PROCESS ASSESSMENT OF BIO-ENERGY OPTIONS (HYDROGEN AND METHANE) USING MICROBES WITH INDUSTRIAL WASTE WATER

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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2015

Dedicated to
My Parents
&
Respected Teachers

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CERTIFICATE

I have the pleasure in forwarding the thesis of **Mr. Virendra Kumar**, entitled **"Process assessment of bio-energy options (hydrogen and methane) using microbes with industrial waste water"** for the award of the **Degree of Doctor of Philosophy** in Department of Environmental Science of the University.

Mr. Virendra Kumar has fulfilled the requirements of the academic ordinance of Babasaheb Bhimrao Ambedkar University, Lucknow. The thesis embodies the result of his investigation conducted during the period, he worked as Ph.D. research scholar under my supervision.

(Dr. Richa Kothari Tyagi)

Supervisor

Declaration

This is to certify that the material embodies in the present work entitled "**Process assessment of bio-energy options (hydrogen and methane) using microbes with industrial waste water**" is based on candidate's original research work. It has not been submitted in part or full for any other diploma or degree of any University. The indebtedness of the candidate to others has been duly acknowledged at relevant places.

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(Candidate)

Countersigned
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Countersigned
(Head of the Department)

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Date:
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Virendra Kumar

Executive Summary

Biological processing of industrial waste materials to produce bioenergy options as a product to fulfill the objective of “green technologies and sustainable development”, as such, is widely researched. Among various bioenergy options like biofuel, biogas, biohydrogen *etc*., biological hydrogen and methane production generates a new arena of waste treatment with simultaneously bioenergy production. Present hydrogen production methods can be grouped into two broad categories: conventional and alternative. Conventional hydrogen production methods mainly involve steam reforming (methane, ethane, butane *etc.*), a process responsible for large amounts of CO_2 emissions whereas, alternative methods of hydrogen production includes electrolysis of water, biophotolysis and biological hydrogen production from organic materials. Among all the novel processes of hydrogen production, biological hydrogen production has two main advantages over the conventional methods; it generate less green house gases and couples the metabolic activity of hydrogen-producing micro-organisms with the use of organic rich human-derived wastes, such as domestic and industrial wastewaters as the substrates for bioenergy (hydrogen and methane) production. So, bioenergy production technologies are very efficient in terms of economical, environmental and sustainable point of view. The lacunas of these technologies are inefficient production rate, unavailability of efficient microbes and optimization of process parameters *etc.*

Anaerobic/dark fermentation and photosynthetic degradation are the two most widely studied biohydrogen production techniques. Among these processes, dark fermentation is more advantageous due to its early hydrogen evolution rate. From literature, we are able to know that inoculums, substrate, reactors type, nutrients concentration, temperature, and pH are the main influencing factors in biohydrogen production routes. The biggest challenge over the next few years in fermentative hydrogen production will be to reduce the cost optimization of substrate concentration (industrial organic waste materials- solid and liquid) and selection of bacterial inoculums (pure/mixed) to improve the biohydrogen yields in particular.

On seeing the scenario of biological hydrogen production, this study aims to analyze the effect of some process parameter with production mechanism and cost-optimization with the advancement in biohydrogen production researches and followed by the fruitful suggestions for the future work of fermentative hydrogen production. Hence, the main objective of this thesis is to assess the process of bioenergy options (hydrogen and methane) using industrial waste (solid and liquid) in

integration with selected pure bacterial strain in general and effect of process and operational parameters on growth, substrate utilization and gas production (hydrogen and methane) in particular with validation through kinetic studies.

The thesis has been divided into following seven chapters:

1. Introduction
2. Review of literature
3. Materials and Methods
4. Selection and optimization of simple sugars with process pH using *Enterobacter aerogens* for bioenergy options
5. Experimental assessment and optimization of industrial waste using *Enterobacter aerogens*: Integration for bioenergy production and treatment options
6. Kinetic analysis to assess the effect of bacterial growth and substrate utilization on bioenergy (hydrogen and methane) production options
7. Conclusions and Future Recommendations

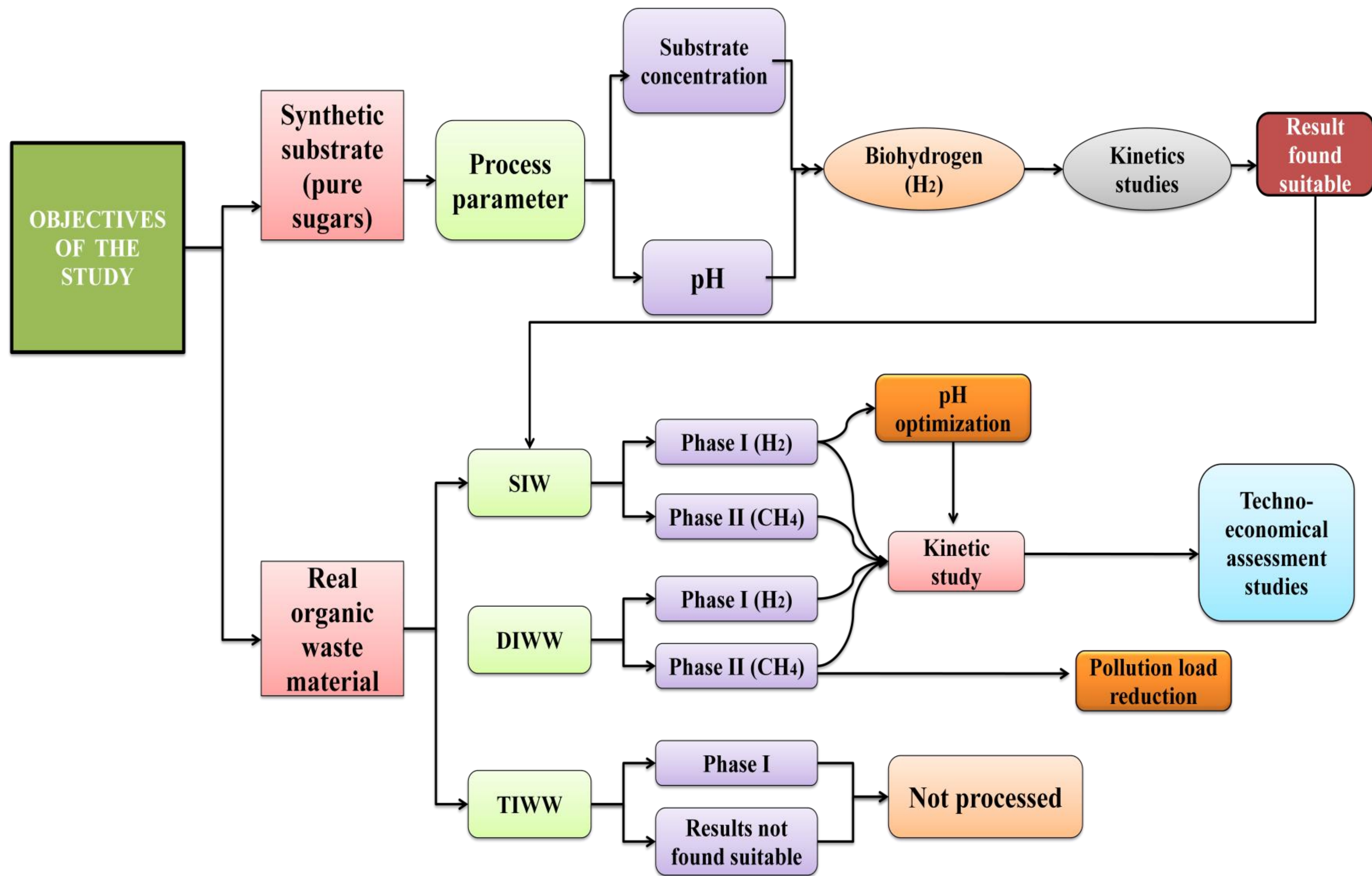
The chapter wise description of the work is as under:

Chapter 1: Introduction

In this chapter, an overview of the various available sources and processes for bioenergy options are discussed. The various processes for bioenergy production and feedstock/substrate compositions reviewed in particular to biogas, biofuel (bioethanol and biodiesel) and biohydrogen. Associated advantages and disadvantages with each are also discussed here to promote these at commercial level.

Chapter 2: Review of Literature

Literature review is broadly categorized into process route for fermentative hydrogen production using type of bacterial strains present in nature. Effects of various operational and process parameters are also discussed here to get optimized conditions for best efficiency in the process route. Operational parameters highlighted in the study with specific conditions are substrate and reactors type, whereas process parameters emphasised in this study are temperature, pH, volatile fatty acids, hydrogen partial pressure, enzymes and bacterial inoculums. Advance approach for bioenergy production options i.e. second stage process or two-stage approach is also reviewed here with their advantages and disadvantages at lab and pilot scale.



Graphical abstract for the study

*SIW- Sugar industry waste; DIWW- Dairy industry wastewater; TIWW- Tannery industry wastewater.

Chapter 3: Materials and Methods

This chapter focuses on the experimental methodology and analytical techniques used for each experimental plan. It outlines the basic information for microbial culture requirements and development for optimized conditions like type of substrate (sugar and industrial waste), effect of substrate concentration, effect of initial pH to assess the rate of biohydrogen production with experimental plans. Kinetic studies are also planned to assess the potential of experimental data in terms of production yield, specific bacterial growth (μ_m), biohydrogen production potential (P), rate (R_m) and lag phase (λ) using gas production models and substrate utilization models. So, this chapter is summarising the basic requirements needed to execute the experimental objectives in general and analytical/instrumental requirements to investigate the findings of Phase -I and Phase –II in particular.

Chapter 4: Selection and optimization of simple sugars with process pH using *Enterobacter aerogens* for bioenergy options

This Chapter deals with to find out the potential of pure simple sugars as the substrate, using facultative anaerobic bacteria *Enterobacter aerogens* for biohydrogen production. The study revealed that glucose is the most compatible and cost effective substrate, among all the selected sugars for the biohydrogen production with the yield of 0.87mol H_2 /mol of glucose consumed. The effect of process pH from initial start-up to final retention time was also analysed and the maximum biohydrogen production was found at the pH 5.5 for the process.

Study revealed that, there is a possibility to use of simple sugars and sugar based organic substrate materials like, organic residues and wastewater from industries for bioenergy production options. It also provides a suitable approach for clean environment in general and minimization of waste by bioenergy production approach in particular.

On the basis of these findings the selected bacterial strain *Enterobacter aerogens* was proposed for bioenergy production options (biohydrogen and methane) in integration with solid waste (organic residual waste) from sugar industry and liquid waste (i.e. wastewater) from dairy industry and tannery industry as the substrate.

Chapter 5: Experimental assessment and optimization of industrial waste using *Enterobacter aerogens*: Integration for bioenergy production and treatment options

This chapter is one step further in respect of previous one, here it is divided in three subsections on the basis of waste selected for study and emphasized on the use of these potential industries waste viz; sugar industry (solid organic residue), dairy industry (wastewater) and tannery industry (wastewater) for biohydrogen production with pure bacterial strain and further methane production from the fermented substrate. The selection of these industries for bioenergy production is based on their potential of waste generation, easy availability and previous studies on energy generation and waste treatment. The techno-economic benefits for bioenergy (hydrogen and methane) production is also evaluated in terms of waste assessment potential as a substrate for energy recovery and cost effectiveness to the other technologies in reference of bioenergy. Energy recovery from all selected industrial waste are also measured in terms of amount can be generated from per unit of substrate utilization and energy value of the produced energy.

5.1. A. Experimental set up plan-I (A): Assessment of sugar industry waste using *Enterobacter aerogens* for bio-energy (hydrogen and methane) production options with optimized parameters

The interactive effects of substrate concentrations (organic industrial residue) and pure bacterial strain for bioenergy production is investigated with cost effective assessment of this experimental plan with waste treatment and energy production approach simultaneously. From the results obtained at this part of chapter, it can concludes that utilization of organic residual substrate with pure strain can help to achieve a biohydrogen yield of 6.02 mM/g sugar with 40 g/l of substrate concentration has the highest gas production in Phase-I of the study. It has been also observed that the optimization of initial pH gave better results with pH 5.5 but growth of bacterial biomass is observed maximum with pH 6.5. The fermented substrate of the Phase -I was remained as soluble fermentation products in the form of solvents such as acetate, propionic acid, butyric acid and ethanol due to low substrate conversion efficiency in hydrogen production process These fermented substrate is further used for methane production and methane yield of 31 ml CH_4/ g sugar reduced with 40 g/l of fermented waste was found.

The maximum yield of bio-energy (biohydrogen and methane) production on per day basis was found in remarkable numbers with the present study (ESP-I, Phase-I and II). The obtained values of economic profit and energy recovery for bioenergy yield are also higher than the values obtained in double stage anaerobic digestion process as reported by other researchers.

5.2. B. Experimental set up plan-II (B): Assessment of dairy industry wastewater using *Enterobacter aerogens* for bio-energy (hydrogen and methane) production and treatment options simultaneously

In this experimental study of ESP-II, optimized substrate concentration was explored with microorganism *Enterobacter aerogens* for production of biohydrogen in phase I and methane in phase II. The study concludes that efficient success of the hypothesis i.e. bioenergy (hydrogen and methane) production and pollutants reduction from industrial wastewater. The best suitable optimum concentration, 75% of dairy industry wastewater was found feasible for bioenergy production with biomass growth as well as simultaneously pollutants reduction results for efficient treatment system such as higher efficiency achieved for phosphate (59% to 86%), sulphate (62% to 100%), nitrate (52% to 76%) at 25%, 50%, 75% and 100%. Similarly, maximum gas production was also achieved at 75% concentration of wastewater with 64% reduction in COD from initial to final HRT for experimental plan.

The maximum yield of bioenergy (biohydrogen and methane) production was found 0.562 L- H_2/ L DIWW, 0.59 L-CH_4./L-FDIWW in the present study (ESP-II, Phase-I and II). The obtained values of economic profit and energy recovery for bioenergy yield are higher than the values obtained in double stage anaerobic digestion process as reported by Park *et al.*, (2009).

Energy recovery from dairy industry wastewater was also measured in terms amount can be generated from per unit of substrate utilization and energy value of the produced bioenergy (MJ/day) provides a sustainable approach for utilization of industrial wastewater for bioenergy production with low cost of system.

5.3. C. Experimental set up plan-III (C): Assessment of tannery industry wastewater using *Enterobacter aerogens* for bio-energy (hydrogen and methane) production options

On the basis of findings, the energy production from tannery industry wastewater could not proceed may be the presence of high value pollutants concentration and

maximum toxicity is due to the presence of chromium ions which inhibit the growth of bacteria. But pretreatment of substrate and maintenance of anaerobic conditions can provide a better option for bioenergy production with tannery industry wastewater.

Therefore, this chapter finally concludes that (a) to achieve the best hydrogen yield an optimum substrate concentration should be obtained, which would be depending upon the substrate utilization capacity of the bacterial strain, (b) low and high pH variations directly affects the bacterial growth but improvement in biohydrogen yields only can attained through optimized process parameters, (c) pretreatment of substrate and maintenance of anaerobic conditions increases the cost of biohydrogen production but it seems to be cost effective and energy efficient at large scale production. Thus, two phase bioenergy production process provide enhanced COD reduction with hydrogen and methane as a product output for all the selected industrial waste materials (solid and liquid), and (d) Techno-economic assessment of energy yield (biohydrogen and methane) and economic bioenergy profit with both selected waste materials provides a better results with sugar industry waste in comparison to dairy industry waste as a substrate for bioenergy options with pure bacterial strain.

Chapter 6: Kinetic analysis to assess the effect of bacterial growth and substrate utilization on bio-energy (hydrogen and methane) production options

In view of the above said kinetic results, this chapter reviewed and analysed the trends of growth of bacteria, bioenergy (biohydrogen and methane) production options and substrate utilization kinetics by the application of different kinetic models. All kinetic models were critically analysed for all parameters taken for study. Following conclusions have been made from the thesis in particular:

- The logistic model applied for the growth of bacteria evaluated the maximum specific bacterial growth rate for sugar industry waste (SIW) and dairy industry wastewater (DIWW) at 40 g/l (0.36 h^{-1}) and 75% (0.52h^{-1}) concentration, respectively.

- Initial process pH on bacterial growth showed specific growth rate at 6.5 and most significant value (0.30 h^{-1}) in respect of biohydrogen production was found with initial pH of 5.5.
- The kinetics for bioenergy production evaluated in terms of biohydrogen and methane.Typical cumulative biohydrogen production curve fitted by the modified Gompertz equation using sugar industrial waste was found most significant (0.9981) with a substrate concentration, 40 g/l. Whereas, for dairy industry wastewater, 75% concentration shows most significant (0.9981) results in integration with *Enterobacter aerogens*. Similar trends of observations were found for both industrial wastes with of same concentrations for methane gas production for regression coefficients.
- Among the various selected models for kinetic studies, regression coefficient (R^2) was best found 0.98 with Moser model from sugar industry waste (molasses) and Hanes model was found best with R^2 value of 0.95 from dairy industry wastewater by selected bacterial strain.

Chapter 7: Conclusions and Future Recommendations

The results obtained are encouraging and study concludes that use of industrial waste materials (solid and liquid) provides a feasible solution with potential gas production in respect of clean energy source. It was also concluded that process parameters viz. substrate concentration and initial pH are very important for the efficient process mechanism. It was also found that industrial wastewater from selected industries can also be treated simultaneously with energy production options. Similarly, kinetic studies are performed with experimental findings also support the approach of sustainable solutions. The work done in thesis presents an answer (at least partially) to the problems associated with the clean and efficient production of bioenergy options. The recommendations for future work are also given in the chapter.

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Abbreviation

AmSBR	Anaerobic membrane sequence batch reactor
AnFBR	Anaerobic fluidized blanket reactor
ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand
Co-A	Co-enzyme A
COD	Chemical oxygen demand
CPCB	Central pollution control board
CSTR	Continuous stirred tank reactor
DIWW	Dairy industry wastewater
DO	Dissolved oxygen
EGSB	Expanded granular sludge bed
EMP	Embden–Meyerhof–Parnas
ESP	Experimental set up plan
Et-OH	Ethanol
FAME	Fatty acid methyl ester
FCFP	The Freedom CAR and Fuel Partnership
FDIWW	Fermented dairy industry wastewater
FSIW	Fermented sugar industry waste
g/l/d	Gram/liter/day
g/l	Gram/liter
HRT	Hydraulic retention time
Kg C/Kg	Kilogram carbon per kilogram
kPa	Kilo pascal
l/l/d	Liter/liter/day
MJ	Mega jule
MJ/kg	Megajoule/kilogram
MJ/L	Mega joule/liter
mM /g	milimol /gram
mmol H_2/L.h	milimol hydrogen/liter hour
NAD+	Aldehyde dehydrogenase
NADH	Nicotinamide adenine dinucleotide
PBR	Plug flow batch reactor
PFL	Pyruate formate lyase
POFR	Pyruvate ferredoxin oxidoreductase
PSI	Photo system first
PS II	Photo system second
SIW	Sugar industry waste
TIWW	Tannery industry wastewater
UNEP	United nation environmental programme
UASB	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acid

Chapter -1

Introduction

1. Introduction

Energy is an essential part of human life as well as an important indicator of socio-economic development for at national and international level. Traditionally we depend on conventional energy sources like oil, coal, and natural gas, which effectively impacts on the economic progress of a country and in most part of the world, it get imported from the Middle East countries. Another side, access use of these resources have resultant adverse impact on environmental conditions around the globe and peril of their exhausting that encouraged intensive research to find out some more efficient and green technology to combat these issues. A wide variety of renewable energy sources are an efficient substitute of this fossil fuel base originated problems. Since environmental protection concerns are increasing, both clean fuel technologies and new renewable energy options are in extensively in search. The recent energy independence and climate change policies encourage development and utilization of renewable energy.

Renewable energy sources are resources that are continually replenished by nature and harvested from the sun as thermal power, photochemical energy, and photo-electric energy, and others in the form of wind, hydropower, tidal energy etc, and bioenergy such as bioethanol, biodiesel, biogas, biohydrogen etc (Barik and Murugan 2015; Yong *et al*., 2015; Gupta and Verma 2015). Renewable energy technologies turn the natural energy sources into usable form of energy like electricity, heat and fuels. The various from of renewable energy that can be harvested from different sources are shown in Fig.1.1.

1.1. Types of Bioenergy

Bioenergy is an efficient option among all existing fuels including solid, liquid and gaseous forms in modern era of technology and in group termed as biofuel (Ullah *et al*., 2015). All forms of biofuel like solid (such as fire wood, wood chips, briquettes, pallets, charcoal etc), liquid (such as bioethanol, biodiesel, butanol, bio oil etc) and gaseous (such as biogas, producer gas, syn-gas biohydrogen etc) forms have been intensively researched and produced (Divya *et al*., 2015; Carlos *et al*., 2014; Passos *et al*., 2014).

Bioenergy technologies could contribute significantly to reductions in green house gas emissions (UNEP, 2014), and they are unique in their potential to serve all three areas of major energy demand: heat, electricity, and transport fuel and

chemicals shown in Fig.1.2 (Ehsan *et al*., 2014; Sinclair *et al*., 2015; Daianova *et al*., 2012; Wulf and Kaltschmitt, 2015). The well established bioenergy technology mainly emphasised on biogas, biofuel (bioethanol and biodiesel) and biohydrogen production (RFA 2014; Hoekman *et al*, 2014; Weiland 2010; Kumar *et al*., 2015; Gopalakrishnan *et al*., 2014) This chapter discuss the role of bioenergy options in general and process mechanism with advantages disadvantages for biogas, biohydrogen and biofuel in particular.

1.1.1. Biogas

Biogas is a flammable gas produced by the degradation of organic matter by the microorganisms in anaerobic environment (Weiland, 2010). It mainly composed of CH_4 (60-70%) and CO_2 (30-35%). The combustible part of biogas is methane, which varied in concentration with substrate and other optimized conditions. As the biogas is well suited technology at industrial level and frequently used for the treatment of industrial waste, municipal waste and sewage wastewater is now used as feedstock. During the last decades with development of technology the biogas can be effectively used as vehicle fuel in internal combustion engine and electricity generation (Lim *et al*., 2015; Yasa *et al*., 2015).

Process routes followed for production of final product in given below:

1.1.1.1 Process mechanism

Biogas production process is composed of four identified phases namely: Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis.

1.1.1.1.1. Hydrolysis

In this stage, aerobic bacteria breaks complex high molecular substances such as protein, carbohydrates, fats and cellulose to low-molecular compounds like monosaccharide, amino acids, fatty acids and water and made available it for acidogenenesis pahse.

1.1.1.1.2. Acidogenesis

This stage is dominant by acid forming bacteria which produce low molecular weight organic acids such as butyric acid, propionic acid, alcohol and partially anaerobic bacteria that consume oxygen present in the reactor to provide suitable environment for methanogenic bacteria. Beside this some amount of gases such as CO_2, H_2S and ammonia is also produced.

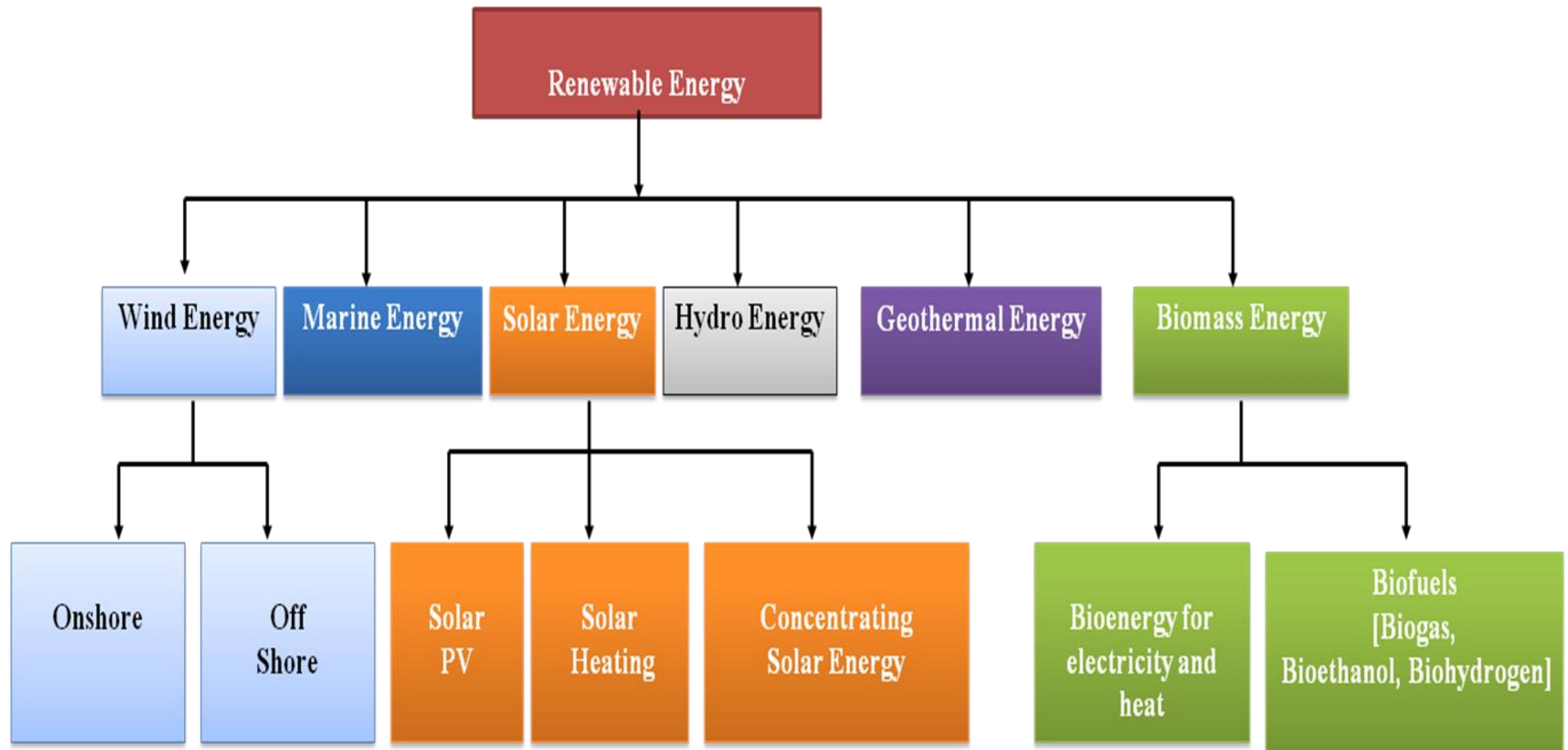

Fig.1.1. Overview of renewable energy source

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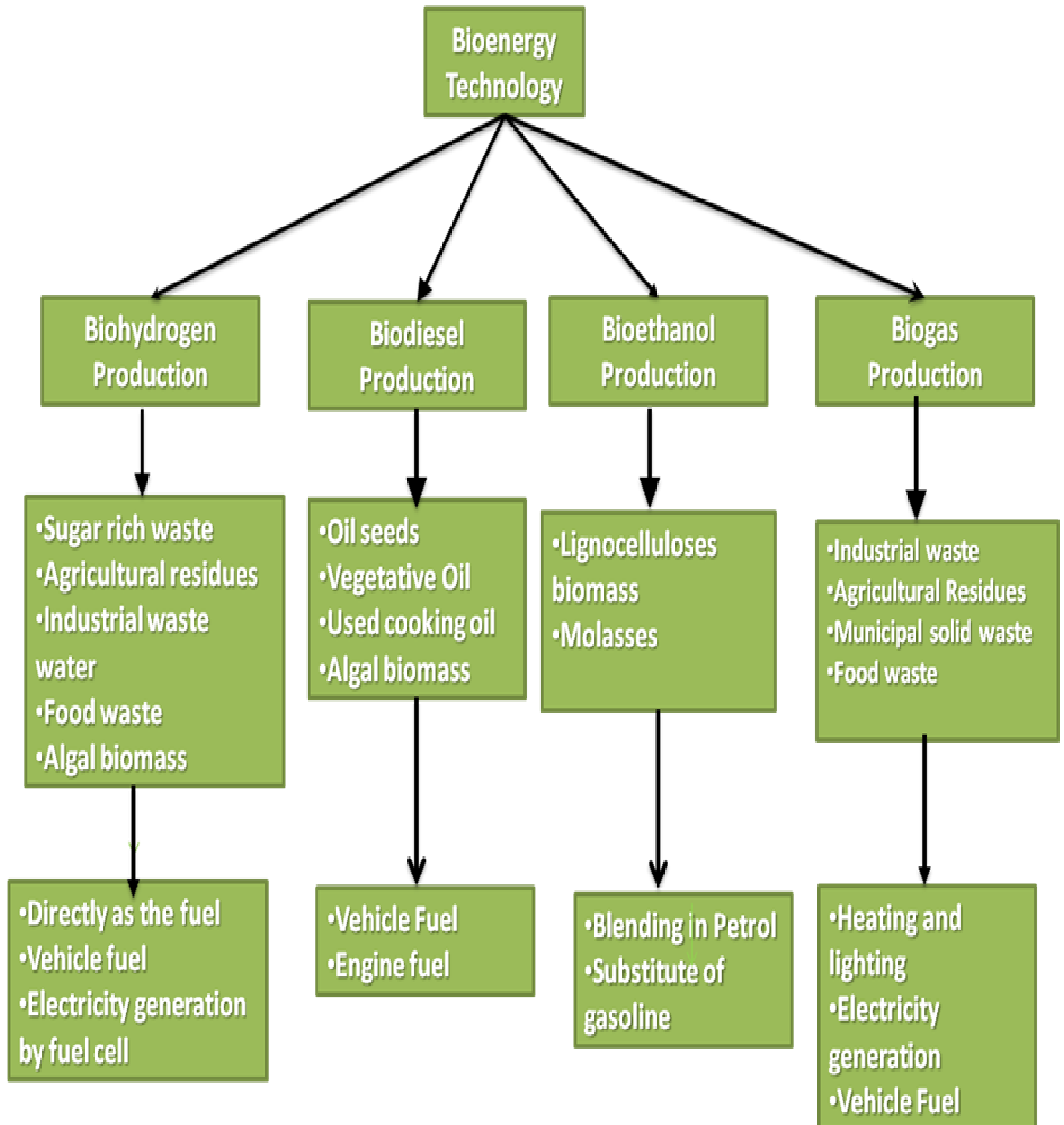

Fig.1.2. Main features of bioenergy technology.

1.1.1.1.3. Acetogenesis

In this stage, organic acids produced in acidogenesis step are converted to acetic acid which acts as the precursor for methane generation when methanogenic bacteria feed on it.

1.1.1.1.4. Methanogenesis

This is the final stage of biogas production process where methanogenic bacteria convert acetic acids to CH_4, CO_2 and water. 90% of methane yield takes place here and 70% of it from acetic acid.

1.1.2. Biofuel

Bio fuels are liquid fuels such as ethanol, biodiesel and bio oil, derived from renewable biological materials. In recent times a wide range of substrates are being used as feedstock for biofuel production such as ligniocellulosic materials for ethanol production (Baeyens *et al*., 2015; Nizaha *et al*., 2014), oil yielding plants such as Karnaja and Jatropha plants and algal biomass for biodiesel production (Knothe, 2001).

1.2.1.1. Bioethanol

Bioethanol is ethanol, produced from vegetative biomass through fermentation. Biomass used for bioethanol production includes food crops (Galbe *et al*., 2011) and lignocellulosic biomass (forests lashes, crop residues, yard trimmings, food processing waste, and municipal organic refuses.) are currently main feedstock (Knothe, 2001). On the basis of feedstock used for ethanol production it is divided in first, second and third generation biofuel (IEA, 2008).

1.2.1.1.1. Process of bioethanol production

Commercial bioethanol is currently produced from starch/ sugar-based crops including sugar cane, sugar beet, sweet sorghum, corn, wheat, barley, potato, yam, and cassava (Table 1.1). One commercially-practiced technology is to produce ethanol by fermenting plant biomass derived simple sugars (i.e., glucose, fructose, and other monosaccharides) (Moser 2009' NREL 2003; Vikari *et al*., 2012). The problem lies with the use of this biomass for ethanol production is it's pretreatments to obtain simple sugars that are bind with hemicellulose and lignin (Krishania *et al*., 2012). Research has been intensively conducted to develop effective pretreatment methods for obtaining simple sugars from lignocellulosic materials (Krishania *et al*

2012, Badger 2002). Bioethanol production from lignocellulosic biomass includes the following processes shown in (Fig. 1.4).

1.2.1.1.1.1. Pretreatment process

Pretreatment methods are generally used for agriculture biomass to remove the lignin. Some common pretreatment methods include: - Concentrated acid hydrolysis process, Dilute acid hydrolysis, Enzymatic hydrolysis, Wet milling processes, Dry milling process.

1.2.1.1.1.2. Saccharification process

It is an enzymatic hydrolysis process where cellulase enzyme degrades the cellulose to sugar. Cellulase producing common fungi are *Aspergillus niger*, *Trichoderma reseesi*. These are frequently used for cellulose degradation and further this sugar goes for fermentation.

1.2.1.1.1.3. Sugar fermentation process

The hydrolysis process breaks down the cellulosic part of the biomass into sugar solutions that can then be fermented into ethanol. Yeast is added to the solution, which is then heated. The yeast contains an enzyme called invertase, which acts as a catalyst and helps to convert the sucrose sugars into glucose and fructose.

1.1.2.1.1.4. Fractional distillation process

The ethanol, which is produced from the fermentation process, still contains a significant quantity of water, which must be removed. This is achieved by using the fractional distillation process. The distillation process works by boiling the water and ethanol mixture. Since ethanol has a lower boiling point (78.3°C) compared to that of water (100°C), the ethanol turns into the vapour state before the water and can be condensed and separated shown in Fig.1.3.

1.1.3 Biodiesel

Biodiesel is a yellowish liquid produced by the transesterification of triglycerides of some lipid contain substrates such as vegetable oils and animal fats. It reacts with monohydric alcohols in the presence or absence of base or acid catalysts or enzymes to obtain biodiesel. In this reaction, the fatty acids of the triglycerides are converted to the corresponding ester, which is called Fatty Acid Alkyl Ester (FAAE). This FAAE is commonly known as biodiesel. When methanol is used as the

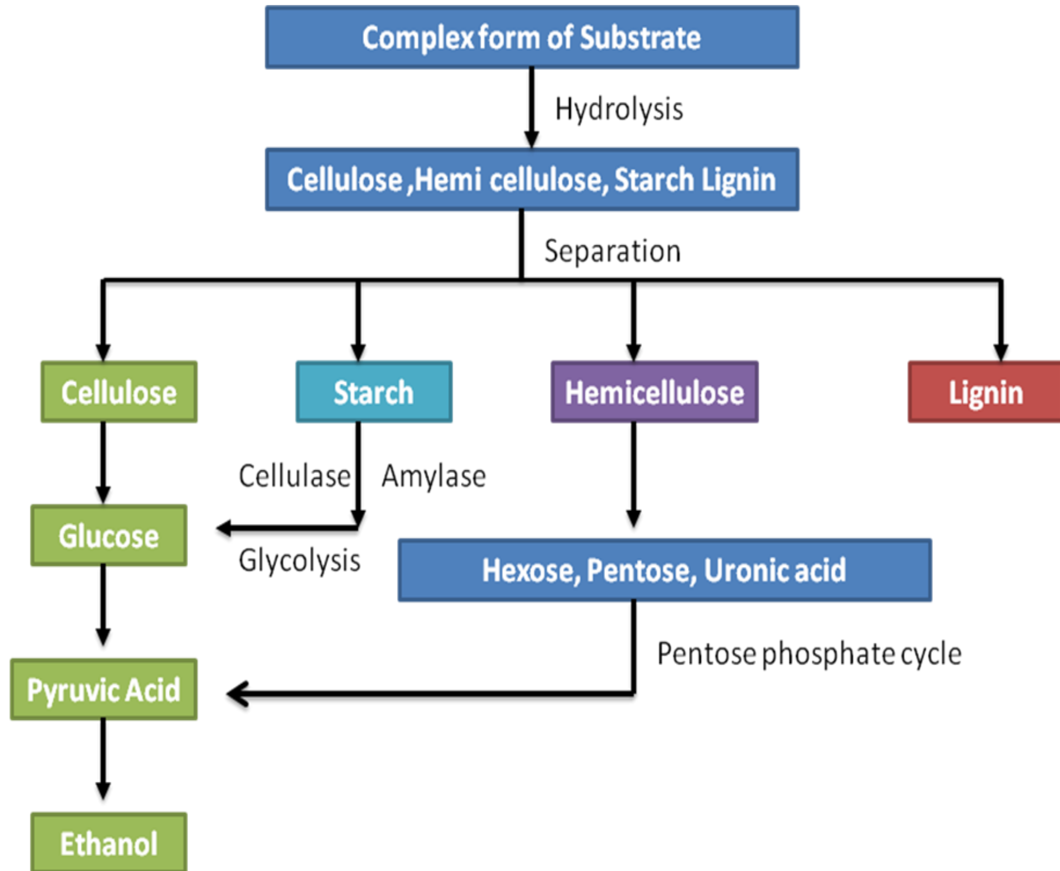

Fig. 1.3. Process of bioethanol production from lignocellulosic biomass**.**

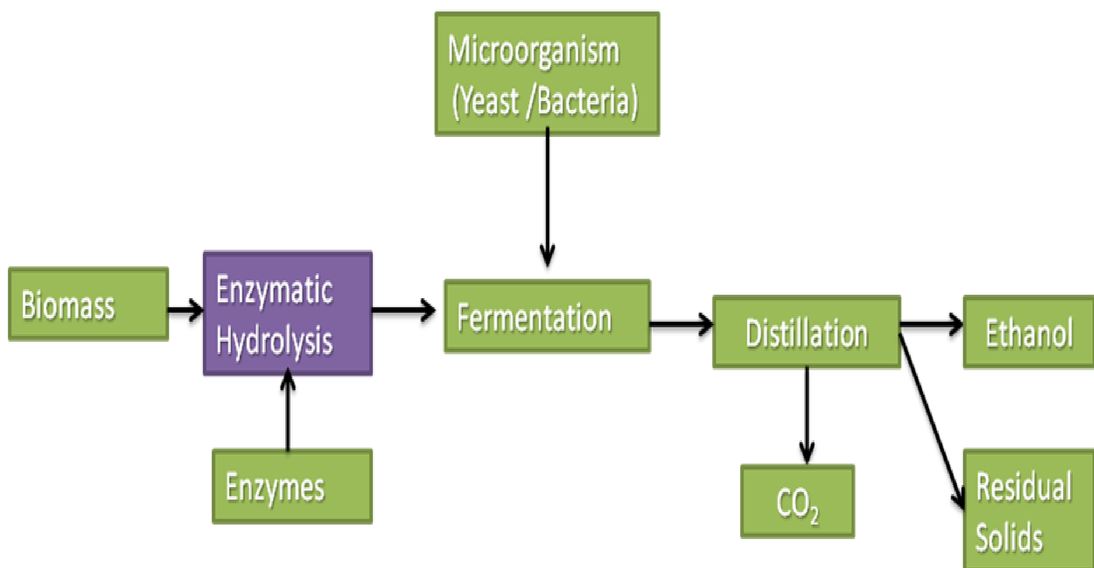

Fig.1.4. Process route of ethanol production from biomass.

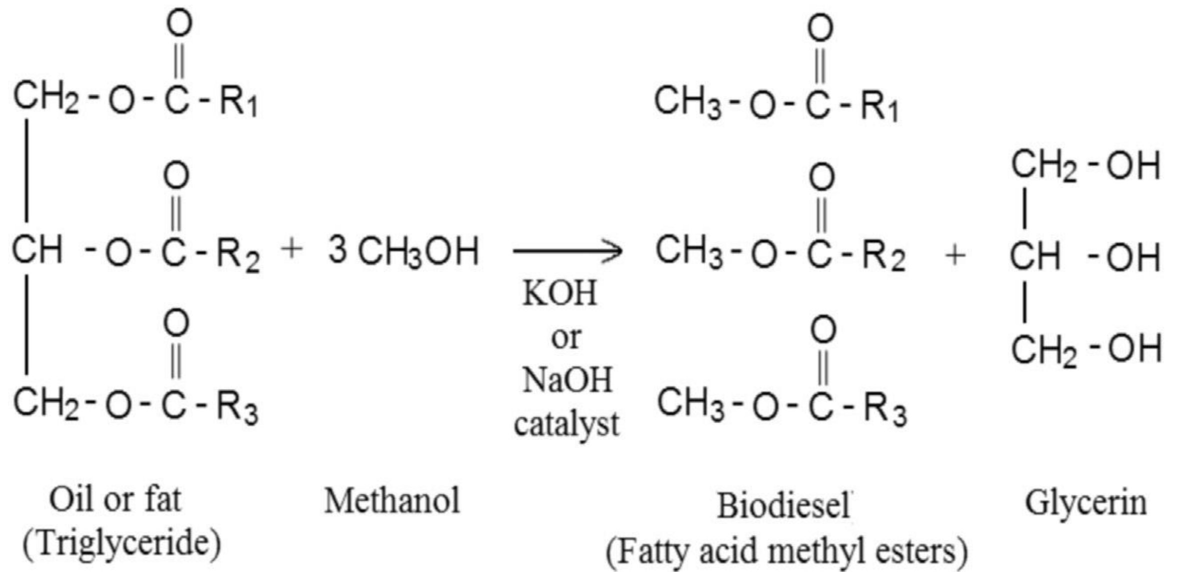

Fig.1.5. Process of biodiesel production**.**

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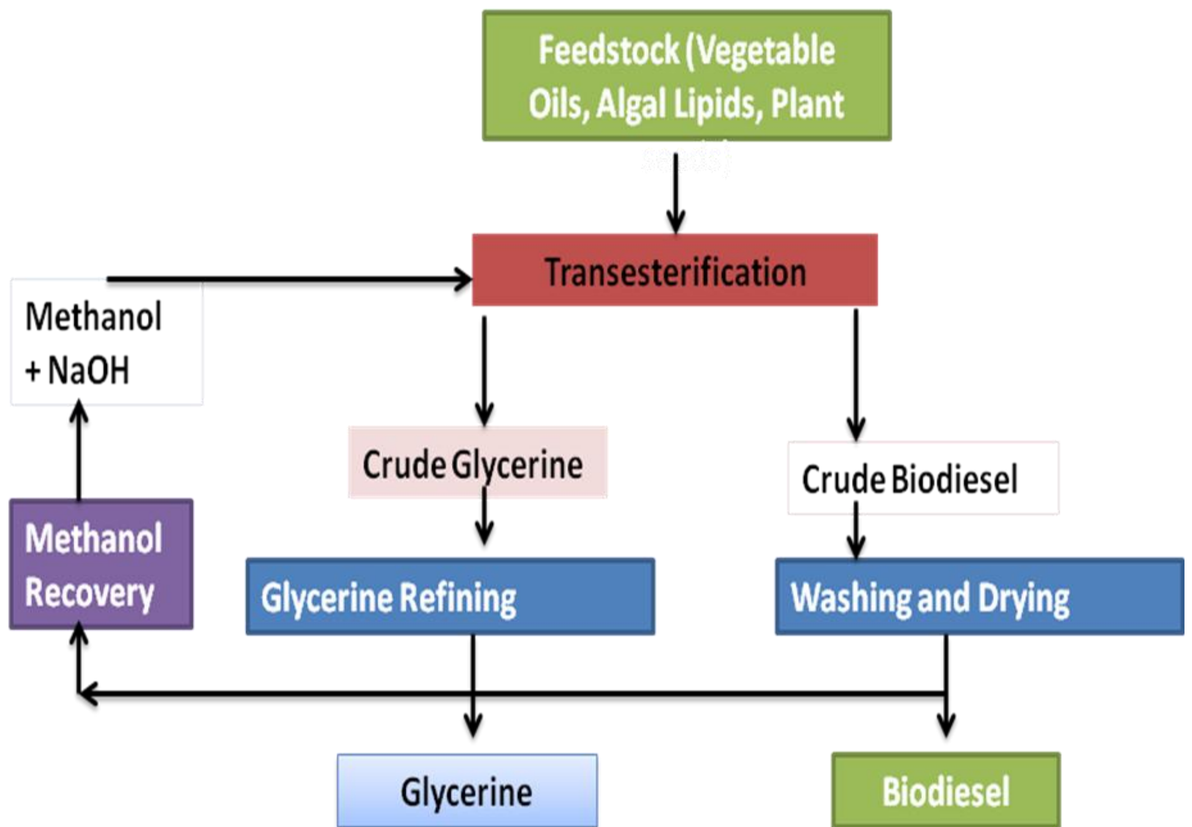


Fig.1.6. Schematic chart of biodiesel production from used vegetable oil

monohydric alcohol, then the resulting fatty acid methyl ester (FAME) is the biodiesel shown in Fig.1.5.

Biodiesel is considered as a possible substitute of conventional diesel fuel. It is quite similar to conventional diesel fuel in its main characteristics (Carla *et al*., 2012). Biodiesel is produced mainly from soybeans. Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil and jatropha oil and biodiesel derived from microalgal oil given in Table 1.1.

The oils obtained from the above said substrates cannot be used as a fuel directly because of some impurities it posses such as free fatty acids, sterols, phospholipids, water, odour etc. To overcome these problems there are some chemical modifications are required like transesterification, pyrolysis and emulsification.

Table 1.1. Substrate used for biodiesel production (Guo *et al*., 2015).

Substrates Types	Examples
Vegetable oils	Yellow grease, used vegetable oil (Castor oil, Corn oil, Hemp oil, Palm oil, Rice bran oil, Safflower oil, Sunflower oil)
Algal lipids	*Chaetoceros calcitrans, Skeletonema costatum, Phaeodactylu mtricor-nutum, Chlamydomonas reinhardtii, Calluna vulgaris, Dunaliella salina, Dunaliella teriolecta, Scenedesmus obliquus,* and *Neochloris oleabundans*
Animal fats	Beef tallow, Pork lard, Chicken fat, etc.
Common oil seed plants	Camelina,(*Camelina sativa*) Canola, Castor (*Ricinus communis*) Bean, Coconut (*Cocos nucifera*) Jatropha (*Jatropha curcas*), Palm, Peanut,(*Arachis hypogaea*) Rapeseed (*Brassica napus*) Soybean (*Glycine max*), Sunflower (*Helianthus annuus*,) and Tung (*Aleurites fordii*)

Among these, the transesterification is the key and foremost important step to produce the cleaner and environmentally safe fuel from vegetable oils. Transesterification or alcoholysis is the displacement of alcohol from an ester by another in a process similar to hydrolysis, except than alcohol is used instead of water. This process has been widely used to reduce the high viscosity of triglycerides. The transesterification reaction is represented by Fig. 1.6. If methanol is used in this process it is called methanolysis. It is one of the reversible reactions and proceeds essentially by mixing the reactants. However, the presence of a catalyst (a strong acid or base) accelerates the conversion as shown in Fig.1.5.

A wide range of lipid containing substrate such as vegetable oils, algal lipids, and animal fats are used for biodiesel production now a day, but commonly oil seed plants used as feedstock to produce biodiesel. In recent years, selected algae species were also studied for cultivating as a biodiesel feedstock. Critical challenges are still exist in cost-effective cultivation of algae, in energy-efficient harvest of algal biomass, and in effectual algal oil extraction.

With the world's growing demand for biofuels, global production and consumption of biodiesel will maintain consistent increase (IEA, 2014). The advantages of biodiesel also include a cleaner emission profile, production simplicity, ease of use, and cost. The promising future of biodiesel, however, also lies in the world's ability to economically produce renewable feedstock such as vegetable oils and algal lipids in sustainable manners, without supplanting land for food production.

1.1.4. Biohydrogen

Hydrogen produced by biological means is called biohydrogen. It can be produced by any sugar rich substrate viz pure sugars, industrial waste, municipal waste, food waste *etc*. Biological method of hydrogen production mainly includes photosynthetic hydrogen production and fermentative hydrogen production. Photosynthetic process includes; biophotolysis (direct and indirect) photofermentation and fermentative biohydrogen production also termed as dark fermentation includes use of microbes for degradation of organic matter for hydrogen production.

