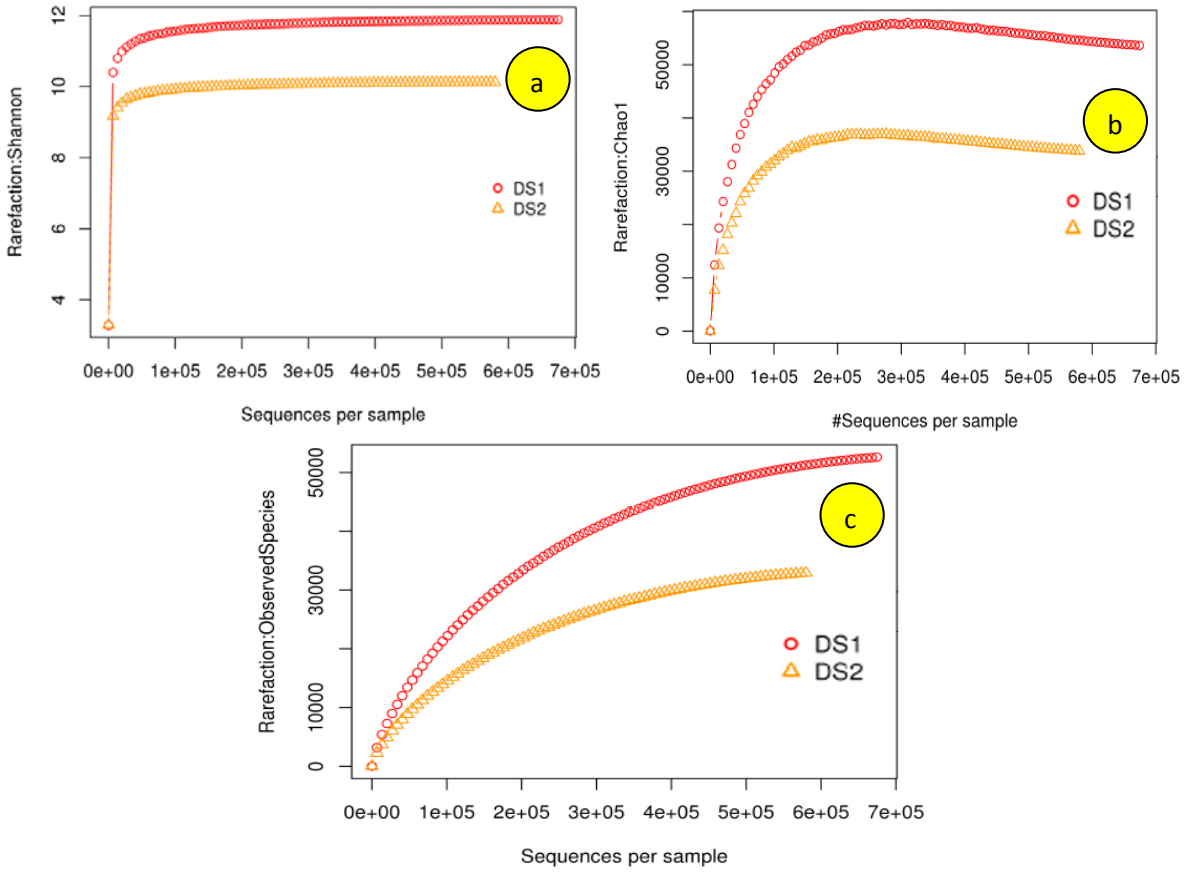
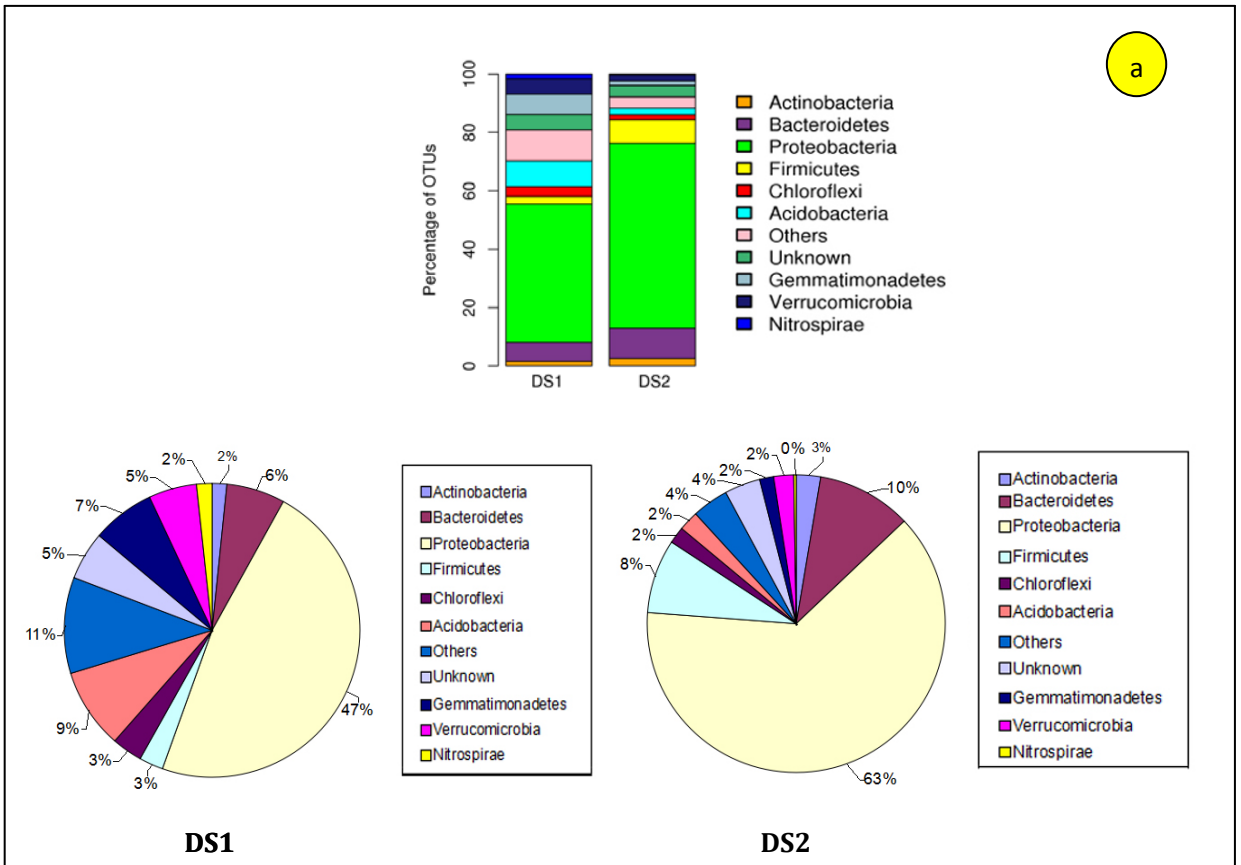



**Fig. 9.10** Rarefaction curves obtained from the DS1 and DS2 (a) Shannon (b) Chao1 and (c) species curve

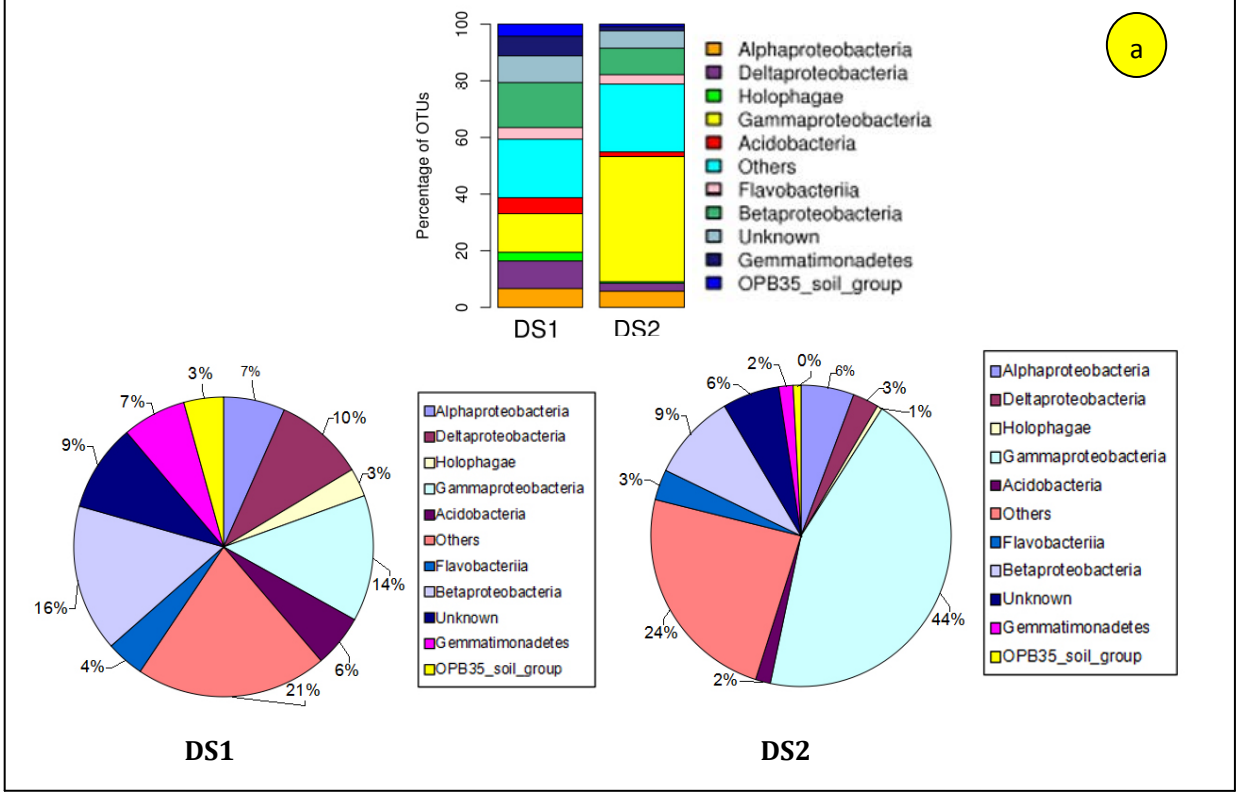
The diversity and composition of microbial communities in the substrate also plays a critical role in the pollutants removal in CWs. The phylum, class, order, family, genus and species of bacterial communities at OUT level is summarised in Fig. 9.11. Only top 10 enriched class categories are shown in the figure. In DS1 and DS2, the ten represented dominant phyla were *Proteobacteria* (47-63%), *Acidobacteria* (9-2%), *Bacteroidetes* (6-10%), *Gematimonadetes* (7-2%), *Verrucomicroia* (5-2%), Unknown (5-4%), *Firmicutes* (3-8%), *Actinobacteria* (2-3%), *Chloroflexi* (3-2%) and *Nitrospirae* (2-0.26%) (Fig. 9.11a). It should be noted that the sequences do not have any alignment against taxonomic database are categorised as Unknown. While, the classes other than top ten results as categorised as Other. However, an unidentified bacterial phylum (other; 11-4%) was also found among top 10 bacterial phyla. At class level in DS1 and DS2, the top ten represented class were *Betaproteobacteria* (16-9%), *Gammaproteobacteria* (13-44%), *Deltaproteobacteria* (10-3%), Unknown (9-6%), *Alphaproteobacteria* (7-6%), *Gemmatimonadetes* (7-1%) *Acidobacteria* (6-2%), *Flavobacteria* (4-3%), OPB35 soil group (4-1%), and *Holophage* (3-1%) (Fig. 9.11b). Overall, 21% (DS1) and 24% (DS2) sequences of total read were not classified at the class level, suggesting that the identities of these bacteria are unknown.

At the order level in DS1 and DS2, the top ten represented order were Unknown (17-12%), *Burkholderiales* (6-7%), *Oceanospirillales* (6-5%), *Myxococcales* (5-2%), *Flavobacteriales* (4-3%), *Nitrosomonadales* (4-1%), *Rhodobacterales* (4-2%), *Pasteurellales* (3-1%), S0134 terrestrial group (3-0.31%), and *Gemmatimonadales* (3-1%), However, an unidentified bacterial order (other; 45-66%) was also found among top 10 bacterial order as shown in Fig. 9.11c. At the family level in DS1 and DS2, the top ten represented family were Unknown (29-16%), *Commanadaceae* (6-1%), *Flavobacteraceae* (4-3%), Uncultured bacterium (4-1%), *Oceanospirillaceae* (4-3%), *Rhodobacteraceae* (4-2%), *Gemmatimonadaceae* (3-1%), *Nitrosomonadaceae* (3-1%), *Pasterurellaceae* (3-1%), and *Cystobacteraceae* (2-1%) (Fig. 9.11d). However, an unidentified bacterial family (Others; 38-70%) was also found among top 10 bacterial families.

Further, at the genus level in DS1 and DS2, the top ten most abundant genus were Unknown (49-31%), Uncultured (9-4%), Uncultured bacterium (6-2%), *Oleibacter* (3-1%), *Alcanivorax (*2-2%), *Thiobacillus* (2-0%), *Actinobacillus* (2-1%), *Anaeromyxobacter* (2-1%), *Streptococcus* (1-0%), and *Nevskia* (1-0%). However, an unidentified bacterial genus (Others; 23-58%) was also found among top 10 bacterial genus (9.11e). While at the species level the top ten most abundant species in DS1 and DS2 were Uncultured *Deltaproteobacterium* (0.49-0.09%), Uncultured bacterium (14-11%), Uncultured *Thiobacillus* sp. (0.45-0.06%), Uncultured prokaryote (1-1%), Uncultured *Betaproteobacterium* (1-0.05%), Uncultured soil bacterium (1-0.28%), Unknown (71-72%), Uncultured organism (2-1%), Uncultured *Alcanivorax* sp. (1-0.37%) and Uncultured *Gemmatimonadetes* bacterium (0.50-0.09%). However, an unidentified bacterial species (Others; 9-14%), was also found among top 10 bacterial species (Fig. 9.11f).




