Study on sustainable and integrated approach for the generation of low-cost algal biomass and biofuel by using agro-industrial wastewater

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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Dedicated to my daughter Ailina and Prof. D.P. Singh

DECLARATION

I hereby declare that the thesis entitled **"Study on sustainable and integrated approach for the generation of low-cost algal biomass and biofuel by using agro-industrial wastewater"** is my own work conducted under the supervision of Prof. D.P. Singh in the Department of Environmental Science, at Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow, and is also approved by Departmental Research Committee (DRC).

I further declare that to the best of my knowledge, the thesis does not contain any part of work, which has been submitted for the award of any other degree either in this university or in any other university/ Deemed University without proper citation.

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CERTIFICATE

This is to certify that the thesis titled **"Study on sustainable and integrated approach for the generation of low-cost algal biomass and biofuel by using agro-industrial wastewater"** submitted by Ms Neha Gupta is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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

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(Neha Gupta)

PREFACE

Rising demand for energy and depletion of fossil fuel has become a major concern for people around the world. Algae can be used to generate a range of renewable fuels including biodiesel, bioethanol, bio-hydrogen, etc. Microalgal biodiesel has been established as one of those sources that can fulfil the ever-increasing demand for fuel for the transport sector. Microalgae have recently gained interest due to its dual function of bioremediation of wastewater and the production of algal biomass that can be used in bioenergy production, pharmaceuticals, fertilizers and animal feed. Microalgae are usually chosen for bioremediation process for its high photosynthetic efficiency, rapid absorption of nutrients, short life span, a low nutritional requirement so that they can be easily cultivated and grown rapidly in both industrial and laboratory conditions. In addition, microalgal wastewater treatment is a more cost-effective and sustainable approach for effectively removing inorganic compounds containing nitrogen and phosphorus, heavy metals, COD and BOD. Microalgae are a single-cell photosynthetic organism capable of assimilating large quantities of carbon, nitrogen and phosphorus for their growth and production of oxygen. It has been reported that a wide range of microalgae such as *Chlorella, Scenedesmus, Phormidium, Botryococcus, Chlamydomonas* and *Spirulina* has been used to treatment of wastewater.

The aims of the present study entitle "**Study on sustainable and integrated approach for the generation of low-cost algal biomass and biofuel by using agro-industrial wastewater**" is to give scientific knowledge about the ability of microalgae *S. vacuolatus* for remediation of wastewater of the agro-industrial waste and biofuel production. Further, the present investigation an attempt to study the DCMU tolerance level, photosynthetic efficiency, biomass and lipid production of the isolated mutant strain compared to wild-type under varying environmental stress conditions. The changes in growth rate, biochemical constituents, photosynthetic efficiency of wild-type and mutant strain were measured by using the UV-Visible spectrometry, FTIR, Chlorophyll fluorescence induction kinetics (OJIP) curve and Liquid chromatography-Mass spectrometry (LC-MS), and study of biodiesel were done by Gas chromatography-Mass spectrometry (GC-MS). The present thesis is divided into eight chapters and each attempt has been made to independence and self-containment of each chapter.

Chapter I on '**Introduction**' presents the outline of the work along with the list of objectives to be accomplished in this thesis.

Chapter II is related with the '**Review of Literature**' of the review and citation of other work done by investigators in the field of microalgae, modification and improvement of algal strain, environmental stress condition, bioremediation and biofuel production.

Chapter III '**Materials & Methods**' is exclusively assigned to the general methodology used during the course of the present investigation.

Chapter IV '**Isolation & characterisation of wild-type and DCMU-tolerant mutant strain**' is assigned to the isolation of microalgal strain and DCMU-tolerant mutant strain, their morphology, and characterisation.

Chapter V '**Effect of light quality & light intensity on photosynthetic efficiency and cell constituents of microalgae *S. vacuolatus***' deals with the efforts to optimize the light intensity and spectral quality of light. Study the changes in biochemical constituents, photosynthetic efficiency and lipid content under both stress conditions.

Chapter VI '**Effect of nitrogen nutrition on the biochemical constituents in the *S. vacuolatus***' deals with the efforts to study the effect of varying nitrogen sources on the growth, biochemical constituents and nitrate reductase activity.

Chapter VII **'Effect of wastewater as a nutrient source on the biomass and biofuel production in *S. vacuolatus*'** deals with the effect of the different nutrient condition on the growth, biochemical constituents, and biodiesel production.

Chapter VIII '**General Discussion**' deal the critical analysis of the various parameters conditions followed by '**Summary and Conclusion**'.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µg	Microgram
µm	Micromolar
µl	Microliter
%	Percent
℃	Degree Celsius
ABS/RC	Absorption of energy flux per active reaction center
Chl ‘a’	Chlorophyll ‘a’
cm	Centimeter
C:N	Carbon:Nitrogen
Conc.	Concentration
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethyl sulfoxide
DTNB	5, 5- dithiobis [2-nitrobenzoic acid
EMS	Ethyl methanesulfonate
etc.	Et cetera (other similar things)
ET_0/RC	Trapping of energy flux beyond QA in the photosystem II
FAME	Fatty acid methyl ester
Fig.	Figure
FSC	Forward scatter
FTIR	Fourier Transform Infra-red
Fv/F_m	Maximum quantum yield of PSII (dark-adapted state)
F_v/F_0	Maximum quantum yield of PSII (more sensitive than F_v/F_m)
g	Gram
GC-MS	Gas Chromatography-Mass spectrometry
h	Hour
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HNO_3	Nitric acid
I_{50}	50% inhibitory concentration
IR	Infra-Red

J&K	Jammu & Kashmir
KBr	Potassium bromide
l	Liter
LC	Light curve
LC-MS	Liquid Chromatography-Mass spectrometry
LED	Light–emitting diode
LI	Light intensity
M	Molar
mg	Milligram
MDA	Malondialdehyde
min	Minute
ml	Millilitre
mM	Millimolar
MUFA	Mono-unsaturated fatty acid
nm	Nanometre
n mole	Nano mole
NPQ	Non-photochemical quenching
NR	Nitrate reductase
OJIP	Chlorophyll fluorescence induction kinetics
OD	Optical density
PAR	Photosynthetically active radiation
PBS	Phosphate Buffer Saline
PI_{ABS}	Performance index on absorption basis
PI	Propidium Iodide
psi	Per square inch
PS I	Photosystem I
PS II	Photosystem II
PUFA	Poly-unsaturated fatty acid
PWW	Poultry wastewater
QY	Quantum yield
RC	Reaction centre
rETR	Relative electron transport rate
RNO	N, N Diethyl p-nitrosoaniline

rpm	Rotation per minute
RT	Retention Time
SEM	Scanning Electron microscope
SFA	Saturated fatty acids
SGR	Specific growth rate
SH	Sulfhydryl
SOD	Superoxide dismutase
SSC	Side scatter
SWW	Soybean wastewater
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TOC	Total organic carbon
TR_0/RC	Efficiency of electron transport leading to reduction of QA
TW	Tap water
UPLC	Ultra-performance liquid chromatography
UV	Ultra Violet radiations
UV-Vis	Ultra Violet-Visible
v/v	volume/volume
Viz.	videlicet
WT	Wild-type
w/v	weight/volume

Chapter- I

GENERAL INTRODUCTION

Increasing energy demand in developing countries has given way to fierce competition for the depleting energy resources of the world (Goh et al., 2019; Pienkos & Darzins, 2009). The consumption of fossil fuels is recognized as undesirable due to loss of natural resources and the significant contribution of these fuels to increased atmospheric concentrations of carbon dioxide (Saravanan et al., 2018). Nevertheless, biofuel production using traditional terrestrial crops can only meet a small fraction of current fuel demand due to additional land requirements for growing such crops (Alaswad et al., 2015; Tyson et al., 2004). Microalgae are a promising new feedstock source for biofuel production. Microalgae photosynthesis is close to that of higher plants, and they are often more effective consumers of solar energy, contributing to the synthesis of useful biochemical products (Udaiyappan et al., 2017). The characteristics of an ideal algal biofuel-producing strain include faster growth speed, higher biomass, high oil content, quick harvesting, and low invasive species susceptibility (Chen et al., 2018; Aransiola et al., 2014). While approximately 500,000 recognized species of algae have been reported to date, but only a few species have been identified as potential sources of biofuels, and none have proved ideal (Naik et al., 2010). It is likely that it will require algae breeding and genetic engineering to develop efficient microalgae strains that are economically viable for biofuel generation. Genetic engineering can, therefore, be used to advance the production of algal biomass and biofuel, including genetic transformation, stable heterologous gene expression, as well as other current and potential molecular techniques (Sajjadi et al., 2018).

The term bioremediation was used to describe the process of removing excess pollutant from the atmosphere through the use of solid waste (leaves, animal manures and agricultural waste). Bioremediation is a method by which, under controlled conditions, organic waste is biologically degraded to a level below the defined limits set by the regulatory authority's concerned (Thassitou & Arvanitoyannis, 2001; Mueller, 1996). However, bioremediation is, by nature, the use of living organisms, mainly microorganisms, to degrade the toxins of the ecosystem into less toxic. This uses bacteria and fungi and microalgae that occur naturally to degrade and detoxify contaminants that are toxic to human health and/or the environment (Mondal & Palit, 2019). Bioremediation is less costly than other technologies used to clean up hazardous waste. Algal remediation is, therefore, the most effective management tool for managing the use of wastewater for microalgal production, which simultaneously eliminates contaminants and generates lower and lipid content biomass. By using

wastewater, microalgal cultivation can significantly reduce the operating costs of algal biomass production (Clarens et al., 2010; Lardon et al., 2009). Microalgal cultivation is more environmentally friendly and economically advantageous for lipid production combined with effluent or wastewater treatment (Soccol et al., 2016). An alternative to making the process more environmentally friendly is to enrich the growth medium of microalgae with industrial effluent wastewater and CO_2 (Ferreira et al., 2018). In short, wastewater can be considered as a source of nutrients for the growth of microalgae but excessive nutrient may have inhibitory effects on the microalgal growth. However, nutrient optimization is thus another critical factor in the development of the output of microalgal biofuel (Osundeko et al., 2019).

1.1 Algae

Algae are photosynthetic organisms which can be either prokaryotic or eukaryotic and they are known to occur in a wide range of habitats (Mata et al., 2010). The main groups of microalgae primarily differ from each other with respect to pigment composition, biochemical constituents, ultrastructure, and life cycle (Xue et al., 2011). The different microalgal groups include (Sheehan et al., 1998).

- Diatoms (Class Bacillariophyceae),
- Green algae (Class Chlorophyceae),
- Golden brown algae (Class Chrysophyceae),
- Prymnesiophytes (Class Prymnesiophyceae),
- Eustigmatophytes (Class Eustigmatophyceae), and
- Blue-green or cyanobacteria (Class Cyanophyceae)

Green microalgae are omnipresent and are located wherever water and sunlight are present. In the presence of sunlight, microalgae absorb carbon dioxide (CO_2) from their environment and convert it into carbohydrates, proteins, lipids, vitamins and pigments (Demirbas & Demirbas, 2011; Amin, 2009; Belarbi et al., 2000; Ajav et al., 1998). In freshwater, brackish, marine or hypersaline water, unicellular photoautotrophic microalgae can usually be grown (Wang et al., 2008; Belarbi et al., 2000). Depending on the species, the average size of microalgae can vary between 1 and 100 micrometres (μm) (He et al., 2003). Figure 1.1 displays typical green microalgae schematically.

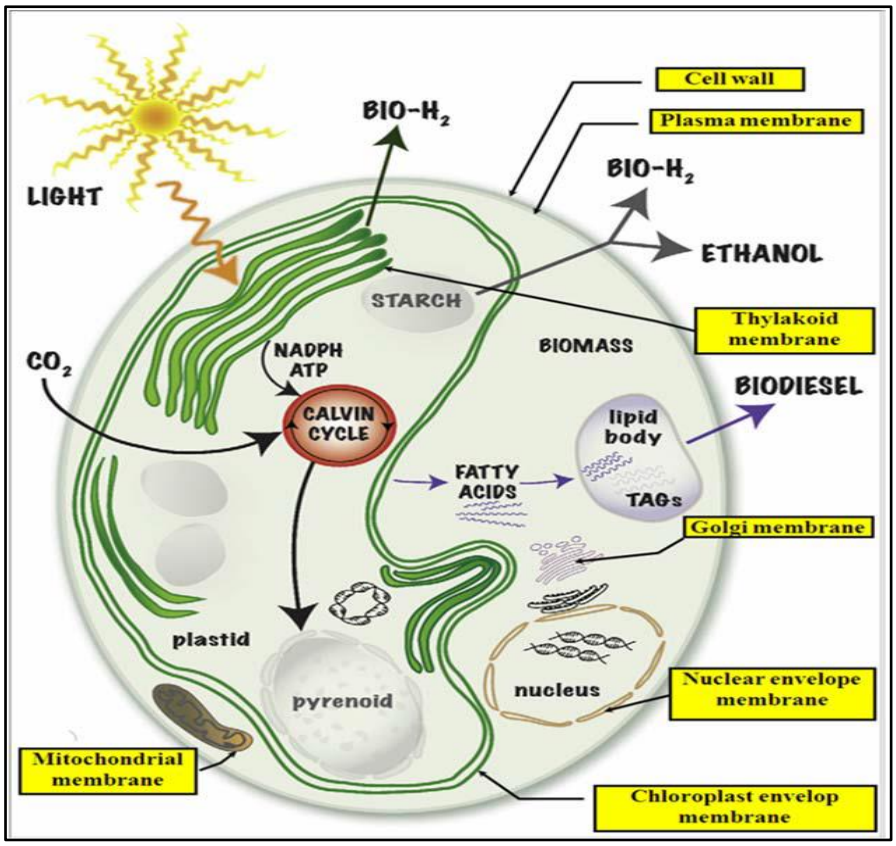

Fig. 1.1 A typical green microalgae cell schematic. **Source**: Beer et al. (2009)

Algae are non-vascular photosynthetic plants with chlorophyll as a pigment (Vonshak & Maske, 1982). The chlorophyll a is the central photochemically active pigment molecule obtaining light for photosynthesis. Photosynthetic activity is therefore regulated by the quality of chlorophyll pigment in microalgae (MacIntyre et al., 2002). The overall concentration of light-harvesting chlorophyll pigment in microalgae has an effect on biomass production as well as biochemical constituents' accumulation (Su et al., 2008).

Photosynthesis is a method of converting CO_2 into glucose and oxygen using sunlight energy. By using light irradiation as a source of energy for metabolism and growth of photoautotrophs, CO_2 is converted into organic matter in this process (Masojidek et al., 2004). Marine phytoplankton accounts for almost half of the earth's overall photosynthesis (Rubio et al., 2003). The productivity efficiency of a photosynthetic organism is mainly associated with light that affects growth and biomass production (Adeniyi et al., 2018).

1.2 Modification and improvement of algal strain

In order to fully exploit the potential of microalgae, it is crucial to invest in the basic researches on algal physiology and reactions to changing environments. In combination with genetic approaches, will enable the exploitation of novel algal strains having maximum growth and biomass production characteristics (Hlavova et al., 2015). The genetic material in living organisms is passed from parents to offspring, forming the basis for inherited characteristics. Genes, the simplest heredity modules, are passed to the next generation in compliance with a set of basic genetic rules (Borowitzka & Moheimani, 2013). After Gregor Mendel's discovery of inheritable genetic traits in the mid-19th century, the breeders of the plant have systematically manipulated these fundamental laws to generate and combine desired traits in the offspring. The researchers involved in the genetic engineering experiment (Blatti et al., 2013) propose that mutations occur as a result of organism encounters with natural environmental effectors such as UV irradiation, creating reactive oxygen species. These mutations, with potential for adaptation, are a major source of genetic variability (Barton et al., 2010; Eyre-Walker & Keightley, 2007).

While generating mutant populations can be a simple task, especially in the case of chemical mutagenesis, selecting mutants with desired phenotypes is the real strength of mutational screening (Kawaroe et al., 2015). The requisite phenotypes can be complex, including increased cell size, increased growth, tolerance to various compounds or improved productivity of a particular compound, all of which may require specific (and different) screening protocols. Because most of them are easy to apply at different doses and their mutagenic potential, chemical and physical mutagens are among the most commonly used, both basic and applied science (Sajjadi et al., 2018; Kim et al., 2012). Alkylating agents such as ethyl methanesulfonate (EMS), methyl nitro nitrosoguanidine (MNNG) are the most widely used chemical mutagens. *Nannochloropsis oculata* were first to be used in mutagenic screening to increase the production of EPA (Chaturvedi & Fujita, 2006) and to improve *Chlorella* sp. growth properties (Ong et al., 2010). Typical physical mutagens involve various types of irradiation with the varying mode of action and mutagenic potentials, such as UV, gamma and heavy-ion. This approach resulted in direct improvements in algal metabolism (Chu, 2017), as well as the production of desired compounds. Eventually, under specific conditions of cultivation, a single strain of organism could be modified

to express multiple novel genes and thus produce different products (Tabatabaei et al., 2011).

1.3 Effect of varying environmental stress conditions on microalgae

Several factors such as growth rate, environmental conditions, and life cycle influence the biochemical composition of microalgae (Richmond, 2004). Light, temperature, available carbon dioxide, pH, and nutritional conditions mainly regulate microalgal growth and chemical composition (Kamyab et al., 2019; Gatamaneni et al., 2018; Zhu et al., 1997). The effects of different conditions of environmental stress on growth and biochemical constituent's enhancement are described in detail below (Fig. 1.2).

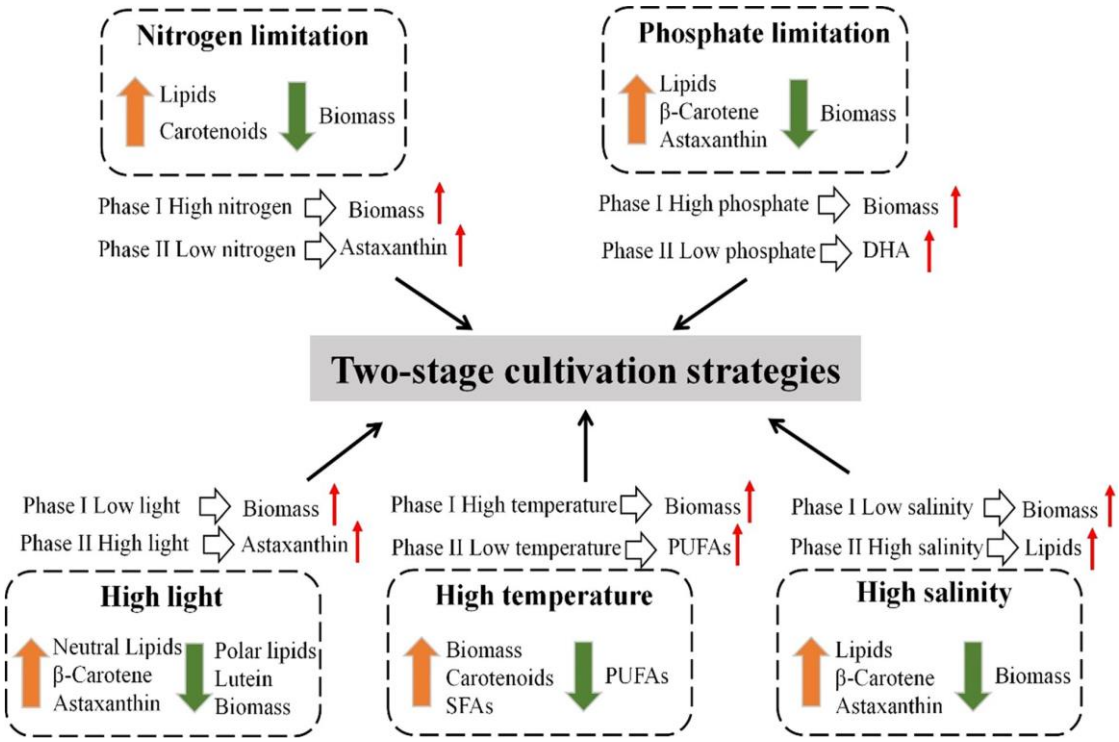

Fig. 1.2 Effects of typical nutrient and environmental stress on the production of growth and biochemical constituents in microalgae and the resulting techniques of two-stage cultivation used to impose the stress- condition to overcome the limitation of biomass. **Source**: Sun et al., (2018)

1.3.1 Light

For photo-autotrophic, light is an important source of energy and is necessary for photosynthetic activity. Research has shown that green algae grow better in the irradiation of blue and red irradiation due to produce high chlorophyll a & b pigment, as main light-harvesting pigments that absorb different spectral wavelengths (Krzeminska et al., 2014). One of the major challenges in microalgal biotechnology is the effective use of light, as it is a key factor in the yield of biomass (Barbosa et al., 2001). Photoacclimation is a process that regulates the adverse effect of light on the biochemical composition of photosynthetic algae. During this cycle, algal cells undergo changes in their cell composition structure, biophysical and physiological properties. (Khoeyi et al., 2011; Dubinsky et al., 1995).

Therefore, algal lipid metabolism and composition are also influenced by light intensity (Sforza et al., 2012). In general, high light intensities usually result in harm to polyunsaturated fatty acids. A study by Fabregas et al. (2004) found in *Nannochloropsis* sp., the degree of unsaturation of fatty acids decreases with increasing irradiance, especially the percentage of the total (n-3) fatty acids decline from 29 to 8% of total fatty acids. Thus, light stimulates the production of algal biomass whereas other studies found that stimulation in chloroplast fatty acid and membrane lipid synthesis as a result of an increase in light intensity is normally expected (Rastogi et al., 2017).

1.3.2 Temperature

The effect of temperature on biochemical reactions influencing the biochemical composition of algae can be observed since the temperature is one of the most important environmental variables (Gatamaneni et al., 2018), each microalgal species has its own optimal growth temperature requirement. The optimal temperature range increases algal growth exponentially, but an increase or decrease in temperature beyond the optimum point slows or even hinders algal growth and metabolic activity (Bechet et al., 2017). For most algal species, the ideal temperature range is 20-30°C (Singh & Singh, 2015). Changes in fatty acid unsaturation are the most common modification found in microalgae membrane lipids due to changes in ambient temperature (Pachiappan et al., 2015). In the green alga, *Dunaliella salina* (Thompson, 1996), showed the low temperature-induced modification of lipid composition. A shift in temperature from 30 to 12°C resulted in a significant increase in lipid unsaturation levels in this alga

(Thompson et al., 1996). In two green algae, *C. vulgaris*, *B. braunii* increased growth temperatures in led to a drop in the relative content of unsaturated fatty acids (Sushchik et al., 2003). Temperature is, therefore, a limiting factor for algal growth and cultivation of biomass as it directly affects the activity of the enzyme and the metabolism of the cells.

1.3.3 pH

The pH of the culture media is another important factor affecting microalgae cultivation. Microalgae species pH requirements vary from species to species. Mostly algal species usually grow well between 6 and 8.76 in the pH range (Bakuei et al., 2015). Various growth media sources of microalgae have different pH values. Therefore, many algal species are sensitive to pH, but few microalgal species can withstand a wide range of pH as wide as tolerating by *C. vulgaris* (Teixeira et al., 2010) but exhibit maximum growth rate and productivity of biomass between pH 9-10 pH (Daliry et al., 2017). An increase in pH can increase the salinity of the algal cell culture media (Juneja et al., 2013). The content of lipids is also affected by severe pH conditions during algae growth. Therefore, alkaline pH stress has been shown to increase the percentage of TAG aggregation and decrease the relative level of membrane lipids in *Chlorella* sp. (Guckert & Cooksey, 1990).

1.3.4 Carbon dioxide (CO_2)

Through burning fossil fuels that produce and accumulate greenhouse gases in our atmosphere, about 85% demand of world's energy fulfil today. The CO_2 condition in the air has risen from 260 to 380 ppm in recent decades. Some recommendations were made and experiments were carried out to mitigate the impact of human activities on the greenhouse gases (Minillo et al., 2013). The biological fixation of carbon dioxide could, therefore, be viewed as helping to alleviate this issue (Salih et al., 2011), or the successful removal of CO_2 from the point source should be noticed (Li et al., 2012). Capturing carbon and biologically sequestrating it is considered safe to reduce carbon dioxide in the atmosphere. Microalgae can be more active than terrestrial plants in trapping carbon dioxide. Selection of microalgal species is crucial to the achievement of operational carbon dioxide removal systems and the selected microalgal species depend on the strategy of carbon sequestration. The amount of carbon dioxide in the air plays a major role in the growth of microalgae, that is, the higher the level of carbon

dioxide, better growth of microalgae (Khairy et al., 2014; Salih et al., 2011). A study on the effect of varying concentrations of carbon dioxide, i.e. control (absence of carbon dioxide), 280, 385, 550, 750 and 1,050 ppm on the *Chlorella gracilis*, observed that increase in the growth up to 385 ppm concentration of CO_2 accompanied by a decrease in growth observed at 550 ppm as microalgae (Khairy et al., 2014).

1.3.5 Salinity

Every microalgae strain shows variations in its ability to adjust to salinity. Stress, caused by high salt concentrations, influences growth and lipid production. Since the two important characteristics which researchers are looking for when selecting a microalgae strain for analysis are often the algae's ability to produce high biomass and lipids, microalgae that thrive in a saline setting are given considerable importance (Asulabh et al., 2012). Saline water, such as seawater or saline groundwater, is the preferred basic source of water for sustainable commercial algae cultivation for biofuels as it does not compete with the need for freshwater or agricultural land for food crop production (Borowitzka & Moheimani, 2010). For most microalgae species, protein, lipids and carbohydrates appear to be slightly affected by a wide range of salinity. Nonetheless, an increase in lipid content at higher salinity was observed in some algal species. Studies by Gireesh et al. (2008) showed that salinity of 25 mM NaCl was ideal for growth and biochemical composition for *Chaetoceros calcitrans*. This study also showed a rise in carbohydrate content at a 35 mM NaCl of salinity while declining lipids and protein content (Gireesh et al., 2008). Another study showed that initial rise in salt concentration in *Dunaliella tertiolecta* from 0.5 M NaCl to 1.0 M resulted in an increase (60 - 67%) in intracellular lipid content (Takagi et al., 2006).

1.3.6 Mixing

Mixing and aeration maintain a consistent distribution in the microalgal culture of nutrients, water and CO_2. These also allow for the penetration and uniform distribution of light within the culture and prevent the settling and accumulation of biomass (Show et al., 2017). If all the other requirements are met but there is no mixing, there will be a significant reduction in the production of biomass. Microalgae cultures, therefore, need to be mixed continuously to hold the cells suspended with free access to light. A proper mixing system in a photo-bioreactor not only enables nutrient dissolution and light penetration into the culture but also provides efficient gaseous exchange (Zeng et al., 2011).

1.3.7 Nutrients

Microalgae cultivation requires high levels of vital ingredients (C, P, N, K, S, Fe, etc.). The emphasis is on the three most important nutrients, carbon, nitrogen and phosphorus; nutrients used for growth are either inorganic or organic compounds other than carbon dioxide and water, and their presence requires for cellular function (Dou et al., 2013). Many algal species allow different organic compounds to be synthesized by other species. Most algae, however, need only inorganic nutrients, and these algae can probably be used as feedstock for the production of biofuel. Nitrogen, phosphorus, silica (for diatoms) and iron are limiting nutrients to algae (Neenan, 1986).

Sulphur, potassium, sodium, iron, magnesium, calcium, and trace elements like boron, copper, manganese, zinc, molybdenum, cobalt, vanadium, and selenium are also essential in algal nutrition (Grobbelaar, 2004). Silicone, found in the cell walls of many algal species, is an important component of diatoms because it is an essential nutrient for their growth and biomass development. (Ren, 2014). It has been shown that nutrient deficiency significantly affects the lipid composition of the *Stephanodiscus minutulus* freshwater diatom when grown under silicon, nitrogen or phosphorus limitation (Lynn et al., 2000). In all nutrient-limited cultures, an increase across TAG accumulation and a decrease in polar lipids (as a percentage of total lipids) were noted (Lynn et al., 2000).

1.3.8 Carbon sources

Microalgae may use both organic and inorganic carbon sources for cell growth, depending on the mode of cell growth (heterotrophic, autotrophic, or mixotrophic) (Feng et al., 2011). Glucose, sucrose, lactose, galactose, acetate, glycerol, ethanol (Liang et al., 2009) and other sugars derived from starch, sugar cane, lignocellulosic biomass are the most important organic carbon sources for heterotrophic and mixotrophic microalgae cultivation (Perez-Garcia-Galan et al., 2011).

1.3.9 Nitrogen

After the carbon source, nitrogen (N) is the most essential nutrient contributing to the higher production of biomass. The nitrogen content of the microalgal biomass can range from 1% to more than 10%, depending on its availability, it can vary among different algal groups (e.g. low in diatoms) and species. (Grobbelaar et al., 2004). Discolouration of algal cells is a prevalent frequent response to nitrogen limitation due to lower

chlorophyll and increased carotenoid content, as well as accumulation of organic carbon compounds such as polysaccharides and certain oils such as polyunsaturated fatty acids (PUFAs) (Becker, 1994). Nitrogen is mostly supplied as nitrate, but ammonia and urea are frequently used, both showing similar growth rates (Kaplan & Richmond, 2017).

1.3.10 Phosphorus

Phosphorus is important for algae growth as well as for many cellular processes such as transferring energy to nucleic acid biosynthesis. Orthophosphate is the preferred form of phosphorus supplied by an energy-dependent method to algae (Grobbelaar et al., 2004). Although less than 1% phosphorus contains algal biomass, it is usually most significant growth limiting factors for algal cultivation (Neenan et al., 1986), might be phosphorus binds with other ions (e.g. carbonate and iron). Whereas phosphorus's insoluble nature makes it impossible for algal absorption (Grobbelaar et al., 2004). Similar effects are observed when algae develop under nitrogen deprivation, accumulate higher lipid content, and decrease in protein, chlorophyll, and nucleic acid content as recorded for cultures deficient in phosphate (Becker, 1994).

1.4 Utilisation of wastewater as a nutrient source for algal growth

Waste management is a major challenge for urban areas around the world. The waste generated from various anthropogenic activities could result in health hazards and have a negative impact on the environment without an effective and efficient waste management program. (Garima & Singh, 2016) Industrial and anthropogenic practices have contributed to agricultural land degradation resulting in biodiversity loss. According to some researchers, European countries generate about 5 billion tons of solid waste per year (Thassitou & Arvanitoyannis, 2001). In addition, Solid waste is probably more important because of its size, not so much because of its volume (Singh & Tripathi, 2007; Thassitou & Arvanitoyannis, 2001).

Today, many degradable methods have tackled the treatment of solid waste produced from many industries such as the agro-food industry, poultry industry, dairy and oil industries. In this study, we concentrate on the process of bioremediation, it is not only a process of removing the pollutant from the atmosphere but also an

environmentally friendly and more efficient process (Singh & Tripathi, 2007). Bioremediation is the most applicable process to detoxify or removes the pollutant from soil and water by using microorganism (Wasi et al., 2008; Talley, 2016). The aim of bioremediation is to use environmentally friendly microorganisms such as bacteria, fungi and microalgae to remove the pollutant from the environment. In this context, microalgae assimilate a significant amount of wastewater nutrients as they require high quantities of nitrogen and phosphorus for the synthesis of proteins, nucleic acid and carbohydrate comprising 45-60% of dry microalgae weight (Munoz & Guieysse, 2006).

In the microalgae reported varies the composition of nitrogen from 1% to 14% dry weight of algae and phosphorus ranges from 0.05% to 3.3% (Richmond, 2004). Several species of algae, including *Botryococcus braunii, Chlamydomonas, Scenedesmus* and *Chlorella* have been documented to be useful for nutrient removal. These contain significant quantities of lipids that can produce biofuels which are non-toxic and highly biodegradable (Abdel-Raouf et al., 2012). Algal cultivation on wastewater for biofuel production sounds quite simple as the algae have relatively low nutritional requirements for growth. Nonetheless, many factors affect the optimal growth of algae and the accumulation of lipids, including micro and macronutrients (availability and concentration), CO_2, temperature, pH and light (Saad et al., 2019).

Agro-industry, particularly the food industry, produces huge quantities of liquid, solid and gaseous waste arising not only from processing operations but also from treatment and disposal (Sadh et al., 2018). The composition and quantity of agro-industrial waste are highly dependent on the raw material origin and the nature of the goods, handling and processing of waste. In general, waste is made up of large quantities of organic material (carbohydrate, protein, fat, oil, etc.) with high levels of (BOD, COD, etc.) and suspended solids. Because of their high nutrient content, if not properly managed or processed, high potential to cause severe pollution problems (Leiva-Candia et al., 2014). Approximately 65%-70% of organic contaminants released in the water bodies. In India food and agro-industries such as distilleries, sugar factories, soybeans, dairies, canned fruit, meat processing and pulp and paper mills (Sara et al., 2012; Rajagopal, 2008) together are potentially a great source of pollution in river and water bodies. However, the released pollutant also offer a rich source of nutrients, if exploited properly (Fig. 1.3).

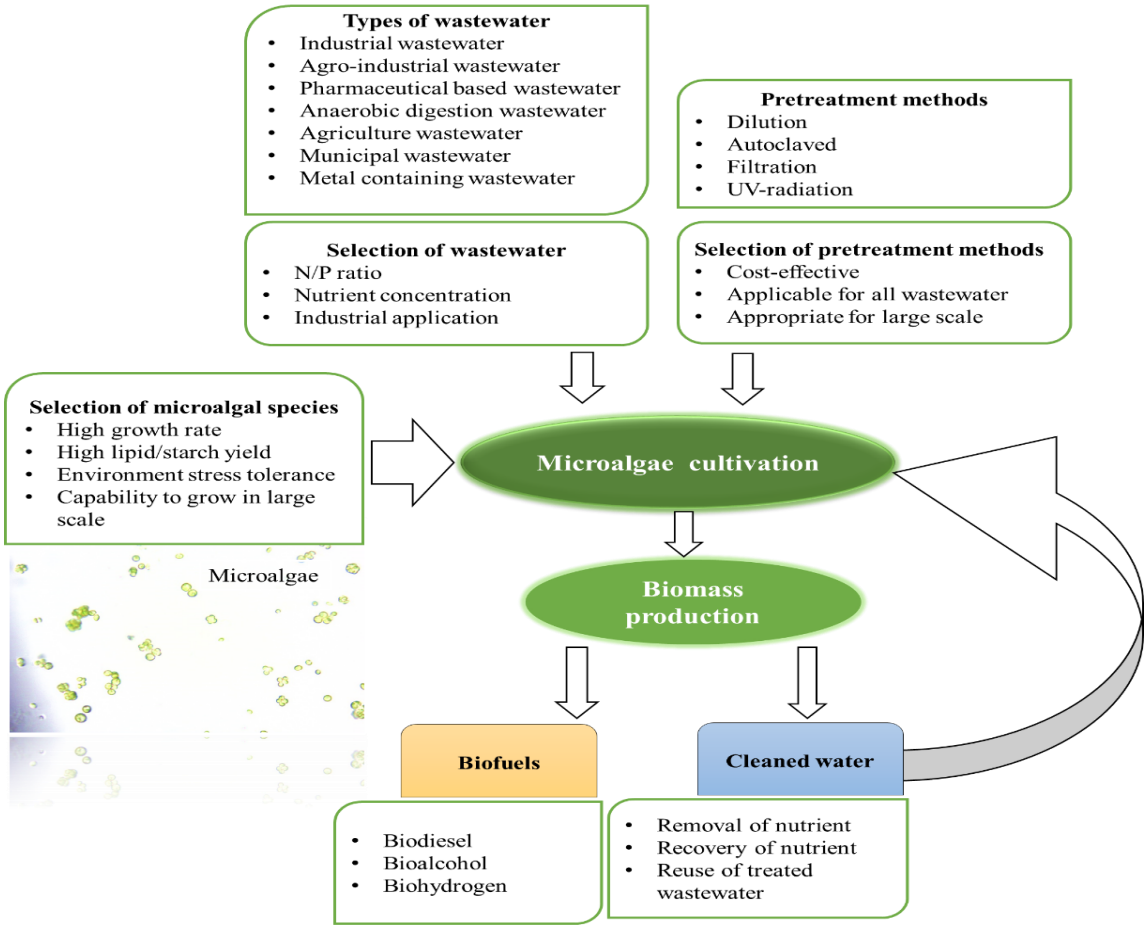

Fig. 1.3 Schematic analysis of simulations of wastewater treatment with microalgal biomass cultivation for the production of biofuel. **Source**: modified, Salama et al. (2017)

It is of great interest to use microalgae for wastewater reclamation. The use of algae in the treatment of agro-industrial waste had already been studied during the 1950s. More recently, a large amount of work was centred on agro-industrial solid waste utilisation by microalgae (Ho et al., 2019). In some cases, there have been records of decreases of 90-95% of nitrogen, phosphorus and other nutrients. Thus, it is important to choose algae with higher tolerance to organic pollutants during their growth on wastewater, as reported for genera *Scenedesmus* and *Chlorella*. (Cheah et al., 2016; Abdel-Raouf et al., 2012). Wen et al. (2017) illustrated *C. vulgaris* strain could be grown in undiluted swine slurry, with the removal of more than 90% TN and TP with high tolerance to COD. Further, various liquid and solid wastes used in the microalgal cultivation include: agricultural and agro-industrial waste (Ferreira et al.,

2018; Jayakumar et al., 2017; Kamarudin et al., 2015), domestic wastewater (Can et al., 2013; Nascimento et al., 2013; Sydney et al., 2011), livestock wastewater (Shen et al., 2008), swine wastewater (Cheng et al., 2019) etc. In addition, the advantages of microalgae used in wastewater treatment as discussed below are:

- Effective cost
- Low energy demand
- Increased output of biomass
- Reduction in sludge formation
- Remove heavy metals
- Algae produce more than 50 % oil of its biomass.
- They provide much higher yields of biomass and biofuel which provide about 10-100 times higher yield compared to other energy crops.
- They can be grown under conditions unsuitable for the growth of conventional crops.