1.1.4.1. Bio-photolysis

Biohydrogen production from biophotolysis of water is of two types:

1.1.4.1.1. Direct biophotolysis

A direct biophotolysis is biohydrogen production process where solar energy and photosynthetic system of algae produces hydrogen from water. Different species of green algae used for the direct biophotolysis. Fresh water green algae and some species of marine algae used for this purpose. *Chlamydomonas reinhardtii* is most common fresh water algae for biohydrogen from algae and others includes *Scenedesmus obliquus* and *Chlorella fusca* (Melis *et al*., 2000; Wünschiers *et al*., 2001; Ghirardi *et al*., 2000).

The two photosynthetic systems are responsible for direct photolysis - Photosystem I (PSI) which produces reluctant for CO_2 and Photosystem II (PSII) which split water to evolve O_2. The two photons obtained from the splitting of water can either reduced to CO_2 by PSI or form H_2 in the presence of hydrogen producing bacteria. In plants due to lack of hydrogenase only CO_2 reduction take place. On the contrary, green algae and cynobacteria contains hydrogenase and thus have the ability to produce H_2. In these organisms, electrons are generated when PSII absorbs light energy, which is then transferred to ferredoxin. A reversible hydrogenase accepts electrons directly from the reduced ferredoxin to generate H_2 in the presence of hydrogenases.

The oxygen is toxic for hydrogenases therefore it is necessary to maintain the low oxygen content (<1%). This reaction is happen in very short span and the rate of the hydrogen production is very low (Bernd *et al*., 1986).

1.1.4.1.2. Indirect biophotolysis

Indirect biophotolysis refers to the coupling of two separates stages of microalgal photosynthesis and fermentation. These involve fixation of CO_2 into storage carbohydrates (*e.g*. starch in green algae, glycogen in cyanobacteria) followed by its conversion to H_2 by dark fermentation process.

Many types of green algae and cynobacteria besides having the ability to fix CO_2 via photosynthesis also have ability to fix nitrogen from the atmosphere and produce enzymes that can catalysed the second hydrogen generating step. Since these nitrogen fixing enzymes nitrogenases are localised within hetrocyst, they provide an O_2 free environment to carry out H_2 evolution reaction.

1.1.4.2. Photofermentation

In photofermentation process, phorosynthetic bacteria (*Rhodobacter sphaeroides*, *Rhodopseudomonas capsulate*, *R. palustris*, *Rhodospirillum rubrum* etc.) produce hydrogen during metabolism by taking organic acids and alcohols substrates. H_2 production process perform by these bacteria due to the presence of nitrogenase under oxygen deficient condition using light energy and reduced organic compounds such as butyrate acetate propionate, lactate and other organic acids.

The main advantage of photofermentation over other process is it lacks of PSII which automatically eliminates the difficulties associated with O_2 inhibition of hydrogen production. But this process lies with the drawback of low efficiency (usually 3-10%). However, rate of hydrogen production can be increases with increasing light intensity Theoretically, H_2 production from one mole of acetic acid, propionic acid and butyric acid are 4,7,10 moles respectively but in actual practice it is 1.6, 2.8 and 4 mol H_2 respectively (Das *et al*., 2006). Photofermentation can be coupled with dark fermentation so the wide variety of industrial waste can be used as substrates which make a sustainable approach for the waste treatment technology.

1.1.4.3. Dark fermentation

Dark Fermentation is the fermentative conversion of organic substrate to hydrogen. It is a complex process manifested by diverse group of bacteria involving series of biochemical reactions, similar to anaerobic conversion. In this process fermentative hydrolytic microorganisms hydrolyze complex organic polymers to monomers, which are further converted to a mixture of lower molecular weight organic acids and alcohol by acidogenic bacteria. The oxidation of substrate by bacteria generates electrons which need to be disposed off in order to maintain the electrically neutrality. Under the anaerobic condition O_2 serves as the electron acceptor while under the anaerobic conditions other compounds such as proton act as the electron acceptor and are reduced to molecular H_2.

1.1.4.4. Hybrid system

Typical hydrogen yield by the dark fermentation process ranges from 1-2 mol H_2/ mol of glucose. There are no naturally occurring biochemical routes for achieving 60-80 % efficiency. Most of the organic fraction remains as soluble fermentation products in the form of solvent such as acetate, propionic acid, butyric acid and ethanol (Khanal *et al*., 2004).

In spite of theoretical conversion efficiency of 33%, only 15% of the energy from the organic source is typically obtained in the form of hydrogen (Mohan *et al*., 2008). It is suggested to find out alternative way to utilized unused substrate. Therefore, the organic acids of dark fermentation process was further use for the photofermentation to complete degradation of organic acids for production of hydrogen or by using remaining organic matter to produce methane (Guwy *et al*., 2011).

Each of biohydrogen production process described above has its own features to exist and they contain some significance in terms of microorganisms used, product form and limitations. The Main features of biohydrogen production process are given in Table 1.2.

1.2. Advantages and disadvantages of bioenergy options

After going through the wide literature on bioenergy technologies, specific advantages and disadvantages associated with them are analysed and findings are given in Table 1.3.

Table 1.2. Main features of biohydrogen production process.

Process	Organism	Advantages	Product	Limitations
Direct biophotolysis	Green algae	Can produce H_2 directly from water	H_2, O_2	O_2 sensitivity to hydrogenase Low light conversion efficiency
Indirect biophotolysis	Cyanobacteria	Can produce H_2 directly from water	H_2, O_2	Enzyme inhibition by O_2 H_2 consumption by an uptake hydrogenase
Photofermentation	Phototrophic bacteria	A wide variety of spectral light energy can be used by bacteria	H_2,CO_2	Increase, H_2 fermentation process becomes thermodynamically unfavorable Product gas mixture contains CO_2 which has to be separated
Dark fermentation	Fermentative bacteria	A wide variety of carbon source can be used as substrate	H_2,CO_2, VFA	Light conversion efficiency is very low, only 1-5 % Inhomogeneity of light distribution
Hybrid System	Fermentative bacteria + Photosynthetic Bacteria	Complete degradation of substrate	CO_2, H_2O	New approach, techno-economical feasible study required

Table 1.3. Advantages and disadvantages of different bioenergy production technologies.

Process	Advantages	Disadvantages
Biogas Technology	Well established technology, Can be used for all types of substrate (liqid and solid). Used at Industrial level	High capital cost, Purification of methane Emission of CO_2 Storage of biogas
Bioethanol Production	Effective substitute of petroleum based vehicle fuel. Organic substrate such as agricultural residue can be used as feedstock. Ethanol can be used as a gasoline substitute to power petrol engines.	Availability of traditional feedstock. Separation of cellulose, hemicelluloses and Lignin Lack of effective “pretreatment” methods and its cost
Biodiesel Production	Effective technology for use of waste materials as used vegetable oils. Effective in mitigate concentration of $CO_{2,}$ if algal biomass is used as feedstock.	Transesterification process is complicated as effective catalysts are not available. Initial cost is high.
Biohydrogen Production	Carbon neutral process. Effectively used for the all types of biomass (solid and liquid)	No commercial plants are available. Effective microorganism bacteria and algae are in search part.

1.3. Conclusions

The bioenergy technologies are very efficient in terms and economical, environmental and sustainable point of view. Among them biological hydrogen production generates a new arena of waste treatment with simultaneously bioenergy production. The lacuna of these technologies is inefficient production rate, efficient microbes and optimized parameters; these are discussed in details in Chapter- 2 (Review of literature).

So, the present study is conducted with keep in mind the above said concept with the following objectives-

1. Evaluation of the characteristics of different organic industries (Food processing, sugar industry and tannery industry) waste (solid and liquid) which are potential substrate for suitable dark fermentation.
2. Identification of the microorganism species with high efficiency to convert organic waste to bio hydrogen.
3. To evaluate the operation performances of system by study the kinetics of dark fermentation for substrate and microorganism used.
4. Process modification and system designing to yield maximum hydrogen production per unit of substrate.
5. Study of system's techno economic feasibility.

Chapter -2

Review of Literature

2.1. Introduction

Renewable energy resources such as water, solar, biomass *etc.,* in the environment are linked together and it requires interdisciplinary research approaches to correctly address our energy preference. In this regard researchers have developed a keen interest for the H_2, an intermediate product during process of biogas production, a process currently in practice at industrial level for methane generation (Chang *et al*., 2002; Kumar and Das, 2000; Nath *et al*., 2008). This process involves three steps to get final output in terms of methane *i.e*. acidogenesis (formation of lower molecular weight organic acids), acetogenesis (formation of acetate) and eventually methanogenesis (Walker *et al*., 2009) shown in Fig. 2.1. Biologically produced hydrogen seems to be more promising energy carrier compared to methane without carbon dioxide and other harmful gases emission during in production as well as combustion process given in Table 2.1 (Wei *et al*., 2010). Furthermore use of H_2 in fuel cell with the efficiency 35-45% than in internal combustion engine 25-30% without co-generation makes it a potential alternative renewable energy fuel for future (Lin *et al*., 2010). In anaerobic digester hydrogen production rate is 10 fold than theoretical methane production rate but it is rapidly consumed by methanogenic bacteria (Das and Veziroglu, 2001).

In the two step digestion process, where, acidogenesis and acetogenesis are carried out in separate vessel than methanogenesis, it is easy to generate desired biogas containing either H_2 or CH_4 depending on operation parameters of the process (Antonopoulou *et al*., 2010). Ding and Wang (2008) in his experimental study found the highest yield mean volumetric hydrogen and methane potential would be in a stoichiometric ratio of 2:2 to 2:3. Hydrogen has been identified as a potential alternative to fossil fuels among all other renewable energy sources due to its carbon neutral and high energy value properties (shown in Table 2.1) (Kothari *et al*., 2010; Kothari *et al*., 2012).

A number of various processes are currently in practice for hydrogen production entitled in Table 2.2. Among all the known processes of H_2 production, biological routes of hydrogen production has more advantage over other conventional processes, as it has less emissions of green house gases and application of biological agent (bacteria and algae) which can simultaneously facilitates the treatment of disposal of human derived waste such as domestic and industrial wastewater when

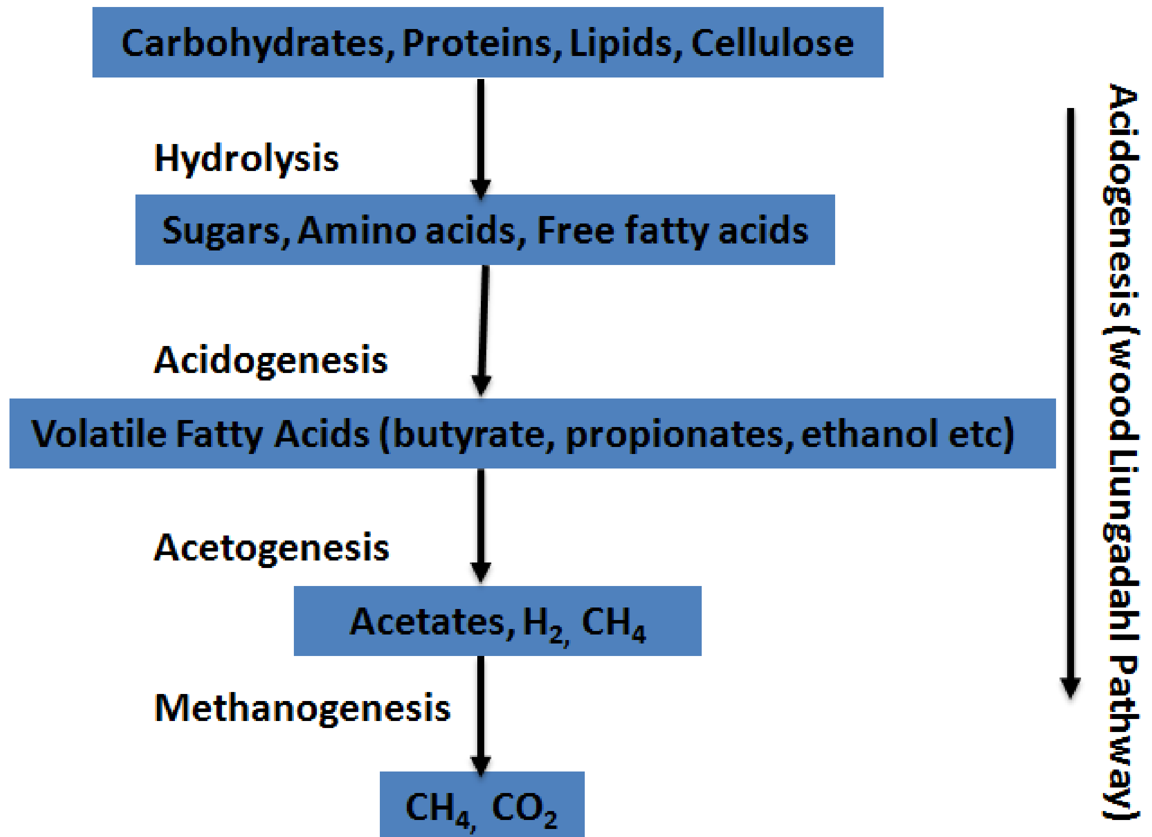


Fig. 2.1. Routes for hydrogen and methane production through anaerobic digestion process.

Table 2.1. Assessment of energy and carbon emission for commercially viable fuels (Kothari *et al*., 2010; 2012)

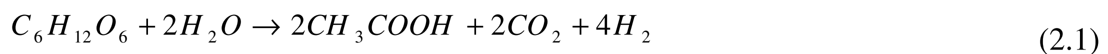
Fuel type	Energy/unit mass, MJ/Kg	Energy /Vol. MJ/l	Carbon emission Kg C/Kg fuel
Hydrogen (gas)	120	2	0
Hydrogen (liquid)	120	8.5	0
Coal(anthracite)	15-19	-	0.5
Natural gas	33-50	9	0.46
Diesel	42.8	35	0.9
Biodiesel	37	33	0.5
Ethanol	21	23	0.5

Used as feedstock substrate. Biological processes of hydrogen production are biophotolysis of water, photo fermentation and dark fermentation among which photofermentation and dark fermentation processes are more in current practices. Over biophotolysis and photofermentation where presence of light is a limiting factor, dark fermentation has high rate of hydrogen evolution rate due to independent for light and use of wide variety of organic waste as substrates. This chapter will incorporate knowledge on advance processes of biohydrogen production with two step process and strategically frame work to assess the yield of alternative options for biohydrogen, presently discuss by researchers to manage energy and environment crisis both.

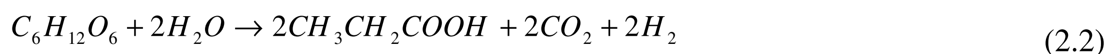
2.1.1. Chemical reactions involved in biological hydrogen processes

The core components involved in biohydrogen production are substrate and microorganism. The interactions between these two components are responsible for many chemical reactions. The conversion process may proceed in the dark or with assistance of light. Some important reactions of the processes are as follows-

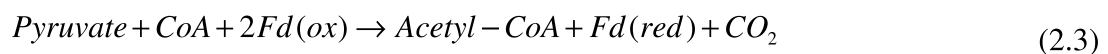
a) Dark fermentation



$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.1)$$



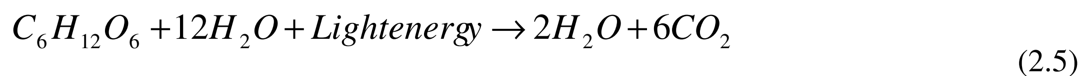
$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (2.2)$$



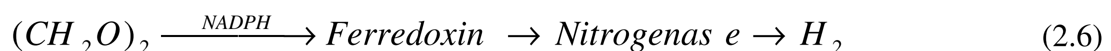
$$Pyruvate + CoA + 2Fd(ox) \rightarrow Acetyl - CoA + Fd(red) + CO_2 \quad (2.3)$$

$$2H^+ + Fd(red) \rightarrow H_2 + Fd(ox) \quad (2.4)$$

b) Photofermentation



$$C_6H_{12}O_6 + 12H_2O + Lightenergy \rightarrow 2H_2O + 6CO_2 \quad (2.5)$$



$$(CH_2O)_2 \xrightarrow{NADPH} Ferredoxin \rightarrow Nitrogenas\ e \rightarrow H_2 \quad (2.6)$$

Table 2.2. Remarkable notes with advantages and disadvantages of hydrogen production process according to literature.

Processes	Advantages	Disadvantages	Remarks
Thermal Processes			
Natural Gas Reforming	Most viable means of hydrogen production in present scenario due to presence of potential infrastructure support.	Capital cost is high for establishment.	Improve catalyst efficiency and Reduction in process of cost is required; Development of low-cost, efficient; separation/purification mechanism.
Bio-Derived Liquids Reforming	Futuristic fuel with existing infrastructure.	High capital cost. Feedstock quantity and quality parameters affect the process.	Low temperature and liquid phase catalysts is needed. Characterization of biomass.
Coal and Biomass Gasification	Low-cost fuel technology.	Feedstock impurities with carbon content affect the system's efficiency. Capital cost is high.	Feedstock storage, preparation, and handling. Emission control measures are required.
Thermo chemical Production Routes	Clean and sustainable route for energy production using solar and nuclear energy in integration with chemicals.	Long term technology but durable and potential.	Development of thermo-chemical storage and heat transfer devices are needed.
Electrolytic Processes			
Water Electrolysis (Splitting Water Using Electricity)	Pollution free fuel cell device can be used for electricity generation.	Solar system can provide a better efficient system, but high cost involves.	Improved photocatalyst with multifunctional materials at low cost assures uniform quality production designs.

Photolytic Processes			
Photo-electrochemical Hydrogen Production route	Clean and sustainable technology with low temperature requirements.	Solar based technologies require high cost.	Long term technology for sustainable development.
Biological Processes			
Direct bio photolysis	Integrated approach for H_2 production using sunlight with water.	Light intensity should be high and O_2 act as inhibitor in the reaction.	More R &D is needed for efficient functioning of microorganism for long term development.
Indirect bio photolysis	Use of blue green algae for hydrogen production from water.	Uptake of hydrogenates is removed	
Photo fermentation	Different range of light spectrum can be optimized to enhance the yield. A wide spectral energy can be used by photosynthetic bacteria	Nitrogenase enzymes get inhibited in presence of small amount of O_2. Light conversion efficiency is low.	Zero-cost method for development of microbes using wastewater as a nutrient resource. Development of efficient bioreactor.
Dark fermentation	Light independent process, several metabolites are produced in process as by-product, various substrate can be used.	Relatively low H_2 yield, process becomes thermodynamically unfavourable.	
Two stage fermentation	Can relatively high H2 yield, first stage metabolites can be converted to H2 and CH4.	Requires continues light source in Dark+ photo fermentation and pH control in anaerobic digestion processes	

Table 2.3. Biological hydrogen production processes (Krupp and Widmann, 2009)

Metabolic Process	Organism	Advantages	Hydrogen yield ($mmolH_2/l.h$)	Product
Direct biophotolysis	Green algae	Can produce H_2 directly from water	0.07	H_2, O_2
Indirect biophotolysis	Cyanobacteria	Can produce H_2 directly from water	0.36	H_2, O_2
Photofermentation	Phototrophic bacteria	A wide variety of spectral light energy can be used by bacteria	0.16	H_2,CO_2
Dark fermentation	Fermentative bacteria	A wide variety of carbon source can be used as substrate	65-75	H_2,CO_2, VFA

2.2. Fermentative hydrogen production process

Fermentative hydrogen production occurs in the anaerobic condition where bacteria degrade organic substrate by oxidation, to provide metabolic energy to the cell by the generation of ATP. In this process, electrons are released. As oxygen is absent in anoxic condition these electrons are accepted by some other compounds as protons, which are reduced to molecular hydrogen and maintains electronic neutrality in the cell.

The process of fermentative hydrogen production is dominated by the bacteria involved in the process mainly of facultative as well as strictly anaerobic bacteria. On the basis of this, the process of fermentative hydrogen production can be facultative or strictly anaerobic hydrogen production process. During the process of glycolysis, catabolism of various organic substrates takes place and as a result of fermentative hydrogen is produced by the anaerobic metabolism of pyruvate formed in the process.

In the process of glycolysis, the simple sugars are converted to pyruvate which is broken down into acetyl co-enzyme A, from which adenosine triphosphate (ATP) can be derived and hydrogen is produced from the product formate or

ferredoxin depend upon the presence of strictly anaerobic or facultative anaerobic bacteria. Facultative bacteria derived hydrogen from formate while strict anaerobes drive it from ferredoxin.

2.2.1. Hydrogen production by facultative anaerobic bacteria

Facultative anaerobic bacteria are gram-negative, rods shaped, with relatively simple nutrient requirements (Chun-xiang *et al*., 2011). Among the facultative anaerobic species that can produce H_2, especially members of the family Enterobacteriaceae like *Escherichia* spp. (*E. coli*), *Enterobacter* spp. *(Enterobacter aerogenes)*. These bacteria ferment sugars to a variety of end products like acetate, formate, lactate, succinate, ethanol, CO_2 and H_2. The degradation of organic matter in anaerobic environments by microbial consortia involves the cooperation of a population of microorganisms that generate a stable, self-regulating fermentation. The facultative bacteria form pyruvate through glycolysis from carbohydrate rich substrates; these bacteria generate acetyl-co A and formate through the enzyme pyruate formate lyase (PFL). Formate is then converted to hydrogen and carbon dioxide the by enzyme activity (Perumal *et al*., 2014).

2.2.2. Hydrogen production by strictly anaerobic bacteria

Strictly anaerobic or obligate bacteria lives in the completely anaerobic environment, a little amount of oxygen becomes too toxic for their growth. In obligate anaerobic bacteria *Clostridium* sp. has been widely studied for hydrogen production. *Clostridia* are spore forming bacteria and have the ability to sustain in adverse conditions (Joshi *et al*., 2012; Wong *et al*., 2014). All *Clostridia* lacks cytochrome system, in the anaerobic oxidation of carbohydrates electrons are generate and which need to dispose of to maintain electrical neutrality.

Table 2.4, representing the pure strains of bacteria belongs to facultative and strictly anaerobic type, which were used by various researchers in previous studies with different substrates. In the oxidative breakdown of carbohydrate NADH-ferredoxin reductase functions as an electron carrier and facilitates pyruvate oxidation to acetyl Co-A and CO_2 as well as proton reduced to molecular hydrogen. The EMP or glycolytic pathway is applied to convert glucose into pyruvate and further to acetyl Co-A associated with the transformation of NADH form NAD^+, in reoxidation of NADH under anaerobic condition in the presence of ferredoxin oxidoreductase and hydrogenase. *Clostridium* sp. can produce 2 mol of hydrogen

when 1 mole of n –butyrate is the end product and 4 mole of H_2 with 2 mol of acetate from 1 mole of hexose.

2.3. Factors affecting biohydrogen production

2.3.1. Substrates

In most of the studies for biohydrogen production simple sugar such as glucose or sucrose have used as model substrate. Only a few studies have looked into agriculture waste (Guo *et al*., 2010; Li *et al*., 2014), industrial waste as distillery wastewater (Mishra and Das, 2014) dairy industry wastewater, food processing and beverage industry (Oh and Logan 2005; Karlsson *et al*., 2008), rice slurry waste water (Fang *et al*., 2006). Beside the industrial wastewater, agriculture waste such as wheat straw (Kongjan *et al*., 2010) corn straw (Li and Chen, 2007), sugar cane molasses (Aceves-Lara *et al*., 2008) are also used for anaerobic fermentation. These forms of organic material seem to be a potential substrate for sustainable hydrogen production.

2.3.1.1. Carbohydrate

Carbohydrate rich substrates are the primary choice of hydrogen producing microbes. Food waste from food processing industry waste such as potato processing industry, rice slurry are rich in carbohydrate content and have been shown suitable for anaerobic fermentation (Oh and Logan 2005; Karlsson *et al*., 2008; Fang *et al*., 2006).

During carbohydrate hydrolysis, hydrolytic bacteria produce simple sugar such as sucrose, glucose, xylose and hexose (Bej *et al*., 2008). At worldwide scale carbohydrate type feedstock used for industrial fermentation processes has been estimated at 4×10^7 tons per year (Vandamme, 2009).

2.3.1.2. Lipids

Sources of lipid are food waste, food processing waste, oils and dairy products (Demirel *et al*., 2005; Pereira *et al*., 2006). Lipid hydrolysis is performed by lipase enzyme found in some bacteria. Its hydrolysis resulted in free fatty acids and glycerol, that can be hydrolyzed to acetyl-CoA and acetate and hydrogen form NADH oxidation during the b-oxidation pathway (Mendes and Castro, 2005; Bruno *et al*., 2008). The oxidation state varies depending upon the number of double bonds in the side chains of the fatty acids.

Table 2.4. Bacterial strains used for biohydrogen production.

Pure bacterial strains	Substrate	References
Clostridium sp.	Glucose, Xylose, Rice	Abdeshahian *et al*. (2014)
Clostridium butyricum CWBI1009,	bran, Pome	Goud *et al*. (2014)
Clostridium perfringens,	sunflower stalks xylan	Hiligsmann *et al*. (2014)
Clostridium perfringens ATCC 13124		Wang *et al*. (2014)
Clostridium		Nasser *et al*. (2014)
saccharoperbutylacetonicum N1-4		Singh *et al*. (2013)
(ATCC 13564)		Monlau *et al*. (2013)
Clostridium butyricum EB6		Rajagopalan *et al*. (2013)
Clostridium strain BOH3		
Enterobacter sp H1,	Glycerol, Microalgal	Maru *et al*. (2013)
Enterobacter cloacae	biomass	Batista *et al*. (2014)
IIT-BT 08	Distillery effluent	Mishra *et al*. (2014)
Enterobacter sp H_2	Glucose	Karthic *et al*. (2012)
Enterobacter aerogens ATCC 13048		Guo *et al*. (2014)
Enterobacter aerogens	Biomass waste	Mahyudin *et al*. (2010)
Enterobacter aerogens MTCC 2822	Cheese whey	Rai *et al*. (2012)
Escherichia Coli	Glucose, oil palm frond	Zulkhairi *et al*. (2012)
Escherichia coli (XL1-Blue)	juice and sewage sludge,	Yasin *et al*. (2013)
Escherichia coli strain HD701	glucose and glycerol,	Trchounian *et al*. (2014)
Escherichia coli WDHL	glycerol	Sanchez-Torres *et al*. (2013)
Escherichia coli O157:H7	Molasses	Sivagurunathan *et al*. (2014
		Morsy *et al*. (2014)
		Rosales-Colunga *et al*. (2012)
		Guan *et al*. (2013)
Citrobacter sp. Y19	Glucose , glucose,	Oh *et al*. (2003)
Citrobacter sp. CMC-1	xylose, galactose,	Mangayil *et al*. (2011),
Citrobacter sp. S-77	mannose, arabinose and	Hamilton *et al*. (2010)
Citrobacter freundii CWBI952	rhamnose,oxidation of	Ainala *et al*. (2014)
Citrobacter amalonaticus Y19	carbon monoxide (CO),	Yanga *et al*. (2011)
Citrobacter freundii		
Klebsiella pneumoniae ECU-15	Glucose, Glycerol	Xiao *et al*., (2013)

Klebsiella sp	,xylose and bamboo	Chookaew *et al*. (2014)
Klebsiella sp. HE1	stalk hydrolysate, crude	Wu *et al*. (2011)
Klebsiella oxytoca HP1	glycerol of biodiesel	Wu *et al*. (2014)
Klebsiella pneumoniae TR17	plant	Chookaew *et al*. (2012)
Klebsiella sp. TR17	Glycerol	Chookaew *et al*. (2014)

The process of hydrogen production from lipid hydrolysis is slower than carbohydrate hydrolysis. Hydrolysis of a lipid is inhibited by the accumulation of volatile fatty acids production causes decrease the pH of the medium (Khanal *et al*., 2006).

2.3.1.3 Protein

Proteins are the polypeptides of amino acids. The source of protein for biohydrogen production consists of food waste, food processing waste from cheese whey, casein, fish meat chicken egg etc (Ramsay *et al*., 2001; Wu *et al*., 2007). In the hydrogen production from proteins bacteria convert it into polypeptides and amino acids by protease enzymes (Herman *et al*., 2006; Carbonaro *et al*., 2012). Further, amino acids are broken down to volatile fatty acid, carbon dioxide and hydrogen. However, there is no much study was done for proteinaceous substrates as biohydrogen production feedstock except by Xiao *et al*., (2013) who proposed the pathway for biohydrogen production from protein (see Fig. 2.2).

2.3.1.4. Cellulose and lignocellulose material

Plants and agricultural biomass including fruits and vegetable waste are the good sources of cellulose, hemicelluloses and lignocelluloses materials. They contain different kinds of sugars that can be used for biohydrogen production (Nowak *et al*., 2005). The only problem with these biomass lies that cellulose is hardly degradable by microbes due to its crystalline structure (Xia *et al*., 2004). It required some pretreatments such as steam explosion, chemical treatment, acidic or alkaline treatment to break rigid structure of cellulose hemicelluloses and lignocelluloses materials to release sugars (Mohammad *et al*., 2008; Cheng *et al*., 2011).

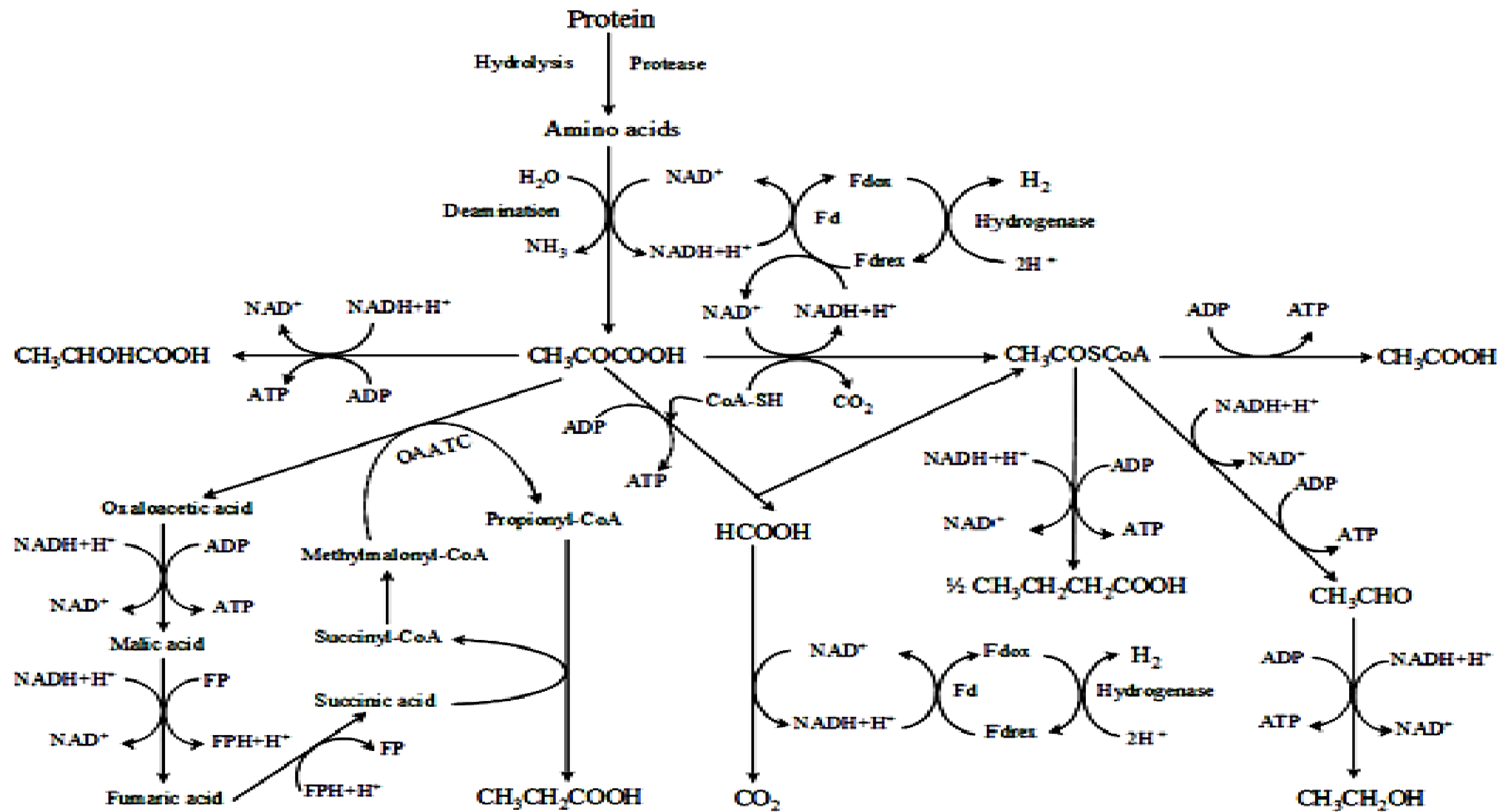


Fig. 2.2. Proposed metabolic pathway for anaerobic bio-hydrogen production from protein (Xiao *et al*., 2013).

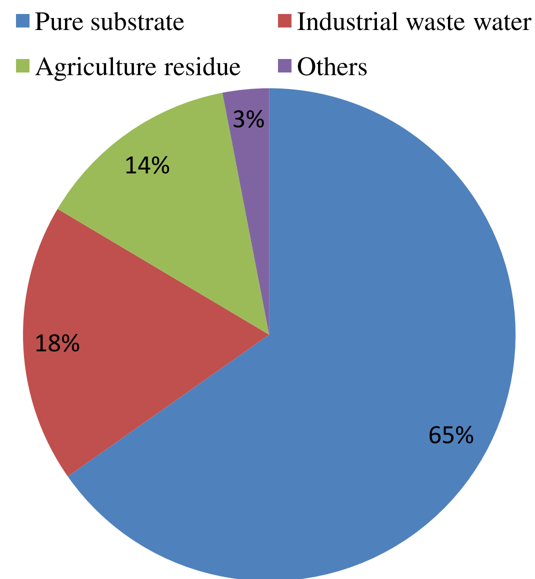
2.3.1.5 Pure/synthetic substrate

Biohydrogen production has been no more applicable for industrial level till now and most the studies is still going on biohydrogen production with pure substrate at lab scale such as glucose, cellulose cellobiose, arbinose, starch, xylose, sucrose and glycerol. Among these, glucose, sucrose and starch are more common substrate of interest while some researcher also worked with glycerol shown in Fig. 2.3 A. However, uses of pure substrates are expensive and not economically viable for biohydrogen process. Fig.2.3 (A & B) is showing the graphical representation of research accomplished on pure and waste substrates according to literature availability.

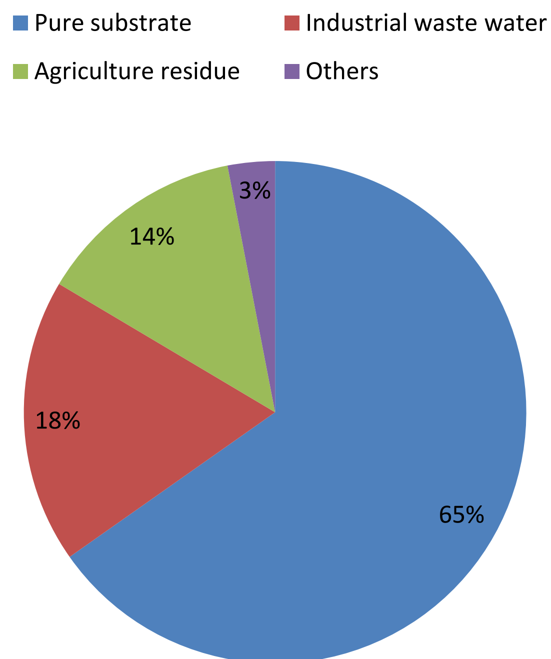
2.3.1.6. Waste materials as substrate

Besides pure substrate industrial wastewater, sludge, municipal solid waste, agriculture waste, domestic wastewater (Van Ginkel *et al*., 2004), paper mill wastewater (Idania *et al*., 2005), molasses based wastewater (Ren *et al*., 2006), glycerol based waste water (Yang *et al*., 2008), chemical wastewater (Venkata Mohan *et al*., 2007), dairy industry process waste (Venkata Mohan *et al*., 2007), distillery wastewater (Vatsala *et al*., 2008, Mishra *et al*., 2014) are well studied for biohydrogen production.

Sewage sludge (Lin *et al*., 2008), municipal waste (Lay *et al*., 1999) can also used as substrate for fermentative hydrogen production as it abundantly available and low cost in nature. It contains several macro and micro nutrients that reduce the cost of addition of nutrients required for microbial growth. However, presence of methanogenic bacteria in this kind of waste interfere the H_2 production process. It needs some treatments prior to use as the substrate for fermentative H_2 production. Use of this kind has dual benefits as generation of bioenergy and waste treatment simultaneously (Kothari *et al*., 2012; Singh *et al*., 2011).



(A)



(B)

Fig.2.3. **(A)** Pure substrates and **(B)** Waste substrates used for biohydrogen production.

2.3.2. Temperature

Temperature is an important operational parameter for fermentative hydrogen production as anaerobic bacteria more sensitive to temperature. The anaerobic fermentation process is lies among four temperature regimes – ambient (15-30°C), mesophilic (30-39°C), thermophilic (50-64°C) and hyper thermophilic (> 64°C) (Munro *et al.*, 2009). Changes in temperature ranges highly affects the hydrogen production rate in general and substrate utilization process, hydrogen yield, formation of liquid products and microbial community of the system in particular (d'Ippolito *et al.*, 2010; Hafez, 2012).Most of the studies on biohydrogen production are done under mesophilic conditions as it is more preferable for economical and technical point of view than thermophilic bacteria and exhibit high yield under stable conditions (Zhang *et al.*, 2003; Munro *et al.*, 2009; d'Ippolito *et al.*, 2010; Schröder *et al.*, 1994). However, mesophilic biohydrogen production process also favors the growth of non-hydrogen producing microbes.

Studies revealed that thermophilic and hyper thermophilic bacterial culture is more proficient in hydrogen production than mesophilic. The highest yield reported by the thermophiles is 4 mol H_2/ mol glucose which are close to the theoretical yield (Schröder *et al.*, 1994). In case of agricultural biomass, it has been recently reported that mesophilic bacteria are unable to use cellulose directly for hydrogen production, an addition of exogenous cellulase enzyme is required for bacterial hydrolysis. On the other hand, thermophilic anaerobic bacteria effectively utilize cellulose without the addition of exogenous cellulase (Liu *et al.*, 2008). In addition to thermophilic condition hydrolysis rate of substrates is also high (Ngo *et al.*, 2012). After literature survey, it has been found that among thermophiles, *Thermoanobacterium sp.* and mesophiles *Clostridium* sp. and *Enterobacter* sp. are most popular species for hydrogen production reviewed in Fig.2.4.

2.3.3. pH

pH has profound effect on fermentative hydrogen production process due to its major role in determination of acidic and alkaline condition of the system, in the limitation of the growth of bacteria and regulation of solvent production (Table 2.5). Solvent generated at the end of fermentation decrease the pH by acids accumulation. The optimum pH for hydrogen production is found between 5.5 and 6.5 avoiding the solvantogenic phase (Khanal *et al.*, 2004). Since, H_2 production is associated with

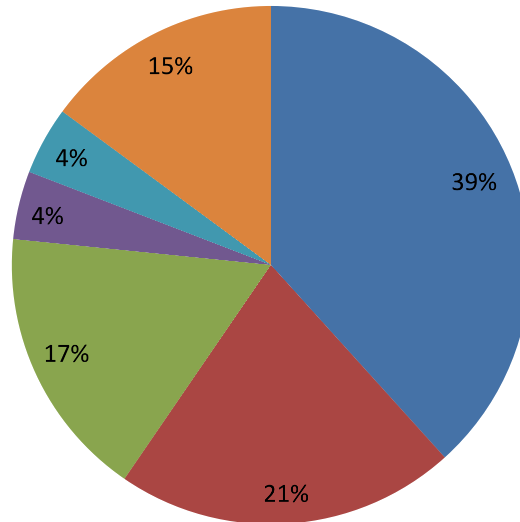
[FeFe] hydrogenase, it may also be possible that low pH affects the activity of this metal containing protein leading to complete inhibition of its activity (Afschar *et al.*, 1986; Dabrock *et al.*, 1992). Inhibition of H_2 production may also be due to the protonation of undissociated weak acid in the medium which can pass through the cell membrane into the cytoplasm and inhibit the growth of microorganisms as well as their abilities to produce hydrogen.

Table 2.5. Hydrogen production from various substrates at optimum pH values.

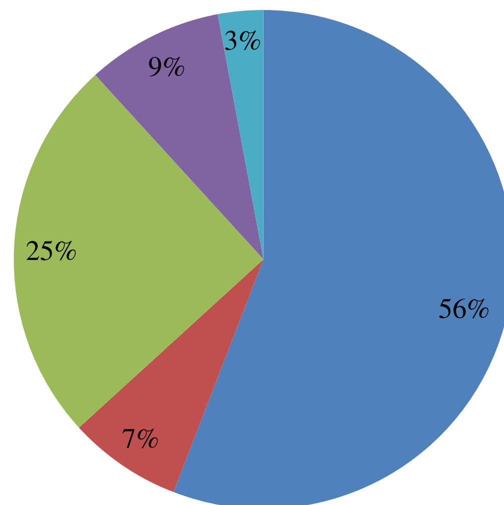
Substrates	Optimum pH	References
Sucrose	4.5	Khanal *et al.* (2004)
Starch	5.0	Davila-Vazquez *et al.* (2008)
Foodwaste water	6.0	Wu *et al.* (2004)
Xylose	6.5	Lin *et al.* (2006)
Corn stalk waste	7.0	Zhang *et al.* (2007)
Cellulose	7.5	Lin *et al.* (2008)
Glucose	6.5	Rodrıguez *et al.* 2008)
Molasses	5-6.5	Nanqi *et al.* (2006)
Food industry waste	7.0	Chu *et al.* (2013)

2.3.4. Volatile Fatty Acids (VFA)

Volatile fatty acid or solvent production is accompanied by hydrogen production in the fermentation process. Their concentration distribution and fractions can be used to monitor the fermentative hydrogen production system. Most of the fatty acids are produced by the hydrolysis process in acidiogenic phase. These acids includes acetic acids, propionic acids, isobutyric, butyric acids , lactic acids and ethanol. In anaerobic treatment process the drop in pH is occurs due to either accumulation of VFA or excessive generation of CO_2 or both. The process occurs for the formations of these acids are as follows (Mariakakis *et al.*, 2012).



(A)



(B)

Fig.2.4. Bacteria strains used for biohydrogen production (A) Thermophilic, (B) Mesophilic.

a) Acetic acid production

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2. \quad (2.7)$$

b) Butyric acid production

$$C_6H_{12}O_6 \rightarrow CH_3CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (2.8)$$

c) Lactic acid production

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad (2.9)$$

d) Succinic acid production

$$C_6H_{12}O_6 + 2CO_2 + H_2 \rightarrow 2(CH_2COOH) + 2H_2O \quad (2.10)$$

e) Formic acid production

$$CO_2 + H_2 \rightarrow HCOOH \quad (2.11)$$

f) Ethanol

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.12)$$

The identification of volatile fatty acids formed during the process gives a valuable indication about the type of metabolic pathway followed by the bacteria. The VFA generation in fermentative hydrogen production process is also affected by change in temperature as at the higher temperature (45°C) acetate and butyrate concentration is high 26-30% than at the mesophilic temperature (30-35°C) with 20-25% concentration of acetate, propionate and butyrate (Lee *et al*., 2006; Lin *et al*., 2008). Ethanol concentration is also important while determine the liquid metabolites, as high ethanol concentration fraction 23-40% were achieved at 30-45°C, which reduces the hydrogen production. Ethanol production consumed electron and favours the propionate formation by directly utilize H_2 which decreases the yield of H_2 (Hawkes *et al*., 2002).

2.3.5. Hydrogen partial pressure

The inhibition created from the hydrogen produced by the bacteria on its own gaseous production is known as hydrogen partial pressure. The increase in the partial pressure of H_2 in the headspace during the fermentation has been associated with a decrease in H_2 production (Das and Veziroglu, 2001; Levin *et al*., 2004). The hydrogen partial pressure increases to a certain level in the reactor headspace lead to the alcohol production. In high concentration of H_2, the H^+/H_2 ratio is lower and the

flow of electron from reduced ferredoxin to molecular H_2 via the hydrogenase system is inhibited.

This leads to the inhibition of enzymes leading to the production of hydrogen through the reduction of proton to the molecular hydrogen (Hawkes *et al.*, 2002; Angenent *et al.*, 2004). Lowering the concentration of the dissolved hydrogen should be one of the key factors for the enhancement of the H_2 production by fermentative microorganisms. This can be achieved by enhancing the liquid-to-gas mass transfer (Pauss *et al.*, 1990). Different studies have shown a correlation between the optimal partial pressure of hydrogen, depending upon the operational temperature used. Lee and Zinder, (1988) obtained 50 kPa as the optimal operational pressure at 60ºC. Similarly, different studies found 20 kPa at 70ºC (Van Niel *et al.*, 2002) and 2 kPa at 98ºC (Adams, 1990; Levin *et al.*, 2004) as the optimal pressure. Hydrogen production becomes thermodynamically unfavourable at H_2 partial pressure greater than 60 Pa. Sparging of inert gases such as nitrogen or argon lowers the dissolved H_2 concentration, resulting increase in yield (Mizuno *et al.*, 2000; Lay *et al.*, 2000; Tanisho *et al.*, 2005).

2.3.6. Enzymes

Two key enzymes take part in dark fermentative hydrogen production. These are Formate hydrogen lyase (FHL) and Fe-Fe hydrogenase.

2.3.6.1. Formate hydrogen lyase (FHL)

In most of the facultative anaerobic bacteria hydrogen production is catalysed by formate hydrogen lyase (Rachman *et al.*, 1997). In anaerobic condition under acidic environment, formic acid is converted to hydrogen. This reaction is catalyzed by the formate hydrogen lyase. FHL consists of formate dehydrogenase, hydrogenase and electron transfer carrier, responsible for formic acid oxidation to CO_2 and H_2. During the reaction formic acid acts as the electron donor and protons are the only electron acceptor leads to the formation of H_2 (Ordal and Halvorson, 1939; Stephenson and Stickland, 1932).

2.3.6.2. Hydrogenase

Hydrogenase enzymes are classified into three groups based on the number and identity of the metal in their active sites [Ni-Fe], [Fe-Fe] and Fe- hydrogenase (Vignais *et al.*, 2001; Vignais and Colbeau, 2004; Meyer, 2007) On the basis of their active site they all contains Fe and Co as a ligand to the Fe atom. Among this

Fe-Fe hydrogenase is known to be most potent in terms of fermentative hydrogen production. These enzymes are monomeric as in *Clostridium* or multimeric as in *Thermotogamaritima* and *Thermoanaerobactertengcon-genesis*, that consist of three and four subunits, respectively. The Fe-Fe hydrogenase are organised into modular domains. The accessory cluster known as the F cluster, functions inter and intra molecular electron transfer centres (Hallenbeck *et al*., 2009). The accessory cluster is linked electronically to the catalytic cluster known as the H cluster. The Fe-Fe hydrogenase have the activity about 10-100 times higher than others hydrogenase. Genomic analysis shows that there is a great deal of varieties in Fe-Fe hydrogenase with some *Clostridia* containing a large no of hydrogenase with different modular structure (Calusinska *et al*., 2010).

2.3.7. Reactors for biohydrogen production

In recent years several different reactors came in exist for biohydrogen production shown in Table 2.6. With the advancement of technology, these became more specify to enhance the yield with utilization of different waste at various loading rate and hydraulic retention time. Among the reactors used for biohydrogen production continuous stirred tank reactors (CSTR) and up-flow anaerobic sludge blanket (UASB) reactor are still in more preference for industries due to high yield and less retention time and application of wide range of waste water. The maximum yield reported are, 2.14 mol/mol hexose by CSTR while, 1.29 mol H_2/mole hexose by UASB from coffee drink manufacturing wastewater. Anaerobic sequencing bioreactor produces 2.53mol H_2/mol, sucrose from carbohydrate-rich wastewater. Anaerobic fluidized bed reactor produces 4.26 mol H_2/mol sucrose from sewage. sludge. Extended Granular Sludge Bed Reactor (EGSB) can produce 3.47 mol/mol sucrose from molasses.