**DS1** **DS2**




**DS1** **DS2**

***Figure conti....***

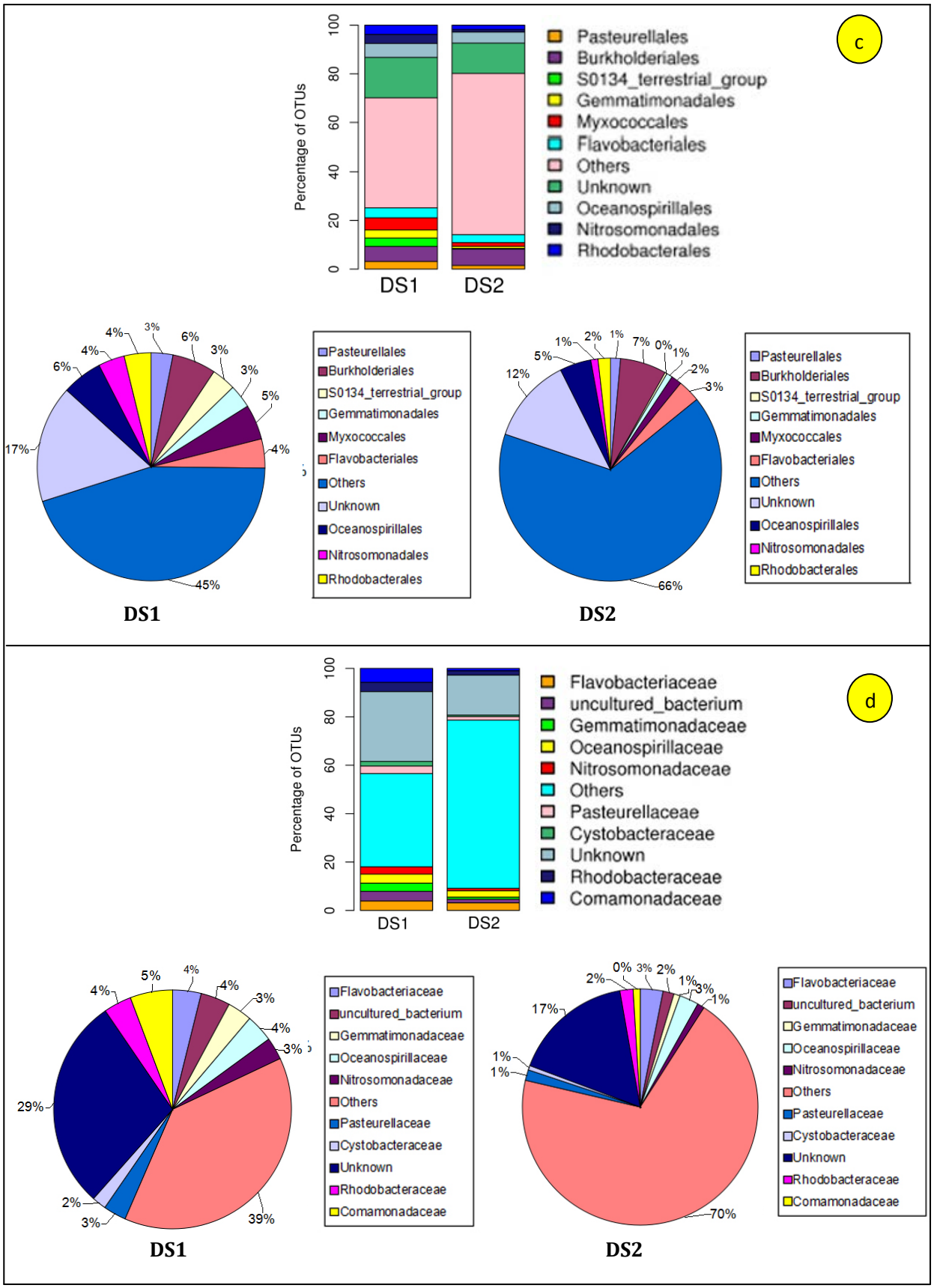



***Figure conti....***

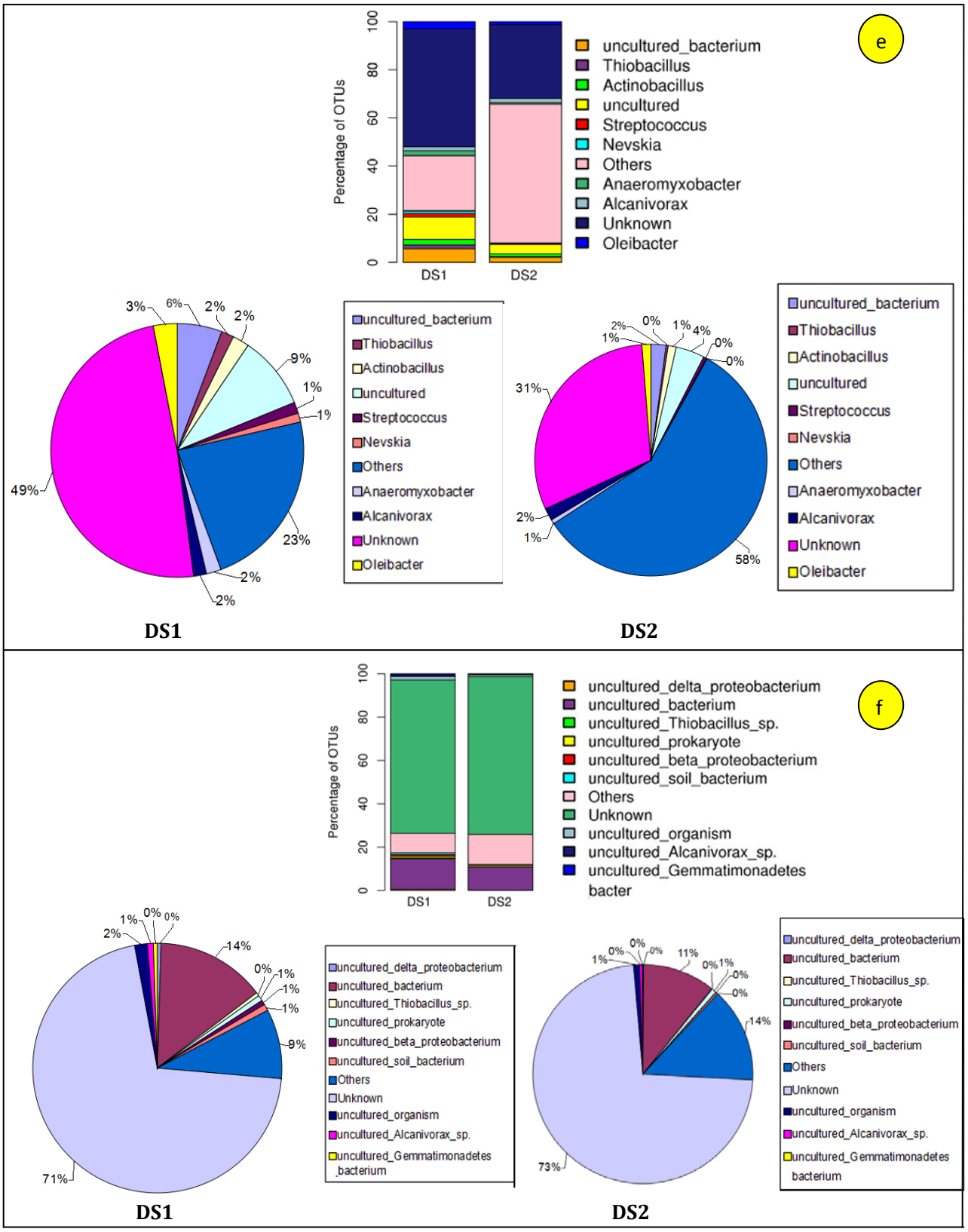



**Fig. 9.11** Pie chart showing taxonomic distribution of OUT at different phylogenetic level; (a) phylum, (b) class, (c) order (d) family (e) genus and (f) species at in DS1 and DS2 based on metagenomic sequencing data. Only top 10 enriched class categories are shown in the figure. The taxonomic classes other than top 10 results are categorised as others. The sequences with very lesser similarity or whose V3-V4 region do not have any alignment hits against taxonomic database are cateogrised as Unknown.

### 9.3.5.2 Microbial community structures at the phylum, class and genus level

For a better understanding of the rhizospheric microbial community structure of *P. communis* in sludge, taxonomic affiliation at different levels was analyzed (Fig.9.12). Using Illumina sequencing of the V3-V4 hypervariable region of bacterial 16S rRNA genes and metagenomic library analysis, we observed conspicuous differences in rhizospheric bacterial community structure of DS1 and DS2. The relative abundance of bacteria at the phylum, class and genus levels among the DS1 and DS2 is shown in Fig. 9.12.The results demonstrated that the root rhizobacteria had significantly different community structures and species abundance. In DS1, *Proteobacteria* (38%) represented the most abundant phylum, followed by *Chlorobi* (15.0%) and *Acidobacteria* (10%). The other predominant phyla in the DS1 were *Chloroflexi* (9%), *Gemmatimonadeted* (8%), *Thaumarchaeota* (5%), *Euryarcheota* (4%), Verrucomicrobia (3%), and *Nitrospirae* (2%) (Fig. a). It was also noted that besides the dominant microbes, as many as 1.96% of total sequences could not be assigned to any phylum. In DS2, the bacterial phyla distributions were *Proteobacteria* (50%), *Bacteriodetes* (33%), *Firmicutes* (5%). The other predominant phyla in the DS2 were *Gemmatimonadetes* (2%), *Choloroflexi* (2%), and *Tenericutes* (2%). *Proteobacteria* was the most abundant phylum in all these sludge samples and they gradually increased by 50% in CW treated PMDE sludge. Proteobacteria are the largest phylum within the bacteria domain and account for the vast majority of the known Gram-negative bacteria (Gupta 2000). *Proteobacteria* encompass an enormous level of morphological, physiological, and metabolic diversity, thus playing a significant role in nutrient recycling (Glowa et al. 2003). Members of the phylum *Proteobacteria* are believed to be involved in the removal of organic pollutants, such as nitrogen, phosphorus and phenolic compounds. *Proteobacteria* is a highly diverse phylum composed of chemolithoautotrophs, heterotrophs, and mixotrophs and subdivided into five classes (Alpha, Beta, Gamma, Delta and Epsilon-*Proteobacteria*). This findings was consistent with a plenty of studies that reported *Proteobacteria* as the dominant phylum in various microorganism sample from the rhizosphere in wetland (Wang et al. 2015; Chen et al. 2015). Similar results were present in previous studies, which found that *Proteobacteria* was the dominant community grown in rhizospheric zone of *P. cummunis* during PMDE treatment (Chandra et al. 2012). In out study, *Bacteriodetes* was the second highest represented phylum found in DS2. Members of the phylum *Bacteroidetes* have colonized many different ecological niches, including soil, ocean, and freshwater, where they display various biological functions. In particular, they are well known degraders of polymeric organic matter (Thomas et al. 2011).

At the class level, the most predominant bacteria in DS1 were affiliated with *Alphaproteobacteria* (14%), *Flavobaceteria* (11%), *Gammaproteobacteria* (11%), *Betaproteobacteria* (9%), *Gemmatimonadetes* (9%), *Acidobacteria* (8%), Anaerolineae (6%), and *Deltaproteobacteria* (6%). The other predominant phyla in the DS1 were *Holophage* (3%), *Halobacteria* (2%), *Nitrospira* (2%), Uncultured Bacterium (1%), and Aradenticatenia (1%). While in CW treated sample (DS2), represented the most abundant bacteria affiliated with *Gammaproteobacteria* (36%) followed by *Sphingobacteria* (19%),

*Aplhaproteobacteria* (11%), *Flavobacteria* (9%), Actinobacteria (6%), *Bacilli* (5%), *Cytophagia* (5%), *Betaproteobacteria* (3%), *Mollicutes* (2%), and *Deltaproteobacteria* (1%). Gammaproteobacteria are a class of medically, and ecologically important groups of bacteria, such as the *Enterobacteriaceae* (e.g., *E. coli*), *Vibrionaceae, Pseudomonadaceae,* and *Xanthomonadaceae* (e.g., *Stenotrophomonas maltophilia*). An exceedingly large number of important pathogens belong to this class, such as *Salmonella, Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. However, members of this group exhibit broad ranges of aerobicity, of trophism, including chemoautotrophism and photoautotrophism, and of temperature adaptation. Hence, within *Proteobacteria*, *Gammaproteobacteria* was the most dominant group in our study (Fig. 9.12). This finding was similar to the results of previous studies, which revealed that the *Gammaproteobacteria* was the predominant one within *Proteobacteria* in constructed wetland treated PMDE in two stage sequential treatment system (Chandra et al. 2012).

Among the assigned taxa at genus level in DS1, the most abundant genera were affiliated with Unknown (51%), followed by Uncultured (16%), bacterium (10%), Uncultured bacterium (10%), *Winogradskyella* (4%), *Actinobacillus* (3%), Oleibacter (3%), *Ruegeria* (3%), *Anaerolinea* (2%), *Thiobacillus* (2%), *Anaeromyxobacter* (2%), and *Nevskia* (2%), and *Alcanivorax* (1%). In DS2, the most predominant bacteria were affiliated with *Rheinheimera* (21%) followed by *Sphingobacterium* (17%), Idiomarina (8%). *Acidothermus* (4%), *Pseudomonas* (2%), *Flavobacterium* (2%), Uncultured bacterium (2%), *Parapedobacter* (2%), *Alcanivorax* (2%), Uncultured (4%), *Bacillus* (3%), *Acholeplasma* (2%), *Hyphomonas* (1%), and *Aquamicrobium* (1%). The genus Rheinheimera, a branch of the *Gammaproteobacteria*, has been detected in our study. Species of *Rheinheimera* are able to grow on and to degrade rapidly the more easily degradable organic matter. This genera present in CWs treating high salinity tannery wastewater reported by Calherios et al. (2010). The second most common genus detected in our study was *Sphingobacterium*. *Sphingobacterium* is the type genus of the family *Sphingobacteriaceae* in the phylum *Bacteroidetes* (Steyn et al. 1998). In our study, *Alcanivorax* was the primary genera found in DS1 and DS2. *Alcanivorax* account for 1.07 % of the sequence obtained from DS1 while it occupied 1.25% in DS2. Hence, our findings indicated that these genera could be well adapted into the stress environments. It should be noted that 29.75 and 22.90% of the DS1 and DS2 sequences were not assigned to any genera, suggesting that many novel microbes were existing in the sludge that needed to be further explored. In DS2, it was showed that 1.32% of total sequence belonged to *Pseudomonas*. The genus of *Pseudomonas* harbors a great deal of metabolic diversity, some of which are able to metabolize diverse chemical pollutants such as *Pseudomonas putida* and *Pseudomonas alcaligenes* (Wu et al. 2011). However, the role of most of the above genera was not well understood but they have been identified using 16S rDNA gene sequencing in our PMDE sample after treatment with constructed wetland.

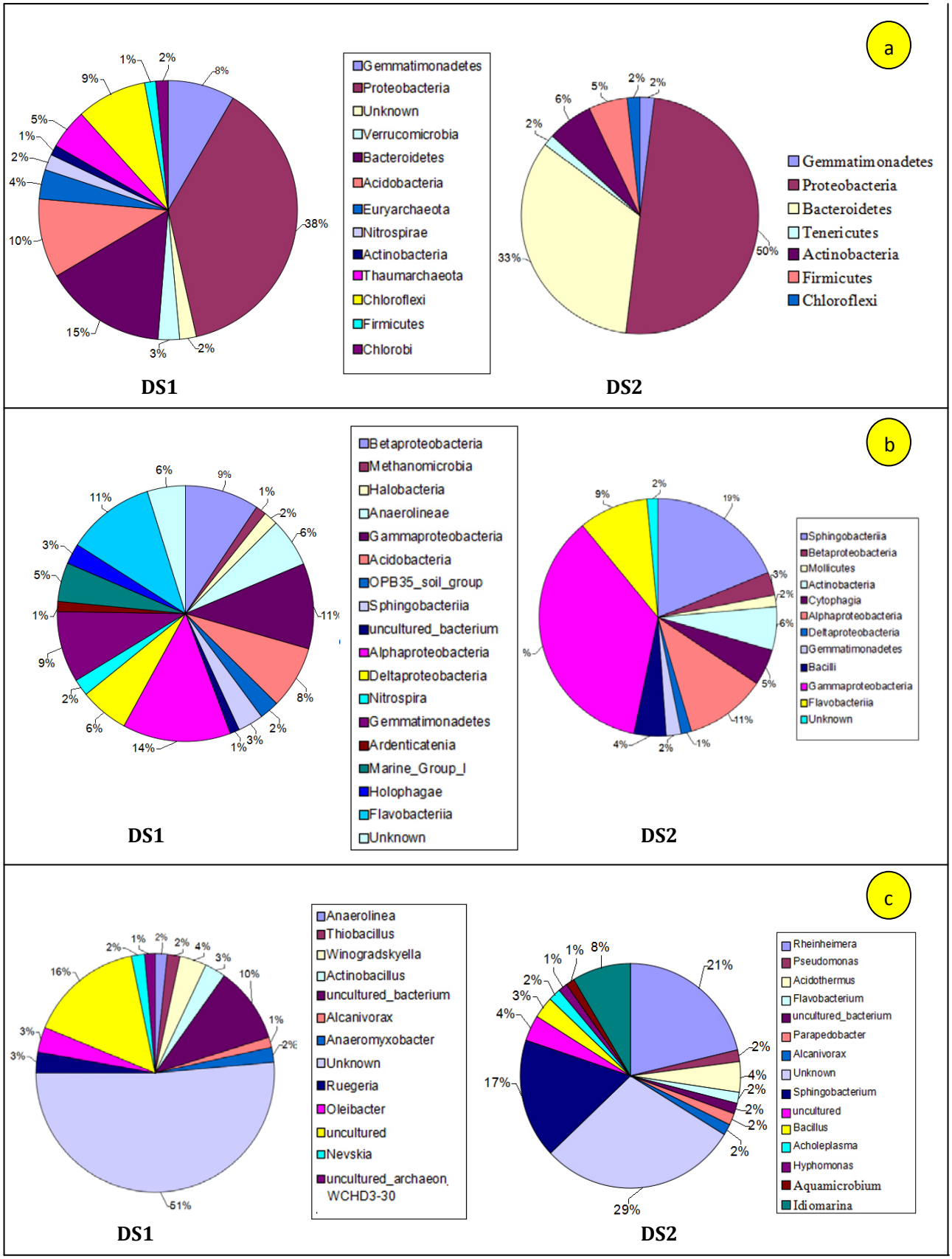



**Fig. 9.12** The composition and relative abundance of the bacterial communities into DS1 and DS2 at the (a) phylum (b) class and (c) genus level. Sequences that could not be classified into any known group were assigned as unknown.