1.5 Biodiesel production from algae

India's growing demand for petroleum-based fuel has challenged the country's energy security, with nearly 90% of its crude oil demand being imported from oil-producing countries (Ferreira et al., 2019). The transport sector consumes approximately 80% of the total energy currently generated from fossil fuels out of the manufacturing, agricultural and domestic industries. The annual diesel consumption was projected at 70 million tons at a growth rate of 6-8% per year, according to 2012-13 estimates (Kumar & Sharma, 2014). The rapid depletion of fossil fuel supplies and rising crude oil prices have made looking for alternative fuels as a replacement for diesel. Because of the similarities in diesel and biodiesel fuel properties, known as a diesel alternative. Government of India has announced Biofuels Policy in the year 2008 to promote the production and the use of biodiesel to achieve the target of blending of biodiesel (20%) with diesel by the year 2017(Kumar & Sharma, 2014).

Biodiesel is a long-chain fatty acid monoalkyl ester derived from vegetable oils or animal fats (Deshmukh et al., 2019; Ganapathy et al., 2011; Fernando et al., 2007). Biodiesel produced by transesterification of triacylglycerols from microalgal biomass is one of the most prominent sources of renewable energy (Mandal & Nirupama, 2011).

The use of microalgae as fuel feedstock was first proposed over 50 years ago for the production of methane gas (Rawat et al., 2013). Microalgae yields for biodiesel production maybe 10 to 20 times higher than those obtained from oleaginous seeds and/or vegetable oils Many microalgae have also shown a suitable non-saponifiable profile for transesterification (Arenas et al., 2017; Maity et al., 2014) which can be used for production of highly oxidized biodiesel and fuel properties (e.g. density, viscosity, acid value, and heating quality) also contained in fossil fuel properties (Sakthivel et al., 2017; Mofijur et al., 2013).

Another benefit is the use of residual microalgal constituents (carbohydrate and protein) after using the lipid component, including the generation of higher-value energy, liquid or gaseous fuels or by-products (Pienkos & Darzins, 2009). Biodiesel has advantages compared to fossil diesel because it is sustainable, biodegradable and reduces GHGs, produces less SOx and particulate emissions when burned, as well as no engine modification required (Raheem et al., 2018; Sheehan et al., 1998). Algal biofuel, on the other hand, helps to reduce waste disposal and residues and also economic and social stability in rural communities is increased. One promising approach is to combine biodiesel production with wastewater treatment since algae can be cultivated successfully in wastewater (Mallick, 2002). Several researchers documented the cultivation of microalgae in swine waste, dairy manure and other animal residues (Mehrabadi et al., 2016; Razzak et al., 2013; Mulbry et al., 2008; Kim et al., 2007; Olguin et al., 2003).

Microalgae can generate various forms of biofuels including microalgae lipid-modified biodiesel (Scott et al., 2010; Schenk et al., 2008), carbohydrate fermentation produce bioethanol (Cheng & Timilsina, 2011; Mussatto et al., 2010), anaerobic digestion produces biomethane (Paolini et al., 2018; Budzianowski et al., 2017) and photosynthesis or fermentation produce biohydrogen (Callegari et al., 2019). In addition, the whole microalgae can be converted into bio-oil (through hydrothermal liquefaction and pyrolysis), hydrochar (through hydrothermal carbonisation) and syngas (through gasification) (Capodaglio et al., 2016; Ho et al., 2014; Kaltschmitt & Streicher, 2009). Fig. 1.4 shows the different forms of algal biofuel.

Fig. 1.4 Different scenarios and conversion processes of algae for the production of biofuels. **Source:** modified, Saad et al. (2019)

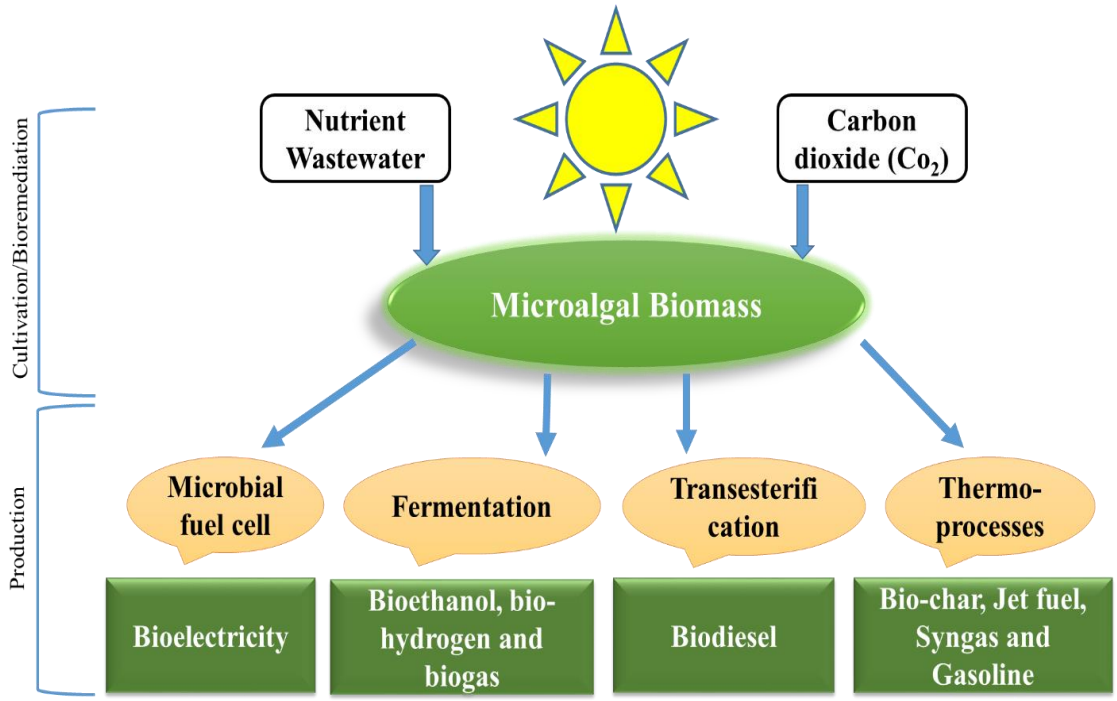

Fig. 1.4 Different scenarios and conversion processes of algae for the production of biofuels. **Source:** modified, Saad et al. (2019)

The residual biomass may be used to produce nutraceuticals, protein supplements, therapeutics, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), biocontrol agents, fertilizers, and animal feed after biofuel production (Saad et al., 2019). Biodiesel is a biodegradable fuel that, while having engine performance similar to petroleum, reduces emissions of sulphur and particulate matter (Hossain et al., 2008). Although microalgal biofuels hold great promise, their marketing poses considerable challenges. More research efforts needed to make microalgal biofuels cost-effective and sustainable technology by selecting and bioengineering of microalgal strains as good quality biofuel producers; optimizing culture conditions for the large amount production of microalgal biomass and biofuels; designing bioreactors suitable for large-scale microalgal cultivation; improving the efficiency of microalgal biomass harvesting and downstream processing with reducing production commercialisation costs and energy consumption (Callegari et al., 2019; Scott et al., 2010). For several reasons, microalgae have gained considerable attention in recent years as a biodiesel feedstock including:

• Accumulation of larger amounts of lipids due to higher efficiency in photosynthesis (He et al., 2003)

• Rapid growth rates resulting in doubling of algae biomass within 24 hours (Chisti et al., 2007)

• Ability to absorb solar energy 10 to 50 times higher than that of terrestrial plants (He et al., 2003).

• Lack of competition with agricultural crops for land requirement (Chisti et al., 2010)

• Improvement of energy security through domestic biomass production ability

• Economic and social development of rural communities

1.6 Challenges in algal biodiesel production

Compared to traditional crops, microalgae offers different advantages for biodiesel production (Chisti et al., 2007). Nonetheless, the microalgae could not be commercially successfully cultivated (Greenwell et al., 2009) and there are many barriers affecting viability on a large scale:

• In algal biofuel study, one of the challenges is to find a species that is suitable across a wide range of factors including environmental sensitivity, high growth rates, high lipid content, and simple harvesting and extraction procedure (Greenwell et al., 2009).

• Biochemical lipid composition is another obstacle to biofuel properties relative to other conventional feedstocks (Chisti et al., 2008).

• The most problematic area of algal biofuel processing engineering is the harvesting, extraction and recovery of biomass from microalgal culture (Greenwell et al., 2009).

• Globally, there are very few companies producing microalgae biofuels on a large scale, which in turn limits the amount of biodiesel available for downstream processing research.

1.7 Following the objectives of this study

i. Isolation and identification of the algal strain.

ii. Physico-chemical and genetic manipulation of the algae to enhance lipid production.

iii. Study on growth characteristics and optimisation of growth condition.

iv. Study of lipid content in the isolated algae under varying physiological and environmental conditions.

v. Study on phycoremediation of agro-industrial wastewater by alga and their effects on algal biomass production (wastewater treatment).

vi. Study on the biofuel production efficiency of algal biomass growing in the presence of agro-industrial wastewater.

Chapter- II

REVIEW OF LITERATURE

Energy security and environmental pollution are a major problem in the changing world that is being addressed jointly by organizations and government around the globe. Microalgae are considered as a possible solution to the twin problems i.e., feedstock for a carbon-neutral fuel and a bio-mitigating agent for wastewater (Chang & Posten, 2016; Mata, 2010). If the resources are sustainably acquired and effective techniques are implemented, microalgae have immense potential to mitigate the pollution load of wastewater. The impacts and performance of biomass production and their uses are regional and site-specific (Popp et al., 2014). Biodiesel is one of the most renewable fuels, as well as non-toxic and biodegradable. However, algal biofuel potential is expected to meet the growing fuel demand (Wu et al., 2012; Amaro et al., 2011; Khan et al., 2009). However, based on its financial and sustainability assessments, this projection is still far from the fact (Kovacevic & Wesseler, 2010). Based on the present understanding, microalgae are likely to generate biofuel that is competitive with fossil fuels and cost-effective without significant technological improvements (Fernandez et al., 2019). Most important improvements in the production of algal biofuels can be anticipated with improvement in the choice of strain, as well as improved cultivation, harvesting and oil extraction technology (Pittman et al., 2011). In order to minimize hurdles and enhance the economic and sustainability parameters of algal biofuel policy, investigation of microalgal biomass production with wastewater has been suggested (Pittman et al., 2011). The integrating microalgal cultivation in the wastewater for remediation of pollutant and production of biofuels can speed up the marketing of algal biofuels in the near future (Saravanan et al., 2018).

Because of the rapid development in technology, elevated biomass yield, lipid and carbohydrate content, microalgae have enormous potential for use in biofuel production as a third-generation feedstock. The biofuels generated by algae includes biodiesel, biogas, bioethanol and bio-methane (Saad et al., 2019; Li et al., 2008). The selection of microalgae for cultivation and biomass production is very significant. Preference be given to species that can grow effectively in the wastewater and generate greater biomass and biofuel (Singh & Gu, 2010). Shriwastav et al. (2014) noted that green microalga such as *Chlorella* are stress-tolerant and have the ability to accumulate higher amounts of lipid. Hence, the choice of stress-tolerant and high lipid-producing species is very crucial for dual applications, i.e. wastewater treatment and biofuel production (Chisti, 2008; Amin, 2009). Algae that grow faster even with low lipid

content will also be ideal as it will reduce production time and energy requirements (Hlavova et al., 2015). During the different stages of growth, microalgae undergo biochemical changes, i.e. from lag phase to late stationary phase. Usually, lipid content in cells is more in late stationary phase than in the logarithmic phase (except for few species such as *Tetraselmis* sp. where lipid content is greater during the logarithmic phase) (Brennan & Owende, 2010; Demirbas, 2010). Table 2.1 shows the lipid content of different microalgae species.

Table 2.1 Lipid content (% dry matter) of microalgal species

Microalgal species	Lipid Content (% dry matter)
Spirulina platensis	4.0-16.6
Spirulina maxima	4–9
Botryococcus braunii	25-80
Phaeodactylum tricornutum	20-30
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35–65
Dunaliella salina	14–20
Dunaliella bioculata	8
Chlamydomonas reinhardtii	21
Chlorella minutissima	57
Chlorella sorokiana	19-22
Chlorella vulgaris	14–40/56
Chlorococcum sp.	19-20
Scenedesmus sp.	19-21
Scenedesmus dimorphus	6–7/16–40
Scenedesmus obliquus	11–22/35–55
Synechococcus sp.	11
Tetraselmis suecica	8.5-23.0
Tetraselmis sp.	12.6-14.7
Porphyridium cruentum	9-14
Prymnesium parvum	22-38

Source: Illman et al. (2000); Chisti, (2007); Wu et al. (2012)

The process of waste management (WM) is considerably different amongst the advanced countries. Due to the lack of adequate waste collection and disposal mechanisms in developing countries. As a result, waste management has become very critical and is given priority owing to gradual environmental degradation and sustainability concerns (Rahman & Muyeed, 2010; Brewer, 2001). Uncontrolled, haphazard and unscientific means of dumping the waste on the outskirts of cities and villages have resulted in overflowing landfills that are impossible to return them to the appropriate condition. This situation but also have solemn environmental consequences in terms of soil and groundwater pollution and contribute to worldwide global warming (Divya & Santhanam, 2019). Therefore, it is very essential to clean the polluted environment in a sustainable manner. In this respect, the function of microorganisms in the biodegradation of contaminants has drawn considerable attention in recent years (Banerjee et al., 2018b; Brar et al., 2017). Various biotechnological technique based on microorganisms such as bioremediation, biodegradation, biocomposting and biotransformation has been used to efficiently collect and degrade the environmental contaminants (Banerjee et al., 2018b). Dahlia & Hassan, (2018) found that *Cladophora* sp. (green algae) has high potential to bioaccumulate toxic metals and thus, it can be used as a powerful tool for alternative waste management. In addition, microbial ecology has a significant role in the ecosystem functioning of the wastewater. The use of microalgae as a sustainable means of wastewater remediation and the production of value-added products has been given considerable attention for decades (Saad et al., 2019; Lohrey & Kochergin, 2012; Ruiz-Marin et al., 2010; Colak & Kaya, 1988).

A broad range of microorganisms such as *Bacillus* sp., *Corynebacterium* sp., *Staphylococcus* sp., *Streptococcus* sp., *Scenedesmus platydiscus*, *S. quadricauda*, *S. capricornutum*, and *Chlorella vulgaris*, have been used efficiently for degradation of environmental contaminants (Brar et al., 2017; Udaiyappan et al., 2017; Abinandan & Shanthakumar, 2015). In addition to its remediation potential, microalgal growth in the wastewater can provide a source of biomass for biofuel production and other future applications such as the cultivation of biomass for use as animal feeds (Salama et al., 2017). Besides the ability to remove contaminants (nitrogen and phosphorus), microalgae provide the opportunity to recycle chemicals in the tertiary treatment phase in an environment friendly and cost-effective manner (An et al., 2003; Aslan & Kapdan, 2006). Further, higher cost of biofuel production could be minimized by combining

wastewater treatment with biomass production (Cheah et al., 2016) and CO_2 sequestration (SundarRajan et al., 2018; Kadir et al., 2018). Improving microalgal strains for efficient cultivation in the presence of wastewater and subsequently for biofuel production under changing environmental conditions, may depend on physico-chemical factors (Banerjee et al., 2018a). The following important factors involved in the regulation of biomass affecting the production biofuels are discussed below.

2.1 Modification and improvement of microalgal strain

As potential tools for producing different important commercial products, including chemicals, pharmaceuticals, food supplements and biofuels, algae have attracted the wide interest of researchers in recent years (Banerjee et al., 2018a). The research on algal physiology research (including algal biotechnology) dates back to 1950s when the commercial potential of algae was first recognized and exploited (Aach, 1952; Geoghegan, 1951). Over the previous, significant development has taken place in the field of algal physiology, growth regulation, optimization of growth condition and improving the energy equilibrium for biofuel production (Chang & Posten, 2016). However, the present knowledge is mostly confined to wild type strains of various origins till to this date. Different strains or species of algae are traditionally used for distinct reasons, so particular fast-growing strains are used for the production of biomass, while others are used for the production of commercial products such as astaxanthin, eicosapentaenoic acid (EPA) and oils (Pulz & Gross, 2004; Trentacoste et al., 2013). An understanding of the physiology of microalgae is necessary for the production of many biotechnologically and economically important products. Therefore, the selective breeding of strains for improved growth of microalgal (Georgianna & Mayfield, 2012). It is therefore essential to invest in basic research on algal physiology particularly with respect to their and responses to environmental stress is necessary for full exploitation of their potential. Combined with genetic methods, this understanding of new algal strains can be exploited with optimized growth and production features. Advances in the molecular biology and genetic tools needed to characterize microalgae sp. have permitted to develop better traits suitable for biofuel production (Banerjee et al., 2016). In recent years, various tools have been created to target plastids and nuclear genomes, such as metabolic selection markers and technique such as ‘CHYSEL’ (Rasala et al., 2014).

The use of a genetic approach to improving the production of desired metabolites in green algae, including the exploitation of basic researches on model organisms, is still in an infancy stage. However, microalgal genetic modification offers possibilities for accurate control of target mechanisms, leading to an increase in lipid accumulation under ordinary growth condition (Chu, 2017). To date, more than 40 microalgae species have been recorded for biofuel production. However, account the difficulties presented by these in the biofuel production is enormous (Gangl et al., 2015; Doron et al., 2016).

Sun et al. (2018) evaluated multiple approaches to enhance the production by microalgal lipids through metabolic engineering and discovered that manipulation of multi-genes is more advantageous than editing of a single gene. In order to reveal new findings on algal starch synthesis in *C. reinhardtii*, the insertion mutagenesis test was carried out. It was found that the mutant lacked a tiny subunit of adenosine diphosphate (ADP)-glucose pyrophosphorylase, resulting in accumulation of less than 0.01% of the starch content present in the wild-type (Zabawinski et al., 2001). Random mutagenesis offers many benefits as it requires only a small amount of an organism's genetic information and it is easier to carry out (Kawaroe et al., 2015). Lee et al. (2014) revealed that random mutagenesis mediated by ethyl methanesulfonate (EMS) is regarded as an appropriate and strong technique inducing point mutations by modulating the A-T to G-C content in the DNA. A study by Anandarajah et al. (2012) recorded an increase in *Nannochloropsis* sp. biomass and lipid production, but without much changes in the structure of fatty acids where the concentration of methyl myristate and methyl palmitate fatty acids increased by 63%. Another research by Ong et al. (2010) showed that the mutant of *Chlorella* sp. showed much higher specific growth rate and lipid productivity are greater relatives on wild-type. Chaturvedi & Fujita, (2006) indicated that concentration of fatty acids increased after mutation of *Nannochloropsis oculata,* and they observed the levels of myristate acid and palmitate acid more prominent than other fatty acids after mutagenesis by EMS. Another research by Doan & Obbard, (2012) showed that lipid content and fatty acid improved the different growth stages (both exponential and stationary phase) of *Nannochloropsis* sp., in comparison to wild type. Whereas Kawaroe et al. (2015) discovered that EMS mutagen did not influence the content of carbohydrates and proteins, but increased the only lipid content of the *Nannochloropsis* sp.

Manipulations in microalgal cells by stressors such as the depletion of nutrients, variable light intensity, temperature, salinity, and pH are traditionally used to increase the accumulation of lipids within the biological boundaries of cells (Bartley et al., 2014; Chu et al., 2015; Suyono et al., 2015). According to Belotti et al. (2013) nitrogen starvation is the most efficient for lipid improvement relative to other stress conditions. Dual-stage cultivation (Doan & Obbard, 2014) and chemical additive with co-culture methods (Singh et al., 2016) help improve lipid output in microalgae.

2.2 Effect of varying environmental stress conditions on microalgae

Although microalgae's growth rate and cell structure differ from species to species. Most of the reports agree that microalgae may grow quicker but produce only a tiny quantity of lipid or carbohydrate under the environmental favourable condition (Kamyab et al., 2019). It has been suggested that lipid or carbohydrate generally start accumulation in microalgal cells only under physico-chemical stress condition, such as extremes of light, temperature, pH, CO_2, accessibility of nutrients, salinity and levels of metals (Chen et al., 2013, Khan et al., 2018). These stresses at a higher intensity, often result in the inhibition of growth resulting in lower productivity of biomass and a higher risk of contamination (Gatamaneni et al., 2018). In order to improve the economic feasibility of using the microalgae for the production of biofuels, greater knowledge is still required as to how fast accumulation of lipids and carbohydrates in microalgae cells with higher cells growth rates. Manipulation of environmental stress during microalgal cultivation (Chen et al., 2017) can a strategy to enhance the lipids and carbohydrates accumulation as shown in Table 2.2 and discussed below.

2.2.1 Light

Light plays a major role in photosynthesis, influencing growth, CO_2 fixation rate, and cell composition of microalgae (Ho et al., 2012, Hu et al., 2008). The effect of light that can fall into one of the three classifications, namely light restriction, light saturation, and light inhibition, immensely affects the microalgal growth (Ho et al., 2012). The growth rate of microalgae species rises with increasing light intensity before reaching the phase of light inhibition, although the magnitude of the required light intensity

differs from species to species. Liu et al. (2012) reported that the biomass productivity of *Scenedesmus* sp. 11-1 was raised from 255 to 452 mg $L^{-1}d^{-1}$ when the light intensity was increased from 50 to 250 μmol $m^{-2}s^{-1}$. Ho et al. (2012) revealed the biomass productivity of *S. obliquus* CNW-N increased nearly 3-fold with an increase in light intensity from 60 to 420 μmol m^{-2} s^{-1}. According to Rismani-Yazdi et al. (2012), exposure of microalgal to an environmental stresses result in a competition between TAG and starch synthesis; and the type of energy-producing compound getting accumulated in the microalgal cell appears to be a species-specific phenomenon.

Sun et al. (2014) suggested that light intensity plays an important role in the accumulation of lipids and carbohydrate in microalgae. As demonstrated in Table 2.2, the accumulation of TAG or carbohydrates increases in the increasing light intensity leads to simultaneous enhancement of cell growth in some microalgae. Typically, a sufficient light leads to decrease in the total polar lipid content and a significant increase in the content of neutral lipids (primarily TAG) as energy-storage compounds (Khotimchenko & Yakovleva, 2005, Sukenik et al., 1989). Unfortunately, the correlation between light supply and carbohydrate synthesis in microalgae remains unclear, although it has been accepted that generation of the precursors for sucrose and starch synthesis can be stimulated by light irradiance (Champigny, 1985). In *Scenedesmus obliquus*, exposure of cells to sufficient light intensity (e.g., 180 μmol $m^{-2}s^{-1}$) strongly increases the percentages of palmitic (C16:0), stearic (C18:0), and oleic (C18:1) acids in total TAG as well as the percentage of glucose in total carbohydrate (Ho et al., 2012). Another study demonstrated that the content of major fatty acids, including palmitic (C16:0), stearic (C18:0), and oleic (C18:1) in the microalga *N. oleoabundans* can be significantly increased by culturing the cells under high-intensity light (300 μmol m^{-2} s^{-1}) (Sun et al., 2014). Based upon these studies, it seems clear that high light intensity can trigger the production of the saturated and monounsaturated fatty acids that are the primary components of neutral lipids (Hu et al., 2008). However, there is still no clear information regarding the dependence of microalgal carbohydrate profiles on irradiance level (Ho et al., 2012).

2.2.2 Temperature

Temperature is one of the most important factors in microalgal cultivation and is highly correlated with the growth rate. Typically, the higher growth rate of microalgae can be achieved by increasing the temperature to its optimal level (Gonzalez-Fernandez &

Ballesteros, 2012). Variations in temperature regime also play an important role in the accumulation of lipids and carbohydrates in many species of microalgae. The lipid content of *Monoraphidium* sp. SB2, *N. oculata*, and *Scenedesmus* sp. LX1 decreases as the temperature are increased from their respective optimum growth temperature (Converti et al., 2009; Wu et al., 2013; Xin et al., 2011). However, James et al. (2013) have shown that the TAG content of *C. reinhardtii* BAF-J5 is positively correlated with the cultivation temperature. Therefore, it can be said that the effect of growth temperature on the lipid content of microalgae varies from species to species. In addition, the suitable temperatures for lipid accumulation often cause the inhibition of cell growth, eventually resulting in lower lipid productivity. As for as carbohydrate accumulation is concerned, it has been reported that the carbohydrate content of *Chlorella vulgaris* SO-26 increases by 20-30% when the ambient temperature is decreased from 20 to 5℃ (Hosono et al., 1994). The enzymes involved in carbohydrate synthesis (e.g., starch synthase and sucrose synthase) are known to be influenced by the ambient temperature condition of the culture (Gonzalez-Fernandez & Ballesteros, 2012). However, some researchers have pointed out that the effect of temperature on the carbohydrate accumulation in microalgae still remains unclear (Chen et al., 2013b). In addition, temperature appears to affect the TAG profile in microalgae, as the proportion of saturated fatty acids increases with increasing temperature (Hu et al., 2008; Wu et al., 2013).

2.2.3 pH

The pH of the culture medium is an important factor, influencing many biological processes associated with microalgal growth, metabolism, and uptake of ions (Khalil et al., 2010). In general, the optimum pH for growth is also species-specific. For instance, Khalil et al. (2010) reported that the optimal pH for the growth of *D. bardawil* is about 7.5, whereas that for *C. ellipsoidea* is about pH10. In addition, if pH of the culture medium is raised, the TAG content increases in some species of microalgae, but this comes at the cost of a sharp decline in the growth rate (Breuer et al., 2013; Gardner et al., 2011; Santos et al., 2012). These data suggest that high-pH condition not only inhibits the cell cycle but effectively triggers the cellular lipid accumulation. Moreover, Santos et al. (2012) also demonstrated that high-pH stress not only increases the lipid content but also improves the lipid quality. At a high pH 10, the major fatty acids produced by *N. oleoabundans* include palmitic (C16:0), stearic (C18:0), oleic (C18:1),

and linoleic (C18:2), all of which are considered suitable for biodiesel production. In contrast, Khalil et al. (2010) found that the carbohydrate content of both *D. bardawil* and *C. ellipsoidea* is highly correlated with biomass concentration, indicating that use of an appropriate pH can significantly improve both growth and carbohydrate accumulation in these species.

2.2.4 Salinity

Similar to other environmental factors, salinity also plays a vital role in the accumulation of lipids/carbohydrates in microalgae. However, high salinity can slow down the growth of some halotolerant species, such as *Chlorococcum* sp. (Harwati et al., 2012), *Dunaliella* sp. (Takagi et al., 2006), and *Botryococcus braunii* (Zhila et al., 2011). The literature shows that lipid/carbohydrate accumulation in microalgae increases in response to an immediate salinity shock. Harwati et al. (2012) found that *Chlorococcum* sp. cultivates for 10 days in the presence of NaCl concentration (0 to 2%) leads to 10.3 to 29.8% increase in the lipid content. Similarly, other reports showed TAG content increases in the microalgae significantly in the presence of NaCl concentration (Takagi et al., 2006; Zhila et al., 2011). Moreover, the starch content of *Chlamydomonas reinhardtii* cultivated in medium containing 100 mM NaCl is 4-fold higher than that obtained with the cultivation of microalgae in the freshwater (Siaut et al., 2011). The fatty acid composition is also affected significantly by changes in the salinity of the culture medium (Zhila et al., 2011). In *Dunaliella* sp. and *Botryococcus braunii*, for instance, an increase in the salinity significantly decreases the polyunsaturated fatty acids (PUFA) content (e.g., linoleic acid (C18:3) and increases the proportions of saturated fatty acid and monounsaturated fatty acids, such as palmitic (C16:0), stearic (C18:0), and oleic (C18:1) acids, a composition that is more suitable for quality biodiesel (Takagi et al., 2006; Zhila et al., 2011). In addition, it has been reported that the sucrose pathway in microalgae can be altered by the addition of NaCl to the culture medium (Gonzalez-Fernandez & Ballesteros, 2012). However, changes in the salinity level also affect the starch accumulation in microalgae and it has been suggested that on yet to be fully elucidated. The increase in salinity leads to the oxidative stress causing an increment in the triacylglycerol (TGA) content. Overall, unfavourable conditions for cell division stimulate the accumulation of neutral lipids. Thus, we have to strike a balance between the growth and lipid content under stress condition, so that quantum of biomass is not reduced.

2.2.5 Carbon dioxide (CO_2)

During photoautotrophic microalgae, CO_2 provide carbon for photosynthesis. For the optimal growth requirement of microalgae needs a sufficient amount of dissolved CO_2. In general, CO_2 concentration increases to an optimal level, biomass and lipid production increases. The aeration of the gas mixture with an appropriate concentration of CO_2 can meet the requirement of photosynthesis. Li et al. (2013) speculated that aeration by pure air resulted in a decline in the growth and lipid production of microalgae *Parachlorella kessleri*. According to Gardner et al. (2013) limited amounts of CO_2 available for microalgae to growth slows down the metabolism, resulting in a decline of lipid. In order to reduce the costs of microalgal biomass production, flue gas (rich in CO_2) can be used as a carbon source in microalgal cultivation systems. Nonetheless, a very high CO_2 concentration can also influence microalgae growth, as unused CO_2 in cultivation can be converted to carbonic acid (H_2CO_3), which can lower the pH of the medium (Sibi et al., 2016). Increased production of biomass and lipids, therefore, requires optimal levels of CO_2 depending on the species of microalgae. Microalga *Chlorella vulgaris* cultivation below 8% (v/v) CO_2, found maximum lipid productivity (29.5 mg L^{-1} day^{-1}) (Montoya et al., 2014). In addition, Widjaja et al. (2009) showed growth and lipid production in *C. vulgaris* increasing with increasing CO_2 concentration. *Chlamydomonas* sp. JSC4 strain showed higher lipid content (65.3%) and productivity (169.1 mg L^{-1} day^{-1}) when providing 4% (v/v) CO_2 (Nakanishi et al., 2014). Ota et al. (2009) reported maximum lipid content (34%) in the microalgae *Chlorococcum littorale* was achieved when the cells cultivated with 5% CO_2 concentration.

However, at high levels of CO_2 (above 20% for many strains) biomass productivity was reduced and in some cases, it ceases depending upon the cell density of the culture and pH condition (Chiu et al., 2009). Some strains such as *Nannochloropsis oculata* grow much more effectively in 2% CO_2 than in air, but above 5% CO_2 level growth is suppressed (Hsueh et al., 2009). In a study by Chiu et al. (2008), *Chlorella* sp. was shown to have higher CO_2 removal capacity but lower productivities at low CO_2 concentrations (Cheng et al., 2006). At higher concentrations of CO_2, more carbon was sequestered and biomass productivity was enhanced, High CO_2 levels (30 to 50%) have been shown to favour the accumulation of total lipids and polyunsaturated

fatty acids in certain microalgae (Chiu et al., 2008; Tang et al., 2011). Xu et al. (2004) reported that high CO_2 concentration inhibited the efficiency of photosystem II. Overall, the CO_2 tolerance of microalgae is dependent on the cell density (Chiu et al., 2008), pH (Olaizola, 2003), nutrients and light (Soletto et al., 2008; Tang et al., 2011). Recently, Hu et al. (2016) screened 10 strains of *Chlorella* for effective CO_2 sequestration and biodiesel production when grown in municipal wastewater. One species of *Chlorella* showed the highest CO_2 removal efficiency (35.5%) in cultures aerated with 10% CO_2, while it also accumulated the highest amount of lipids (58.5% dry weight).

2.2.6 Nitrogen

Nitrogen source is the most critical factor in terms of its effect on lipid and carbohydrate accumulation in microalgae (Chen et al., 2013b; Chisti, 2007). A wide variety of studies have demonstrated that microalgae tend to allocate their carbon molecules to energy-rich lipids or carbohydrates when they encounter conditions of nitrogen deficiency (Hu et al., 2008; John et al., 2011; Siaut et al., 2011). Nitrogen starvation causes the microalgae cells to synthesize lipid in the form of triglyceride fatty acids that are used as carbon and energy storage (Van Vooren et al., 2012). Nitrogen limitation may cause a sharp increase in microalgal lipid/carbohydrate content, along with a marked drop in the protein and chlorophyll content of cells in response to extreme condition (Ho et al., 2012; Sun et al., 2014). Su et al. (2011) reported that 48% lipid content in the microalga *N. oculata* was achieved when the organism was cultured under nitrogen-depletion condition for 4 days. In addition, cultivation of *S. obliquus* CNW-N and *C. vulgaris* FSP-E under nitrogen-limiting conditions resulted in a dramatic increase in the cellular carbohydrate content, from 21 to 49% and 15 to 51%, respectively (Ho et al., 2013a; Ho et al., 2013b). *Scenedesmus* sp. grown on nitrogen or phosphorus starved condition showed higher lipid content (30% and 53%, respectively) (Xin et al., 2010). According to Illman et al. (2000) lipid content (40%) of *Chlorella vulgaris* was increased significantly in nitrogen limiting condition. For higher lipid/carbohydrate accumulation, nitrogen limitation showed a strong influence on both the lipid (fatty acids) and carbohydrate profiles of many microalgae (Ho et al., 2012). Several reports have shown that nitrogen depletion leads to major increases in the proportions of saturated (e.g., palmitic acid) and monounsaturated (e.g., oleic acid) fatty acids, which are primary constituents of biodiesel (Siaut et al., 2011).