Table 2.6. Different reactors available for biohydrogen production

Reactor type	Substrate used	OLR	Yield (H_2)	HRT/SRT	References
CSTR	Kitchen waste	20 g COD/L d	2 mmol/g COD	4d	Shiue-Lin Li (2008)
	Rice winery wastewater,	34 g COD/l d	2.14 mol/mol hexose	1d	Hanqing Yua (2002)
	Corn starch	26.7 g COD/ld,	0.92 mol H_2/glucose	18h	Muhammad Farhan *et al.* (2008)
	Fruit waste water	5-15 kg COD/m^3d	56% or 5.4- 4.2 mol/kg COD	15-5h	V.Diamantis *et al.* (2011)
UASB	Citric acid wastewater	10.0 to 75.0 kg COD/m^3 d	0.84 molH_2/mol hexose	12h	Badshah *et al.* (2012)
	Sludge of coffee drink g wastewater	-	1.78 mol H_2 to 2.76 l H_2/l	12h	Jung *et al.* (2011)
	Coffee drink manufacturing wastewater	20 g COD/l d	1.29 mol H_2/mol hexose	6h	Jung *et.al* (2010)
	Cheese whey	5- 20 g COD/l d	0.38 and 0.36 l H_2/l	6h	Carrillo-Reyes *et.al.* (2012)
AnSBR					
	Alcohol waste water	68 kg/m^3 d	130ml H_2/g COD	21.3h	Intanoo *et al.* (2012)
	Chemical waste water	6.3 kg COD/m^3 d	0.297 - 0.483 mol H_2/kg COD	24h	Mohan *et al.* (2007)
	Synthetic Sample	75 g-COD/l d	60–74 mLH_2/g COD	8h	Cheong *et al.* (2006)
	Carbohydrate rich substrate	22.5 g COD/l d	2.53 molH_2/mol sucrose	16h	Chena *et al.* (2008)

AnFBR	Synthetic wastewater	2.36 l/l h	4.34 mmol-H_2/g VSS h	4h	Barros *et al*. (2010)
	Sewage sludge	20 g COD/l	4.26 ± 0.04 mol H_2/mol sucrose	6h	Lin *et al*. (2008)
	Glycerol waste	0.70 g/l d	-	-	Yang *et al*. (2008)
	Extracted sunflower flour	9.3 g COD/l d	-	1.1d	Borja *et al*. (2001)
	Sewage sludge	20 g COD/l	4.26 ± 0.04 mol H_2/mol sucrose	6h	Lin *et al*. (2008)
EGSB	Starch waste water	1.0 g-starch/l d	1.64 l/l d	4h.	Guoa *et al*. (2010)
	Molasses	8 kg COD/m^3 d	3.47 mol/mol sucrose	1-6h	Guoa *et al*. (2010)
	Brewery wastewater sludge	5 g COD/l.	500 ml/d	13h	Abreu *et al*. (2011)
	Brown Sugar	97.2kg COD/m^3 d.	5.73l / l d	2h	Li *et al*. (2010)
	Compost leachate	1.4-16.7 g/l d	460 ml/l d	30h	Liu *et al*. (2009)

***CSTR (Continuous stirred tank reactor), UASB (Upflow anaerobic sludge blanket), AnSBR (Anaerobic sequencing batch reactor), AnFBR (Anaerobic fluidized bed reactor), EGSB (Expended granular sludge blanket).**

2.4. Strategies employed to enhance biohydrogen production

There are various techniques are employed to enhance the biohydrogen yield some of them are as follows:

2.4.1. Pretreatment of bacterial inoculums

Fermentative hydrogen production process employed the use of mixed bacterial culture as inoculums. The presence of the hydrogen consuming bacteria which either inhibit the process or forms methane make the process difficult (Goud *et al*., 2014; Luo *et al*., 2010). Therefore, to remove the hydrogen consuming bacteria various pretreatments of the inoculums are required. Heat pretreatment, alkali pretreatment, acid pretreatment, ultrasonic, chloroform, chemical treatment and combined treatment *etc*., are the most frequently used pretreatment methods to increase H_2 production yield (Setlow 2003; Guerra *et al*., 2002 ; Oremland *et al*., 1988; Lehninger *et al*., 1975; Chidthaisong *et al*., 2002; Sprott *et al*., 1988). Among these, heat shock treatment and chemical inhibitor treatment are considered to be more effective and taken as a part in this section of study.

2.4.1.1. Heat-shock treatment

Some microbial species such as *Bacillus* and *Clostridium* have the capacity to sporulate when environmental conditions become hostile such as heat shock, changes in nutrients status, presence of deleterious chemicals, among others (Sparling, 1997). The spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals. In anaerobic environments, the main spore-forming microorganisms are several genera of acidogenic bacteria. This fact has been used by several authors to eliminate or kill non-spore-forming microorganisms, mainly methanogens, by means of a heat treatment with inoculums typically at 100°C for 15–120 min (Valdez-Vazquez *et al*., 2005). This treatment simultaneously selects spores of acidogenic bacteria that will germinate and produces H_2 when conditions become favourable.

2.4.1.2. Chemical Inhibitors

In literature, there have been reports of many chemical substances that inhibit methane formation or methanogenic archaea. These compounds have been applied to study the importance of methanogenesis in the environment. chloroform, fluoroacetate and acetylene are some examples of inhibitors for methanogens (Valdez-Vazquez *et al*., 2009). Chloroform ($CHCl_3$) is known to block the function

of corrinoid enzymes and to inhibit methyl-coenzyme M reductase. $CHCl_3$ not only inhibits methanogenesis, but also inhibits partially acetate-dependent sulfate reduction and possibly H_2 dependent homoacetogenesis (Oremland, 1998).

2.4.2. Application of co-culture

Examining the available literature, it is found that there are many advantages of employing co-cultures over mono-cultures for economical as well as the technical point of view. From the economy view, co-cultures can help maintain anaerobic conditions for strict high hydrogen producers and eliminate the need for an expensive reducing agent. From the technical view, co-cultures can improve the hydrolysis of complex sugars and plant biomass, and can provide a wider range of pH for bacteria to ferment hydrogen. Table 2.7 is explaining the potential of co-culture with other important operational parameters, managed after reviewing the several research reviews and experimental studies available on scientific database. Elsharnouby *et al*., (2013) reported that there are primarily three different types of co-culture of pure isolates. The co-cultures involves strict or obligate and facultative anaerobes, cellulose degrading anaerobes and high hydrogen producers via fermenting simple sugars and aciduric hydrogen producing microorganisms and high hydrogen producers.

2.4.3. Engineering tools involved in process

2.4.3.1. In pathway

In the anaerobic fermentative hydrogen production glucose is oxidised in two step:1) glyceraldehydes 3-phosphate to, 1,3-biphosphoglycerate and., 2) Pyruvate to acetyl –Co A. The metabolic engineering required to increase yield of H_2 is so for possible at the pyruvate step (Wang *et al*., 2010).

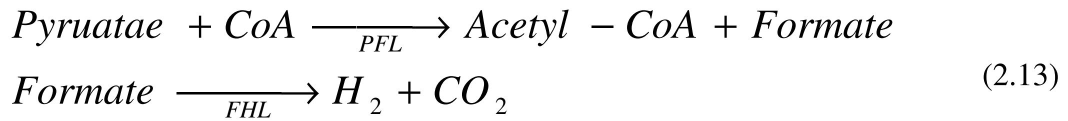
Three types of biochemical reactions are involved in the generation of H_2. The first one is found in the family of Enterobactereacae (Hallenbeck and Benemann, 2002; Hallenbeck and Ghosh, 2009; Kim *et al*., 2009). It employs two major enzymes Pyruvate formate lyase (PFL) and Formate hydrogen lyase (FHL) (Hallenbeck, 2005). PFL acts upon splitting of Pyruvate into acetyl-Co A and formate in anaerobic condition while FHL cleaved formate to H_2 and CO_2.

The second type of H_2 producing reaction involves pyruvate ferredoxin oxidoreductase (PFOR) and Fd- dependent hydrogenase (hydA) (Hallenbeck, 2005).

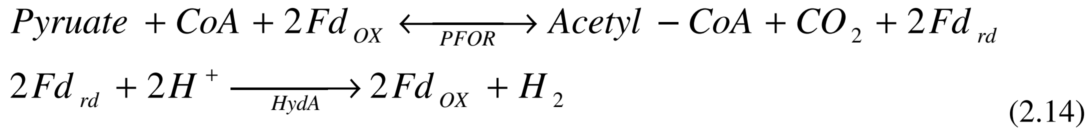
Table 2.7. Various pure strains bacterial co-cultures for biohydrogen production

Cultural Conditions	Cultures	T (°C)	Substrate	Substrate concentration (g/l)	pH	Hydrogen Yield (mol/mol)	Hydrogen Production rate(l/l/d)	References
Co-cultures involves strict or obligate and facultative anaerobes,	*C.butyricum* and *E.coli*	37	Glucose	3.0	6.5	2.09	0.41	Seppala *et al*. (2004)
	Enterobacter aerogens and *C.butyricum*	37	Starch	-	6.5	2.0	-	Yokoi *et al*. (1998)
	Enterobacter aerogens HO *and C. butyricum*	37	Sweet potato	-	5.25	2.7		Yokoi *et al*. (2002)
	B.thermoamylovorans and C. beijerinckii L9.	40	Brewery yeast waste	18.75	-	91.6 (ml H_2) from a 80-ml co-culture	-	Chang *et al*. (2008)
Cellulose degrading anaerobes and high hydrogen producers via fermenting simple sugars	*Thermoanaerobacteriumthermosacchrolyticum* GD17 and *C. thermocellum* JN4	60	Cellulose	5.0	4.4	0.8	0.01	Liu *et al*. (2008)
	Clostridium butyricum-NRRL 1024 and Clostridium pasteurianum-NRRL B-598	30	Wheat starch	10	5.5	109 ml H_2 g TS	1.8	Ozmihci and Kargi (2011)

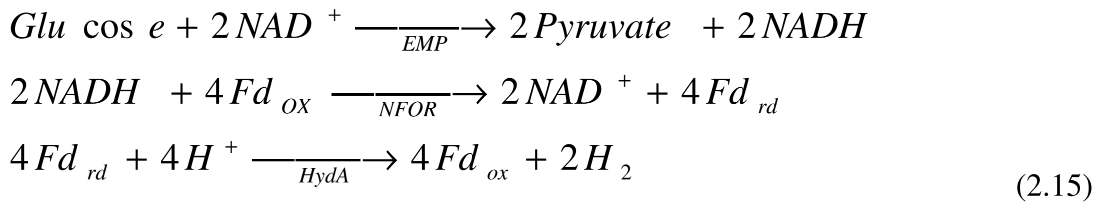
	C. acetobutylicum x9 and *Ethaniolegenes herbinese*	37	Microcrystalline cellulose	10	5.0	1.32	11.06	Wang *et al*. (2008)
	C. thermocellum and *C. thermosacchro-lyticum*	55	Corn stalk waste	10	7.2	-	0.34	Li *et al*. (2012)
	C. thermocellum DSM1237 and *C. thermopalmarium* DSM 5974	55	cellulose	9	7	1.36	0.42	Geng *et al*. (2010)
Aciduric hydrogen producing microorganisms and high hydrogen producers	*Enterobacter aerogens* W23 and *Candida matosa* HY 35	35	Glucose	5	6.5	2.59	6.27	Lu *et al*. (2007)



$$Pyruatae + CoA \xrightarrow[PFL]{} Acetyl - CoA + Formate$$
$$Formate \xrightarrow[FHL]{} H_2 + CO_2 \quad (2.13)$$



$$Pyruate + CoA + 2Fd_{OX} \xleftrightarrow[PFOR]{} Acetyl - CoA + CO_2 + 2Fd_{rd}$$
$$2Fd_{rd} + 2H^+ \xrightarrow[HydA]{} 2Fd_{OX} + H_2 \quad (2.14)$$



$$Glu\cos e + 2NAD^+ \xrightarrow[EMP]{} 2Pyruvate + 2NADH$$
$$2NADH + 4Fd_{OX} \xrightarrow[NFOR]{} 2NAD^+ + 4Fd_{rd}$$
$$4Fd_{rd} + 4H^+ \xrightarrow[HydA]{} 4Fd_{ox} + 2H_2 \quad (2.15)$$

Then, third type reaction of hydrogen production NAD(P)H utilizes by bacteria to evolve H_2. This reaction is catalyzed by two major enzymes: NAD(P)H-ferredoxin oxido reductase (NFOR) and HydA (Wang *et al.*, 2010).So far, all the systematic and quantitative analysis pathway approaches to evolve more H_2 by increasing flow of electron to H_2 producing pathway, increasing substrate utilization efficiency and investigation of more efficient and oxygen resistant enzymes. The metabolic engineering in of native hydrogen producing pathway mainly focuses on the increase of yield by maximum utilization of carbon source. This includes over expression of several enzymes and redirection of carbon flux by eliminating competitive reaction in production pathway.

The process discussed above gives the maximum theoretical yield of 2 or 4 mole H_2 as per the presence of facultative and strictly anaerobic bacteria. But in several extreme thermophiles 3.3 to 4 mole H_2/mol of glucose can be achieved naturally. These bacteria utilize both NFOR and PFOR for H_2 production (Kanai *et al.*, 2005; Verhaart *et al.*, 2010). From the thermodynamic perspective, the H_2 production from NAD(P)H is unfavourable but the high yield indicates that NFOR and HydA function efficiently in some thermophilic bacteria at elevated temperature.

2.4.3.2. On enzyme hydrogenase

Another way to potentially increase H_2 production would be through the heterologous expression of hydrogenase. Many early attempts to express [Fe-Fe] hydrogenases in *E. coli* by over expression of hydA from an organism such as

Clostridium were unsuccessful and remained unreported. Later it was shown that in order co-express maturation gene hydE, hydF and hydG that are required for H-cluster maturation and insertion of the organism does not possess these enzyme i.e. *E.Coli* (Keseler *et al*., 2005; Maeda *et al*., 2007) On the other hand, heterologous expression of hydA is simpler and possible without the heterologous expression of the accessory genes if these are encoded by the host genome. Some recent works reported for the expression hydrogenase gene hydA in *Enterobacter colace* IIT BT 08 that expressed high hydrogen yield from the strain of *E. aerogens* (ATCC 13408) which doubling the hydrogen yield (Zhao *et al*., 2010).

2.4.3.3. In microbes

Besides these most of the attempts have been made to work with genetically modified bacteria like *E. Coli* (Zhao *et al*., 2010, Wells *et al*., 2011; Maeda *et al*., 2007) *Clostridium* sp. (Tripathi *et al*., 2010; Olson *et al*., 2010) and some species of *Enterobacter* (Zhang *et al*., 2011) is used as genetically modified organisms for high yield of biohydrogen production.

2.5. Second stage processes: advance approach

Theoretically, maximum 4 mole of H_2/mole of glucose (~33% of substrate concentration) is possible during dark fermentation but only 2 mole H_2/mole of glucose (~17% substrate conversion) is achieved during the process. Presently hybrid approaches are invented by researchers to improve the hydrogen yield during production by combining the dark fermentation with photofermentation/Methanogenesis/microbial electrolysis of cells to get more substrate degradation efficiency. (Kyazze *et al*., 2010; Sharma *et al*., Claassen *et al*., 2007; Mohanakrishna *et al*., 2010).

2.5.1. Photofermentation

Several studies showed that mixed photofermentative microflora enriched from a reservoir could be used to convert acetate and butyrate effectively to H_2. Previous work had concentrated on pure cultures. If cost-effective photobioreactors were available, the two-stage dark and light H_2 fermentation process using mixed microflora would be a promising method as it has a theoretical maximal molar H_2 yield of 12 mole/mole hexose converted in the two-stage process (Lo *et al*., 2010; Ding *et al*., 2009; Azbar and Cetinkaya, 2010).

2.5.2. Microbial fuel cell

Microbial fuel cells (MFC) can utilise acetate and butyrate to produce electricity (Cheng and Logan, 2007; Rozendal *et al*., 2007; Manuel *et al*., 2010]. It has been considered the utilisation of the VFAs from the dark biohydrogen reactor, with a single chamber MFCs as a second energy generating stage. Volatile fatty acid of dark fermentation stage has feasibility to use in electricity generators and simultaneously achieved good COD (approx. 80%) and turbidity (approx. 65%) removal rates, so improving the overall system efficiency (Mohanakrishna *et al*., 2010; Tront *et al*., 2008).

2.5.3. Anaerobic digestion

Two-stage anaerobic digestion is an elegant approach to the completely utilization of organic substrate for H_2 and CH_4 generation. In dark fermentation process, the acids produced can be a precursor for methane generation (Di Stefano and Palomar, 2010). The advantage of two-stage process over dark fermentation and methane generation is that the process can be operated at high organic loading rate (Cohen *et al*., 1980). Furthermore, it employs treatment and conversion of a wide range of wastes including sewage sludge dairy wastewater, food waste (Han and Shin, 2004) and agro-industrial wastes (Rincón *et al*., 2009).

2.6. Conclusions

Biohydrogen production methods for energy generation have many application and for instance, the development of cost-effective production technology may affect the system efficiency. From the above literature, we know that inoculums, substrate, reactor type, nutrients concentration, temperature and pH are main influencing factors in biohydrogen production routes. Effect of every factor with production mechanism and protocol optimization were discussed with advancement in researches and followed by fruitful suggestions for the future work of fermentative hydrogen production. The biggest challenge over the next few years in fermentative hydrogen production will be to reduce the cost optimization of substrate concentration and composition of bacterial inoculums to improve the biohydrogen yields in particular.

Hence, this research work provides an illustration for all the challenges like material and methodology adopted to carry out the experimental plans discussed in *Chapter-3,* impacts of substrate composition (simple to complex sugars) with pure

culture is discussed in *Chapter-4,* whereas, *Chapter-5* is explaining the potential of industrial waste for bioenergy options (hydrogen and methane) with experimental database. Integrated approach for possible technologies of wastewater treatment with clean energy production is also highlighted with pure bacterial strain. Similarly, chapter -6 provides an overview on kinetics mechanisms for biohydrogen production, yields and substrate degradation efficiency with growth kinetic models. *Chapter 7* provide a concluding remarks for research problem selected and future recommendation for research in concern area.

Chapter -3

Materials and Methods

3.1. Introduction

This chapter describes the experimental methodology. The materials and methods used for the study are described in detail, explaining the purpose of each experiment and the analytical techniques were used. It outlines the basic information for microbial culture requirements and development (*Enterobacter aerogens*) for optimized conditions like type of substrate (sugar and industrial waste), effect of substrate concentration, effect of initial pH and controlled pH to assess the rate of biohydrogen production with experimental plans. Kinetic studies were also planned to assess the potential of experimental data in terms of production yield, specific bacterial growth (μ_m), biohydrogen production potential (P), rate (R_m) and lag phase (λ) using gas production models and substrate degradation models.

3.2. Regents, Chemicals and Glassware

In order to minimize contamination problem, all reagents were prepared with distilled/deionised water. All glassware and plastic containers were used by after proper washing followed by rinse with Milli-Q water. Generally, all chemicals were of analytical reagent grade. All the aqueous solutions were also prepared in Milli-Q water.

3.3. Microorganism, medium, and culture conditions

A bacterial culture of *Enterobacter aerogens* was brought from Microbial Technology and Culture Centre (MTCC, Chandigarh Punjab, India) for compilation of this study. Two media were used for the study, one for bacterial culture development and another for biohydrogen production for the selected bacterial strain termed as growth medium and fermentation medium respectively. The culture was grown in media at 30^oC temperature in an incubator shaker (New Brunswick Scientific Innova 43) for 24 hours incubation period.

3.4. Selection of substrate

Selection of proper substrate is very important since the substrate cost and its availability is a major contribution to the expenditure of the production of biohydrogen, the final product (Anderson *et al*., 2003).

Table 3.1. Media preparations for *Enterobacter aerogens.*

Composition of growth medium	Composition of fermentation medium
Beef Extract- 1 g/l	$(NH4)_2SO_4$- 4.0 g/l
Yeast Extract -2 g/l	KH_2PO_4- 4.0 g/l
Peptone -5 g/l	Na_2HPO_4- 4.0 g/l
NaCl -5 g/l	Yeast extract 1.0 g/l
	$MgSO_4$- 0.20 g/l
	Trace elements solution -2.0 ml/l
	HCl- 1.0 ml/l
	$MnCl_2.4H_2O$- 100 mg/l
	$ZnCl_2$ -70 mg/l
	H_3BO_3 -60 mg/l
	$CoCl_2.6H_2O$-200 mg/l
	$CuCl_2.2H_2O$- 10 mg/l
	$NiCl_2$- 20mg/l
	$Na_2MoO_4.2H_2O$-30 mg/l

As described in *section 2.3* of *Chapter-2,* there are many choices available varying from low to high quality, low to high cost, less available to abundantly available pure substrate to mixed substrates. The main focus of this research is to find out the compatibility of pure bacterial strain, *Enterobacter aerogens* with simple sugars and industrial waste materials, to seek for bioenergy production options.

Simulated substrate mimicked the real industrial waste from sugar industry (molasses) in terms of contents. So, the next step would be to investigate real organic residue and wastewater from industries for bioenergy production. Therefore, to

promote the waste to energy concept for sustainable development, three potential industries namely; major contribution for pollutants discharge in the environment are sugar industry, dairy industry and tannery industry waste (solid and liquid) were selected as the substrate materials for the study. A brief general description of raw materials used, water consumed and production of wastewater and waste in each industry is given below.

3.4.1. Simple sugars

Different types of simple sugars (monosaccharide and disaccharides) viz; lactose, maltose, sucrose, L-arabinose, D-xylose, glucose and cellobiose were of analytical grade (Hi media) used for biohydrogen production by using selected bacterial strain *Enterobacter aerogens*. Details relevant to experimental plan were given in of the research work in *Chapter-4 section 4.2.*

3.4.2. Industrial waste

After extensive survey of literature, waste (solid and liquid) of three potential industry viz ; sugar industry, dairy industry and tannery industry were selected as substrate.

3.4.2.1. Sugar Industry Waste (SIW)

Sugar industry activities generate large quantities of organic solid waste and by-products (*e.g.,* leaves from cane or beet, molasses from the final crystallization, press mud or cachaza, bagasse, fibre from the cane, mud and soil arriving at the plant with the raw material, and lime solids from the juice clarification. The generation of higher quality waste can provide opportunities for reprocessing of discarded raw materials into commercially viable by-products (*e.g.* paper making and particle board manufacturing).

Generally, the solid waste generated in sugar factory can be broadly categorized as Bagasse, Press mud and Molasses. Molasses is produced in the last operational steps of separating sugar from the mother liquor in centrifuges. It has been found that average production of molasses is 4.2% of the cane crushed. The characteristic features of the molasses for selecting as the substrate for the experimental study was its organic nature, high sugar contents, less availability of toxic metals but rich in micro nutrients, facilitate the growth of bacterial as well as

biohydrogen production. Sugar industry waste (molasses) used for the study was collected from the sugar industry located at Jalndhar (Punjab) India. The process flow sheet of the industry is given in Fig.3.1. Details relevant to experimental set-up plan (ESP-I.A) were given in the *section 5.1.A* of the *Chapter 5* and brief descriptions of plan are given in Table 3.2.

3.4.2.2. Dairy Industry Wastewater (DIWW)

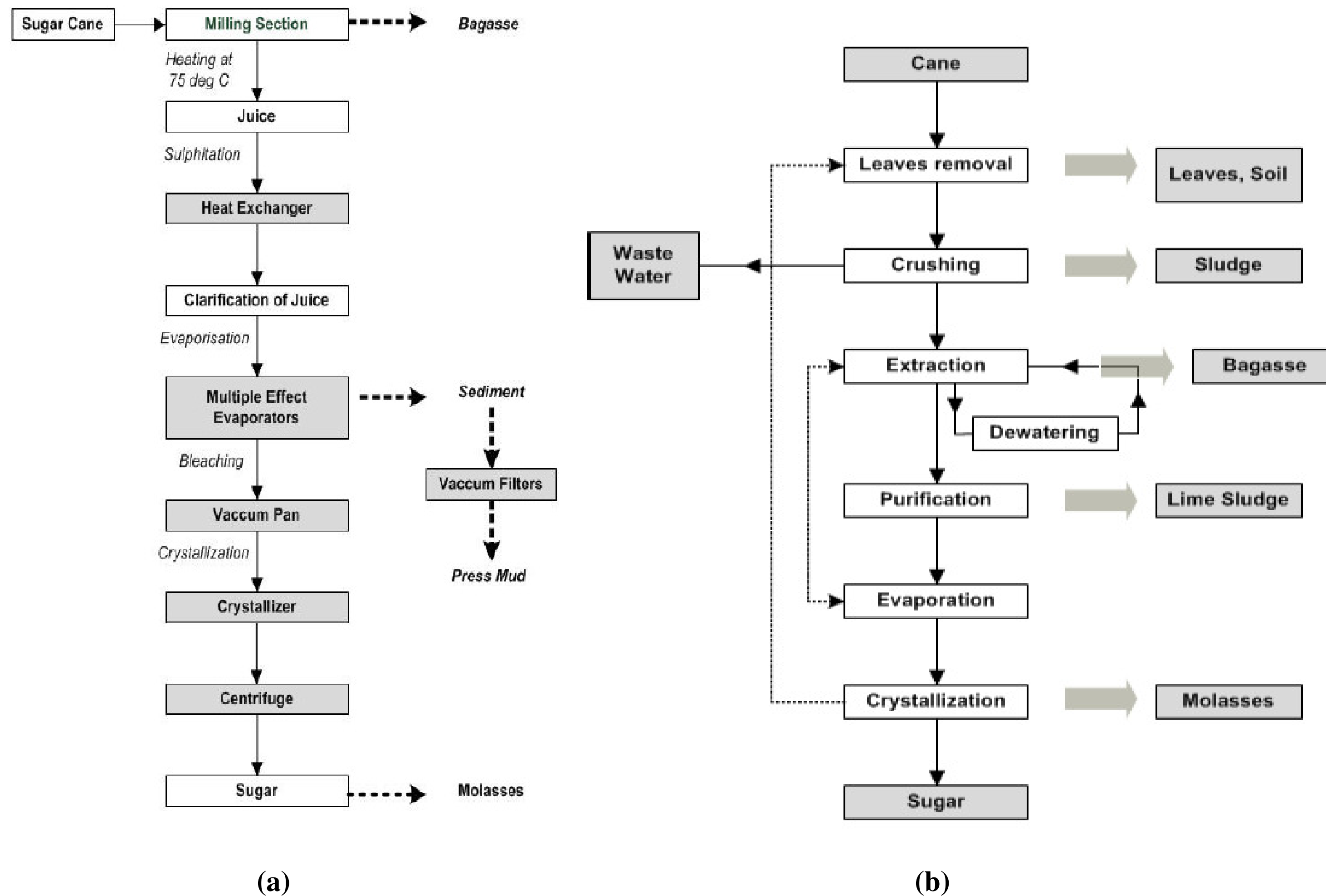
Dairy is one of the major agriculture industries and its wastewater problem is larger in developing countries because all milk is processed at industrial level. This industry is one of the important industries during the last two decades due to enormous increase in the milk production. It is also one of the industries, producing wastewater rich in organic matter and thus leading to creation of odorous and high COD containing water among the major industries in India (Kothari *et al.,* 2012). The organic nature of the dairy industry wastewater makes it feasible for bioenergy production.

Dairy industry wastewater used for the study was collected from Verka dairy Industry, Jalandhar (Punjab) India. Process layout of the industry is given in Fig. 3.2. Details relevant to experimental set-up plan (ESP-II.B) is given in *Chapter-5* of the thesis in *section 5.2.B* and brief descriptiosn of the experimental study are given in Table 3.2.

3.4.2.3 Tannery Industry Wastewater (TIWW)

Tannery waste consists of large amount of wastewater, and solid waste flashings and waste skin trimmings. This waste is mostly composed of lipids and proteins. Gaseous efficiency from fats is estimated to be higher than those of carbohydrates and proteins, therefore lipid rich waste can be regarded as a large potential renewable energy source (Cirne *et al*., 2007).

Besides this, wastewater generated from the tannery industry contains huge amount of chemicals and metals that make industrial wastewater treatment expensive. The aim behind the use of tannery industry waste is to provide the solution for its wastewater treatment rather than energy generation. The tannery wastewater used for the study was collected from the Leather complex, Jalndhar (Punjab), India. Details relevant to experimental set-up plan is given in were given in *Chapter-5 of section 5.3.C* and brief descriptions of the experimental study are given in Table 3.2.



(a) (b)

Fig. 3.1. (a) Manufacturing process of sugar industry; (b) Manufacturing of sugar from sugarcane (TGM- ILS 2010).

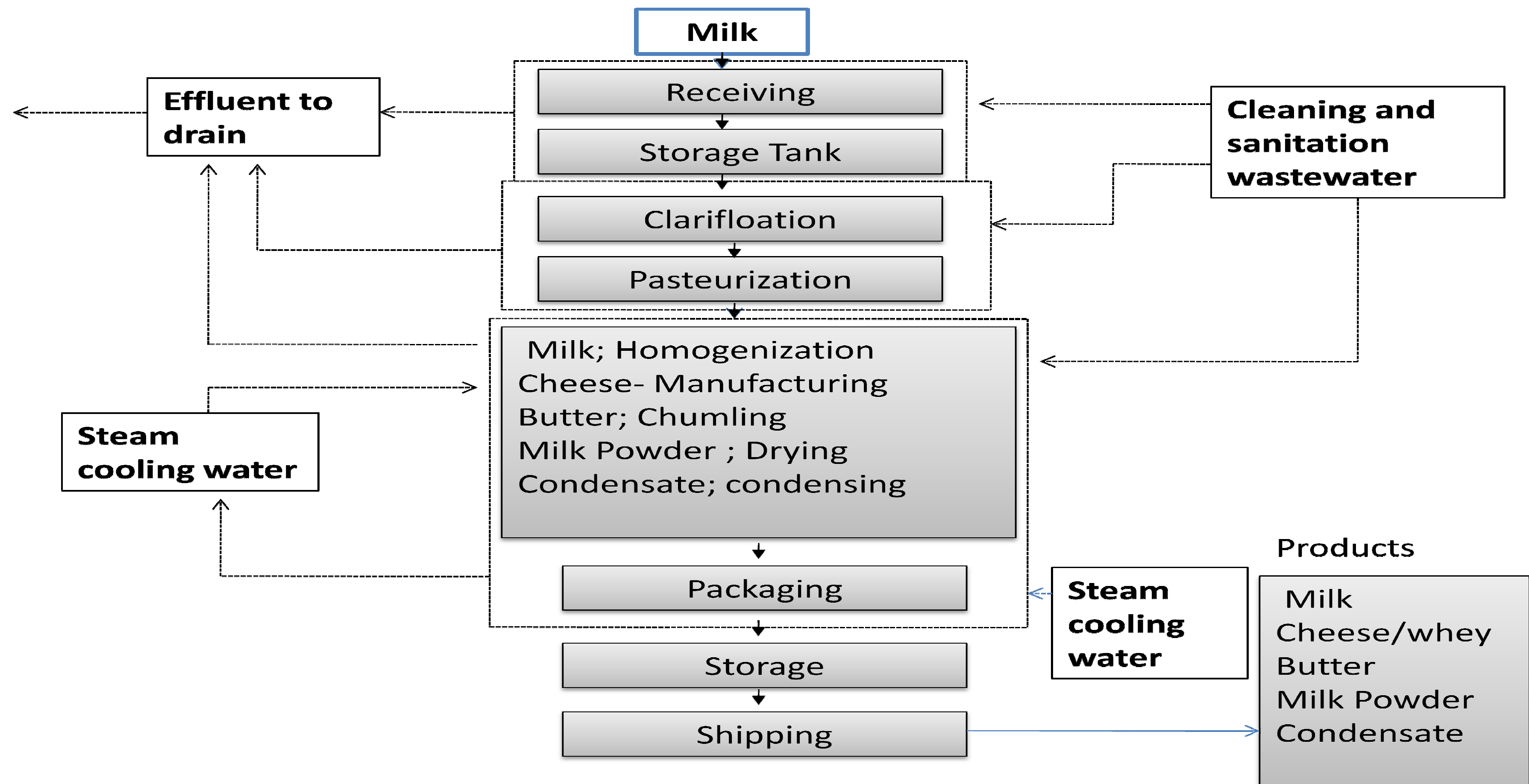


Fig.3.2. Wastewater generation sources in dairy industry (FAO 2009).

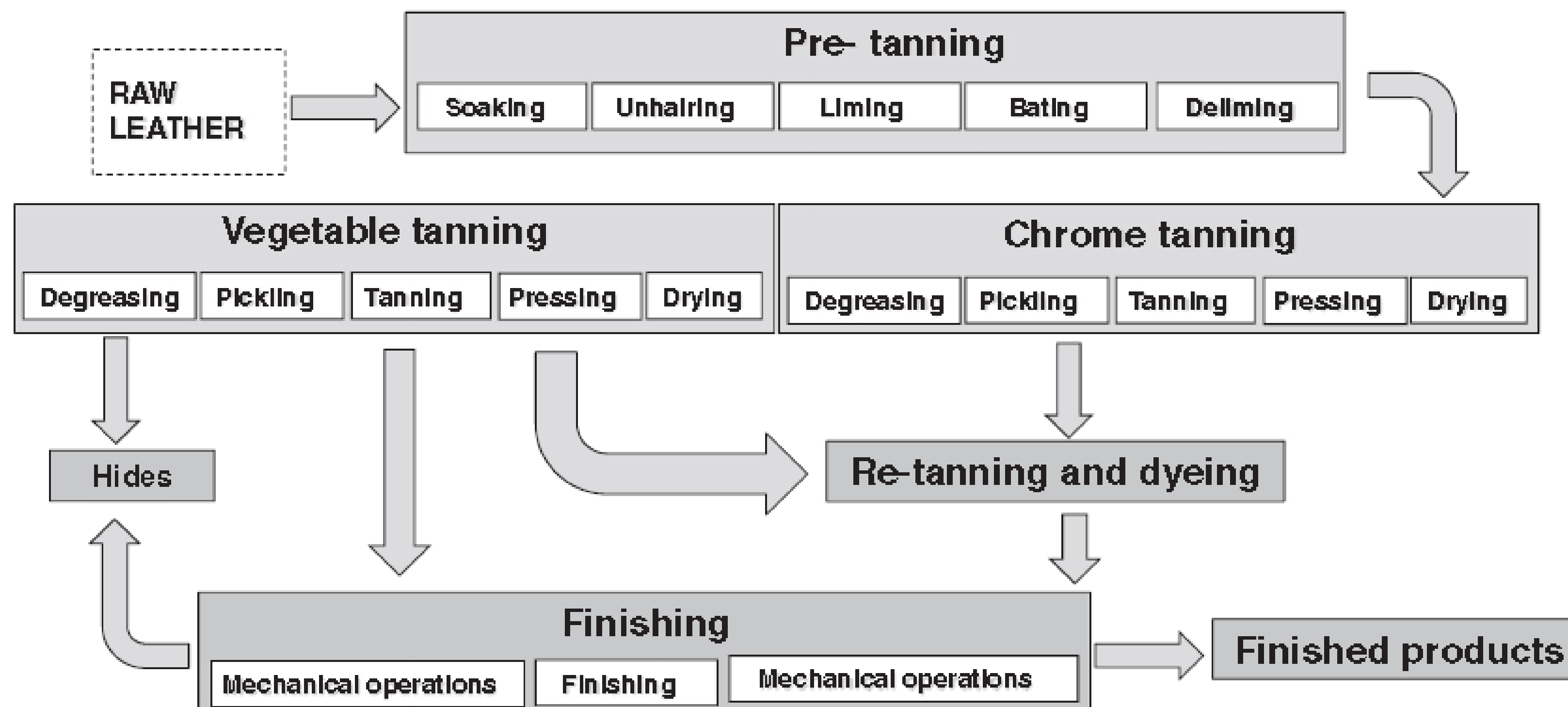


Fig.3.3. A typical process flow sheet in an integrated leather tannery industy.

Table 3.2: Experimental set up plan (ESP) layout for research work.

S.N.	ESP-I (A) SIW		ESP-II (B) DIWW		ESP-III (C) TIWW	
1.	**Phase I** Hydorgenic phase (H_2 Production)	**Phase II** Methanogenic phase(CH_4 production)	**Phase I** Hydrogenic phase (H_2 Production)	**Phase II** Methanogenic phase (CH_4 production)	**Phase I** Hydrogenic phase (H_2 Production)	**Phase II** Methanogenic phase (CH_4 production)
2.	Energy recovery and economic profit capacity		Energy recovery and economic profit capacity		Energy recovery and economic profit capacity	

3.5. Designing of bioreactor

A two litre batch reactor was used for the gas production analysis and biochemical parameter analysis. The reactor contains following part

- **a)** Aspirator bottle
- **b)** Stirrer
- **c)** Thermometer
- **d)** Gas collection port
- **e)** Liquid sample collection port

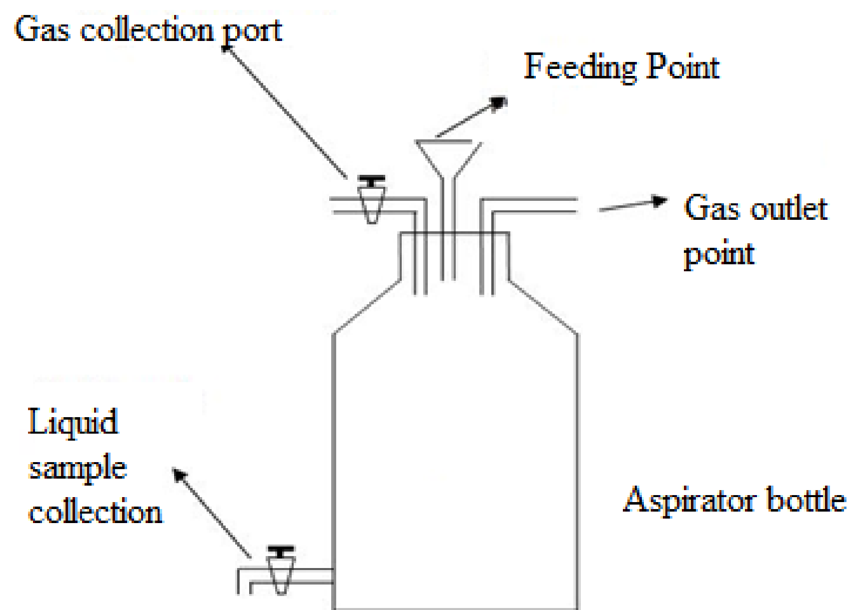


Fig.3.4. Schematic layout of batch reactor for experimental work.

Experimental setup plan (ESP) was divided according to the objectives (*Chapter-1*) in the three plans (ESP-I, ESP-II and ESP-III). Complete experimental plan layout of ESP-I, II and III were proposed in *Chapter-5 (Table 5.1)* with phases according to the study (Phase-I and Phase II) for each.

Table 3.3. Specifications of reactor operation for Phase-I and Phase-II study.

Mode of Operation	Biohydrogen	Methane Production
Reactor Environment	Anaerobic	Anaerobic
HRT	30 hours	7 Days
Total Volume	2 L	2L
Feed volume	1L	1L
Operating Temperature	30 °C	Ambient (30-35 °C)
Initial pH	6.5	6.5

3.6. Analytical Methods

3.6.1. Process parameters

3.6.1.1. pH

The pH unit measures the degree of acidity or basicity of a solution. pH of the substrate was taken with the pH meter of Thermo scientific. The disered pH of the fermntation medium was adjusted by the addtion of 5N HCl and 5N NaOH. The variation in pH has been done to optimized the [H^+] in simulated waste and real industrial waste to improve the gas production. The four different pH (4.5, 5.5, 6.5, and 7.5) were selected for the study.

3.6.1.2. Sugar estimation

The sugar estimation in the feedstock at each phase of the process was done by the Anthrone methods (Loewus, 1952). Detailed protocol is given in Annexure-1.

3.6.1.3. Gas production analysis

Gas production analysis was done by water displacement methods with batch set-up (Fig. 3.5) as proposed by Walker *et al.,* (2009). Total volume of aspirator bottles were 2L was taken for the study having working volume 1 L .

3.6.1.4. Volatile fatty acids Analysis

The volatile fatty acids produced during the process were measured for all types of substrate used in the study. The detail of the procedure for estimation of volatile fatty acids is given in Appendix-1.

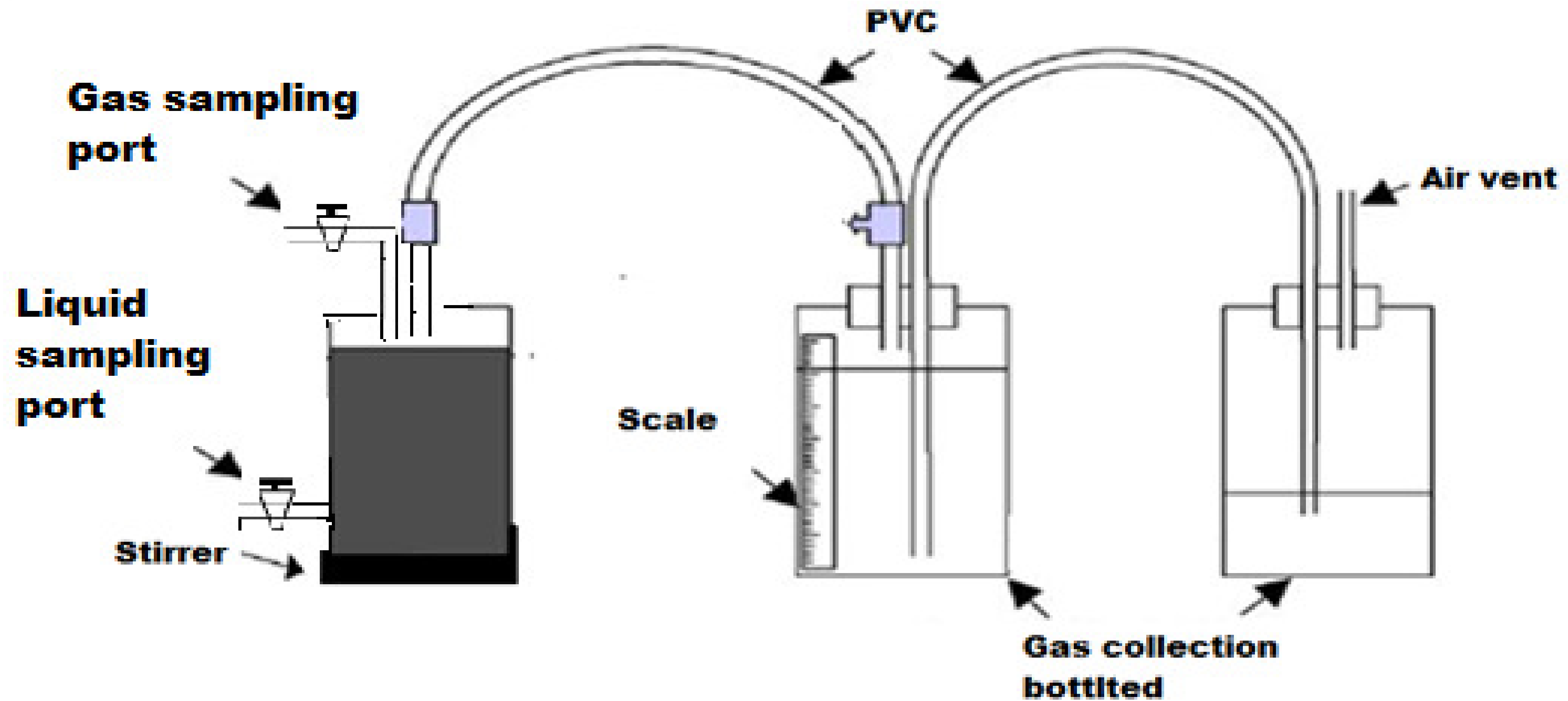


Fig.3.5. Batch experimental set up for production of bioenergy options with modifications.

3.6.2. Characteristics of wastewater

The selected wastewater of the industries were characterised for various physico- chemical parameters viz; pH, Total Solids (TS), Chloride (Cl^-), Nitrate (NO_3^-), Nitrite (NO_2^-), Ammoniacal nitrogen (NH_4^+-N), Phosphate (PO_4^{-3}), Sulphate (SO_4^{-2}), Biological oxygen demand (BOD), and Chemical oxygen demand (COD). The detail methodology for the estimation of each parameter is given in Appendix-1.

3.7. Instrumental Analysis

3.7.1. Gas Chromatograph

Hydrogen content in the gas was determined with Gas Chromatograph (GC 5765 Nucon India Make) equipped with Thermal Conductivity Detector (TCD) a stainless steel column packed with porapak Q (80/100 mesh). The operational temperature of the oven, injector port and detector were 70°C, 120°C and 120°C respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min.

3.7.2. High Performance Liquid Chromatography (HPLC)

At particular interval, fermented broths (in triplicate flasks) were removed and the contents were analyzed for total sugar and ethanol production. Glucose and ethanol was analyzed by high-performance liquid chromatography (HPLC) using Hi-Plex H column at 57°C with 1mM H_2SO_4 as the mobile carrier at a flow rate 0.7 ml min^{-1} and detected by refractive index detector.

3.8. Kinetic studies

A variety of bacterial growth and kinetic models, describing substrate utilization pattern have been developed, modified and used by many researchers. Growth kinetics is a relationship between specific growth rate and the concentration of substrate compositional variation. Bacterial growth often shows a phase at a value of zero, where specific growth rate starts and then exceeds to a maximum value (μ_m) in a particular period of time and when bacteria acclimatized itself in environment and no significant growth was observed was results a lag time (λ). Among the various proposed kinetic models, the Logistic bacterial growth model (Mu *et al.*, 2006), Monod model (Monod, 1949), Moser model (Ardestani, 2011), Hanes-woolf model (Hanes, 1932) and Eadie-Hofestee model (Hofstee, 1959) were evaluated in the

present study for bacterial growth and substrate consumption for both phases of experiment (Phase-I and II.)

The kinetic study used to analyse the experimental data is divided in three sections:

3.7.1. Kinetics for growth of bacteria
3.7.2. Kinetics for gas production (hydrogen and methane)
3.7.3. Kinetics of substrate utilization

A detail of all these kinetics with specific models used to study is given in *Chapter-6*. Relevant mathematical derivation with process variables is only summarised here to introduce the concept of kinetics for experimental phases.

3.8.1. Kinetics for growth of bacteria

Logistic bacterial growth model and modified logistic model (Eq.1) can easily describe the progress of bacterial growth in batch experiments.

$$\frac{dX}{dt} = \mu X \qquad (3.1)$$

Where X is the bacterial biomass, μ is the specific growth rate of bacteria. By integrating Eq.1 and using boundary conditions, we have:

$$X = X_0 e^{(\mu . t)} \qquad (3.2)$$

3.8.2. Kinetics for gas production (hydrogen and methane)

The cumulative hydrogen and methane production obtained in the anaerobic reactor were followed by the modified Gompertz equation (Lay *et al.,* 1998).

$$H(t) = P \exp\left\{- \exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \qquad (3.3)$$

Where, H(t) represents the cumulative volume of gas (hydrogen and methane) production (ml) for phase I and II respectively, as a function of time; P, the gas production potential (ml); R_m, the maximum production rate (ml /h); , the lag time (h); t, incubation time (h); e, the exponential constant having value 2.718. The typical cumulative hydrogen production curve was nonlinearly modeled by the equation 7. Parameters (P, R_m and λ) were estimated using non linear curve fitting tool of Origin 8.5. Analysed results for cumulative hydrogen production and methane production were given in *Chapter-4 and Chapter-5.*

3.8.3. Kinetics of substrate utilization

The growth of microorganism is highly depended on the availability of substrate. A specific amount of substrate can enhance on retard the growth of organism. The kinetic models discussed here are the growth model which depicts the interaction of bacteria to the concentration of substrate.