**Table 9.5** Major genera with the sequence percentage above 1% in only one sludge sample

| **Bacteria Genus** | **Abundance %** | | **Bacteria Genus** | **Abundance %** | |
|---|---|---|---|---|---|
| | **DS1** | **DS2** | | **DS1** | **DS2** |
| *Aquamicrobium* | 0.00 | 1.00 | *Flavobacterium* | 0.00 | 1.25 |
| *Anaerolinea* | 1.25 | 0.00 | *Hyphomonas* | 0.00 | 0.00 |
| *Acidothermus* | 0.00 | 3.44 | uncultured_bacterium | 7.9 | 1.19 |
| *Bacillus* | 0.00 | 2.44 | ***Alcanivorax*** | **1.07** | **1.25** |
| *Rheinheimera* | 0.00 | 16.72 | *Anaeromyxobacter* | 1.55 | 0.00 |
| *Thiobacillus* | 1.33 | 0.00 | **Unknown** | **39.75** | **22.90** |
| *Pseudominas* | 0.00 | 1.32 | *Ruegeria* | 2.15 | 0.00 |
| *Parapedobacter* | 0.00 | 1.32 | *Spingobacterium* | 0.00 | 13.52 |
| *Winogradskyella* | 2.80 | 0.00 | *Oleibacter* | 2.63 | 0.00 |
| *Nevskia* | 1.38 | 0.00 | **Uncultured** | **12.03** | **2.92** |
| *Idiomarina* | 0.00 | 6.75 | Uncultured archaeon WCHD3-30 | 1.09 | 0.00 |
| *Actinobacillus* | 2.19 | 0.00 | | | |

### 9.3.5.3 Interactive metagenomic visualization in a Krona diagram

Based on the taxonomic classification of representative sequences from all OTUs, the composition of rhizospheric bacterial communities was revealed at phylum level (Fig. 4). Therefore, the Krona taxonomy visualization diagram was constructed to observe the distribution of OTUs among the sludge samples (DS1 and DS2) (Fig. 9.13). The analysis result of species annotation is visually shown by Krona, where circles from inside to outside stand for different classification levels, and the area of sector means respective proportion of different OTUs annotation results for each treatment. In addition, Krona were able to store the various metagenomic data sets and its compatibility with popular metagenomic analysis tools among the all samples in a single document. For metagenomic point, Krona provides the answer of those questions regarding the relative abundance of taxa across multiple levels of the hierarchy simultaneously. Krona is a new visualization tool that allows intuitive exploration of relative abundances and confidences within the complex hierarchies of metagenomic and bacteria diversity classifications as well as store the data from numbers of samples in a one documents. The analysis across rhizospheric samples is better left to traditional heat-map and differential bar chart visualizations. Furthermore, to the best of our knowledge, application of Krona is the first bioinformatics tool built completely on numbers of compost samples and serves as a demonstration of the power of emerging web technologies for creating widely allow to explore the relative abundance and highly accessible visualization tools for identification of bacterial diversity.

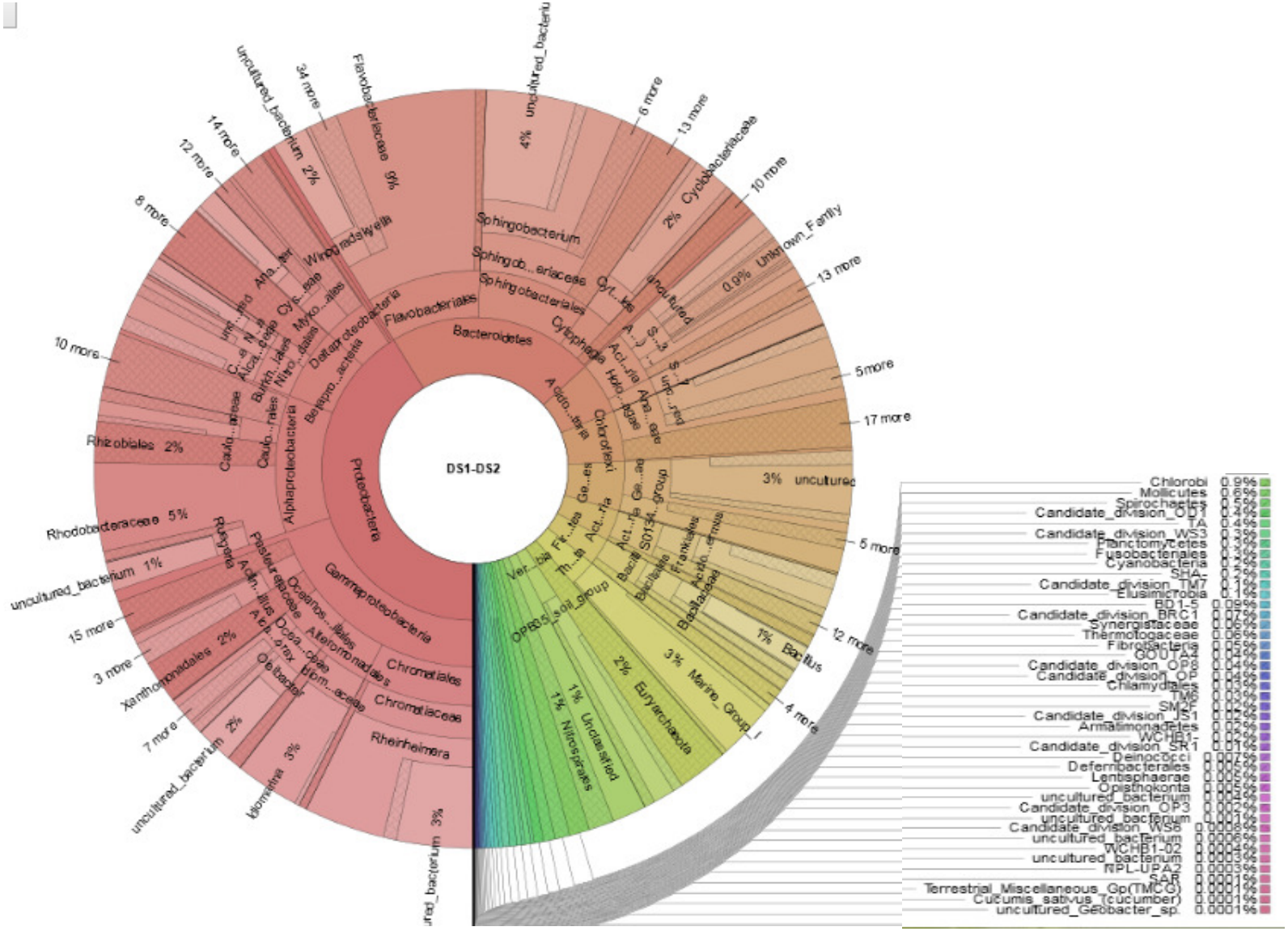
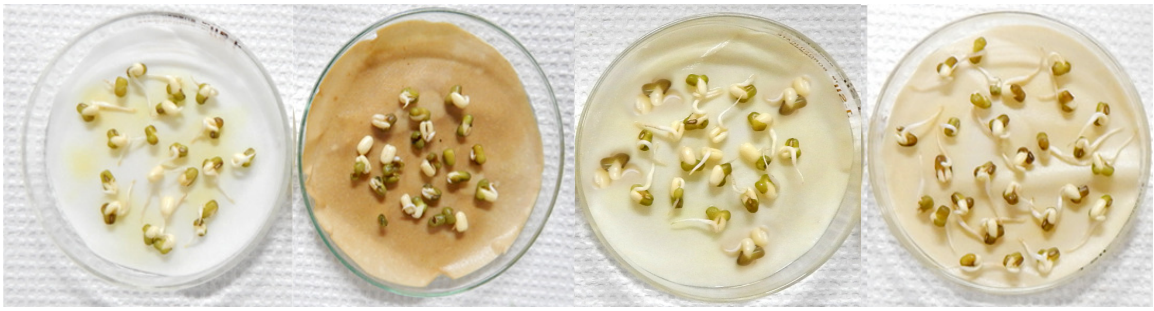



**Fig. 9.13** Krona is taxonomy web visualization; circles from inside to outside stand for different classification levels, and the area of sector means respective proportion of different OTUs annotation results.

### 9.3.6 Effect of bacteria and CW plant treated PMDE on seed germination and seedling growth of *Phaseolus mungo* L.

The seed germination test of *P. mungo* L. showed inhibitory effects of untreated, bacterial decolurised, and constructed wetland plant treated PMDE at 10 % concentrations in terms of seed germination and growth parameters of seedlings (Table 9.6 and Fig. 9.14). In our previous study (chaper no. 04) no seed germination was observed at 10% concentration of PMDE. But, after treatment with bacteria, the toxicity of PMDE was reduced extensively and showed 80% germination at same concentration of PMDE. Suppression of germination by PMDE might be due to the occurrence of highly toxic organic pollutants which absorbed by the seed before germination and affecting different biochemical and physiological process of seed germination (Bharagava and Chandra 2010a). However, the bacterial treated sample after treatment with constructed wetland 100% seed germination was observed after 24 h. Various parameters were studies showed germination index, radical length, stress tolerance index, phytotoxicity, relative toxicity percent with untreated, bacterial treated, and constructed wetland treated PMDE compared with control.These results clearly indicated that the toxicity of PMDE was reduced appreciably after bacterial treatment followed by constructed wetland plant treatment process.



Control (tap water) | PMDE (Untreated) | PMDE (Bacterial Treated) | PMDE (CW Treated)

**Fig.9.14** Effect of PMDE on seedling growth of *Phaseolus mungo* L. seeds

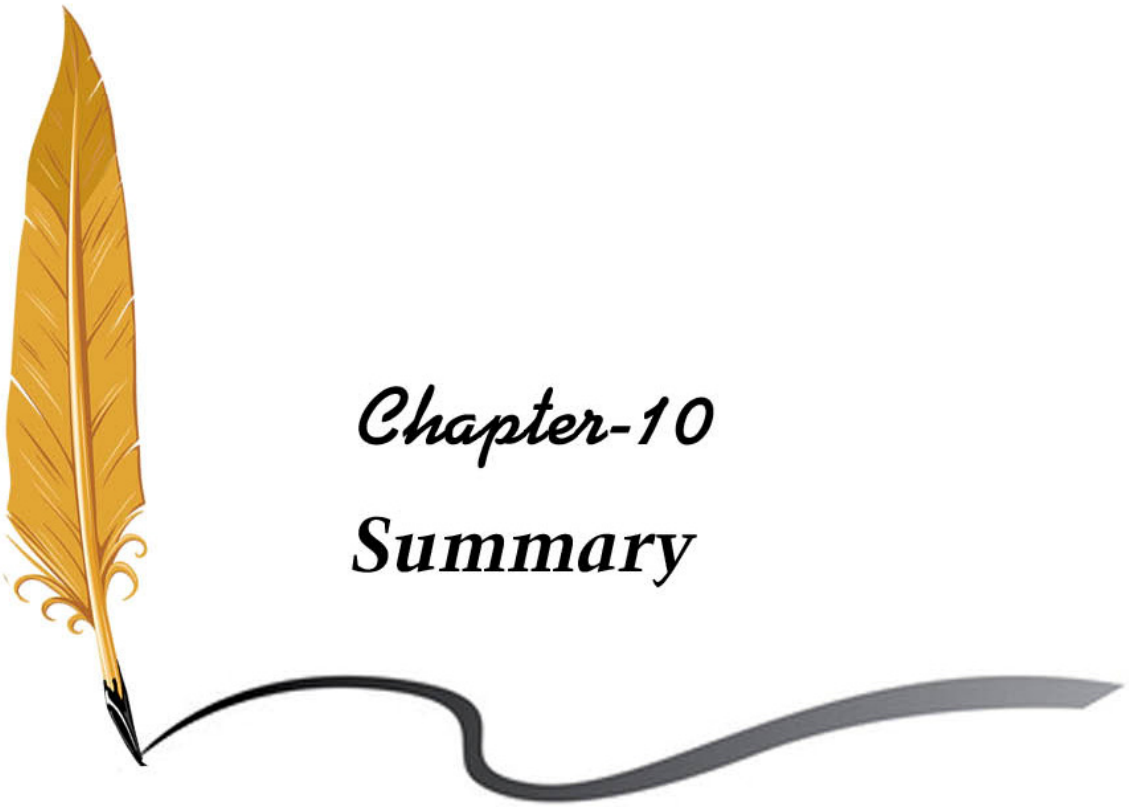
**Table 9.6** Effects of PMDE on the growth of *Phaseolus mungo* L.

| Parameters | Control | Untreated | BT | CW treated |
|---|---|---|---|---|
| No. of seed shown | 10 | 10 | 10 | 10 |
| Germination (%) | 100±0.00 | NG | 80±0.00 | 100±0.00 |
| Germination index | 00 | -- | 0.66±0.00 | 00 |
| Stress tolerance index | 00 | -- | 0.83±0.01 | 00 |
| Radical length (cm) | 18.12±0.82 | NG | 15.11±0.54 | 18.12±0.01 |
| Radical length inhibition (cm) | NI | -- | 3.01±0.02 | NI |
| Reduction in germination (%) | NI | NG | 20±0.00 | NI |
| Phytoxicity | 00 | -- | 16.61±0.52 | 00 |
| Relative toxicity (%) | 00 | -- | 25±0.00 | 00 |

All values are mean of three replicate ± S.D; NG: No germination; NI: No inhibition; BT: Bacterial treated; CW: Constructed wetland

## Conclusion

This study has revealed that untreated PMDE carry very high concentration of BOD, COD, and TDS along with various toxic metals, which were reduced significantly after bacteria and constructed wetland plant treatment process. Further, the GC-MS and other spectrophotometric analysis have shown that most of the compounds detected in untreated PMDE were diminished from bacterial and wetland plant treated samples. The disappearance of compounds from bacteria and wetland plant treated sample could be related with degradation and color removal from PMDE. The Illumina MiSeq analysis through metagenomic approach showed the presence of *Rheinheimera* (21%), *Sphingobacterium* (17%), *Idiomarina* (8%). *Acidothermus* (4%), *Pseudomonas* (2%), *Flavobacterium* (2%), Uncultured bacterium (2%), *Parapedobacter* (2%), *Alcanivorax* (2%), Uncultured (4%), *Bacillus* (3%), *Acholeplasma* (2%), *Hyphomonas* (1%), *Aquamicrobium* (1%), as dominant rhizospheric bacterial communities of *Phragmites* communis plant which were beneficial for the degradation of toxic constituents present in the PMDE. This is the first time to identify the microbial communities of *Phragmites communis* during PMDE treatment in HSSF-CW plant treatment system by Illumina high-throughput sequencing. This study provides valuable data for the potential growth of wetland plant and bioremediation of distillery effluent. Such approaches can be implemented for a feasible technology in wetland plant treatment system for pollutant bioremediation.



# Chapter-10

# Summary

# Chapter-10


## Summary

Sugarcane-molasses based post methanated distillery effluent (PMDE) is a threat to environment for its safe disposal due to presence of complex polymer containing heterocyclic nitrogenous compounds of aldehyde-amines, various heavy metals, phenolic compounds and plant derived resins and fatty acids. Melanoidin is a major colorant of distillery effluent. It has also been reported that melanoidins have net negative charges; therefore, various heavy metals strongly binds with melanoidins to form large organo-metallic complex molecules. Among the different process available for treatment of spent wash, biomethanation is a popular anaerobic convention process which produces methane to meet a part of the power requirement in distilleries. The effluent after anaerobic treatment (methanogenesis) becomes more viscous and dark due to complexation of organic and inorganic pollutants present in spent wash. The discharged effluent after anaerobic treatment causes environmental problems in aquatic and soil ecosystem which ultimately affect to aquatic flora and fauna. Therefore, adequate treatment of the distillery effluent is warranted prior to its discharge into environment. Hence, the detailed chemical properties of various pollutants present in distillery effluent before and after anaerobic treatment have yet to be elucidated.