Nutrient limitations will affect the photosynthetic capacity of microalgae, and it also exerts severe limitation on cell division and triglyceride lipid synthesis (Reitan et al., 1994). Some species are better adapted than other species to the under nutrient limiting conditions. For example, *Nannochloropsis gaditana* is able to retain its chloroplast structure and photosynthetic capacity for a longer period of nitrogen starvation than *Neochloris oleoabundans* (Klok et al., 2014). Species with high biomass productivity under unfavourable conditions is beneficial for lipid production (Breuer et al., 2012). Roopnarain et al. (2015) found that nitrogen concentration is directly proportional to biomass productivity. Due to this, the overall lipid productivity under nitrogen-starved conditions may actually be lower than normal growth conditions (Chu et al., 2013). In addition, molecular and cellular responses related to nitrogen deficiency may provide potential gains by driving the metabolic flux toward lipid production. Similar studies have been conducted with oleaginous microalgae, such as *Nannochloropsis oceanica* IMET1 (Li et al., 2014; Dong et al., 2013), *Nannochloropsis gaditana* (Carpinelli et al., 2013), *Nannochloropsis* sp. (Liang et al., 2013) and *Neochloris oleoabundans* (Rismani-Yazdi et al., 2015) to elucidate the mechanism of oil synthesis in a better way.

2.2.7 Phosphorus

Besides nitrogen, phosphorus is another critical essential nutrient which determines the growth and biomass as it is a key element for nucleic acids, adenosine triphosphate (ATP) and phospholipids (Solovchenko et al., 2016). Microalgae utilise phosphorus in two steps; the first step is to use the phosphorus for construction of organic cellular components such as phospholipids, and in the second step, the residual phosphorus is used to produce inorganic polyphosphate granules (Schmidt et al., 2016). Wastewater contains nitrogen and phosphorus that can be converted into biomass and other bio-products by microalgae. Phosphorus starvation has lesser effects on photosynthesis in microalgae in comparison to nitrogen starvation (Kamalanathan et al., 2015). In a study on the *Chlamydomonas reinhardtii* growing under phosphorus-limiting condition, it is observed that the number of ribosomes is reduced to balance the protein synthesis and polyphosphate storage granules (Chen et al., 2017). In another case, using phosphate starvation of *Monodus subterraneus*, induces a six-fold increase in the lipid production (Khozin-Goldberg et al., 2006). Meanwhile, Chu et al. (2013) have demonstrated that

the biomass production of *Chlorella vulgaris* under phosphorus sufficient nitrogen-starvation condition exhibited response similar to that of nitrogen-sufficient condition, where maximum lipid content (58.39 mg/L/day) was achieved. Phosphorus limitation results into increase lipid content, particularly the TAG as observed in case of *P. tricornutum, Chaetoceros sp., Isochrysis galbana* and *Pavlova lutheri*, but the lipid content in *Nannochloris atomus* and *Tetraselmis* sp. was reduced under phosphorus starved condition (Reitan et al., 1994). Due to phosphorus deprivation, production of C16:0 and C18:1 was increased and production of C18:4ω3, C20:5ω3 and C22:6ω3 was decreased (Reitan et al., 1994).

Table 2.2 Effect of various environmental stress conditions on carbohydrate/lipid content of microalgae. **Source**: Ho et al. (2014c).

Microalgal strain	Environmental stress	Products	Improvement	References
S. obliquus CNW-N	Light 60 to 420 µmol $m^{-2}s^{-1}$	Carbohydrate	With an increase in the light intensity, carbohydrate content raised up to 15 to 38% Around 3 fold increase in the biomass productivity with increasing light intensity from 60 to 420 µmol m^{-2} s^{-1}	Ho et al., 2012
N. oleoabundans HK-129	Light 50 to 200 µmol $m^{-2}s^{-1}$	TAG	With increasing light intensity from 50 to 200 µmol m^{-2} s^{-1}, biomass increased from 1.2 to 1.7 g L^{-1} TAG content also increased from 19 to 25% with an increase in the light intensity	Sun et al., 2014
Scenedesmus sp. 11-1	Light 50 to 250 µmol $m^{-2}s^{-1}$	Lipid/TAG	Amount of lipid and TAG increased from 26% to 41% and from 16% to 32% respectively, with an increase in the light intensity Biomass was increase from 2.5 to 3.6 g L^{-1} with increasing light intensity	Liu et al., 2012
Monoraphidium sp. SB2	Temperature 25 to 35°C	Lipid	With increasing temperature lipid content decreased from 33 to 29%, but biomass increased with increase in temperature up to 35°C. Further increase in temperature led to a decline in biomass	Wu et al., 2013
N. oculata	Temperature 15 to 25°C	Lipid	Lipid content increased by 14% at 25°C temperature and showed decrease in the lipid content from 15 to 8% with increase in temperature from 15 to 20°C Specific growth rate was enhanced with increase in	Converti et al., 2009

			the temperature from 15 to 20℃, but decreased with further increase in temperature 25℃	
Scenedesmus sp. LX1	Temperature 10 to 30 ℃	Lipid	With an increasing temperature of 20 to 30℃, lipid content declined from 35 to 22%, Biomass was enhanced with an increase in temperature from 10 to 25℃. A further increase in temperature beyond 30℃ decreased in biomass	Xin et al., 2011
Chlorella vulgaris SO-26	Temperature 5 to 25℃	Carbohydrate	With an increase in the temperature from 5 to 20℃ carbohydrate content decreased from 70 - 50%, while specific growth rate increased But further increase in temperature to 25℃ led to a decline in the growth rate	Hosono et al., 1994
N. Oleoabundans	pH 8.1-10	TAG	TAG content increased from 13 to 35% with increasing pH Whereas growth rate decreased with an increase in pH	Santos et al., 2012
S. obliquus UTEX 393	pH 6-8	TAG	TAG content increased with increase in the pH of the medium beyond optimal pH range (6-8)	Breuer et al., 2013
Coelastrella sp. PC-3	pH 6-10	TAG	TAG content enhanced with increase in pH, whereas growth rate decreased	Gardner et al., 2011
C. ellipsoidea	pH 6-10	Carbohydrate	For optimal growth and biochemical constituents, pH 9 and 10 were more favourable	Khalil et al., 2010
N. oleoabundans HK-129	Nitrogen depletion	TAG	After 3 days of nitrogen depletion, TAG content increased from 8 -26%, Biomass productivity decreased from 220 to 197 mg L^{-1} d^{-1}	Sun et al., 2014
N. oculata	Nitrogen depletion	Lipid	After 4 days of nitrogen starvation, lipid content increased up to 10 - 48%	Su et al., 2011

S. obliquus CNW-N	Nitrogen depletion	Carbohydrate	After 2 days of nitrogen depletion, carbohydrate content increased from 21to 49% Growth rate was slightly decreased	Bondioli et al., 2012
Nannochloropsis sp. F&M-M24	Nitrogen depletion	Lipid	Lipid content increased (39- 69%) and biomass productivity decreased with nitrogen starvation	Bondioli et al., 2012
Scenedesmus sp. LX1	Phosphorus limitation	Lipid	Lipid content increased up to 53%, Lipid productivity decreased under P starved condition	Xin et al., 2010
T. obliquus	Phosphorus limitation	Lipid	Lipid content increased up to 30% under P starvation	Mandal & Mallick, 2009
C. reinhardtii	Sulphur starvation	TAG	Metabolic carbon was diverted from protein to TAG synthesis	Sato et al., 2014
B. braunii Kutz IPPAS H-252	Salinity 0 to 0.7 M NaCl	TAG	TAG content increased 5-31%, With increasing NaCl concentration from 0-0.7 M, growth rate significantly decreased	Zhila et al., 2011
Chlorococcum sp.	Salinity 0 to 2.0 % NaCl	Lipid	Lipid content increased by 10-30% Biomass significantly decreased by approx. 4-fold with an increase in salinity from 0-2%	Harwati et al., 2012
D. tertiolecta ATCC 30929	Salinity 0.5 to 1.0 M NaCl	TAG	TAG content increased (40-57%)	Takagi et al., 2006

2.3 Utilisation of wastewater for microalgal biomass and biofuel production

Bioremediation is a technology that involves the metabolic ability of microorganisms to clean up contaminated locations such as ground or surface waters, soils, sediments, wastewater and air in the atmosphere (Boopathy, 2000). Bioremediation method also involves detoxification, mineralization and biotransformation of waste into simpler forms such as carbon dioxide, water, and methane etc. (Kshirsagar, 2013). The term

bioremediation also covers both microbial remediation as well as phytoremediation. In turn, microbial remediation involving the use of bacteria, fungi and algae for remediation purposes is comprised of multiple steps of different enzymatic reactions (Das et al., 2019). Phycoremediation has developed in recent years as one of the most sustainable ways of eliminating toxic compounds in the environment. Phycoremediation is selected over other biological remediation methods (such as phyto or myco remediation) due to non-pathogenic nature, easy growth conditions, and the ability of microalgae to withstand the adverse impact of a variety of toxic waste. Phycoremediation involves the use of microalgae and cyanobacteria to remove nutrients and xenobiotics from the wastewater, mitigate the excess CO_2 level in the air, and transform inorganic and organic pollutants (Olguin & Sanchez-Galvan, 2012; Dominic et al., 2009). Microalgal species exhibit sensitivity to these pollutants that makes them efficient environmental indicators for assessing the toxicity chemicals, genotoxicity and environmental risk (Tigini et al., 2011).

Many review articles are available on the application of using microalgae for wastewater treatment (Udaiyappan et al., 2017; Wang et al., 2016; Abinandan & Shanthakumar, 2015; Cai et al., 2013) which offer the advantage of simultaneous removal of nutrients and removal of pollutants. The nutrients load of wastewater, become feed for the microalgae as utilized and accumulated effectively (Ferreira et al., 2018; Abdel-Raouf et al., 2012). Photoautotrophic microorganisms are preferred for their use as environmental friendly bioremediation without creating secondary pollution as long as the generated biomass is reused (Mulbry et al., 2008). Furthermore, photoautotrophic microorganisms are considered as most significant bio-resources which presently gaining tremendous popularity owing to their capacity to grow fast, their potential for cultivation on non-arable land, less water and energy requirement (Singh & Olsen, 2011). *Chlorella, Scenedesmus, Phormidium, Botryococcus, Chlamydomonas, Spirulina, Oscillatoria, Desmodesmus, Arthrospira, Nodularia, Nostoc, Cyanothece* etc. are the species that are used for phycoremediation (Dubey et al., 2013; Rawat et al., 2011). Table 2.3 depicts the use of various microalgal species to remediation of wastewater.

Table 2.3 Remediation of wastewater by microalgae.

Wastewater source	Strains	Nitrogen removal (%)	Phosphorus removal (%)	COD removal (%)	References
Municipal wastewater (secondary effluent)	*S. obliquus* *C. vulgaris*	- -	55-83 53-80	-	Ruiz-Marin et al., 2010
Soybean processing wastewater	*C. pyrenoidosa* *Spi. platensis*	88 -	70 93	41 94	Hongyang et al., 2011
Piggery manure digested and diluted 25×	*Chlorella sp.*	75-82	62-74	28-38	Wang et al., 2010a
Food processing wastewater	*Chlorella* sp.	90	62	63	Gupta et al., 2016
Dairy wastewater	*Scenedesmus quadricauda*	86	89	64	Daneshvar et al., 2018
Dairy wastewater + 6% BDWG (biodiesel industry waste glycerol)	*Chlorococcum* species RAP13	-	-	93.9	Ummalyma & Sukumaran, 2014
Alcohol wastewater + starch anaerobic digested wastewater	*C. pyrenoidosa*	91.6	90.7	75.78	Yang et al., 2015
Olive washing water	*Sphaeropleales* (Cyanobacteria)	-	-	85.86	Maza-Maequez et al., 2017
Bio industrial waste	*C. sorokiniana*	100	100	86	Podevin wt al., 2017
Aquaculture runoff	*Chlorella sp., Pediastrum sp., Gloeothece sp.,*	100	84-95	-	Garcia-Galan et al., 2018
Mixed piggery-brewery wastewater	*C. vulgaris*	32-96	28-95	83-93	Zheng et al., 2018

Typically, wastewater includes organic carbon, nitrogen, phosphorus and other compounds, making it unsuitable for human consumption, but appropriate for algae cultivation. Algal growth in wastewater relies on multiple critical variables including pH, temperature, light accessibility, CO_2, O_2 and levels of nutrients in particular. According to Pittman et al. (2011) & Rawat et al. (2011), primary settled sewage water substantially enhanced microalgal growth during lengthy photoperiods with the

addition of CO_2. The preconditioned microalgae such as nitrogen starvation could enhance the nitrogen removal from the wastewater and therefore wastewater was remediated within a short period. Rao et al. (2011) revealed that growth of *Chlorella vulgaris* on the leather-processing chemical plant effluent, could remove heavy metals, pigments, calcium, and magnesium and reduce the ammonia, nitrite, BOD and COD concentrations. A study by Hernandez et al. (2013) showed that wastewater from the potato processing industry and pig manure were treated by using two mixed procedures microalgae *Chlorella sorokiniana* and aerobic bacteria in 5 L photobioreactors, the total COD was reduced by 82.7% and 62.3% respectively.

Ayodhya & Kshirsagar, (2013) demonstrated that *Chlorella vulgaris* and *Scenedesmus quadricauda* removed BOD after 15 days treatment of domestic wastewater by 70.91, and 89.21 respectively, whereas COD was reduced by 80.64% to 70.97% respectively. A study by Zhang et al. (2008) found that *Scenedesmus* sp. efficiently removes the inorganic nutrients from domestic effluents. Whereas Kim et al. (2007) using fermented swine wastewater with a phosphorus concentration of 120 mg/L and they found that removal of phosphorus by *Scenedesmus* sp. was about 83%. Olguin, (2012) demonstrated using seawater with anaerobically digested piggery wastewater for growth of *spirulina* and for production of high-value products such as biogas, biodiesel, hydrogen. In both small and large-scale culture of microalgae, use of wastewater to improve biomass and lipid production has been demonstrated. For example, *Chlorella vulgaris* grown on swine wastewater showed higher biomass productivity (0.247 g L^{-1} d^{-1}) and fatty acid than conventional growth medium source (0.165 g L^{-1} d^{-1} biomass and 0.058 g L^{-1} d^{-1}) (Nam et al., 2016). In another study by Woertz et al. (2009), lipid productivity by microalgal culture was enhanced when grown on dairy and municipal wastewater. Hence, the successful operation of integrating wastewater treatment with large-scale biofuel production has been reported for various microalgae.

Microalgae cultivation integrated with effluent treatment for lipid production can be environmentally and economically beneficial (Soccol et al., 2016). An alternative to this can be supplementing the culture medium with industrial wastewater, flu gases CO_2 or urban wastewater (Ferreira et al., 2018). Besides, important factor already discussed is the strain selection, i.e., choice of algae with high tolerance to organic pollutants and environmental stress for higher growth, and lipid production such as in case of microalgal *Scenedesmus* and *Chlorella* sp. (Abdel-Raouf et al., 2012; Guldhe et al., 2017; Salama et al., 2017). More researches are revealed that there are many problems that need to be tackled for success in large-scale production of algae on

wastewater. It is also necessary to prove that the ultimate success of the technology is dependent on the economic viability (Wu et al., 2012). Many reactor design studies focused on algae are limited to the laboratory level and their commercial or large-scale implementation is still a dream. However, up-scaling of the system poses challenges to environmental scientists regarding the organisms used, growth requirements and performance. Finally, phycoremediation work needs to overcome a range of limitations before it can be used for commercial advantage (Sharma et al., 2018).

2.4 Biodiesel production

Each microalgal cell contains a complex mixture of various constituents such as lipids, carbohydrates, proteins, pigments and other constituents of the cells. Out of these; lipid productivity is an important prerequisite for determining microalgae's suitability for commercial production of biofuel (Sun et al., 2018). Overall, the lipid content of microalgal cells in the range of 30-80% (Chisti, 2007). Energy storage is the primary function of the neutral lipid, whereas polar lipids form cell membrane structural elements (Halim et al., 2012). The most prevalent neutral lipids found in the cell cytoplasm as small oil droplets are triacylglycerols (TAGs) (Bajhaiya et al., 2010).

Further, transesterification is the method where triglycerides react with alcohol (frequently methanol or ethanol) in the presence of an acidic or basic catalyst to provide biodiesel and glycerol (Machado et al., 2012). Figure 2.1 shows the transesterification reaction of biodiesel. The transesterification process relies mainly on the type of alcohol, type of catalyst, and proportion of molar ratio. This processing method is essential for biodiesel so that it can be directly applied to motors (Adeniyi, 2018). Conventional transesterification process includes extraction of lipids and its esterification. Lipase enzyme catalyzes both esterification and transesterification simultaneously as a catalyst in the transesterification process and helps to exclude product regeneration (Antczak et al., 2009; Khan et al., 2009).

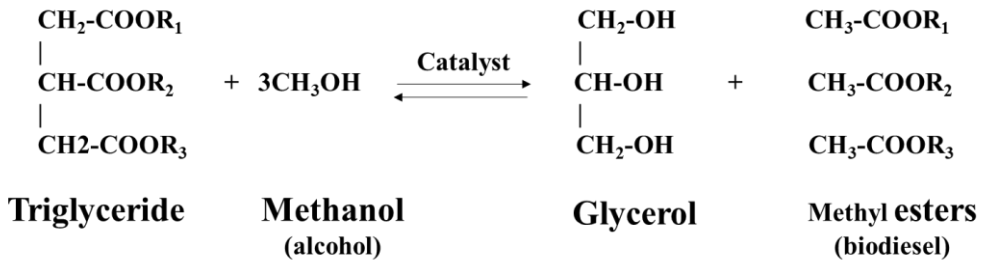

Fig. 2.1 Transesterification reaction in biodiesel production. Chisti, (2007)

Biodiesel consists of long-chain fatty acid methyl esters (mainly C12-C18 groups) derived from triglycerides found in a number of biological feedstocks such as oleaginous plants, algae and animal fat. (Yen et al., 2013). Figure. 2.2 depicts the biodiesel production process. However, fatty acids in microalgal are common carboxylic acids of 4 to 36 carbon-containing hydrocarbon chains (D'Alessandro et al., 2016). They are usually divided between fatty acids that are saturated, monounsaturated and polyunsaturated (Soccol et al., 2016). The most common saturated fatty acids (SFA) in microalgae range from butanoic (C4:0) to octanoic (C28:0), palmitic (C16:0) acid. Monounsaturated fatty acids (MUFA) may also have different chains of hydrocarbons (mainly 14 to 24 carbons), but oleic acid (C18:1), also known as omega-9, is usually the most commonly found in microalgae (Khan et al., 2009). The high-value lipid components, such as linoleic (C18:2) and linolenic (C18:3) acids, also known as omega-6 and 3, are polyunsaturated fatty acids (PUFA). Mostly omega-3 rich microalgal species are from seawater, such as *Schizochytrium* sp., and *Nannochloropsis* sp. Nonetheless, an important source of omega-3 long-chain PUFA, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids from freshwater i.e., *Desmodesmus* sp (Sharam et al., 2018; Ho et al., 2014a).

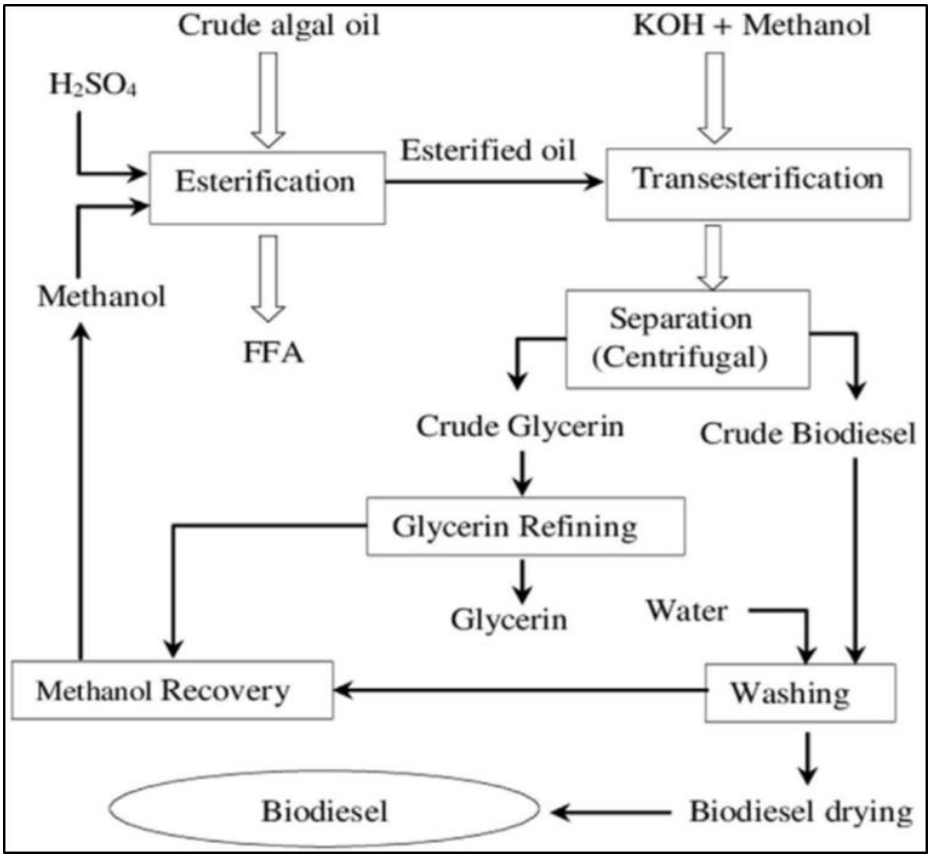

Fig. 2.2 Flow diagram for biodiesel production. **Source**: Rahman et al. (2017)

Scharff et al. (2015) evaluated the effect of the photoperiod on the FA profile, making it possible driving longer photoperiods (24:0, 22:2, and 20:4). They showed a reduction in the synthesis of α-linolenic acid but an increase in the synthesis of linoleic acid, as more expressively observed for *C. vulgaris* than *S. obliquus*. On the other hand, Chandra et al. (2017) found that the content of both linoleic and α-linolenic acids increased simultaneously with an increase in the light intensity as a continuous light source with optimum photoperiod. Therefore, it is important to mention that the fatty acid profile can differ depending on the conditions of cultivation or the nature of the culture medium for the same microalgae species (Chu, 2017). The key species of microalgae used in the production of biodiesel such as *Scenedesmus* (Ho et al., 2012; Xia et al., 2013), *Chlorella* (Chen et al., 2013), *Botryococcus* (Rao et al., 2012), *Chlamydomonas* (Nakanishi et al., 2014; Siaut et al., 2011), *Nannochloropsis* (Bartley et al., 2013) and *Dunaliella* (Moheimani, 2013; Tang et al., 2011). Nevertheless, the fatty acid profile of microalgal lipid plays an important role for the quality of biodiesel produced, that affect the combustion efficiency and heating capacity of engine (Singh & Gu, 2010, Talebi et al., 2013).

Calixto et al. (2016) examined four types of microalgae grown in sewage and agro-industry wastewater, the result showed an increase in SFAs content (36%), and PUFAs (69%) as compared to synthetic medium (Zarrouk). Another study by Fernandez-Linares et al. (2017) found that *C. vulgaris* grown on wastewater contains reduces the content of oleic acid, while linolenic acid was increased. However, the content of palmitic, stearic, and linoleic acid were contrasting. A fatty acid profile may, therefore, vary not only with the medium origin but also with the differences in the microalgal strains (Singh & Gu, 2010). In addition, Han et al. (2016) compared the fatty acid profile of *S. obliquus* grown on wastewater without aeration and fatty acid profile when cells aerated with 2% CO_2 enriched air, the result showed a decrease in SFA content, a rise in MUFA and a slight decrease in PUFA (mostly C18, FA). Although it is possible to induce lipid accumulation by enriching the medium with waste resources, and the fatty acid composition will not necessarily be negatively affected. Several wastewater studies showed an increase in the omega-3 fatty acid, such as *Chlorella* and *Scenedesmus quadricanda*, showed increased in C18:3 content almost by three-fold as compared to synthetic medium grown culture (Saad et al., 2019; Goh et al., 2019). Therefore, CO_2 injection improved lipid production significantly, which will also be

environmentally beneficial for reducing global warming (SundarRajan et al., 2019). Growth conditions must be evaluated after strain selection in order to optimize biomass and lipid productivity along with PUFA content. Some studies have already approached this issue, bicarbonate combined with nitrogen limitation, alkaline pH as well as inorganic carbon supply, are expected to have a positive effect on long-chain PUFA production (Guiheneuf & Stengel, 2013).

For the choice of biodiesel production, a systematic study of the structure of the fatty acid methyl ester (FAME) and comparative fuel properties would be important. The most common microalgae fatty acids are palmitic (hexadecanoic C16:0), stearic (octadecanoic C18:0), oleic (octadecenoic C18:1), linoleic (octadecadienoic C18:2) and octadecatrienoic (C18:3) acids (Knothe, 2009). Most algae have eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6) in smaller amounts. Nevertheless, these polyunsaturated fatty acids (PUFAs) can get accumulate in significant quantities in some species of different genera, depending on the conditions of cultivation (Huerlimann et al., 2010). Diatoms and eustigmatophytes generally make significant amounts of EPA, while dinoflagellates and haptophytes normally produce both EPA and DHA (Brown, 2002). In the late stationary growth phase, higher levels of SFAs (C16:0) and MUFAs (C16:1 and C18:1) are achieved with concurrent reduction of PUFA (C18:3, C18:4 and C20:5). It is clear that the composition of saturated and unsaturated fatty acids varies significantly among similar microalgal groups (Deshmukh et al., 2019).

Good quality of biodiesel should have C16:1, C18:1 and C14:0 fatty acid ratio of 5:4:1 as suggested by Schenk et al. (2008). The increase in biodiesel use has contributed to the need to standardize alkyl ester fuel quality requirements. Biofuel properties such as cetane number, melting point, boiling point, calorific value and viscosity show variation with changes in chain length of fatty acid. Combustion heat, melting point, and biodiesel viscosity increases with the carbon length of fatty acid chain and inversely associated with unsaturation (Knothe, 2012), increasing autoxidation and lubricity with increasing unsaturation (Dunn & Bagby, 1995). Polyunsaturated fatty acids (PUFAs) are not desirable for biodiesel due to their susceptibility to oxidation, saturated lipids tend to increase the cloud and viscosity of biodiesel (Knothe, 2012), so monounsaturated fatty acids (MUFAs) are the most

needed lipids for biofuel (James et al., 2013). In all these properties, the increase in the number of double bonds indicates a decrease in the quality of fuel properties (Knothe, 2005). Thus, the composition of fatty acids in biodiesel is significant from the perspective of biodiesel properties as a transport fuel as demonstrated by previous studies (Pinzi et al., 2013; Hellier et al., 2015).

Comparing the features of different industries, it can be concluded that, in order to expand microalgae business applications, efforts are required to generate low-cost biomass, below 10 euro/kg, in large quantities of more than 1000 t/year, which may enable the manufacturers to enter in the emerging markets requiring medium and minimum production security (Fernandez et al., 2019). In order to reduce the price of microalgal biomass production, new techniques are needed to boost the present production ability to 30,000 t/year. New procedures and techniques are needed even for these industries. Integrating the production of microalgae with wastewater treatment is the most viable way which would a considerably boost the production ability of microalgal biomass while decreasing the cost of biomass production (Saravanan et al., 2018).

Without government support, the development of microalgal biodiesel industries cannot be sustained. Currently, biodiesel production is economically feasible due to multiple policies that promote marketing through tax credits, subsidies, tariffs on imports and set objectives (Zivkovic et al., 2017). Reducing machinery taxes, temporary training and teaching sessions for farmers and other subsidies are essential for the effective industrialization of microalgal biodiesel (Su et al., 2017). Microalgae produce high-value organic products that can be extracted and utilized, such as pigments, vitamins and antioxidants (Chew et al., 2017). The industrial facility of bio-refinery can be useful for the utilization of biomass in a various way and convert it into valuable products such as chemicals, energy and materials products (Zhu, 2015; Sun et al., 2017). Lee et al. (2011) consider that algal biofuel could provide 7.1% of the energy demand of the developed world by 2040 and 0.5% energy requirement of developing countries with government support.

More industrial plants integrating the microalgal production and waste treatment are expected to opt for commercial use of microalgae in the near future, thereby increasing the availability of microalgal biomass for non-human uses and

reducing the cost of biomass production (Callegari et al., 2019). The techno-economic assessment carried out showed that the uses of wastewater as the growth medium enables the reduction in the price of biomass production nearer to the critical value of 1.0 Euro/kg (Basavaraj et al., 2012). In addition, concurrent wastewater treatment provides extra financial and environmental advantages. However, in order to achieve the low-cost biomass, it is also necessary to improve the technology for maximizing the productivity of biomass, reducing the cost of reactor/technology and minimizing the manpower required for the operation of the facility. However, current technology cannot produce a large amount of biomass required from markets more than 100 kt/year for a single facility at a lower cost which is less than 1.0 Euro/kg (Saravanan et al., 2018; Basavaraj et al., 2012).

Five main strategies for successful marketing of algae biofuels have been recognized (Thurmond, 2009a). These strategies are fatter, quicker, cheaper, easier, and fractional marketing methods to assist manufacturers to cut expenses and speed up the marketing of biodiesel, bio-crude, and drop-in fuels (Thurmond, 2009b). The marketing of algal biofuel also depends on the achievement of new technology and ease of application. With regard to economic advantages, algae can be used through an integrated sequential bio-refinery method to extract co-products of industrial importance (Saravanan et al., 2018). In this situation, it is necessary for the public and government to regulate and use the biofuel effectively, transparent and simple policies that benefit the industries. Some governments have provided the privileges of cost-sharing to reduce investment risk and also to reduce subsidies from full biodiesel production, i.e. from feedstock experiments to biofuel industrialization (Su et al., 2015). Consequently, the achievement of the biofuel program is driven by efficient and effective biofuel policies. Following Brazil's achievement and other developing countries, including India, there is need to prepare a roadmap to support biofuel initiatives, blend objectives, and provide subsidies through mandates, tasks, and biofuel policies (Fernandez et al., 2019). An in-depth assessment of the legal framework regulating biofuel in India has been made in September 2008, the Government of India adopted the ‘National Biofuels Policy’ on 24 December 2009. The Ministry of Petroleum and Natural Gas has recently set up an industry-based working group in cooperation with the Petroleum Conservation Research Association to raise awareness and encourage biofuels in India (PTI, 2015).

2.5 Future prospective of biofuel production

Factors that affect microalgal biodiesel production, include efficient photosynthesis to produce adequate biomass. Efficient algal biomass production needs high-photosynthetic performance, sufficient nutrients and CO_2 as well as ideal light conditions to drive photosynthesis at the ideal temperature (Wolf et al., 2016). In addition to offering biologically ideal production circumstances (e.g. temperature and nutrient supply), different engineering factors (e.g. dilution, surface area and volume) of microalgal culture need to be optimized.

In addition, microalgae biomass harvesting, dewatering and oil extraction include the greatest downstream processing algal biofuels in terms of the high cost. Biofuel production by using lipid/carbohydrate-rich microalgae is a very promising solution to biofuel production (Sun et al., 2014). In order to considerably enhance the microalgal based biofuel, engineering approaches need to be established that will efficiently increase growth as well as lipid/carbohydrate content and should be optimized by using favourable environmental factors and appropriate operating techniques. Several innovative operating strategies for suitable environmental stresses have been critically evaluated here for higher biofuel production in the near future (Ferreira et al., 2019). It is possible to select the appropriate engineering approach for biofuel production by comparing the biological features of microalgal species under varying environmental conditions their response (Sukla et al., 2019). Hopefully, the development of microalgal based biofuel production processes is economically viable in the coming years by enhancing the cultivation process by using metabolic and genetic engineering tool.

Chapter- III

MATERIALS & METHODS

This chapter addresses the common procedures used in these investigations. In the relevant chapters, other specialized methods have been included.

3.1 Sterilization

All the glassware and the growth medium for the routine culture of *Scenedesmus vacuolatus* were sterilized in an autoclave operated at a steam pressure of 15 lb/inch^2 with a temperature of 121℃. The mouth of the Erlenmeyer's flasks containing medium were tightly closed with cotton plugs and wrapped with aluminium foil before placing them in the autoclave.

3.2 Growth medium

Scenedesmus vacuolatus is grown in BG-11 medium as described by Stainer et al. (1971). The nutrient medium's composition is as follows:

Macro-elements	Concentration (g L^{-1})
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
Na_2CO_3	0.02 g
Trace metal mix A5	1.0 mL
Distilled water	1.0 L

Trace metal mix A5	Concentration (g L^{-1})
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

For the preparation of BG-11 medium, macro-elements were mixed together and autoclaved. The pH of the medium was maintained at pH 7.2.

3.3 Culture conditions

The axenic culture of *Scenedesmus vacuolatus* was routinely grown in Erlenmeyer's flasks (1000 mL) containing 500 mL sterile BG-11 growth medium and the culture flask were maintained in an air-conditioned culture room with a temperature of 25±2℃. The culture flasks were illuminated by using cool white fluorescent tubes (10 Wm^{-2}) with 16/8 hour's light/ dark cycle. The cultures flasks were shaken thrice daily to deflect settling and sticking of the cells to the bottom of the flask.

3.4 Determination of growth

The growth of *S. vacuolatus* cells was performed in Erlenmeyer flask (500 mL) containing BG-11 medium (250 mL). The flask was inoculated with inoculum density of 0.1 OD. The flasks were kept under the favourable condition of growth such as temperature and light. The turbidity of microalgal cell suspension was monitored periodically at 680 nm up to 20 days by using a double beam UV-visible spectrophotometer (Shimadzu, Japan 1601). The specific growth rate (μ, d^{-1}) was calculated using the following formula as suggested by Yu et al. (2017):

$$\textbf{Specific growth rate } \mu, (d^{-1}) = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)}$$

Where, X_1= Initial density of population at t_1 time;

X_2= Final density of population at t_2 time

3.5 Measurement of cell constituents

3.5.1 Estimation of Protein

Protein content in the cell suspension was estimated by the method of Lowry et al. (1951) modified by Herbert et al. (1971).

Reagents

I. 1N – NaOH

II. (i) 5% (w/v) solution of Na2CO3 (sodium carbonate)
(ii) 0.5% (w/v) solution of CuSO4 (copper sulphate)
(iii) 1% (w/v) solution of Na-K tartarate

For making the reagent II, 50 mL of solution (i) was mixed with 1 mL each of solution (ii) and (iii).

III. 1 N Folin Phenol (ciocalteau
reagent)

Procedure

To 0.5 mL of the cell suspension, 0.5 mL of 1 N- NaOH was added and the mixture was placed in a boiling water bath at 100℃ for 10 min. After sufficient cooling of the mixture 2.5 mL of reagent, II was added and the mixture was thoroughly shaken and was left for 10 min at room temperature. Thereafter, 0.5 mL of Folin-Phenol reagent (1N) was added to the mixture. After 15 min of the incubation at room temperature, the colour intensity of the mixture was read at 650 nm by a UV-visible spectrophotometer (UV –1601, Shimadzu, Japan) against an appropriate blank. The concentration of protein in the unknown solution was determined by using a calibration curve prepared by using Bovine Serum Albumin (BSA).

3.5.2 Estimation of carbohydrate

The total carbohydrate content in the cell suspension was measured by the phenol-sulfuric method as described by Dubois et al. (1956).

Reagents

I. 5% (w/v) of phenol solution

II. Sulphuric acid (96% reagent grade)

Procedure

To 1.0 mL of the cell suspension, 0.5 mL of phenol reagent was added to each tube and it was shaken well. Thereafter 2.5 mL of 96% sulphuric acid was added to each tube, using the sidewalls of the test tube. The reaction mixture was shaken well and left for 20 min to form the green coloured. The colour intensity of the reaction mixture was measured at a wavelength of 490 nm in a UV-visible spectrophotometer (UV–1601, Shimadzu, Japan) against appropriate blank. The glucose solution was used for the preparation of the standard curve. The total carbohydrate content of the sample solution was calculated by using the standard graph and expressed in terms of µg carbohydrate/mL^{-1}.