3.8.3.1. Monod model

The Monod equation is used in the present case as the fundamental basis for the kinetic study and expressed as:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{3.4}$$

Where, μ is the specific growth rate; K_s is the half-saturation coefficient and $\mu_{\max}$, is the maximum specific growth rate of the bacteria in the batch system.

3.8.3.2. Moser model

Moser model is a modified Monod equation with power function of substrate concentration.

$$\mu = \mu_{\max}\left(\frac{S^n}{K_s + S^n}\right) \tag{3.5}$$

3.8.3.3. Hanes-Woolf model

Hanes- Woolf plot is a graphical representation of kinetic parameters in which the ratio of the initial substrate concentration [S] to the specific growth rate of bacteria is plotted against [S].

$$\frac{[S]}{\mu} = \frac{1}{\mu_{\max}}[S] + \frac{K_s}{\mu_{\max}} \tag{3.6}$$

3.8.3.4. Eadie-Hofstee model

Eadie- Hofstee diagram is a graphical representation of kinetic parameters in which maximum specific growth rate of bacteria is plotted as a function of the specific growth rate of bacteria against the substrate concentration and specific growth rate ratio

$$\mu = -K_s \frac{\mu}{S} + \mu_{\max} \tag{3.7}$$

These models were used to compare the data obtained and putting it on a graph. The value of y intercept is the value of $\mu_{\max}$ while the slope is K_s.

3.9. Economical evaluation of energy production process from industrial waste

The economic analysis of the bioenergy production (hydrogen and methane) was done. The evaluation was made on the basis of comparative analysis of selected coupled substrate-strain system for hydrogen production processes reported earlier by several researchers. The analysis was done on the basis of substrate consumption (molasses) and COD removal of the industrial waste dairy industry wastewater.The finding of the process is compared it with other energy production processes and amount that can be generated by utilizing waste. Sections *of Chapter-5 (section 5.3.3.5.A. and 5.4.3.2.4.B)* are proposing the full picture of economical assessment of concept “zero waste discharge”.

3.10. Conclusions

This chapter is summarising the basic requirements needed to execute the experimental objectives in general and analytical/ instrumental requirements to investigate the findings of ESP-I, II and ESP-III in Phase-I and Phase-II in particular.

Chapter -4

Selection and optimization of simple sugars with process pH using Enterobacter aerogens for bioenergy options

4.1. Introduction

Biohydrogen production can be used a wide variety of organic substrates as feedstock like pure sugars, agricultural waste by-products, food processing waste and other organic industry waste, but most of the studies at lab scale were done with the pure sugars like glucose, sucrose and starch (Nanqi *et al*., 2011; Wang *et al*., 2009). Researchers show that almost in 80% biohydrogen production process pure sugars such as monosaccharides (glucose, xylose, fructose, arabinose, mannose and ribose), disaccharides (sucrose, cellobiose, maltose and lactose) and polysaccharides (starch, cellulose, glycogen) used as feedstock. Among them, simple sugars are a preferable feedstock for biohydrogen production due to their simple structure and easily degradable qualities. However, use of pure sugars for biohydrogen production is costly at large scale (Hu and Chen 2007; Hafez *et al*., 2007) but these are the model substrates to study the process at the lab scale.

Literature on fermentative biohydrogen production involve the use of bacterial inoculums that can be either taken from sludge of wastewater treatment plant (Wang and Wan, 2008) or pure culture (Walid *et al*., 2008) and mixture of more than one bacteria (Chang *et al*., 2008; Ozmihci and Kargi, 2011). There is an extensive search is going on find out the suitable bacterial strain for fermentative hydrogen production and most of the studies are involved the use of pure culture of *Clostridium* sp. Fermentative anaerobic hydrogen production is completely anaerobic process and maintaining the anoxic conditions during whole process is very difficult and required addition of some expensive reducing chemicals like cysteine or cysteine hydrochloride that enhance the cost of process (Elsharnouby *et al*., 2013). To avoid the toxicity of oxygen during the process, use of some facultative anaerobic bacteria is a novel approach. The bacteria belong to the family Enterobactereacae like *Enterobacter* sp. and *E. Coli* has capabilities to produce hydrogen without the addition of some reducing chemicals.

Facultative anaerobic bacteria are gram-negative, rod shaped bacteria with the relatively simple nutrient requirement. Among the hydrogen producing anaerobic bacteria (*E. coli*, *P. vulgaris, E. aerogenes)*, ferments sugars to a variety of end products like acetate, formate, lactate, succinate, ethanol, CO_2, and H_2. The degradation of organic matter in anaerobic environments by microbial consortia involves the cooperation of a population of microorganisms that generate a stable,

self-regulating fermentation process. The facultative anaerobic bacteria convert carbohydrate- rich substrate to pyruvate through the process glycolysis. These bacteria generate acetyl-co A and formate through the enzyme pyruvate formate lyase (PFL). Formate is then converted to hydrogen and carbon dioxide by enzyme formate hydrogen lyase (FHL) complex (Fig.4.1). The facultative anaerobes, especially members of the family Enterobacteriaceae, can metabolize pyruvate to formic acid and other products in a process sometimes called the formic acid fermentation. Then, fermentative bacteria form organic acids, H_2 and CO_2 from monomeric molecules. At that point, H_2 and acetate can be utilized and/or produced by several microbial groups. Thus, acetate is generated during acetogenesis from CO_2 reduction and H_2 by autotrophic acetogens via the Wood– Ljungdahl pathway, a process named homoacetogenesis.

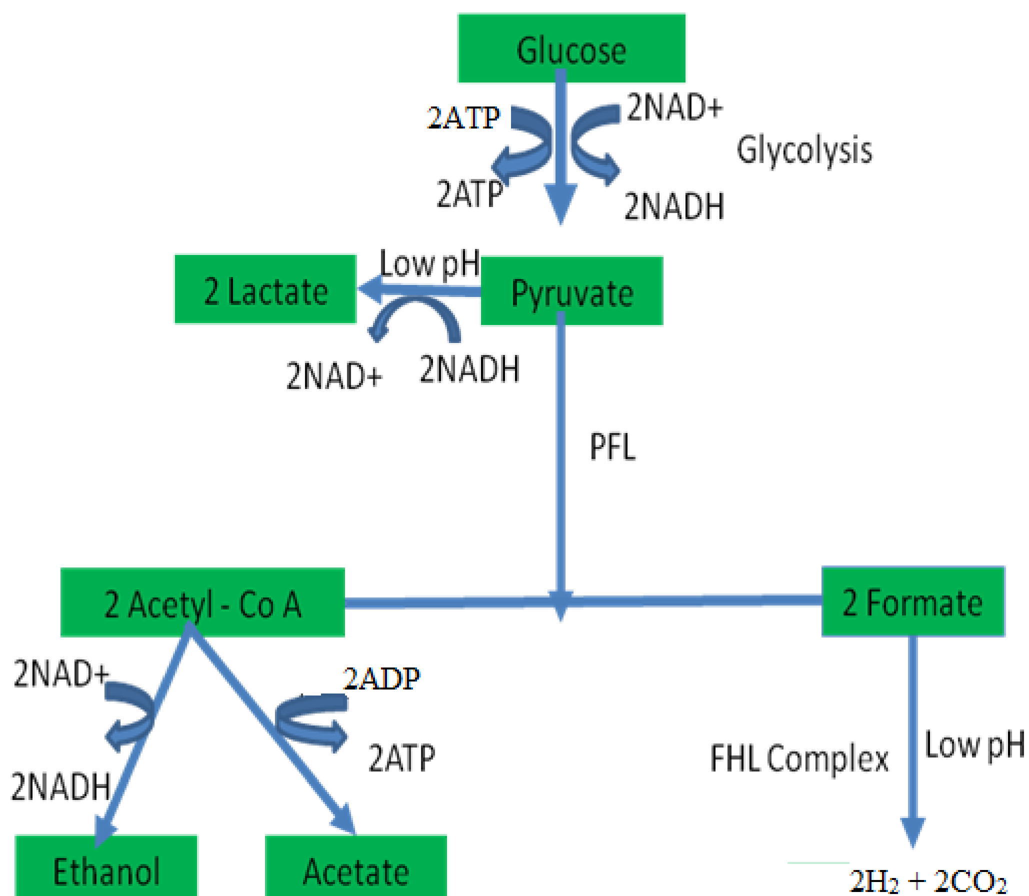


Fig.4.1. Biohydrogen production process by facultative anaerobic bacteria (Hellenbeck, 2004).

This chapter emphasized on the analysis of facultative anaerobic bacteria *Enterobacter aerogens* to utilize different sugars for biohydrogen production and further to observe the pattern of biohydrogen production from glucose, its consumption and effect of pH.

4.2. Materials and methods

4.2.1. Selection and preparation of bacterial inoculums

Various facultative anaerobic bacteria are present in environment but as per our specific need to fulfil the research study after extensive literature survey *Citrobacter* sp, *Enterobacter aerogens* and *E. Coli* found to be more suitable. Among these, *Enterobacter aerogens* has more advantages in comparison to other bacterial strains i.e. its short doubling time (0.25h) and consequent fast rate of hydrogen evolution (Table 4.1). The yield of hydrogen from glucose however was smaller at 1 mol than other bacteria, such as *Clostridium butyricum*, which yielded 2.3 mol H_2 from 1 mol of glucose (Wood, 1961). Table 4.2 is showing the metabolic properties of selected bacterial strain as mentioned in literature.

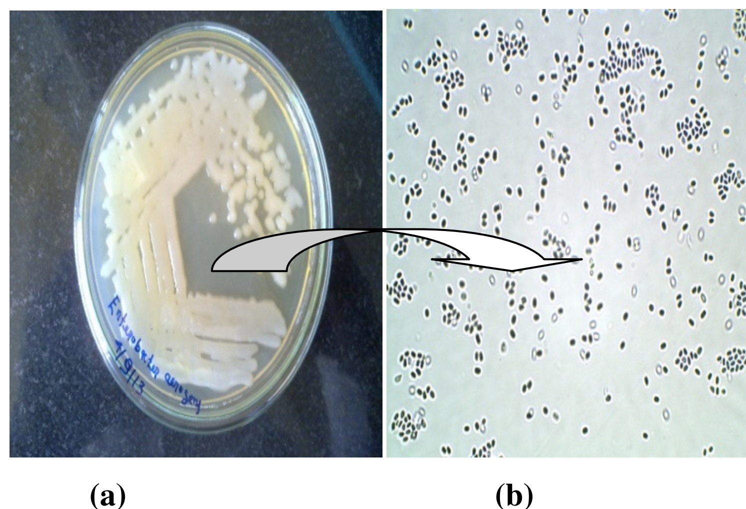
Table 4.1. Rates of hydrogen evolution from aerobic and anaerobic bacteria (Oskar,1998).

Category	Doubling Time (h)	H_2 evolution rate mmol H_2/l.h	H_2 evolution rate mmol H_2/g.h
Fermentative evolution	0.16-2		
Strict anaerobe			
Clostridium butyricum			
Closridium beijerinckii AM 21B		17	25
Facultative anaerobes			
Citrobacter intermedius		11	9.5
Enterobacter aerogens E. 8205	0.25	36	17

Table 4.2. Properties of *Enterobacter aerogens* (Tanisho *et al*., 1987).

Metabolism	**Facultative anaerobes**
Suitable pH	
For growth	7.0
For H_2 production	5.5-6.0
Temperature suitable for growth	30-40C
Yield of H_2	
From glucose	1.0 mol H_2/mol
From sucrose	2.5 mol H_2/mol
Evaluation rate of H_2	
Batch cultivation	17mmol/g dry cell/h
	21mmol/L culture/h
Continuous cultivation	36 mmol /L culture/h

A bacterial culture of *Enterobacter aerogens (E. aerogens)* (MTCC no.8100) was brought from Microbial Technology Culture Centre (MTCC), Chandigarh, Punjab, India and grown on prescribed media. Preparation and constituents of the growth media is discussed in *Chapter-3* of Table 3.1.



(a) **(b)**

Fig.4.2. A microscopic view of *Enterobactor aerogens* (a) & (b.)

4.2.2. Preparation of fermentation medium

All the chemicals were used of analytical grade. Anaerobic liquid media used for experimental study in *Chapter -3,* Table 3.1.

4.2.3. Experimental set-up

The gas production measurement was done by water displacement methods. Experiments were done in anaerobic aspirator bottle with batch set-up plan as shown in *Chapter 3*, (Fig.3.4). The total volume of the reactor was 1000 ml for the study with working volume of 600 ml. The fermentation medium sample with 10% v/v microbial seed culture was kept for study with adjusting the initial pH at 6.5.

4.2.4. Analytical methods

The analytical methods required for gas production and composition analysis and sugar estimation was done by the standard methods. The details procedure of each method is given in Appendix-1.

4.3. Results and Discussion

4. 3.1. Growth of Bacteria

Most facultative anaerobes produce hydrogen through breakdown of glucose to pyruvate forwarded by volatile fatty acid formations during fermentation (Elsharnouby *et al*., 2013). The growth pattern of the bacteria in growth medium was analyzed after each hour for OD_{600}. The growth of selected bacteria *Enterobacter aerogens* was started after the lag phase of 2 hours in growth media and the exponential phase ended at 24 hours. The stationary phase of the bacteria was achieved after 24th hour in 30 hours retention time.

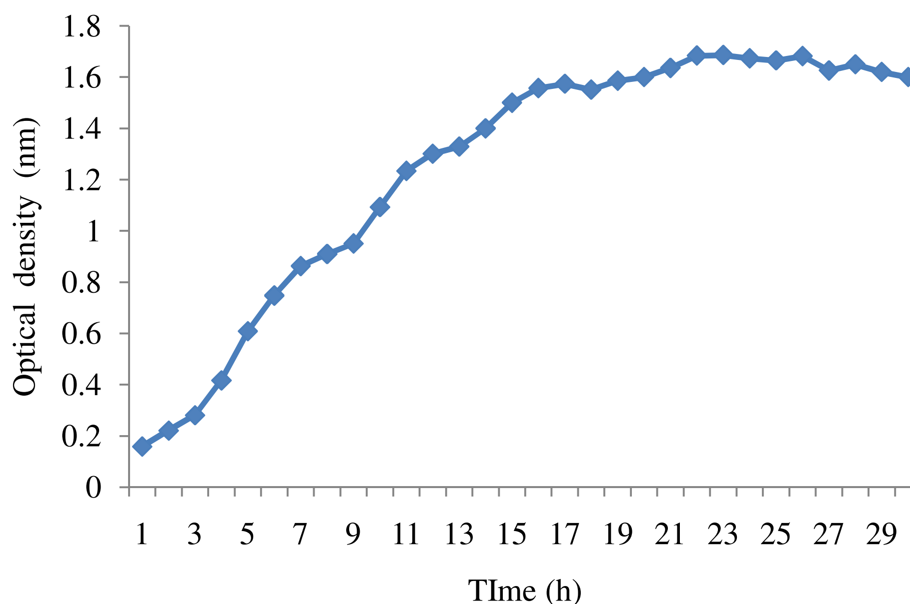


Fig. 4.3. Growth curve of bacteria in medium at OD_{600}.

4.3.2. Biochemical assay for the carbohydrate fermentation

The sugar fermentation pattern may be unique to a particular bacterial species or strain. The biochemical analysis of bacteria for the fermentation of carbohydrate (glucose and sucrose) was done in lab (Aneja, 2010). The results obtained were given in Table 4.3. All test tubes in duplicate for assay's observation.

Change of medium into yellow colour means organism ferment the given sugars (glucose and sucrose) and produce organic acids and gas. Gas production was observed by the presence of small bubbles in the sugar consisted tubes. The change in colour may be due to acidic pH of the medium (S.No. 3 and 4), whereas no change in colour of medium (red) represents that bacteria cannot utilized the provided carbohydrate (control i.e. no sugar) but the organism continue to grow in the medium using other energy sources in the medium (S.No. 1 and 2). No change in colour is due to neutral or basic pH of the medium Fig 4.4.

Table 4.3. Fermentation of sugar assay by selected microbial species

S. No.	Composition	Results	Observation
1	Media + Phenol red (1)*	_	No colour change
2	Media + Bacteria + Phenol red (1)*	_	No colour change
3	Media + Bacteria + Phenol red Glucose (2)*	+	Yellow colour appeared + gas bubbles seen
4	Media + Bacteria + Phenol red Sucrose (2)*	+	Yellow colour appeared + gas bubbles seen

*Number of test tube used

Enteric organisms on fermentation of carbohydrates produce lactic acid, formic acid succinic acid as well as ethanol and gases, CO_2 and H_2. Gas production during the process in carbohydrate containing growth medium, typically is CO_2 and H_2 only.

4.3.3. Hydrogen gas production

4.3.3.1. Effect of various sugars

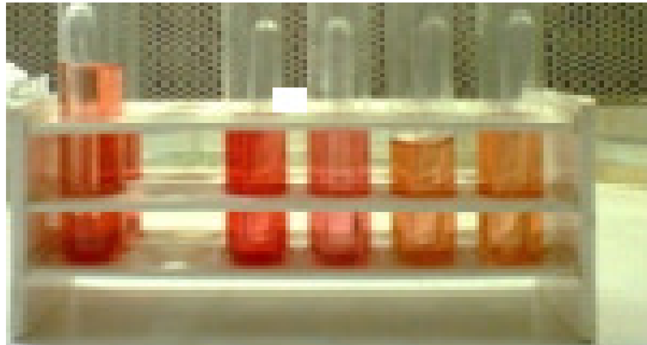
Keeping in mind above biochemical results, the feasibility of the selected bacterial strain *Enterobacter aerogens* for biohydrogen production was also observed with different simple sugars. The bacteria showed hydrogen production with all

sugars taken, but it seems more compatible with simple sugars as the maximum production was achieved from cellobiose (2.13 mol/mol) followed by glucose (0.87 mol/mol) and least production was occurs with lactose (0.33 mol/mol) shown in Table 4.4. It shows that the bacteria present a better adaptation to cellobiose than to glucose. The reason maybe that cellobiose was found to be chief product in cellulolysis by various bacterial strains (Hungate, 1944; Hulcher and King 1958, a and b). However, better yield obtained on cellobiose due to the phosphrylation in the cellobiose phosphorylase. Similar results of higher yields were also obtained by Giallo *et al.,* (1983) with the *Clostridium* strain.

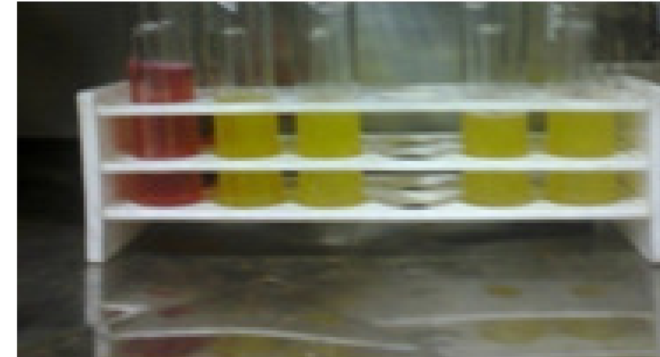
Hence, glucose and cellobiose both have potential for gas production with selected bacterial strain, but due to cost limitation with cellobiose (INR 14852 per 100 gm; Sigma Aldrich), we selected glucose (INR 2,773 per 100 gm; Sigma Aldrich) as a model substrate for experimental plan.

Table 4.4. Hydrogen production from different pure sugars.

Sugars (10 g/l)	Sugar type	HPR (ml/l.h)	H_2 Yield (mol/mol)
Lactose	Disaccharide ($C_{12}H_{22}O_{11}$)	9.1	0.33
Maltose	Disaccharide ($C_{12}H_{22}O_{11}$)	23.3	0.85
Sucrose	Disaccharide ($C_{12}H_{22}O_{11}$)	20.0	0.73
L-Arbinose	Monosaccharide ($C_5H_{10}O_5$)	36.6	0.58
D-Xylose	Monosaccharide ($C_5H_{10}O_5$)	24.1	0.38
Glucose	Monosaccharide ($C_6H_{11}2O_6$)	50.8	0.87
Cellobiose	Disaccharide ($C_{12}H_{22}O_{11}$)	58.3	2.13



(a)



(b)

(c)

Fig.4.4. Biochemical assay of selected microorganism (a) initial phase (b) fermentation phase (c) gas production phase.

Each experimental last for around 30 hours, initial lag period was observed for this study in the range of 4-5 hours for the substrate (glucose) concentration, 10 g/l. The composition of H_2 gas was 35% as per gas chromatograph analysis (Fig.4.6). So, the H_2 was 210 ml (600 ml water displaced) or 105 ml H_2/g glucose (0.105 l/g glucose) i.e. 0.87mol/mol of glucose. The yield of facultative anaerobic bacteria by dark fermentation is 2 mol H_2/ mol of glucose or 0.248 l H_2/g glucose at standard temperature and pressure (Hallenbeck, 2004) as per the previous research database. This low yield was due to decrease in the pH of the medium, during the fermentation. Although VFA (volatile fatty acid) concentration were not observed during the study due to instrumental limitations but from the literature we can say that decrease in pH was indication of increase in volatile fatty acids concentration and thus hydrogen production was somewhat inhibited (Khanal *et al.,* 2004).

Initial concentration of glucose taken was 10 g/l and after fermentation only 6g/l was observed in the fermented solution of the reactor, Hence, 4 g/l (40%) of glucose was consumed by the bacterial organisms and 0.87 mol H_2/mol of glucose was obtained as a hydrogen yield and rest may be converted to other fermentation products. Hallenbeck and Benemann (2002), reported that facultative anaerobic bacteria generate acetyl coenzyme-A and formate by utilizing glucose. The formate is then converted to hydrogen and carbon dioxide in acidic condition/environment.

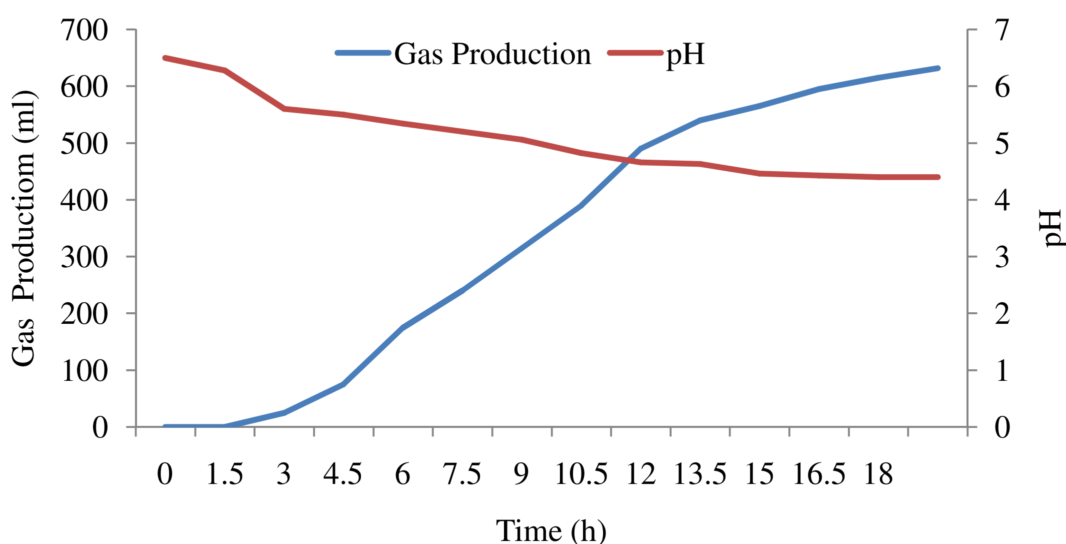


Fig.4.5. Effect of pH and cumulative gas production of the bacteria during process.

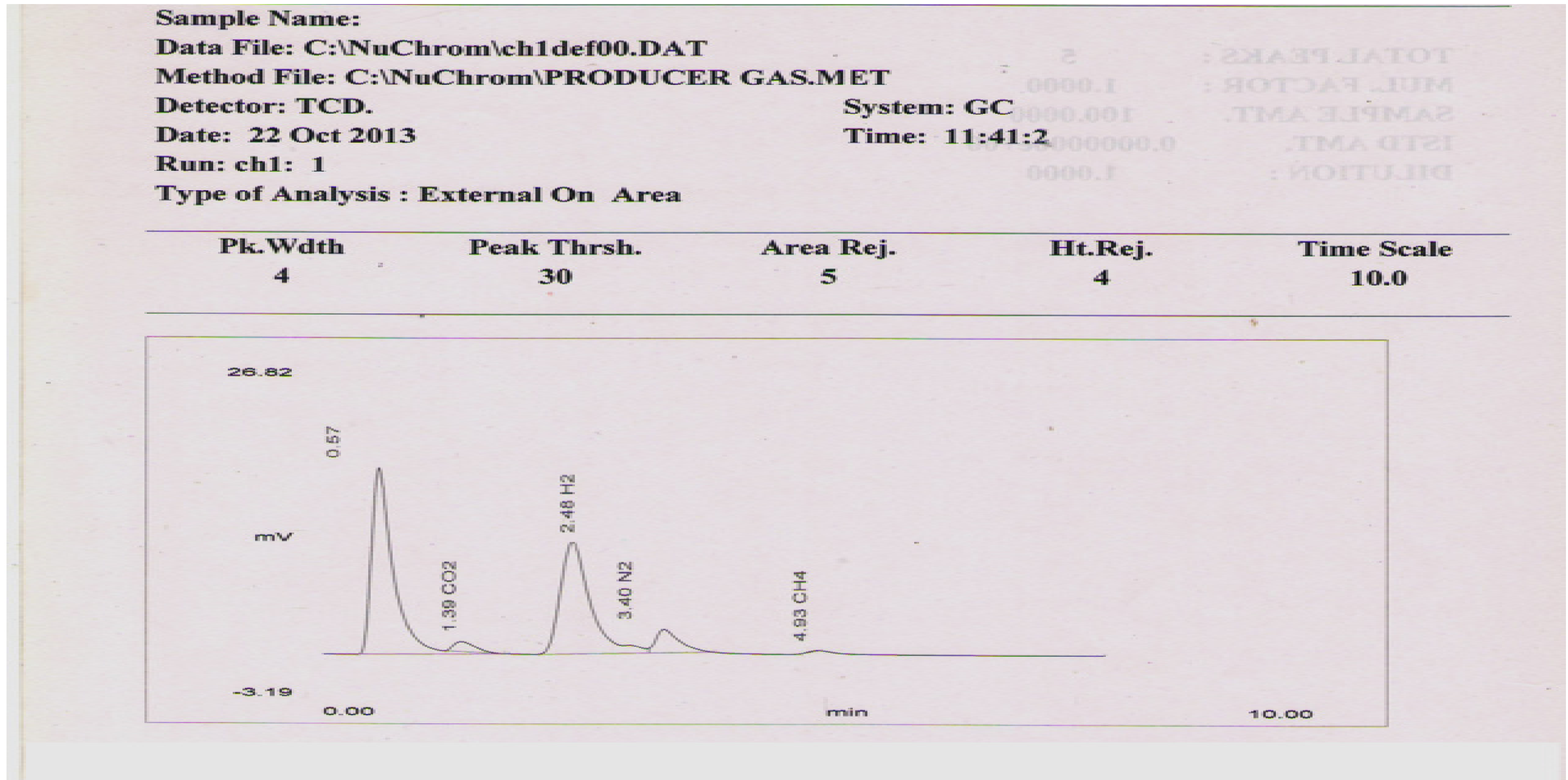


Fig.4.6. Composition of biohydrogen produced from glucose.

4.3.3.2. Effect of pH

The effect of initial pH on biohydrogen production was investigated from 6.5 to 4.5 as shown in Fig.4.5. From the graphical representation, results can be summarised that hydrogen production and yields were pH dependent. In the initial hours of the fermentation the gas production was high (pH 6.5 to 5.0). Values below the pH 5 do not support the gas production and it was decreased drastically. The findings may comparable with the findings of Tanisho *et al.,* (1987), who works on *Enterobacter aerogens* strain E.82005 with glucose (15 g/l) as the substrate however, it is in close contradiction with the works of Wei *et al.,* (2010) and Lin et al., (2010) where, initial pH 6.5 was found optimum for hydrogen production. Hence, it can be said that substrate concentration and composition with organisms used in their study may not be comparable with the present study.

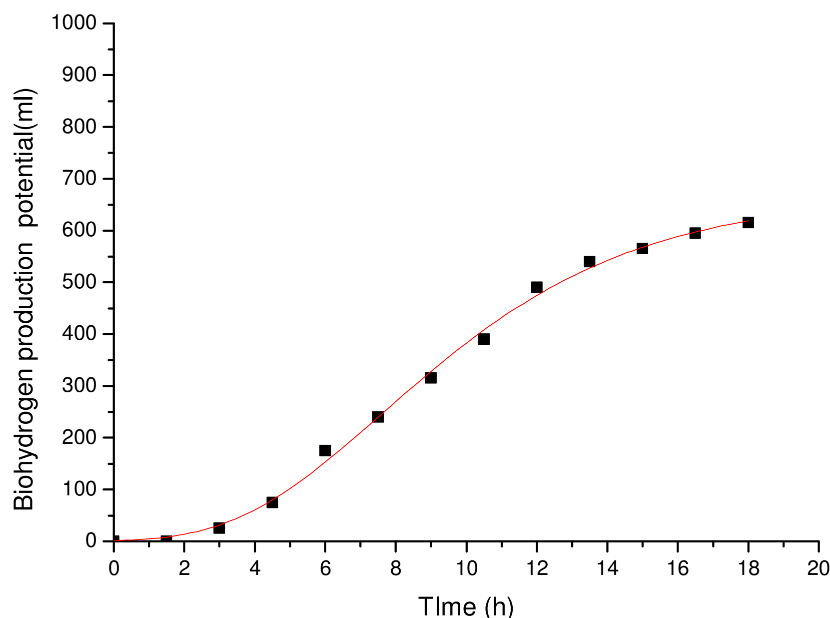


Fig.4.7. Typical cumulative hydrogen production curve fitted by the modified Gompertz equation with glucose as substrate**.**

To determine the effect of initial pH on the best fitting of cumulative hydrogen production data using the modified Gompertz equation (Table-4.5) investigated with kinetic parameters P, R_m and λ. Where, data points (X) represent experimental data and line represents non-linearly modelled using in the Fig 4.7. Regression was obtained in the range of 0.998 provides a strong correlation between the experimental data produced and the fit.

Table4.5. Comparative review of kinetic parameters as obtained by modified Gompertz equation.

Organism	Substrate used	Optimal pH	P (ml)	R_m (ml/h)	λ(h)	References
Clostridium butyricum	Sucrose	5.5	-	-	-	Chena *et al.* (2005)
Clostridium butyricum CWBI1009	Glucose	5.1	-	-	-	Masset *et al.* (2010)
Caldimonas taiwanensis On1	Starch	7.5	-	-	-	Chena *et al.* (2008)
Clostridium sp. YM1	Glucose	6.5	-	-	-	Abdeshahian, *et al*. (2014)
E. cloacae IIT-BT 08	Glucose	6.5	889.38	13.97	4.04	Nath *et al.* (2006)
R. sphaeroides 0.U.001	Glucose	-	615.65	16.15	14.3	Nath and Das (2006)
E. cloacae IIT-BT 08	Glucose	pH 6.5 (Initial)	343.7	29.8	25	Khanna *et al.* (2011)
E. cloacae IIT-BT 08	Glucose	pH 6.5 (Regulated)	796.1	72.1	25	Khanna *et al.* (2011)
E. cloacae IIT-BT 08	Glucose supplemented with Na+	pH 6.5 (Initial)	560.2	47.1	8.6	Khanna *et al.* (2011)
Enterobacter aerogens	**Glucose**	**6.5**	**670**	**36.7**	**7.6**	**This study**

Lower hydrogen production at higher pH (6.5, 6.3, 5.5) may be attributed to solvent toxicity due to lower reaction rate of hydrogenase enzyme responsible for H_2 production. From the results it is also observed that the acidic condition in the reactors favours the H_2 production, however, an initial pH 4.6 onwards seem to decrease in production trend. This may be due to the metabolic activities of *Enterobacter aerogens* which could not be maintained at low pH ranges and as a results inhibition in H_2 production.

Similar findings have been reported by Xiao *et al.,* (2013), Gioannis *et al.,* (2014) Xiao *et al.,* (2010), Faloye *et al.,* (2014), and Khanna *et al.,* (2010), who support statement with finding that the extreme high and low pH values affects the biohydrogen production. Reduction in H_2 production may also be explained on the basis of production of weak acids, in the reactor, which pass through the cytoplasm and control the growth of as well as their production potential of hydrogen.

4.4. Conclusions

From the above study, it can be concluded that the selected species *Enterobactor aerogens* show the feasibility to produce hydrogen from various simple sugars and it is most compatible with glucose.

This chapter highlights the importance of substrate composition (simple sugar i.e. glucose) with initial pH for the rate of hydrogen production using *E. aerogens*. It has been observed and concluded that initial pH (5.6 to 5.1) gave better results as compared to the extreme high and low ranges. This finding was found significant with the modified Gompertz equation model. The experiment also showed that the *E. aerogens* utilizes glucose and convert it into H_2 and organic acids strongly dependent on the bacterial isolate. Thus, there is a possibility to use glucose and glucose based organic substrate materials, residues and wastewater form industries for the biohydrogen production, although probably optimization of bacterial strains or their genetic modification will be desired. On the basis of these findings the selected bacterial strain *Enterobacter aerogens* was proposed for bioenergy production options (biohydrogen and methane) in integration with solid waste (organic residual waste) from sugar industry (*Chapter 5; Part-5.1*) and liquid waste from dairy industry and tannery industry i.e. wastewater (*Chapter 5; Part-5.2 and 5.3*) in the further chapters of this thesis.

Chapter -5

Experimental assessment and optimization of industrial waste using Enterobacter aerogens: Integration for bioenergy production and treatment options

5.1. Introduction

An enormous quantity of organic waste is available from various sources such as domestic, agricultural and industrial sectors, which is composed of a reasonably good biodegradable carbon fraction associated with inherent net-positive energy. The regulatory need for their treatment prior to disposal makes them an ideal commodity to produce energy by the biological treatment. Utilizing wastes as a potential source for energy generation through biological routes has investigated considerable interest due to its sustainable nature and further opening up a new avenue for the utilization of renewable and inexhaustible energy sources (Li and Fang, 2007; Van Ginkel *et al*., 2005; Venkata Mohan, 2009; 2010; Zhang *et al*., 2007).

Waste materials from natural and anthropogenic sources are recently become substrate for renewable bioenergy production by the application of different thermal and biochemical technology (Kothari *et al*., 2012). This idea also felicitates the reducing cost of waste treatment and simultaneously production of energy and some value-added products (Das, 2011). Recently biological treatment of waste has been gaining importance due to its sustainable nature. Biological processes are generally preferred to treat the industrial waste as they are technically feasible, simple, economical, and eco friendly. In another way scientists are also approaching for gradually shifting their focus from “pollution control” to “resource exploitation from waste”.

Municipal and industrial waste (solid and liquid) along with the waste generated from agriculture and food-processing industries contains enough organic loads which can be tapped beneficially if utilized appropriately (Fig. 5.1). Integration of biohydrogen and methane production with an existing effluent treatment plant is the futuristic goal envisage with the uses of wastewater as the primary feedstock (Venkata Mohan *et al*., 2007; 2012).

Thus, this chapter is divided in three subsections on the basis of waste selected for study and emphasized on the use of these potential industries waste viz; sugar industry, dairy industry and tannery industry waste for biohydrogen production with pure bacterial strain and further methane production from the fermented substrate. The selection of these industries for bioenergy production was based on their potential of waste generation, easy availability and previous studies on energy generation and waste treatment.

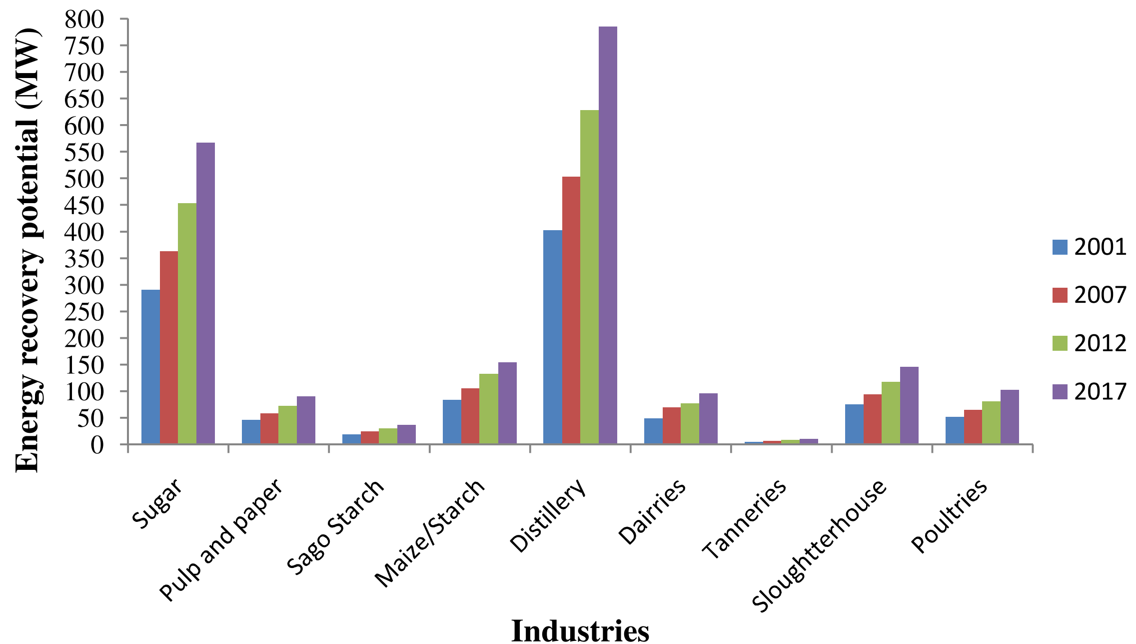


Fig. 5.1. Energy potential of various industrial waste (MNES 2006).

5.2. Materials and Methods

It outlines the essential requirements for microbial culture development (*Enterobacter aerogens*) for experimental assessment and optimization (process parameters-pH and substrate) of industrial waste an integrated approach for treatment and bioenergy production options (hydrogen and methane).

5.2.1. Selection of bacterial strain and preparation of inoculums

Details related to selection and preparation of bacterial culture of *Enterobacter aerogens* (MTCC no.8100) was given in *section 3.2 of Chapter-3*. Same preparations for the culture development were followed for assessing the potential of selected industrial waste (sugar industry waste, dairy industry waste and tannery industry waste). 10% (w/v) of digested slurry of cow dung was also used as inoculums to process the methanogenic activity in second phase. Second phase of batch experiment was also operated at room temperature 35 °C ±2 at HRT of 2 days and 7 days respectively for experimental set up plan (ESP), ESP-I and ESP-II.

5.2.2. Experimental set-up

Experimental setup plan (ESP) is divided according to the objectives (*Chapter-1*) in the three steps ESP-I (A), ESP-II (B) and ESP-III (C)) and optimization of process parameter such as initial pH and substrate concentration to improve the biohydrogen and methane production (shown *Chapter 3, Table 3.2)* with pollutants reduction simultaneously. Physicochemical analysis of all waste materials taken as the substrate from selected industries was also carried out to estimate the parametric load, given in Table 5.1.

5.2.2.1. Two phase bioenergy (biohydrogen and methane) production

The anaerobic digestion process was performed in two phases for biohydrogen and methane production using sugar industry waste (SIW), dairy industry wastewater (DIWW) and tannery industry waste (TIWW). Varying concentrations were selected for observation of substrate utilization efficiency of *Enterobacter aerogens* for biohydrogen production in Phase-I whereas, in the Phase II (methanogenic) of the experiment bioenergy production in form of methane by using fermented/digested substrate from Phase-I of the experiment in ESP-I, II and III. Optimization of process parameter (initial pH and substrate) was also observed to improve the biohydrogen production in ESP-I only due to limitations of instrumental part. Simultaneously, pollutant removal from DIWW was also observed in the ESP II of the study. Other details related to experimental plan (ESP-I, II and III) were discussed in *Chapter-3 (section 3.5.1)*.

5.2.3. Analytical methods

Analytical and instrumental methodology adopted for Phase-I and Phase-II of the study in ESP-I, II and III were given with detail description at *Chapter-3 (section 3.5 and 3.6)* and Appendix-1.

Table 5.1. Physicochemical analysis of different selected industrial waste.

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Parameters	Sugar Industry (Diluted molasses 50 g/l)	Dairy industry wastewater	Tannery industry wastewater
pH	8.5	6.2	5.7
TS	2452	2200	10800
TDS	1585	2010	10200
TSS	866	190	600
Chloride	36.7	385	2550
Nitrate	50	66.4	53
Nitrite	31	0.94	-
Ammonium	22	24	250
Phosphate	16	21	-
Sulphate	89	137	2600
COD	15452	11200	4800

Except pH, all parameters are in mg/l

5.3. Experimental set up plan-I (A): Assessment of sugar industry waste using Enterobacter aerogens for bioenergy (hydrogen and methane) production options with optimized parameters

5.3. Experimental set up plan-I (A): Assessment of sugar industry waste using *Enterobacter aerogens* for bioenergy (hydrogen and methane) production options with optimized parameters

5.3.1. A. Overview

Sugar industry activities generate large quantities of organic solid waste and by-products (*e.g*. leaves from cane or beet, molasses from the final crystallization, press mud or cachaza, bagasse fibre from the cane, mud and soil arriving at the plant with the raw material, and lime solids from the juice clarification). The amount of waste generated depends on the quality of the raw materials themselves and on the initial cleaning in the field. The generation of higher quality waste can provide opportunities for reprocessing of discarded raw materials into commercially viable by-products (*e.g.* paper making and particle board manufacturing). Other solid wastes from the sugar manufacturing process include spent filter material (e.g. active carbon, resins from the ion exchange process, acids from chemical cleaning of equipment, vinasse or spent wash from the distillation of fermented molasses-sugar juice, and ashes from the steam boiler plant (shown in Fig.3.1of *Chapter 3*). Generally, the solid waste generated in sugar factory can be broadly categorized as Bagasse, Press mud and Molasses.

Molasses is produced in the last operational steps of separating sugar from the mother liquor in centrifuges. It has been found that average production of molasses is 4.2% of the cane crushed. But there is quite a large fluctuation in this. Molasses has very high pollution characteristics. Even though molasses is a commodity under excise control; it is often observed that molasses gets spoiled due to improper storing facilities provided by the factory. Even with the excise regulations and the consent conditions given by the Pollution Control Boards (PCBs), many industries still follow the practice of strong molasses in unlined pits locally termed as kutcha pits. During rainy season and also owing to groundwater table conditions, molasses gets diluted and becomes unsuitable for fermentation. This diluted molasses has a BOD concentration varying between 50000 and 80000 mg/l, which are to be disposed off to factories conveniently, let it out to the natural water bodies thereby causing heavy pollution. However, such molasses serves as the raw material for the distillery industry and is transported to the distilleries unit at frequent intervals.

Many studies were done on the energy production from sugar industry waste particularly on sugar industry molasses. Sugarcane molasses is a feasible feedstock for hydrogen production due rich in sugar contents 40-50% and lack of toxic elements. However, it is a main feedstock for distillery industry and around 80-90% of the molasses was used in alcohol production. This practice is very feasible for the sugar industries which have distilleries integrated with it but for others, to handle molasses is a big challenge. The high content of sugar in molasses makes it attractive to many pathogens as well as diseases during improper handling and storing and it's dumping in open on land or dispose in water bodies is not good practice and increase pollution load in water channels and on leaching from soil. Besides this the industries have to also bear the cost of transportation if it transported to the site of discharge or to the distillery. On analysis the physicochemical characteristics of sugarcane molasses (as per the literature, Table 5.3.1.A), it is found a feasible feedstock for biohydrogen production (Ozgur *et al*., 2010; Lay *et al*., 2010).

5.3.2. A. Experimental Plan

` The sugar industry waste (SIW) organic residue, sugarcane molasses was taken as substrate in different concentration (10, 20, 40 and 50 g/l) for biohydrogen and methane production in two phases of the study. The composition and preparation for experiments carried out with different variables were given in the previous section of *Chapter-3* and the brief description of the experimental plan is given in Table 5.3.2.A.

5.3.3.A. Results and Discussions

The biohydrogen production from industrial waste (organic residue) is a new phenomenon in terms of the waste treatment and energy production approach simultaneously. The reason behind this approach is very clear that a very limited number of studies are available on the sugar cane molasses which provides a significant process for biohydrogen production and hence, selected as substrate feed for biomass growth of bacteria, *Enterobacter aerogens*. Although wastes from number of industries i.e. food processing industries such as distillery waste and dairy waste are kept under study by few researchers for fermentative hydrogen production (Zhao and Ruan, 2014; Mohan *et al*., 2007; Kalia, 1994; Chen *et al*.)

Table 5.3.1. A. Pollution characteristics of sugarcane molasses (Ozgur et al., 2010; Lay *et al.,* 2010).

Parameters	Molasses Characteristics	Permissible limits to discharge in water bodies
pH	3.5-4.5	5.5-9.0
Colour	Dark Brown	Colourless
Total Dissolve Solid (TDS)	20,0000 to 320000	2100
Chloride	32000	600
Sulphate	15000	1000
DO	Nil	5
BOD	440000	30
COD	960000	25

Except pH and colour, all parameters are in mg/l.

Table 5.3.2.A. Plan of experiments carried out with different variables (brief description)

S.N.	Aim of experiment	Testing variables	Values of variables	Experimental conditions
1.	Phase I (Biohydrogen production)	Concentration of substrate (g/l)	10, 20, 40, 50	HRT-30 hours Temp. -30 °C
2.	Phase II (Methane Production)	Fermented residue from Phase-I	40 g/l	HRT-2 days Temp. -30 °C
3.	Initial pH optimization	Initial pH	4.5, 5.5, 6.5, 7.5	HRT-7 days Temp. -30 °C
4.	Economic profit and energy recovery	Biohydrogen production and Methane production	For H_2- $/Ton of molasses For CH_4- $/ m^3-FSIW	Theoretical assessment

But the sugar-rich industrial wastes, available from the waste materials have the potential/feasibility to act as raw material for biohydrogen production. Therefore, sugar cane molasses which contain 40-60% sugar are found to be feasible and promising for the production of biohydrogen, using dark fermentation process with variation in process parameters (Kiviharju *et al.*, 2007; Rena *et al.*, 2006). Thus, this study emphasized on the production of biohydrogen with variation in substrate concentration for the maximum retention time of 24 hours. The growth of bacterial strain in different substrate concentrations and their effect on the production of biohydrogen (rate and yield) were calculated for each set of experimental conditions using different kinetic models and the discussion of results is given in further sections.

5.3.3.1. A. Effects of substrate concentrations on bacterial growth

The relationship between biomass growth and time with respect to different substrate concentration is shown in Fig. 5.3.1.A. In this study, bacterial biomass attributed maximum 3.5 g/l at 40 g/l substrate concentration and retarded over it as at 50 g/l. It has also been found a profound effect of substrate concentration on specific growth rate of bacteria. It has been also seen that in lower substrate concentrations, there is limited bacterial growth with lower substrate concentration that prevailed deficient carbon source while at higher concentration it become inhibitory for microbial growth.