The above information has been systematically elaborated in the first chapter of thesis as introduction. Subsequently, second chapter has mentioned objectives of thesis. Further, the review of literature of the topic has been elaborated in chapter three, while chapter four has mentioned the physico-chemical analysis and identification of various persistent organic compounds present in PMDE before and after anaerobic treatment. The result has revealed that DSW showed high BOD (42,000), COD (90,000), TDS (77,776), TS (83,084), chloride (2200), phenol (4.20), sulfate (5760), and phosphate (5.36 mg $L^{-1}$) levels with acidic pH (4.07), and dark brown color. In addition, several major heavy metals were detected, including Fe (163.947), Mn (4.556), Zn (2.487), and Ni (1.175 mg $L^{-1}$). Subsequently, the physico-chemical characteristics of PMDE showed high BOD (6500), COD (10,864), TDS (10,764), TS (12,248), chloride (19,993), phenol (3.98), sulfate (4850), and phosphate (5.12 mg $L^{-1}$) levels with alkaline pH (8.17), and dark brown color. The GC-MS analysis of DSW revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy-[(TMS)oxy], TMS ester; and tricarballylic acid 3TMS), which are listed as endocrine-disrupting chemicals. While, The GC-MS analysis of PMDE revealed the presence of hexadecanoic acid, TMS ester; octadecanoic acid, TMS ester; β–Sitosterol TMS ester; benzeneacetic acid, α,4-bis[(TMS)oxy], TMS ester; benzoic acid, 2,5-bis(TMSoxy)-TMS ester; Stigmasta-5,22-Dien-3-ol (3β, 22E); phenol,4,4'-thiobis[2-(1,1dimethylethyl)-6-methyl. The detected compounds have been listed as potential EDCs by the USEPA. Further, phytotoxicity assay of DSW and PMDE

with *Phaseolus mungo* L. and *Triticum aestivum* revealed the presence of toxic organic compounds.

Due to presence of sucrose and glutamic acid, which abundantly present in sugarcane juice and represent the major composition of molasses-melanoidins, we have selected sucrose and glutamic acid for the degradation study. The sucrose glutamic acids-Maillard reaction products (SGA-MRPs) are predominantly present in PMDE responsible for color of distillery effluent as environmental pollutant due to its recalcitrant nature. The chapter five of thesis has mentioned the characterisation of potential manganese peroxidase (MnP) and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard reaction products at different nutritional and environmental conditions. The result revealed that twenty four morphologically different aerobic bacterial strains (IITRCS01 to IITRCS024) were isolated by nutrient enrichment technique from distillery sludge by streak plate method. Further, these bacterial strains were screened on the basis of MnP and laccase and melanoidins tolerance activity. Out of 24 bacterial strains, four aerobic bacterial strains IITRCS01, IITRCS06, IITRCS07, and IITRCS11 were showed maxium MnP and laccase producing activity on phenol red amended GPM medium and guiacol amended B&K agar medium plated. Further, these bacterial strains were also showed higher melanoidins (3500 mg $L^{-1}$) tolerance activity. On the basis of 16S rRNA gene sequence analysis potential MnP and laccase producing bacterial strains IITRC01, IITRC06, IITRC06, and IITR11, were identified as *Klebsiella pneumoniae* (KU726953), *Salmonella enterica* (KU726954), *Enterobacter aerogenes* (KU726955), *Enterobacter cloaceae* (KU726957), respectively. The identified aerobic bacterial consortium consisting *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), *E.* cloaceae (KU726957) showed optimum production of MnP and laccase at 120 and 144 h of growth, respectively. The potential bacterial consortium showed decolourisation of SGA-MRPs up to 70% in presence of glucose (1%), peptone (0.1%) at optimum pH (8.1), temperature (37 °C) and shaking speed (180 rpm) within 192 h of incubation. The reduction of colour of Maillard product correlated with shifting of absorption peaks in UV–Vis spectrophotometry analysis. UV-Vis spectrophotometric analysis of SGA-MRPs showed many absorption peaks between 200 and 450 nm and their absorption maximum peak was noted at 250 nm in spectrophotometric detection. Further, the changing of functional group in FT-IR data showed appearance of new peaks and GC–MS analysis of degraded sample revealed the depolymerisation of complex MRPs. The toxicity evaluation using seed of *P. mungo* L. showed reduction of toxicity of MRPs after bacterial treatment. Thus, this consortium might be useful for decolourisation of industrial wastewater containing high concentration of melanoidins.

Based on potentiality of isolated bacterial strains the developed bacterial consortium was used to assess degradability of molasses-melanoidins. The study was conducted for optimal decolourisation and degradation of molasses-melanoidins under optimised environmental and nutritional conditions which constitute as chapter six of my thesis. The result revealed that molasses-melanoidin extracted from PMDE with mixture of isopropanol and PMDE (1:1 v/v) showed presence of Mn (8.20), Cr (2.97), Zn (16.61), Cu (2.55), Fe (373.95), Pb (2.59) and Ni

(4.18 mg $L^{-1}$) along with mixture of other organic compounds which have endocrine-disrupting chemicals (EDCs) properties as per USEPA. The aerobic bacterial consortium consisting with four bacterial strains *K. pneumoniae* (KU726953), *S. enterica* (KU726954), *E. aerogenes* (KU726955), and *E. cloacae* (KU726957) isolated from distillery sludge sample to degrade synthetic SGA-MRPs was used in this study. A consortium of aerobic bacteria comprising *K. pneumoniae* (KU321273), *S. enteric* (KU726954), *E. aerogenes* (KU726955), and *E. cloacae* (KU726957) in ratio of 2:1:2:2 showed the optimum decolourisation of molasses-melanoidins up to 81% through co-metabolism in presence of glucose (1.0%) and peptone (0.2%) as a carbon and nitrogen source, respectively. The absorption spectrum scanning by UV-Visible spectrophotometer between 200-700 nm revealed reductions of absorption spectrum of organic compounds present in bacterial degraded sample of melanoidins in range of 200-450 nm compared to control. The degradation and decolourisation of melanoidins by bacterial consortium was noted by induction of manganese peroxidase and laccase activities in sample supernatant. Further, the TLC and HPLC analysis of bacterial decolourised melanoidins also showed degradation and reduction of absorption peak at (295nm), respectively. Furthermore, FT-IR and GC-MS analysis also showed the change of functional group and disappearance of ion peaks. This indicated the degradation and depolymerisation of melanoidins and cleavage of C═C, C=O and C≡N conjugated bonds which resulted in reduction of colour. The metabolic analysis also showed the disappearance of some organic compounds and generation of new metabolites. Further the seed germination test using *P. mungo* L. showed toxicity reduction in decolorized effluent. Thus, the result revealed that the developed bacterial consortium could be used to scale up the decolourisation, degradation and detoxification process of PMDE for industrial application.

The nature of complex pollutants at distillery waste contaminated sites and the potential for microbial strains to grow such environment are still unknown. A study was conducted to reveal the endemic bacterial communities growing in specific environments that might be responsible for amelioration of these pollutants, eventually leading to biological succession and bioremediation for eco-restoration of polluted sites. Therefore, the chapter seven of thesis has mentioned the dominant bacterial communities and metabolic products of spent wash and post methanated distillery sludge during *in*-situ bioremediation. The result revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy--[(TMS)oxy], TMS ester; and tricarballylic acid 3TMS) in spent wash, which are listed as potential EDCs. Subsequently, the dominant autochthonous bacterial communities were investigated by the RFLP method using a metagenomic approach to reveal the microbial niche in this polluted environment. Bacterial community analysis by RFLP revealed that *Bacillus* and *Stenotrophomonas* were dominant autochthonous bacterial communities belonging to the phylum *Firmicutes* and *γ-Proteobacteria,* respectively grown in distillery spent wash. The presence of *Bacillus* and *Stenotrophomonas* species in highly acidic environments indicated its broad range

adaptation. Further, phytotoxicity assay of DSW with *P. mungo* L. and *T. aestivum* revealed that *T. aestivum* was more sensitive than *P. mungo* L. in the seed germination test. Moreover, GC-MS analysis of distillery sludge and leachate both showed dodecanoic acid, octadecanoic acid, n-pentadecanoic acid, hexadecanoic acid, β-sitosterol, stigmasterol, β-sitosterol trimethyl ether, heptacosane, dotriacontane, lanosta-8, 24-dien-3-one, 1-methylene-3-methyl butanol, 1-phenyl-1-propanol, 5-methyl-2-(1-methylethyl) cyclohexanol, and 2-ethylthio-10-hydroxy-9-methoxy-1,4 anthraquinone as major androgenic and mutagenic organic pollutants along with heavy metals (mg $kg^{-1}$): Fe (2403), Zn (210.15), Mn (126.30, Cu (73.62), Cr (21.825), Pb (16.33) and Ni (13.425). In a simultaneous analysis of bacterial communities using the RFLP method the dominance of *Bacillus* sp. followed by *Enterococcus* sp. as autochthonous bacterial communities growing in this extremely toxic environment was shown, indicating a primary community for bioremediation. A toxicity evaluation showed a reduction of toxicity in degraded samples of sludge and leachate, confirming the role of autochthonous bacterial communities in the bioremediation of distillery waste *in*-situ. These findings indicated that these autochthonous bacterial communities were pioneer taxa for *in*-situ remediation of hazardous distillery waste during ecological succession. The results of this study may be useful for monitoring and toxicity assessment of sugarcane molasses-based distillery waste at disposal sites.

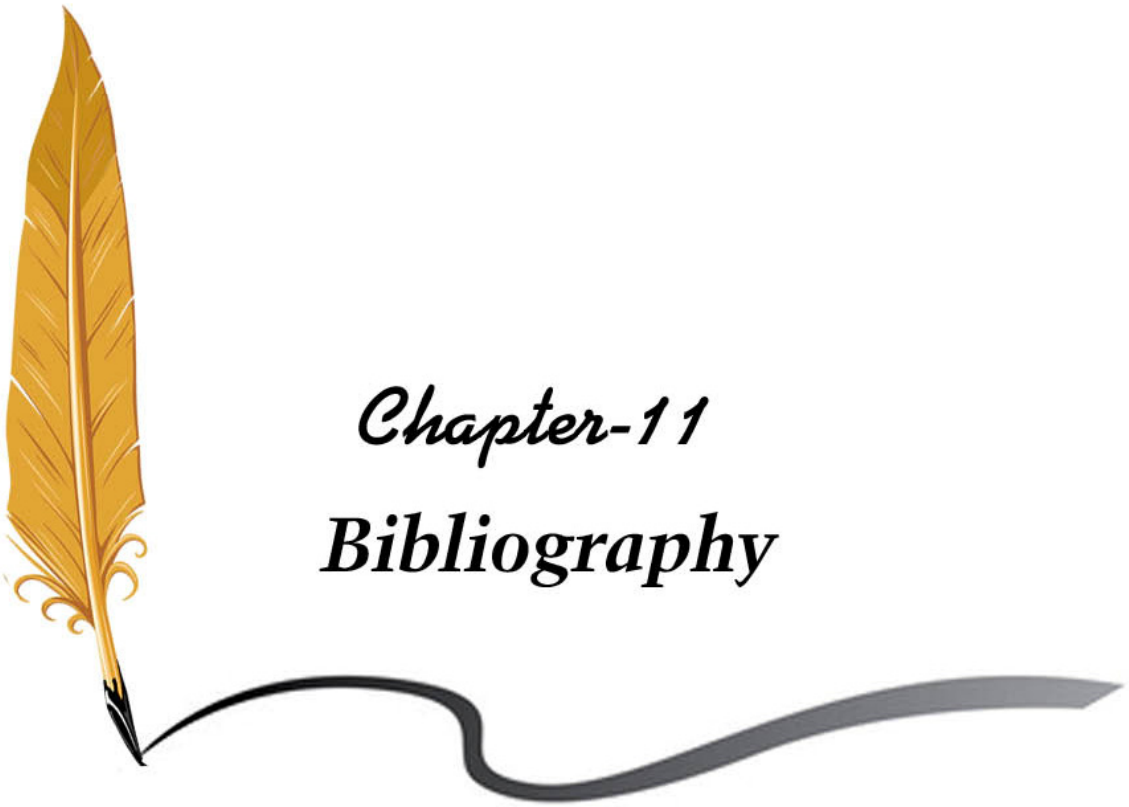
Since, the phytoremediation of complex industrial waste by native plants is an emerging green technology for eco-restoration of polluted site. Hence, before construction of wetland plant treatment system some potential native plants have been assessed for phytoextraction of heavy metals from stabilised post methanated distillery sludge. Therefore, in chapter eight of thesis has showed in detail result of phytoextraction of heavy metals by twenty four native plants species (weeds and grasses) (i.e. *Dhatura stramonium, Achyranthes* sp., *Kalanchoe pinnata, Trichosanthes dioica, Parthenium hysterophorous, Cannabis sativa, Amaranthus spinosus* L. *Croton bonplandianum, Solanum nigrum, Ricinus communis, Setaria viridis, Blumea lacera, Argemone mexicana, Saccharum munja, Cynodon dactylon, Pennisetum purpureum, Chenopodium album, Rumex dentatus, Tinospora cordifolia, Calotropis procera,* and *Basella alba).* These plants were collected based on dominant species luxuriantly growing on disposed distillery sludge. These native plants species were uprooted with associated sludge samples for the analysis of accumulated heavy metal in different parts of growing plants. Besides, the fresh disposed dried distillery sludge cakes were collected in clean pre-sterilized polythene bags from sludge dumping site of distillery plant located inside the premises of industry. This study revealed that distillery sludge contains not only mixture of complex organic pollutants but also retains high quantity of Fe (5264.49), Zn (43.47), Cu (847.46), Mn (238.47), Ni (15.60), and Pb (31.22 mg $kg^{-1}$) which enhances the toxicity of sludge to the environment. The major identified organic compounds were benzene, 1-ethyl-2-methyl, benzene, 1-ethyl-4-methyl benzoic acid, 3,4,5-tris(TMS oxy), TMS ester; hexanedioic acid, dioctyl ester; stigmasterol TMS ether; 5α-cholestane,4-methylene; campesterol TMS; β-sitosterol and lanosterol. These compounds are listed under the EDCs also as per USEPA. However, the phytoextraction potential of growing

native weeds and grasses revealed the high accumulation of Fe, Zn, Cu, Mn, Ni, and Pb in their root and leaves compared to shoot. This indicated high accumulation and translocation capabilities of these plants. Further, the bioaccumulation coefficient factor (BCF) and translocation factor (TF) was found >1 for majority of plants for various metals. Thus, this given strong evidence for hyperaccumulation tendency of these native weeds and grasses from complex polluted sites. Anatomical observations through TEM in the root of various potential native plants showed apparent formation of multi-nucleolus, multi-vacuoles and deposited metal granules in the cellular components of plants. This indicated the variable adaptive characteristics of these plants growing at a hazardous waste polluted site. Hence, these native plants may be used as a tool for *in*-situ phytoremediation and eco-restoration of industrial waste contaminated site.