3.5.3 Estimation of Total Organic Carbon (TOC)

The total organic carbon content (TOC) of the cell suspension was measured by the Walkley-Black method as described by Grobler, (1979).

Reagents

I. 49.04 g of $K_2Cr_2O_7$ dissolved in 1.0 L distilled water (DW) (dichromate solution)

II. $Fe(NH_4)_2(SO_4)_2.6H_2O$ (1961.1 g) dissolved in 800 mL DW containing 200 mL sulphuric acid (H_2SO_4) and made up to 1L. This solution was standardised against the dichromate solution.

Procedure

100 mL microalgal culture was concentrated and the pellet was evaporated to dryness at 80℃ for overnight. The dry biomass sample was taken in a boiling tube with 10 mL potassium dichromate solution (v/v) followed by 20 mL concentrated sulphuric acid with gently swirling and mixing. The reaction mixture was left to cool down at room temperature for 30 min before making the final volume up to 50 mL in a volumetric flask. The turbidity of the reaction mixture could be cleared by either leaving the mixture to stand overnight or by centrifugation at 2000 rpm for 20 min. The concentration of the chromium- III ions chromophore was determined by measuring the colour intensity of the green colour at a wavelength of 649 nm in a UV-visible spectrophotometer (UV – 1601, Shimadzu, Japan) against an appropriate blank. The standard prepared curve was with a known concentration of chromium-III ion. The total organic carbon content of the sample was calculated by using a standard curve and TOC value was expressed in terms of mg L^{-1}.

3.5.4 Estimation of pigments by whole-cell scan

For the extraction of photosynthetic pigments, an aliquot of cells suspension (4.0 mL) was withdrawn at regular interval and was subjected to repeated freezing and thawing to break the cells. The broken cell pellet was suspended in equal volume (4.0 mL) of acetone (80%, v/v) and was shaken well. The suspension was kept at 4℃ for 24 h. After thawing of the broken, the cells suspension was centrifuged for 5 min at 4000 rpm to collect the supernatant. The collected supernatant was scan (400-700 nm) in a double beam UV-visible spectrophotometer (UV – 1601, Shimadzu, Japan) by using a light path of 1 cm. The pigments were quantified by using the following equation (Lichtenthaler, 1987) as mentioned below:

Chlorophyll 'a' (µg mL^{-1})	$12.25A_{663.2} - 2.79A_{646.8}$
Chlorophyll 'b' (µg mL^{-1})	$21.50A_{646.8} - 5.10A_{663.2}$
Total Chlorophylls (µg mL^{-1})	$7.15\ A_{663.2} + 18.71A_{646.8}$
Total Carotenoids (µg mL^{-1})	$(1000A_{470} - 1.82C_a - 85.02C_b)/198$

3.6 Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy was used to detect the cells constituents in the cells grown under conditions. The microalgal biomass was harvested by centrifugation (5000 rpm, 10 min) and the pellet (approximately, 1 mg) was air-dried for 24 h and powdered by using pestle and mortar before mixing it with IR grade potassium bromide (KBr, 1:10, w/w ratio). Pellets of microalgal samples and KBr were formed under vacuum hydraulic press (150 lbs), by using spectra lab pelletizer. The IR absorbance spectrum was recorded within the range of 4000-400 cm^{-1} by using FTIR (Thermoscientific, Nicole 6700, USA). The baseline correction of the data for the background absorbance by KBr was performed by using Omnic 8.0.342 software (Thermoscientific). The biochemical constituents were assessed based on the characteristic IR absorbance of different macromolecules (Duygu et al., 2012; Laurens et al., 2011) and monitored by the analysis of total lipid, lipid/carbohydrate (L/C ratio) and lipid/protein (L/P ratio), amide I/amide II ratio, unsaturation/saturation ratio (Dean et al., 2010; Mahapatra et al., 2013).

3.7 Flow cytometer analysis

The intracellular lipid of microalgae were analysed by using high-speed flow cytometer (BD FACS Influx^{TM}, Becton Dickinson, San Jose, CA, USA). The data were analysed by software BD FACS ^{TM} Software 1.2.0.87. The Nile red (9-diethylamino-5H-benzo [α] phenoxy- phenoxazine-5- one), fluorescent dye purchased from Sigma-Aldrich, USA, was used for the indirect measurement of neutral lipid, using a concentration of 0.1 mg mL^{-1}. The stationary phase cultures were centrifuged at 5000 rpm for 10 minute and washed three times with PBS (Phosphate buffered saline) to remove the debris before the measurement of lipid. Subsequently, the microalgal cells suspension (300 µL) was taken in falcon tube followed by addition of 10% (v/v) DMSO (Dimethyl sulfoxide) (500 µL) solution for enhancing the permeability as standardized previously. Thereafter, the cells suspension was supplemented with PBS (185 µL) solution and working solution of Nile red dye (15 µL). The mixture was vortexed for 1.0 min and thereafter the suspension was incubated for 15 min at room temperature under darkness (Dashkova et al., 2016; Satpati & Pal, 2015). The solid-state blue laser of flow cytometer was used to record excitation maxima/emission maxima (488nm, 580/30 nm respectively) of fluorochrome in the Nile red-stained cells. A total of at least 10,000 events for each sample were recorded for both the control (without dye) and treated cells. The unstained cells were used as auto-fluorescence control. The total fluorescence emission intensity was corrected for autofluorescence emitted by the unstained cells (without Nile red) and data were expressed as fluorescence emission in arbitrary unit and percentage (%) of the stained cell population. The lipid content was showed in terms of fluorescent intensity (%) as compared to control.

3.8 Statistical analysis

All experiment was performed in triplicate and demonstrated as mean ± SD with error bars. The sample paired Student‘t’ test was conducted to evaluate the mean difference between the strains (wild-type and mutant strain) at *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns-non-significant. Using SPSS ver. 21.0 (IBM Co., Armonk, NY, USA) for the statistical analysis in all the experiment.

3.9 Equipment

I. Spectrophotometric readings were taken by a Double beam UV- visible spectrophotometer (Shimadzu, 1601, Japan).

II. pH of the solution was measured by using a pH meter of Toshniwal Inst. Mfg. Pvt. Ltd., Ajmer.

III. The microscopic study was done by using Multifunctional digital microscope (model, US3).

IV. Fluorescence spectra of cells were taken by Spectro-fluorometer (Cary Eclipse 100, India).

V. Infra-red spectra of the samples were recorded on Fourier Transform Spectrophotometer (Nicolet 6700, Thermo Scientific, USA).

VI. Accumulation of intracellular lipid of microalgae was analysed by high-speed flow cytometer (BD FACS Influx^{TM}, Becton Dickinson, San Jose, CA, USA).

VII. Chlorophyll a fluorescence transient was measured by using Pulse Amplitude Modulated (PAM) (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic).

VIII. LC-MS/MS (liquid chromatography-mass spectroscopy/mass spectroscopy) analysis was performed by Aquity UPLC (Waters) and coupled with electrospray ionisation (ESI) mass spectrometry (API 4000 triple quadrupole) (AB Sciex).

IX. GC-MS (gas chromatography-mass spectroscopy) analysis was performed by using Trace GC Ultra TSQ Quantum XLS Mass spectrometer (Thermo, USA).

3.10 Chemicals

All chemicals used in this study are analytical Reagent (AR) grade products of Thermo Fisher Scientific (US), HiMedia (France), Sigma Chemicals (USA) or Merck (Germany), SD-Fine Chemicals (India), and Loba Chemicals Ltd. (India).

Chapter- IV

ISOLATION & CHARACTERIZATION OF WILD-TYPE AND DCMU-TOLERANT MUTANT STRAIN

4.1 Introduction

Microalgae have several advantages in comparison to plants such as higher growth rate, ability to grow on the wastelands with low-quality water and also their ability to take up excess nutrients from the wastewater (Tan et al., 2019; Slade & Bauen, 2013). Despite these significant advantages, use of microalgae as clean energy resources still remains an unfulfilled dream due to several practical bottlenecks in the biomass production process (Williams & Laurens, 2010; Sheehan et al., 1998). In addition, oleaginous microalgae have been regarded as a potential source of next-generation feed-stocks for production of bioethanol and biodiesel due to their higher photosynthetic efficiency and greater lipid accumulation, when compared with other than terrestrial crops (Rahman et al., 2014; Naik et al., 2010). At present, there is a need to develop an inexpensive way for the production of biofuel from the oil-rich microalgal biomass. The real cost of producing biodiesel can be reduced by the adoption of modern technique, and also by improvement in the capabilities of microalgae to produce oil-rich biomass (Bajpai, 2019; Demirbas, 2010; Chisti, 2007). Presently, two general approaches are being made to enhance lipid accumulation in microalgae; selection of better strains for the accumulation of lipids and direct genetic manipulation of the microalgal strains to improve the production of microalgal diesel. In the current scenario, genetic manipulation of microalgae is rising as a significant tool to enhance biofuel production (Sun et al., 2018; Stephens et al., 2010).

Mass scale culturing of microalgal cells is mainly limited due to nutritional and environmental conditions (Gendy & Temtamy, 2013). The overall biomass productivity of microalgae is far from achieving the desired photosynthetic efficiency and biomass production due to limitations imposed by environmental stresses; such as light, salinity, and temperature etc. (Manandhar-Shrestha et al., 2009; Kao et al., 2003). Thus, there is a shift in focus in favour of microalgal strains with improved photosynthetic characteristics which may be useful to overcome the limitations imposed by environmental stresses (Murata et al., 2007; Smith et al., 1990). To facilitate genetic manipulation of the strains, rapid progress made in the field of microalgal genomics and transcriptomics during the last two decades would be highly useful (Rismai-Yazdi et al., 2012; Radakovits et al., 2012; Worden et al., 2009). Genetic manipulation involving the method of direct introduction of known genes which interfere with the

specific target genes is a quite expensive process (Beacham et al., 2017; Borowitzka, 2013). The genetic manipulation of strains in terms of random chemical mutagenesis also offers many advantages, such as no needs to know the genetic information of an organism, screening of superior strains, easier and cost-effective process to obtain novel promising strains (Khan et al., 2018; Medipally et al., 2015). The fundamental source of mutation is genetic variability as it is able to produce new adaptive alleles (Lee et al., 2014). Thus, the mutagenesis is considered to be an important tool for the genetically modified organism (Beacham et al., 2017; Larkum, 2010; Scott et al., 2010).

There are several other examples of successful random mutagenesis to generate mutants exhibiting an enhanced lipid accumulation. The process of chemical mutagenesis by using EMS (ethyl methanesulfonate) is a suitable and powerful tool to induce the mutation by modulating A-T to G-C in a DNA (Mobini- Dehkordi et al., 2008; Kodym & Afza, 2003). The advantages associated with EMS mutation includes wider application range, high frequency of mutagenesis and its efficiency (Mck & Neuffer, 1987). Moreover, the EMS induced mutants are usually dominant, which helps in the screening of the target mutants (Zhang et al., 2016; Mck & Neuffer, 1987). It has been reported that EMS can be used to generate pigment mutant, mutants with enhanced biofuel production, autoflocculating mutants in eukaryotes like *Saccharomyces cerevisiae* and cyanobacterium *Arthospira platensis,* respectively (Kim et al., 2013; Huesemann et al., 2009). It has been reported that EMS treated microalga *Nannochloropsis* sp. produces higher lipid and biomass, particularly under nitrogen deprived condition (Anandarajah et al., 2012). Successful EMS mutagenesis leading to generate starch-less mutant of *Chlamydomonas reinhardii* with higher lipid content has been reported, which exhibits about 10 fold increase in the production of TAG due to a defect in ADP-glucose pyrophosphorylase (Li et al., 2010; Work et al., 2010). Kawaroe et al. (2015) have also shown that 0.1µM of EMS could increase the cell size of *Dunaliella sp.* by about three times larger than the parent cells. The attempts made by Beachama et al. (2015) on random mutagenesis with the help of EMS and UV on *Nannochloropsis salina* CCAP849/3 showed about 156% increase in fatty acid synthesis (FAME analysis) in the mutant when compared with the wild-type. The enhanced lipid productivity in water surface floating microalgae *Botryosphaerella* sp. and *Chlorococcum* sp., using chemical mutagen ethyl methanesulfonate, has been reported by Nojima et al. (2017). Most of the studies on EMS treatment of microalgae

showed major changes in the organisms and greater output in terms of growth, biomass and biochemical composition (Kumar et al., 2017; Baker, 1991).

In the present study, the procedure of random chemical mutagenesis of *S. vacuolatus* by using EMS was adapted. The mutants were screened by using a sub-lethal concentration of DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea) a herbicide (also known as diuron, direx, karmex). DCMU is known to inhibit the photosynthetic electron transport by blocking the electron transfer at the level of QB site of photosystem II (Manandhar-Shrestha et al., 2009; Huber & Edwards, 1975). Earlier Microalgal resistance to DCMU has been correlated with the expression of the PSB-A gene product, i.e. D1- protein, which is required for photoautotrophic growth of the organism (Vermaas & Ikeuchi, 1988; Golden et al., 1986). Hence, DCMU-tolerant mutant of *S. vacuolatus* may be a useful tool for studying the effect of PSII modifications on the production of biomass and lipid accumulation (Rutherford & Krieger-Liszkay, 2001). Further, the present investigation is an effort to study the physiological changes in the mutant leading to a compositional alteration in the cell constituents of both the wild-type (WT) and mutant strain.

4.2 Materials and Methods

4.2.1 Sample collection

Sample of microalgae was collected from Pangong Lake, Leh valley Ladakh, India. The sample was brought to BBAU (Lucknow), laboratory and stored in the refrigerator at 4°C in the presence of light.

4.2.2 Isolation and identification of microalgae

The sample of algae washed with deionized water and near-pure population of algae were then serially diluted and spreading over the agar plates containing BG-11 medium (Stanier et al., 1971). After 3 weeks, the pinhead colonies appearing on the plates were picked up by using sterilized capillary tubes under a trinocular microscope and transferred in 100 mL Erlenmeyer flask containing BG-11 nutrient medium and maintained white fluorescent light (~ 4000 lux) for 16:8 h light to the dark period at 25 °C.

The axenic culture of wild-type (WT) was identified from the National Collection of Industrial Microorganism (NCIM), CSIR- National Chemical Laboratory (NCL), Pune, by using 18S rRNA sequencing technique.

4.2.3 Mutagenesis and isolation of mutant strain

The axenic culture of wild-type was grown in the presence of BG 11 medium as described in chapter III of Materials and Methods, at a density of ~ 2.57×10^6 cells mL^{-1}. The exponentially growing cells were harvested by centrifugation (5min ×5000 rpm) and the pellet was washed twice with sterilized potassium phosphate buffer (0.1 M, pH 7.0). The pellet was subsequently suspended in 5 mL of BG-11 medium to an approx. cells density of 10^5 to 10^6 cells / mL. An aliquot of 2 mL cell suspension was withdrawn from the flask and volume was maintained to 5 mL by adding sterilized distilled water and 0.1M ethyl methanesulfonate (EMS) as described by Anandarajah et al. (2012) and the suspension was kept in dark for 60 min and 120 min at room temperature. After mutagenic treatment, sodium thiosulfate (10%, w/v) added and the suspension was incubated for 15 min to terminate the process of mutagenesis. Thereafter, the cell suspension was centrifuged and washed thrice with the same phosphate buffer (0.1 M, pH 7.0) and finally, the cells were suspended in 20 mL BG-11 medium. Small aliquots (5 mL) were withdrawn from the cells suspension (both 60 and 120 min treated cells) incubated with 75 µM DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea) in the dark at room temperature for 24 h. The DCMU treated mutagenized cells were again washed with the same phosphate buffer (0.1 M, pH 7.0) and were streaked on the solid agar plates (1.8 % agar in BG-11 medium) containing 50 µM DCMU. The control plate was simultaneously prepared (without DCMU), using the mutagenized microalgal cells with the same approximate density of the culture. At last, both DCMU treated and untreated plates were kept under white fluorescent light (10 w/m^2) for 16:8 h light/dark cycle until the pinhead colonies appeared on the plates.

After 20-25 days, sparsely growing pin head colonies were picked up by using sterilized glass capillary tubes under a trinocular microscope and were transferred to test tubes containing 5 mL BG-11 medium. Then, they were allowed to grow until sufficient turbidity was observed in the growth media. Thereafter, reversibility of the DCMU-tolerance in the mutant strain was checked for 5-6 successive generation in the BG-11 medium.

4.2.4 Morphological identification

The morphology and cells size of both the wild-type (WT) and mutant strain was examined by using the SEM (Scanning Electron Microscope, JSM-6490LV, JEOL, Japan) at Babasaheb Bhimrao Ambedkar University, Lucknow. India. Dried biomass of the microalgal algal cells (5.0 mg) of both the WT and mutant strain were fixed in 2.5% (v/v) of glutaraldehyde for 4 h at 4℃ and it was washed with phosphate buffer (0.01 M, pH 7.0). Thereafter, the cells were dehydrated for 5 min by using the gradient of ethanol solution (v/v) of different concentrations (10%, 30%, 50%, 70%, 95% and 100%) (Kumar et al., 2016). The final dehydration was carried out in 100% (v/v) ethanol solution for 30 min. The dehydrated algal sample was mounted on aluminium stub with the help of carbon tape.

4.2.5 Cell size and cell cycle analysis by flow cytometer

The cells size and cells cycle of both the WT and mutant strain were analysed by high-speed flow cytometer (BD FACS Canto II, BD Bioscience, USA). For the cell size analysis, exponential phase cells of both WT and mutant strain were harvested by centrifugation at 5000 rpm (10 min) and the pellet was washed thrice with PBS (Phosphate buffer saline) solution. A small aliquot of 300 µL of algal cells transferred to a round-bottom tube and 300 µL PBS solution was added and vortexed for 2 min. The fluorescent intensity of 10,000 events was recorded by using the BD FACS Diva™ software. The solid-state blue laser of the flow cytometer was used for excitation wavelength (488 nm).

The cell cycle analysis was performed by flow cytometry by using propidium iodide (PI) dye (Pozarowski & Darzynkiewicz, 2004). The exponential grown microalgal cells of WT and mutant strain were harvested and washed thrice with PBS to remove the debris. An aliquot of 300 µL of cells was supplemented with 300 µL DMSO (10%) solution to enhance the permeability of PI dye. After centrifugation, the pellets were resuspended in 200 µL PBS solution. The microalgal cells of both strains were stained with PI (10 µg/mL) for 30 min at room temperature. The cell cycle analysis was performed with FACS Canto™ II, BD Biosciences, San Jose, CA, USA) equipped with a 488 nm laser. The software used for analysis was BD FACS Diva ™ Software v 6.1.2.

4.2.6 Growth kinetics

Growth of microalgal cells was calculated in terms of optical density at 680 nm for 0-20 days, using a double beam UV-visible spectrophotometer (UV-A 1601, Shimadzu, Japan) and also calculated in terms of specific growth rate (μ) as described in chapter III of Materials and Methods. Another growth kinetics parameter such as absolute growth rate (AGR), doubling time (T_d), generation time (T_g) and maximum yield (Y_{max}) was calculated by the following equation (Liao et al., 2017):

Absolute growth rate (AGR) = X_2-X_1/t_2-t_1

Where, X_2 and X_1 represent the biomass concentration of biomass at t_2 and t_1 time, respectively

Doubling time (T_d, days) = ln (2)/specific growth rate (μ) (Luo et al., 2016)

Generation time (T_g) = 0.6931/ specific growth rate (μ) *24 (calculate in hours)

Maximum yield (Y_{max}) = $e^{LN\ value}$

Where e = 2.718 and LN value represent the cell number

The growth of wild-type and the mutant strain was optimized under different pH (6.8-9.0) and temperature condition (5, 10, 15, 25, 30 ℃). The growth was calculated in terms of specific growth rate (μ) and biochemical constitutes (protein, carbohydrate), and pigment was analysed as described earlier in chapter III Materials and Methods.

4.2.7 Characterisation of mutant strain of microalga *S. vacuolatus*

4.2.7.1 Optimization of DCMU tolerance level and growth inhibitory concentration (I_{50})

The effect or tolerance level of DCMU (10-100 μM) was optimized in WT and mutant strain of microalgae *S. vacuolatus.* The tolerance level was calculated in terms of specific growth rate (μ), biochemical constitutes (protein, carbohydrate), and pigment analysis as described in chapter III, Materials and Methods.

Plot V_0/V of 2 was used to calculate the 50% growth inhibitory concentration (I_{50}) as described by Samuel & Bose, (1987). V_0 is the initial growth rate without inhibitor and V represented the growth rates at different concentration of DCMU. The growth inhibitory concentration corresponding to the value of V_0/V of 2 was taken as the concentration required for 50% inhibition of growth.

4.2.7.2 Measurement of fluorescence spectra of DCMU

Wild-type and mutant grown cells were incubated in the presence of DCMU (75 µM) for 1 h and keep in dark at room temperature before the measurement of the fluorescence emission spectrum (650-750 nm). The chlorophyll fluorescence was measured in the presence of +DCMU (treated) and in the absence of DCMU in both the strains by using Fluorescence Spectrophotometer (EL08013454, Cary Eclipse 100, India). The excitation wavelengths were at 435 nm and emission wavelength was at 675 nm respectively by using a slit width of 5 mm and a light path of 1 cm.

4.2.7.3 Measurement of chlorophyll fluorescence induction kinetics (OJIP)

Chlorophyll a fluorescence transient was measured by using Pulse Amplitude Modulated (PAM) (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic). The 7 days grown cells were incubated in the presence of DCMU (75 µM) for 1 h in the presence of light. For the measurement of chlorophyll fluorescence induction curve (OJIP), the cells without DCMU and with DCMU were dark-adapted for 10 min at room temperature. The induction kinetics of chlorophyll fluorescence was recorded with an excitation wavelength of 450 nm. The fluorescence parameters (quantum yield F_v/F_m, F_v/F_o, ET_0/RC, TR_0/RC, ABS/RC, and PI_{ABS}) were determined as described by Strasser et al. (2000).

- **F_v/F_m** = Maximum quantum yield of PSII (dark-adapted state)
- **F_v/F_0** = Maximum quantum yield of PSII (more sensitive than F_v/F_m)
- **ET_0/RC** = M_0 $(1/V_j)$. Ψ_0 (trapping of energy flux beyond QA in the photosystem II)
- **TR_0/RC** = M_0 $(1/V_j)$ (efficiency of electron transport leading to reduction of QA)
- **ABS/RC** = M_0. $(1/V_J)$. $(1/\varphi\_P_0)$ (Absorption of energy flux per active reaction center)
- **PI_{ABS}** = $(\varphi\_P_0/(1- (\varphi\_P_0)x$ (Performance index on absorption basis)

4.2.7.4 Flow cytometer analysis

The lipid content of both wild-type and the mutant strain was analysed by high-speed flow cytometer (BD FACS Canto II, BD Bioscience, USA). The different phase of

microalgal cells (lag, exponential, stationary) were harvested by centrifugation at 5000 rpm for 10 min and washed thrice with PBS (Phosphate buffer saline). The procedure described in chapter III of Materials and Methods.

4.2.8 Statistical analysis

The performed statistical analysis was described in Materials and Methods of Chapter III.

4.3 Results

4.3.1 Isolation and molecular identification of microalgal strain

The unicellular microalga was isolated from the Pangong Lake, Leh Ladakh, in the state of J & K, India. The microalgae were growing under high light intensity in the Pangong Lake, Leh Ladakh (J & K), India. However, Pangong Lake situated at a height of about 4,350 m. whereas Leh is a town in the Leh district of the state Jammu and Kashmir (India) situated at longitude 34°08'43.43'N and latitude 77°34'03.41'E.

The isolated microalga was routinely grown on BG-11 medium. The isolated unicellular microalga was identified, using 18S rRNA gene sequencing technique (White et al., 1990) from the National Collection of Industrial Microorganism (NCIM; CSIR-NCL, Pune, India), the microalgal strain (wild-type) was identified as *Scenedesmus vacuolatus*. The gene sequence was submitted to the NCBI (National Center for Biotechnology Information) for the accession number; the provided NCBI GenBank accession number is MH459062 for *Scenedesmus vacuolatus*.

The universal primer NS1_NS4 (1021 bp) were used for amplification as detail given in Table 4.1. Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between gene sequences. The gene sequencing and blast analysis showed that *Scenedesmus vacuolatus* (MH459062) (96%) exhibited similarities with *Chlorella Zofingiensis* (X74004.1) (95%) and *Bracteacoccus bullatus* (JQ259930.1) (94%). Based on the NCBI database, the similarities of *S. vacuolatus* with other different species are present in the form of the phylogenetic tree as shown in (Fig. 4.1)

Table 4.1 Details of primers used in the gene sequencing of *S. vacuolatus*.

Locus	Primer	Direction	Primer sequence (5' 3') →	Bases
18S	NS1	Forward	GTAGTCATATGCTTGTCTC	19
18S	NS4	Reverse	CTTCCGTCAATTCCTTTA	18

- Gene sequence of *S. vacuolatus*

>NS1_NS4_SeqrRNA

TCCGTGGCCTTAGGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAA
TCAGTTATAGTTTATTTGGTGGTACCTTCTTACTCGGAATAACCGTAAGAAAATTAG
AGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATATATTAGATAAAAG
GCCGACCGGGCTCTGCCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCA
TGGCCTTGCGCCGGCGCTGTTCCATCAAATTTCTGCCCTATCAACTTTCGATGGT
AGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTC
CGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCG
CAAATTACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAATACCGGGC
ATTTCATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCAT
TGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTAT
ATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGGGTTTCAGCG
GTCCGCCTATGGTGAGTACTGCTGTGGCCTTCCTTACTGTCGGGGACCTGCTTCT
GGGCTTCATTGTCCGGGACAGGGATTCGGCATGGTTACTTTGAGTAAATTAGAGT
GTTCAAAGCAGGCTTACGCCGTGAATACTTTAGCATGGAATAACATGATAGGACT
CTGCCCTATTCTGTTGGCCTGTAGGAGTGGAGTAATGATTAAGAGGAACAGTCGG
GGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACT
ACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGG
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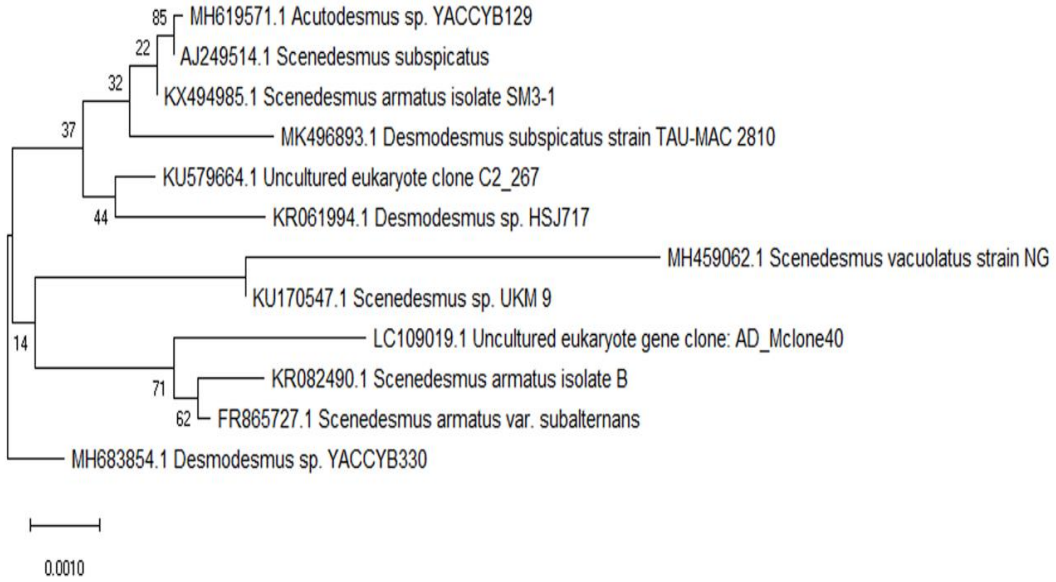

Fig. 4.1 Phylogenetic analysis of *Scenedesmus vacuolatus* inferred from 18s rRNA gene sequencing and references sequence of its nearest relatives based on the NCBI database.

4.3.2 Characteristics of *Scenedesmus* species

Scenedesmus spices are unicellular, colonial and non-motile microalga belonging to family Scenedesmaceae. In the present time, approx. 74 taxonomic species of *Scenedesmus* such as *acutodesmus, dimorphus, acuminatus, vacuolatus* etc. are known to exist (Guiry, 2015). This is the most common freshwater alga genera and present in diverse morphologies within species that make difficult to identify.

Scientific classification

Phylum: Chlorophyta
Class: Chlorophyceae
Order: Sphaeropleales
Family: Scenedesmaceae
Genus: *Scenedesmus*

4.3.3 Selection of mutant strain

The isolated mutants was selected amongst many DCMU tolerant mutant clones due to its fastest growth on the BG-11 medium. Further, other characteristic features of the mutant was compared with the parent strain (wild-type).

4.3.4 Morphological identification of microalga *S. vacuolatus*

The morphological identification of microalgae *S. vacuolatus* of both wild-type and mutant strain in terms of shape and cells size were carried out, using SEM (Scanning Electron Microscope, JSM-6490LV, JEOL, Japan) analysis at BBA University, Lucknow. The results demonstrated that the morphology of *S. vacuolatus* mutant strain was very closely similar to the WT cells. The results of SEM analysis showed that the average size of the wild-type cells (1.745 µm) was larger than the mutant cells (1.3725 µm). Thus, the results revealed that the mutation in *S. vacuolatus* cells led to the smaller size of the isolated mutant strain when compared with the WT (Fig. 4.2).

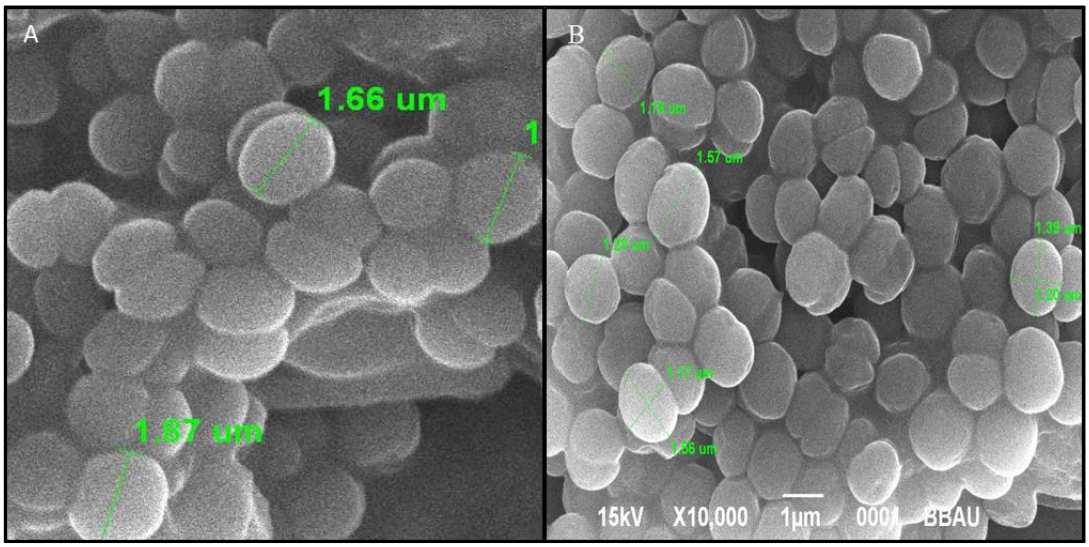

Fig. 4.2 The microscopic examination of both wild-type (Fig. 2A) and mutant strain (Fig. 2B) of *S. vacuolatus* was performed under SEM (JEOL, Japan).

4.3.5 Cells size and cell cycle analysis by flow cytometer

The cells size analysis of microalgal cells was also confirmed by the flow cytometer. The result demonstrated that mutant strain cells size smaller than WT cells. The fluorescence signal of both WT and mutant strain was obtained using a solid-state laser. Cells morphology including cells size and granularity were correlated with two

scattering signals, forward scattering (FSC) which determines the size of the cell and side scattering (SSC) represents the cellular granularity. The fluorescent intensity of WT cells was higher (6.35%) than the fluorescent intensity of mutant cells (2.40%), which clearly indicated that WT cell size was bigger than the mutant cells (Fig. 4.3).

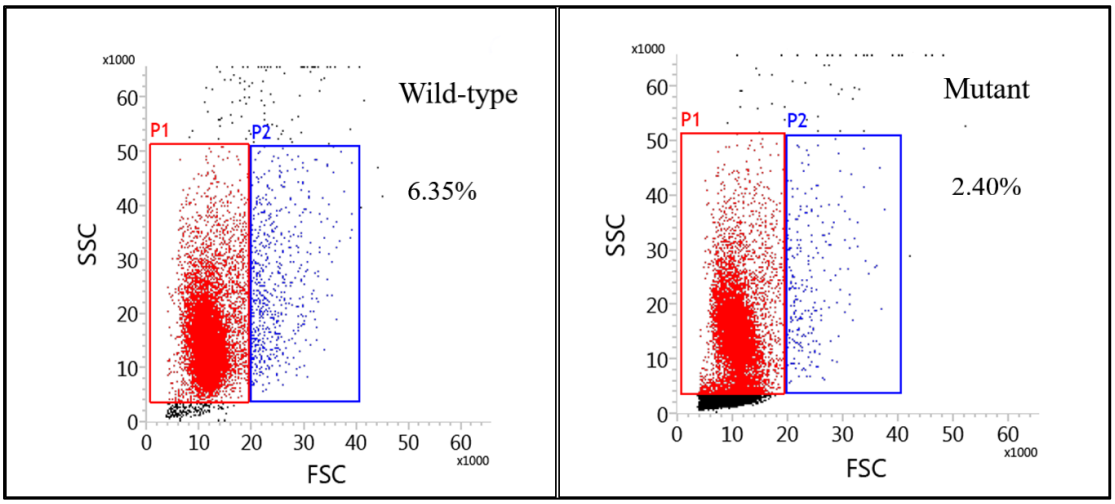

Fig. 4.3 Cell size analysis by flow cytometer of both wild-type and mutant strain of *S. vacuolatus*.

The cell cycle analysis of both WT and mutant was done by flow cytometer. The result of cell cycle analysis of WT and mutant strain has revealed that there is an increment in the cell proliferation in the mutant strain (without DMSO, with DMSO (10%, v/v) as compared to WT (-DMSO and +10 % (v/v) DMSO) cell cycle analysis. The results revealed fast shifting from G_1 phase (2n) to the S phase of the cell cycle in the mutant strain than the WT. The S phase (Synthesis Phase) of the cell cycle has been earmarked for DNA replication and the DNA content and it is between G_1 phase and G_2M phase. Thus, on the basis of cell cycle analysis by flow cytometer, it is concluded that the mutant strain is growing faster than the WT (Fig. 4.4).