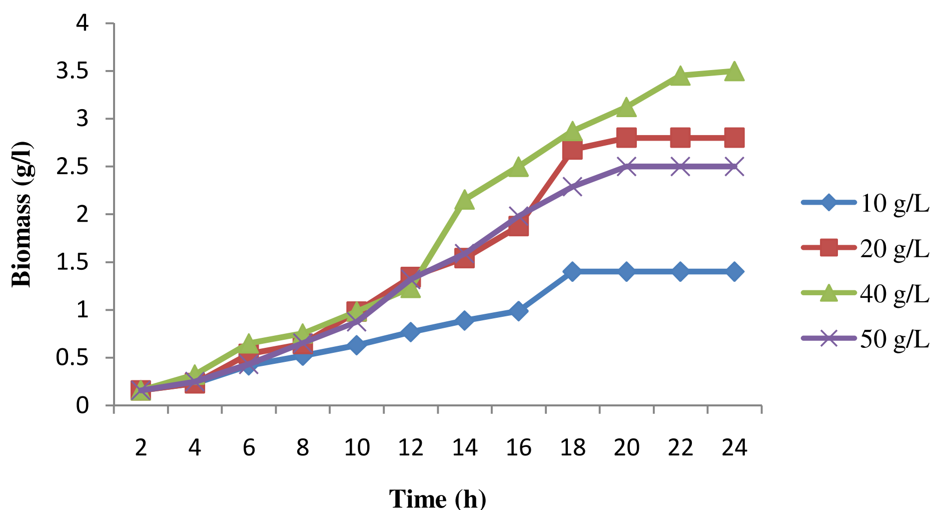

Fig. 5.3.1.A. Effect of various substrate concentrations on bacterial growth.

5.3.3.2. A. Effect of various substrate concentrations on biohydrogen production

The effect of varying substrate concentration (10, 20, 40 and 50 g/l) was analyzed for biohydrogen production with initial pH (6.8). A small change in substrate utilization rate was observed in batch set up with increasing substrate concentration (10, 20, 40 and 50 g/l), similar trend was also reported by Yu *et al.*, (2006), however, the substrate utilization rate decreased at substrate concentration of 50 g/l with decrease in pH at final stage of 24th hour.

As shown in Table 5.3.3.A, biohydrogen production increases with an increase in substrate concentration but the substrate utilization rate in terms of sugar consumption showed decreasing trend with the increase in substrate concentration. This may be due to the decrease in lag time of bacterial cell growth which can be attributed to less availability of the carbon source. However at higher substrate concentration, bacterial cell growth supported to satisfy it, whereas, at higher concentration beyond 40 g/l i.e. 50 g/l, it could be attributed to the phenomena of inconsequential growth. This showed that the pure culture used in this study has a greater potential to degrade industrial waste in comparison to mixed cultures with pure synthetic sugars. It was analysed that the higher concentration of sugar become inhibitory for the bacterial cell as well as for production also (Tanisho and Ishiwata, 1994). At the concentration of 40 g/l, maximum biohydrogen production achieved that provide an optimum concentration for the bacterial cell biomass growth and biohydrogen production shown in Fig. 5.3.2.A

Table 5.3.3.A. Bioydrogen production with various substrate concentrations.

Substrate Concentration (g/l)	pH		Sugar consumption (%)	Hydrogen yield (mM/g sugar)	Gas Production (ml)	Total VFA (mg/l)
	Initial	Final				
10	6.8	4.5	73	7.9	800	620
20	6.8	4.6	68	6.29	1050	1100
40	6.8	4.8	64	6.02	1520	2110
50	6.8	4.2	45	4.5	900	3400

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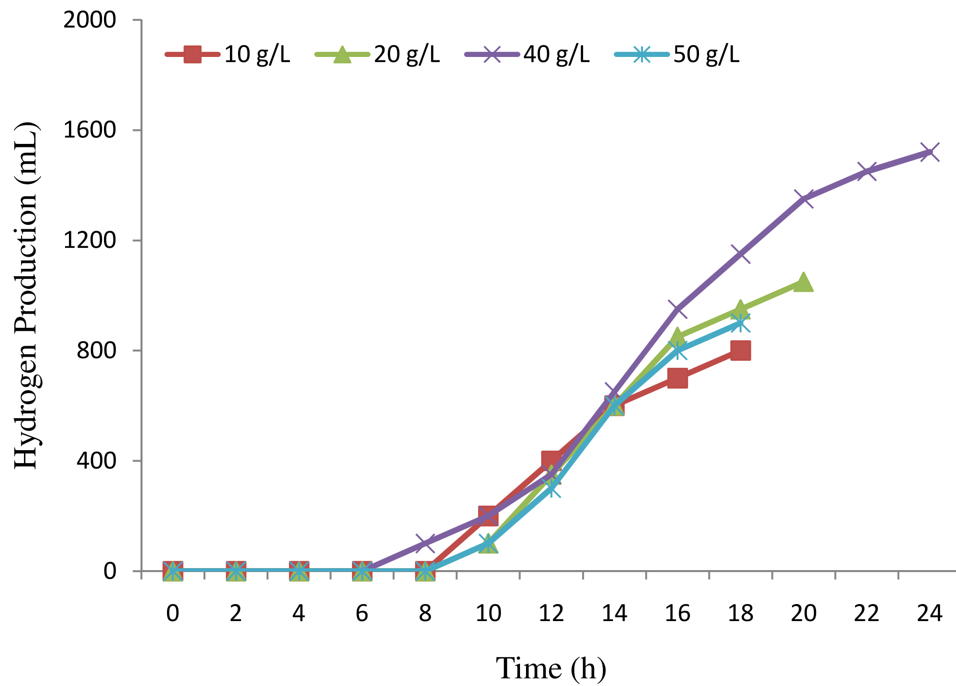


Fig. 5.3.2.A. Effect of various substrate concentrations on biohydrogen production

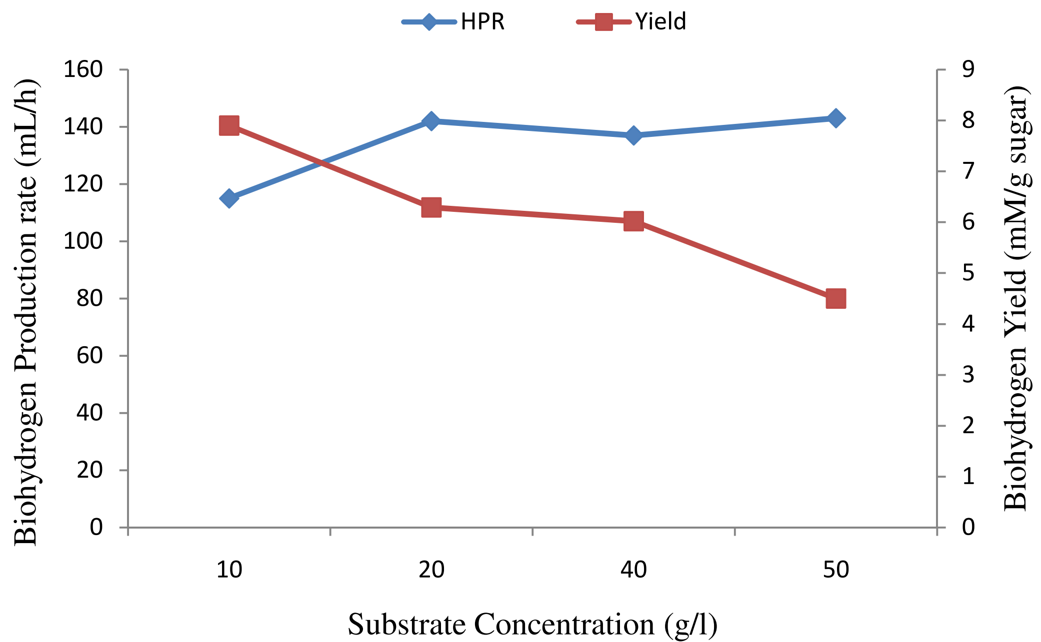
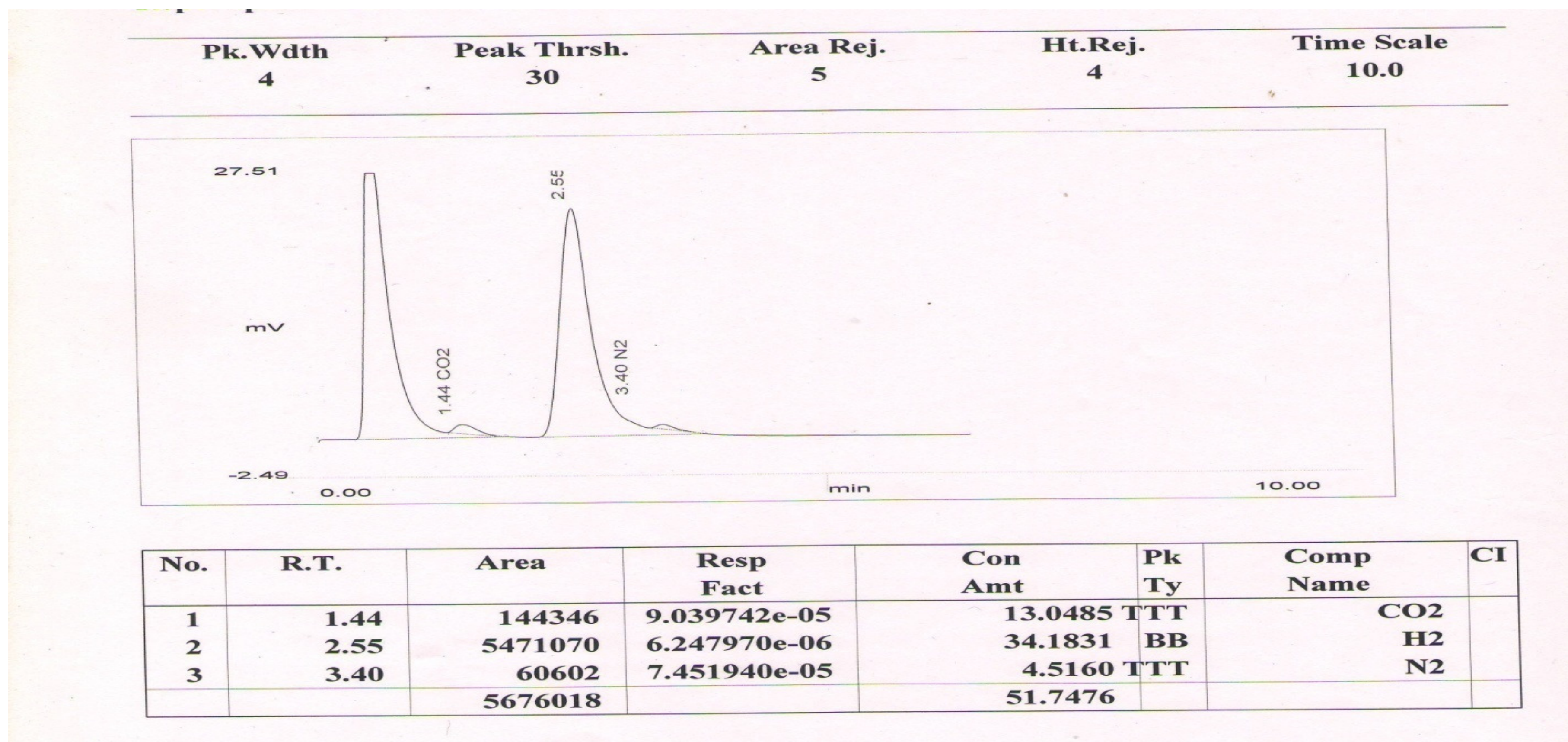


Fig. 5.3.3.A. Effect of various substrate concentrations on biohydrogen yield and production rate.



Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	10.0

No.	R.T.	Area	Resp Fact	Con Amt	Pk Ty	Comp Name	CI
1	1.44	144346	9.039742e-05	13.0485	TTT	CO2	
2	2.55	5471070	6.247970e-06	34.1831	BB	H2	
3	3.40	60602	7.451940e-05	4.5160	TTT	N2	
		5676018		51.7476			

Fig.5.3.4.A. Composition of biohydrogen produced from fermented SIW.

Fig.5.3.4.A, showed the effect of substrate concentration on biohydrogen yield and production rate. The result showed a decreasing trend in biohydrogen yield (7.5-4.5 mM/g sugar) in terms of sugar consumed. This followed the trend where other pure strain of *Enterobacter aerogens* showed biohydrogen yield, 3.3 mol/mol of glucose and 1.12 mol/mol of glycerol in biodiesel waste (Chen *et al*., 2005; Yan and Lin, 2009). This suggests that biohydrogen production is strongly affected by the substrate concentration via the bacterial metabolism and microbial community structure (Wu and Lin *et al*., 2004). This may be due to the increasing substrate concentration could increase the hydrogen yield during fermentative hydrogen production process.

5.3.3.3. A. Effect of initial pH on biohydrogen production process

Among the different substrate concentration of sugarcane molasses, the best optimized concentration for biohydrogen production was 40 g/l. This substrate concentration was further optimized for different initial pH 4.5 to 7.5, focusing on the improvement of biohydrogen production (yield and rate) generated from organic residue from sugar industry with facultative anaerobic bacteria, *Enterobacter aerogens,* with an approach for potential to generate more energy-rich process.

5.3.3.3.1.A. Effect of initial pH on bacterial growth and biohydrogen production

The biohydrogen gas production was also targeted at different initial pH 4.5, 5.5, 6.5 and 7.5 values. Results showed that the process pH 5.5 is an optimum pH to improve the hydrogen production with *Enterobacter aerogens* but not biomass production as given and discussed in previous section of this study. On quantitative part, best findings was observed with initial process pH 5.5 with maximum gas production (1800 ml) and sugar consumption (76%) with biomass total VFA concentration (1350 mg/l) as given in Table 5.3.4.A The reason behind this pattern of biomass and biohydrogen production was that the acidic pH favors the fermentation while neutral pH favors the growth of bacteria.

The anaerobic facultative bacteria follows the acetate pathway (Tanisho *et al.,*1998) and main metabolites of process are acetate, formate and ethanol (solvantogenesis process). The concentration of these liquid metabolites (Acetate, Formate and Ethanol) at different initial pH values were also analysed with their potential effects on biohydrogen production, yields and biomass production (Table

5.3.4.A). The highest concentration of ethanol is followed by acetate and formate to be observed with all initial pH values. The concentration of formate was very low so it could not be detected in chromatograph. The results may be supported and discussed on the part of unfavorable pH, which directly affects the higher concentration of these solvents in the system resulted low yields of H_2, as higher concentration of these solvents affects the activity of hydrogenase enzyme responsible for hydrogen production. It was also observed that in unfavorable initial pH the concentration of H^+ ions affects the ionization state of the functional group of the amino acids involved in the enzyme affect the biohydrogen production rate. There is also possibility of existence of some other metabolites as lactate because the total VFA concentration was more than some of the all acids detected. The obtained result also conclude that hydrogen production is higher in acidic conditions but too low initial pH (pH 4.5) do not support the process and selected bacteria *Enterobacter aerogens* could not maintain its metabolic activity at such low process pH and biohydrogen production process get very slow. The rate of H_2 production at low process pH may be due to the generation of more protons of undissociated weak acids in the system which can pass through cell membrane into the cytoplasm and inhibit the growth of microorganism and biohydrogen production as well. It was also reported that low pH affects the [FeFe] hydrogenase enzymes activity.

The rate of H_2 production at low process pH may be due to the generation of more protons of undissociated weak acids in the system which can pass through cell membrane into the cytoplasm and inhibit the growth of microorganism and biohydrogen production as well. It was also reported that low pH affects the [FeFe] hydrogenase enzymes activity. The same results were also reported by Zhang *et al.*, (2007), Khanal *et al.,* (2004) and Mohd Yasin *et al.*, (2011) who also observed total inhibition of biohydrogen production at extreme high or low initial pH with inhibition of hydrogenase activity. It has been also observed that the optimization of initial pH gave better results with pH 5.5 but growth of bacterial biomass was observed maximum with pH 6.5. Thus, it can be concluded that low and high pH variations directly affects the bacterial growth but improvement in biohydrogen yields only can attained through optimized process parameters.

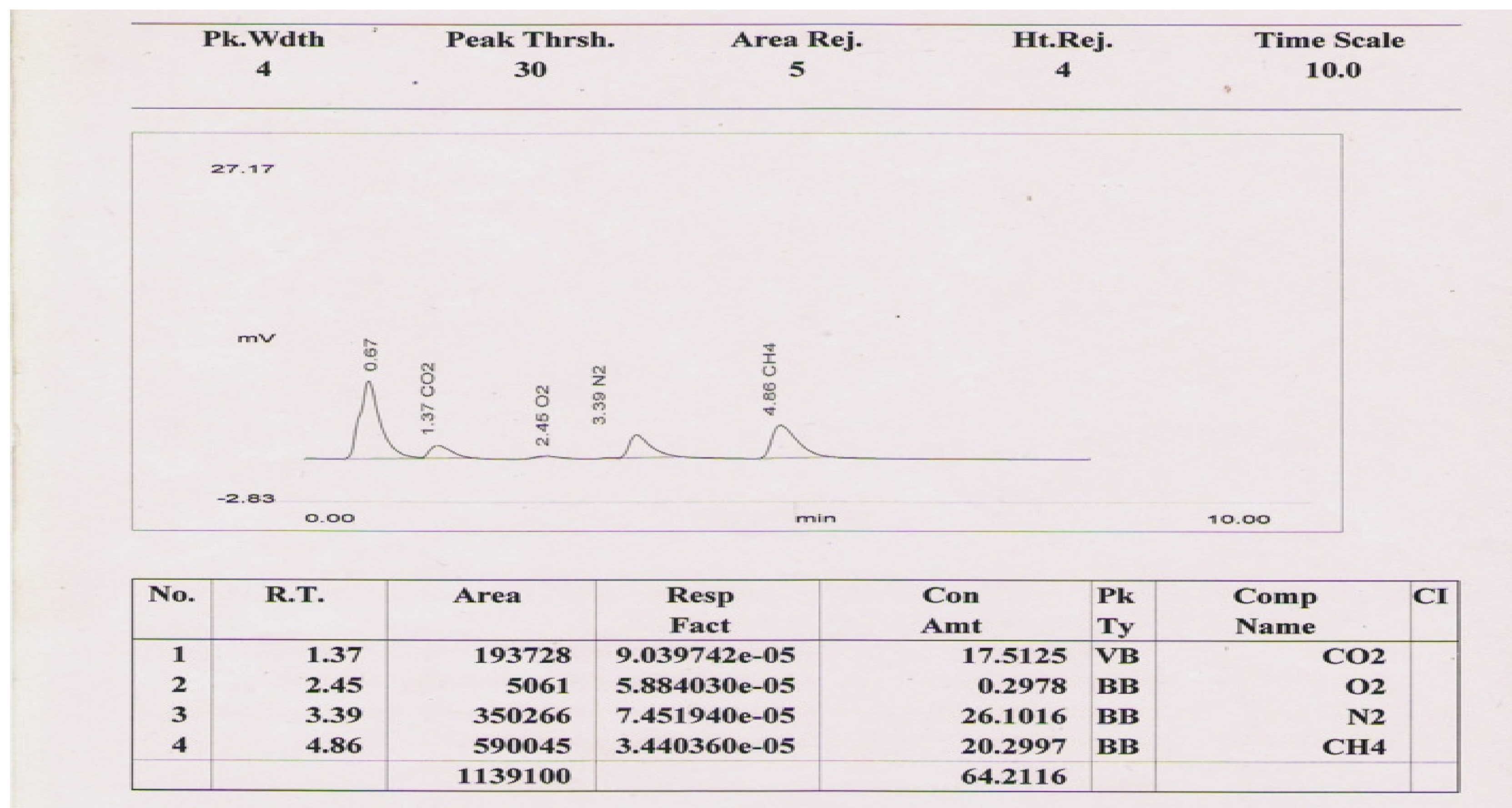
5.3.3.4. A. Effect of optimized substrate concentration of fermented SIW on methane production

The 40 g/l, molasses was best suited for hydrogen production and was further fermented for methane production. For methane production, 10% (w/v) cowdung was inoculated in fermented molasses with neutralized pH. The advantage of this phase was to use remaining organic matter for methane production which remains as soluble fermentation products in the form of solvents such as acetate, propionic acid, butyric acid and ethanol due to low substrate conversion efficiency in hydrogen production process (Karlsson *et al.,* 2008; Koutrouli *et al.,* 2009). After first Phase of the experiment from 40 g/l molasses, which is equal to 24 g/l sugar only 18.4 sugar was consumed and 7.6 gm sugar was left for methane production. Total gas production from fermented molasses was 700 ml and methane production is 224 ml, within 2 days and the gas production gets seized. So, the methane yield was 224 ml CH_4/ 7.2 g sugar is equal to 31 ml CH_4/ g sugar reduced.

The composition of produced gas was analysed with chromatograph shown in Fig.5.3.5.A.

5.3.3.5. A. Techno-economic analysis of bioenergy production profit and energy recovery capacity with selected substrate-strain system

The techno-economic benefits for bioenergy (hydrogen and methane) production was evaluated in terms of waste assessment potential as a substrate for energy recovery and cost effectiveness to the other technologies in reference of biohydrogen only. Energy recovery from sugar industry waste was measured in terms amount can be generated from per unit of substrate utilization and energy value of the produced energy MJ/day (Park *et al.,* 2010 and Das, 2009).



Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	10.0

No.	R.T.	Area	Resp Fact	Con Amt	Pk Ty	Comp Name	CI
1	1.37	193728	9.039742e-05	17.5125	VB	CO2	
2	2.45	5061	5.884030e-05	0.2978	BB	O2	
3	3.39	350266	7.451940e-05	26.1016	BB	N2	
4	4.86	590045	3.440360e-05	20.2997	BB	CH4	
		1139100		64.2116			

Fig.5.3.5.A. Composition of methane produced from fermented SIW.

Table 5.3.4.A. Biohydrogen and metabolite production at selected initial pH.

pH	Biohydrogen production (ml)	Sugar consumption (%)	Biomass production (gm)	H_2 yield (ml/g sugar)	Total VFA (mg/l)	Acetate (mg/l)	Ethanol (mg/l)
4.5	800	29	1.21	47.3	140	20	32
5.5	1800	76	1.64	105.8	1128	322	406
6.5	1200	52	2.26	70.5	1350	214	574
7.5	900	38	1.40	52.9	1778	355	445

5.3.3.5.1.A. Assessment for biohydrogen production

Maximum yield of the hydrogen at optimized substrate concentration

=**6.02 mM/g sugar = 134.8 ml/g-sugar reduced**

Sugar content in the substrate (40 g/L of molasses) = **18.4 g**

Reduction in sugar = 64% (64% of 18.4 g sugar i.e. 11.2 g sugar) =**24.8 g molasses reduced**

Hydrogen yield = **134.8 ml/g-sugar reduced × 24.8 g/40-g molasses = 3.34 L H_2/40 g molasses = 83.5 L H_2/kg- molasses= 116.9 L H_2/ L molasses**

Economic profit of bioenergy produced = **0.835 m^3×0.39$/$m^3$= 0.032 $/kg-molasses**

= 32 $/Ton of molasses

Energy recovery (MJ/L-molasses) = Energy Density of Hydrogen × Yield of Hydrogen/ L molasses (Park *et al*., 2014) = **0.0108×116.9= 1.26 MJ/L-molasses**

Annual production of molasses in India **= 10881 MT = 10.88 ×10^6 Kg = 15.23×10^6 L/Annum** (ISMA, 2014).

Annual bioenergy profit on hydrogen produced (using all the molasses for hydrogen energy production) = **0.1169×15.23×10^6 ×0.39$/$m^3$= 7.2×$10^5$ $/Annum**

Energy recovery per annum = **1.26× 15.23 ×10^6 = 19.8 ×10^6 MJ = 18.7 ×10^6 MBTU/per annum**

Taking plant operating expenditure: **$1545 per day (Das, 2009)**

Cost of Energy generation from H_2= **$1545/8523MBTU= $0.175/MBTU**

Cost of natural gas **= $5.69/MBTU (EIA, 2015).**

Cost of gasoline = **$17.81/MBTU (EIA, 2015).**

The data obtained from the comparison of cost of biohydrogen production to the others fuel based process is very low ($ 0.175/MBTU) that make our study more significant.

Keeping the same trend of hydrogen production in present experimental condition, a two- stage anaerobic digestion process would be more cost effective to scale up the process. Hence, the present results provide a baseline for improvement of biohydrogen yield using pure strain under optimized concentration of substrate. The maximum yield of biohydrogen production on per day basis was found higher in the present study than the other reported study shown in Table-5.3.5.A. The similar result is also reported by Liang (2009), who achieved maximum hydrogen production rate of 3.63 L/L/Day using 40 g/L condensed molasses but with mixed culture consortium.

5.3.3.5.2. A. Assessment for methane production

Methane production from Fermented sugar industry waste (FSIW) = **220 ml**

Sugar left in the medium after H_2 production = **Sugar present in the 40 g/l molasses (18.4) - sugar consumed for biohydrogen production (11.2) = 7.2 g**

Methane yield of from fermented SIW= **31ml/g sugar reduced**

Total sugar in fermented SIW = **7.2 g/ 40 g molasses = 180 g/kg molasses**

Reduction in COD= **35.6%= 1.56 g COD reduced/L-Fermented SIW (FSIW)**

Methane yield = **31 ml/g×180 g/ kg FSIW= 5580 ml/ kg-FSIW = 5.58 m^3 CH_4/ Ton FSIW**

Economic Profit of Methane produced= **0.133×0.56$/$m^3$= 0.076$/ L-FSIW = 76$/ m^3-FSIW**

Energy recovery (MJ/L FSIW) = Energy Density of methane × Yield of methane / L-FSIW

= 0.0378MJ/L × 133.32 L/L-FSIW = 5.04 MJ/L-FSIW

Table 5.3.5.A. Comparative assessment for maximum biohydrogen production rate with various coupled substrate-strain systems

Substrate	Reactor Type	Species	Culture conditions			Maximum biohydrogen production rate (l/l/day)	Reference
			Temperature (°C)	pH	HRT (hour)		
Glucose	CSTR	Mixed Culture*	35	5.5	9.6	2.14	Michael *et al*. (2007)
Sucrose	CSTR	Mixed Culture*	35	5.2-5.3	12	11.57	Kyazze *et al.* (20007)
Molasses (28 g/l)	PBR	Mixed culture (*Clostridium butyricum Clostridium leptum Clostridium drakei*)	35	5.5	6	2.80	Liang *et al.* (2009)
Molasses (40 g/l)	CSTR	Mixed Culture*	35	5.5	8	3.63	Park *et al.* (2014)
Molasses (40 g/l)	**CSTR**	**Pure culture *Enterobacter aerogens***	**30**	**N.C.**	**24**	**3.41**	**This study**

N. C. = Not controlled, *species not identified

5.3.3.6. A. Conclusions

The interactive effects of substrate concentrations (organic industrial residue) and pure bacterial strain for bioenergy production was investigated with cost effective assessment of this experimental plan with waste treatment and energy production approach simultaneously. From the results obtained at this study, it can conclude that utilization of organic residual substrate with pure strain can help to achieve a biohydrogen and methane yield of 6.02 mM/g sugar and 31 ml CH_4/ g sugar reduced respectively with 40 g/l of substrate concentration with the highest gas production.It has been also observed that the optimization of initial pH gave better results with pH 5.5 but growth of bacterial biomass was observed maximum with pH 6.5. The maximum yield of bioenergy (biohydrogen and methane) production on per day basis was found higher in the present study (ESP-I, Phase-I and II) than the other reported study. The obtained values of economic profit and energy recovery for bioenergy yield are higher than the values obtained in double stage anaerobic digestion process as reported by Park *et al.,* (2009).

5.4. Experimental set up plan-II (B): Assessment of dairy industry wastewater using Enterobacter aerogens for bio-energy (hydrogen and methane) production and treatment options simultaneously

5.4. Experimental set up plan-II (B): Assessment of dairy industry wastewater using *Enterobacter aerogens* for bioenergy (hydrogen and methane) production and treatment options simultaneously

5.4.1. B. Overview

The Indian food processing industry is one of the largest industries in the world in terms of production, consumption, export and growth prospects. Increase industrialization with literacy and affluence has given a considerable push to the food processing industry growth. Food processing industries can be divided in to four major sectors: i.e. meat, poultry and sea food, fruit and vegetable, dairy and beverage. To run these industries main resources needed are include water, raw materials and energy. Among them water has been main consumed natural resources and industry has been a large user. Water is used as an ingredient, an initial and intermediate cleaning source, an efficient transportation conveyor of raw materials, and the principal agent used in sanitizing plant machinery and areas. Although water use will always be a concern part of the food-processing industries, it has become the principal target for pollution prevention, source reduction practices. Wastewater generated from these industries depicts wide variation in strength and characteristics. This variation is due to the amount of water usage, type of vegetable and process used type of product and different additives like salt, sugar, gelatin, colurs, oil and preservatives added also leads to the pollution load in the wastewater but this wastewater is non toxic in nature because it comprises less hazardous compounds. Almost 50% of the water utilized in food processing industry is for washing and rinsing purposes. Water being the primary ingredient is widely used as a cleaning agent in food processing industry. This wastewater has been reported to be treated by aerobic and anaerobic biological techniques.

The dairy industry in India on an average has been reported to generate 6-10 litres of waste water per litre of the milk processed (Kushwaha *et al*., 2011). The dairy waste is basically organic and slightly alkaline in nature, when discharged in to streams without treatment, result in rapid depletion of dissolved oxygen (DO) and encourage the growth of algae i.e. eutrophication.

Due to the overuse of surfactants in dairy, the waste can become unsuitable to the biological treatment. Characteristics of industrial wastewater varies from industry to industry and within industries also there are variations in the quality depending

Table 5.4.1.B. Plan of experiments carried out with different variables (brief description)

S.No.	Aim of experiment	Testing variables	Values of variables	Experimental conditions
1.	Phase-I (Biohydrogen production)	Concentration of substrate (%)	25%, 50%, 75%, 100%	HRT-30 hours Temp. -30 °C
2.	Phase –II (Methane production)	Fermented residue from Phase-I	75%	HRT-7 days Temp. -30 °C
3.	Study of reduction in pollution load of DIWW	pH, Chloride, Nitrate, Nitrite, Ammonium, Phosphate, Sulphate, COD	25%,50%,75%, 100%	HRT-30 hours Temp. -30 °C
4.	Economic profit and energy recovery	Biohydrogen production and Methane production	For H_2- $/m^3-DIWW For CH_4- $/ m^3-FDIWW	Theoretical assessment

upon the processes, for example quality of wastewater coming out from a cooling tower will be quite different then the wastewater coming out from any chemical process on the other hand there are limited variations in the quality of sewage depending upon season, sewerage system, lifestyle of people etc.

The aim of this research work is to evaluate the utilization of dairy industry wastewater as a substrate for bioenergy options (hydrogen and methane) to retain biomass and reduction in pollution load simultaneously at lab scale to treat dairy industry wastewater. The research was done with two phases i.e. hydrogen generation in first phase with treatment options simultaneously and methane generation in second phase under anaerobic conditions. Pollution reduction rates and

treatment rates are determined on the basis of COD. Economical assessment of the bioprocess routes is also calculated for this experimental study.

5.4.2. B. Experimental plan

The experiments were carried out taking dairy industry wastewater (DIWW) as the substrate for bioenergy production (biohydrogen and methane) by using pure strain of bacteria *Enterobacter aerogens*. The detailed of the experimental plan is given in the *Chapter-3* and brief description of the experiments is given in Table 5.4.1.B.

5.4.3. B. Results and Discussions

The dairy industry wastewater (DIWW) was taken for the analysis of biohydrogen and methane production in two phases of the study at different concentration (25, 50, 75 and 100%). In the Phase-I, the biohydrogen production with simultaneously pollution load reduction was analysed with bacteria *Enterobacter aerogens.* Among them the best feasible concentration for biohydrogen production was further analysed for methane production with cow dung as the inoculums in Phase-II of the study. The detail of the each finding is given below**-**

5.4.3.1. B. Effects of substrate concentrations on bacterial growth

The growth of selected bacterial strain was analysed in four different concentrations of dairy industry wastewater and it was found, the bacterial biomass was well grown in 25, 50 and 75% concentrations of substrate while it decreased at 100% concentration. The study revealed that initial concentration of nutrient favours the growth of bacteria and 75% is the optimum substrate concentration where maximum biomass 2.5 g/l was achieved whereas at 100% substrate concentration, growth of bacteria get inhibited with the retention time of 30h. The lag phase of the bacteria was observed 6-7 hours as till this period no growth in bacterial community observed at regulated environment of reactor.

5.4.3.2. B. Effect of substrate concentration on biohydrogen production

Hourly variation of accumulated hydrogen production, at various dairy industry waste water (DIWW) concentrations is shown in Fig 5.4.2.B. Hydrogen production did not increase instantly with substrate concentration of wastewater. Substrate concentrations of 25% and 50% provided low hydrogen production, whereas, 75% results showed a maximum (580 ml) hydrogen production. Higher substrate concentration lowers the yield of hydrogen production; hence the present

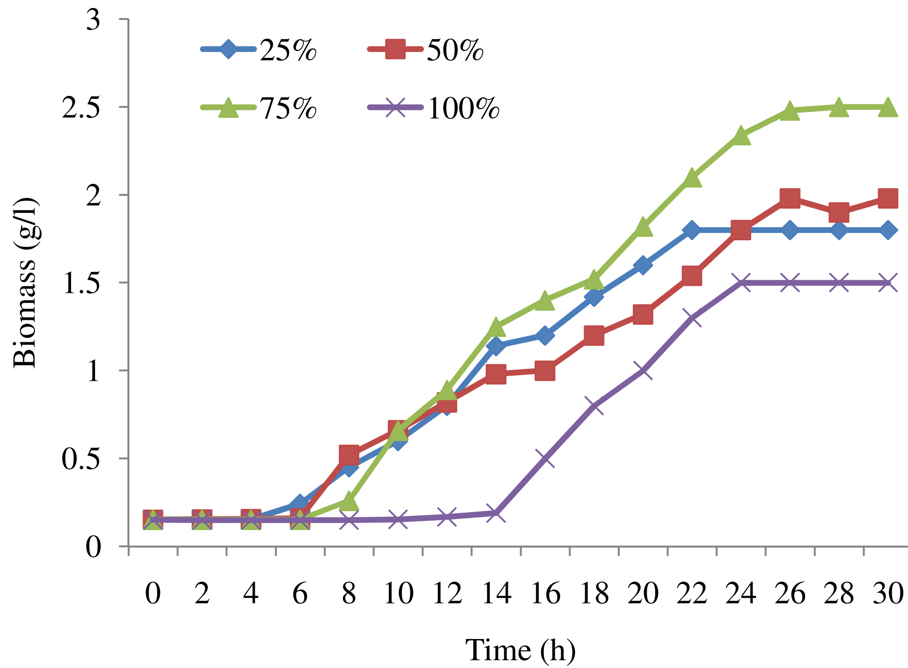


Fig.5.4.1.B. Effect of various substrate concentrations on bacterial growth.

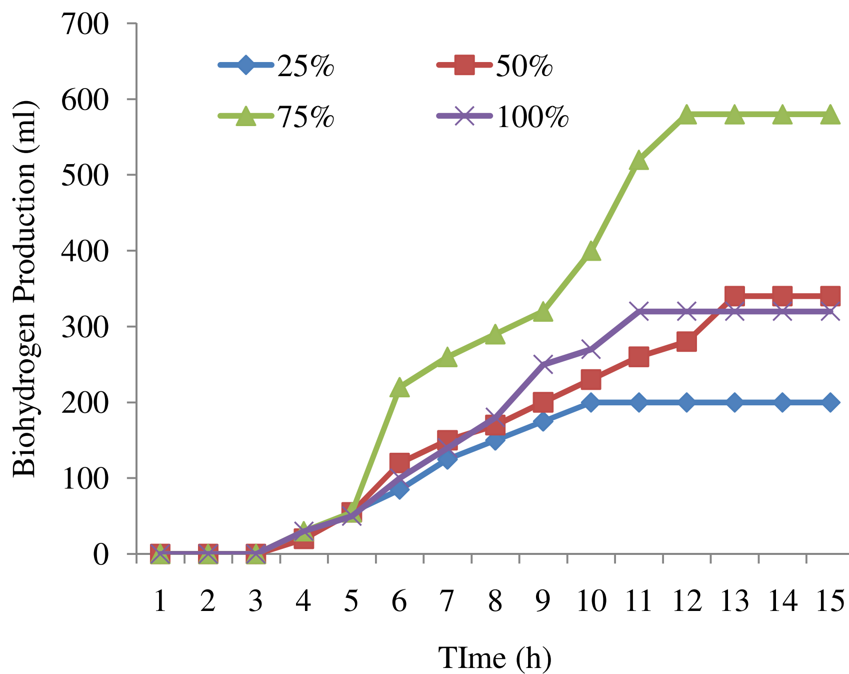


Fig.5.4.2.B. Effect of various substrate concentrations on biohydrogen production.

study and other studies suggested optmized substrate concentration for higher hydrogen yield (Van Ginkel *et al*., 2005). Hydrogen production could be improved by adding optimized amount of surfactants, however extra amount of dosage could diminish the cell wall of bacterial biomass and distract the process performance of reactor (Guo *et al*., 2007). It was also reported that saline concentration also inhibit substrate removal efficiency which alters the metabolic pathways to lactic and propionic acid generation and causes decrease hydrogen production rate (Kim *et al*., 2009).

COD removal rate and hydrogen production was observed simultaneously at initial and final hour to measure the performance of reactor in terms of biohydrogen yield. The initial pH was maintained at 6.5 for all the concentration of feed while the pH at detention time showed a slight variation to 4.5 to 4.0 with 25-100% concentration, respectively.

Results showed that there is very low hydrogen yield at final pH 4.0 with 100% substrate concentration (45 ml/g COD), indicating that hydrogen production from DIWW was inhibited at low pH (Table 5.4.2.B). The operational pH mainly depends on the substrate and the composition of microbial population in a hydrogen producing bioreactor (Karaday *et al*., 2014). The variation in COD reduction (%) in terms of substrate consumption follows decreasing pattern with increasing concentration according to experimental results obtained at 25% (low) to 100% (high) concentration (Fig.5.4.3.B). The reason for this pattern of substrate utilization was effective consumption of food at lower concentration; however the substrate consumption efficiency goes down at higher concentration.

It was also observed that the maximum substrate degradation efficiency was for 25% concentration of substrate followed by 69% for 50% and 64% for 75% substrate concentration (Table 5.4.2.B). The region behind this pattern of substrate was due to at low substrate concentration the bacteria utilized the food very efficiently while increasing the substrate concentration the efficiency of utilization get decreased. The biohydrogen yield of the system depends on the availability substrate and its consumption and gas production.

Table 5.4.2.B. Biohydrogen production at various substrate concentrations.

Substrate concentration (%)	pH		Substrate consumption (COD in %)	Hydrogen Gas Production (ml)	Biohydrogen Yield (ml/g COD)	SDR (g/l/d)
	Initial	Final				
25	6.5	4.5	90	200	79	0.63
50	6.5	4.5	69	340	86	1.95
75	6.5	4.2	64	580	105	4.05
100	6.5	4.0	62	320	45	7.0

As for 75% concentration maximum optimum COD was available 11200 mg/l and 7000 mg get consumed so the enhanced biogas production 580 ml was achieved during the experiment with yield 105 ml/g COD reduced Fig.5.4.3.B.

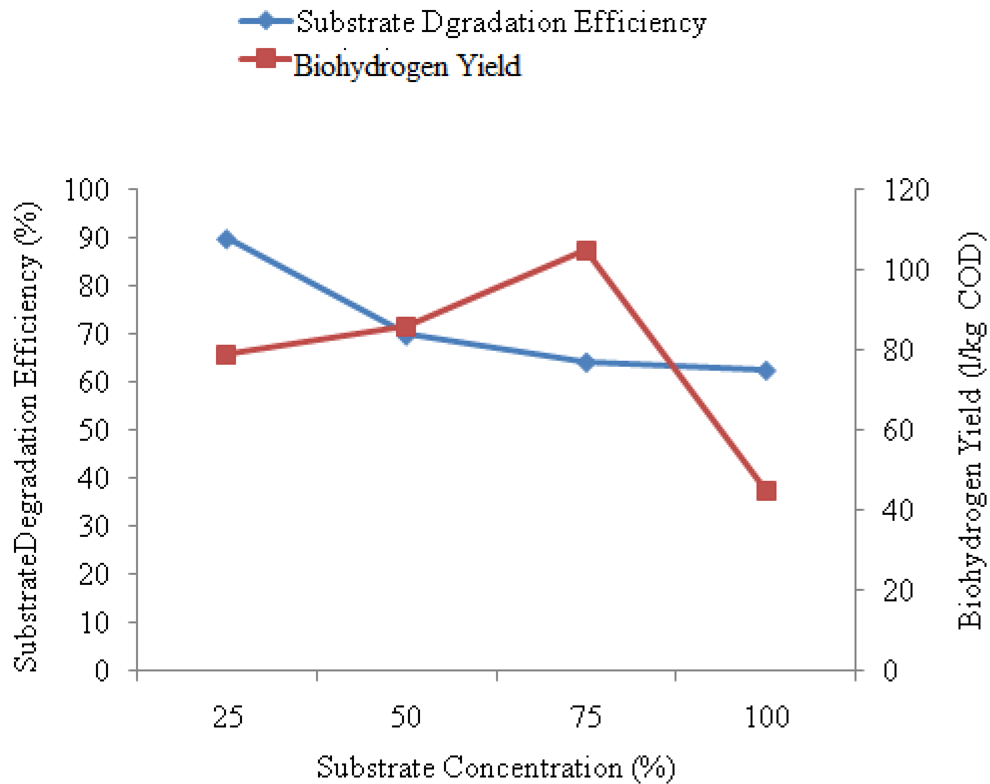


Fig.5.4.3.B. Substrate degrdation efficiency and biohydrogen yield.

5.4.3.3.B. Effect of optimized concentration of fermented DIWW on methane prodution

A batch reactor used for the bioprocess of second phase of study was exactly the same as used in the first phase. The fermented organic waste obtained at 75% of DIWW was utilized for a second phase process to produce methane. The substrate was inoculated with cow dung to introduce methanogenic bacteria for methane production. The HRT of the reactor was set for 7 days. The initial pH was set to 7.0 and no pH control system was provided in second phase of study. The methane production was observed without any strategy used for enrichment of methanogenic bacteria as used in first phase. The biogas production started from first day of inoculation and seized on eighth day consequently (Fig.5.4.4.B) with a production of 984 ml. The possibility of growth of hydrogenic bacteria cannot be neglected as it can flow from fermented DIWW in second phase of study.

Initially, hydrogen gas is also evolved in biogas production, however the methane concentration after the completion of second phase of study was found about 60% (Fig.) which is equivalent to 590.4 ml. The another advantage of second phase is to reduce COD of wastewater remained after first phase of study. Initially, the COD of fermented waste was about 4400 mg/l with an assumption of zero COD of completely digested cowdung. According to the stoichiometric conversion, the methane production is directly related to organic degradation i.e. 395 ml methane equals 1 g COD removal (Speece, 1996). This relation can be used for measurement of efficiency of second phase of study. Considering this relation, about 1494.8 mg of COD is removed in second phase which is equivalent to 27.1%.

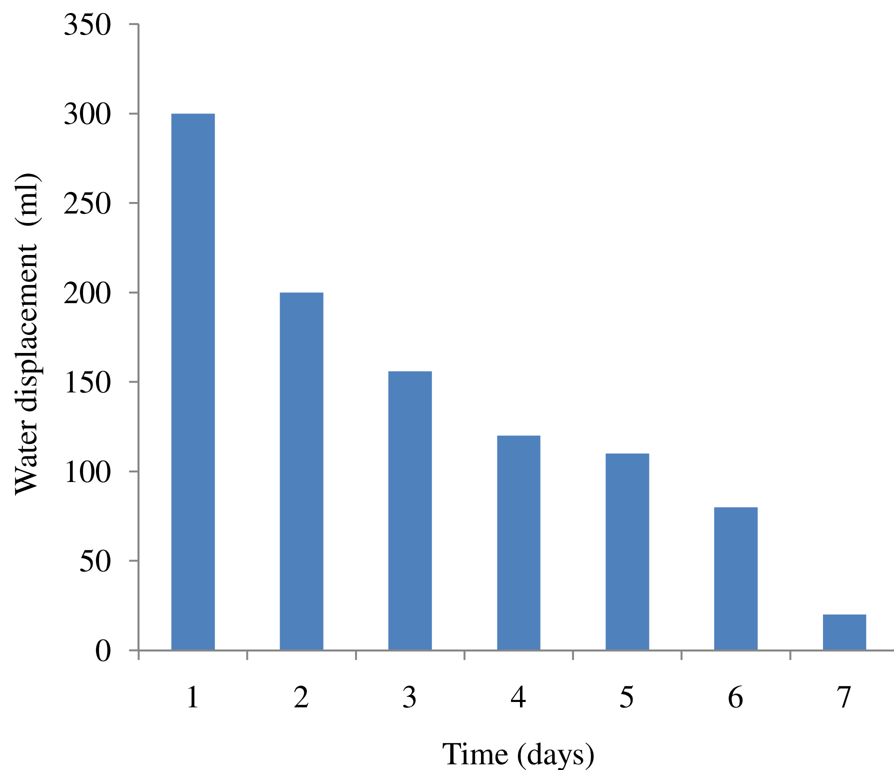
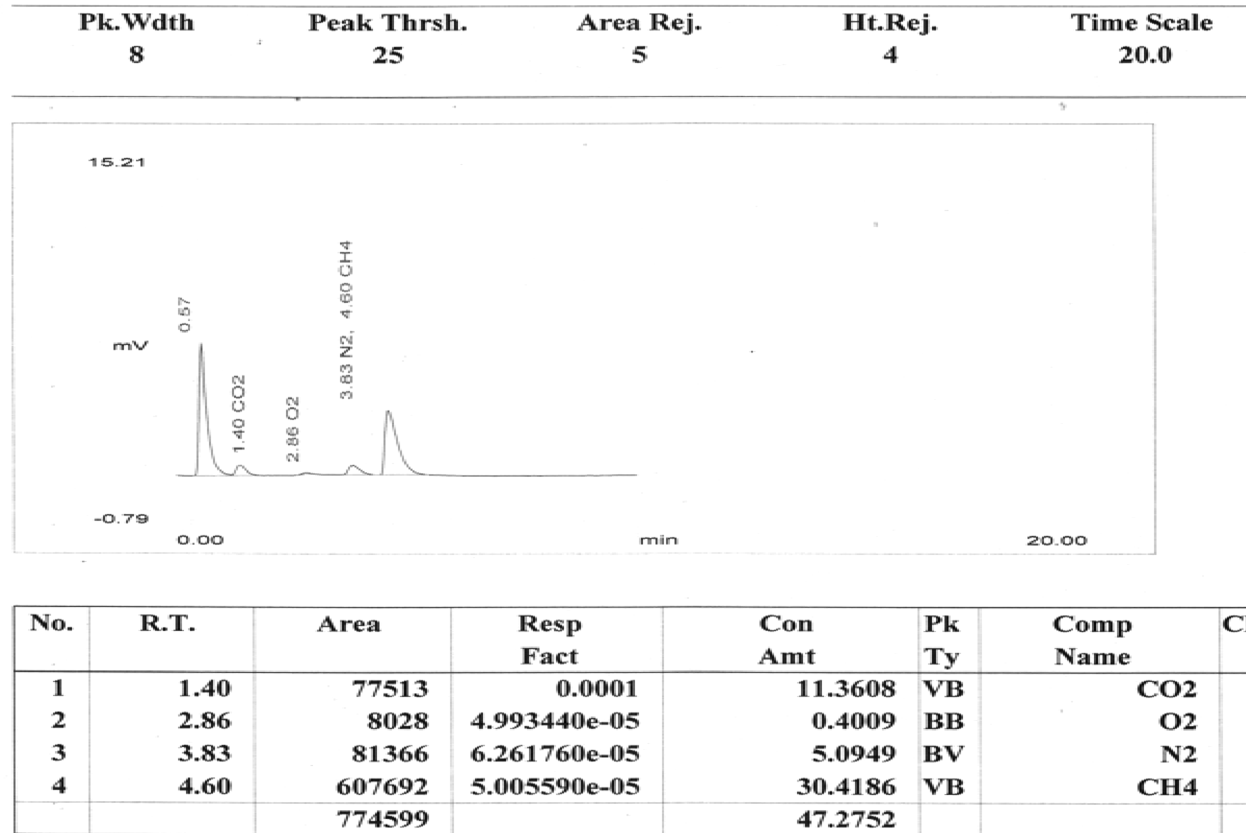


Fig.5.4.4.B. Biogas production from fermented dairy industry wastewater.



Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
8	25	5	4	20.0

No.	R.T.	Area	Resp Fact	Con Amt	Pk Ty	Comp Name	CI
1	1.40	77513	0.0001	11.3608	VB	CO2	
2	2.86	8028	4.993440e-05	0.4009	BB	O2	
3	3.83	81366	6.261760e-05	5.0949	BV	N2	
4	4.60	607692	5.005590e-05	30.4186	VB	CH4	
		774599		47.2752			

Fig.5.4.5.B. Composition of methane produced from FDIWW.

5.4.3.2.4. B. Techno-economic analysis of bioenergy production profit and energy recovery capacity with selected substrate-strain system

The techno-economic analysis for energy recovery and cost effectiveness has been carried out for bioenergy (hydrogen and methane) production from dairy industry wastewater. Energy recovery from dairy industry wastewater was measured in terms amount can be generated from per unit of substrate utilization and energy value of the produced energy MJ/day. The assumption is based on experimental data obtained in the present study and methodology followed by Park *et al*., (2010) and Das, (2009).

5.4.3.2.4.1.B. Assessment for biohydrogen production

COD concentration of substrate (at 75%) = **8400 mg/l= 8.4 g/l**

Reduction in COD = 64%=

(64% of 8.4 g COD) = 5.37 g COD reduced/L- DIWW)

Maximum yield of the hydrogen at optimized substrate concentration

= 105 ml/g COD reduced

Hydrogen yield = 105 ml × 5.36 g COD = 562 ml H_2/L- DIWW

= 0.562 L- H_2/ L DIWW

Economic profit of hydrogen produced ($-L DIWW) =

0.562 m^3×0.39$/$m^3$=0.22$/m^3 DIWW

Energy recovery (MJ/L DIWW) = Energy Density of Hydrogen × Yield of Hydrogen/ L DIWW **= 0.01079 MJ/L × 0.562 L-H_2/L DIWW**

= 0.006 MJ/L DIWW

5.4.3.2.4.2. B. Assessment for methane production

Methane production from fermented dairy industry wastewater (FDIWW) **= 590 ml**

COD of fermented DIWW after biohydrogen production = **3.1 g/L**

Methane yield of from FDIWW= **190 ml/g-COD reduced**

Methane yield = 190 ml H_2× 3.1 g COD = **590 ml /L-FDIWW**

= 0.59 L CH_4 / L-FDIWW

Economic Profit of Methane produced= **0.59 m^3×0.57$/$m^3$= 0.336$/ m^3-FDIWW**

Energy recovery (MJ/L DIWW) = Energy Density of methane × Yield of methane / L DIWW **= 0.0378MJ/L × 0.573 L/L-FDIWW= 0.216 MJ/L FDIW**

5.4.3.3. B. Pollution reduction in DIWW

Dairy industry wastewater provides suitable substrate for growth of microflora which alters wastewater composition by degradation of organic substances. In present study, pre and post characterization of DIWW has shown the efficiency of microbial strain for reduction in pollutants concentration at different selected concentration of wastewater. The parameters of pollution load are given in Table 5.4.4.B, rapid decrease in pH (from 6.5 to 4.5) was observed throughout the whole study with all concentrations (25, 50, 75 and 100 %). The decrease in pH, negatively affects the biomass growth and gas production, hence pH control system for constant pH is recommended. Significant reduction was achieved in chloride, nitrate, nitrite, ammonaical nitrogen, phosphate, sulphate and COD at all selected concentrations of waste water (25 %, 50%, 75% and 100%).

An equal variation in pH was observed at every concentration of DIWW. However, reduction in other parameters varies with concentration of wastewater. The highest reduction (78%) in chloride was achieved at 100% wastewater concentration while reduction in nutrient load such as nitrate, nitrite, phosphate shows different trends. Reduction in nitrate ranges from 69% to 76% and highest reduction was achieved at 25% concentration of DIWW. Nitrite is absent in post characterization of all concentration of DIWW, which shows its complete removal or conversion in other forms of nitrogen. Reduction in ammonical nitrogen ranges from 12 to 32% with highest reduction at 50% DIWW concentration. The present experimental results showed higher efficiency of microbial strain for phosphate reduction which ranges from 59% to 86%. Sulphate, at 25% the concentration DIWW was reduced completely while at 50% , 75% and 100% it is reduced by 55% , 62% 43%, respectively. In our study maximum gas production was achieved at 75% concentration of wastewater with 64% COD reduction of industrial waste water. Our results showed that 75% DIWW concentration is optimum for bacterial biomass growth.

Table 5.4.3.B. Pollutants reduction from dairy industry wastewater with simultaneously bioenergy options.

Parameters	Concentrations											
	25%			50%			75%			100%		
	Initial	Final	R*	Initial	Final	R*	Initial	Final	R*	Initial	Final	R*
pH	5.5	4.2	23	5.5	4.2	23	5.5	4.2	23	5.5	4.2	23
Chloride	96.25	50.1	80	192.5	73.21	61	228.75	93.5	49	385	83.65	38
Nitrate	16.6	3.91	76	33.25	8.12	75	49.85	15.23	69	66.4	16.12	52
Nitrite	0.25	-	-	0.5	-	-	0.7	-	-	0.94	-	-
Ammoniacal -N	6.0	4.67	38	12	-	32	18	12.96	28	24	21.11	12
Phosphate	5.25	2.12	59	10.5	2.64	74	16	2.73	82	21	2.8	86
Sulphate	34.25	-	-	68.5	30.26	55	102.7	38.3	42	137	77.32	30
COD	2800	280	90	5600	1689	69	8400	3000	64	11200	4200	62.5

***Except pH, all values are in mg/l; R*= Reduction (%).**

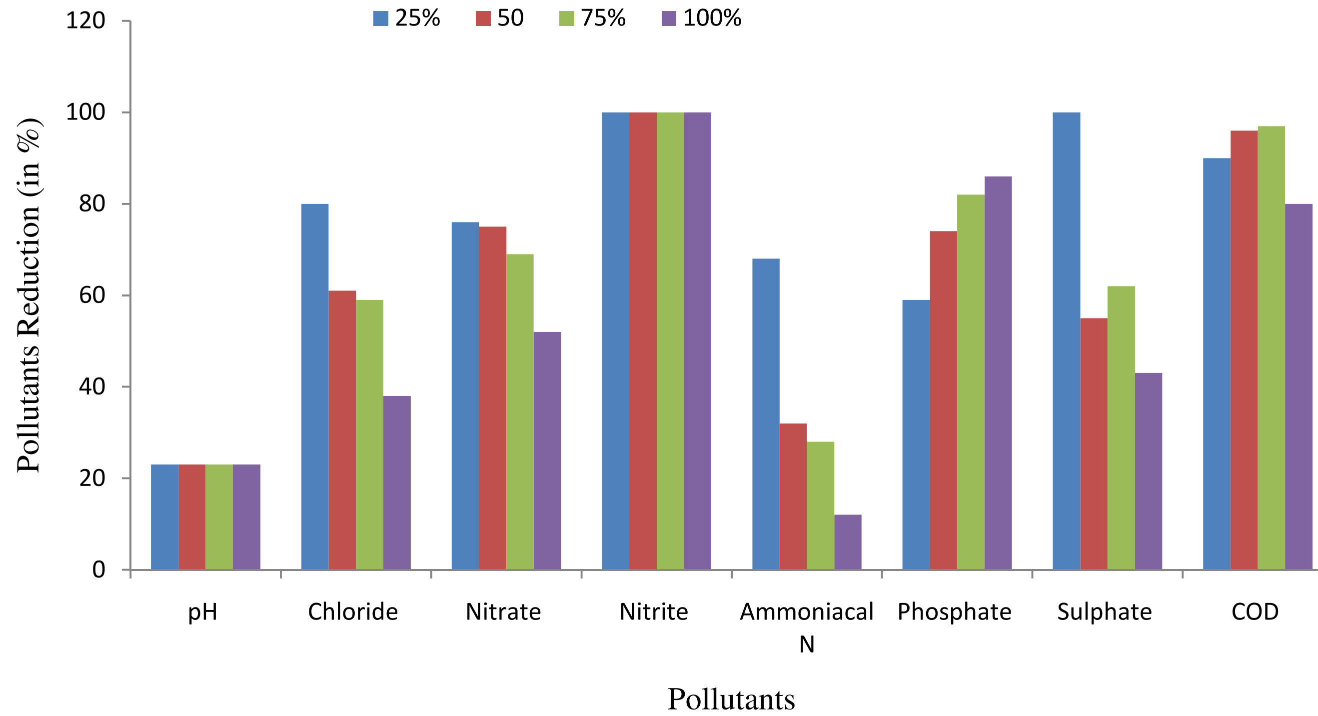


Fig.5.5.6.B. Pollutants reduction in DIWW.

5.4.4. B. Conclusions

In this experimental study of ESP-II, optimized substrate concentration was explored with microorganism *E. aerogens* for production of hydrogen in phase I and methane in phase II. The study concludes that efficient success of the hypothesis i.e. bioenergy (hydrogen and methane) production and pollutants reduction from industrial wastewater. The best suitable optimum concentration, 75% DIWW was found feasible for bioenergy production with biomass growth as well as simultaneously pollutants removal results for efficient treatment system such as higher efficiency achieved for phosphate (59% to 86%), sulphate (62% to100%), nitrate (52% to 76%) at 25%, 50%, 75% and 100%. Similarly, maximum gas production was also achieved at 75% concentration of wastewater with 64% reduction in COD from initial to final HRT for experimental plan.

The maximum yield of bioenergy (biohydrogen and methane) production was found 0.562 L- H_2/ L DIWW, 0.59 L-CH_4./L-FDIWW in the present study (ESP-II, Phase-I and II). The obtained values of economic profit and energy recovery for bioenergy yield are higher than the values obtained in double stage anaerobic digestion process as reported by Park *et al.,* (2009).

Energy recovery from dairy industry wastewater was measured in terms amount can be generated from per unit of substrate utilization and energy value of the produced bioenergy (MJ/day) provides a sustainable approach for utilization of industrial wastewater with zero/low cost of system.

5.5. Experimental set up plan-III (C): Assessment of tannery industry wastewater using Enterobacter aerogens for bioenergy (hydrogen and methane) production options

5.5. Experimental set up plan-III (C): Assessment of tannery industry wastewater using *Enterobacter aerogens* for bioenergy (hydrogen and methane) production options

5.5.1. C. Overview

The conventional leather tanning technology is highly polluting as it produces large amounts of organic and chemical pollutants. Wastes generated by the leather processing industries pose a major challenge to the environment. According to conservative estimates, more than 600,000 tons per year of solid waste are generated worldwide by leather industry and approximately 40–50% of the hides are lost to shavings and trimmings.

An average of 30–35 m^3 of wastewater is produced per ton of raw hide. However, wastewater production varies in wide range (10–100 m^3 per ton hide) depending on the raw material, the finishing product and the production processes (Tunay *et al*., 1995). Organic pollutants are originated from skins (it is calculated that the raw skin has 30% loss of organic material during the working cycle) or they are introduced during processes.

Tannery waste consists of wastewater, and solid waste flashings and waste skin trimmings, the former two being composed mostly of lipids and proteins. Gaseous efficiency from fats is estimated to be higher than those of carbohydrates and proteins, therefore lipid rich waste can be regarded as a large potential renewable energy source (Cirne *et al*., 2007). For example, 1250 liter (68% CH_4, 31% CO_2, 1% other) of biogas was estimated to be produced from 1 kg (dry solids) of fat, while 790 liter and 704 liter of biogas was produced from the same amount of carbohydrates and proteins, respectively (Urbaniak, 2006).

Anaerobic digestion (or biomethanation) systems are mature and proven processes that have the potential to convert tannery wastes into energy efficiently, and achieve the goals of pollution prevention/reduction, elimination of uncontrolled methane emissions and odour, recovery of bio-energy potential as biogas, production of stabilized residue for use as low grade fertilizer. Anaerobic digestion of tannery wastes is an attractive method to recover energy from tannery wastes.

5.5.2. C. Experimental plan

The tannery industry wastewater (TIWW) was taken as the substrate at different concentration (25, 50, 75 and 100%) to analyse its potential for bioenergy (hydrogen and methane) production after physicochemical analysis (Table 5.1).The experiment plan was followed same as with the previous substrate. The detail of the experiments is given in the Table 5.5.1.C.

Table 5.5.1.C. Plan of experiments carried out with different variables (brief description)

S. No.	Aim of experiment	Testing variables	Values of variables	Experimental condition
1	Phase I	Concentration of substrate (in %)	25%, 50%, 75%, 100%.	HRT-30 hours Temp. -30 °C
2	Phase II	Not proceed	Nil	Nil

Table 5.5.2.C. Variation in bacterial biomass growth with selected TIWW concentrations.

Wastewater concentrations (in %)	Tannery Industry wastewater	
	Initial Biomass (g/l)	**Final Biomass (g/l)**
25	0.152	0.534
50	0.152	0.231
75	0.152	0.254
100	0.152	0.156

This method degrades a substantial part of the organic matter contained in the sludge and tannery solid wastes, generating valuable biogas, contributing to alleviate the environmental problem, giving time to set-up more sustainable treatment and disposal routes. Digested solid waste is biologically stabilized and can be reused in agriculture. Until now, biogas generation from tannery wastewater was considered that the complexity of the waste water stream originating from tanneries in

combination with the presence of chroming would result in the poisoning of the process in a high loaded anaerobic reactor**.**

5.5.3. C. Results and discussions

The experiment was planned for the biomass growth of bacteria at different concentration viz; 25, 50, 75 and 100% of tannery industry wastewater as shown in Table 5.5.2.C. The results, showed that there was no significant growth of biomass occurred in all four concentrations of tannery industry wastewater except only in 25 % concentration, with the biomass growth 0.534 g/l in 30 hours study. As available in various literatures, tannery industry wastewater has more pollutants concentration and maximum toxicity is due to the presence of chromium (Tare *et al*., 2012; Samanta *et al*., 2010) which inhibit the growth of bacteria. On the basis of these findings from tannery industry wastewater, experimental process could not proceed for further studies of bioenergy production options and parametric optimization.

5.5.4. C. Conclusions

On the basis of these findings, the biohydrogen production form tannery industry waste water could not proceed may be the presence of high value pollutant concentrations and maximum toxicity is due to the presence of chromium ions which inhibit the growth of bacteria. But pretreatment of substrate and maintenance of anaerobic conditions can provide a better options for bioenergy options with tannery industry wastewater.

5.6. Comparison with other two stage production processes in respect of SIW and DIWW

Separation of acidogenesis and methanogenesis process was initially proposed by Pohland and Ghosh (1971), later this concept was investigated by various researchers. The sequential hydrogen and methane production was observed in two stages serial continuous stirred tank reactor (CSTR) system.. However, in ESP-I (A) and ESP-II (B) experiments are followed the batch operation for anaerobic digestion process. Most of the researchers have suggested operating pH 5.5 for acidogenic reactor and 7.0 for methanogenic reactor. While in present experiments the initial pH for biohydrogen production was 6.5 for ESP-I (A) and ESP-II (B) in phase-I and for methane production it was 7.0 for ESP-I (A) and ESP-II (B) in phase-II maximum hydrogen and methane yield obtained in ESP-I (A) and ESP-II (B) were 116 H_2 l/l SIW, 0.562 l H_2/l DIWW and 5.58l CH_4 /l FSIW , 0.59 l CH_4/ FDIWW.

Hence, the results seem to be more significant than yield obtained with olive pulp at 55 °C with findings of Koutrouli *et al.,* (2009). Substrates like sucrose, glucose, and molasses have been reported for high hydrogen and methane production process in double stage anaerobic digestion process in CSTR. So, the present experimental results have shown the potential of DIWW for bioenergy production in respect of SIW. Table 5.2 is highlighting the use of molasses as for the hydrogen production with production yield, economic profit and energy recovery in comparable to two stage anaerobic digestion. The obtained value of economic profit for hydrogen yield is higher than the value obtained in double stage anaerobic digestion process investigated by Park *et al.,* (2014) shown in Table 5.2. Whereas Table 5.3.clearly indicates that DIWW has more potential for hydrogen production in comparison to molasses using pure strain of hydrogenic bacteria. Presence of fat substances inhibit the methanogenic activity, hence methane production is lower from DIWW. The result is highly applicable in context of India, where shortage of molasses has been recognized due to 5% ethanol blending target in Petroleum products (National Biofuel Policy, 2009).

Table 5.2. Bioenergy profit and energy recovery from hydrogen produced using molasses.

Process	Production Rate (L/L-molasses/day)			Economic profit of bioenergy produced ($/L-molasses/day)			Energy Recovery (MJ/L-molasses)			References
	EtOH	**H_2**	**CH_4**	**EtOH**	**H_2**	**CH_4**	**EtOH**	**H_2**	**CH_4**	
Alcoholic fermentation	0.375	--	---	0.196	---	---	9.0	---	---	Park *et al*. (2014))
Two stage anaerobic digestion	---	27	342	---	0.011	0.195	---	0.29	0.206	Das, (2014)
Single stage anaerobic digestion	**---**	**116.9**	**---**	**---**	**0.033**	**---**	**---**	**1.26**	**----**	**This study**

Table 5.3. Comparative analysis of economic of bioenergy production with SIW and DIWW.

Substrate	Production Rate (L/L-substrate/day)		Economic profit of bio-energy produced ($L/substrate/day)			References
	Hydrogen (H_2)	**Methane (CH_4)**	**Hydrogen (H_2)**	**Methane (CH_4)**	**Total**	
Molasses	27	342	0.011	0.195	0.206	Park *et al*. (2010)
SIW (Molasses)	**116.9**	**5.58**	**0.032**	**0.076**	**0.108**	**Present study**
DIWW	**57.85**	**0.59**	**0.22**	**0.33**	**0.55**	**Present study**

5.7. Conclusions

The major conclusions of this extensive experimental study can be summarized as follows:

- Hence, it may be finally concluded that to achieve the best hydrogen yield an optimum substrate concentration should be obtained, which would be depending upon the substrate utilization capacity of the bacterial strain.
- It can be concluded that low and high pH variations directly affects the bacterial growth but improvement in biohydrogen yields only can attained through optimized process parameters.
- Although pretreatment of substrate and maintenance of anaerobic conditions increases the cost of biohydrogen production but it seems to be cost effective and energy efficient at large scale production. Thus, two phase digestion process provide enhanced COD reduction with hydrogen and methane as a product output for all the selected industrial waste materials (solid and liquid).
- Techno-economic assessment of energy yield (biohydrogen and methane) and economic bioenergy profit with both selected waste materials provides a base for Reduce, Reuse and Recycle concept at commercial ground. Here, sugar industry waste provides a better opportunity in comparison to dairy industry waste as a substrate for bioenergy options with pure bacterial strain.

Chapter -6

Kinetic analysis to assess the effect of bacterial growth and substrate utilization on bioenergy (hydrogen and methane) production options

6.1 Overview

Kinetic models are used to analyse the pattern of a biochemical reaction occurs for the formation of the products. The experimental work was validated with mathematical models for the meaningful and quantitative estimations of the results. Kinetic models validate the results significantly and make it more authentic. There are several types of kinetic models available for different purpose but they are varying from segregated and non-segregated tostructured andunstructured.

The microbial process is very complex that made very tough to select particular types of kinetic models to validate the results. The selection of a particular kinetics for the microbial study depends on the difference between cells in the reactor, metabolic reaction and formation of products (Neeleman, 2002).

6.1.1 Classification of kinetics models

6.1.1.1. Segregated

A segregated type of kinetic modelstake care of each cell separately, and distinguish each cell is different from one another. These models depict different morphologyof cells, age of cells and explain the interactions between different cells of a population.

6.1.1.2. Non-segregated

Non-segregated models are applied on the culture of cells or lump of cell population.These models depict interaction of the cells with the external environment. External environment influences cell response and can give new growth characteristics on the cell. Non-segregated models are mathematically simpler than the segregated models(Avhandling, 1999; Ochoa *et al*., 1999). Non segregated models are further divided into structured and unstructured models.

Structured and unstructured models are being used for kinetic study in microbial processes. Structured models describe somebasic physiological characterization of cells,like cell structure, their function and composition while unstructured models consider total cellularconcentration.

6.1.1.3. Structured Models

Structured models consider the internal states of the cells. It incorporates genetic, morphological, or biochemical attributes that collectively determine the physiological state of the biomass. These models are used to analyse the growth and biomass of the bacteria.(Kayombo*et al*., 2003; Liao*et al*., 1988). Structured models

helps in analyses concentration of metabolites, enzymes, DNA and/or RNA and lag or transient phase of the bacterial biomass (Di *et al.*, 2001;Jeppsson, 1996; Lei *et al.*, 2001; Panikov 1995).

6.1.1.4. Unstructured Models

In unstructured model, the kinetic reaction is described on the basis of the reaction rate of the process but it is independent of cell concentration and residence time of the biomass, because the whole cell mass is taken as uniform unit. These models describe the kinetics of the cell growth on the basis of cell and nutrient profile. These are the simplest models describing the relationship between exponential growths, nutrient profile and product formation (Di Serio*et al.*, 2001;Coons *et al*., 1995; Majewsky*et al.*, 1990).

6.2.Kinetic models used for study

Various growth models were used to observe the pattern of microbial growth and substrate consumption in batch culture which includes, Logistic models,Monod model, Moser model, Hanes-woolf modeland Eadie-Hofstee model. The Monod model is one of the best-known kineticmodel describing microbial growth, which shows a functional relationship between the specific growth rate and an essential substrate concentration. It is based on the Michaelis-Menten equation used for enzyme kinetics.

6.2.1. Logistic Model

Usually bacterial cultures grow by binary fission in presence of unlimited food

1 > 2 > 4

Cell increase can be rated by differential equation; $\frac{dx}{dt} = \mu X$ (6.1)

On solving the above equation: $X = X_0 e^{\mu t}$ (6.2)

Where, X, is the number of cells or concentration of cell in solution; µ. is the specific growth rate of bacteria; X_o, is the number of bacterial cells at initial phase i.e.; t = 0

6.2.2. Monod Model

The Monod model was supported by the Michaelis–Menten equation, derived from the mechanism of enzyme reaction and it was developed from a curve fitting exercise, which is an example of an empirical correlation. The Michaelis–Menten kinetics for enzymatic reactions gives mechanistic meanings to the constants involved, but none of those meanings can be applied readily to a substrate-cell system as described by the Monod equation, even though, the Monod relationship can provide the most generally satisfactory curve fitting of the growth data. However, the Monod equation and Hill or Moser equation are purely empirical and theoretical derivation of these models has not been readily available in the literature.

Monod equation is used in this study worked as a fundamental basis of the kinetic study. This is expressed by following equation:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{6.3}$$

Where, μ is the specific growth rate; K_s is the half-saturation coefficient and $\mu_{\max}$, is the maximum specific growth rate of the bacteria in the batch system. Monod model based equation explore from the MichaelisMenten equation is not very satisfactory due to two major reasons; firstly the graph obtained from the data is not following the proper mathematical model. Second, the graph is following the tangent model by this, it becomes difficult to calculate an exact $\mu_{max}$and K_s. Keeping in mind above, back draw of Michaelis -Menten model,Lineweaver and Burk (1934) made some modification to improve it as follows:

$$\mu = \frac{\mu_{\max}[S]}{K_s + [S]} \tag{6.4}$$

Equation-4 can be inverted,

$$\frac{1}{\mu} = \frac{(K_s + [S]}{\mu_{\max}[S]} \tag{6.5}$$

$$\frac{1}{\mu}=\frac{K_s}{\mu_{\max}[S]}+\frac{[S]}{\mu_{\max}[S]} \tag{6.6}$$

Equation 6 can be rearranged as

$$\frac{1}{\mu}=\frac{K_s}{\mu_{\max}}\frac{1}{[S]}+\frac{1}{\mu_{\max}} \tag{6.7}$$

This form of equation resembles the straight line equation, y-mx+c. Here, 1/μ is the y-axis co-ordinate and 1/[S] is the x-axis co-ordinate. K_m/μ_{max} is the slope for equation and $1/\mu_{max}$ is the intercept shown in Fig.6.1.1.

6.2.3. Moser Model

Moser model is a modified Monod model equation with power function of substrate concentration. The value of power determines the degree of inhibition. However, it does not indicate any critical substrate concentration or inhibition constant.

The model is derived from the basic Monod model, where values of specific growth rate of bacteria (μ), half saturation coefficient (K_s) maximum specific growth rate of bacteria μ_{max}can be calulated from the curve fitting shown inFig.6.1.2.

The Moser equation is as follows:

$$\mu=\mu_{\max}(\frac{S^n}{K_s+S^n}) \tag{6.8}$$

Where, *n* is the coefficient of adaptation.

6.2.4. Hanes-Woolf Model

Hanes-Woolf plot is a graphical representation of kinetic parameters in which the ratio is plotted for the initial substrate concentration [S] and the specific growth rate of bacteria(μ).It is based on the rearrangement of the Lineweaver-Burk plot where, the equation is multiplied by the factor, initial substrate concentration,[S]. The equation was first described by Barnet Woolf and Charles

Samuel Hanes, subsequently pointed out that use of liner regression to determine the kinetic parameters.

$$\frac{1}{\mu}[S]=\frac{K_s}{\mu_{\max}}\frac{1}{[S]}[S]+\frac{1}{\mu_{\max}}[S] \tag{6.9}$$

$$\frac{[S]}{\mu}=\frac{K_s}{\mu_{\max}}+\frac{1}{\mu_{\max}}[S] \tag{6.10}$$

$$\frac{[S]}{\mu}=\frac{1}{\mu_{\max}}[S]+\frac{K_s}{\mu_{\max}} \tag{6.11}$$

To get maximum efficient results, perfect data will yield a straight line of slope $1/\mu_{max,}$ y-intercept of K_s/μ_{max}, and an x- intercept of $-K_s$ shown in Fig.6.1.3.

Like other techniques, that linearize the Michaelis –Menten equation, the Hanes- Woolf plot was used historically for rapid determination of the important kinetic parameters- K_s,μ_{max} and μ_{max}/K_s but it has been superseded by non-linear regression methods that are significantly more accurate and no longer computationally inaccessible.

6.2.5.Eadie- Hofstee model

Eadie-Hofstee model is a graphical representation of kinetic parameters in which maximum specific growth rate of bacteria is plotted as a function of the specific growth rate of bacteria against the substrate concentration and specific growth rate ratio. This plot is derived from Lineweaver –Burk plot as the starting point. The equation is multiplied by μ, μ_{max} on both sides shown in Fig.6.1.4.

$$\frac{1}{\mu}(\mu\times\mu_{\max})=\frac{K_s}{\mu_{\max}}\frac{1}{[S]}(\mu\times\mu_{\max})+\frac{1}{\mu_{\max}} \tag{6.12}$$

$$\mu_{\max}=K_m\frac{\mu}{[S]}+\mu \tag{6.13}$$

$$\mu=\mu_{\max}-K_m\frac{\mu}{[S]} \tag{6.14}$$

Equation (6.14) can be rearranged as:

$$\mu = -K_m \frac{\mu}{[S]} + \mu_{\max} \tag{6.15}$$

Hence, kinetic parameters are of great importance for any experimental study. Taking this as one of the prime objective for this research study, all the experimental set-up plans (Table- 3.1, Experimental set-up plan) were carried out in *Chapter-3 (section-3.3.2)* and analysed here again to kinetic parametric observations. ESP-III C from the *Chapter-3* and *Chapter-5* was not considered for kinetic analysis, due to negative finding of all experimental studies carried out to fulfill the proposed objectives.

Table 6.1. Experimental plan for kinetic studies.

S.N.	Kinetic analysis of substrate	Kinetic parameters	Kinetic model used
1.	Sugar industry waste (SIW)	Growth of bacteria	Logistic model
		Biohydrogen and Methane production	Modified Gompertz equation
		Substrate utilization	Monod model Moser model Eadie- Hofstee modal Hanes model
2.	Dairy industry wastewater (DIWW)	Growth of bacteria	Logistic models
		Biohydrogen production and Methane production	ModidfiedGompertz equation
		Substrate utilization	Monod model Moser model Eadie- Hofstee modal Hanes model

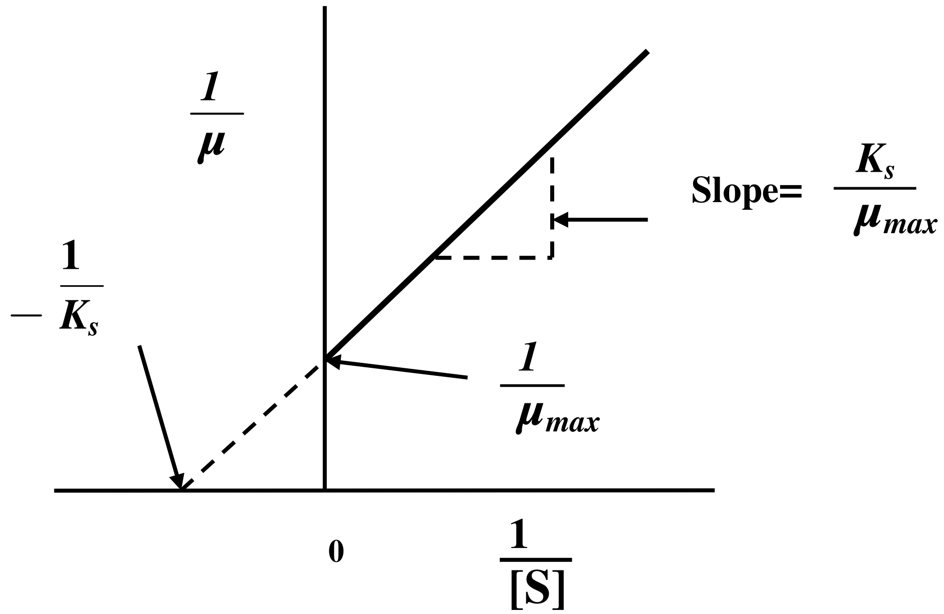


Fig. 6.1.1. The Lineweaver-Burk Plot

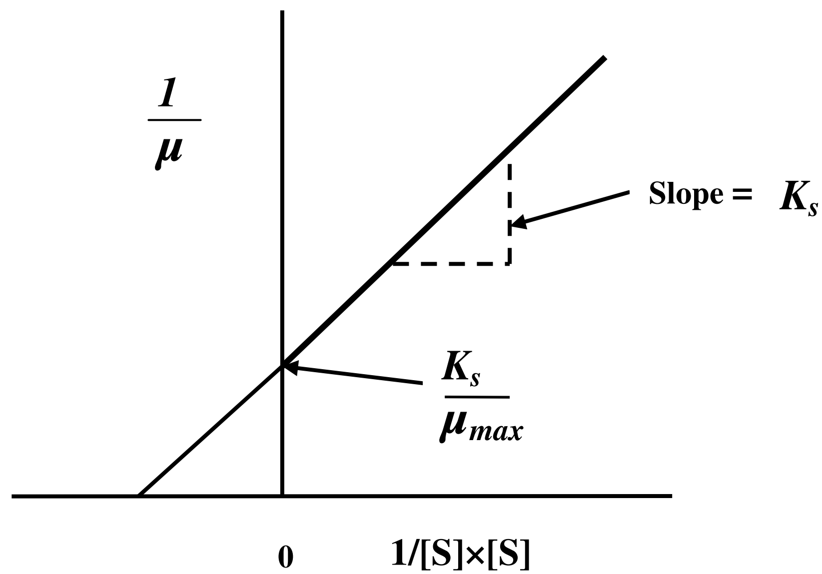


Fig.6.1.2. Moser Model.

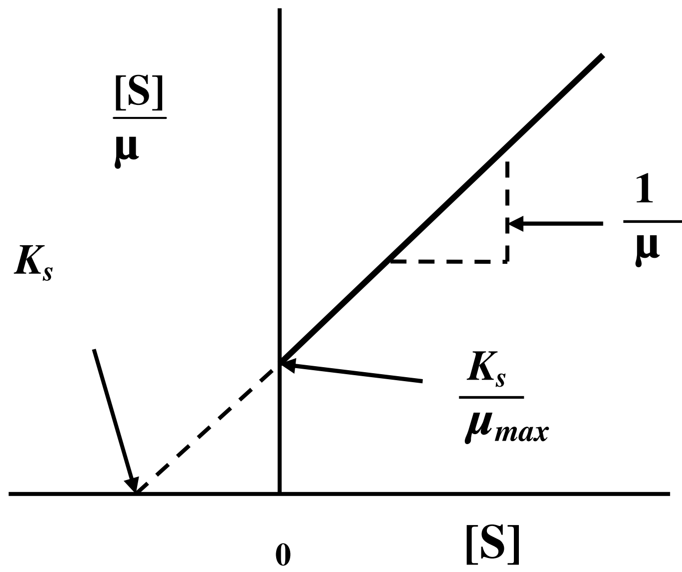


Fig. 6.1.3. Hanes Plot.

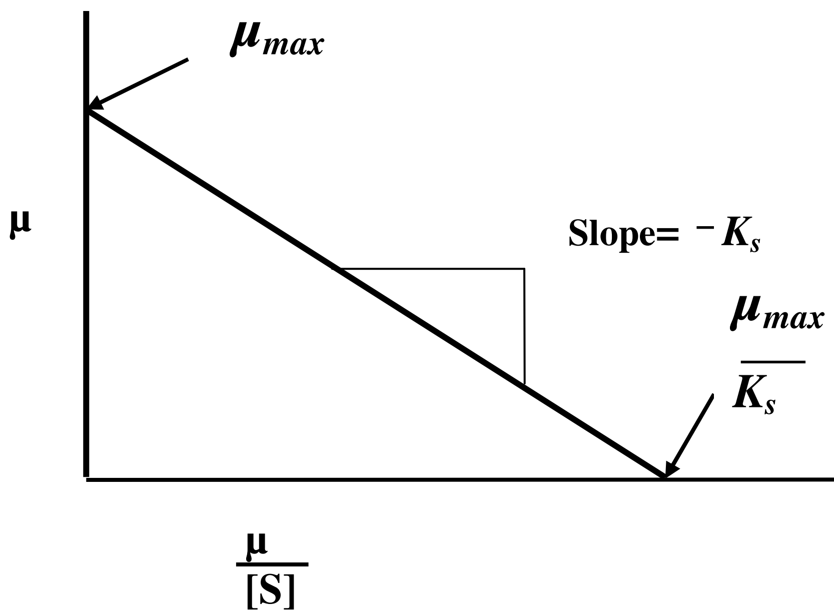


Fig. 6.1.4. Eadie-Hofstee Plot.

Thus, to observe and assess the kinetic relationship between industrial waste with pure bacterial strain *Enterobacter aerogens* and their effects on growth, substrate utilization and production of bioenergy options (H_2 and CH_4) taken as the main aim of this chapter. So, this chapter is divided into two main sections on the basis of potential findings observed in *chapter-5 (Section-5.6)* for industrial waste and brief layout for this study is described in Table 6.1.

6.2.6. Kinetics for gas production (hydrogen and methane)

The cumulative hydrogen and methane production obtained in the anaerobic reactor were followed by the modified Gompertz equation (Lay *et al.,* 1998).

$$H(t) = P \exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \qquad (6.15)$$

Where, H(t) represents the cumulative volume of gas (hydrogen and methane) production (ml) for phase I and II respectively, as a function of time; P, the gas production potential (ml); R_m, the maximum production rate (ml /h); , the lag time (h); t, incubation time (h); e, the exponential constant having value 2.718. The typical cumulative hydrogen production curve was nonlinearly modeled by the equation 6.15. Parameters (P, R_m and λ) were estimated using non linear curve fitting tool of Origin 8.5. Analysed results for cumulative hydrogen production and methane production were given in chapter 4 and chapter 5.

6.3. Kinetic studies with industrial waste

From the three selected industries, only two showed the potential of bioenergy production. The kinetic studies for these industries were done on the basis of parameters like, growth of bacteria, bioenergy production options and substrate utilization by the application of above said models.

6.3. A. Sugar industrial waste (SIW) : Kinetic parametric analysis

Several investigators have studied kinetic models for scaling up of reactors and deducting the optimal operational conditions for energy production by using pure or mixed cultures. In this part of the study, emphasis has been made on the efficacy of facultative anaerobic bacteria, *Enterobacter aerogens* for green energy production options taking sugarcane molasses (an organic industrial residual waste)

as the substrate. The aim of this study was to find a suitable model and accompanying kinetic parameters for scaling up of a batch reactor on the basis of experimental results obtained in *Chapter-5 (section-5.3.3.A).* The study was categorised in three phases as for growth of bacteria, initial pH, utilization of substrate with various product formation kinetics biohydrogen and methane.

6.3.1. A. Results and Discussions

Kinetic studies has been performed on the basis of experimental results done in ESP-I,A using sugar industrial waste (cane molasses).Kinetic parametric results obtained after analysis are discussed here witheffect on growth of bacteria, initial pH, utilization of substrate with various product formation kinetics biohydrogen and methane.

6.3.1.1. A. Effect of substrate concentration on growth of bacteria

The relationship between biomass growth and time with respect to different substrate concentration is shown inFig.6.3.1.A. In this study bacterial biomass, attributed maximum 3.5 g/l at 40 g/l substrate concentration and retarded over it as at 50 g/l. It has also been found a profound effect of substrate concentration on specific growth rate of bacteria. It has been also seen that in lower substrate concentrations, there is limited bacterial growth with lower substrate concentration that prevailed deficient carbon source while at higher concentration it become inhibitory for microbial growth.

Specific bacterial growth rate at different substrate concentrations were given in Table 6.3.1.A, based on the experimental data of Fig. 6.3.1.A. The peak specific growth rate of the pure bacterial culture (0.36 h^{-1}) was obtained at a concentration of 40 g/l. Therefore, the general growth models, Monod, Moser, Hanes and Eadie-Hofstee were used in this study for curve fitting of bacterial growth rate with substrate utilization.

6.3.1.2.A. Effect on bioenergy production options

Experimental results are validate through only kinetic studies, so to get the bioenergy production options i.e. biohydrogen and methane in its efficient way provides a better yields, using modified Gompertz equation, which discussed here in proper way.

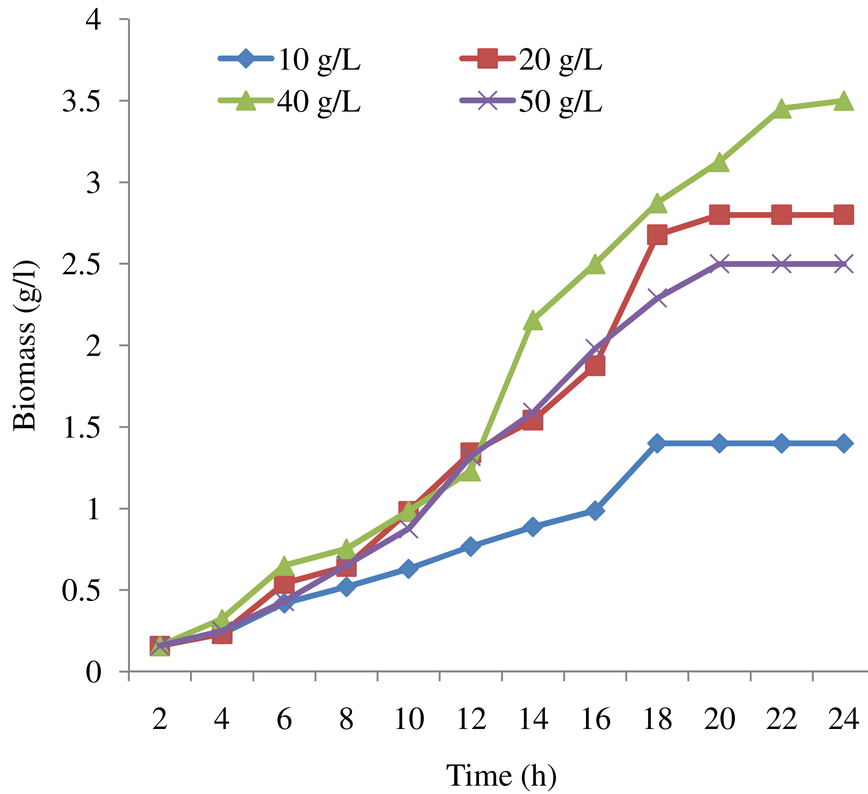


Fig.6.3.1.A. Effect of various substrate concentrations on bacterial growth.

Table 6.3.1.A. Specific growth rate of bacteria at various substrate concentrations.

Substrate (g/L)	t (h)	dx/dt	μ (h^{-1})
10	18	0.16	0.12
20	20	0.89	0.32
40	24	1.26	0.36
50	20	0.75	0.31

.

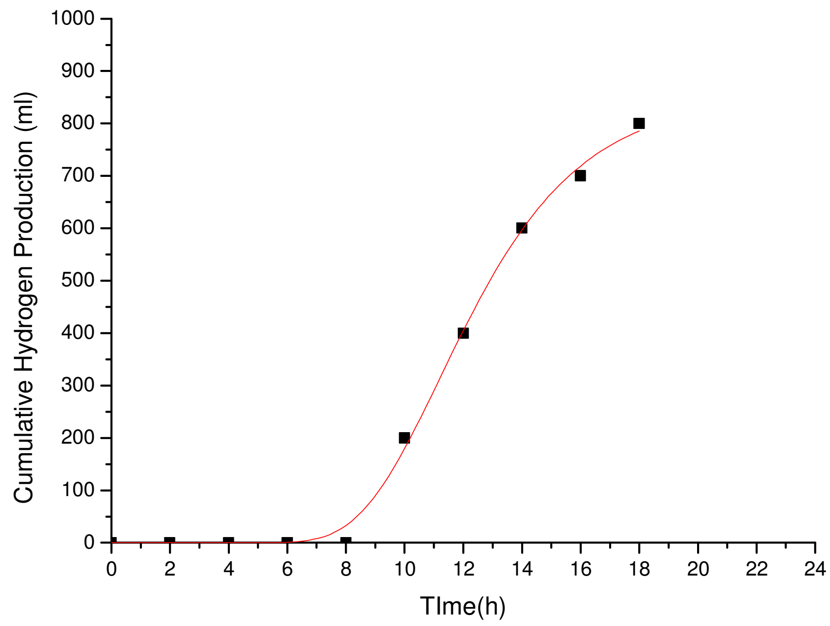
Table 6.3.2.A. Bioydrogen production with various substrate concentrations.

Substrate Concentration (g/l)	pH Initial	Final	Sugar consumption (%)	Hydrogen yield (mM/g sugar)	Gas Production (ml)	Total VFA (mg/l)
10	6.8	4.5	73	7.9	800	620
20	6.8	4.6	68	6.29	1050	1100
40	6.8	4.8	64	6.02	1520	2110
50	6.8	4.2	45	4.5	900	3400

6.3.1.2.1.A. Biohydrogen production kinetics with modified Gompertz equation

The effect of varying substrate concentrations (10, 20, 40 and 50 g/l) were analyzed for biohydrogen production with initial pH (6.8). A small change in substrate utilization rate was observed in batch set up with increasing substrate concentration (10, 20, 40 and 50 g/l), similar trend was also reported by Yu *et al.,* (2006), however, the substrate utilization rate decreased at substrate concentration of 50 g/l with decrease in pH at final stage of 24th hour.

As shown in Table 6.3.2.A, biohydrogen production increases with an increase in substrate concentration but the substrate utilization rate in terms of sugar consumption showed decreasing trend with the increase in substrate concentration. This may be due to the decrease in lag time of bacterial cell growth which can be attributed to less availability of the carbon source. However, at higher substrate concentration, bacterial cell growth supported to satisfy it, whereas, at higher concentration beyond 40 g/l i.e. 50 g/l, it could be attributed to the phenomena of inconsequential growth. This showed that the pure culture used in this study has a greater potential to degrade industrial waste in comparison to mixed cultures with pure synthetic sugars. It was analysed that the higher concentration of sugar become inhibitory for the bacterial cell as well as for production also (Tanisho and Ishiwata, 1994). At the concentration of 40 g/l, maximum biohydrogen production achieved that provide an optimum concentration for the bacterial cell biomass growth and biohydrogen production.



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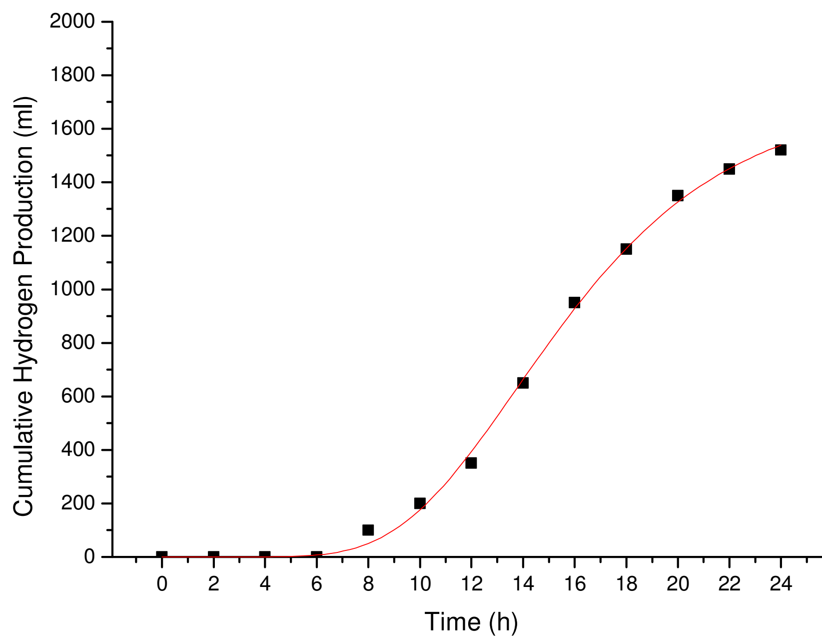

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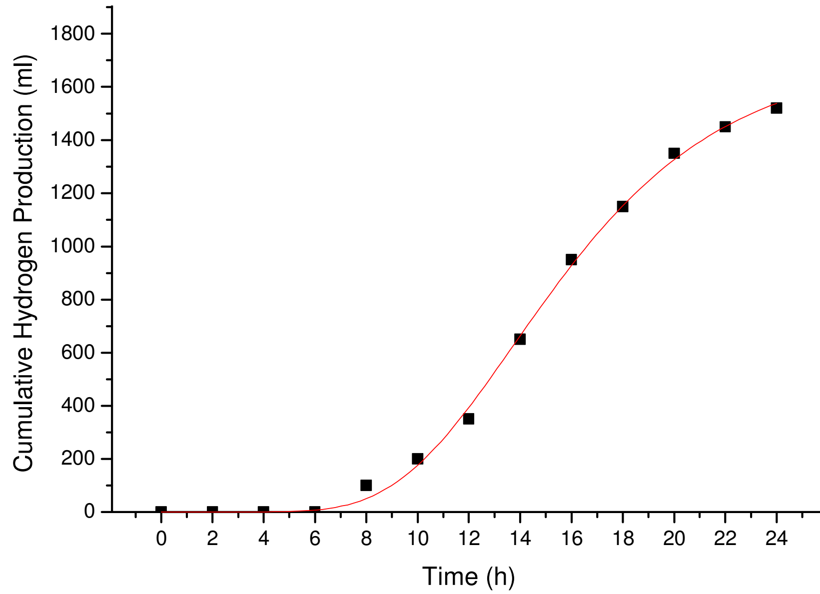

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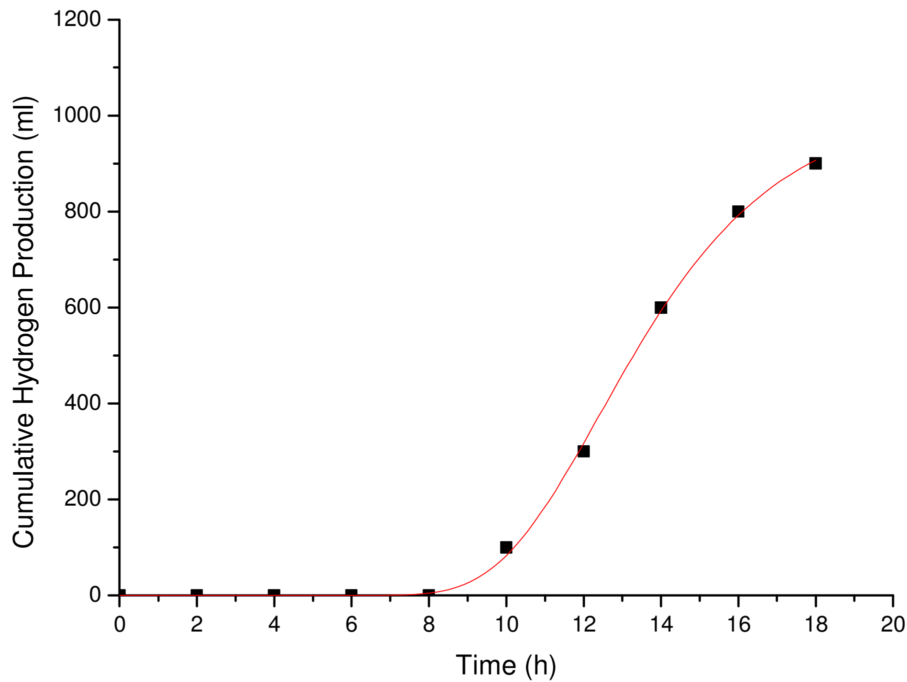


(d)

Fig.6.3.2.A. Typical cumulative biohydrogen production curve fitted by the modified Gompertz equation at different substrate concentration (a) 10 g/l, (b) 20 g/l, (c) 40 g/l and (d) 50 g/l.