Further, as per objective of study, the bacterial degraded PMDE was integrated with designed horizontal subsurface flow-constructed wetland (HSSF-CW) plant treatment system. The PMDE degradation was assessed at different treatment stages. Therefore, the chapter nine of thesis has mentioned the dominant rhizospheric bacterial communities of *Phragmites communis* characterised through metagenomic approach to reveal the microbial community structure of rhizobacteria of *P. communis* during PMDE degradation and decolourisation in HSSF-CW plant treatment process. It was also interesting to note that the bacterial pretreatment (for 168 h) followed by phytoremediation with *P. communis* wetland plant rhizosphere (for 168 h) has also improved the physico-chemical properties of effluent resulting reduction in colour, BOD, COD, TS, TDS, TSS, Total nitrogen, chloride, sulfate, and phenol up to 84.03, 91.81, 92.84, 94.09, 94.93, 91.66, 92.19, 88.83, and 88.51%, respectively. Moreover, the constructed wetland plant rhizosphere treated PMDE sample has also shown reduction of various heavy metals such as Fe, Zn, Ni, Mn, Pb, Cu, and Cd up to 97.16, 89.01, 86.66, 82.84, 98.57, 99.75, 0.00%. The absorption spectrum scanning by UV-Visible spectrophotometer between 200-700 nm revealed reductions of absorption spectrum of organic compounds present in bacterial degraded and constructed wetland rhizosphere treated sample of PMDE in range of 200-450 nm compared to control. The HPLC analysis confirmed the reduction of peak lead to colour reduction. Further, the GC-MS analysis has shown that most of the compounds detected in untreated PMDE were diminished from bacterial and wetland plant treated samples. The disappearance of compounds from bacteria and wetland plant treated sample could be related with degradation and color removal from PMDE. The sludges from the rhizopheric zone of *P. communis* were collected and the microbial communities were analyzed by Illumina high-throughput sequencing of V3-V4 hypervariable reagions of 16S rDNA. The most abundant phylum was *Proteobacteria* (50%) followed by *Bacteriodetes* (33%), *Firmicutes* (5%), *Gemmatimonadetes* (2%), *Choloroflexi* (2%), and *Tenericutes* (2%). At the class level, the most predominant bacteria affiliated with *Gammaproteobacteria* (36%) followed by *Sphingobacteria* (19%), *Aplhaproteobacteria* (11%), *Flavobacteria* (9%), Actinobacteria (6%), *Bacilli* (5%), *Cytophagia* (5%), *Betaproteobacteria* (3%), *Mollicutes* (2%), and *Deltaproteobacteria* (1%). At the genus level, the Illumina MiSeq analysis showed the presence of *Rheinheimera* (21%), *Sphingobacterium* (17%), Idiomarina

(8%). *Acidothermus* (4%), *Pseudomonas* (2%), *Flavobacterium* (2%), Uncultured bacterium (2%), *Parapedobacter* (2%), *Alcanivorax* (2%), Uncultured (4%), *Bacillus* (3%), *Acholeplasma* (2%), *Hyphomonas* (1%), and *Aquamicrobium* (1%), as dominant rhizospheric bacterial communities of *P. communis* plant which were beneficial for the degradation of toxic constituents present in the PMDE. This is the first time to identify the microbial communities of *P. communis* during PMDE treatment in HSSF-CW plant treatment system by Illumina high-throughput sequencing. The decolourization of PMDE is combined phenomenon by *P. communis* and rhizosphere bacteria. Bacteria helped in the degradation of toxic wastes and plant accumulates the heavy metals in its tissues. Both of them played an important role in the bioremediation of environmental pollutants. Constructed wetland can be an effective technology for bioremediation of organic and inorganic pollutants in two step treatment of PMDE.

Based on work, six original research papers have been published in high impact journal and one research paper is under consideration. While eleven conference papers presented in national and international conferences.



# Chapter-11

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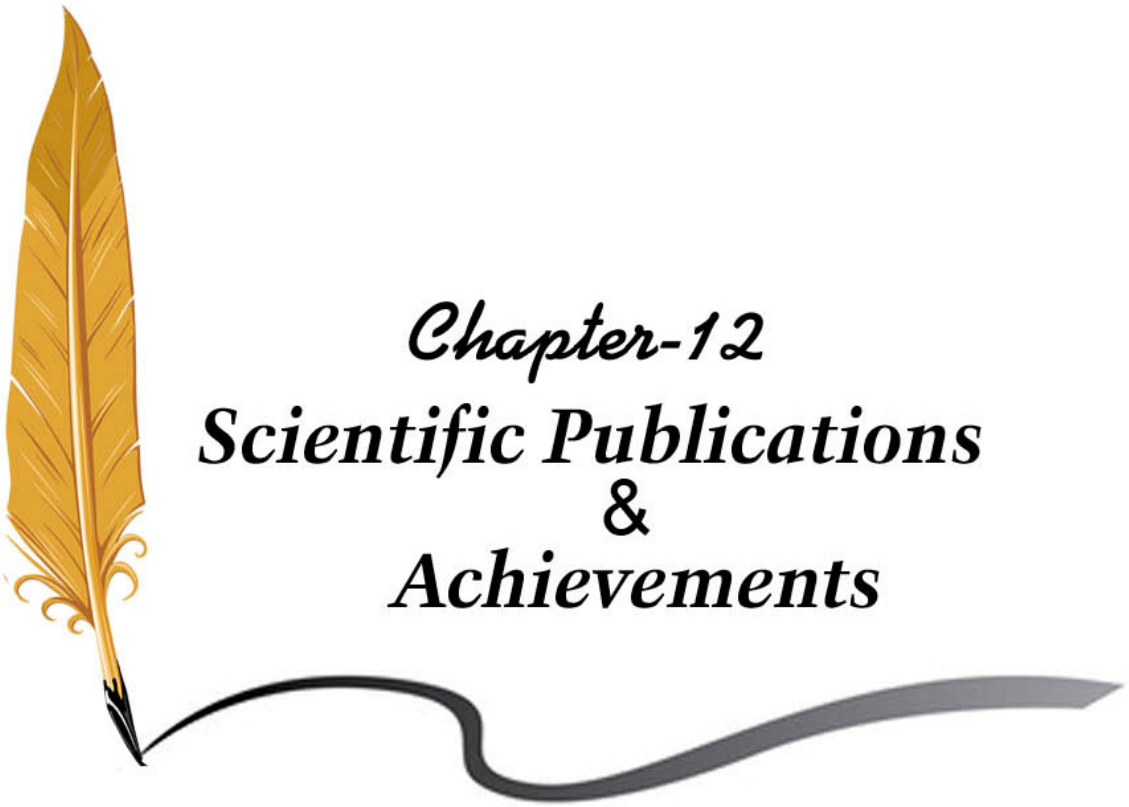
**Zhou J, Bruns MA, Tiedje JM (1996)** DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62(2): 316-322.



# Chapter-12

# Scientific Publications & Achievements

# Chapter 12

## Scientific Publications and Achievements:

### (A) Research Papers published high impact Journals of International Repute: Six

**(1)** Ram Chandra, **Vineet Kumar,** Sonam Tripathi **(2018).** Evaluation of molasses-melanoidins decolourisation by potential bacterial consortium discharged in distillery effluent. **3 Biotech**

**(2) Vineet Kumar,** Ram Chandra **(2018).** Characterisation of MnP and laccase producing bacteria capable for degradation of sucrose glutamic acid-maillard products at different nutritional and environmental conditions. **World Journal of Microbiology & Biotechnology**

**(3)** Ram Chandra, **Vineet Kumar**, Sonam Tripathi, Pooja Sharma **(2018).** Heavy metal phytoextraction potential of native weeds and grasses from endocrine-disrupting chemicals rich complex distillery sludge and their histological observations during in-situ phytoremediation. **Ecological Engineering** 111:143-156.

**(4)** Ram Chandra, **Vineet Kumar (2017).** Detection of androgenic-mutagenic compounds and potential autochthonous bacterial communities during in-situ bioremediation of post methanated distillery sludge. **Frontiers in Microbiology** 8:887.

**(5)** Ram Chandra, **Vineet Kumar (2017).** Detection of *Bacillus* and *Stenotrophomonas* species growing in an organic acid and endocrine-disrupting chemicals rich environment of distillery spent wash and its phytotoxicity. **Environmental Monitoring and Assessment** 189:26.

**(6)** Ram Chandra, **Vineet Kumar (2017).** Phytoextraction of heavy metals by potential native plants and their microscopic observation of root growing on stabilised distillery sludge as a prospective tool for in-situ phytoremediation of industrial waste. **Environmental Science and Pollution Research** 24:2605-2619

### (B) Research Paper Communicated: One

**(1) Vineet Kumar,** Ram Chandra **(2018).** Analysis of dominant rhizospheric bacterial communities from *Phragmites communis* growing in constructed wetland plant treatment system for decolourisation and detoxification of bacterial treated post methanated distillery effluent. **Bioresource Technology.**

### (C) Book Chapters: Eleven

**(1) Vineet Kumar** and Ram Chandra **(2018).** Bacterial assisted phytoremediation of industrial waste pollutants and eco-restoration. In: Phytoremediation of Environmental Pollutants, Ram Chandra, N.K. Dubey, Vineet Kumar (Eds) CRC Press, USA, pp-1-42.

**(2)** Ram Chandra, **Vineet Kumar (2018).** Hyperaccumulator versus nonhyperaccumulator plants for environmental waste management**.** In: Phytoremediation of Environmental Pollutants, Ram Chandra, N.K. Dubey, Vineet Kumar (Eds), CRC Press, USA, pp- 43-80.

**(3)** Ram Chandra, **Vineet Kumar,** Sonam Tripathi, Pooja Sharma **(2018).** Phytoremediation of industrial pollutants and life cycle assessmen. In: Phytoremediation of Environmental

Pollutants, Ram Chandra, N.K. Dubey, Vineet Kumar (Eds), CRC Press, USA, pp-441-469.

**(4)** Ram Chandra, **Vineet Kumar (2018).** Phytoremediation: A green sustainable technology for industrial waste management. In: Phytoremediation of Environmental Pollutants, Ram Chandra, N.K. Dubey, Vineet Kumar (Eds), CRC Press, USA, pp 159-199.

**(5)** Ram Chandra, **Vineet Kumar**, Sheelu Yadav **(2017).** Extremophilic ligninolytic enzymes. In: Extremophilic Enzymatic Processing of Lignocellulosic Feedstocks to Bioenergy (R. Sani, R.N. Krishnaraj (Ed.)., Springer International Publishing AG, DOI:10. 1007/978-3-319-54684-1_8

**(6)** Ram Chandra, **Vineet Kumar (2016)** Persistent Organic Pollutants (POPs): Health Hazards and Challenges for their Bioremediation in Environment. In: Bioremediation of Industrial Pollutants, Ram Naresh Bharagava, Gaurav Saxena, (Ed.) Write and Print Publications, New Delhi, pp 1-51

**(7)** Ram Chandra, **Vineet Kumar (2015)** Biotransformation and biodegradation of organophosphates and organohalides. In: Environmental Waste Management, Ram Chandra (Ed.) CRC Press, USA. pp 475-524.

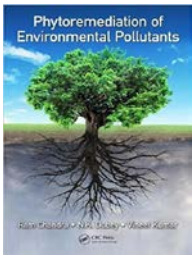
**(8)** Ram Chandra, Sheelu Yadav, **Vineet Kumar (2015)** Microbial Degradation of Lignocellulosic Waste and Its Metabolic Products. In: Environmental Waste Management, Ram Chandra (Ed.) CRC Press, USA, pp-249-298.

**(9)** Ram Chandra, **Vineet Kumar (2015)** Mechanism of wetland plant rhizosphere bacteria for bioremediation of pollutants in an aquatic ecosystem. In: Advances in Biodegradation and Bioremediation of Industrial Waste, Ram Chandra (Ed.), CRC Press, USA, pp. 329-379

**(10)** Ram Chandra, Gaurav Saxena, **Vineet Kumar (2015)** Phytoremediation of Environmental Pollutants: An Eco-Sustainable Green Technology to Environmental Management. In: Advances in Biodegradation and Bioremediation of Industrial Waste, Ram Chandra (Ed.) CRC Press, USA, pp-1-29

**(11)** Ram Chandra, Sangeet Yadav, **Vineet Kumar (2015).** Two step treatment by bacteria and rhizofiltration for bioremediation of complex industrial wastewater: A Novel Approach for Safe Disposal. In: Plant-Microbe Interactions, K. Ramasamy and K. Kumar (Ed.) New India Publishing Agency, New Delhi, pp-483-519

## (D) Book Co-editor: One

| Book | Book Detail |
|---|---|
|  | **Title: Phytoremediation of Environmental Pollutants**<br>Editor: Ram Chandra, N.K. Dubey, **Vineet Kumar**<br>Year of Publication: **2017**<br>Publisher: CRC Press (Taylor & Francis Group), USA<br>ISBN: 9781138062603<br>Pages: 510 |

## (E) Popular Scientific Magazine Article in Hindi: Three

(1) राम चन्द्रा, **विनीत कुमार, (2016).** आसवनी अपशिष्ट एवं कागज उद्योग उत्स्राव के कारण पर्यावरण प्रदूषण तथा भारी धातुओं से खाद्य श्रृंखला एवं मानव स्वास्थ्य पर खतरा, विषविज्ञान संदेश, पेजः4–14, आई0एस0एस0न0 0972–1746

(2) **विनीत कुमार** एवं राम चन्द्रा **(2016).** आसवनी अपशिष्ट को अनॉक्सीकृत प्रक्रिया द्वारा शोधनोपरान्त पर्यावरण मे सुरक्षात्मक निस्तारण की चुनौतियाँ, विषविज्ञान संदेश, पेजः34–49, आई0एस0एस0न0 0972–1746

## (F) Paper in Conference Proceedings (one)

**(1)** Ram Chandra, **Vineet Kumar,** Sonam Tripathi **(2016)** Environmental Health Hazards of Post Methanated Distillery Waste and its Detoxification. In: Proceeding of 74th Annual Convention of STAI, ISBN-81-85871-83-3, New Delhi, pp-684-699.

## (G) Awards

**(1)** Awarded **3rd prize** for best poster presentation in 4th Lucknow Science Congress (LUSCON) organised by BBAU, Lucknow in 2017

**(2)** Awarded Merit Certificate for Best Academic Contribution by BBAU, Lucknow in **2013**

**(3)** Rajiv Gandhi National Fellowship Awarded by UGC in 2012

## (H) Memberships of Scientific Societies

**(1)** Life Member of **Association of Microbiologists of India (AMI)**

**(2)** Life Member of **The Biotech Research Society, India (BRSI)**

**(3)** Life Member of **Indian Science Congress Association (ISCA)**

## (I) Research Paper Presented in National /International symposium and conferences

**(1) Vineet Kumar** and Ram Chandra **(2017).** Degradation and decolourisation of molasses-melanoidins by potential bacterial consortium extracted from anaerobic digested distillery effluent at different nutritional and environmental conditions. Presented in "**International Conference** organized by **Association of Microbiologist of India (AMI)** held from Nov 16-19, 2017 at B.B. Ambedkar University, Lucknow

**(2)** Ram Chandra and **Vineet Kumar (2017).** Environmental Health Hazards of Post Methanated Distillery Waste and Development of New Technology on Decolourisation and Detoxification of Post Methanated Distillery Effluent for its Recycling and Re-use. Presented in "**International Conference** organized by **Association of Microbiologist of India (AMI)** held from Nov 16-19, 2017 at B.B. Ambedkar University, Lucknow

**(3)** Ram Chandra and **Vineet Kumar (2017).** Use of Industrial Wastewater in Agricultural Practices: Challenges and Applications. Presented in **"National Symposium"** organized by B.B. Ambedkar University Lucknow held from August 29-30, 2017

**(4)** Ram Chandra, **Vineet Kumar (2017).** Detection of organic pollutants and indigenous bacterial community growing in the endocrine-disrupting chemicals and heavy metal rich environment of sugarcane molasses based distillery waste. Presented in **"National Conference"** organized by B.B. Ambedkar University Lucknow held from March 2-3, 2017

**(5) Vineet Kumar,** Ram Chandra **(2017).** Isolation and characterization of manganese peroxidase and laccase producing bacteria capable for degradation and decolourisation of sucrose-glutamic acid maillard products (SGA-MPs). Presented in **"National Conference"** organized by B.B. Ambedkar University Lucknow held from March 2-3, 2017

**(6)** Ram Chandra, **Vineet Kumar (2016).** Detection of indigenous bacterial community growing in the endocrine-disrupting chemicals and heavy metal rich environment of sugarcane molasses based distillery waste. Presented in "**International Conference** organized by **Association of Microbiologist of India (AMI)** held from Nov 24-27, 2016 at University of Gauhati, Assam