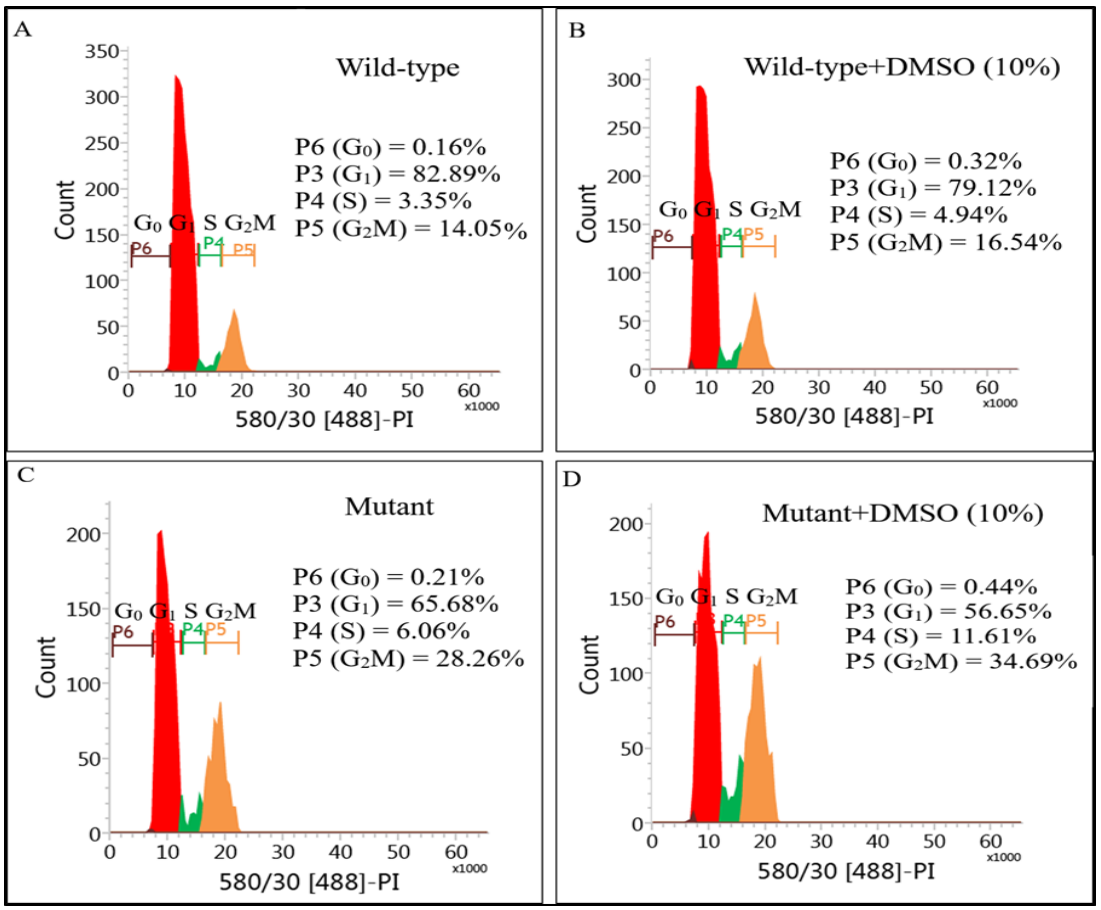

Fig. 4.4 Cell cycle analysis by flow cytometer of both wild-type and mutant strain of *S. vacuolatus* in the presence of DMSO and control (without DMSO).

4.3.6 Growth kinetics

The growth measurement of WT and the mutant strain was determined at a regular interval in terms of increases in the optical density (OD) of the cell suspension at 680 nm for 0-20 days (Fig. 4.5) by using a double beam UV-visible spectrophotometer (UV-A 1601, Shimadzu, Japan). The time-dependent growth curve showed that continuous growth in both strains till 16th days of growth followed by suddenly decline in the growth between 16-20 days in both WT and mutant strains. Further, overall growth in the mutant strain was higher in the WT. Other growth parameters detail such as absolute growth rate (AGR), specific growth rate (μ), doubling time (T_d), generation time (T_g) and maximum yield (Y_{max}) are given in Table 4.2.

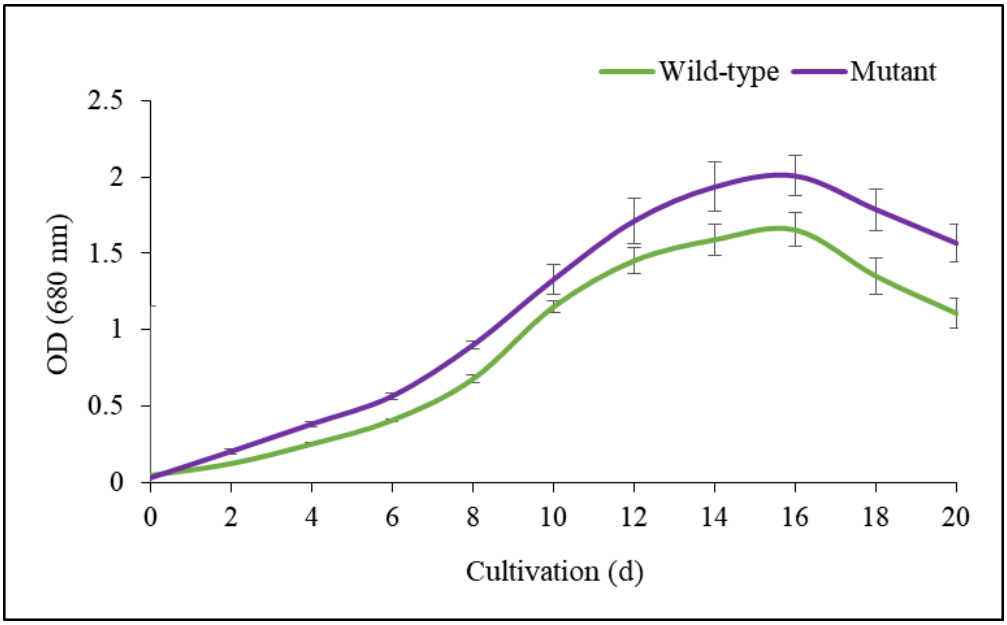

Fig. 4.5 Optimization of growth of wild-type and mutant strain of *S. vacuolatus* in terms of optical density (OD) measured by spectrophotometer. Error bar showed the mean±SD.

Table 4.2 Study of growth kinetics of wild-type and mutant strain of *S. vacuolatus.* Data depicted as mean±SD.

Growth Kinetics	Absolute growth rate (AGR)	Specific growth rate (μ), d^{-1}	Doubling time (T_d)	Generation time (T_g) (24 h)	Maximum yield (Y_{max})
Wild-type	0.072±0.002	0.167±0.0072	4.14±0.021	99.5±6.2	9.8±0.36
Mutant strain	1.34±0.082	0.204±0.011	3.39±0.011	81.3±4.1	11.08±0.08

Further, optimization of different growth conditions under ranging pH, temperature and light conditions were performed in both WT and mutant strain as discussed below.

4.3.6.1 Effect of varying pH conditions

Favourable pH plays an important role in microalgal growth. Growth of both WT and mutant strain of microalgae *S. vacuolatus* was studied in pH range between pH 6.8-9.0.

Effect of varying pH conditions (6.8-9.0) on the specific growth rate (μ) of WT and mutant strain, as shown in Fig. 4.6, showed that specific growth rate of WT (0.16±0.011 d^{-1}) and mutant strain (0.20±0.020 d^{-1}) was maximum at pH 7.8. Further increase in the pH towards the alkaline range, beyond the optimum pH, showed a

gradual decline in the specific growth rate of both WT and mutant strain. However, the second-best specific growth rate of WT (0.14±0.0056 d^{-1}) and mutant strain (0.17±0.011 d^{-1}) was observed at pH 8.2, when compared with respective specific growth rated of both the WT and mutant strains at pH 8.8 and 9. The lowest value of the specific growth rates of WT (0.063±0.006 d^{-1}) and mutant strain (0.095±0.005 d^{-1}) was recorded at pH 6.8. However, the overall results indicated that the growth of both the strains of microalgae *S. vacuolatus* was the highest at alkaline pH (7.8) as compared to growth rates in the acidic (pH 6.8) and alkaline pH conditions (pH 8.5 and pH 9.0). The statistical analysis was performed by sample paired Student't'-test to determine the mean difference of specific growth rate of WT and mutant strain under different pH condition.

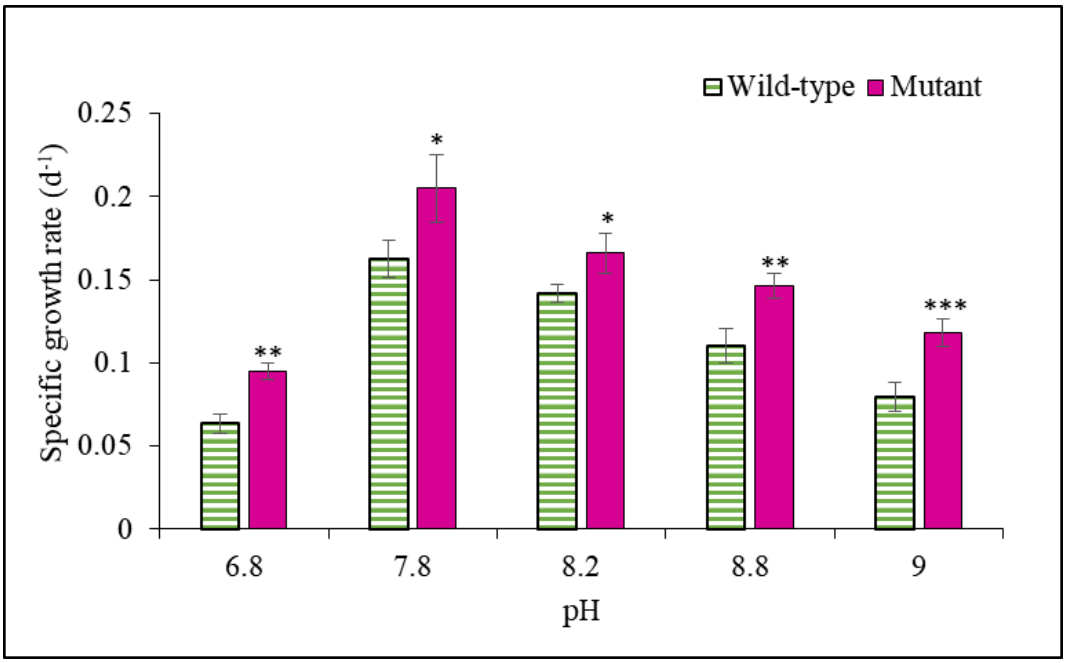

Fig. 4.6 Specific growth rate (μ) of wild-type and mutant strain of *S. vacuolatus* in the presence of varying pH condition (6.8-9.0). Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

The result in Fig. 4.7 (a, b) depict the effect of varying pH conditions (6.8-9.0) on the biochemical constituents (protein and carbohydrate content) of both WT and mutant strain. It was observed that the protein content of WT (128±8.1 μg mL^{-1}) and mutant strain (176±6.5 μg mL^{-1}) was maximum at pH 7.8. A further rise in the pH of growth medium beyond the optimum pH (7.8) resulted in a pH-dependent gradual

decline in the protein content of both WT and mutant strain (Fig. 4.7, a). Similarly, the maximum carbohydrates content was recorded at pH 7.8 in both WT (145±6.0 µg mL^{-1}) and mutant strain (181±6.5 µg mL^{-1}), as compared to other pH condition (8.2-9.0 µg mL^{-1}) (Fig. 4.7, b). However, the overall result showed that biochemical constituents like protein, carbohydrate in the mutant strain were higher than that in the WT. The mean significant difference level between biochemical constituents of both WT and mutant strain was determined by sample paired Student't' test under all the selected pH conditions (6.8-9.0).

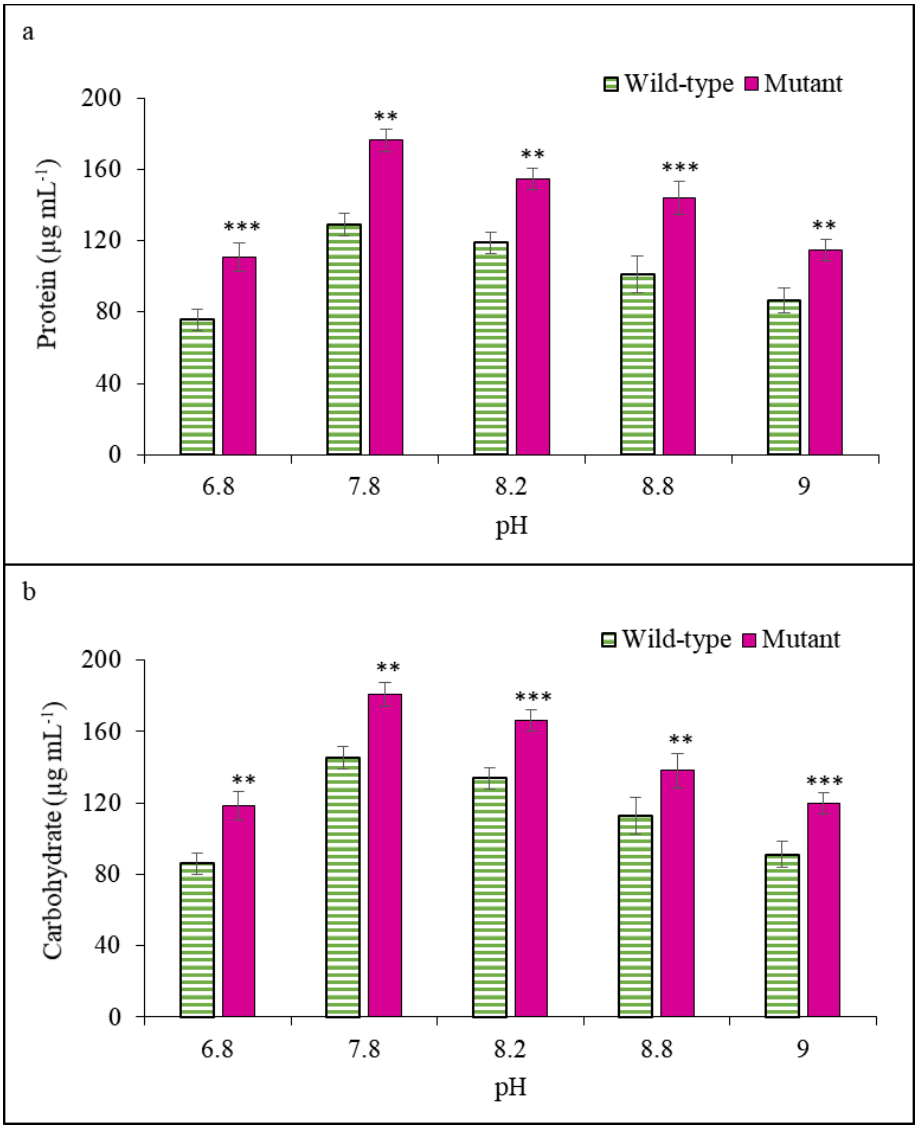

Fig. 4.7 Changes in biochemical constituents protein (a) and carbohydrate (b) of wild-type and mutant strain of *S. vacuolatus* in the presence of varying pH condition (6.8-9.0). Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

Effect of varying pH (6.8-9.0) conditions on the pigment content (total chlorophyll and carotenoid) of both the WT and mutant strain, as depicted in Fig. 4.8 (a, b), revealed an increase in the total chlorophyll and carotenoid content of WT (3.48±0.34, 1.30±0.08 µg mL^{-1}, respectively) and mutant strain (4±0.44, 1.52±0.075 µg mL^{-1}, respectively) at pH 7.8. A further increase in the pH of the medium showed a pH-dependent decline in the total chlorophyll and carotenoid level in both WT and mutant strains. The lowest level of chlorophyll and carotenoid content were recorded at pH 6.8 and pH 9.0 in both the strains. The overall pigment content in the mutant strain was higher than the WT under all the selected pH conditions. The significance mean difference between the pigment of WT and mutant strain was analyzed by sample paired Student't' test under varying pH conditions.

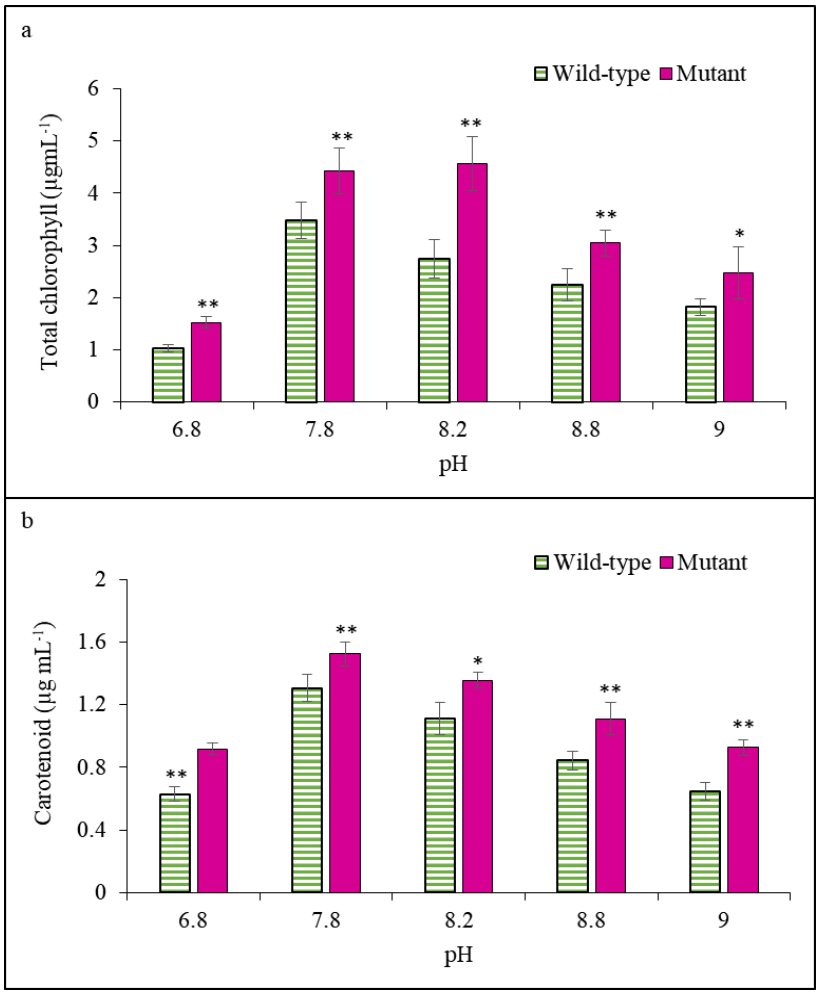

Fig. 4.8 Changes in total chlorophyll (a) and carotenoid (b) of wild-type and mutant strain of *S. vacuolatus* in the presence of varying pH condition (6.8-9.0). Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

4.3.6.2 Effect of different temperature conditions

Temperature is another parameter which plays an important role in the growth of microalgae, as it affects the metabolic and physiological condition of microalgae. The favourable temperature favours higher growth and lipid content in the microalgae. In the present study, different temperature (5, 10, 15, 25, 30 ℃) conditions were used for the optimisation of optimum microalgal growth as measured in terms of specific growth rate (μ), biochemical constituents (protein, carbohydrate) and pigment analysis.

The specific growth rate of both WT and mutant strain was recorded at different temperature (5-30 ℃). The result showed that specific growth rate of WT (0.162±0.01 d^{-1}) and mutant strain (0.199±0.010 d^{-1}) was maximum at temperature 25℃, whereas the specific growth rate of WT and mutant strain (0.142±0.01, 0.17±0.009 d^{-1} respectively) gradually declined with increase in temperature (Fig. 4.9). Further, the results showed that the specific growth rate of WT and mutant strain was reduced with decreasing temperature (5, 10, 15 ℃). The growth rate of mutant strain at 25℃ was found to be comparatively better than the corresponding growth of WT. The statistical analysis was performed by sample paired Student‘t’ test to determine the mean difference on the effect of temperature of WT and mutant strain.

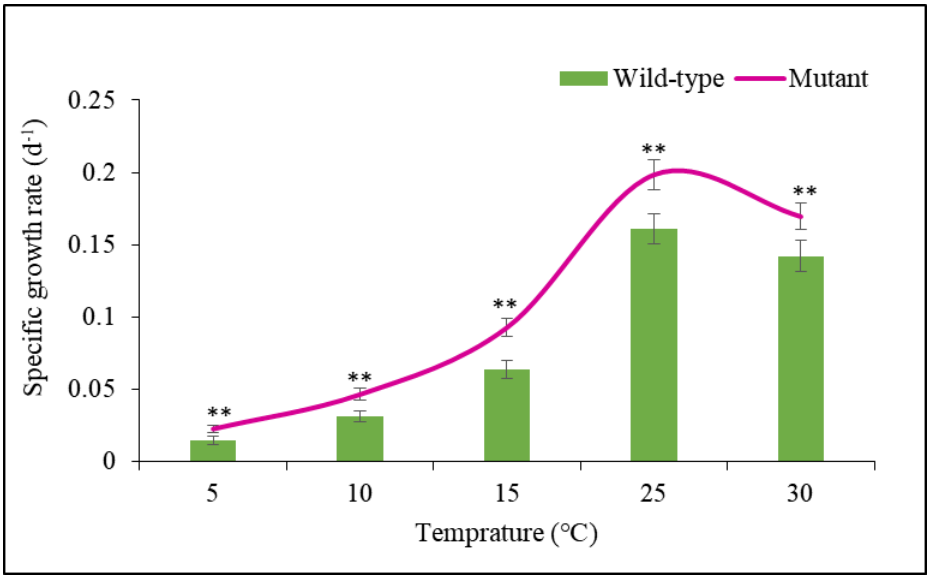

Fig. 4.9 Specific growth rate (μ) of wild-type and mutant strain of *S. vacuolatus* under different temperature conditions (5-30℃). Student‘t’ test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

Results on the effect of different temperature (5-30°C) on biochemical constituents (protein, carbohydrate) of WT and mutant strain, as shown in Fig. 4.10 (a, b), showed the highest protein content in both the WT (129±6.5 µg mL^{-1}) and mutant strain (165±5.0 µg mL^{-1}) at 25°C temperature. Further increase in the temperature beyond 25°C showed a temperature-dependent gradual decline in the protein content of both WT and mutant strain. Similarly, the carbohydrates content in both the WT (140±7.1 µg mL^{-1}) and mutant (177±8.3 µg mL^{-1}) strain was found to be the highest at 25°C temperature (Fig. 4.10 b). However, the overall results showed higher biochemical constituents in the mutant strain than the WT under all the temperature conditions. Student't' test used to determine the significant difference between the biochemical constituents of WT and mutant strain under all the temperature conditions.

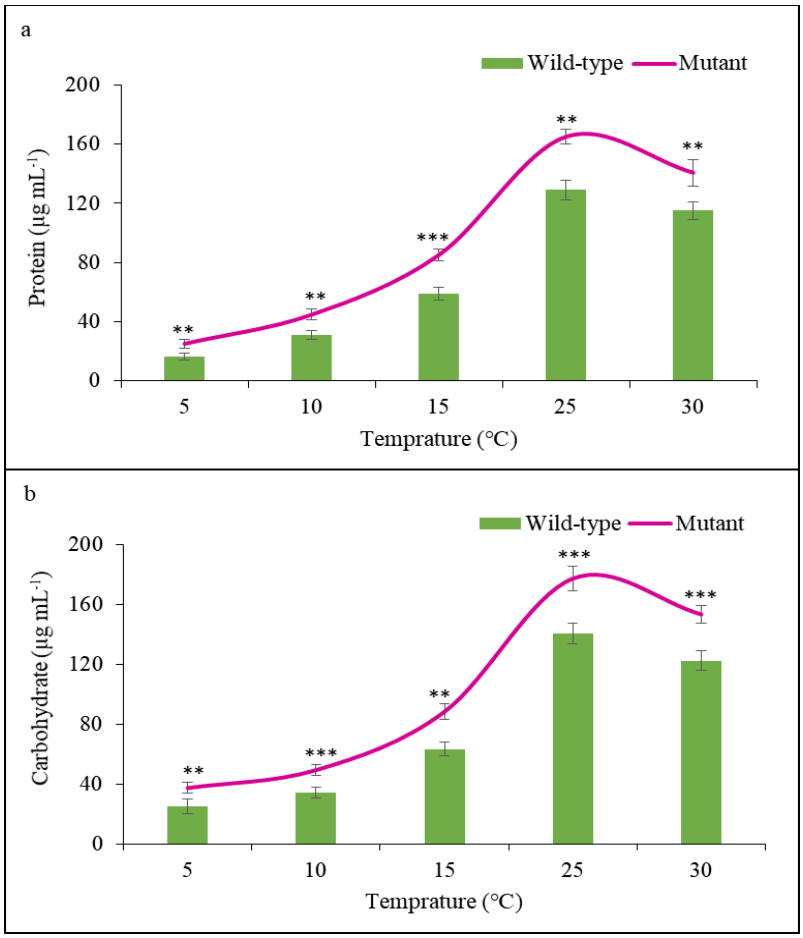

Fig. 4.10 Changes in biochemical constituents protein (a) and carbohydrate (b) of wild-type and mutant strain of *S. vacuolatus* different under temperature conditions (5-30°C). Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

The result in Fig. 4.11 (a, b) exhibited the effect of varying temperature (5-30°C) conditions on the pigment content (total chlorophyll and carotenoid) of both the WT and mutant strain. These results showed that total chlorophyll and carotenoid level in the WT (3.42±0.35, 1.2±0.10 µg mL^{-1} respectively) and mutant strain (4.6±0.32, 1.6±0.05 µg mL^{-1} respectively) was maximum at 25°C temperature. Further increase in the temperature beyond the optimum growth temperature showed a decline in total chlorophyll and carotenoid content of both WT and mutant strain. On the other hand, the pigment content in both the strains was reduced at lower growth temperatures (5-15°C). The significance of data was supported by sample paired Student t-test on the effect of varying temperature conditions on the pigment content of WT and mutant strain.

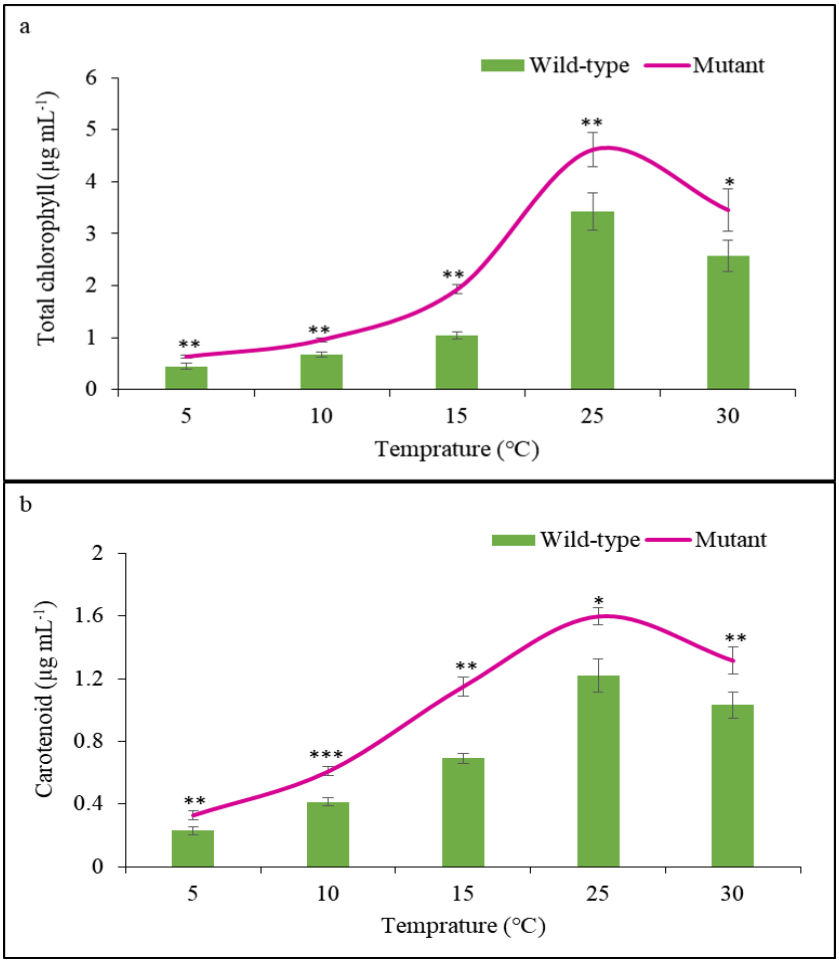

Fig. 4.11 Changes in total chlorophyll (a) and carotenoid (b) of wild-type and mutant strain of *S. vacuolatus* under different temperature conditions (5-30°C). Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

4.3.7 Characterisation of mutant strain of microalga *S. vacuolatus*

4.3.7.1 Optimization of DCMU tolerance level and Plot of V_0/V of 2 (growth inhibitory concentration (I_{50}) of DCMU)

Effect of DCMU (10-100 µM) on the specific growth rate, biochemical constituents and pigment content in the WT and mutant strain of microalgae *S. vacuolatus* revealed a little decrease in the specific growth rate (µ, d^{-1}) of WT (0.07±0.0097 d^{-1}) and mutant strain (0.11±0.011 d^{-1}) at 10 µM concentration of DCMU (Fig. 4.12, a), when compared with the control (without DCMU, WT (0.16±0.0095 d^{-1}) and mutant strain (0.21±0.011). A further increase in DCMU concentration (10-100 µM) showed a concentration-dependent fast decline in the specific growth rate of both the WT and mutant strain (Fig 4.12, a). There was drastic inhibition of growth in both WT and mutant strain at 100 µM concentration of DCMU. DCMU sensitivity of both WT and mutant strain towards DCMU (10-100 µM) was compared by V_0/V of 2 plot of their sensitivity to DCMU (Fig. 4.12, b). The 50% growth inhibitory concentration (I_{50}) of DCMU was found to be 9 and 20 µM for the WT and mutant strain, respectively. Thus, it was observed that mutant strain showed about 2 fold higher tolerance towards DCMU as compared to the WT. The statistical analysis was performed by sample paired Student't' test on the specific growth rate of WT and mutant strain in the presence of a different concentration of DCMU.

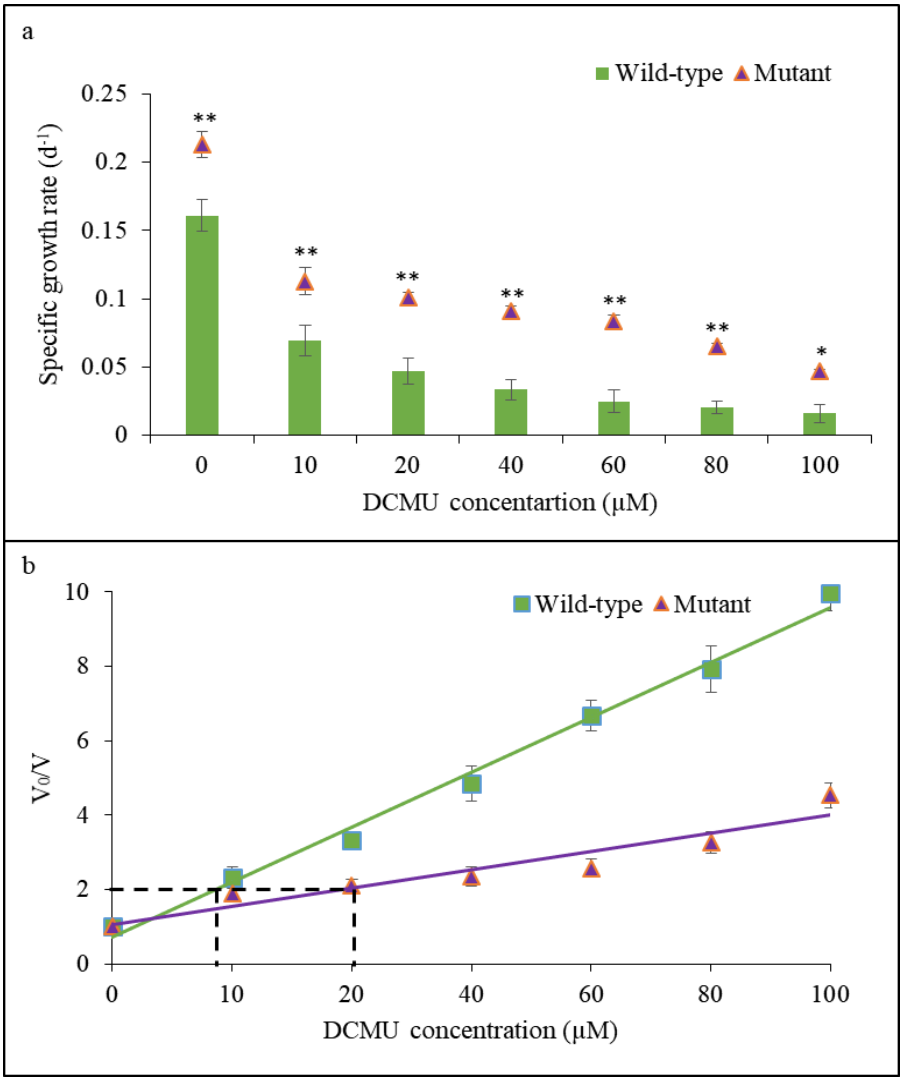

Fig. 4.12 Specific growth rate (a) Plot of V_0/V of 2 (indicating I_{50} inhibitory concentration) (b) of wild-type and mutant strain of *S. vacuolatus* in the presence of a different concentration of DCMU. Student't' test was applied on specific growth rate to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

The effect of different concentration of DCMU (10-100 µM) was recorded on biochemical constituents (protein and carbohydrate) of WT and mutant strain as shown in Fig. 4.13 (a, b). The results revealed that protein content in both the WT (128±6.0 µg mL^{-1}) and mutant strain (171±8.0 µg mL^{-1}) in the absence of DCMU (control) was higher in comparison to that observed under the effect of varying concentrations of DCMU. The result showed DCMU concentration-dependent decline in the protein content of both the WT (66±4.5 µg mL^{-1}) and mutant strain (97±5.0 µg mL^{-1}). A complete cessation of protein synthesis was observed in both the strain was

observed between 60-100 µM concentrations of DCMU (Fig. 4.13, a) Similarly, the carbohydrate content of WT and mutant strain also declined in response to increasing concentration of DCMU (Fig. 4.13, b), when compared with their respective control (without DCMU). The carbohydrate content at 10 µM concentrations of DCMU in the WT (76±5.0 µg mL^{-1}) and mutant strain (102±6.5 µg mL^{-1}) was reduced by 55.4% and 58%, as compared to respective control (100%). The overall results showed that biochemical constituents were higher in the mutant strain than the WT in response to varying concentration of DCMU. Student't' test was applied to show significant difference level of WT and mutant strain on the effect of biochemical constituents in the presence of a different concentration of DCMU.