The modified Gompertz equation was also used to fit the cumulative biohydrogen production, data obtained from batch experimental set up by using non-linear curve fitting tool of Origin 8.5 to obtain the value of H, R_m and λ (Fig. 6.3.2.A. and Table 6.3.2.A). The coefficient of determination (R^2) of all the fitting were all over 0.9901 to 0.9991, which indicates that the progress of cumulative biohydrogen production in the batch set up for this study was successful but the value of biohydrogen potential (P) showed significant evaluation of effective optimum substrate concentration with 40 g/l (1710 ml) only. This demonstrated that in an appropriate range, increasing substrate concentration could increased the biohydrogen production potential during fermentative hydrogen production, but substrate at much higher concentration could decrease it with increasing concentration whereas, biohydrogen production rate (R_m) is maximum with 40 g/l (142 ml/h) substrate concentration.

The maximum biohydrogen production rate (142 ml/h) was achieved at the substrate concentration 40 g/l and decreased at 50 g/l as shown in Table 6.3.3.A. Experimental data demonstrated that in the appropriate range, increasing substrate concentration could increase the biohydrogen production, at much higher concentration it becomes inhibitory (Akutsu *et al*., 2009; Kopsahelis *et al*., 2007).

6.3.1.2.2.A. Methane gas production kinetics with modified Gompertz equation

The methane content of the biogas produced during the second phase of experiment was about 60 % as obtained by the GC analysis. Kinetics of biogas production from the fermented sugar industry waste (FSIW) was analysed by the use of modified Gompertz equation (Eq.6.15.). The parameters of the process, biogas potential, P (247ml); production rate,R_m(16 ml/h) and lag phase, λ (4 hours) was obtained from equation (6.15). Fig. 6.3.3.A shows the curve fitted by the modified Gompertz equation with fermented SIW. Data points (X) represent experimental data and line represents nonlinearly modeled used in study.

Table 6.3.3.A. Values biohydrogen potential (P), production rate (R_m) and lag phase (λ) for various concentrations of substrate.

Substrate Conc.(g/l)	P(ml)	Rm (ml/h)	λ (t)	R^2
Biohydrogen				
10	852	115	8.5	0.9967
20	1125	137	9.6	0.9991
40	1710	142	9.2	0.9981
50	1020	130	9.8	0.9901
Methane				
40g/l from Phase-I	247	16	4.0	0.9820

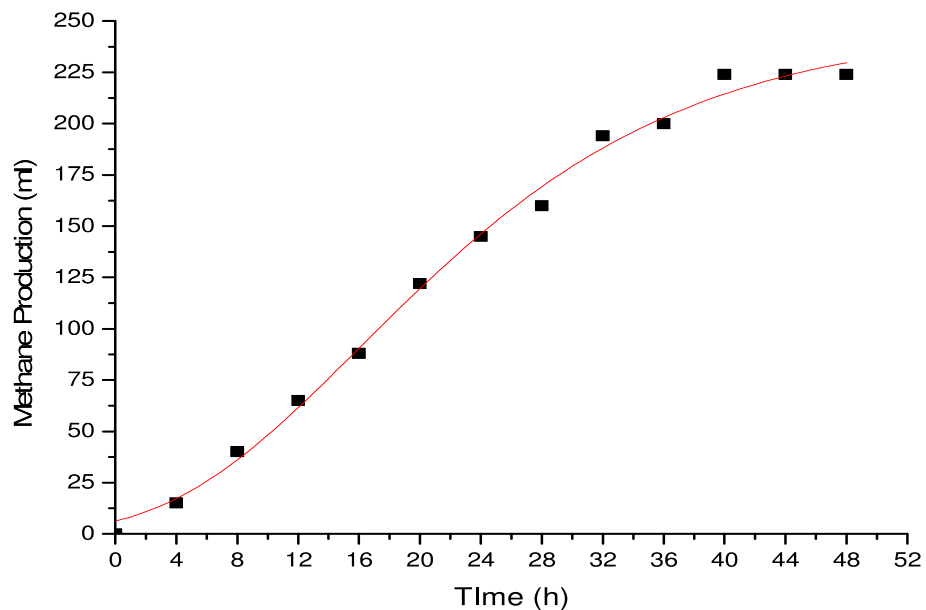


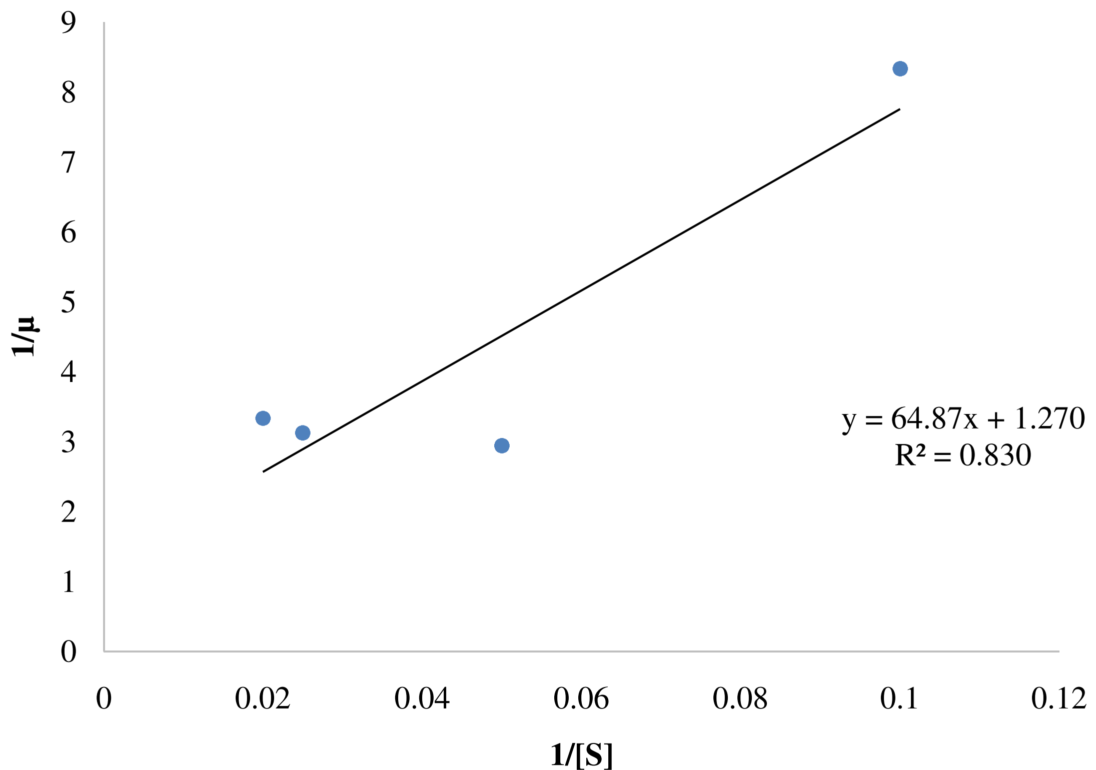
Fig. 6.3.3.A. Effect on production kinetics (methane) with modified Gompertz equation.

6.3.1.3. A. Kinetic models for process parameters

The kinetics for the bioenergy process parameters was evaluated with the substrate utilization during the process and initial pH of the process. The analysis was based on the substrate utilization at various substrate concentrations of SIW and growth of bacteria and biohydrogen production for the initial pH.

6.3.1.3.1A. Effect of specific growth rate of bacteria on substrate utilization

Different models for the growth of bacteria and substrate utilization in biohydrogen production were established to depict the bacterial growth kinetics at various substrate concentrations. The best suitable models were analysed with maximum specific growth rate of bacteria (μ_{max}), half-saturation coefficient (K_s) and value of R^2 in various equations.



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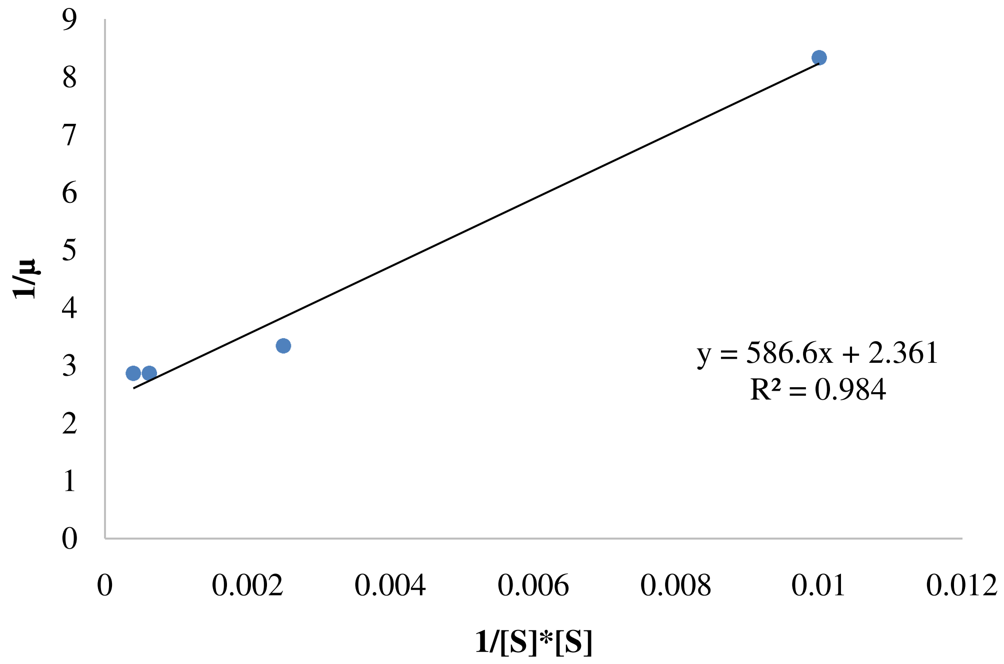

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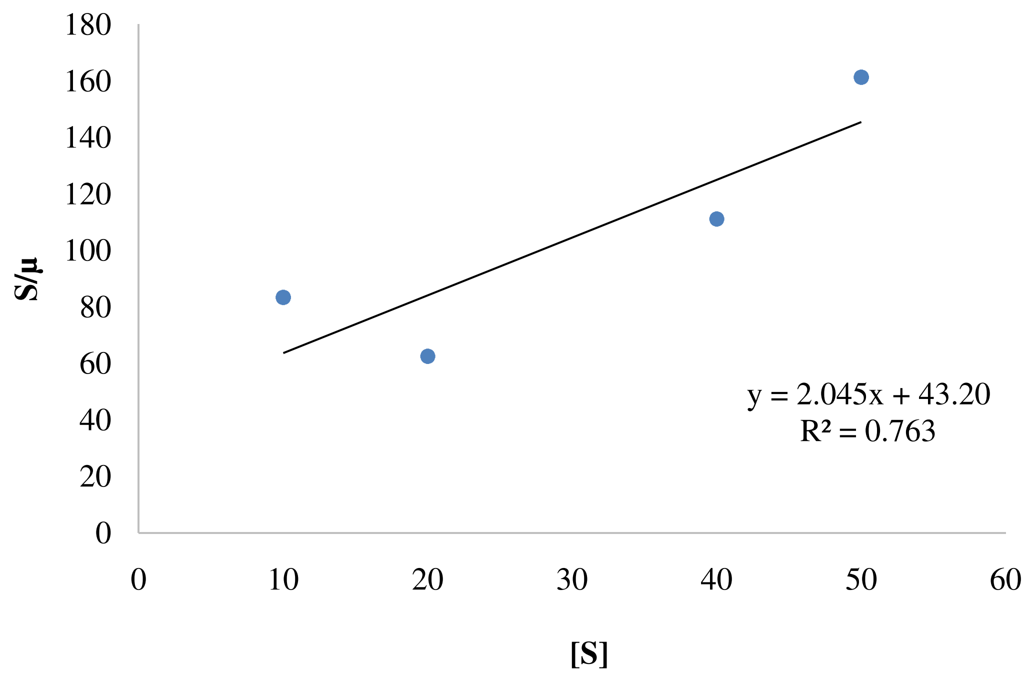

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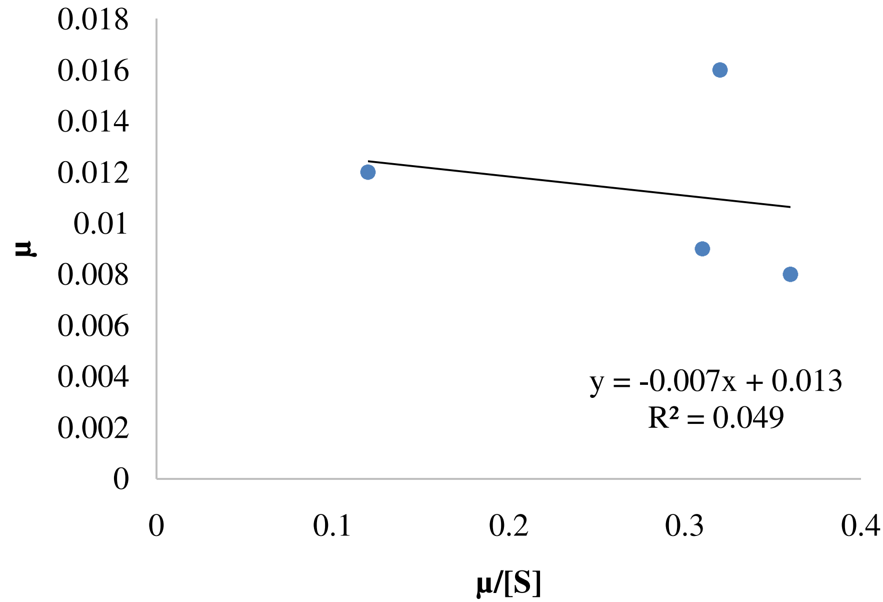

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Figure 6.3.4.A. Fitting of the experimental data with growth models and substrate utilization kinetics (a) Monod model, (b) Moser model, (c) Hanes model (d) Eadie-Hofstee model.

Table 6.3.4.A. Estimated values of growth kinetic parameters with various models.

Model used	μ_{max}	K_s	R^2
Monad Model	0.79	51	0.83
Moser Model	0.42	248.73	0.98
Hanes Model	0.49	21.12	0.76
Eadie-Hofstee Model	0.014	0.02	0.049

Kinetic studies on rate of specific growth of bacteria at various substrate concentrations indicated by Fig. 6.3.4.A. The experimental data revealed that different R^2 values for different models was not satisfactory except linearised Monod (Lineweaver-Burk) equation model and Moser model with values of 0.83 and 0.98 respectively (Table-6.3.4.A). The kinetics of batch experiment for hydrogen production was estimated by fitting the data to the well knownmodels like Monod model, Moser model, Hanes model and Eadie-Hofsteemodel. These models were used to describe the kinetic of specific cell growth rate as a function of

substrate and product formation in the process and the corresponding constant were estimated. The results showed that regression coefficient (R^2) values obtained with Moser equation (0.98) only..

6.3.1.3.2. A. Kinetics for effect of various initial pH on production

pH has been found to be crucial to the distribution of acidogenic products in a biohydrogen production.Hence,this is focusing on the improvement of biohydrogen production (yield and rate) generated from organic residue from sugar industry with facultative anaerobic bacteria, *Enterobacteraerogens,* with an approach for potential to generate more energy-rich process at variations in initial pH from 4.5 to 7.5.

6.3.1.3.2.1.A. Effect of initial process pH on bacterial growth

Among the others parameters, operational initial pH plays an important role in enhancing or decreasing the hydrogen yields, as they directly influence the fermentation end products (Lay, 2000) with bacterial biomass growth. The effect of initial process pH with optimized substrate concentration (40 g/l of sugarcane molasses) on biohydrogen production was investigated at various initial pH values (4.5, 5.5, 6.5 and 7.5) using *Enterobacter aerogens,* in this section. As seen from the results summarized in Table 6.3.5.A, three-fold increase in bacterial growth was observed with change in initial pH range from highly acidic environment (4.5) to low acidic environment(6.5) whereas, biomass growth rate showed decreasing trend with increase in one scale of initial pH i.e. 7.5. It can be said that during stationary phase (starting from pH 6.5onwards), bacterial population growth ceases may be due to depletion of carbon source or accumulation of toxic waste product or due to the production of pH-lowering acids during metabolism (solvantogenesis process), which inhibit their own growth overtime. However, at lower sides of process pH (4.5 and 5.5) or concentration of free proton [H^+] in a substrate medium not provide suitable nutrients for the proper growth, so less growth was observed at initial pH 4.5 (0.1h^{-1}) and 5.5 (0.15 h^{-1}). The maximum biomass was found 2.26 g/l on initial pH 6.5 with specific growth rate 0.30 h^{-1} while maximum hydrogen yield 105.8 ml/g sugar was recorded with initial pH 5.5 (Table6.3.5.A and Fig.6.3.5.A). This type of trend in results may be comparable with the findings of Khanna*et al.,* (2011)

who also reported that the best bacterial growth do not correspond the high biohydrogen yield as during metabolic shift of gas production to VFA production only favors biomass growth not gas production.

6.3.1.3.2.2.A. Effect of initial pH on biohydrogen production.

Biohydrogen production from sugarcane molasses with 40 g/l concentration at different initial pH variations was also evaluated by using modified Gompertz equation, a gas production model (Table 6.3.6.A and Fig. 6.3.6.A). The equation was evaluated for different parameters like biohydrogen potential (P,(ml)), biohydrogen production rate ($R_{m,}$(ml/h)) and lag phase (λ, (h)). At different initial process pH, the values of P, R_m and λ are different and the best value was analyzed with determination coefficient, R^2. The best value for R^2 (0.9982) was found at pH 5.5 with the values of 1944, 64.7and 4 for P, R_m, λ, respectively.

This kinetic study also highlighting the improvement of biohydrogen production with the optimization of initial pH using *Enterobacter aerogens* (pure bacterial strain) at proper substrate concentration, results obtained from previous chapter-5, of the thesis.

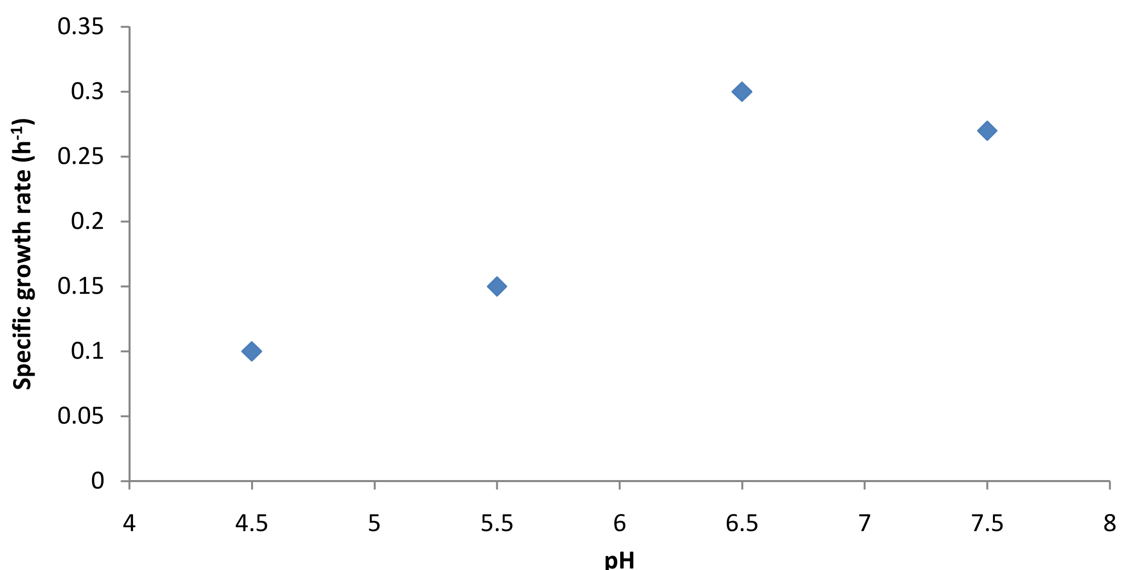
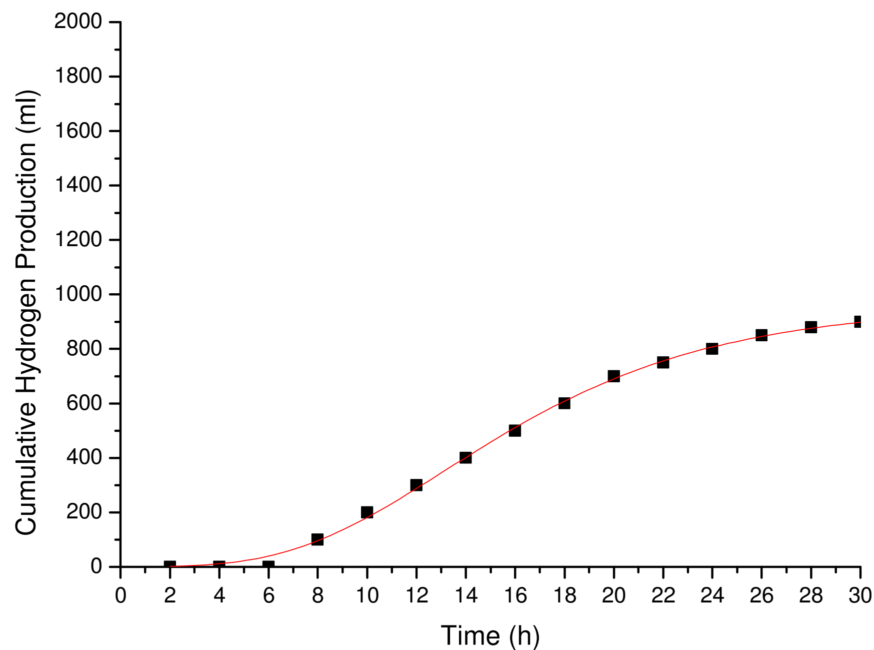


Fig. 6.3.5.A. Effect of initial pH (4.5 to 7.5) on specific growth rate of bacteria.

Table 6.3.5.A. Effect on bacterial biomass growth rate with low (4.5) and high pH (7.5).

pH	t (h)	dx/dt	$\mu(h^{-1})$	Fold increase
4.5	12	0.12	0.10	Nil
5.5	20	0.24	0.15	Approx one fold
6.5	24	0.97	0.30	Three fold
7.5	24	0.78	0.27	Approx two fold



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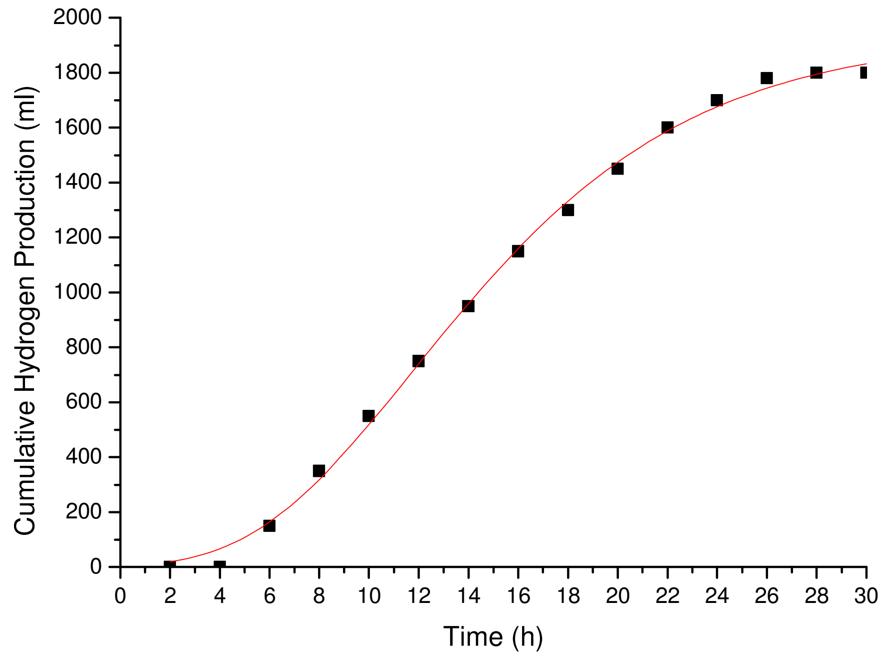

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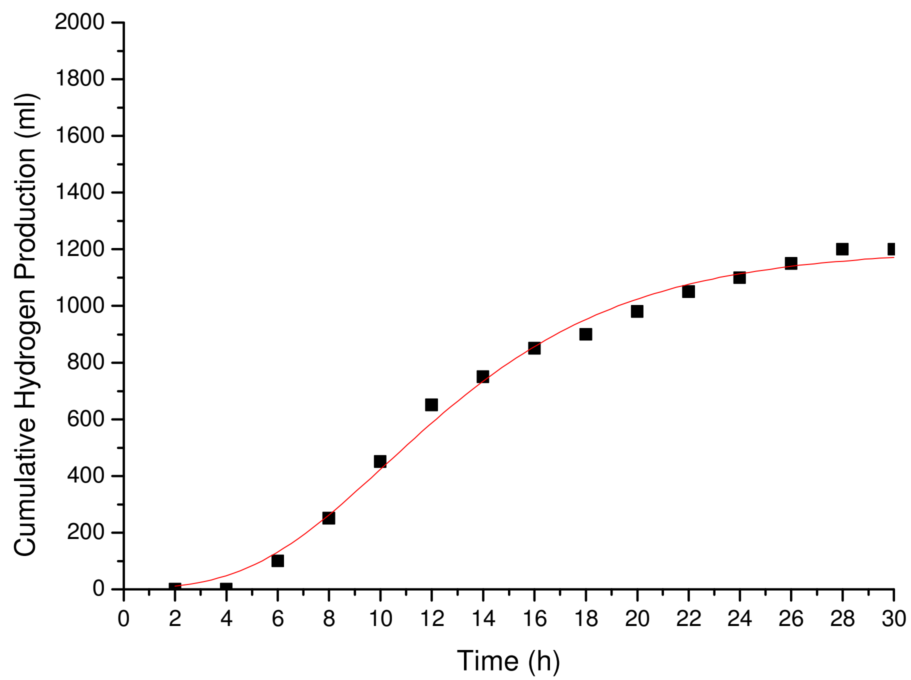

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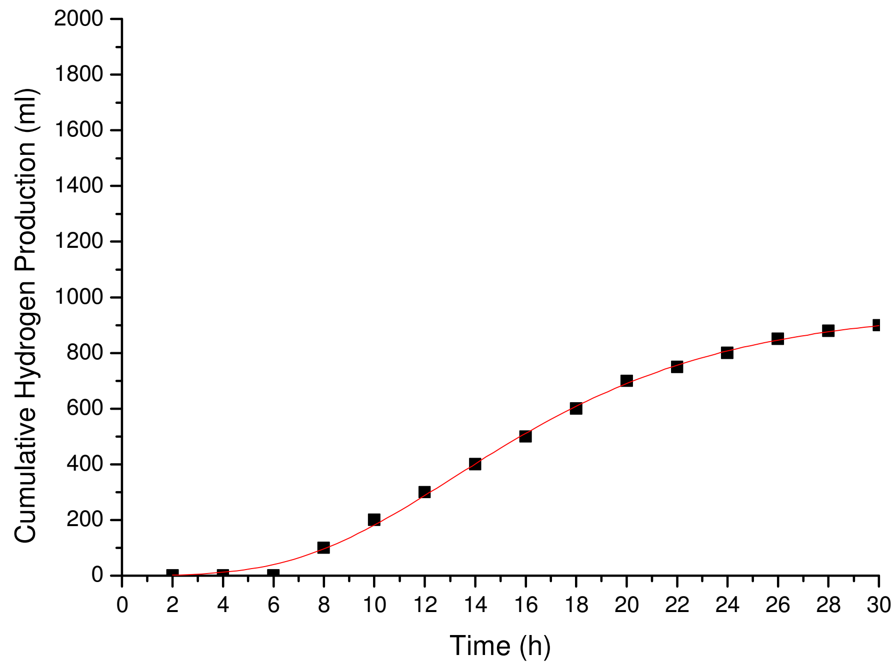

(d)

Fig.6.3.6.A. Typical cumulative biohydrogen production curve fitted by modified Gompertz equation at different initial pH (a) 4.5, (b) 5.5, (c) 6.5, (d) 7.5.

Table 6.3.6.A: Values of biohydrogen production potential (P), production rate (R_m) and lag phase (λ) at different intila pH of the substrate.

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pH	P (ml)	R_m(ml/h)	λ (h)	R^2
4.5	894	29.8	6	0.9975
5.5	1941	64.7	4	0.9982
6.5	1199	39.9	2	0.9978
7.5	957	31.9	4	0.9976

B. Dairy Industry wastewater (DIWW): Kinetic parametric analysis

It has been observed that the optimization of initial pH gave better results with pH 5.5 but growth of bacterial biomass was observed maximum with pH 6.5. Kinetic study results are totally supported by the experimented data obtained from the study.

6.3.2. B. Results and Discussion

Kinetic studies has been performed on the basis of experimental results done in ESP-II,B using dairy industry wastewater. Kinetic parametric results obtained after analysis are discussed here with effect on growth of bacteria, utilization of substrate with various product formation kinetics biohydrogen and methane.

6.3.2.1. B. Effect of bacterial growth with substrate concentrations

The growth of *E. aerogens* was analysed at varying wastewater concentrations. The results showed that biomass was well grown in 25, 50 and 75% concentrations of substrate (wastewater) while it was decreased at 100% concentration. The growth rate of bacteria can be positively correlated with substrate (Fig. 6.4.1.B). At low concentration of substrate at (25 %), bacteria feed up promptly and showed their stationary phase within 22 hours with a specific growth rate of 0.14 h^{-1}. Similarly, for the 50 %, specific growth rate was 0.17 h^{-1}. The maximum specific growth rate was obtained with 75% substrate concentration (0.52 h^{-1}) shown in Table 6.4.1.B. However, 100% concentration of substrate neither favours the biomass growth nor the specific growth rate of bacteria. This was due to the inhibitory effect of toxicants present in higher amount in the

6.3.2.2. B. Effect on bioenergy production options

Experimental results are validate through only kinetic studies, so to get the bioenergy production options i.e. biohydrogen and methane in its efficient way provides a better yields, using modified Gompertz equation, which discussed here in proper way.

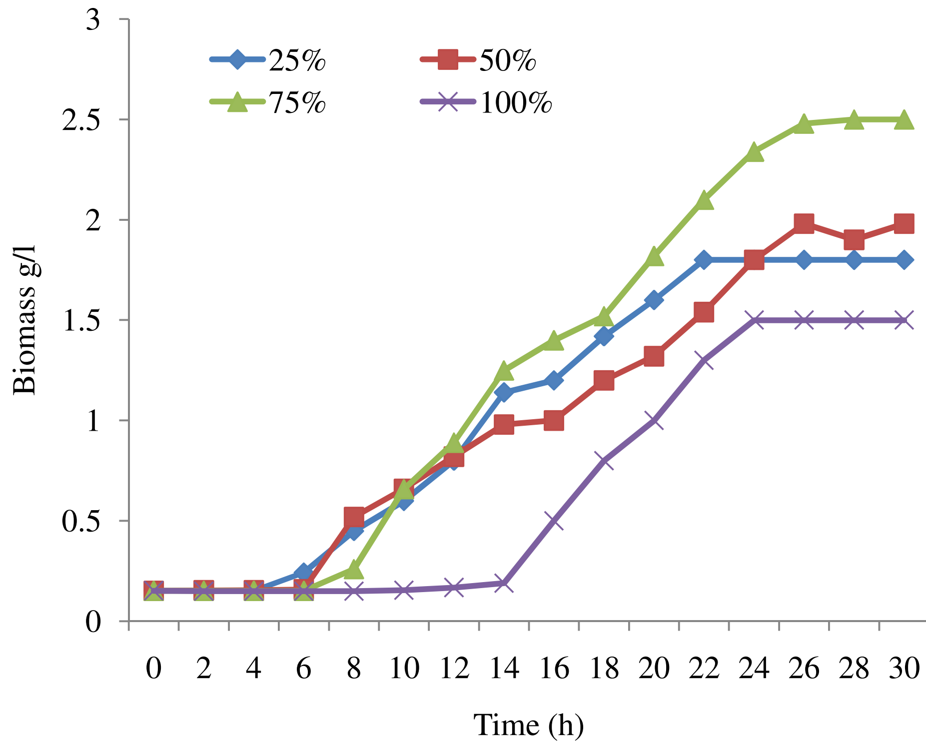
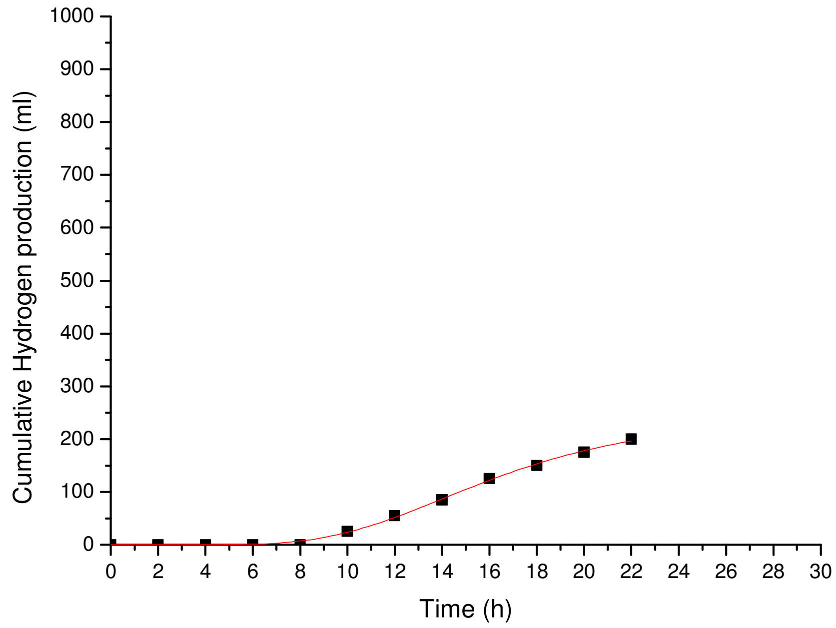


Fig. 6.4.1.B. Growth of bacteria at variation in substrate concentrations.

Table 6.4.1.B. Specific growth rate of bacteria in DIWW.

Substrate Conc. (%)	**t (h)**	**dx/dt**	**μ (h^{-1})**
25	22	0.25	0.14
50	30	0.33	0.17
75	28	0.52	0.21
100	24	0.27	0.18



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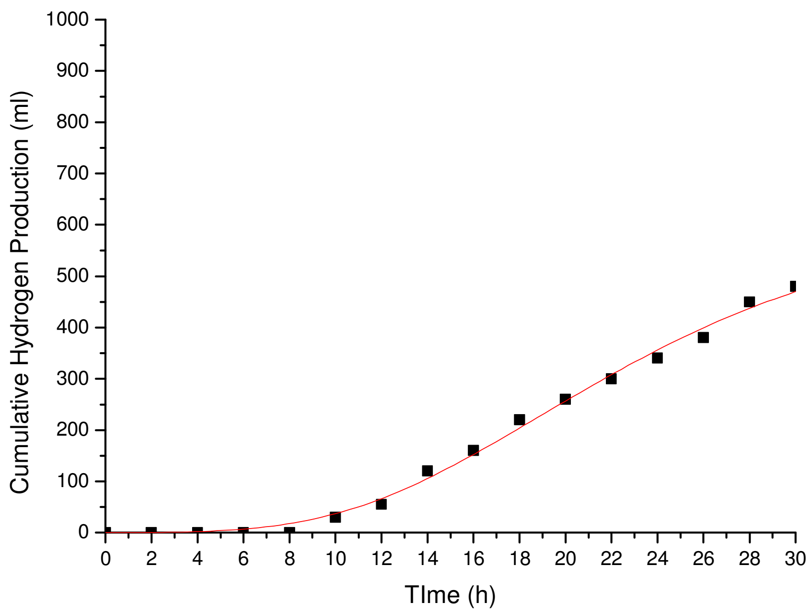

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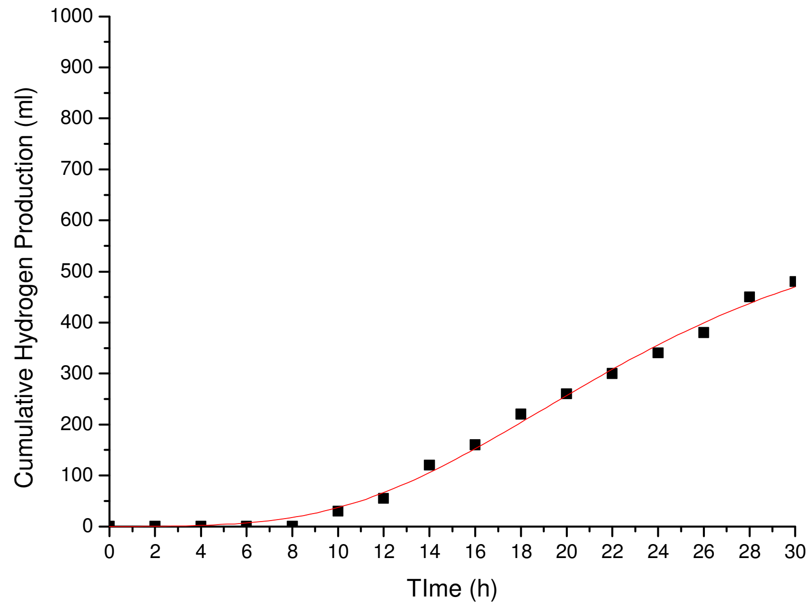

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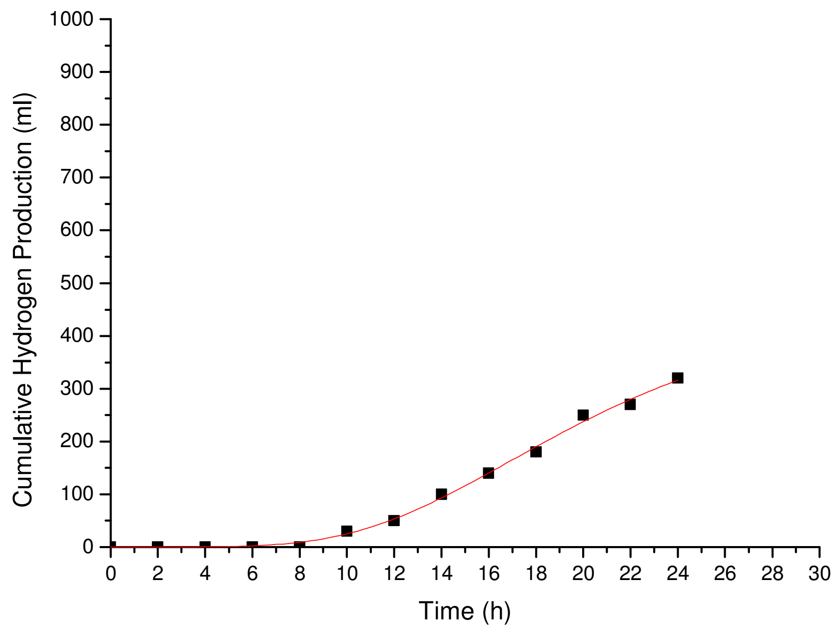


(d)

Fig.6.4.2.B. Typical cumulative biohydrogen production curve fitted by modified Gompertz equation at different substrate concentration (a) 25%, (b) 50% (c) 75% (d) 100%.

6.3.2.2.1.B. Biohydrogen production kinetics with modified Gompertz equation

The modified Gompertz equation was used to fit the quantitative and cumulative hydrogen production with experimental data obtained from biohydrogen production process, as given in *Chapter-5 (Section-5.4.3.2.1.B).* The progress of cumulative hydrogen production from the batch experiment is shown in Table 6.4.2.B with variables such as hydrogen production potential (P (ml)) and hydrogen production rate (R_m (ml/h)). R^2 for all parameters was obtained beyond the 0.90 which indicate that the parameters were statistically significant (Fig.6.4.2.B).

Nutrient availability in substrate permits the bacterial growth within some extent only while at higher concentration it becomes inhibitory for growth of bacteria. Therefore, in present study 75% substrate was the optimized concentration for bacterial growth. Different values of P were obtained for different substrate concentrations for instance 238 ml, 306 ml, and 617 ml for 25, 50 and 75% respectively, which again indicate the feasibility of 75% concentration for maximum biohydrogen production from dairy industry wastewater. Therefore, it is the optimum concentration (75%) as indicated by R2 value, 0.998 obtained by fitting the experimental data in modified Gompertz equation.

6.3.2.2.2.B. Methane gas production kinetics with modified Gompertz equation

The methane content of the biogas produced during the second phase of experiment was about 60 % as obtained by the GC analysis. Kinetics of methane gas production from the fermented dairy industry wastewater was analysed by the use of modified Gompertz equation. The kinetic parameters of the methane production potential, P (604ml); production rate, R_m (86 ml/d) and lag phase, λ (1.5 days) were obtained from equation (6.15).Fig. 6.4.3.B and Table 6.4.2.B shows the curve fitted by the modified Gompertz equation with fermented DIWW. Data points (X) represent experimental data and line represents nonlinearly modeled used in study.

6.3.2.3. B. Kinetic models for process parameters

Substrate utilization in respect of specific growth rate of bacteria has been the part of kinetic study taken as a process parameter with established kinetic models in this section.

Table 6.4.2.B. Variables of Gompertz equation obtained at various substrate concentrations.

Substrate Conc. (%)	P(mL)	R_m (ml /h)	λ(h)	R^2
Biohydrogen				
	238	14	7.5	0.9867
25				
50	306	14	7.8	0.9791
75	617	18	7.2	0.9981
100	444	16	7.8	0.9960
Methane				
FDIWW	604	86 ml/d	1.5 days	0.998

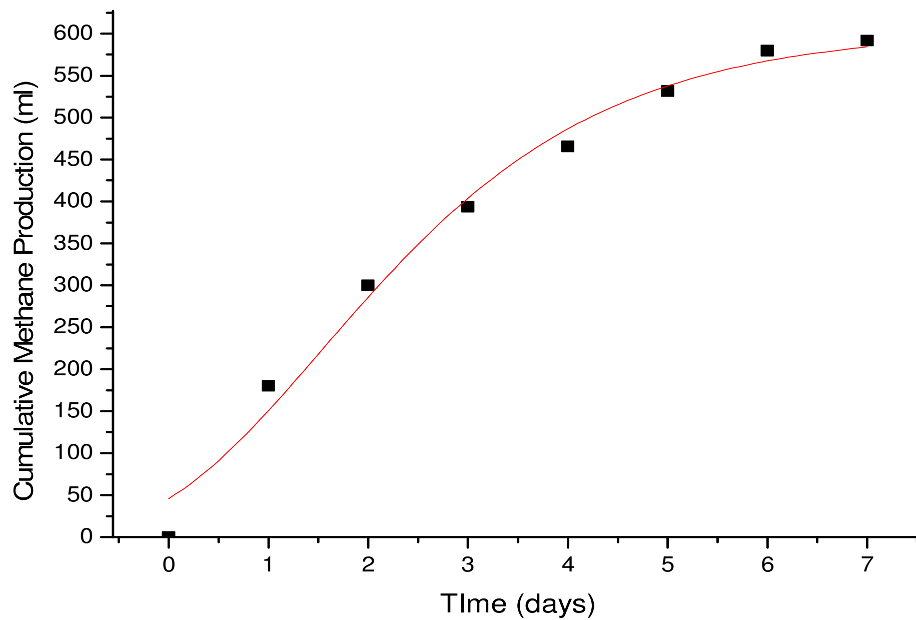
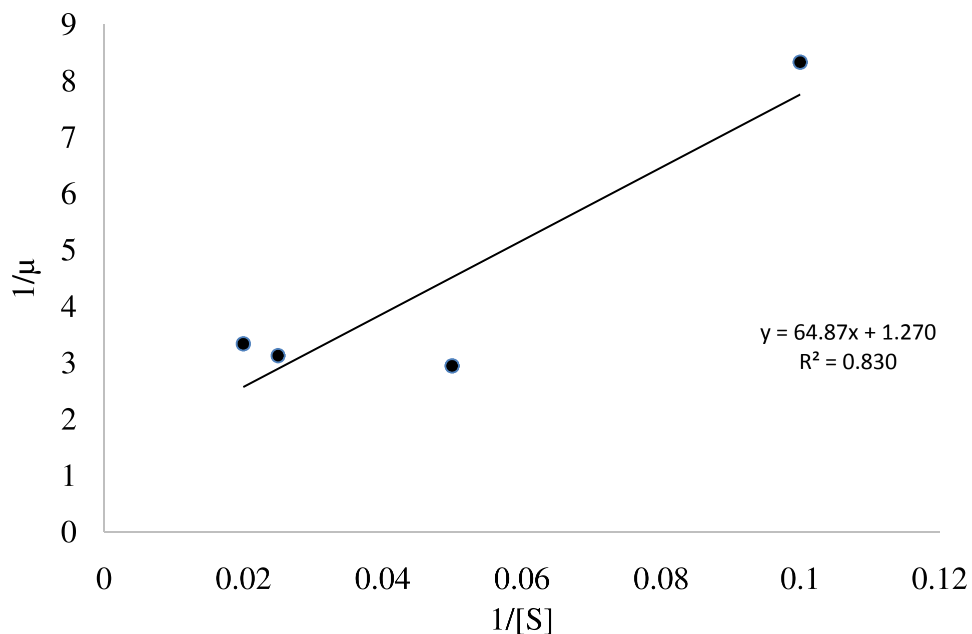


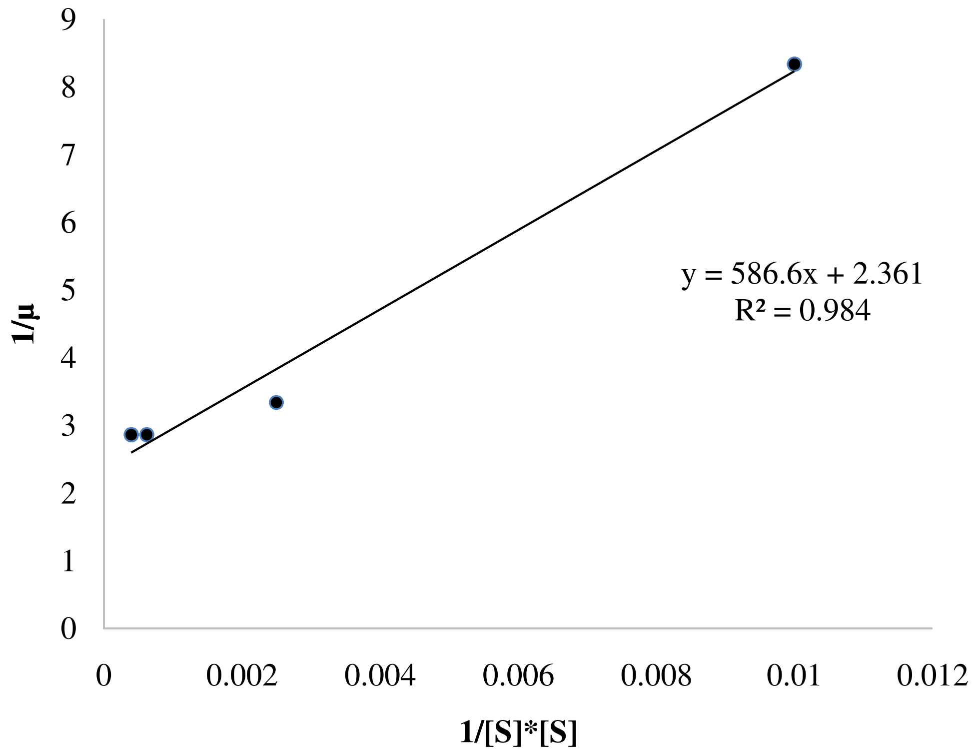
Fig. 6.4.3.B. Typical cumulative methane production curve with modified Gompertz equation.