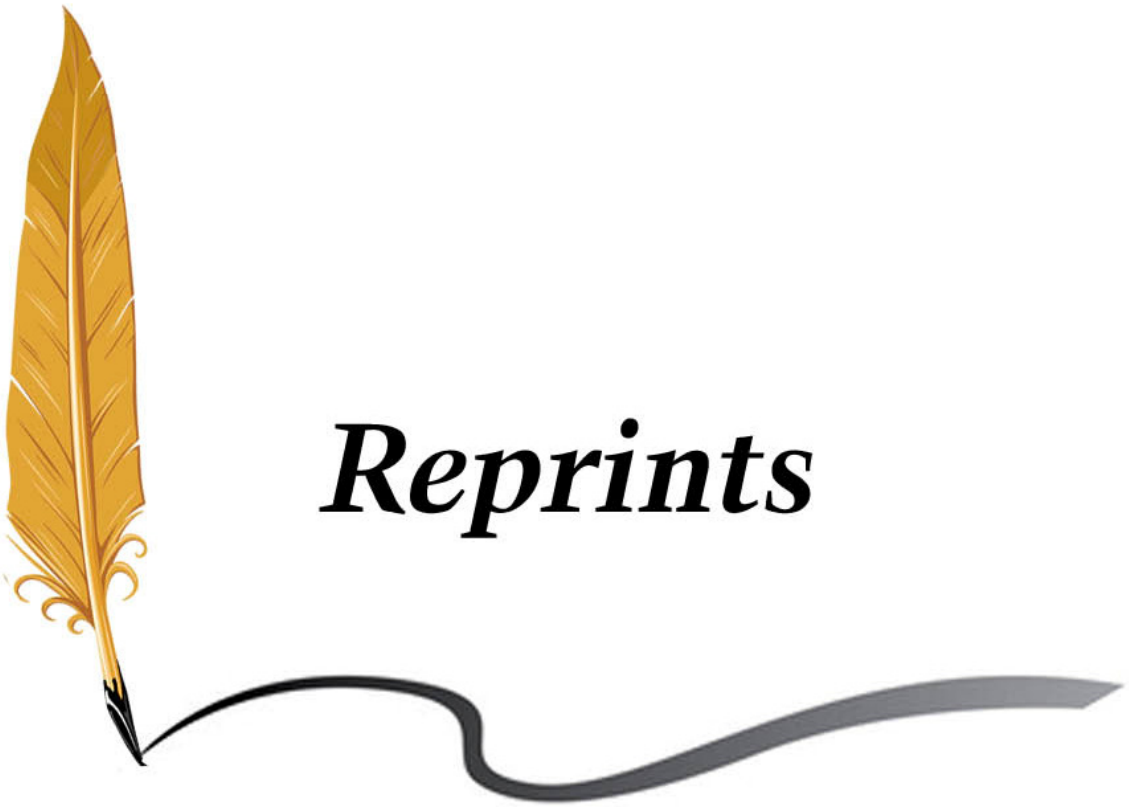
**(7) Vineet Kumar,** Ram Chandra **(2016).** Isolation, screening and characterization of MnP and laccase producing bacteria capable for degradation and decolourisation of sucrose-glutamic acid maillard products. Presented in "**International Conference** organized by **Association of Microbiologist of India (AMI)** held from Nov 24-27, 2016 at University of Gauhati, Assam

**(8)** Ram Chandra, **Vineet Kumar (2016).** Environmental Health Hazards and Food Contamination from Post Methanated Distillery Waste and its Safety. Presented in "**National Conference** on Food Safety and Consumer Awareness" organized by Innovation Centre on Food Processing & Food Technology held from **February 21-22, 2016** at University of Lucknow, Lucknow.

**(9)** Ram Chandra, **Vineet Kumar (2015).** Detection of recalcitrant toxic organic pollutants and Identification of *Bacillus* and *Enterococcus Sp.* by RFLP method as dominant autochthonous firmicutes growing in sugarcane molasses based post methanated distillery sludge. Presented in **International Conference** organized by **Association of Microbiologist of India (AMI)** held from Dec 7-10, 2015 at Jawaharlal Nehru University, New Delhi

**(10)** Ram Chandra, **Vineet Kumar (2014).** Phytoextraction and bioremediation of heavy metals by potential native plants growing on post methanated distillery effluent sludge dumping sites. Presented in **International Conference** organized by The Biotech Research Society, India (BRSI) held from Nov 08-10, 2014 at Jawaharlal Nehru University, New Delhi

**(11)** Ram Chandra, **Vineet Kumar (2014).** Characterization of antibiotic and heavy metal resistant bacteria isolated from the rhizosphere of native plant growing on distillery effluent methane reactor sludge dumping site. Presented in **International Conference** organized by **Association of Microbiologist of India (AMI)** held from Nov 12-14, 2014 at Tamil Nadu Agricultural University, Coimbatore



# *Reprints*

3 Biotech (2018) 8:187
https://doi.org/10.1007/s13205-018-1205-3

ORIGINAL ARTICLE

# Evaluation of molasses-melanoidin decolourisation by potential bacterial consortium discharged in distillery effluent

Ram Chandra[1] · Vineet Kumar[1] · Sonam Tripathi[1]

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## Abstract

The extracted sugarcane molasses-melanoidins showed the presence of Mn (8.20), Cr (2.97), Zn (16.61), Cu (2.55), Fe (373.95), Pb (2.59), and Ni (4.18 mg $L^{-1}$) along with mixture of other organic compounds which have endocrine-disrupting chemicals (EDCs) properties. A consortium of aerobic bacteria comprising *Klebsiella pneumoniae* (KU321273), *Salmonella enteric* (KU726954), *Enterobacter aerogenes* (KU726955), and *Enterobacter cloacae* (KU726957) showed the optimum decolourisation of molasses-melanoidins up to 81% through co-metabolism in the presence of glucose (1.0%) and peptone (0.2%) as a carbon and nitrogen source, respectively. The absorption spectrum scanning by UV–visible spectrophotometer between 200 and 700 nm revealed reductions of absorption spectrum of organic compounds present in bacterial degraded sample of melanoidins in range of 200–450 nm compared to control. The degradation and decolourisation of melanoidins by bacterial consortium was noted by induction of manganese peroxidase and laccase activities in sample supernatant. Furthermore, the TLC and HPLC analysis of bacterial decolourised melanoidins also showed degradation and reduction of absorption peak at (295 nm), respectively. Furthermore, FT-IR and GC–MS analysis also showed the change of functional group and disappearance of ion peaks. This indicated the degradation and depolymerisation of melanoidins and cleavage of C=C, C=O and C≡N conjugated bonds which resulted in reduction of colour. The metabolic analysis also showed the disappearance of some organic compounds and generation of new metabolites. Furthermore, the seed germination test using *Phaseolus mungo* L. showed toxicity reduction in decolourized effluent.

**Keywords** Melanoidins · Metabolites · PMDE decolourisation · Endocrine-disrupting chemicals · Toxicity reduction

## Introduction

Melanoidins are negatively charged, complex, heterogenous non-linear polymers produced through non-enzymatic Maillard reaction (MR) between amino compounds and carbohydrates, and are highly resistant to biological and chemical degradation (Wedzicha and Kaputo 1992; Wang et al. 2011). It is a major colouring constituents of dark-brown effluent released from sugarcane molasses-based ethanol producing distillery industries. During the distillation process, an average of 12–15 L of effluent is generated per litre of ethanol production (Chandra and Kumar 2017a). Dark coloured effluent from the distilleries is one of the most obvious indicators of water pollution. Apart from the colour, the discharged distillery effluent also contains high amount of total dissolved solids (TDS) and recalcitrant pollutants in form of complex polymer containing heterocyclic nitrogenous compounds of aldehyde-amines, various heavy metals, phenolic compounds and plant derived resins and fatty acids which behave as endocrine-disrupting chemicals (EDCs) (Chandra and Kumar 2017a). The major metallic constituents in distillery effluent is reported as iron (Fe), copper (Cu), manganese (Mn), lead (Pb), zinc (Zn), cadmium (Cd), and nickel (Ni). Furthermore, it has also been reported that melanoidins have net negative charges; therefore, various heavy metals strongly bind with melanoidins to form large organo-metallic complex molecules (Migo et al. 1997). Therefore, discharged effluent after anaerobic

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s13205-018-1205-3) contains supplementary material, which is available to authorized users.

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ORIGINAL PAPER

# Characterisation of manganese peroxidase and laccase producing bacteria capable for degradation of sucrose glutamic acid-Maillard reaction products at different nutritional and environmental conditions

Vineet Kumar[1] · Ram Chandra[1]

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**Abstract**
Maillard reactions products (MRPs) are a major colorant of distillery effluent. It is major source of environmental pollution due to its complex structure and recalcitrant nature. This study has revealed that sucrose glutamic acid-Maillard reaction products (SGA-MRPs) showed many absorption peaks between 200 and 450 nm. The absorption maximum peak was noted at 250 nm in spectrophotometric detection. This indicated the formation of variable molecular weight Maillard products during the SGA-MRPs formation at high temperature. The identified aerobic bacterial consortium consisting *Klebsiella pneumoniae* (KU726953), *Salmonella enterica* (KU726954), *Enterobacter aerogenes* (KU726955), *Enterobacter cloaceae* (KU726957) showed optimum production of MnP and laccase at 120 and 144 h of growth, respectively. The potential bacterial consortium showed decolourisation of Maillard product up to 70% in presence of glucose (1%), peptone (0.1%) at optimum pH (8.1), temperature (37 °C) and shaking speed (180 rpm) within 192 h of incubation. The reduction of colour of Maillard product correlated with shifting of absorption peaks in UV–Vis spectrophotometry analysis. Further, the changing of functional group in FT-IR data showed appearance of new peaks and GC–MS analysis of degraded sample revealed the depolymerisation of complex MRPs. The toxicity evaluation using seed of *Phaseolus mungo* L. showed reduction of toxicity of MRPs after bacterial treatment. Hence, this study concluded that developed bacterial consortium have capability for decolourisation of MRPs due to high content of MnP and laccase.

**Keywords** Melanoidins · Manganese peroxidase · Laccase · Metabolites · GC–MS analysis

## Introduction

Melanoidins are complex, dark coloured, amino-carbonyl polymeric compounds with high molecular weight, formed by non-enzymatic browning Maillard reaction (MR) during several industrial processes (Hayase 2000; Echavarría et al. 2014; Bharagava and Chandra 2010). The major sources of melanoidins in environment are sugarcane-molasses based distilleries and fermentation industries all over the world Due to its complex unknown organic pollutants and high total dissolved solids (TDS), the safe disposal of distillery waste is a paramount challenge for industrialist (Chandra and Kumar 2017). The toxicity of distillery waste is well documented in terrestrial and aquatic environment due to presence of heavy metals and recalcitrant organic compounds (Chandra et al. 2008a, b; Hemavanthi et al. 2015) The majority of molasses-based distilleries are located in tropical and subtropical regions of world and generate ~ 12–15 liters of effluent per liter of alcohol production. Currently, there are more than 397 distilleries are operating in India producing approx. $3.25\times10^{10}$ liters of ethanol and generating $40.90\times10^{15}$ liters of effluent annually (AIDA 2016)

Various physico-chemical methods such as column filtration (Satyawali and Balakrishnan 2008), flocculation (Liang

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# Heavy metal phytoextraction potential of native weeds and grasses from endocrine-disrupting chemicals rich complex distillery sludge and their histological observations during in-situ phytoremediation

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Native weeds
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β-Sitosterol

ABSTRACT

Sugarcane-molasses based distillery waste is a threat to environment for its safe disposal due to complexation of endocrine-disrupting chemicals (EDCs) containing mixture of organic pollutants. This study revealed that distillery sludge contains not only mixture of complex organic pollutants but also retains high quantity of Fe (5264.49), Zn (43.47), Cu (847.46), Mn (238.47), Ni (15.60), and Pb (31.22 mg $kg^{-1}$) which enhances the toxicity of sludge to the environment. The major identified organic compounds were benzene, 1-ethyl-2-methyl, benzene, 1-ethyl-4-methyl benzoic acid, 3,4,5-tris(TMS oxy), TMS ester; hexanedioic acid, dioctyl ester; stigmasterol TMS ether; 5α-cholestane,4-methylene; campesterol TMS; β-sitosterol and lanosterol. These compounds are listed under the EDCs also as per U.S. Environmental Protection Agency. However, the phytoextraction potential of growing native weeds and grasses i.e. *Argemone mexicana, Saccharum munja, Cynodon dactylon, Pennisetum purpureum, Chenopodium album, Rumex dentatus, Tinospora cordifolia, Calotropis procera* and *Basella alba* revealed the high accumulation of Fe, Zn, Cu, Mn, Ni, and Pb in their root and leaves compared to shoot. This indicated high accumulation and translocation capabilities of these plants. Further, the bioaccumulation coefficient factor (BCF) and translocation factor (TF) was found > 1 for majority of plants for various metals. Thus, this given strong evidence for hyperaccumulation tendency of these native weeds and grasses from complex polluted sites. Furthermore, the ultrastructural observations of root tissues also revealed the deposition of heavy metals at various cellular components without any apparent toxic effects. This indicated the variable adaptive characteristics of these plants growing at a hazardous waste polluted site. Thus, the study given a strong evidence for application of these weeds and grasses as tools for in-situ phytoremediation and eco-restoration of polluted sites.

## 1. Introduction

Sugarcane-molasses based distillery waste is well known as source of complex environmental pollutants due to various heavy metals containing complex organic pollutants (Chandra et al., 2008; Chandra and Kumar, 2017a, 2017b). In India, there are more than 397 sugarcane molasses based distilleries releasing approximately $3.5 \times 10^{13}$ kL spent wash annually (AIDA, 2016). There is an average sludge generation of 1500 tons per day during anaerobic digestion of spent wash (Kansal et al., 1998). This reflects the magnitude of the environmental pollution caused by the waste generated from distillery sector all over India. The sludge generated from distilleries also contain mainly dodecanoic acid, octadecanoic acid, *n*-pentadecanoic acid, hexadecanoic acid, β-sitosterol, stigmasterol, β-sitosterol trimethyl ether, heptacosane, dotriacontane, lanosta-8, 24-dien-3-one, 1-methylene-3-methyl butanol, and 1-phenyl-1-propanol as androgenic and mutagenic compounds (Chandra and Kumar, 2017a), which are listed under the endocrine-disrupting chemicals (EDCs) list of USEPA (2012). The study has revealed that these organic pollutants makes organo-metallic complex with various heavy metals which are mainly iron (Fe), zinc (Zn), copper (Cu), chromium (Cr), cadmium (Cd), manganese (Mn), nickel (Ni), and lead (Pb) present in high quantity i.e. (Fe: 2403.64), (Zn: 210.624), (Cu: 73.63), (Cr: 21.84), (Cd: 1.446), (Mn: 126.292), (Ni: 13.425), (Pb: 16.332 mg $kg^{-1}$) (Chandra and Kumar, 2017c). The concentrations of these metals are for above than the prescribed limit in environment as per USEPA (2002) and European Union (2002).

Generated effluent after distillation process also a major source of aquatic pollution due to high level of maillard products generated due

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frontiers in Microbiology

ORIGINAL RESEARCH
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# Detection of Androgenic-Mutagenic Compounds and Potential Autochthonous Bacterial Communities during *In Situ* Bioremediation of Post-methanated Distillery Sludge

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Sugarcane-molasses-based post-methanated distillery waste is well known for its toxicity, causing adverse effects on aquatic flora and fauna. Here, it has been demonstrated that there is an abundant mixture of androgenic and mutagenic compounds both in distillery sludge and leachate. Gas chromatography-mass spectrometry (GC-MS) analysis showed dodecanoic acid, octadecanoic acid, *n*-pentadecanoic acid, hexadecanoic acid, β-sitosterol, stigmasterol, β-sitosterol trimethyl ether, heptacosane, dotriacontane, lanosta-8, 24-dien-3-one, 1-methylene-3-methyl butanol, 1-phenyl-1-propanol, 5-methyl-2-(1-methylethyl) cyclohexanol, and 2-ethylthio-10-hydroxy-9-methoxy-1,4 anthraquinone as major organic pollutants along with heavy metals (all mg $kg^{-1}$): Fe (2403), Zn (210.15), Mn (126.30, Cu (73.62), Cr (21.825), Pb (16.33) and Ni (13.425). In a simultaneous analysis of bacterial communities using the restriction fragment length polymorphism (RFLP) method the dominance of *Bacillus* sp. followed by *Enterococcus* sp. as autochthonous bacterial communities growing in this extremely toxic environment was shown, indicating a primary community for bioremediation. A toxicity evaluation showed a reduction of toxicity in degraded samples of sludge and leachate, confirming the role of autochthonous bacterial communities in the bioremediation of distillery waste *in situ*.