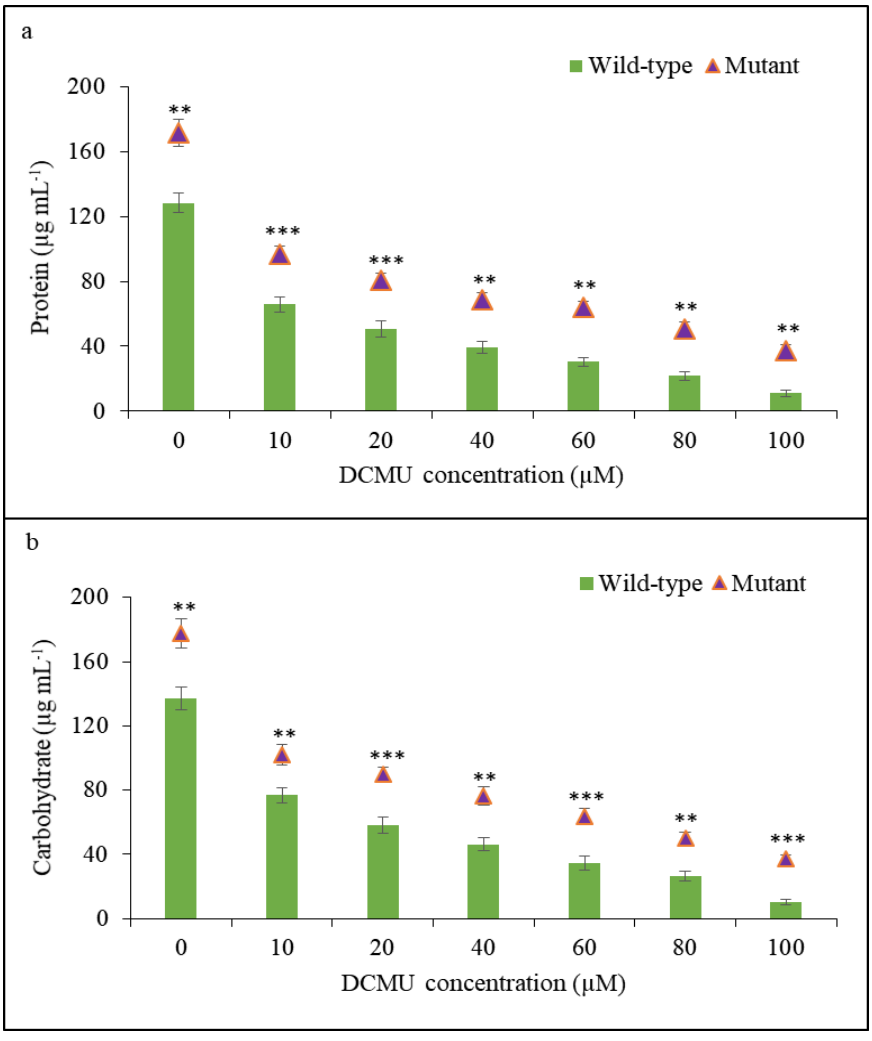

Fig. 4.13 Biochemical constituents of protein (a) and carbohydrate (b) content of wild-type and mutant strain of *S. vacuolatus* in the presence of different DCMU concentration. Student't' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

Similarly, the effect of different concentration of DCMU (10-100 µM) was observed on photosynthetic pigments (total chlorophyll and carotenoid content) of both the WT and mutant strain as given in Fig. 4.14 (a, b). The results showed that total chlorophyll and carotenoid content of WT (1.5±0.11, 0.67±0.066 µg mL^{-1}, respectively) and mutant strain (2.5±0.36, 1.03±0.065 µg mL^{-1}, respectively) were reduced by 38 & 48% and 52 & 68%, respectively at 10 µM concentration of DCMU (Fig. 4.14, a, b), when compared with the control (without DCMU, 100%). The overall chlorophyll and carotenoid content in the WT (4.0±0.16 & 1.3±0.095 µg mL^{-1}, respectively) and mutant strain (5.09±0.45 1.53±0.11 µg mL^{-1}, respectively) in the absence of DCMU were higher than that observed in the DCMU treated strains. However, result further exhibited that with an increase in the DCMU concentration, pigment content in both the WT and mutant strain showed a declining pattern. The paired sample Student't' test showed significant difference level ($p<0.05$) between WT and mutant strain related to pigment content in the response to different concentration of DCMU.

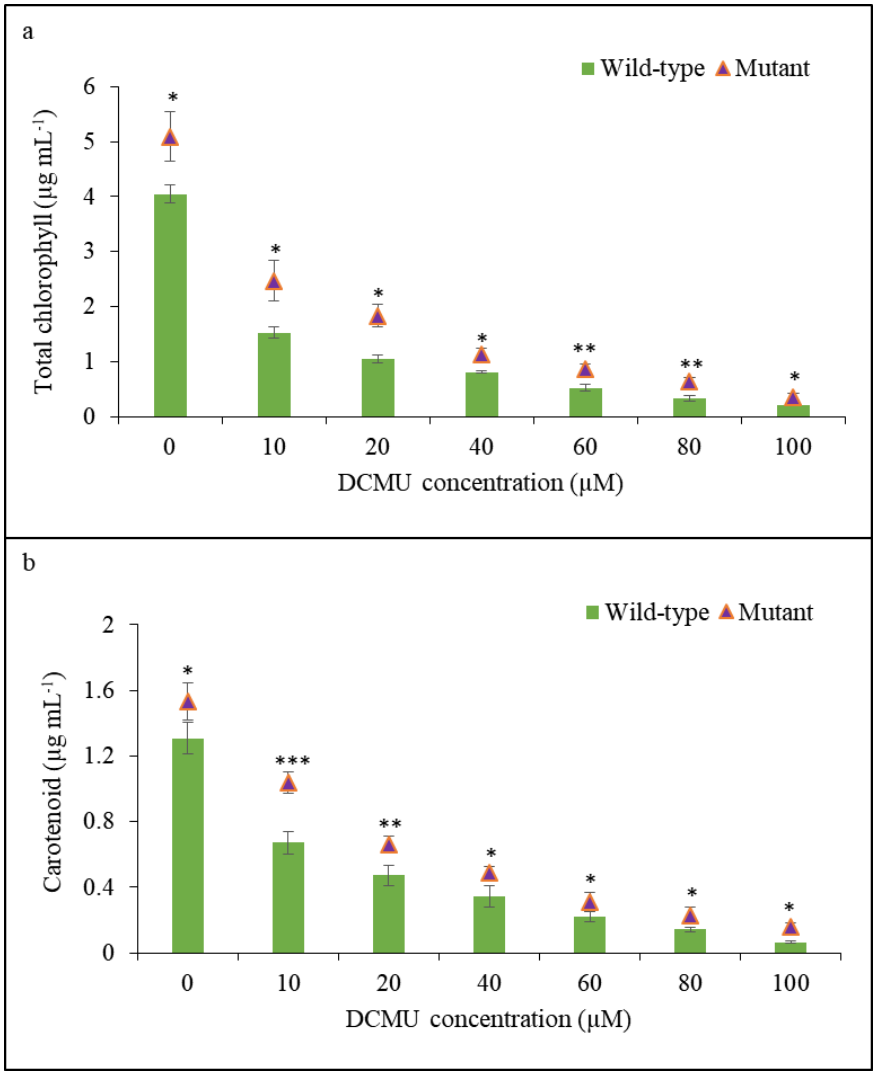

Fig. 4.14 Total chlorophyll (a) and carotenoid (b) content of wild-type and mutant strain of *S. vacuolatus* in the presence of different DCMU concentration (10-100 µM). Student'*t*' test was applied to determine significance difference level at *p<0.05, ** p<0.01, ***p<0.001, ns- non-significant. Error bar showed the mean±SD.

4.3.7.2 Measurement of fluorescence spectra of DCMU

The fluorescence emission spectra (600-750) of both WT and mutant strain was recorded in the absence (–DCMU) and presence of 75 µM of DCMU (+DCMU). A characteristic peak of chlorophyll was observed at 686 nm, emanating from chlorophyll associated with Photosystem II reaction center. The result showed that chlorophyll fluorescence intensity at 686 nm in case of WT and mutant strain were enhanced by 118.1% and 50.5% in the presence of 75 µM of DCMU as compared to their respective control (without DCMU, 100%) (Fig. 4.15). Based on the results, the results may be interpreted as a percent increase in the chlorophyll fluorescence in both the strains was

in proportion to DCMU induced inhibition of their photosystem II activity. Thus, a lesser inhibitory effect of DCMU on PSII due to its enhanced tolerance to DCMU might have resulted in relatively less increase in the chlorophyll fluorescence. Therefore, results suggested that WT cells are more sensitive to DCMU as compared to mutant strain

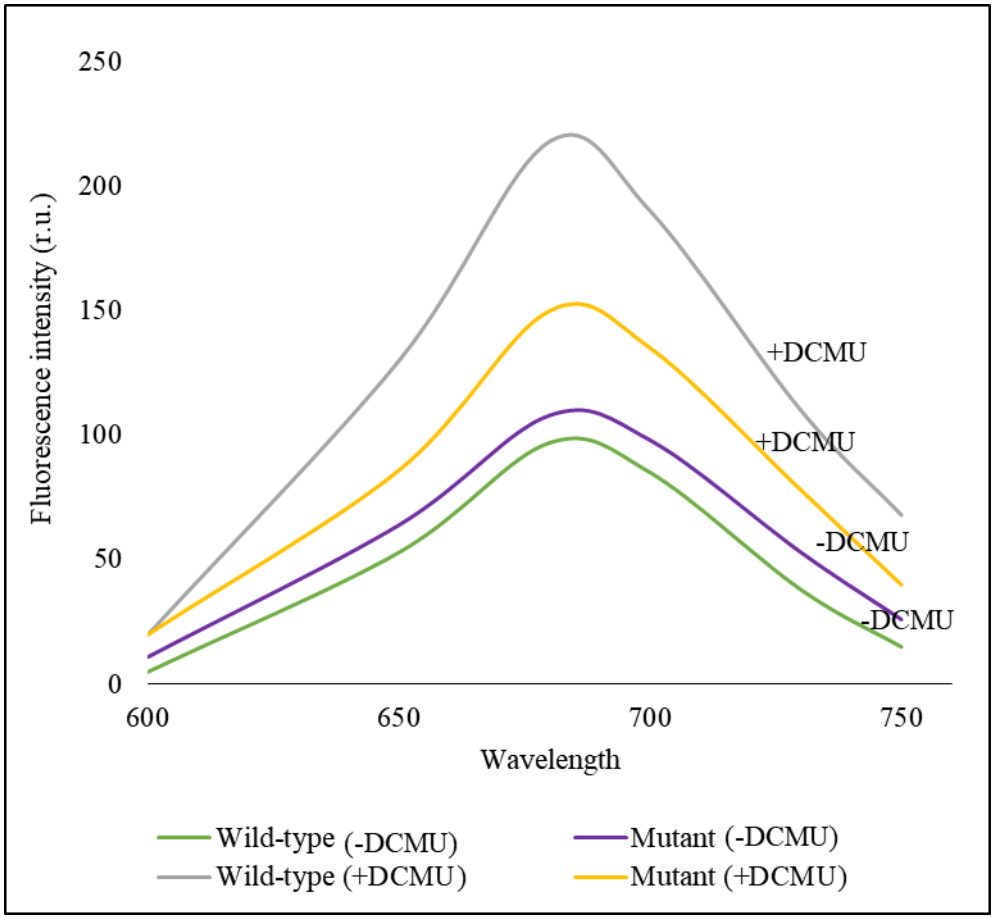

Fig. 4.15 Chlorophyll fluorescence spectra (600-750 nm) of wild-type and mutant strain of *S. vacuolatus* in the absence and presence of DCMU (75µM) treated cells.

4.3.7.3 Chlorophyll fluorescence induction kinetics (OJIP) analysis

Effect of DCMU on the Chlorophyll fluorescence induction kinetics (OJIP) in both the WT and mutant strain was analyzed to ascertain the health of the photosynthetic apparatus. Data pertaining to changes in the OJIP parameter are presented in Table 4.3. Different parameters such as F_v/F_m, F_v/F_0, ET_0/RC, TR_0/RC, ABS/RC, and $PI_{ABS,}$ derived from OJIP curve, showed a major difference in PSII photochemistry of DCMU grown cells of WT and mutant strain (Fig. 4.16) when compared with control (-DCMU). The maximum

quantum yield of photosystem II (F_v/F_m) in the dark-adapted WT and mutant strain in the absence of DCMU (0.56±0.031, 0.61±0.042 respectively) was higher than the quantum yield (F_v/F_m) observed in the presence of DCMU (75 µM) for both the strains (0.35±0.015, 0.39±0.02, respectively). The quantum yield of DCMU supplemented cells of both the strains was reduced by about 40% as compared to untreated control. The result showed that F_v/F_0 ratio of WT (1.27±0.11) and mutant strain (1.59±0.12) was higher in the absence of DCMU as compared to DCMU supplemented WT and mutant strains (0.53±0.041, 0.64±0.030, respectively). However, overall results also showed that quantum yield in the mutant strain was better than the WT under both DCMU added and control (-DCMU) conditions. However, other photosynthetic parameters also exhibited a decline in the photosynthetic performance of both the strains, when treated with DCMU (Fig. 4.16).

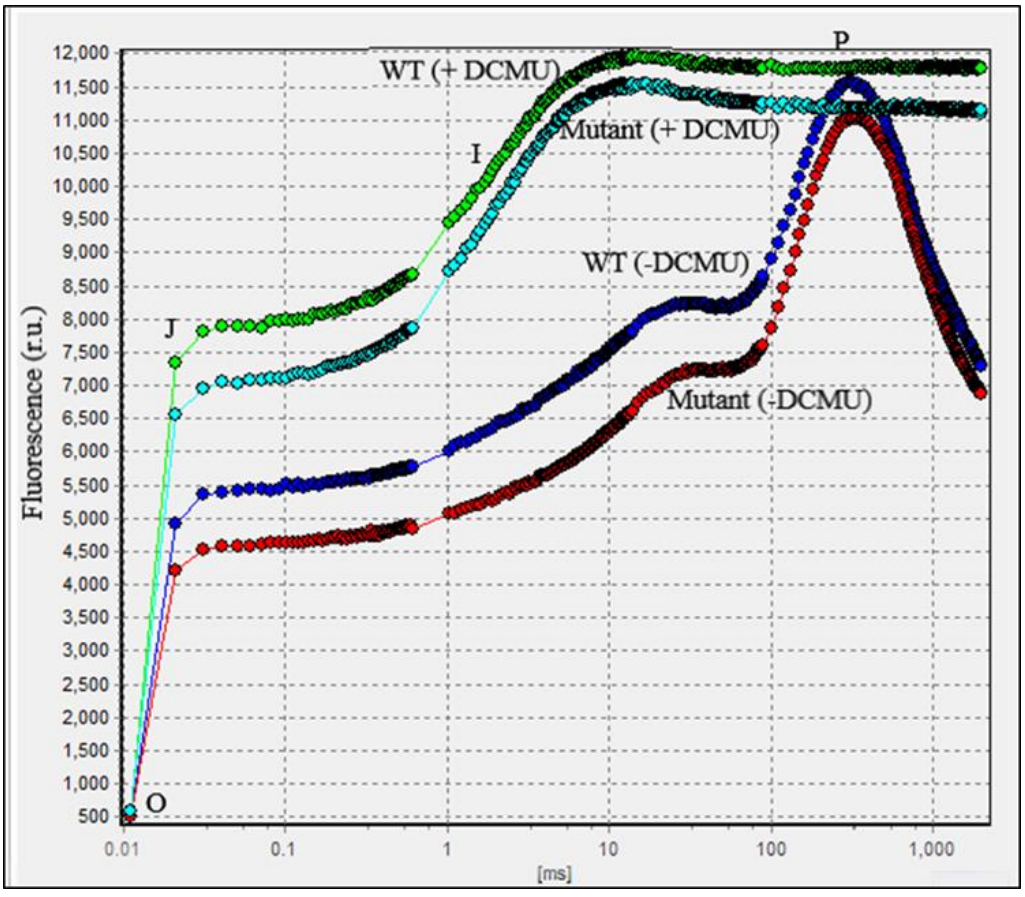

Fig. 4.16 Chlorophyll induction kinetics curve (OJIP) of wild-type and mutant strain of *S. vacuolatus* grown cells in the presence (+DCMU) and absence of (-DCMU).

Similarly, results on the TR_0/RC (denoting the respective efficiency of electron transport leading to reduction of QA) and ET_0/RC (trapping of energy flux for electron carriers beyond QA) showed higher values in the mutant (0.69±0.04, 0.95±0.036, respectively) strain than the WT (0.83±0.04, 1.07±0.10, respectively). The values of TR_0/RC and ET_0/RC in the DCMU supplemented WT and mutant strains (0.59±0.02, 0.59±0.025 and 0.231±0.011, 0.21±0.01, respectively) were reduced as compared to control (Table 4.3).

Table 4.3 Changes in OJIP parameters derived from chlorophyll induction fluorescence induction kinetics of the wild-type and mutant strain of *S. vacuolatus* grown cells in the presence of (+DCMU) and absence of (-DCMU). Data depicted as Mean±SD.

***S. vacuolatus* strain**	**F_v/F_m**	**F_v/F_0**	**TR_0/RC**	**ET_0/RC**	**ABS/RC**	**PI_{ABS}**
Wild-type (-DCMU)	0.56±0.030	1.3±0.110	0.834±0.051	0.699±0.040	1.49±0.131	4.40±0.210
Mutant (-DCMU)	0.61±0.042	1.6±0.121	1.068±0.110	0.951±0.036	1.73±0.152	7.52±0.611
Wild-type (+DCMU)	0.35±0.015	0.54±0.041	0.594±0.022	0.231±0.011	1.69±0.133	0.20±0.012
Mutant (+DCMU)	0.43±0.02	0.65±0.030	0.587±0.025	0.214±0.011	1.49±0.126	0.25±0.011

Similarly, ABS/RC, denoting absorption of energy flux per active reaction center, showed higher values in the untreated (-DCMU) WT and mutant strain (1.49±0.13, 1.73±0.15 respectively) than the corresponding values of ABS/RC in the DCMU added WT and mutant strain (1.69±0.13, 1.49±0.12, respectively). Although, the performance index of PSII on absorption basis (PI_{ABS}) in WT and mutant strain in the absence of DCMU (4.4±0.21, 7.5±0.61 respectively) was higher than that in the DCMU added WT and mutant strains (0.20±0.010, 0.25±0.011 respectively). These results clearly demonstrated that the photosynthetic performance of the mutant strain was better than the WT. However, the photosynthetic performance of both the strain was relatively better in the absence of DCMU herbicide (Table 4.3). These results suggested that the photosynthetic yield and electron transport in both the strains were higher in the absence of DCMU. However, photosynthetic efficiency of mutant was relatively better than the WT

4.3.7.4 Flow cytometer analysis

The flow cytometer analysis of neutral lipid content in both WT and mutant strains was carried out during different growth phases (lag phase, exponential and stationary phases). The results showed that lag phase of growth in both the WT and mutant strain less accumulation of lipid content in both the WT and mutant strain (12.24%, 14.08%, respectively) as compared to the exponential phase growth of WT and mutant strain (14.58%, 16.87% respectively) (Fig. 4.17, 4.18). Whereas the stationary phase of growth showed maximum lipid content (20.32% & 28.23% respectively) in both the WT and mutant strains. Thus, the results exhibited variation in the lipid content under different phases of growth cycle microalgae. However, the overall results also revealed a higher accumulation of lipid in the mutant strain than the WT during all the phases of the growth cycle.

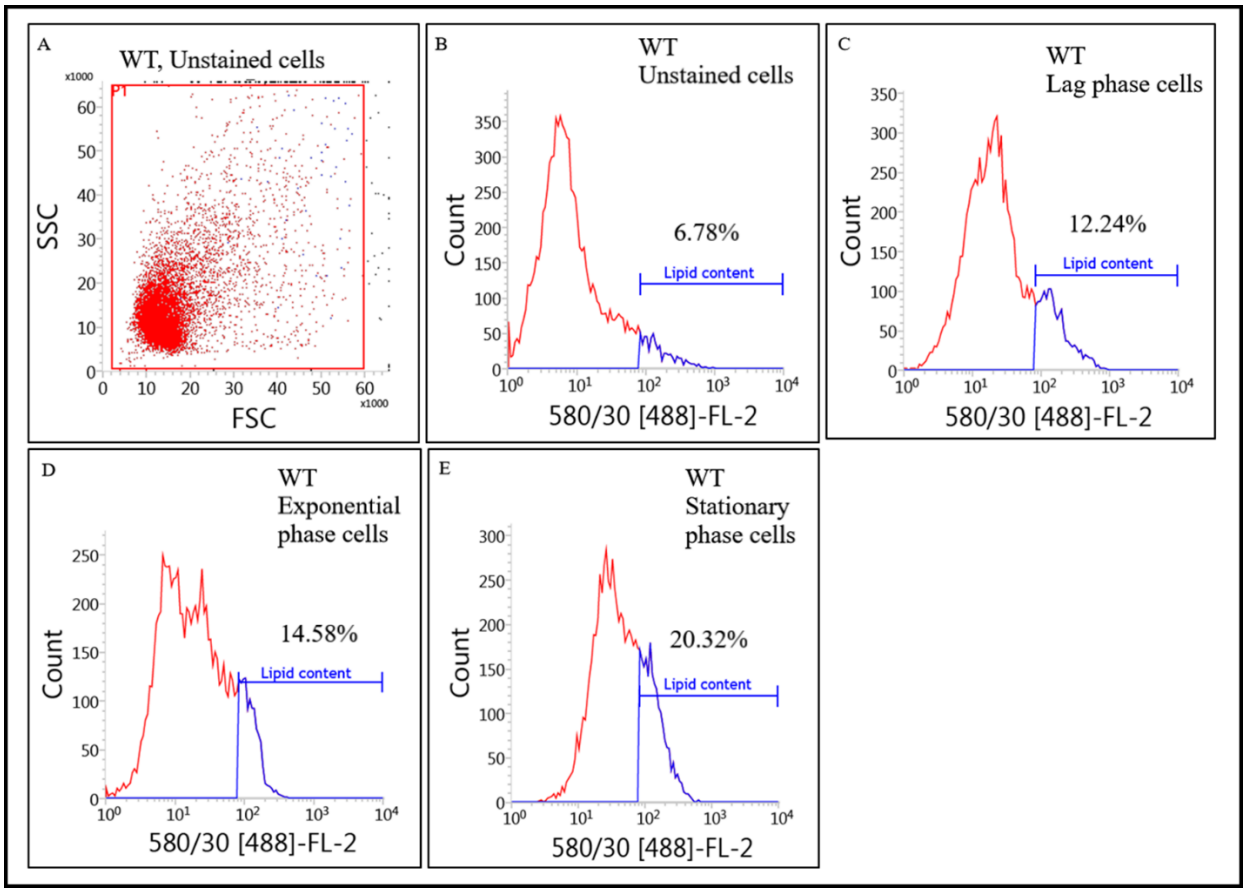

Fig. 4.17 Flow cytometry analysis of neutral lipid by using Nile red dye stained cells of wild-type grown cells of *S. vacuolatus* of different phases of growth.

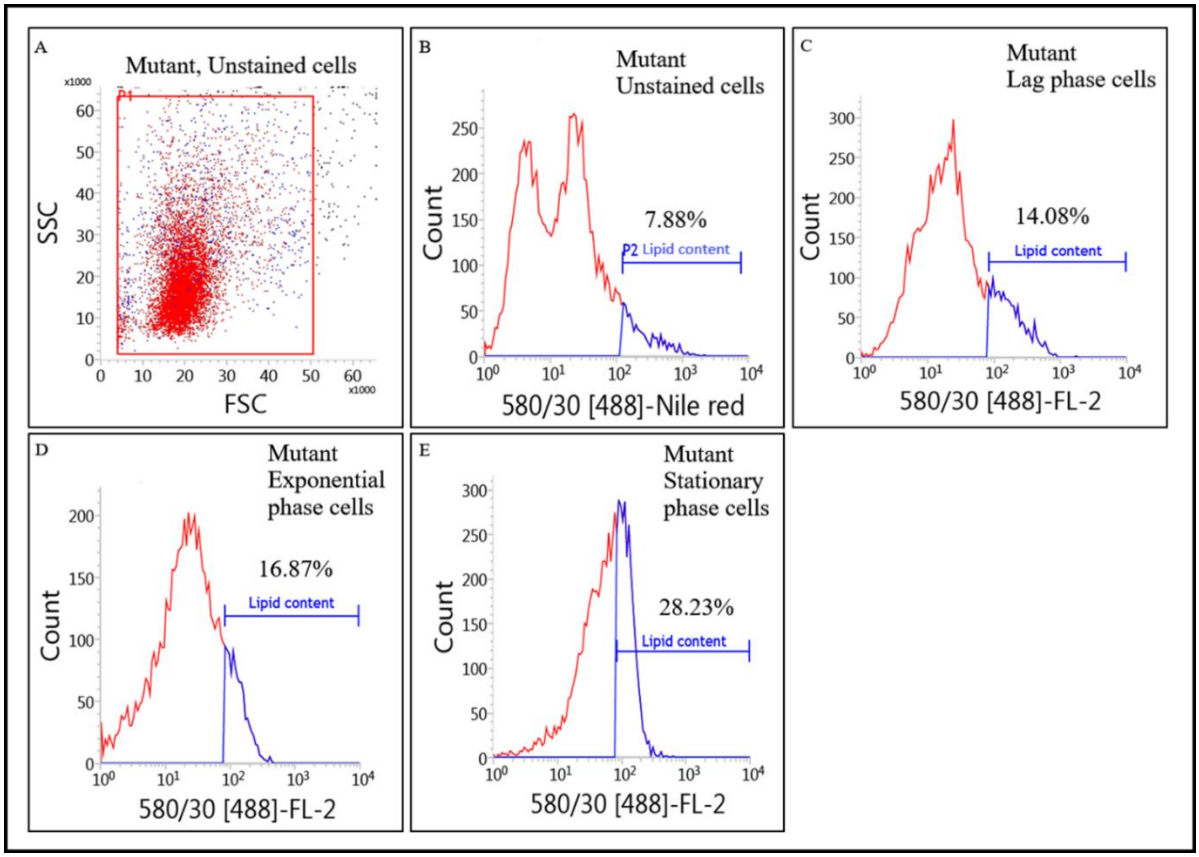

Fig. 4.18 Flow cytometry analysis of neutral lipid by using Nile red dye stained cells of mutant strain grown cells of *S. vacuolatus* of different phases of growth.

4.4 Discussion

Mutagenesis is an effective tool to select mutant strains of microalgae, which are capable of synthesizing higher lipid content (Larkum et al., 2012; Stephens et al., 2010). EMS mutagenesis is a procedure which is not specifically dependent on the DNA repair or initial DNA damage, it can create a stable mutation in DNA which can be transcribed and translated into protein (Sharma et al., 2018; Kawaroe et al., 2015). The objectives of the present study were to isolate a DCMU-tolerant mutant strain of microalgae *S. vacuolatus,* which has the potential to sequester more carbon into lipid content as a consequence changes in the photosynthetic apparatus of the microalgae. Because Photosystem II is considered a major site that is sensitive to different environmental conditions (Barber, 1995; Gilmour et al., 1984), it was imperative to modify the photochemistry of PSII to overcome the various limiting conditions imposed on the cells (Endo et al., 1995). The present result

showed that the mutant, despite its smaller cell size, exhibited faster growth and higher accumulation of lipids than the WT. However, the optimum pH and temperature condition (7.8 pH and 25 ℃) were the same for both the algal strains as indicated earlier (Pawlita-Posmyk et al., 2018; Rai & Rajashekhar, 2014; Guedes et al., 2011). A plot of V_0/V of 2 indicated about 2 fold greater DCMU-tolerance in the mutant strain as compared to WT. The overall result showed that mutant exhibited higher DCMU-tolerance than the WT. The mutant was further evaluated with respect to its photosynthetic characteristics by measuring the fast chlorophyll fluorescence induction parameters (OJIP curve) in the dark-adapted cells of both the strains, using the PAM fluorimeter.

The results revealed that the photosynthetic performance of the mutant strain was better than the WT in the presence and absence of DCMU. It is a well-known fact that herbicide -DCMU blocks the electron transfer between Q_A and Q_B and it binds to D1 protein at the Q_B site (Antonacci et al., 2018; Trebst, 2007; Astier et al., 1984). It has also been suggested that herbicide binding in the photosynthetic organisms, not only inhibits forward electron transfer to PQ, but it also leads to charge recombination between $P680^+$Pheo radical pair and formation of a chlorophyll triplet as well as singlet oxygen ($^1O_2$) (Trebst, 2007; Krieger-Liszkay, 2005; Fufezan et al., 2002; Rutherford & Krieger-Liszkay, 2001). The ability of mutant strain to tolerate DCMU might be due to alteration in the photosynthetic apparatus. Earlier studies have also shown that the mutant of *Aphanocapsa* 6714 was resistant to DCMU because of an alteration in the photosynthetic apparatus near PS II reaction center (Laczko & Kaiseva, 1987; Astier et al., 1979) and change in turnover of D1 protein (Antonacci et al., 2018; Chiu et al., 2009; Erickson et al., 1989). A higher degree of DCMU-tolerance in the mutant strain of *S. vacuolatus* might be associated with changes in the DCMU binding 33-kDa D1 protein and turn over in the photosynthetic apparatus (Matto et al., 1984; Astier et al., 1984; Erickson et al., 1984). In many photosynthetic organisms, the degree of DCMU induced damage to PSII under various environmental stresses has been correlated with a decrease in the level of the D1 protein (Roncel et al., 2007; Przibilla et al., 1991).

In order to elucidate the impact of DCMU on microalgal cells in terms of the photochemistry of PSII, various photosynthetic parameters in the WT and mutant strain of *S. vacuolatus* were investigated. The transient chlorophyll fluorescence induction kinetics (OJIP) was used to evaluate the alteration in the photosynthetic parameters (F_v/F_m, F_v/F_o, ET_0/RC, TR_0/RC, ABS/RC, and PI_{ABS}) (Stirbet & Govindjee, 2012; Zaghdoudi et al., 2011). The maximum photochemical efficiency of PS II of the mutant strain showed a change in F_v/F_m that indicate an alteration in photochemical efficiency of PS II. These result demonstrated that mutant strain has a higher ability to maintain photosynthetic activity and make more efficient use of the absorbed energy compared to WT (Strasser et al., 2000). As evident from the OJIP curves, it was observed that a shorter time was taken by the mutant strain than the WT to reach the fluorescence maxima (F_m), indicating an efficient electron transfer in the mutant strain on the reducing side of PSII, which might be due to reduced probability of closure of PSII reaction center (Strasser & Govindjee, 1992). Reduced value of F_v/F_m in case of DCMU treated WT cells indicated a reduced quantum yield of PSII and damage to the photosynthetic apparatus as compared to mutant strain (Lichtenthaler et al., 2005; Strasser et al., 2004). A longer duration of time taken by the WT to reach Fm value along with reduced quantum yield (F_v/F_m) might be contributed by sluggish electron transfer on the donor side of PS II or partial inactivation of the reaction center (Buchel & Wilhelm, 1993). The present results showed a decrease in all these photosynthetic parameters (ABS/RC, TR_0/RC, and ET_0/RC) in the DCMU treated WT and mutant cells as compared control (without DCMU). However, the effect of DCMU was more pronounced on ABS/RC, TR_0/RC, and ET_0/RC in the WT than the mutant strain. On another hand, a decrease in ET_0/RC under the presence of DCMU cells could be due to the adverse impact of DCMU on PS II system (Roach & Krieger-Liszkay, 2014). The present findings suggested reduced energy flux per reaction center and inactivation of PSII centers. Wang et al., (2010) have also reported that stress factor decreased the number of active reaction centers because of inactivation of the activity of the water-splitting complex. The result on ET_0/RC, TR_0/RC, and ABS/RC suggested a reduction in energy flux parameters for electron transport, trapping of electrons, absorption of photons per active reaction center in PS II are reduced in the presence of DCMU as suggested earlier (Kumar et al., 2015; Lu & Vonshak, 2002). The performance index PI_{ABS} value in the mutant

strain under both the presence and absence of DCMU suggested better light utilisation efficiency of the mutant than the WT. However, the mutant strain showed the increase in the value of ABS/RC, ET_0/RC etc. due to its better light utilization efficiency rather than inactivation of PS II reaction center as reported by earlier workers (Roach & Krieger-Liszkay, 2014; Takahashi & Badger, 2011). Taken together, it was inferred that DCMU-tolerant mutant was endowed with better photosynthetic efficiency resulting in greater biomass and lipid synthesis.

4.5 Conclusion

- The isolated algae form Pangong Lake, Leh Ladakh (J&K), India was identified as *Scenedesmus vacuolatus* (MH459062).
- The DCMU-tolerant mutant strain was isolated by random chemical mutagenesis by EMS and a sub-lethal dose of DCMU was used for screening of the mutant strain.
- The results of plot V_0/V of 2 and chlorophyll fluorescence spectra observed that mutant strain showed greater tolerance of DCMU compared to WT.
- Various photosynthetic parameters obtained from the OJIP curve revealed that the photosynthetic efficiency of mutant strain was better than the WT due to its ability to handle the excess photons, largely due to alteration in the photosynthetic apparatus.
- Flow cytometer analysis of both WT and mutant strain exhibited higher lipid content in the stationary growth phase.
- Finally, changes in the photosynthetic system of the mutant were responsible for its enhanced DCMU tolerance.

Chapter- V

EFFECT OF LIGHT QUALITY & LIGHT INTENSITY ON PHOTOSYNTHETIC EFFICIENCY AND CELLS CONSTITUENTS OF MICROALGA *S. VACUOLATUS*

5.1 Introduction

Microalgae are considered to be the most promising source of biofuel production due to the easier method of their cultivation, photoautotrophic nature and faster growth, which facilitate large-scale production of biomass with higher lipid content (Zhu et al., 2016). Currently, photosynthetic organisms are gradually receiving greater attention for their potential application as third-generation biofuel (Cordara et al., 2018; Simionato et al., 2013). One of the major factors influencing the algal growth is light: which drives the process of photosynthesis required for greater biomass productivity (Gonzalez-Camejo et al., 2019; Hannon et al., 2010). Due to photoautotrophic nature of microalgae, both light quality and intensity, duration of light irradiance, plays an important role in biomass production (Cheirsilp & Torpee, 2012). Light intensity directly affects the photosynthetic rate and excess of light irradiance leads to photooxidative damage, influencing biomass productivity (Mandotra et al., 2016; Yeesang & Cheirsilp, 2011). It has been reported that high light condition triggers the accumulation of neutral lipids in the microalgae *Scenedesmus abundance*, *Nanochloropsis sp*. and *Botryococcus braunii* (Wahidin et al., 2013). Generally, it is considered that required spectral quality of light for algae is dependent on the absorption spectrum (light wavelength) by photosynthetically active pigments like chlorophyll a, b, c, d and carotenoids (Teo et al., 2014). The microalgae mainly absorb different spectral regions of light energy for photosynthesis through chlorophyll (450-475 nm, 630-675 nm) and carotenoids (400-550 nm) (Carvalho et al., 2011).

A long term variation in the light energy irradiance is known to induce changes in the composition, structure and function of the photosynthetic organisms (Orefice et al., 2016). But most of the photosynthetic organisms including microalgae are equipped with a cellular defence system against the high light-induced photooxidative damage (Shi et al., 2017; Singh & Verma, 1995). There is a delicate balance between the light intensity-dependent photooxidative condition and antioxidative defence system (Shi et al., 2017; Yang et al., 2013). Among the photoautotrophs, another mechanism to protect themselves against excess light intensity irradiance includes the down-regulation of photosynthetic machinery by photo-inhibition of PSII reaction centre (Tyystjarvi & Aro, 1996). However, the molecular mechanism of PSII photo-inhibition is not yet fully understood. Thus, efforts are required to improve the light energy harvesting efficiency of microalgae under high

light stress without the adverse effect of photooxidative damage and PSII photo-inhibition, which limits the biomass productivity and biofuel.

Photo-destruction of the photosynthetic components under higher irradiance occurs due to a variety of photosynthetically generated oxygen radicals (Nishiyama et al., 2001). However, higher levels of ROS may cause cellular damage including DNA and membranes lipids (Chandrasekaran et al., 2014; Niyogi, 1999). The microalgae are known to have strong antioxidative defence system such as presence of carotenoids, superoxide dismutase enzyme and ascorbate-glutathione peroxidase system (Snoeijs et al., 2012; Singh & Verma, 1995), which can offset the adverse effects reactive oxygen species (ROS) generated under the high-intensity light irradiance (Shi et al., 2017; Garg & Manchanda, 2009). The glycolate pathway is also a system which minimizes the impact of oxidative stress (Pope, 1975). Earlier workers have also suggested that high light intensity can influence the cost of carbon sequestration and its allocation by altering cell metabolism (He et al., 2015; Jungandreas et al., 2014; Jakob et al., 2007). However, a deeper understanding of the molecular basis of the light use efficiency and cellular defence mechanism against high light intensity is seminal to optimize microalgal cultivation and biofuel production (Orefice et al., 2016; Singh et al., 2015).