6.3.2.3.1.B. Effect of specific growth rate of bacteria on substrate utilization

For the analysis of effective utilization of substrate,bacterial growth model was applied on the experimental data obtained (Fig.6.4.4.B). Four kinetic bacterial growth models namely Monod, Moser, Hanes and Eadie-Hofsteemodels were showed different maximum specific growth rate (μ_{max}) and substrate inhibition coefficients (K_s). The significant model was selected on the basis of the value of determination coefficient, R^2. The value of R^2 near to 0.99 is most suitable. Table 6.4.3, showed the different values of μ_{max} and K_s for different models and it was found that the value of determination coefficient, R^2 for Monad, Moser, Hanes and Eadie-Hofstee was 0.81, 0.83, 0.95 and 0.57 respectively. On the basis of this the best suited model for substrate utilization is Hanes model.



(a)



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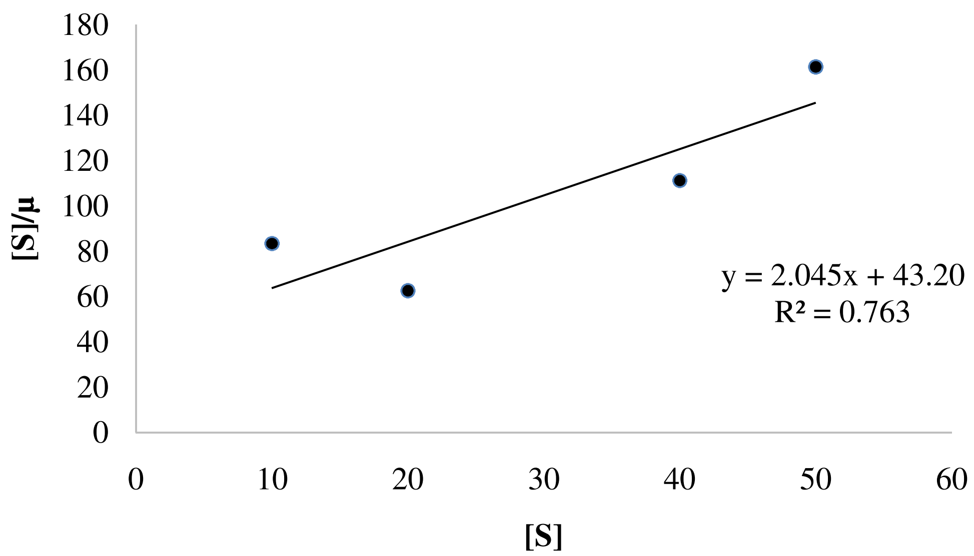

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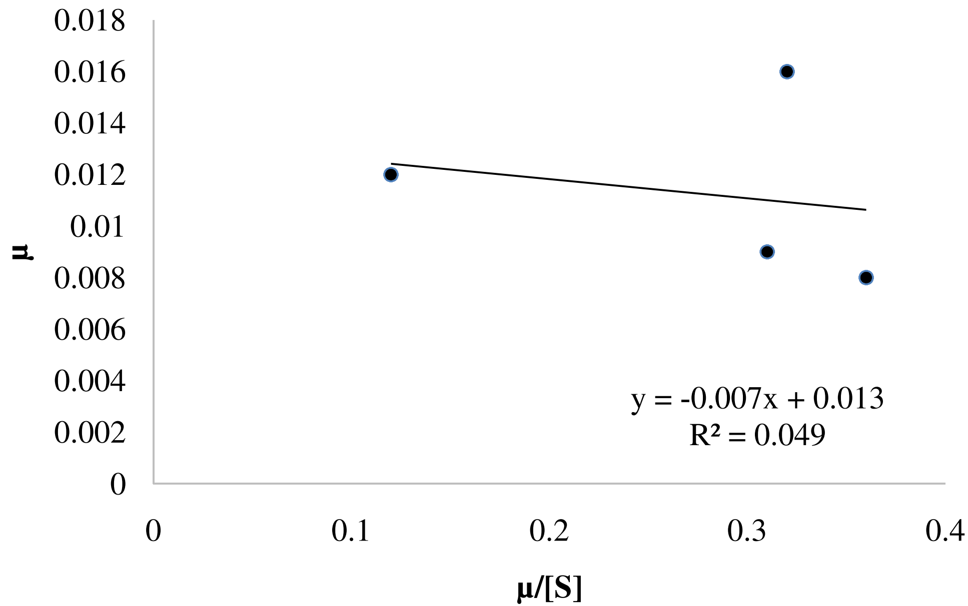

(d)

Fig. 6.4.4.B. Fitting of the experimental data with growth models and substrate utilization kinetics (a) Monod model, (b) Moser model, (c) Hanes model (d) Eadie-Hofstee model.

Table 6.4.3.B. Kinetic parameters obtained from experimental data at different substrate concentration.

Model used	μ_{max}	K_s	R^2
Monod	0.22	14	0.81
Moser model	0.19	253.73	0.83
Hanes equation	0.02	0.099	0.95
EadieHofstee	0.0042	0.042	0.57

6.5. Conclusions

In view of the above said kinetic results, this chapter review and analysed the trends of growth of bacteria, bioenergy (biohydrogen and methane) production options and substrate utilization kinetics by the application of different kinetic models. All kinetic models were critically analysed for all parameters taken for study. Following conclusions have been made from this thesis in particular:

- The logistic model applied for the growth of bacteria evaluated the maximum specific bacterial growth rate for SIW and DIWW at 40 g/l(0.36 h^{-1}) and 75% (0.52h^{-1}) concentration respectively.
- Initial process pH on bacterial growth showed specific growth rate at 6.5 and most significant value (0.30 h^{-1}) in respect of biohydrogen production was found with initial pH of 5.5.
- The kinetics for bioenergy production evaluated in terms of biohydrogen and methane.Typical cumulative biohydrogen production curve fitted by the modified Gompertz equation using sugar industrial waste was found most significant (0.9981) with a substrate concentration, 40 g/l Whereas, for dairy industry wastewater, 75% concentration shows most significant (0.9981) results in integration with *E. aerogens*.

  Similar trends of observations were found for both industrial waste with of same concentrations for methane gas production for regression coefficients.
- Among the various selected models for kinetic studies, regression coefficient (R^2) was best found 0.98 with Moser model from sugar industry waste (molasses) and Hanes model was found best with R^2 value of 0.95 from dairy industry wastewater by selected bacterial strain.

Chapter -7

Conclusions and Future Recommendations

7.1. Conclusions

In view of the objectives decided for the thesis, work has been completed successfully by their experimental validation and their kinetic analysis. The experimental results were analyzed with optimization of process parameters (substrate and initial pH) using industrial waste materials (solid and liquid) with pure facultative anaerobic bacterial strain, *Enterobacter aerogens* as a part of this work and the effect of kinetic parameters with established models for growth, substrate utilization and gas production had been studied. Though conclusions are given in the end of each chapter of the thesis, however, the major conclusions were drawn from the thesis can be summarized as given below:

- The bioenergy technologies are very efficient in terms of economy, environment and sustainable point of view. Among various options at commercial level energy production, bioenergy options for hydrogen and methane production provide a new arena of waste treatment and clean energy production. Hence, various organic industrial waste materials (solid and liquids) are evaluated here for characterization. Three industries (sugar industrial waste, organic residue i.e. molasses, Dairy industry wastewater i.e. influent and Tannery industry wastewater i.e. influent) are selected for experimental assessment for bioenergy options and pollutants loading rate for treatment approach using pure bacterial strain *Enterobacter aerogens*.
- Effect of *Enterobacter aerogens* with real industrial waste for production of bioenergy (biohydrogen and methane) options is also taken into account to improve the yield. For this, synthetic substrate material (glucose) was used to analyse the feasibility of selected microorganism (*E. aerogens*) and its optimized growth in selected concentrations of industrial waste. 40 g/l of sugar industry waste (SIW) and 75% concentration of dairy industry wastewater (DIWW) are found suitable for growth at maximum side.
- Effect of inoculums, substrate, reactor types, nutrients concentrations, temperature and pH are found to be most influencing factor in the process of dark fermentation for the production of bioenergy options which are discussed and concluded in *Chapter-2* of the thesis. Among those, designing of reactor with process parameters (substrate concentration and pH) is taken

as objectives for research and optimized results are achieved to get improved yields of biohydrogen and methane with selected waste and microorganism in the process routes of dark fermentation.

- Industrial waste is one of the eminent source of pollution as well as it is rich in organic loads and high ionic concentration of dissolve solids like, chemical oxygen demand, biochemical oxygen demand, phosphate, sulphate, nitrate etc. From the study, it was also concluded that the selected industrial wastes were a potential substrates for bioenergy options. Taking pollution factor into consideration simultaneously with bioenergy production, by dark fermentation process of wastewater can be an environment friendly process because after fermentation samples showed a remarkable reduction (about 90%) in pollution load of parameters like phosphate, nitrate, sulphate, COD etc with dairy industry wastewater.
- Trends of growth of bacteria, bioenergy production options and substrate utilization kinetics with the application of different kinetics model are also analysed for improvement in systems working process. Best trend are observed with the concentration of SIW (40 g/l) and DIWW (75%) for bioenergy production options in integration with pure strain *of E. aerogens*. R^2 are also found significant with molasses (Moser model) and DIWW (Hanes model).
- Techno-economics of the proposed experimental set up plans (ESP-I and ESP-II) has been evaluated and it shows the positive scale at commercial level with concept of "reduce, reuse and recycle" and zero effluent discharge. This leads to the conclusions that the use of industrial waste is more useful in case of renewable energy production options.

The work done in the thesis presents an answer (at least partially) to the problem associated with the clean and efficient production of bioenergy options. It also s generated a new paradigms for the future energy scenario and waste management approach as the energy deamand and pollution reduction are the two main problems that the whole world is going to face.

7.2 Future recommendations

For future work, it is advised to use of other organic rich materials from industries for bioenergy options with pure bacterial strain and mixed cultures to improve the yields of rate and production.

It is recommended to treat the waste materials (solid and liquid) prior to use as substrate for production of bioenergy options. The physical pretreatments followed by chemical pretreatment are suggested to get best results.

- The optimum values of process parameters like substrate concentration, pH, temperature ranges, should be used to get the best results.
- It is recommended to use of industrial waste as a solution to problem of environmental pollutants and energy crisis in rural and urban areas.

On the other hand, the techno-economic bioenergy profit assessment is also an important factor. In this respect, the choosing of the substrate is an important factor on cost. Hence, the whole process of optimization with various variables, as done here, can be tried with other different substrates and bacterial strains and can be applied in field to get useful results.

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Appendix-1

Appendix-I

Methodology adopted for the analysis of physicochemical parameters analysis (APHA, 2010), process parameters (Loewus, 1952; Maiti, 2004, Wang 2010; Reiner, 2012) and instruments used (Daniel *et al.,* 1997) was followed as the prescribed in the particular protocols is given in details :

1. pH

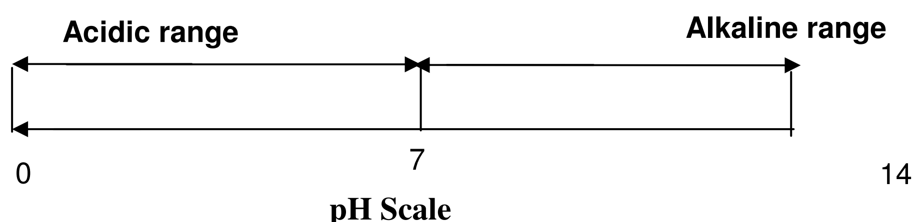
The pH unit measures the degree of acidity or basicity of a solution. pH of most natural water fall within the ranges of 4 to 9. The majority of waters are slightly basic (i.e. generally over 7.0) because of the presence of the carbonate and bicarbonates. The pH increases (acidic) during day time due to photosynthesis activity. By definition pH is the negative logarithm of hydrogen ion concentration, more precisely hydrogen ion-activity.

$$pH = -\log 10\ [H^+] \quad \text{or} \quad pH = \log 10\ 1/[H^-]$$

The conc. range suitable for the existence of most biological life is quite narrow and critical (typically 6 to 9). Wastewater with an extreme conc. of hydrogen ion is highly affecting the discharge point and if the conc. is not altered before discharge to the environment, it generates some adverse condition to the flora and fauna of discharge point. The allowable pH range usually varies from 6.5 to 8.5.

The hydrogen ion conc. in water is connected closely with the extent to which water molecules dissociate. Water will dissociate into hydrogen ion as follows

H_2O $H^+ + OH^-$



pH Scale

Estimation of pH:-

Estimation of pH can be done by two methods:-

(i) By using paper: simple, inexpensive, and inaccurate.

(ii) By using electronic pH meter: accurate and free from interferences, gives reading with accuracy of ± 0.05 pH.

2. Total Solids

Total solids can be determined as the residue left after evaporation at 103 to 105°C of the unfiltered sample.

Apparatus

1. Evaporating dishes: Dishes of 100 ml capacity made up of either porcelain or platinum.
2. Desiccator, provided with a desiccant containing a colour indicator of moisture ($CuS0_4.5\ H_2O$).
3. Drying oven or hot air oven, for operation at 103 to 105°C.
4. Analytical balance, capable of weighing to 0.1 mg.

Procedure

1. Take an evaporating dish or clean beaker (400 ml capacity) of suitable size and dry at 103 to 105°C for 1 h. Store and cool the dish in desiccator until needed. Weigh immediately before use. Note the initial weight (Wi) in mg.
2. Put 250-300 ml unfiltered well-mixed sample in it.
3. Put in hot air oven at 103°C to 105°C for 2 h up to dryness.
4. Cool in desiccator and take the final weight (Wt) in mg.
5. Repeat cycle of drying, cooling, desiccating and weighing until a constant weight is obtained, or weight change is less than 4% of previous weight or 0.5 mg, whichever is less. When weighing dried sample, be alert to change in weight due to air exposure and lor sample degradation. Duplicate determination should be within 5% of the average.

CALCULATION

TS (mg/l)= (Wf - Wi) x 1000/Volume of sample, ml

3. Electrical Conductivity

Conductivity is a measure of the ability of water to pass an electrical current. In water it is affected by the presence of inorganic dissolved solids such as chloride, nitrate, sulfate, and phosphate anions (ions that carry a negative charge) or sodium, magnesium, calcium, iron, and aluminium cations (ions that carry a positive charge). Organic compounds like oil, phenol, alcohol, and sugar do not conduct electrical current very well and therefore have a low conductivity when in water. Conductivity

is also affected by temperature: the warmer the water, the higher the conductivity. For this reason, conductivity is reported as conductivity at 25° C.

The same suspension used for pH measurement was also used for EC measurement. EC was measured using conductivity meter (Hanna Euro 04) in dSm^{-1}.

4. Chemical Oxygen Demand (COD)

The COD test is used to measure the oxygen equivalent of the organic material in waste water that can be oxidized chemically using dichromate in an acid solution. It would be expected that the value of the ultimate carbonaceous BOD would be as high as the COD, this is seldom the case, some of the reason for the observed differences as follows –

1. Many organic substances which are difficult to oxidize biologically, such as lignin, can be oxidized chemically.
2. Inorganic substances that are oxidized by the dichromate increase the apparent organic content of the sample.
3. Certain organic substances may be toxic to the microorganisms used in the BOD test.
4. High COD value occurs because of the presence of inorganic substances with which the dichromate reacts.

Reagent

1. Standard potassium dichromate solution (0.250 N) – dissolve 12.259 gm. of $K_2Cr_2O_7$ in dissolved in 1000 ml distilled water.
2. Ferroin indicator- Dissolve 1.485 gm., 1, 10 phenothalein monohydrate and 695 mg $FeSO_4$. $7H_2O$ in dissolved in 100 ml distilled water.
3. Standard ferrous ammonium sulphate titrant (0.25 N) – dissolve 98 gm. $Fe(NH_4)_2(SO_2)_2.6H_2O$ (FAS) in distilled water and add 20 ml. conc. H_2SO_4, cool it and dilute in 1000 ml.
4. Mercuric sulphate

Procedure

1. Take 20 ml sample in conical flask.
2. 10 ml. $K_2Cr_2O_7$ in each sample and add 1 pinch Mercuric sulphate then add 1 pinch silver sulphate in sample.
3. Add 30 ml. conc. H_2SO_4 in each sample.

4. And then put all samples in COD digester for 2 Hours in 150°C.
5. After digestion of sample, add the ferroin indicator in 1-3 drops.
6. Titrate with 0.25 N FAS
7. Then colour change with green to wine red.

Calculation:

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

A = vol. of FAS used for blank conc.

B = Vol. of FAS used for sample

N = Normality of FAS

5. Nitrate

Reagents

1. Stock solution of KNO_3: 0.1gm KNO_3 dissolved in 100 ml distilled water.
2. 5 % Salicylic acid: 5 gm salicylic acid dissolved in 100 ml conc. H_2SO_4.
3. 2N NaOH: 20 gm NaOH dissolved in 250 ml distilled water.

Procedure

Standard with KNO_3

Reference: 0.1 ml of distilled water + 0.4 ml of 5 % salicylic acid + 9.5 ml 2N NaOH.

1. Take 0.1 ml of sample in test tube.
2. Add 0.4 ml 5 % salicylic acid and then added 9.5ml 2N NaOH.
3. Orange-yellowish colour appeared after 20 minute.
4. Take OD at 410 nm.

Calculation:

Nitrate (mg/L) = K- factor x Absorbance (O.D.)

K- Factor = Absorbance (O.D.) / Concentration

6. Nitrite

Reagents:

1. $NaNO_2$ stock solution: dissolved 0.001 gm of NaNO2 in 100ml distilled Water.
2. 1% sulphamilamide (LR) solution (4 amono benzene sulphonamide $C_6H_8N_2O_2S$): Dissolved 1 gm sulphanilamide in 100 ml 1N HCl.
3. 0.01 % NED (N -1-napthyl ethyldiamine dihydrochloride (GR) $C_{12}H_{16}C_{12}N_2$: Dissolved 0.01 gm of NED in 100 ml distilled water .

Procedure: - standard with $NaNO_2$

Reference: 0.1 ml distilled water + 1 ml sulphanilamide + 1 ml 0.02 % NED

1. Take out 0.1 ml of water sample.
2. Add 1 ml of 1 % sulphanilamide
3. Add 1 ml of 0.02 % NED (N -1-napthyl ethyldiamine dihydrochloride).
4. After 10 minutes pink colour developed
5. Take OD at 540 nm.

Calculation

Nitrite (mg/l) = K-factor x Absorbance (O.D.)

K- Factor = Absorbance (O.D.) / Concentration.

7. Ammonical Nitrogen

Principal

In water and wastewaters, the forms of nitrogen of greatest interest are, nitrate, ammonia, nitrite and organic nitrogen. All of these forms are biochemically inter-convertible and are components of the nitrogen cycle and are of interest for many reasons. The measurement of ammonia concentration in waters by Nessler method depend on that the graduated yellow to brown colour produced by the Nessler-ammonia reaction is strongly absorbed over a wide range. (λ= 400-500nm)

Reagents

1- Ammonia free water: about 2liters by simple distillation (Boil dH_2O).

2- Standard NH_3-N solution (1000ppm); weight 3.819gram of anhydrous NH_4Cl, dilute in 1000 ml (1L) ammonia free water anhydrous NH_4Cl, dry in oven 100 °C for 1 hour.

3- K-Na Tartarate: (Rochelle salt solution): 50gram KNa -Tartarte in 100 ml dH_2O (free ammonia) you can boil this solution to expel ammonia.

4- Nessler Reagent:

A-(100 gram Hg I2+70 gram KI) dilute in small quantity of NH_4+ free water (about 50 ml)

B-160 g NaOH dilute to 50 ml NH_4+ free water

Add (A) to (B) stir gently, dilute the final volume to 1 litre. Store in borosilicate bottle and out of sunlight. It will stay about 1 year.

Procedure

1. Prepare the standards NH_3-N as shown

Stock solution→5 ml/100 ml dH_2O (50mg/L) 1000 mg/l 1ml/50dH_2O 1mg/L

2. Take 50 ml of each standard 50 ml of dH_2O (free ammonia as blank) 50 ml of sample

3. Add 1 ml of K-Na Tartarate (Filter before use K-Na Tartarate)

4. Add 1 ml of Nesslers reagent, wait for 5 minutes

5. Read at 425 nm

6. Plot strait curve

8. Chloride

Chloride is the common anion found in water and sewage. The concentration of chloride in natural waters varies from a few milligrams to several thousand milligrams per litre. Higher concentration of chloride may be due to the contamination by sea water, brines, sewages or industrial effluents such as those from paper works, galvanizing plates, water softening plants and petroleum refineries. Titrimetric estimation method is used for analysis of chloride. This method involves silver nitrate titrimetric method.

Reagents

1) Standard silver nitrate titrant (0.0282 N) - Dissolve 4.971 g silver nitrate in distilled water and make up to 1000 ml in a volumetric flask.

2) Potassium chromate indicator- Dissolve 25g potassium chromate in 100 ml distilled water. Add drop wise silver nitrate solution until a slight red precipate is formed. Allow to stand for 12 hours, filter and dilute the filtrate to 500 ml.

3) Sodium hydroxide (1N) – Dissolve 40g NaOH in distilled water dilute o 1000 ml.

4) Sulphuric acid (1N) - Place 27.7 ml conc. H_2SO^4 in 500 ml distilled water in a 1000 ml volumetric flask. Make up to the mark with distilled water.

Procedure

1. Place 100 ml of sample in beaker.

2. Add 1 ml potassium chromate indicator solution.

3. Titrate against standard silver nitrate solution until a slightest precipiTable reddish coloration persists.

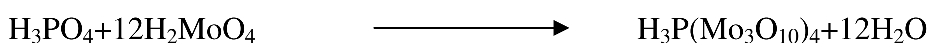
Calculations-

$$\text{Chloride mg/l} = \frac{(\text{ml } AgNO_3 \text{ solution for sample} - \text{ml } AgNO_3 \text{ solution for blank}) \times \text{Normality of } AgNO_3 \times 35.5 \times 1000}{\text{ml sample taken for estimation}}$$

9. Phosphate

Principal

Phosphate in extract is measured by the reaction of phosphate with ammonical molybdate in an acid medium to form molybdohpsphoric acid .the molybdophosphoric acid is then reduced to a pink coloured complex and these blue coloured compound detected through at absorbence 640 nm using spectrophotometer.

$H_3PO_4+12H_2MoO_4$  $H_3P(Mo_3O_{10})_4+12H_2O$

Reagent

1) Ammonium molybedate $(NH4)_2MoO_4$

2) $SnCl_2$

Procedure

1. Take 10 ml sample in a test tube.
2. Add 0.4 ml ammonium molybedate $(NH4)_2MoO_4$ in a test tube
3. Then add 2 drop $SnCl_2$
4. Take OD at 680 nm.

Calculations-

Phosphate (mg/l) = K-factor x Absorbance (O.D.)

K- factor = Absorbance (O.D.) / Concentration

10. Sulphate

Principle

Sulphate ions ($SO4^{-2}$) are precipitate in an acetic acid medium with barium chloride ($BaCl_2$) so as to form barium sulphate ($BaSO_4$) crystals of uniform size. Light absorption of the $BaSO_4$ suspension is measured by photometer and the sulphate concentration is determined by comparison of reading with standard curve.

Apparatus

1) Magnetic stirrer

2) Spectrophotometer

3) Measuring spoon

Reagent

1- Conditioning reagent – Mix 50 ml glycerol with a solution containing 30 ml conc. HCL, 300 ml distilled water, 100 ml 95% ethyl alcohol and 75 gm sodium chloride.

2- Barium chloride crystals 'AR grade'.

3- Standard Sulphate solution – Prepare by diluting 10.41 ml of the standard 0.02 NH_2SO_4 to 100 ml with distilled water.

Procedure

1. Formation of barium sulphate turbidity – Measure 100ml sample or a suitable aliquot made up to 100ml into a 250ml Erlenmeyer flask. Add exactly 5 ml conditioning reagent and mix in the stirring apparatus. While the solution is being stirred add a spoon full of barium chloride crystals stir.
2. Measurements of barium sulphate turbidity – Immediately after the stirring period is over, pour some of the solution into the absorption cell of the photometer and measure the absorption at fifth minute. Maximum turbidity is usually achieved within 2 min. and the reading remains constant thereafter for 3-10 min.
3. Read mg SO_4 present in the sample on the calibration curve prepared by standard solutions.

Calculation

$$SO4\ ^{-2}\ mg / l^{-} = mg\ SO4^{2-} \times 1000 / ml\ sample$$

11. Estimation of sugars

11.1. Anthrone Method

Principal

The anthrone reaction is the basis of a rapid and convenient method for the determination of hexose, aldopentose and hexuroninc acids either free or present in polysaccharides.

The following reactions are taking place during the process:

Carbohydrates are dehydrated by concentrate sulphuric acids to form furfural.

Furfural condenses with anthrone (10 keto -9, 10 dehydroantherocene) to form a blue green colured complex which is measured calorimetry at 630nm.

Reagents and Procedure

1. Dissolve 200 mg anthrone in 100 ml ice cold 95% H_2SO_4. Prepare fresh before use.
2. Prepare standard of 10 to 100 micrograms/ml glucose concentration.
3. Take the sample 05 to 1.0 ml
4. Add 4 ml anthorne reagent.
5. Heat for 8 minutes in boiling water bath.
6. Cool rapidly and reed green to dark green colour at 630nm.

11.2. HPLC Method

At particular interval, fermented broths (in triplicate flasks) were removed and the contents were analyzed for total sugar, ethanol production. Glucose and ethanol was analyzed by high-performance liquid chromatography (HPLC) using Hi-Plex H column at 57°C with 1mM H_2SO_4 as the mobile carrier at a flow rate 0.7 ml min-1 and detected by refractive index detector.

12. Estimation of Volatile Fatty Acids (VFA)

12.1. Distillation Method

Principal

Volatile fatty acids (VFAs) are classified as water soluble fatty acids that can be distillate at atmosphere pressure. This procedure can recover acids containing upto six-carbon atoms. In this procedure heat distillation followed by titration of the condensate with strong base such as NaOH using phenolphthalein as an indicator. The VFAs are estimated on the basis of acetic acid.

Apparatus

- Centrifuge, with head to carry 50 ml tubes
- Distllation assembly : Distllation flask, 500ml; condenser ; adapter
- Titration assembly: burette, Flask etc.

Reagents

1. Sulphuric acid
2. Standard NaOH
3. Phenolphthalein indicator

Procedure

1. Take 200 ml sample and centrifuge it for 5 minutes
2. Collect supernatant, and place exactly 100 ml supernatant liquor in a 500 ml distillation flask
3. Add 100 ml distilled water, few glass baeads or similar materials to prevent bumping, and 5ml H_2SO_4.
4. Mix well so that acid does not remain in the bottom of the flask.
5. Connect distillation flask to a condenser and adapter and distillate at the rate of about 5ml/min.

6. Discard first 15 ml distillate (Note: H_2S and CO_2, librate during distillation and titration, will give a positive error. Discarding first 15 ml distillate eliminate this error).
7. Collect exactly 150 ml distillate in a 500 ml conical flask.
8. Titrate the distillate with 0.1N NaOH, using phenolphthalein as an indicator. At the end point colour changes to pink.

Calculation - VFA as acetic acid, mg/L = $\frac{mLNaOH \times 60 \times 1000}{mLofsample}$

Where N = Normality of NaOH

12.2. GC Method

The concentrations of VFA, including formate, acetate, propionate, and butyrate in the digestate were determined by the gas chromatograph (GC 5765 Nucon India Make) equipped with a flame ionization detector and 10% SP-1000/1% H_3PO_4 on 100/120 Chromosorb® WAW column. The liquor samples were first centrifuged at 12000 rpm for 5 min, and were then acidified by formic acid and filtrated through 0.2 lm membrane and finally measured for free acids. The temperatures of the injector and detector were 250° C and 300 °C, respectively. The initial temperature of oven was 70 °C for 3 min followed with a ramp of 20 °C min1 for 5.5 min and to final temperature of 180 °C for 3 min. Helium was used as carrier gas with a flow rate of 2.6 ml min.

13. Dry cell weight

1mL of the aseptically obtained sample was centrifuged and washed three times with phosphate buffer; thereafter it was dried in pre-weighed eppendorf tubes at 95 °C till three consecutive constant readings were obtained.

Procedure

1. Dry in an oven an empty aluminum weighing pan or a sheet of cellulose acetate filter membrane, 47mm in diameter, 0.45µm in pore size. Weigh them and store them in a desiccator lined with drierite (anhydrous $CaSO_4$).
2. Stir the flask to suspend the culture evenly. Pour out 100 ml of the culture into a graduated cylinder.
3. Separate the cells from the broth either by centrifugation at 10,000 g for 5 minutes or by filtration.

4. In the case of centrifugation, carefully discard the clear broth and scrape the cell paste from the centrifuge tube into a weighing pan.
5. Rinse the centrifuge tube with a few ml of water. Pour the rinse water into the weighing pan, as well. In the case of filtration, the culture is poured into the holding reservoir fitted on the filter membrane.
6. A vacuum is applied to pull the liquid through the membrane. Rinse the reservoir with a few ml of water and scrape any paste adhering to the glassware. The wet weight of the culture is measured immediately after all the water has been pulled through.
7. Dry the cell paste in an oven set at 100ºC. The cells will be charred and the filter membrane will be burned if the temperature of the oven is set too high.
8. Measure the weight of the pan/filter plus the cell paste periodically until there is no further decrease in the dry weight. It will take 6-24 hours to dry the sample completely, depending on the oven temperature and the thickness of the paste.
9. Calculate the difference in the weight, and express the dry weight in g/l.

14. Biochemical assay for the carbohydrate fermentation

Principal

In the fermentation process the acids are formed by the utilization of carbohydrates by the microorganism in the medium. The presence of these acids can be detected by the pH indicator. The fermentation process mostly resultants the formation of acids like lactic acids, formic acids, acetic acids, butyl alcohol, acetone, ethyl alcohol and gases like CO_2 and H_2. Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests because most of the end-products of carbohydrate utilization are organic acids. The whole experiment constitutes the following steps-

i) Media inoculation by bacteria

The fermentation medium was inoculated with the selected bacteria, *Enterobacter aerogens* with the inoculating tube in the test tubes containing Durhum tubes in it. Make some precaution while inoculating the medium with bacteria as the inoculating tube should not touch with to the Durhum tube.

ii) Incubation

After inoculation, incubate the test tube at 30 °C in the incubator for 24 hours and observe the change in colour in and gas production.

iii)Observations of results

The change in the colour of the medium in the test tubes from red to yellow in the presence of phenol red depicts the formation of acids in the fermentation process due to consumption of sugar by the bacteria inoculated in the medium. The formation of acids lower the pH generally acidic (< 7). During the fermentation with the production of acids some amount of gas production is also occurred that trapped in the Durhum tubes in the form of bubbles.

List of Publications

Published

1. **Virendra Kumar**, Richa Kothari & Sohini Singh. Dark Fermentation: a green way to produce hydrogen and methane. International Journal of Science, Technology & Society*;* Vol. 1, No. 1, January-June, 2015. 39-42. (ISSN - 2395-1605)

2. Richa Kothari, Ravindra Prasad, **Virendra Kumar**, D.P. Singh. Production of biodiesel from microalgae *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater. Bioresource Technology 2013; 144:499-503. (Impact Factor-5.06)

3. Richa Kothari , Vinayak V Pathak, **Virendra Kumar**, D.P. Singh Experimental study for growth potential of unicellular alga *Chlorella pyrenoidosa* on dairy waste water: An integrated approach for treatment and biofuel production, Bioresource Technology 2012:16:466–470. (Impact Factor-5.06)

4. Meena Krishania, Virendra Kumar, Virendra Kumar Vijay, Anushree Malik. Analysis of different techniques used for improvement of biomethanation process: A review. Fuel 2013;106:1-9 (Impact Factor-3.04)

5. Meena Krishania **Virendra Kumar**, Virendra Kumar Vijay and Anushree Malik Opportunities for improvement of process technology for biomethanation processes, Green Process Synth 1 (2012).

6. Kothari Richa, **Kumar Virendra**, Tyagi V V. Assessment of waste treatment and energy recovery from dairy industrial waste by anaerobic digestion. International Journal of the Institute of Integrative Omics and Applied Biotechnology (IIOAB). special issue on environmental management for sustainable development 2012.

Book Chapters:

1. Richa Kothari **Virendra Kumar**, N L Panwar, V V Tyagi. Municipal Solid-Waste Management Strategies for Renewable Energy Options: 1st edited by Lijun Wang, 2014. pages 263-283; CRC Press., ISBN: 978-1-4665-0552-0.

2. **Virendra Kumar** and Richa Kothari, Biological hydrogen production by facultative anaerobic bacteria *Enterobacter aerogens* (MTCC 8100) recent advances in bioenergy research volume III, ISBN 978-81-927097-2-7 .

Publications in international conferences:

1. **Virendra Kumar**, Richa Kothari, Shamshad Ahmad, S. K. Tyagi. Improvement of biohydrogen production with optimized initial pH using industrial organic residue (molasses) with *Enterobacter aerogens*. International Conference on “Advances on power generation from renewable energy sources (APGRES-2015)” June 15th & 16th 2015 Rajasthan Technical University, Kota, India.

2. K Meena, **V Kumar**, VK Vijay. Anaerobic technology harnessed fully by using different techniques: Review. IEEE First International Conference on Clean Energy and Technology (CET), 2011:78-82, University of Malaya, Kualalampur, Malaysia.

Communicated

1. **Virendra Kumar**, Richa Kothari, SK Tyagi, Vinnayak V Pathak. Optimization of substrate concentration and pH for biohydrogen production from sugar cane molasses with pure culture. (*communicated in* ***International journal of Ecological Engineering*** **Impact Factor 3.47**)

2. **Virendra Kumar**, Richa Kothari, SK Tyagi. Optimization of simple sugars and process pH for effective biohydrogen production using *Enterobacter aerogens*: An experimental study (communicated in **Fuel**; **Impact Factor- 3.04**)

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4. Richa Kothari, **Virendra Kumar**, Ochieng Aoyi, V.V. Tyagi. Advancement in Biohydrogen Production Processes: A Review (*Communicated in International Journal of* ***Energy for Sustainable Development*** **(Impact Factor- 2.04**)

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Dark Fermentation: a green way to produce hydrogen and methane

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Abstract

Dark fermentation is an efficient way for the degradation of organic matter with the help of microorganism. It basically deals with the organic matter present in the solid or liquid waste. The anaerobic microbes such as Clostridium, Enterobacter, Escherichia etc. are found to be potential to degrade the organic matter in the useful product such as hydrogen and methane. The enormous type of waste such as industrial waste water , industrial sludge, and other organic waste can be degrade in the useful product such as hydrogen, alcohol and methane by dark fermentation but it is mostly perform for the production of hydrogen as well as methane. Conversion of substrate to hydrogen perform by a series of biochemical reactions and microorganisms and it is still inefficient. In the fermentative process most of the organic fraction remains as soluble fermentation products such as acetic, propionic, butyric acid and ethanol. In spite of theoretical conversion efficiency of 33% only 15% of the energy from the organic source is typically obtained in the form of hydrogen and remaining 67-85% of the substrate remains unused. One way to utilize the remaining organic matter in a usable form for energy production is to produce methane.

Keywords: *Biohydrogen, Methane, Organic substrate, Microorganism, Fermentation.*

1. Introduction

As the world resource of fossil fuel energy are depleting day by day and demand of it increasing vigorously there is need to find some alternative way for generation of energy. The increasing use of the fossil fuels pollutes the environment. The energy generation from renewable sources is only way to combat these problems. Biological routes of energy production are taking more attention nowadays because it generates energy as well as treat the waste without harming the environment. Biogas, bio-hydrogen, bio-ethanol, bio-butanol are some examples of the new forms of energy that are produced by biological routes.

Hydrogen is a promising alternative carbon based fuel compared to others because it is a clean renewable source of energy having calorific value 122 Kj/g and produces water as only by product after burning (chang *et al.,* 2002)[1] Biological production of Hydrogen is one of the alternative methods where process can be operated at ambient temperature and pressure and are less energy intensive and more environment friendly. Broadly hydrogen production processes can be classified as biophotolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria and fermentative hydrogen production from organic compounds.

Among all the known process of biological hydrogen production dark fermentation is more advantageous for mass production of hydrogen by microorganism with different types of organic industrial waste (municipal solid waste, sewage sludge) and agricultural waste that can be used as substrate for biohydrogen production (Das and Veziroglu, 2002)[2].

Table1: Comparison of biological hydrogen production processes [3, 4]

Metabolic Process	Organism	Advantages	Hydrogen yield ($mmolH_2/l.h$)	Product
Direct biophotolysis	Green algae	Can produce H_2 directly from water	0.07	H_2, O_2
Indirect biophotolysis	Cyanobacteria	Can produce H_2 directly from water	0.36	H_2, O_2
Photofermentation	Phototrophic bacteria	A wide variety of spectral light energy can be used by bacteria	0.16	H_2,CO_2
Dark fermentation	Fermentative bacteria	A wide variety of carbon source can be used as substrate	65-75	H_2,CO_2, VFA

Fermentative conversion of substrate to hydrogen is generally manifested by diverse group of bacteria such as anaerobes (Clostridia, Methylotrophs, Rumen bacteria)

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Experimental study for growth potential of unicellular alga *Chlorella pyrenoidosa* on dairy waste water: An integrated approach for treatment and biofuel production

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ABSTRACT

This communication presents an integrated approach to study the potential of *Chlorella pyrenoidosa* for treatment of dairy wastewater (DWW) and biofuel extraction. The experiment was set up in two steps. The step-1 of the experiment was designed for treatment of dairy wastewater. The physical and chemical parameters of wastewater quality such as nitrate, phosphate, chloride, fluoride, hardness, etc., were studied. The level of nitrate and phosphate known, agents of eutrophication in water bodies was reduced by 60% and 87% in influent, 49% and 83% in the effluent, respectively. The step-2 of the experiment was designed for biofuel extraction by harvesting the biomass (algal strain) grown in dairy waste water. The result of this study shows that algal strain *C. pyrenoidosa* is not only an agent for mitigation of pollutant load, but it can also be used as potential agent for biofuel production.

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1. Introduction

Dairy industry is one of the major industry have economic importance in various industries of agricultural sector. Among the various milk producing countries, India has attained the first rank in milk production. India is sharing about 13.1% of the total milk produced in the world (Kumbhar Vijay, 2010). There are about 286 large and small scale dairy industries in India responsible for large number of waste production (solid and liquid). Specifically dairy industry is noted as one of the significant contributor to water pollution. Dairy waste is basically biodegradable, but produces undesirable color and odor. It is estimated that about 110 million tonnes of milk and about 275 million tonnes of wastewater are being generated annually from the Indian Dairy Industries by the year 2010 (Kushwaha et al., 2011). Dairy waste water is characterized by strong color, offensive odor, high BOD (40–48,000 mg/l), high COD (80–95,000 mg/l) (Kushwaha et al., 2011) and variable pH (Kothari et al., 2011). It also contains sufficient nutrient like N (14–830 mg/l) and P (9–280 mg/l) required for biological growth (Rico Gutierrez et al., 1991; Gavala et al., 1999). Normally for treatment of dairy wastewater, physical and chemical methods are involves, which are often very costly. There has been an increasing interest in the treatment of dairy waste water (Wang et al., 2010). Most of the studies have concentrated on the use of fungi and bacteria for reducing the organic load of dairy waste water (Tastan et al., 2010; McMullan et al., 2001). In recent years the use of microalgae in treatment and recycling of waste water has attracted great interest due to their central role in carbon dioxide fixation. Waste water is considered to be potentially sustainable growth medium for the algal feedstock (De la Noue et al., 1992). Algal biomass caused by nutrient leakage can often be traced to large scale operations that process agricultural and animal products (Kebede-Westhead et al., 2006). Recently it has been demonstrated that most of algal species such as *Spirogyra* (Khalaf et al., 2008), *Caulerpa lentillifera* (Marungrueng et al., 2006), *Caulerpa scalpelliforis* (Arvindhan et al., 2007), *Chlorella vulgaris* (Acuner et al., 2004) are effective agents of color removal from the wastewater either by biosorption or bioconversion. Microalgae also have potential to generate significant amount of biomass considered as third generation feedstock and are suitable agent for conversion to biodiesel as they synthesize TAGs (triglycerides). Production of biodiesel from biomass can provide non-polluting and environment friendly source of energy (Barnwal et al., 2005). It is growing concern that the use of food crop for biodiesel and other renewable fuels may be an uneconomical long term solution (Patzek et al., 2005). Algae have higher biomass productivity than the crop plant in terms of land area required for biomass generation and lower cost of growth (Brennan et al., 2010). The potential value of microalgal photosynthesis to produce biofuels is widely recognized in recent years (Hu et al., 2008; Rodolfi et al., 2009). The idea of using microalgae as a source of fuel is not new and it is being taken seriously because of the rising price of petroleum and, more significantly emerging concern about global warming which is associated with burning of fossil fuel (Gavrilescu et al., 2005). However efforts are required to reduce the high cost of biofuels. Keeping in view the high cost of biomass production and environmental concerns, the present work is an effort to integrate remediation of dairy waste water

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Production of biodiesel from microalgae *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater

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HIGHLIGHTS

- Phyco-remediation for treatment of dairy wastewater and its potential for biofuel.
- *Chlamydomonas polypyrenoideum* used for integrated approach.
- Micro alga showed significant reduction in pollution load.
- Special significance to nitrate removal.
- Higher production of crude bio-oil was achieved in respect of control media used.

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ABSTRACT

This study involves a process of phyco-remediation of dairy industry wastewater by algal strain *Chlamydomonas polypyrenoideum*. The results of selected algal strain indicated that dairy industry wastewater was good nutrient supplement for algal growth in comparable with BG-11 growth medium. Alga grown on dairy industry wastewater reduced the pollution load of nitrate (90%), nitrite (74%), phosphate (70%), chloride (61%), fluoride (58%), and ammonia (90%) on 10th day of its growth as compared to that of uninoculated wastewater. The lipid content of algal biomass grown on dairy wastewater on 10th day (1.6 g) and 15th day (1.2 g) of batch experiment was found to be higher than the lipid content of algal biomass grown in BG-11 growth medium on 10th day (1.27 g) and 15th day (1.0 g) of batch experiment. The results on FTIR analysis of the extracted bio-oil through transesterification reaction was comparable with bio-oil obtained from other sources.

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1. Introduction

Industrial wastewater has now become one of the major sources of water pollution. Apart from organic carbon, wastewater also contains significant amount of organic/inorganic nutrients like nitrogen, protein, ammonia, nitrate, and phosphate, etc. (Narkis et al., 1979) which cause eutrophication in aquatic bodies, methaemoglobinaemia in infants and interference with the cardiac function in the humans (Sawyer and McCarty, 1978). Various physico-chemical treatment methods require high amount of chemicals, energy, and man power, but the biological treatment of wastewater is considered more eco-friendly and sustainable approach (Durrant et al., 1999; Franta and Wildere, 1997). Use of microalgae in biological treatment methods has attracted genuine interest due to its central role in carbon sequestration and pollution mitigation by removing major pollutants such as N (41%), P (30%), and S (30%) (Bhatnagar et al., 2010).

Wastewater can be potentially a sustainable growth medium for the algal feed stock. Use of microalgae in treatment and recycling of waste water has attracted a great deal of interest because of excessive biomass generation at cheaper cost without extra input of nutrients (Kothari et al., 2012). Microalgal biomass is considered as third generation feedstock due to presence of sufficient amount of TAGs (triglycerides). Many workers have demonstrated that most of algal species such as *Scendesmus obliquous* (Mata et al., 2012), *Arthrospira pcrtenix* (Markou et al., 2012), *Botryococcus braunii* (An et al., 2003), *Azolla microphylla*, (Arora and Saxena, 2005), *Chlorella vulgaris* (Feng et al., 2011), and *Chlorella pyrenoidosa* (Kothari et al., 2012; Wang et al., 2010a,b) can be effective tool for wastewater treatment. Use of algae can remove major inorganic contaminants like nitrogen (50%) and phosphorus (90%) from the wastewater (Wang et al., 2012).

The idea of using algal biomass as a source of fuel is not new that gained momentum due to rising cost for exploration, processing, and transportation of traditional fuels including the environmental cost (Gavrilescu and Chisti, 2005). A successful application of algal biomass for biofuel production will largely

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Title: Optimization of simple sugars and process pH for effective biohydrogen production using Enterobacter aerogens: An experimental study

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Abstract: This communication aims to find out the potential of pure simple sugars (10 g/l) as the substrate, using facultative anaerobic bacteria Enterobacter aerogens for biohydrogen production. The study revealed that glucose is the most compatible and cost effective substrate, among all the selected sugars for the biohydrogen production with the yield of 0.87mol H2 /mol of glucose consumed. The effect of process pH from initial start-up to final retention time was also analysed in the study and the maximum biohydrogen production was found at the pH 5.5 for the process. The obtained data was fit in the modified Gompertz equation and the regression coefficient (R2) was found in the range of 0.998 which provides a strong correlation between the experimental data produced and the curve fit. Hence, there is a possibility to use simple and sugar based organic substrate materials like, residues and wastewater from industries provides a suitable approach for clean environment in general and minimization of waste reduction for energy (fuel) approach in particular.

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Abstract: This communication presents the waste substrate optimization and kinetics for improvement in biohydrogen yield by anaerobic digestion of cost effective product of the sugarcane industry i.e. cane molasses inoculated with the pure culture of facultative anaerobe Enterobacter aerogens. The variation in the substrate concentrations such as 10, 20, 40 and 50 g/l were taken for the optimization of biohydrogen production. The study prevailed that the rate of hydrogen production enhanced significantly using the modified Gompertz equation. The maximum hydrogen production rate was found to be 142 ml/h from 40 g/L of condensed molasses at 30°C and initial pH 6.8. The maximum specific growth rate (μmax) of the pure bacterial culture (0.36 h-1) was also obtained with the substrate concentration of 40g/l. Various kinetics models were fitted for bacterial growth and substrate utilization in which Moser model (R2=0.98) was the best-suited model. Finally, economical bioenergy profit assessment in optimized conditions was found cost effective over other production processes (fossil fuel based) with this selected coupled substrate-strain experimental plan. Therefore, the present study strengthens the approach to use pure bacterial strain with optimized substrate concentration for the treatment and utilization of sugarcane molasses for bioenergy production.