**Keywords: distillery sludge, toxicity, β-sitosterol, *Bacillus* sp., *Enterococcus* sp., RFLP**

## INTRODUCTION

Various forms of industrial waste are major sources of environmental pollution due to the release of several unidentified toxic pollutants. The majority of agro-based industries, i.e., distilleries, tanneries, and pulp paper are major sources of aquatic and soil pollution. Due to the release of huge amounts of waste water and sludge, sugarcane-molasses-based distilleries are among the most polluting industries in India. Distilleries release 12 to 15 l of spent wash per liter of alcohol produced. Currently, there are more than 319 distilleries in India, reflecting the magnitude of the problem due to the presence of various complex pollutants in post-methanated distillery effluent (PMDE) and post-methanated distillery sludge (PMDS). The detection and detoxification

Environ Monit Assess (2017) 189:26
DOI 10.1007/s10661-016-5746-9

# Detection of *Bacillus* and *Stenotrophomonas* species growing in an organic acid and endocrine-disrupting chemical-rich environment of distillery spent wash and its phytotoxicity

**Ram Chandra · Vineet Kumar**

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**Abstract** Sugarcane molasses-based distillery spent wash (DSW) is well known for its toxicity and complex mixture of various recalcitrant organic pollutants with acidic pH, but the chemical nature of these pollutants is unknown. This study revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy-4-[(TMS)oxy], TMS ester; and tricarballylic acid 3TMS), which are listed as endocrine-disrupting chemicals. In addition, several major heavy metals were detected, including Fe (163.947), Mn (4.556), Zn (2.487), and Ni (1.175 mg $l^{-1}$). Bacterial community analysis by restriction fragment length polymorphism revealed that *Bacillus* and *Stenotrophomonas* were dominant autochthonous bacterial communities belonging to the phylum *Firmicutes* and γ-*Proteobacteria*, respectively. The presence of *Bacillus* and *Stenotrophomonas* species in highly acidic environments indicated its broad range adaptation. These findings indicated that these autochthonous bacterial communities were pioneer taxa for in situ remediation of this hazardous waste during ecological succession. Further, phytotoxicity assay of DSW with *Phaseolus mungo* L. and *Triticum aestivum* revealed that *T. aestivum* was more sensitive than *P. mungo* L. in the seed germination test. The results of this study may be useful for monitoring and toxicity assessment of sugarcane molasses-based distillery waste at disposal sites.

**Keywords** *Bacillus* sp. · *Firmicutes* · Spent wash · Molasses · Endocrine-disrupting chemicals

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## Introduction

Sugarcane molasses-based distillery spent wash (DSW) is characterized by a high biological oxygen demand (BOD; 42,000 mg $l^{-1}$), chemical oxygen demand (COD; 90,000 mg $l^{-1}$), total dissolved solids (TDS; 77,000 mg $l^{-1}$), and the presence of heavy metals, sulfur and nitrogen containing complex organic pollutants with low pH (Chandra et al. 2004; Saliha 2005). The majority of molasses-based distilleries are located in tropical and

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RESEARCH ARTICLE

# Phytoextraction of heavy metals by potential native plants and their microscopic observation of root growing on stabilised distillery sludge as a prospective tool for in situ phytoremediation of industrial waste

**Ram Chandra**[1,2] · **Vineet Kumar**[2]

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**Abstract** The safe disposal of post-methanated distillery sludge (PMDS) in the environment is challenging due to high concentrations of heavy metals along with other complex organic pollutants. The study has revealed that PMDS contained high amounts of Fe (2403), Zn (210), Mn (126), Cu (73.62), Cr (21.825), Pb (16.33) and Ni (13.425 mg $kg^{-1}$) along with melanoidins and other co-pollutants. The phytoextraction pattern in 15 potential native plants growing on sludge showed that the *Blumea lacera*, *Parthenium hysterophorous*, *Setaria viridis*, *Chenopodium album*, *Cannabis sativa*, *Basella alba*, *Tricosanthes dioica*, *Amaranthus spinosus* L., *Achyranthes* sp., *Dhatura stramonium*, *Sacchrum munja* and *Croton bonplandianum* were noted as root accumulator for Fe, Zn and Mn, while *S. munja*, *P. hysterophorous*, *C. sativa*, *C. album*, *T. dioica*, *D. stramonium*, *B. lacera*, *B. alba*, *Kalanchoe pinnata* and *Achyranthes* sp. were found as shoot accumulator for Fe. In addition, *A. spinosus* L. was found as shoot accumulator for Zn and Mn. Similarly, all plants found as leaf accumulator for Fe, Zn and Mn except *A. spinosus* L. and *Ricinus communis*. Further, the BCF of all tested plants were noted <1, while the TF showed >1. This revealed that metal bioavailability to plant is poor due to strong complexation of heavy metals with organic pollutants. This gives a strong evidence of hyperaccumulation for the tested metals from complex distillery waste. Furthermore, the TEM observations of root of *P. hysterophorous*, *C. sativa*, *Solanum nigrum* and *R. communis* showed formation of multi-nucleolus, multi-vacuoles and deposition of metal granules in cellular component of roots as a plant adaptation mechanism for phytoextraction of heavy metal-rich polluted site. Hence, these native plants may be used as a tool for in situ phytoremediation and eco-restoration of industrial waste-contaminated site.

**Keywords** Phytoremediation · Bioconcentration factor · Transmission electron microscopy · Multi-vacuoles · Hyperaccumulator plants

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## Introduction

Heavy metals are a major source of water and soil pollution, generated as either geogenic activities or industrial waste discharge (Hawari and Mulligan 2006; Li et al. 2009). Currently, several industrial waste discharges are a mixture of heavy metals along with various complex organic waste which are a major source of environmental pollution due to its complexity and slow degradation. Various agro-based industries, i.e. distilleries, pulp and paper mills and tanneries are also a source of heavy metals in the environment due to discharge of their huge amounts of complex waste (Sinha et al. 2007; Yadav and Chandra 2011; Singh et al. 2008). During the course of alcohol production, around 12–15 L of effluent is generated per litre of alcohol production. In India, there are more than 319 distilleries producing ~$3.25 \times 10^9$ L of alcohol and generating ~$40.4 \times 10^{10}$ L spent wash annually (Chandra et al. 2008a; Pant and Adholeya 2007). The spent wash after

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Ram Chandra and Vineet Kumar

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# Chapter 8
# Extremophilic Ligninolytic Enzymes

**Ram Chandra, Vineet Kumar, and Sheelu Yadav**

**What Will You Learn from This Chapter?**

Extremophilic microorganisms have developed a variety of physiological strategies that help them to survive on different ecological niche such as extreme temperature, pH, salt concentration and pressure. It has been demonstrated that these microorganisms produce extracellular isoenzyme capable to degrade the ligninocellulosic waste and other related compounds for their growth and survival. These are known as extremophilic ligninolytic enzyme. The extremophilic enzymes are considered superior than normal enzyme because they allow the performance of industrial processes even under adverse condition in which conventional proteins are completely denatured. The common extremophilic ligninolytic enzymes are manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase. These enzymes predominantly have been reported in fungus but their occurrence and role for decolourisation and detoxification of various environmental pollutants also have been reported in bacteria and actinomycetes. Biochemically, MnP and LiP are glycosylated haem protein with molecular weight (MW) ranging from 38 to 62.5 kDa (MnP: 38–62.5 kDa; LiP: 38–46 kDa) while laccases are monomeric, dimeric and trimeric glycoprotein with MW range from 50 to 97 kDa. The optimum activity at pH range for MnP and LiP in fungus is 3.0–5.0 while in bacteria pH range

Chapter 1

# Persistent Organic Pollutants (POPs): Health Hazards and Challenges for their Bioremediation in Environment

RAM CHANDRA[1] AND VINEET KUMAR

SUMMARY

Persistent organic pollutants (POPs) have become a great concern due to their toxicity, transformation and bioaccumulation property. Therefore, this book chapter highlights the types, sources, classification, health hazards and mobility of organochlorine pesticides, industrial chemicals and their by-products. Moreover, with the signing of Aarhus Protocol and Stockholm Convention on POPs, there is an increased demand to identify and characterise such chemicals from industrial wastes and environment, which are toxic in nature or to existing biota. Due to their long life and persistent nature, they enter into body through food chain and transfer to all tropic levels of ecological unit. In addition, POPs are lipophilic in nature and accumulate in lipid containing tissues and organs, which further indicates the adverse symptoms

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# 1

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**Ram Chandra, Gaurav Saxena and Vineet Kumar**

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CHAPTER - 26

# Two Step Treatments by Bacteria and Rhizofiltration for Bioremediation of Complex Industrial Wastewater: A Novel Approach for Safe Disposal

**Ram Chandra*, Sangeeta Yadav and Vineet Kumar**
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**Abstract**

*Complex industrial wastewater by using conventional treatment method is a significant challenge for its safe disposal. Recently, two step treatments by aerobic bacteria using activated sludge process followed by rhizofiltration has been recommended as a promising technology for detoxification of complex industrial waste. In activated sludge process recalcitrant compounds are biotransformed along with biodegradation of organic waste. Aerobic treatment may be activated sludge process, trickling filter or aerobic lagoon process, which one is used for treatment it is based upon the characteristics of wastewater. Carbon and nitrogen ratio along with other organic compound is also play important role for microbial growth and degradation of pollutants. During degradation of various pollutants, metal microbes interaction play significant role for its bioremediation either through adsorption or bioaccumulation. Subsequently, in rhizofiltration by constructed wetland treatment enhances the degradation as a result of multi mechanism process i.e. bidegradation, accumulation, adsorption, biofilteration and biotransformation for detoxification. Siderophores contributes very important role for the establishing the bacteria and root association for the formation of root matrics associated microbe's biofilter. Besides, siderophores also facilitate the nutrient availability to plant and microbes through solubilizing the complex compounds. This technique is*


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# आसवनी अपशिष्ट एवं कागज उद्योग उत्स्राव के कारण पर्यावरण प्रदूषण तथा भारी धातुओं से खाद्य श्रृंखला एवं मानव स्वास्थ्य पर खतरा

**राम चन्द्रा एवं विनीत कुमार**
पर्यावरणीय सूक्ष्मजैविकी विभाग, बाबा साहेब भीमराव अम्बेडकर विश्वविद्यालय,
विद्या विहार, रायबरेली रोड़, लखनऊ, उत्तर प्रदेश–226025

आसवनी अपशिष्ट एवं कागज उद्योगो के उत्स्राव पर्यावरण मे प्रदूषण के मुख्य स्त्रोत है। इनके द्वारा प्रक्षेपित अपशिष्टों मे बहुतायत मात्रा मे भारी धातुओं के साथ–साथ हानिकारक हठधर्मी कार्बनिक प्रदूषक भी मिले होते है। आसवनी अपशिष्ट मे शोधनोपरान्त भी भारी धातुओ की मात्रा अधिक होती है जैसे– मैंगनीज (0.98–126.30) आयरन (35.967–2403) जिंक (3.11–210.15) निकिल (1.233–13.425) लेड़ (0.08–16.33) तथा क्रोमियम (4.124– 21.825)। इसी प्रकार से कागज उद्योग अपशिष्ट मे अत्यधिक मात्रा मे भारी धातुएं जैसे– मैंगनीज (0.102–11.00) जिंक (0.198–13.90) निकिल (0.198–3.30) लेड़ (0.005–1.05) क्रोमियम (0.020–2.30), तथा कैडमियम (0.00–0.255) आदि पायी जाती है। इन प्रदूषकों के कारण हमारे पारिस्थितिक तंत्र मे जल एवं मृदा प्रत्यक्ष रूप से प्रभावित होते है जिसके फलस्वरूप जलीय जीव–जन्तुओ द्वारा मानव खाद्य श्रृंखला प्रभावित होती है। अध्ययन मे यह भी पाया गया है कि बहुत से हानिकारक धातुओं जैसे–कैडमियम, मैंगनीज, आयरन, जिंक, निकिल, लेड़, तथा क्रोमियम इत्यादि जलीय जीव–जन्तुओ एवं खाद्य पौधों से संचयित होकर हमारे स्वास्थ्य

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# आसवनी अपशिष्ट को अनॉक्सीकृत प्रक्रिया द्वारा शोधनोपरान्त पर्यावरण मे सुरक्षात्मक निस्तारण की चुनौतियाँ

**विनीत कुमार एवं राम चन्द्रा**
पर्यावरणीय सूक्ष्मजैविकी प्रभाग, पर्यावरण विषविज्ञान विभाग
सी.एस.आई.आर.–भारतीय विषविज्ञान अनुसंधान संस्थान
विषविज्ञान भवन, 31 महात्मा गांधी मार्ग, लखनऊ, 226001, उत्तर प्रदेश, भारत

## 1.प्रस्तावना

आसवनी उद्योग भारत के मुख्य 17 प्रदूषण फैलाने वाले उद्योगों में एक है तथा इन आसवनी उद्योगों का एल्कोहल उत्पादन में एशिया में प्रथम स्थान है। एल्कोहल को मुख्यतः शीरे के किण्वन द्वारा यीस्ट कोशिकाओं (खमीर) की सहायता से 50 से 55 घंटे में 25 से 30 डिग्री सेन्टीग्रेड तापमान पर बनाया जाता है जिसमें एल्कोहल की मात्रा लगभग 8 से 10 प्रतिशत होती है। किण्वनोपरान्त एल्कोहल युक्त इस विलयन को आसवन विधि द्वारा एल्कोहल तथा सह उत्पादकों जैसे मिथेनाल, ब्यूटेनाल, प्रोपेनाल आदि मे तथा वेस्टवाटर बनने की प्रक्रिया को चित्र 1 द्वारा दर्शाया गया है।

अखिल भारतीय आसवनी उद्यमी संगठन के आंकड़ों (2005) के अनुसार हमारे देश मे लगभग 319 आसवनी उद्योग प्रतिवर्ष लगभग $3.25 \times 10^9$ लीटर एल्कोहल का उत्पादन करते हैं जिसके फलस्वरूप $40.4 \times 10^{10}$ लीटर स्पेन्टवास उत्प्रवाह के रूप में विसर्जित होता है। यह स्पेन्टवास अम्लीय प्रकृति का होता है जिसमे मुख्यतः मिलेन्वाइडिन्स, फिनोलिक्स, कैरामल, सल्फाइड तथा भारी धातुएँ पायी जाती हैं जो कि स्पेन्टवास के गाढ़े भूरे रंग के लिए उत्तरदायी

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Environmental health hazards of post methanated distillery waste...
Proceedings of 74th Annual Convention of STAI: 684 – 699 2016

# ENVIRONMENTAL HEALTH HAZARDS OF POST METHANATED DISTILLERY WASTE AND ITS DETOXIFICATION

Ram Chandra*, Vineet Kumar & Sonam Tripathi

***ABSTRACT***

*The sugarcane molasses based distillery waste water is major source of environmental pollutant due to its huge generation and complex nature. The post Methanated distillery effluent (PMDE) is being used indiscriminately by farmers also in country as irrigating water without knowing its adverse effect. The study has revealed that 1-10% PMDE showed stimulatory effect on Phaseolus mungo L. growth parameter, while above 15-20% distillery effluent showed toxic effect on plant growth parameter. Similarly, PMDE sludge having concentration ≤ 10% (w/w) act as a fertilizer. However, above than 10% of PMDE sludge in soil (w/w) both caused adverse effect on plant growth parameters and soil fertility. The research revealed that accumulation of heavy metal at ≥ 40% (w/w) concentration was highest in the root except iron and manganese. Further, the biodegradation of distillery wastewater (DWW) by using aerobic bacterial consortium comprising Bacillus licheniformis (DQ79010), Bacillus sp. (DQ779011), and Alcaligenes sp. (DQ779012) were showed that the bacterial consortium was efficient for 70% color removal in presence of glucose (1.0%) and peptone (0.1%) at pH 7.0 and temperature 37 0C. The metabolic products were also characterized by LC-MS/MS analysis. This confirmed that bacterial consortium was capable for biodegradation. Further, the seed germination test for environmental safety showed that the PMDE after bacterial treatment showed growth promoting effect.*

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58th Annual Conference of
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**Degradation and decolourisation of molasses-melanoidins by potential bacterial consortium extracted from anaerobic digested distillery effluent at different nutritional and environmental conditions**

**Vineet Kumar and Ram Chandra**

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In this study melanoidin was extracted with 1:1 (v/v) mixture of isopropanol and post methanated distillery effluent (PMDE). The melanoidins was revealed as organo-metallic complex. The major heavy metals were detected as Mn (8.20), Cr (2.97), Zn (16.61), Cu (2.55), Fe (373.95), Pb (2.59) and Ni (4.18 mg $L^{-1}$) along with mixture of other organic compounds with endocrine-disrupting chemicals (EDCs) category list. A consortium of aerobic bacteria comprising *Klebsiella pneumoniae* (KU321273), *Salmonella enteric* (KU726954), *Enterobacter aerogenes* (KU726955), and *Enterobacter cloacae* (KU726957) showed the optimum decolourisation up to 81% in presence of glucose (1.0%) and peptone (0.2%) as a carbon and nitrogen source. The scanning of absorption spectrum by UV-Vis spectrophotometer between 200-700nm showed major reductions of absorption spectrum in range of 200-450nm compared to control. The degradation and decolourisation of melanoidin by bacterial consortium was noted in presence of MnP and Laccase activity in sample supernatant. Further, the TLC and HPLC analysis also showed degradation and reduction of absorption peak at (295nm), respectively. Furthermore, FT-IR and GC-MS analysis also showed the change of functional group and disappearance of ion peaks. This indicated the degradation and depolymerisation complex polymer, which resulted in reduction of colour. The metabolic analysis showed the production of as major metabolic products. Further the seed germination test using *Phaseolus mungo L.* showed toxicity reduction in decolorized effluent.