Whereas the spectral quality of light influences many physiological and morphological changes associated with the quality and duration of light irradiance (Romero-Romero et al., 2017). When there is an insufficient supply of light or change in the quality of light, the growth of microalgal cells experiences kind of photo-limiting condition (Al-Qasmi et al., 2012). The photosynthetically active radiation (PAR) means the region of the light wavelength used by the photosynthetic organisms to initiate the process of photosynthesis (Perrine et al., 2012). Thus, photosynthetic active radiation (PAR) in case of photoautotrophically grown microalgal cells does not necessarily represent the total spectrum of absorbed radiation. There is need to understand the role of an indifferent array of light quality in the cultivation of microalgae, which bring about compositional changes for their better utilization (Teo et al., 2014; Korbee et al., 2005; Shu et al., 2011). Wu (2016), reported that some key enzyme associated with photosynthesis that regulated in the presence of blue and red light. Thus, it has been suggested that the effect of spectral quality of light and synthesis of macromolecule is species-specific (Godinez-Ortega et al., 2008). Thus, the effect of the spectral composition of light on microalgae, though recognised as an essential factor in controlling the growth and productivity, is little understood (Vadiveloo et al., 2015).

Light-emitting diodes (LEDs) can now be used to obtain the particular specific quality of light due to several advantages such as lower heat dissipation, longer lifespan and lower emission rate (Schulze et al., 2014; Cuaresma et al., 2011). LEDs with different spectral ranges are therefore a valuable source of light not only for the growth of microalgae in photo-bioreactors but also for achieving the desired quality and quantity of commercially available cell constituents (Zhao et al., 2013; Blanken et al., 2013). Fu et al. (2013) demonstrated the synthesis of β-carotene and lutein in the *Dunaliella* green algae and found that growth was enhanced under blue light irradiance. Nevertheless, in a combination of a red LED (75 %) and blue LED (25 %), there was a significant accumulation of both β-carotene and lutein material. Another research carried out by Mohsenpour & Wiloughby, (2013) on the impact of spectral quality on green algae showed that the synthesis of photosynthetic pigments depended on the colour of the light under which microalgae were grown. In addition, the study also found that the red luminescent photo-bioreactor increased the production of algal biomass; however, the overall photosynthetic pigmentation level in algal cells increased under the green light. Kwon et al. (2013) study showed that benthic microalgae growth was stimulated under blue light but the growth was suppressed under the yellow and red LEDs light. By comparison, another study reported that red light irradiance favoured cell growth, cell division, and carbon accumulation in red algae (Carvalho et al., 2011). A recent study by Hwang & Maier, (2019) showed that the maximum growth rate under red light-emitting diodes for *Neochloris oleoabundans*. Intriguingly, multiple studies have reported conflicting results on the effect of spectral quality on growth and cell constituents in the algal system (Ra et al., 2018; Vadiveloo et al., 2015).

In recent biotechnology advancement have shown that metabolic networks can be transferred to lipid synthesis and biofuel production under different stress conditions (Yang et al., 2014; Lei et al., 2012) such as oxidative stress, salt stress and nutritional stress etc. (Fan et al., 2014; Wang et al., 2004). The stress-based metabolic strategies are now widely adopted as environmentally sustainable approaches to induce lipid overproduction (Fan et al., 2014; Sharma et al., 2012). Hence, the foregoing study on light suggested that growth of photosynthetic species can be directly affected by light intensity and quality of light (Yilancioglu et al., 2014; Lu & Zhang, 2000). It suggested that light intensity and spectral light quality could be interlinked with cell growth and metabolic performance, both factors crucial to the synthesis of cell constituents. Therefore, it is important to elucidate the

mechanism underlying the influence of excess light intensity and light colours on the growth and constituents of the microalgae.

5.2 Materials and Methods

5.2.1 Experimental design

In the present study, the experiment proceeds by the growth of a monoclonal culture of the wild-type (WT) and mutant strain. BG-11 medium (Stanier et al., 1971) (pH 7.4±0.2) containing in an Erlenmeyer flask (500 mL) and inoculated both flask of WT and mutant strain with cells of 0.05 OD, and maintained in 25±2 °C for 07 days, as assessed by fluorescent tube light (10 watts / m^2) with light/dark cycle of 16/8 h. Five times a day, the culture flasks shake to prevent settling.

The experiment was done under different light intensity (10-100 µmol m^{-2} s^{-1}) exposed to WT and mutant cells grow in the BG-11 medium (pH 7.4±0.2). The light intensity supplied by the LED Floodlight (Philips, India Ltd). The light source provided beneath the culture flask by fixing the light intensity position of each flask by solarimeter (KM-SPM-530). Each culture flasks inoculated with the WT and mutant strain exponential phase cells of approx. (0.1 OD), and kept 25±2℃, by maintained 16/8 h light/dark cycle. In addition, the experiment was conducted for 20 days in triplicate under similar conditions. The culture flask was shaken five times a day to prevent sticking and settling of the cells.

The Erlenmeyer flasks (250 mL) containing the BG-11 medium were inoculated with exponential phase culture of both the WT and mutant cells, approximately initial 0.1 optical density (OD) for spectral quality of light experiments. Then, under different spectral quality of light irradiances such as blue (410–550 nm), green (480–620 nm), orange (585–620 nm), yellow (500–640 nm) and red (570–700 nm), each culture flask was incubated using LEDs (Philips India Ltd) fitted on top-open hard plastic box (Hultberg et al., 2014). With the light / dark cycle of 16/8 h and 25±2 °C, the culture flask maintained. The flasks were mounted horizontally from a distance of 10-12 cm to provide equal spectral irradiance (100 µmol m^{-2} s^{-1}) to each flask. The experiment was conducted in triplicate for both the WT and mutant strains under a similar set of growth

conditions for 0-20 days. The culture flasks were shaken five times a day to avoid settling and sticking in the cells.

5.2.2 Estimation of growth, biochemical constituents and pigment analysis

The turbidity of microalgal cell suspension (WT and mutant strain) of different light intensity (10-100 µmol m^{-2} s^{-1}) and spectral quality of light (Blue, Green, Yellow, Orange & Red) was monitored at regular interval for 0-20 days at 680 nm by using a double beam UV- visible spectrophotometer (Shimadzu, Japan 1601). The procedure of the specific growth rate (µ) was described in chapter III of Materials and Methods.

Effect of different light intensity (10-100 µmol m^{-2} s^{-1}) and spectral quality (Blue, Green, Yellow, Orange and Red) of light on the biochemical constituents like protein, carbohydrate, pigments and total organic carbon (TOC) in *S. vacuolatus* grown cells of wild-type (WT) and mutant in BG-11 medium were measured as described below:

5.2.2.1 Estimation of protein

Protein content in the cell suspension was estimated by the method of Lowry et al. (1951) modified by Herbert et al. (1971) as described in chapter III of Materials and Methods.

5.2.2.2 Estimation of carbohydrate

The carbohydrate content was quantified by the phenol-sulphuric acid method of Dubois et al. (1956), as described earlier in chapter III of Material and Methods.

5.2.2.3 Estimation of total organic carbon (TOC)

Total organic carbon (TOC) of microalgal cells was analysed by the Walkley-black method as described by Grobler & Davies, (1979), the detail is shown in chapter III of Materials and Methods.

5.2.2.4 Estimation of pigment

The microalgal cells were homogenised and broken by repeated freezing and thawing before the measurement of cell constituents. For estimation of total chlorophyll and carotenoids, the procedure described earlier Material and Methods in chapter III.

5.2.3 Flow cytometer analysis

Flow cytometer (BD FACS Influx^{TM} Becton Dickinson, San Jose, CA, USA) were measured the neutral lipid content of WT and mutant strain. The procedure describes earlier in chapter III of Materials and Methods.

5.2.4 Measurement of RNO bleaching

Exponential phase cells of the WT and a mutant strain of *S. vacuolatus* were centrifuged at 5000 × g for 10 min and washed with HEPES buffer (pH 7.5, 20 mM). The pellet was suspended in the same buffer before ultrasonic breakage of the cells for 5 min. The unbroken cell was removed by low-speed centrifugation (1000 x g for 5 min). The supernatant was supplemented with RNO (N, N Diethyl p-nitrosoaniline) (10 µg/mL) prepared and incubated under different light intensity for 15 min. The light-induced bleaching of RNO was recorded at 440 nm in a UV- Visible spectrophotometer (UV-A 1601 Shimadzu, Japan) as described by Joshi & Pathak, (1984). The rate of RNO bleaching was expressed as ΔOD_{440}nm/mg protein/min.

Radical quenchers such as histidine, sodium formate, and sodium azide (5 mM each) were added to cell suspension before adding the RNO. The reaction mixture was incubated at 60 µmol m^{-2} s^{-1} light intensity for 15 min. The rate RNO bleaching was recorded at 440 nm as described above. A control set of RNO bleaching without radical quenchers was run in parallel.

5.2.5 Measurement of glycollate activity

The glycollate content was measured in the WT and mutant cells of *S. vacuolatus* by the method of Takahashi, (1972). The cells suspension 150 µL was mixed with 3 mL of reagent (0.1% (w/v) solution of 2.7- dihydroxy naphthalene) (Sigma, USA), in conc. H_2SO_4. The reaction mixture was kept in a boiling water bath for 20 min. After cooling down the mixture at room temperature and collecting the condensed water vapours inside the walls of the test tube, the reaction mixture was centrifuged at 5000 × g for 5 min. to remove the turbidity. The absorbance of clear supernatant was read at 540 nm in a double beam UV-Visible spectrophotometer (UV-A 1601 Shimadzu, Japan) against an appropriate blank. Total glycollate content was expressed as µmol glycollate/mg protein.

5.2.6 Measurement of superoxide dismutase (SOD) activity

The SOD activity was analysed by the method of (Nishikimi & Rao, 1972). The SOD enzyme assay was based on the inhibition of formation of NADPH-Phenazine methosulfate-nitroblue tetrazolium formazon. The colour formation at the end of the reaction was measured at 560 nm in a double beam UV- Visible Spectrophotometer. One unit of the enzyme activity is defined as the amount of enzyme required to inhibits the 50% of NBT reduction in one min.

5.2.7 Estimation of thiol (-SH) groups

The wild-type (WT) and mutant cells of *S. vacuolatus* (3 mL each) placed under different light intensities (10-100 µmol m^{-2} s^{-1}) were centrifuged at 5000 × g for 10 min. The pellet was suspended in Tris- HCl buffer (100 mM, pH 8.6), and it was supplemented with 1 mM of 5, 5- dithiobis [2-nitrobenzoic acid] (DTNB) reagent (Ellman, 1959) and it was then incubated for 20 min. The cells suspension was centrifuged at 5000 × g for 10 min and the absorbance of the supernatant was read at 440 nm. The stock solution of DTNB was prepared in the Tris buffer after dissolving the DTNB in a minimum amount of ethanol (0.1%, w/v). The absorbance of the supernatant was compared with a standard curve prepared by using known concentrations of cysteine. Total sulfhydryl content in the cells was expressed as n mole –SH groups/mg protein.

5.2.8 Estimation of lipid peroxidation activity

The lipid peroxidation activity in the exponential phase cells of both the WT and mutant strain of *S. Vacuolatus* was measured in terms of malondialdehyde (MDA) formation. An aliquot of 4 mL of cell sample withdrawn at regular interval from different light intensity incubated cells were supplemented with a pinch of sand grains as a boiling aid and supplemented with 2 mL of TBA (Thiobarbituric acid) stock solution. The TBA stock solution was prepared by using 100 mL of (20 %, w/v) of trichloroacetic acid (TCA) and 0.5 g of TBA. The mixture was incubated at 95°C for 25 min. Thereafter, the reaction mixture was centrifuged (5000×g, 5 min.) to remove the cloudiness of the solution. Absorption of the coloured solution was recorded at 532 nm and the turbidity of the solution was measured at 600 nm in double beam UV-Visible-spectrophotometer. The absorption value obtained at 600nm was subtracted from the absorbance recorded

at 532 nm. The amount of MDA was calculated using the extinction coefficient of 155 mM/cm^3 (Heath & Packer, 1968). The rate of lipid peroxidation was expressed in terms of µmol MDA formed/mg protein.

5.2.9 Measurement of chlorophyll fluorescence induction kinetics (OJIP), non-photochemical quenching (NPQ) and light curve (LC)

Chlorophyll a fluorescence transient was measured by using Pulse Amplitude Modulated (PAM) (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic). Seven days grown cells of different light intensity (10-100 µmol m^{-2} s^{-1}) and spectral quality (Blue, Green, Yellow, Orange and Red) were dark-adapted for 10 min at room temperature. The induction kinetics of chlorophyll fluorescence was recorded with an excitation wavelength of 450 nm. The chlorophyll fluorescence parameters (F_v/F_m, F_v/F_0, $\Delta F/F_m$' ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS) were determined as reported by Strasser et al. (2000).

- **Fv/F_m** = Maximum quantum yield of PSII (dark-adapted state)
- **F_v/F_0** = Maximum quantum yield of PSII (more sensitive than F_v/F_m)
- **$\Delta F/F_m$' =** Actual quantum yield of PSII photochemistry
- **ET_0/RC** = M_0 $(1/V_j)$. Ψ_0 (trapping of energy flux beyond QA in the photosystem II)
- **TR_0/RC** = M_0 $(1/V_j)$ (efficiency of electron transport leading to reduction of QA)
- **ABS/RC** = M_0. $(1/V_J)$. $(1/\varphi\_P_0)$ (Absorption of energy flux per active reaction center)
- **RC/ABS** = φP_o/ (1- φPo). Ψo/(1-Ψo) (Performance index on absorption basis of PIabs)

- Non-photochemical quenching (NPQ) value in both strains was determined (Lichtenthaler et al., 2005). Seven days grown cells of different light intensity (10-100 µmol m^{-2} s^{-1}) and spectral quality (Blue, Green, Yellow, Orange and Red) were used for the analysis. Further, the cells were dark-adapted for 10 min at room temperature for NPQ analysis (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic). The NPQ fluorescence calculated by the following formula: NPQ = $_NF/F_m$' ($_NF=F_m-F_m$')

Non-photochemical quenching (NPQ) is based on a measuring light flash to obtain a minimum fluorescence level (F_0). To achieve maximum fluorescence (F_m) level in the dark-adapted cells, a short saturating light flash is applied. After a brief dark period, the sample is again exposed to actinic irradiance for 20 seconds to elicit a transient of the Kautsky effect. Moreover, a series of saturating flashes is applied on top of the actinic light to probe the NPQ and effective quantum yield (QY) of photosynthesis in the light-adapted state.

- Light-Response Curve relating to the rate of photosynthesis to photon flux density was based on the successive exposure of the samples to a stepwise increase in the light intensity (100, 200, 300, 500 µmol m^{-2} s^{-1}). The relative photosynthetic electron transport rate (rETR) value was determined in WT and mutant strain the in low and high light (Malapascua et al., 2014). Before analysis, the cells were dark-adapted for 10 min at room temperature. The rETR value at different light intensity was calculated by the formula: $rETR= Y_{II}*E_{PAR}$

5.2.10 Fourier transform infrared (FTIR) analysis

The study of the biochemical composition of microalgal biomass of both WT and mutant strain by using FTIR. The procedure describes earlier in Materials and Methods chapter III.

5.2.11 Statistical analysis

The performed statistical analysis method was described in the Materials and Methods of chapter III.

5.3 Results

(A) Effect of light intensities on the microalga *S. vacuolatus*

5.3.1 Effect of light intensity on growth and biochemical constituents

The result of the specific growth rate of both the WT and mutant strain was recorded over a period of 20 days in response to varying light intensities (10-100 µmol m^{-2} s^{-1}). The maximum specific growth rate of WT (0.079±0.007 d^{-1}) was achieved at 40 µmol m^{-2} s^{-1} light intensity (Table 5.1), whereas the mutant strain showed optimum specific growth rate (0.135±0.12 d^{-1}) at 60 µmol m^{-2} s^{-1} (Table 5.1). The results showed about 50% higher light

tolerance in the mutant strain than the WT. However, the lowest specific growth rate of the wild-type (0.022±0.002 d^{-1}) and mutant (0.051±0.003 d^{-1}) strain were recorded at 100 µmol m^{-2} s^{-1}. The overall growth of mutant strain under varying light intensities was higher than the wild-type. Analysis by paired-sample 't' test showed a significant difference between the result of wild-type and mutant strain.

Table 5.1 Comparison of specific growth rate, protein and carbohydrate content of wild-type (WT) and mutant strain of *S. vacuolatus* exposed to different light intensities (10-100 µmol m^{-2} s^{-1}). Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. The data presented as mean±SD.

Light intensity (10-100 µmol m^{-2} s^{-1})	**Specific growth rate (µ) (d^{-1})**		**Protein (µg mL^{-1})**		**Carbohydrate (µg mL^{-1})**	
	Wild-type	**Mutant**	**Wild-type**	**Mutant**	**Wild-type**	**Mutant**
10	0.042±0.004	0.060±0.004**	152±13.52	232±19.51**	120±10.50	162±11.67**
20	0.061±0.005	0.092±0.006***	222±20.55	318±26.00**	152±14.84	251±18.58***
40	0.079±0.007	0.110±0.010**	250±24.26	340±33.17**	205±10.58	283±20.29**
60	0.064±0.005	0.135±0.012**	210±15.50	390±36.11**	154±14.05	319±24.70**
80	0.041±0.003	0.081±0.005***	150±12.25	267±21.63**	115±10.96	204±16.09**
100	0.022±0.002	0.051±0.003**	87.6±7.00	191±16.09**	74±5.560	151±11.50**

Results on different biochemical constituents showed the highest protein content in the WT cells (250±24.26 µg mL^{-1}) at 40 µmol m^{-2} s^{-1} light intensity, followed by the protein level (210±15.50 µg mL^{-1}) at 60 µmol m^{-2} s^{-1} light intensity. On the other hand, the maximum protein content in the mutant strain was observed in the cells grown under 60 µmol m^{-2} s^{-1} (390±36.11 µg mL^{-1}), followed by 40 µmol m^{-2} s^{-1} (340±33.17 µg mL^{-1}) (Table 5.1). Similarly, results on carbohydrate content in the WT cells (205±10.58 µg mL^{-1}) was maximum at 40 µmol m^{-2} s^{-1}, followed by the wild-type cells (154±14.05 µg mL^{-1}) grown at 60 µmol m^{-2} s^{-1} light intensity. But the mutant strain showed the maximum level of carbohydrate in the cells (319±24.70 µg mL^{-1}) grown at 60 µmol m^{-2} s^{-1} light intensity, followed by the mutant cells (283±20.29 µg mL^{-1}) grown at 40 µmol m^{-2} s^{-1} (Table 5.1). These results revealed that the optimum level of protein and carbohydrate contents in both the strains were found in the cells grown at their growth saturating light intensities.

5.3.2 Effect of light intensity on photosynthetic pigments

The result on total chlorophyll content (2.06±0.18 µg mL^{-1}) in the WT strain was found to be maximum at 40 µmol m^{-2} s^{-1} grown cells, followed by the cells grown at 60 µmol m^{-2} s^{-1} light intensity grown cells (1.51±0.12 µg mL^{-1}). Whereas total chlorophyll contents in the mutant strain (3.62±0.33 µg mL^{-1}) were higher at 60 µmol m^{-2} s^{-1} light intensity, followed by 40 µmol m^{-2} s^{-1} light intensity grown cells (2.93±0.26 µg mL^{-1}) (Table 5.2). The carotenoid content in the wild-type strain (2.99±0.26 µg mL^{-1}) was found to be the highest at 40 µmol m^{-2} s^{-1}, followed by the cells (2.40±0.20 µg mL^{-1}) grown at 60 µmol m^{-2} s^{-1}. Similarly, the carotenoid content in the mutant strain (5.60±0.52 µg mL^{-1}) was maximum at 60 µmol m^{-2} s^{-1} light intensity, followed by the cells (4.56±0.41 µg mL^{-1}) grown at 40 µmol m^{-2} s^{-1} light intensity (Table 5.2). The level of photosynthetic pigments in both the strains was found to be the lowest in the cells grown at 100 µmol m^{-2} s^{-1} light intensity. The statistical analysis also showed a significant difference value between WT and mutant. The overall results indicated a higher level of pigments and other cell constituents in the mutant strain, in spite of its higher level of high light tolerance.

Table 5.2 Total chlorophyll and carotenoids content of the wild-type (WT) and mutant strain of *S. vacuolatus* in response to varying light intensities (10-100 µmol m^{-2} s^{-1}). Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. The data presented as mean±SD.

Light Intensity (10-100 µmol m^{-2} s^{-1})	**Total chlorophyll (µg mL^{-1})**		**Carotenoids (µg mL^{-1})**	
	Wild-type	**Mutant**	**Wild-type**	**Mutant**
10	0.816±0.065	1.60±0.133**	1.44±0.105	2.76±0.208**
20	1.16±0.065	2.24±0.215**	2.23±0.217	3.46±0.321**
40	2.06±0.180	2.93±0.265*	2.99±.0260	4.56±0.416**
60	1.51±0.120	3.62±0.335**	2.40±0.200	5.60±0.529***
80	0.816±0.06	1.68±0.135**	1.23±0.100	2.76±0.216*
100	0.313±0.025	0.913±0.08***	0.66±0.493	1.51±0.110**

5.3.3 Flow cytometer analysis

Flow cytometric analysis of lipid content of both the WT and mutant strain is grown under varying light intensities exhibited light intensity-dependent increase in the lipid content of both the strains, depending upon their respective metabolic response. Initially, the level of lipid increased in an intensity-dependent manner in both the strains up to their respective growth saturating light intensity.

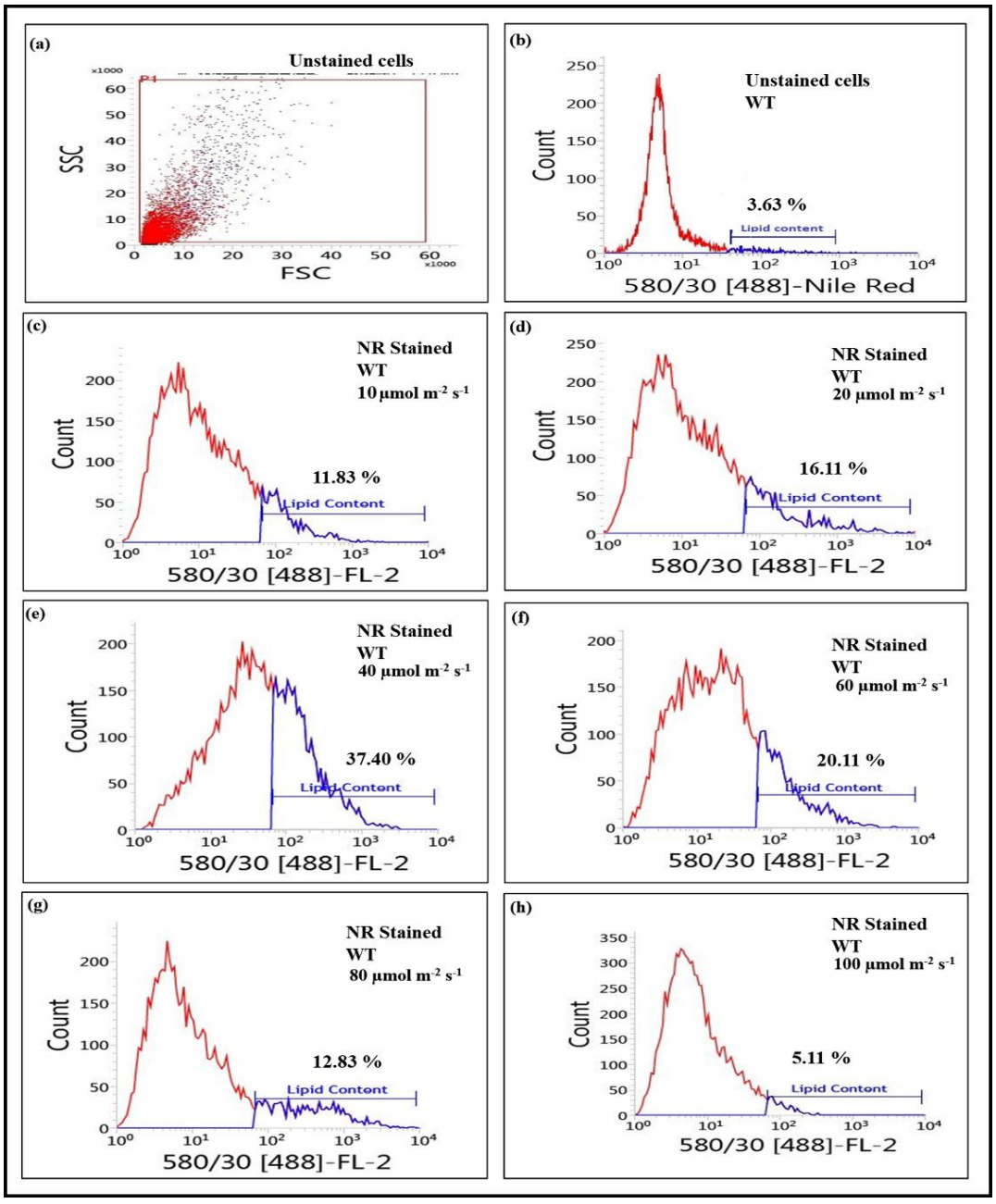

Fig. 5.1 Flow cytometer analysis of neutral lipid by using Nile red (NR) stained cells of wild-type (WT) of *S. vacuolatus* grown under different light intensity.

The results revealed a maximum percentage of lipid yield in the WT (37.40 %) at 40 µmol m^{-2} s^{-1} as compared to unstained cells (without the Nile red) (Fig. 5.1). However, the mutant cells grown at 60 µmol m^{-2} s^{-1} showed maximum lipid content (46.87 %) as shown in (Fig. 5.2). An increase in the light intensity beyond the tolerance limit of both the WT and mutant strain (80-100 µmol m^{-2} s^{-1}) showed an intensity-dependent declining pattern. In addition, the overall result showed that lipid content was recorded maximum in the mutant strain than the WT.

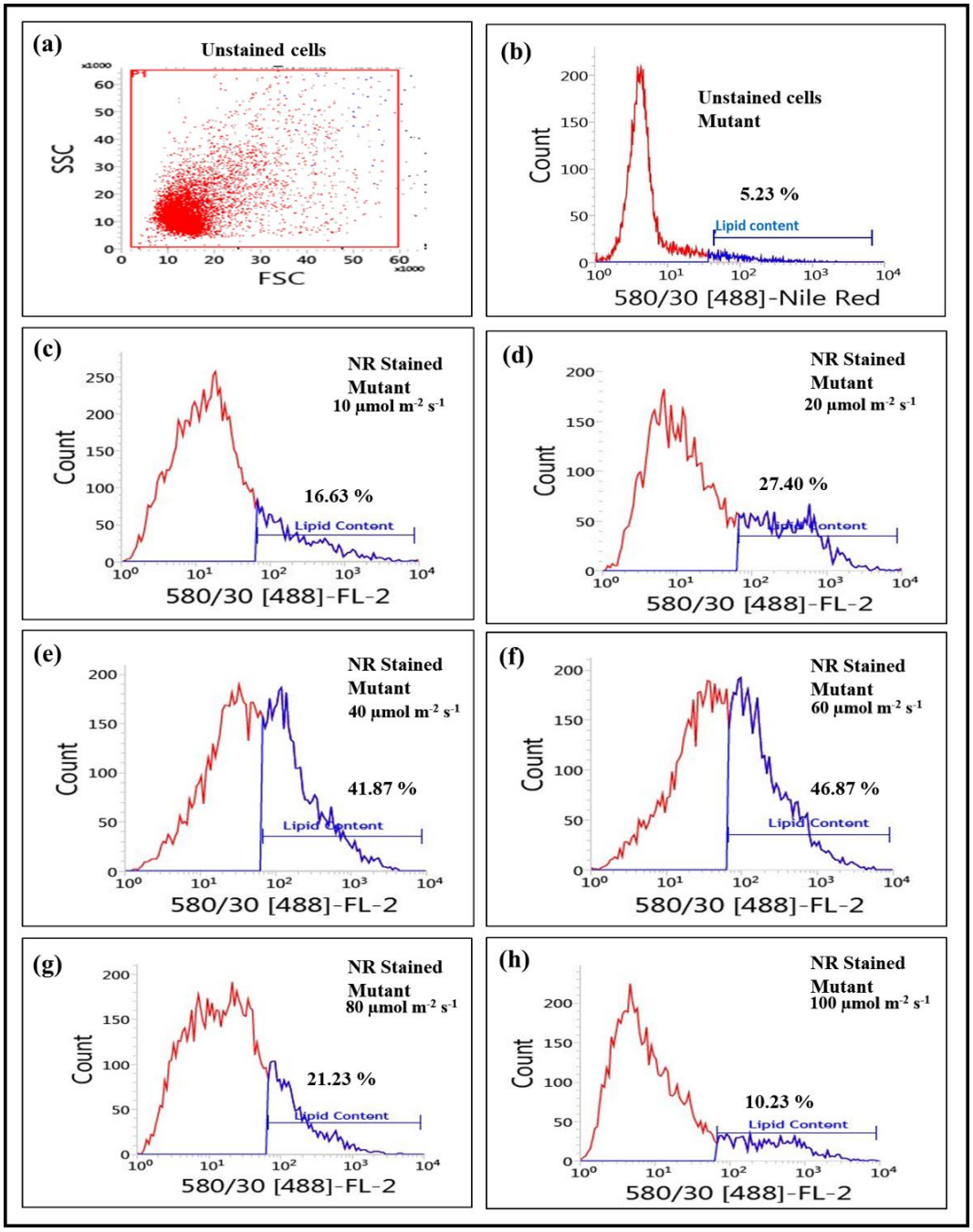

Fig. 5.2 Flow cytometer analysis of neutral lipid by using Nile red (NR) stained cells of a mutant strain of *S. vacuolatus* grown under different light intensity.

5.3.4 Effect of RNO bleaching, -SH group, glycollate, lipid peroxidation, and SOD activity on light intensity

The rate of RNO bleaching in the cells grown under varying light intensities (10-100 µmol m^{-2} s^{-1}) demonstrated light intensity-dependent increase in the rate of RNO bleaching in both the WT and mutant strain (Fig. 5.3, a). The highest rate of RNO bleaching was recorded at 100 µmol m^{-2} s^{-1} light intensity in both the strains (8.34±0.70, 3.94±0.21 ng/mg protein/min respectively). The results revealed the two-fold higher rate of RNO bleaching in the WT cells than the mutant cells. This clearly suggested that photo-induced production of oxyradicals was about two-fold higher in the WT than the mutant. The RNO bleaching were measured in incubated cells of WT and mutant strain in the presence of different radical quenchers like histidine, sodium formate, sodium azide (5 mM, each) at 60 µmol m^{-2} s^{-1} light intensity showed maximum inhibitory effect of histidine (38.8 and 40.6%, respectively), followed by the effect of formate (59.7 and 65.5%, respectively) and sodium azide (86.1 and 87.5%, respectively) (Table 5.3). The inhibitory effect of radical quenchers was compared with the control (100%, RNO bleaching without radical quenchers). Since the histidine and formate are basically quenchers of superoxide and hydroxyl radicals, the results suggested for photooxidative damage in both the strains, mainly caused by the superoxide radicals, followed by hydroxyl radicals.

Table 5.3 Effect of radical quenchers on RNO bleaching in the wild-type (WT) and mutant cells incubated under 60 µmol m^{-2} s^{-1} light intensity for one hour. The values given in the parenthesis represent percent activity of RNO bleaching. The data presented as mean±SD.

Name of radicals quenchers	**Rate of RNO bleaching (ΔOD_{440}nm/mg protein/min)**	
	Wild-type	**Mutant**
Control (without quencher)	7.2±0.40 (100)	3.2±0.24 (100)
Histidine (5mM)	2.8±0.25 (38.8)	1.3±0.10 (40.6)
Sodium formate (5mM)	4.3±0.41 (59.7)	2.1± 0.20 (65.6)
Sodium azide (5mM)	6.2±0.40 (86.1)	2.8± 0.17 (87.5)

The results on total sulfhydryl grouped (-SH) in both the WT and mutant strains under varying light intensities showed a light intensity-dependent gradual decline in the total thiol groups. The maximum decline in the –SH groups in both the strains was observed at 100 µmol m^{-2} s^{-1} light intensity (87.7±6.1, 139±10.5 nmol/mg protein, respectively) (Fig. 5.3, b). However, the lowest sulfhydryl content was observed in the WT than the mutant through the range of light intensities used for the experiment, perhaps due to greater photooxidative stress being experienced by the WT.

The result on glycollate content in both the strains incubated under varying light intensities showed the maximum glycolate content in the WT (153±11.7 µM glycollate/mg protein/h) grown at 40 µmol m^{-2} s^{-1} light intensity and the mutant strain (273±16.6 µM glycollate/mg protein/h) grown at 60 µmol m^{-2} s^{-1} (Fig. 5.3, c). A further increase in the light intensity beyond the light intensity tolerance limit showed a gradual decrease in the glycollate content. However, the overall glycollate content in the mutant was relatively higher than the WT throughout the range of light intensity used.

Effect of light intensities on lipid peroxidation measured in terms of MDA (malondialdehyde) formation, was measured to assess the photo-oxidative damage to both the WT and mutant strain under varying light irradiance. The results showed light intensity-dependent increases in the rate of lipid peroxidation activity in both the WT (0.21±0.019 nmol MDA formed/mg protein) and mutant cells (0.119±0.009 nmol MDA formed/mg protein) up to their respective light tolerance limit (40 and 60 µmol m^{-2} s^{-1}, respectively) (Fig. 5.4, a). A further increase in the light intensity resulted in a gradual decrease in the rate of lipid peroxidation in both the strains. However, the per-oxidative damage was more severe in the WT cells than the mutant throughout the range of light intensity used for incubation.

The result on SOD activity in both the WT and mutant strain exhibited the highest rate of SOD activity in the WT (223±16.2 Unit/g) at 40 µmol m^{-2} s^{-1} light intensity and in the mutant (414±35.6 Unit/g) at 60 µmol m^{-2} s^{-1} light intensity. Further, an increase in the light intensity beyond the tolerance limit showed a gradual decreasing pattern in the activity of SOD enzyme (Fig. 5.4, b). However, the overall rate of SOD activity was higher in the mutant strain than the WT.