**Keywords:** Melanoidins, Metabolites, PMDE decolourisation, Endocrine disrupting chemicals, Toxicity reduction.

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The sugarcane molasses based distillery wastewater is major source of environmental pollution all over country due to its huge generation and complex nature. There is production of 12-15 litre of spent wash generation in production per liter of alcohol. The spent wash of distillery even after anaerobic digestion which is known as post methanated distillery effluent (PMDE) remains toxic and became more dark black colour. This not only increases the BOD, COD, and color to receiving aquatic ecosystem but also adds some toxic heavy metals and organic pollutants containing the mutagenic and endocrine-disrupting chemicals (EDCs) to the environmental organisms. The PMDE is being used indiscriminately by some farmers in country as irrigating water without knowing the adverse effect. Therefore, the effect of PMDE was evaluated with Phaseolus mungo L. by irrigating 1- 100% distillery effluent in pot culture experiment. The results showed that up-to 1-10% distillery effluent only showed stimulatory effect on plant growth parameters, while above than 15-20% distillery effluent showed toxic effect on plant growth parameter. Further, in field condition irrigation with PMDE for agricultural crop also showed health hazards in Triticum aestivum, and Brassica compestris by accumulating the heavy metals in seed and stem. Furthermore, during the in-situ and ex-situ phytoremediation of heavy metals from distillery effluent contaminated site by Typha angustifolia and Phragmites cummunis also showed cellular toxicity in presence of higher concentration of melanoidins and phenols. Hence, the biodegradation of distillery wastewater has been developed by IITR using aerobic bacterial consortium comprising of Bacillus licheniformis (DQ79010), Bacillus sp. (DQ779011), and Alcaligenes sp. (DQ779012) showed decolourisation and detoxification of PMDE which was environmentally safe for disposal. The study also recommended the T. angustifolia, P. cummunis, Cyperus esculantus and other native plants can be used as bio-tool for in-situ bioremediation of distillery waste pollutants and phytoremediation of heavy metals from polluted site. Further, in process to development of innovative technology the integration of potential bacterial consortium and sub-surface flow constructed wetland treatment system in two step treatment process after TDS reduction of PMDE has been developed and optimized in laboratory scale level. It was noted that the decolourised PMDE can be re-used for sustainable development without causing any adverse effect to environment. Further, the pilot scale optimization and field scale demonstration of the developed technology is under process.

Keywords: Distillery waste, Bioremediation, Decolourisation, Recycling, Endocrine-disrupting chemicals




**Use of Industrial Wastewater in Agricultural Practices: Challenges and Applications**

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Safe disposal of agrobased industrial waste i.e. distilleries, pulp and paper industries, and tanneries are major challenge for country due to its huge generation of wastewater containing complex organic pollutants. There is production of 12-15 liter of spent wash per liter of alcohol production. Similarly, approx. 200 cubic gallon of wastewater is generated per ton of paper production. These effluents remain toxic and dark color even after the available treatment technology. Consequently, these are major source of aquatic and soil pollution. Several places these industrial wastes are indiscriminately being used by farmers as irrigation water in various crops without knowing the adverse effect. The studies have revealed that post methanated distillery effluent (PMDE) showed stimulatory result upto 1-10% concentration on *Phaseolus mungo* L. in irrigated water, while above than 15-20% of effluent showed adverse effect on plant growth parameters. Similarly, soil amended with 10% (w/w) distillery sludge also induced the growth in root, shoot length of *P. mungo* L. while above than 20% (w/w) sludge amended soil showed adverse effect on plant growth of *P. mungo* L. Based upon these studies, it was recommended that sludge having concentration upto 10% (w/w) act as a fertilizer. However, above than 10% of PMDE v/v in water and sludge in soil (w/w) both causes adverse effect on plant- growth parameters and soil fertility. Further, in field condition irrigation with PMDE for agricultural crop also showed health hazards in *Triticum aestivum* & *Brassica compestris* by accumulating the heavy metals in seed and stem. Furthermore, during the in-situ and ex-situ Phytoremediation of heavy metals from distillery effluent contaminated site by *Typha angustifolia* and *Phragmites cummunis* also showed cellular toxicity in presence of higher concentration of melanoidins and phenols. Hence, the biodegradation of distillery wastewater by aerobic bacterial consortium comprising of *Bacillus licheniformis* (DQ79010), *Bacillus sp.* (DQ779011), and *Alcaligenes* sp. (DQ779012) showed decolourisation and detoxification of PMDE which was environmentally safe for disposal. The study also recommended the *T. angustifolia, P. cummunis* and *Cyperus esculantus* can be used as biotool for monitoring of heavy metal pollution at industrial waste contaminated site in aquatic resources upto certain concentration for sustainable development. Moreover, the wastewater discharge from various industrial waste have mixture of endocrine-disrupting chemicals (EDCs) and carcinogenic compounds. But, the detail knowledge regarding the nature of pollutants and their life cycle is unknown. Therefore, there must need to reveal the toxic load of discharging various industrial waste and evaluation of their effect on various crops in irrigation during the variable seasons and geographical conditions due to variation of soil and environmental parameters.


**LT-15: Detection of indigenous bacterial community growing in the endocrine-disrupting chemicals and heavy metal rich environment of sugarcane molasses based distillery waste**

**Ram Chandra and Vineet Kumar**

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Sugarcane-molasses-based distillery spent wash (DSW) is well known for its toxicity and complex mixture of various recalcitrant organic pollutants with acidic pH, but the chemical nature of these pollutants is unknown. This study revealed the presence of toxic organic acids (butanedioic acid bis(TMS)ester; 2-hydroxysocaproic acid; benzenepropanoic acid, α-[(TMS)oxy], TMS ester; vanillylpropionic acid, bis(TMS)), and other recalcitrant organic pollutants (2-furancarboxylic acid, 5-[[(TMS)oxy] methyl], TMS ester; benzoic acid 3-methoxy-4-[(TMS)oxy], TMS ester and tricarballylic acid 3TMS), which are listed as endocrine-disrupting chemicals. In addition, several major heavy metals were detected, including Fe (163.947), Mn (4.556), Zn (2.487), and Ni (1.175 mg $l^{-1}$). Bacterial community analysis by restriction fragment length polymorphism revealed that *Bacillus* and *Stenotrophomonas* were dominant autochthonous bacterial communities belonging to the phylum *Firmicutes* and γ-*Proteobacteria*, respectively. The presence of *Bacillus* and *Stenotrophomonas* species in highly acidic environments indicated its broad range adaptation. Similarity, the post methanated distillery analysis also showed dodecanoic acid (RT:20.73), octadecanoic acid, n-pentadecanoic

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INNOVATION CENTRE ON FOOD PROCESSING AND FOOD TECHNOLOGY
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**Environmental Health Hazards of Post Methanated Distillery Waste and Contamination of Food and its Safety**

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The sugarcane molasses based distillery waste water is major source of environmental pollutant due to its huge generation and complex nature. The post methanated distillery effluent (PMDE) is being used indiscriminately by farmers also in country as irrigating water without knowing the adverse effect. The study has revealed that 1-10% PMDE showed stimulatory effect on *Phaseolus mungo* L. growth parameter, while above 15-20% distillery effluent showed toxic effect on plant. Similarly, experiment were conducted to study the effect of distillery sludge amendments with garden soil (10, 20, 40, 60, 80 and 100 %) on seed germination and growth parameters of *P. mungo* L. Result revealed that PMDE sludge having concentration ≤ 10% (w/w) act as a fertilizer. However, above than 10% of PMDE sludge in soil (w/w) both caused adverse effect on plant- growth parameters and soil fertility. The research revealed that accumulation of heavy metal at ≥ 40% (w/w) concentration was highest in root the except iron and manganese. This indicated a source of food contamination with heavy metals due to industrial effluent irrigation. The biodegradation of distillery wastewater (DWW) by using aerobic bacterial consortium comprising *Bacillus licheniformis* (DQ79010), *Bacillus sp.* (DQ779011), and *Alcaligenes* sp. (DQ779012) were showed that the bacterial consortium was efficient for 70% color removal in presence of

# Association of Microbiologists of India & International Symposium on Microbes and Biosphere: What's New What's Next

**November 24-27, 2016**

**EM-95: Isolation, screening and characterization of MnP and Laccase Producing Bacteria Capable for degradation and decolourisation of sucrose-glutamic acid maillard products**

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Melanoidins are complex amino carbonyl compound produced by non-enzymatic browning maillard reaction in several industrial process. It is a major complex colouring constituents of sugarcane-molasses based distillery effluent, which retains a dark color even after anaerobic treatment, and hardly changes in extended aeration tanks, due to its recalcitrant nature. In this study, the four potential aerobic bacterial strains IITRCS01, IITRCS0106, IITRCS0107, and IITRCS011 were isolated from distillery sludge, identified as *Klebsiella pneumoniae* (KU321273), *Salmonella enteric* subsp. *arizonae* (KU726954), *Enterobacter aerogenes* (KU726955), and *Enterobacter cloacae* (KU76957), respectively by 16S rRNA gene sequence analysis. These identified bacterial strains were found capable to produce manganese peroxidase (MnP) and Laccase (lac) enzymes. The synthesized sucrose glutamic acid- maillard products (SGA-MPs) showed major absorbance spectra in range of 200-450nm after 6 hrs polymerization in laboratory conditions. While the absorbance maxima was noted 240nm with minor changes. This indicated the mixture compound products of all three reaction stages of maillard products

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**Detection of Recalcitrant Toxic Organic Pollutants and Identification of*Bacillus* and *Enterococcus Sp.* by RFLP Method as Dominant Autochthonous Firmicutes Growing in Sugarcane Molasses Based Post Methanated Distillery Sludge**

Ram Chandra[1], Vineet Kumar[2]

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Sugarcane molasses based post methanated distillery sludge is characterised by high content of several unknown recalcitrant organic and inorganic pollutants and relatively unexposed unique microbial environment. Various organic solvent was used in solid-liquid to extract the broad range of persisting organic pollutants and RFLP technique was used to identify autochthonous bacterial communities growing in toxic environment of distillery sludge. The GC-MS analysis showed that dodecanoic acid (RT: 20.73), Octadecanoic acid (RT: 30.34), n-pentadecanoic acid (RT:25.28), hexadecanoic acid (RT:27.47), β-sitosterol (RT:41.57), stigmasterol (RT:41.55), β-sitosterol trimethyl ether (RT:42.67), heptacosane (RT:21.62), dotriacontane (RT:22.13) lanosta-8, 24-dien-3-one (RT:42.14), Bis (inolyl)cyclopentane (RT:9.64), 1-methylene-3-methyl butanol (RT:11.10), 1-phenyl-1-propanol (RT:14.84), 5-methyl-2-(1-methylethyl)cycohexanol (RT:12.57), 2-Ethylthio-10-hydroxy-9-methoxy-1,4 anthraquinone (RT:12.90) as a major organic pollutants present in sludge along with various toxic metals i.e. Fe (2403), Zn (210.15), Mn (126.30, Cu (73.62), Cr (21.825), Pb (16.33), Ni (13.425), and Cd (1.440 mg kg-1). These compounds are not only toxic to environment but also reported as androgenic as well as DNA fragment inducer also. Further, the bacterial community analysis through culture independent




IBF-173

**Phytoextraction and bioremediation of heavy metals by potential native plants growing on post Methanated distillery effluent sludge dumping sites**

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Post Methanated distillery effluent (PMDE) retains very high TDS and color even after anaerobic treatment. Therefore, disposal of PMDE sludge is environmental threat due to high concentration of heavy metals and other persistent organic pollutants. The physico-chemical analysis of PMDE sludge showed high contents of Fe (2403), Zn (210.15), Cu (73.62), Cr (21.825), Cd (1.440), Mn (126.30), Ni (13.425), Pb (16.33) along with high concentration of different salts like $Cl^-$(1825), $Na^+$ (56), $NO_3^{2-}$ (110), $NH_4^+$ (190) mg/L at pH 8.0 and other parameters like electric conductivity (2.30) µs/cm, salanity (1.1) ppt, moisture (57.727) and TDS (1560) mg/l. But, growth of various potential native plants on PMDE dumping sludge indicated the capacity for phytoextraction of heavy metals and other residual pollutants present in PMD sludge. The periodic analysis of various metals from different parts of native plants, for monitoring of phytoextraction and bioremediation potential of these plants were done. During the analysis it was noted that *Dhatura alba* accumulated high quantity of Cu, Cd, and Pb in root, Mn and Ni in shoot. But, Cr, Zn and Fe accumulated in leaf. Similarly, *Achyranthus* sp. accumulated high quantity of Zn and Cd in root, Mn, Ni, Cu and Fe accumulated in shoot. *Kalanchoe Pinnata* accumulated high quantity of Mn in root, Zn and Cu in shoot and Fe, Cd in leaf. *Trichosanthes dioica* accumulated Zn and Ni in root, Cr, Cu, Cd Pb in shoot, Mn and Fe in leaf. *Parthenium indicum* accumulated high quantity of Zn and Cd in root. *Cannabis sativa* accumulate high quantity of Zn, Cd in root. *Amaranthus* sp. accumulated high quantity of Zn, Pb and Cd in root, shoot and leaf, respectively. *Croton bonplandianum* accumulated high quantity of Ni in root, while Zn, Mn, Cu, Fe, Cd and Pb accumulated in shoot. *Salanum nigrum* accumulated high quantity of Zn and in root and shoot, respectively. *Ricinus cummunis* accumulated high quantity of Mn and Fe in root. *Sacchrum munja* accumulated high quantity of Cr, Zn, Mn, Ni, Cu, and Fe in leaf. *Basella rubra* accumulated high quantity of Cr, Zn, Mn, Ni, Cu, and Fe in root. *Setaria glauca* accumulated high quantity of Cr, Ni, Fe, and Pb in root while Zn, Mn, Cu accumulated in shoot. *Chenopodium* accumulated high quantity of Zn and Mn in root. *Trianthema monogyna* accumulated high quantity of Zn, Mn, Cu, Fe and Pb in leaf. *Blumea lacera* accumulated high quantity of Cr, Zn, Mn, Ni, Cu and Pb in root. These plants showed successive reduction of various heavy metals from PMDE sludge in periodic analysis. Thus, these plants were found as potential bioremediater of toxic distillery sludge containing the high quantity of heavy metals and salts.

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