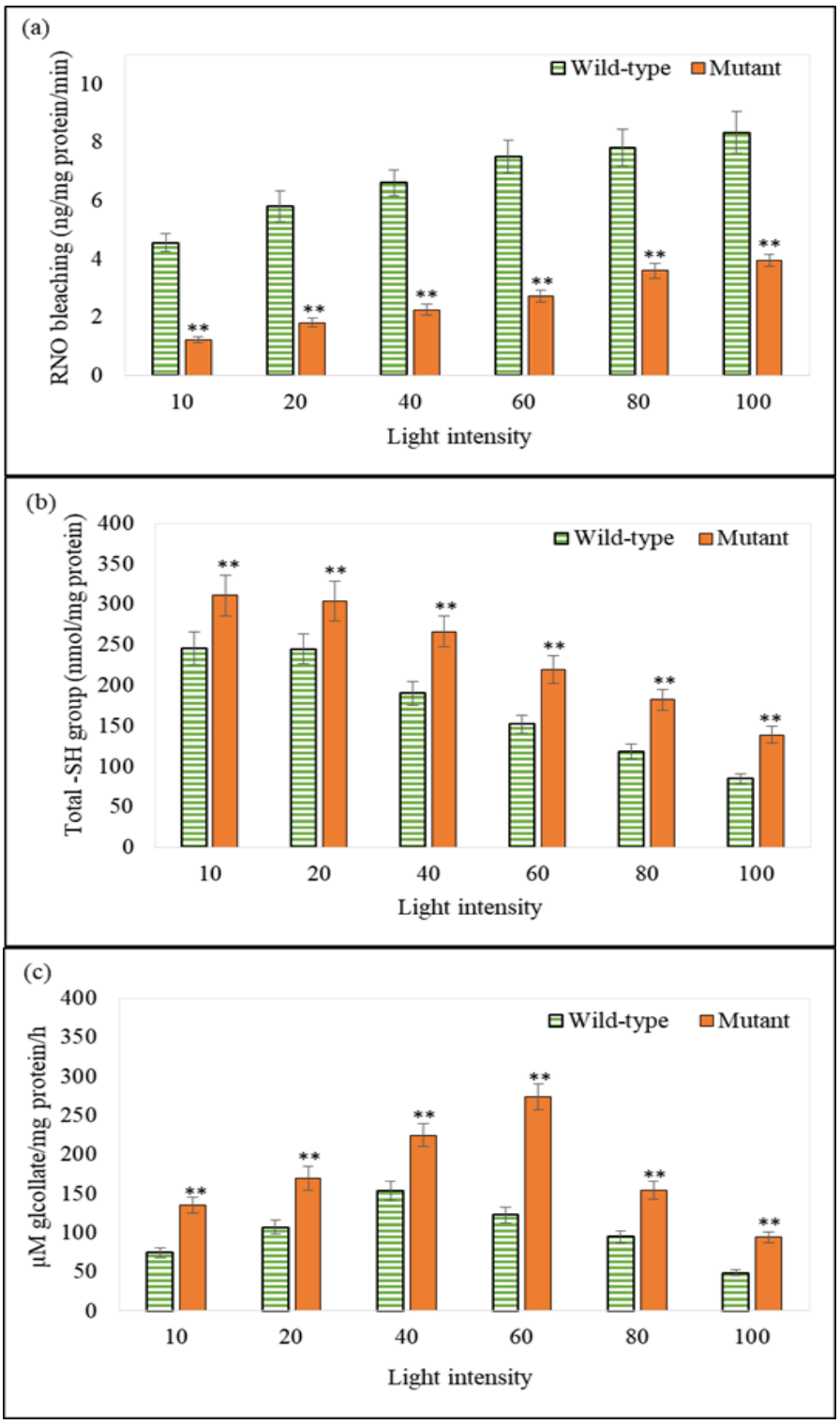

Fig. 5.3 Effect of different light irradiance (10-100 µmol m^{-2} s^{-1}) on the (a) RNO bleaching (b) SH-group (c) glycollate activity in both wild-type (WT) and mutant strain of *S. vacuolatus.* Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. Error bar showed the mean±SD.

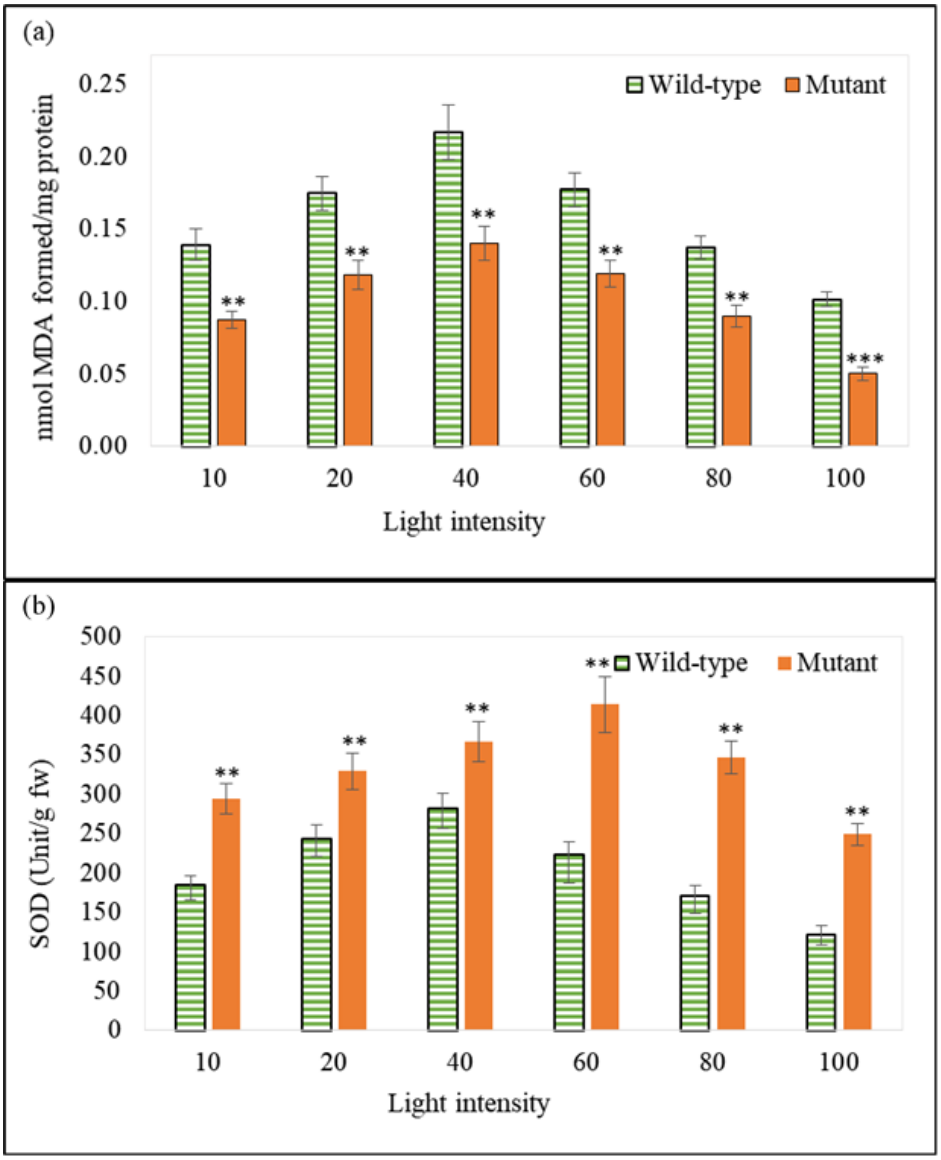

Fig. 5.4 Effect of different light irradiance (10-100 µmol m^{-2} s^{-1}) on the (a) lipid peroxidation (MDA formation) (b) SOD activity in both wild-type (WT) and mutant strain of *S. vacuolatus.* Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. Error bar showed the mean±SD.

5.3.5 Effect of light intensity on chlorophyll fluorescence induction kinetics (OJIP), non-photochemical quenching (NPQ) and light curve (LC) analysis

Impact of light intensity (10-100 µmol m^{-2} s^{-1}) on the PSII photochemistry of *S. vacuolatus* cells was analyzed in terms of Chlorophyll fluorescence induction kinetics, non-photochemical quenching (NPQ) and light curve (LC). Data pertaining to changes in the OJIP parameter are presented in Table 5.4. Different parameters such as F_v/F_m, ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS derived from OJIP curve showed an increasing trend with increasing light intensity in both the WT mutant cells up to their respective light tolerance limits (40 and 60 µmol m^{-2} s^{-1}, respectively) (Fig. 5.5, 5.6).

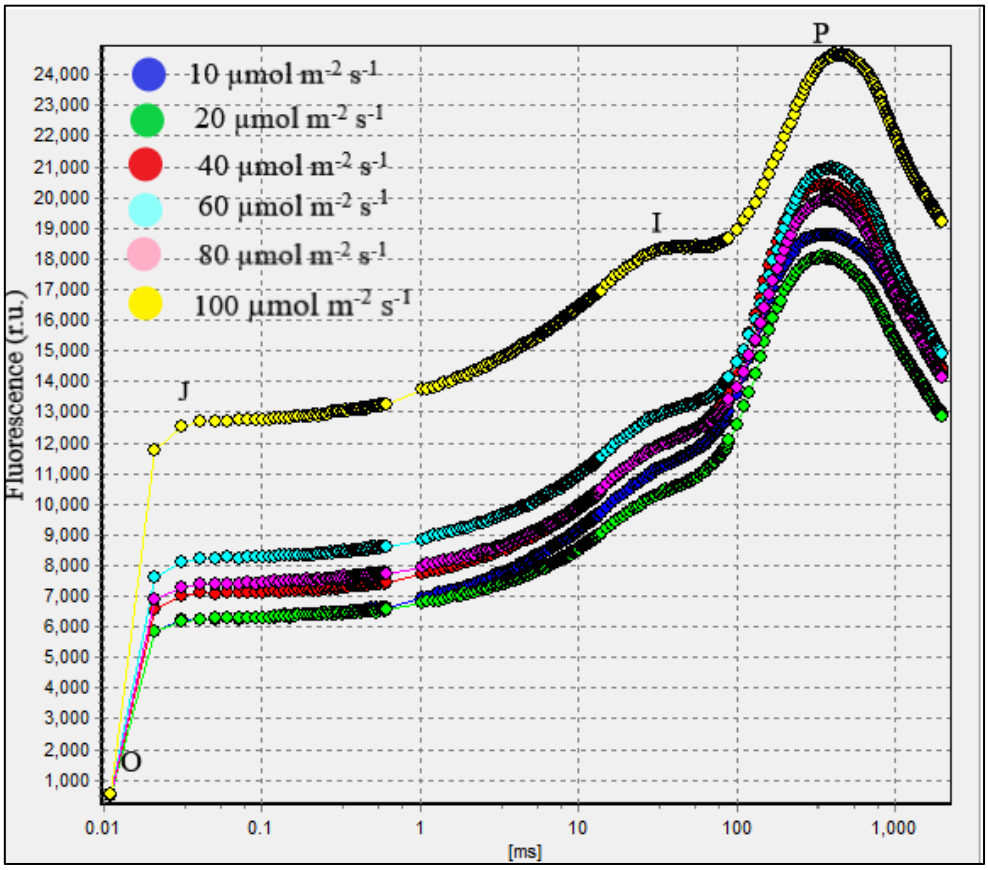

Fig. 5.5 Chlorophyll α fluorescence induction curve (OJIP) of wild-type cells of *S. vacuolatus* grown under varying light intensity (10-100 µmol m^{-2} s^{-1}). The O, J, I, P stand for O, (F_0) fluorescence intensity at 50 µs; J, (Fj) fluorescence intensity at J-step (at 2 ms); I, (Fi) fluorescence intensity at I step (at 60 ms); P, (Fm) maximal fluorescence intensity.

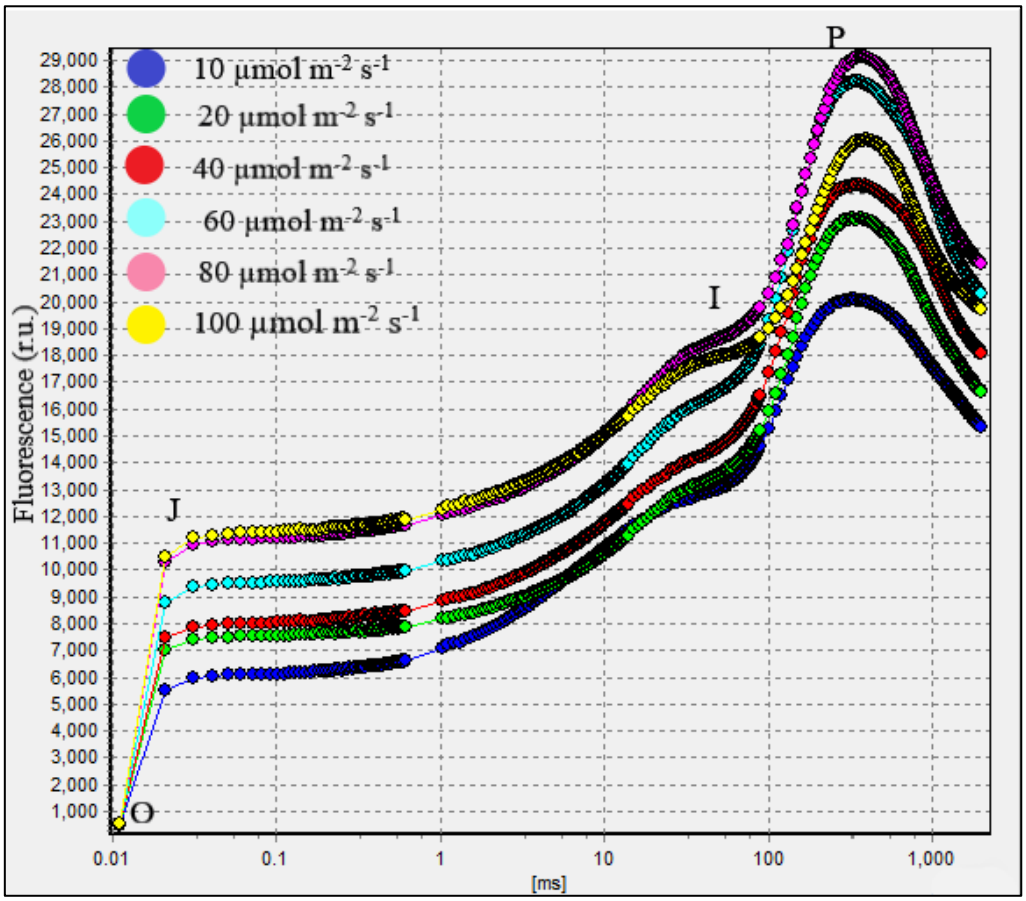

Fig. 5.6 Chlorophyll α fluorescence induction curve (OJIP) of mutant strain of *S. vacuolatus* grown under varying light intensity (10-100 µmol m^{-2} s^{-1}). The O, J, I, P stand for O, (F_0) fluorescence intensity at 50 µs; J, (Fj) fluorescence intensity at J-step (at 2 ms); I, (Fi) fluorescence intensity at I step (at 60 ms); P, (Fm) maximal fluorescence intensity.

A maximum increase in the quantum yield (F_v/F_m) of PSII of WT (0.762±0.05) was observed at 40 µmol m^{-2} s^{-1}, while the quantum yield in the mutant cells (0.911±0.05) was the highest at 60 µmol m^{-2} s^{-1} (Fig. 5.7). The ABS/RC, denoting absorption of energy flux per active reaction centre, showed the highest value in the WT cells (1.34±0.13) at 40 µmol m^{-2} s^{-1}, whereas ABS/RC value in the mutant strain (1.52±0.10) was maximum at 60 µmol m^{-2} s^{-1}. Similarly, the values of TR_0/RC and ET_0/RC, denoting respective efficiency of electron transport leading to reduction of QA and beyond in the photosystem II, were found to be maximum in both the WT and mutant strain at 40 and 60 µmol m^{-2} s^{-1}, (0.92±0.04, 1.07±0.10 respectively) (Table 5.4). These results suggested that the photosynthetic yield and electron transport in both the strains were maximum at their respective light-tolerance limit. In both the strains, the light

intensities (80-100 µmol m^{-2} s^{-1}) beyond their respective photosynthesis saturating light intensities showed down-regulation of the photosynthetic system. However, the photosynthetic parameters derived from the OJIP curve showed better light utilization efficiency and higher quantum yield in the mutant strain than the WT throughout the range of applied light intensities. Further, the result showed the OJIP curves, it was found that a shorter time was taken to reach the fluorescence maxima (F_m) by the mutant strain than the WT, suggesting an efficient transfer of electron in the mutant strain on the reduction side of PSII, which could be due to decreased probability of closure of PSII reaction centre. Whereas sluggish electron transfer on the donor side of the PS II reaction center could contribute to a longer period of time taken by the WT to reach Fm value and reduced quantum yield (F_v / F_m).

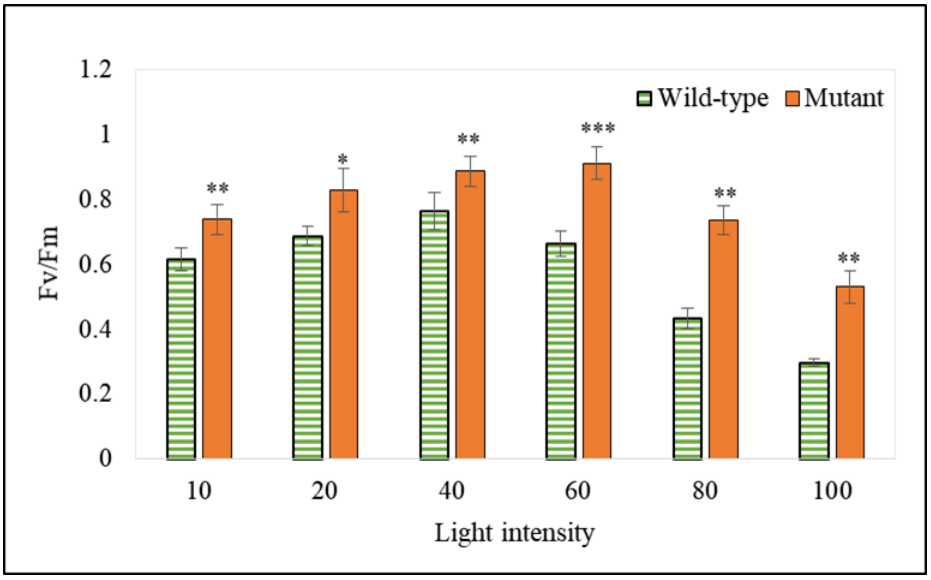

Fig. 5.7 Changes in the PS II photosynthetic yield (F_v/F_m) of *S. vacuolatus* cells of wild-type (WT) and mutant strain exposed to varying light intensity. Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. Error bar showed the mean±SD.

The results on non-photochemical quenching (NPQ) measured by PAM fluorometer at two light intensities i.e., low light (LL) intensity (10 µmol m^{-2} s^{-1}) and high light (HL) intensity (60 µmol m^{-2} s^{-1}) showed relatively higher NPQ values in WT than the mutant strain. But the NPQ value in the high light (60 µmol m^{-2} s^{-1}) grown cells of both the WT and mutant strain (0.36±0.03 and 0.22±0.02, respectively) registered significant decline as compared to corresponding NPQ values obtained in the

low light-grown cell (0.44±0.031 and 0.29±0.017, respectively) (Fig. 5.8). However, overall NPQ value in the mutant strain was significantly lower than the WT. The results clearly indicated greater dissipation of absorbed light energy in the WT than the mutant strain.

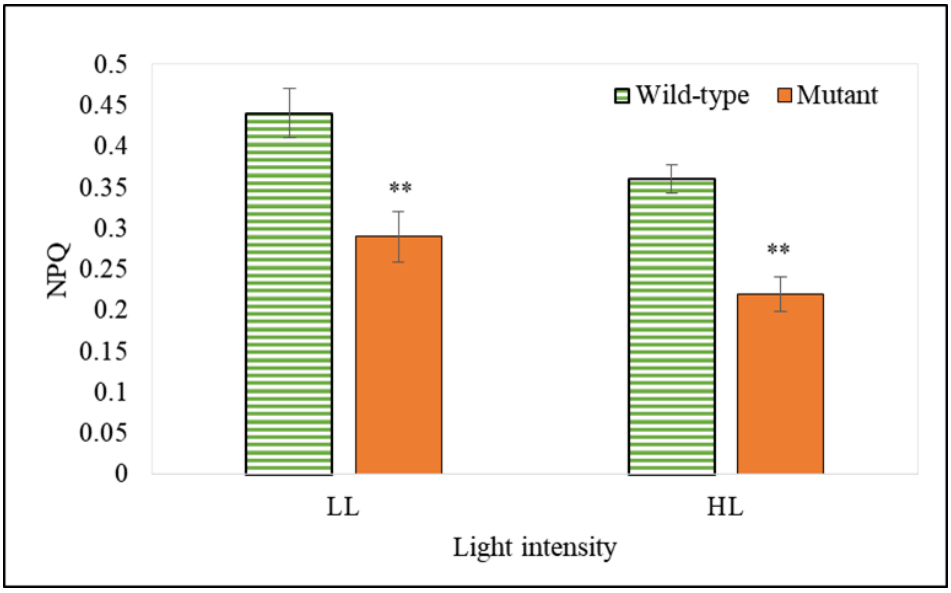

Fig. 5.8 Non-photochemical quenching (NPQ) of *S. vacuolatus* cells of wild-type (WT) and mutant strain exposed to low light (LL) (10 µmol m^{-2} s^{-1}) and high light (HL) (60 µmol m^{-2} s^{-1}) intensities. Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. Error bar showed the mean±SD.

The light response curve of both the WT and mutant strains was recorded in the cells, particularly grown under two light conditions i.e., high light (60 µmol m^{-2} s^{-1}) and low light conditions (10 µmol m^{-2} s^{-1}). The relative electron transport rate (rETR), was calculated from the light curve (LC) obtained under different actinic light intensities (100, 200, 300, 500 µmol m^{-2} s^{-1}). The rETR values in both the WT and DCMU-tolerant mutant strains exhibited a different level of photosynthetic yield under the range of light irradiance used for the light curve. The rETR value for the WT showed its maxima at (200 µmol m^{-2} s^{-1}) which was about 33% lower than that recorded for the mutant strain (300 µmol m^{-2} s^{-1}) (Fig. 5.9). The rETR values of both the strains indicated their respective photosynthetic performance, depending upon their light tolerance and light harvesting efficiencies. Beyond their photosynthesis saturating light intensities, both the WT and mutant dissipate the excess light energy in the form of heat. Thus, all the

photosynthetic parameters clearly indicated better photosynthetic performance by the mutant strain than the WT even at a higher light intensity.

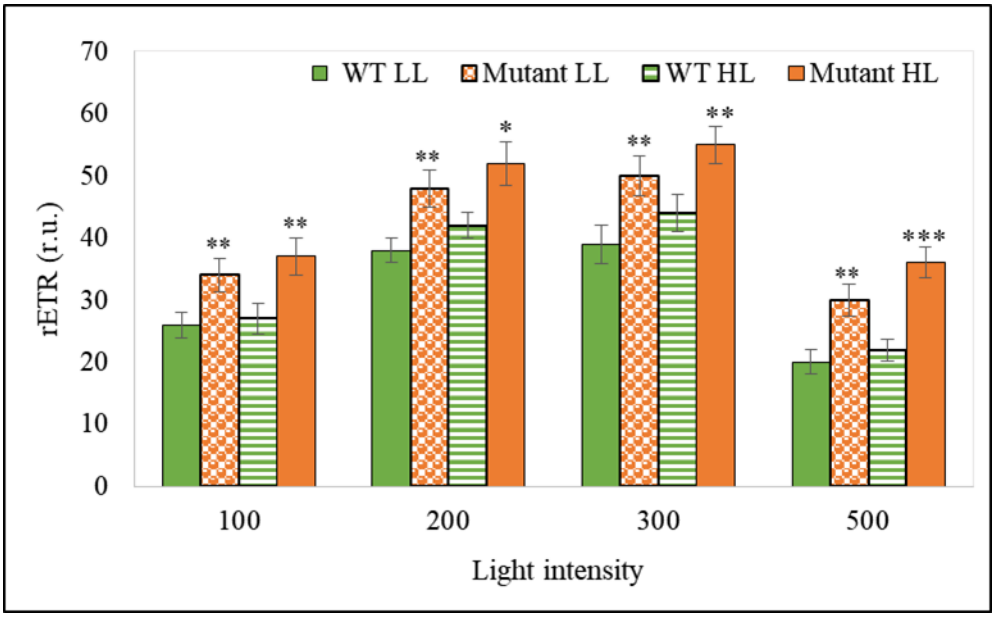

Fig. 5.9 Response light curve (LC) effect on wild-type (WT) and mutant strain of *S. vacuolatus* cells exposed to varying irradiance (100-500 µmol m^{-2} s^{-1}). Both strains were adapted to low light (LL) (10 µmol m^{-2} s^{-1}) and high light (HL) (60 µmol m^{-2} s^{-1}) intensities. Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. Error bar showed the mean±SD.

Table 5.4 Changes in the OJIP parameters derived from chlorophyll fluorescence induction kinetics of the wild-type (WT) and mutant cells (dark-adapted) of *S. vacuolatus* exposed to varying light intensities. Student't' test showing significance difference level, *p<0.05, **p<0.01, ***p<0.001. The data presented as mean±SD.

Light intensity (µmol m^{-2} S^{-1})	**Wild-type**				**Mutant**			
	ET_0/RC	**TR_0/RC**	**ABS/RC**	**RC/ABS**	**ET_0/RC**	**TR_0/RC**	**ABS/RC**	**RC/ABS**
10	0.62±0.02	0.64±0.05	1.03±0.06	17.6±1.5	0.77±0.59*	0.74±0.06**	1.20±0.10*	23.4±1.5***
20	0.81±0.03	0.83±0.04	1.20±0.09	20±1.8	0.99±0.09*	0.959±0.07*	1.326±0.10**	25.4±1.8***
40	0.919±0.09	0.92±0.04	1.31±0.13	23.3±1.9	1.0±0.10*	1.03±0.06*	1.452±0.11*	27.2±1.0*
60	0.87±0.08	0.86±0.05	1.34±0.11	20.6±2.0	1.1±0.08***	1.07±0.10*	1.52±0.10**	29.1±1.5**
80	0.61±0.03	0.63±0.06	1.16±0.10	14.0±1.4	0.81±0.03**	0.87±0.06***	1.37±0.11**	18.1±1.7**
100	0.48±0.04	0.48±0.06	0.73±0.05	6.4±0.8	0.70±0.02*	0.75±0.06**	1.15±0.12*	12.2±1.5**

5.3.6 FTIR analysis

FTIR spectra of cell biomass of both the wild-type and mutant strain grown under varying light irradiances showed changes in the IR absorption bands over the wavelength range 4000-400 cm^{-1}. Each IR absorption band assigned to the specific molecular group of biochemical constituents (protein, lipids and carbohydrate) and the associated functional groups exhibited light intensity-dependent changes (Fig. 5.10, 5.11). IR bands attributed to (C=O) stretching of amide I (1655 cm^{-1}), (N–H) bending of amide II (1545 cm^{-1}), (C=O) of ester group of lipid and fatty acid (1740 cm^{-1}), (C-O-C) stretching associated with polysaccharides/carbohydrate (1040 cm^{-1}), and lipid at 2920 cm^{-1} and 3011 cm^{-1} were considered for interpretation of results (Stehfest et al., 2005; Murdock & Wetzel, 2009). The compositional changes in both the WT and mutant strain of *S. vacuolatus* were in terms of total lipid (2920/3011cm^{-1}), lipid/carbohydrate (L/C) ratio (1740/1040cm^{-1}), carbohydrate/protein (C/P) ratio (1040/1650cm^{-1}), and lipid/protein (L/P) ratio (1740 /1650cm^{-1}) (Dean et al., 2010; Laurens et al., 2011) (Table 5.5 a, b). The result observed that total lipid ratio in both the WT and mutant strain was grown in the presence varying light intensities (10-100 µmol m^{-2} s^{-1}) initially showed an intensity-dependent gradual

increase, followed by a decline at a higher light intensity (80-100 µmol m^{-2} s^{-1}). The maximum level of total lipid ratio in both the WT (1.3±0.1) and mutant (1.8±0.08) cells was observed at their growth saturating light intensities (40 and 60 µmol m^{-2} s^{-1}, respectively). Similar observations were made for carbohydrate/protein (C/P) ratio lipid/carbohydrate (L/C) ratio, the overall ratios showed that C/P (0.81±0.05) and L/C (1.1±0.08) in the wild-type cells was found to be the highest at 40 µmol m^{-2} s^{-1} and in mutant cells (0.9±0.05, 1.7±0.10 respectively) at 60 µmol m^{-2} s^{-1} light intensity. Similarly, the L/P ratio was found to be the highest in the WT (1.3±0.10) cells grown at 60 µmol m^{-2} s^{-1}, whereas the mutant strain showed L/P ratio (1.9±0.15) in the cells grown at 60 µmol m^{-2} s^{-1} (Table 5.5 a, b). These results, together, suggested significant compositional alterations in the lipid, carbohydrate and protein content of both the strains in commensuration with their light tolerance abilities.

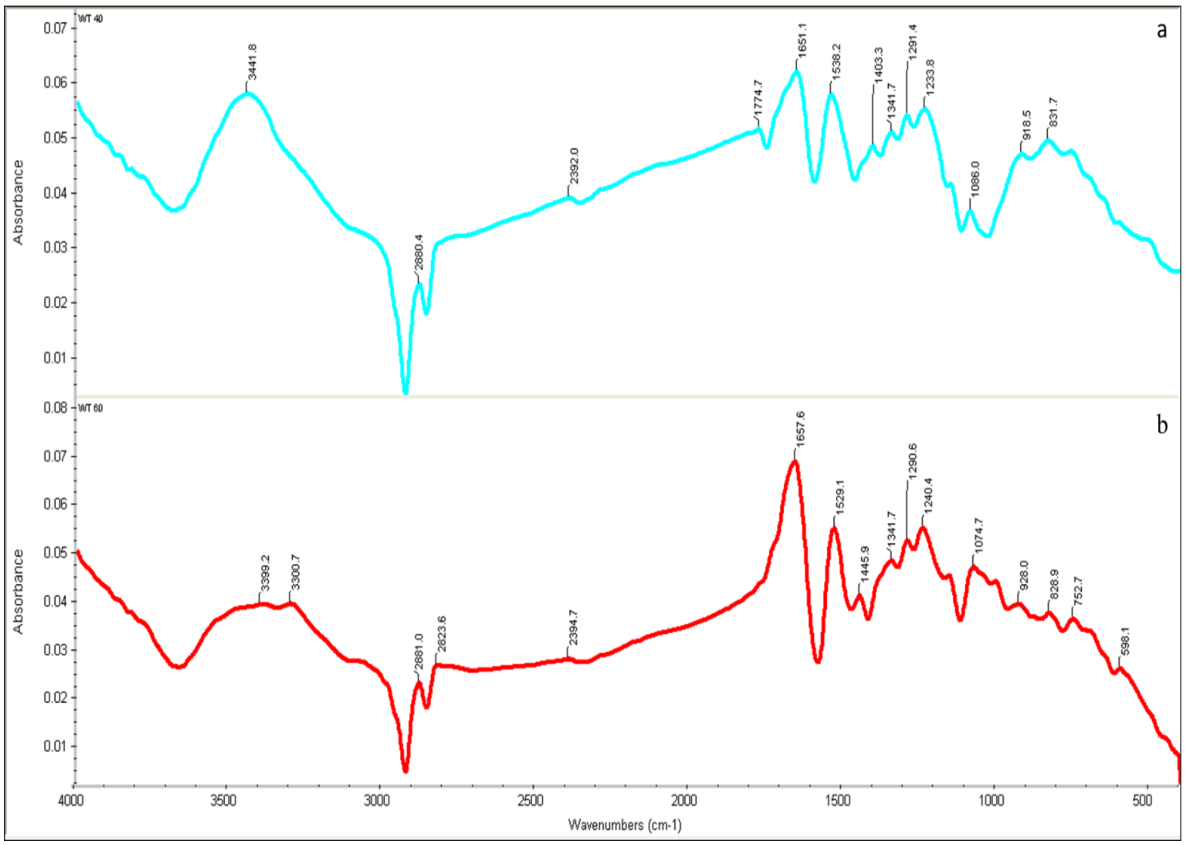

Fig. 5.10 FTIR analysis of microalgal biomass of wild-type of *S. vacuolatus* in the presence of 40 µmol m^{-2} s^{-1} (a) and 60 µmol m^{-2} s^{-1} (b) light intensity.

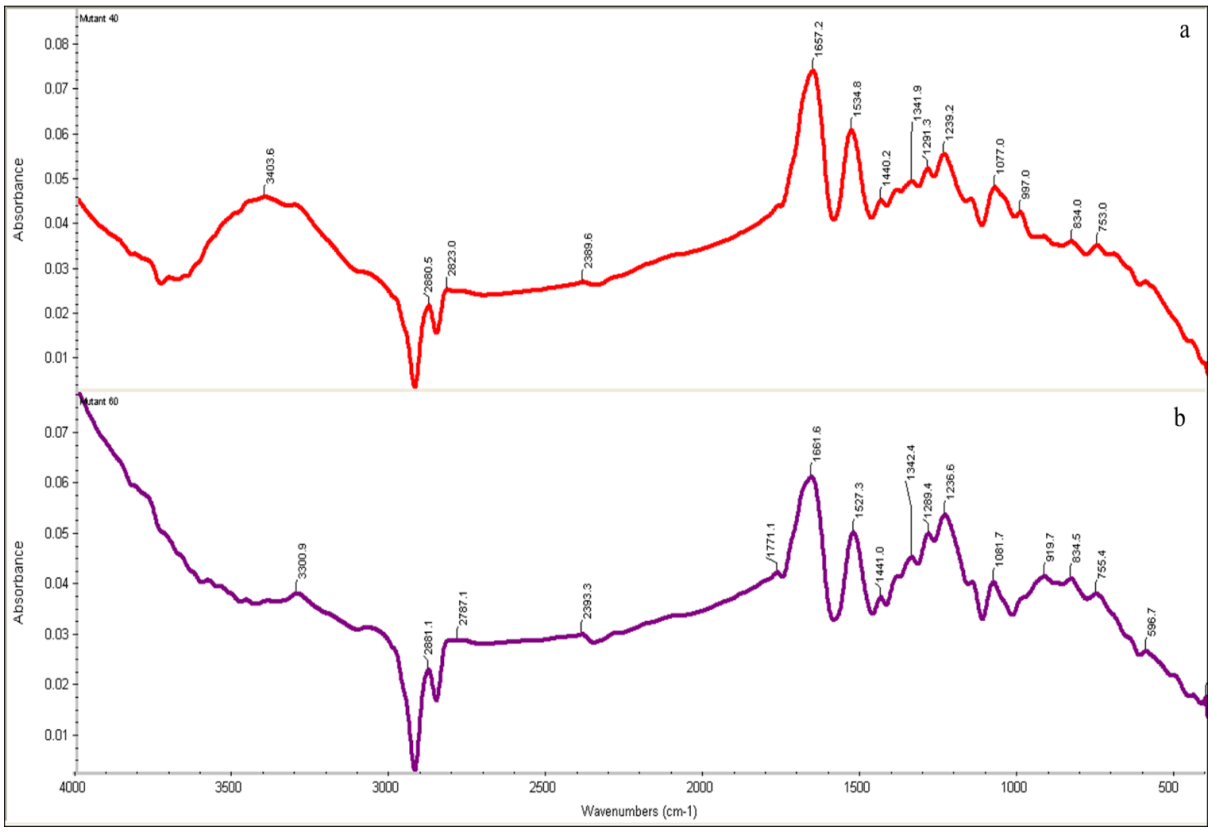

Fig. 5.11 FTIR analysis of microalgal biomass of mutant strain of *S. vacuolatus* in the presence of 40 µmol m^{-2} s^{-1} (a) and 60 µmol m^{-2} s^{-1} (b) light intensity.

Table 5.5 (a) Total lipid, Carbohydrate/Protein (C/P), Lipid/Carbohydrate (L/C) and Lipid/Protein (L/P) ratios derived from the FTIR spectra of cell biomass of wild-type (WT) of *S. vacuolatus* under varying light irradiances (10-100 µmol m^{-2} s^{-1}). The data presented as mean±SD.

Light Intensity (µmol m^{-2} s^{-1})	Total lipid	Carbohydrate/Protein (C/P)	Lipid/Carbohydrate (L/C)	Lipid/Protein (L/P)
10	1.0±0.07	0.51±0.04	0.7±0.03	0.5±0.04
40	1.3±0.1	0.81±0.05	1.1±0.08	0.81±0.05
60	0.7±0.05	0.7±0.03	0.91±0.05	1.3±0.10
100	0.51±0.04	0.3±0.02	0.61±0.04	0.8±0.03

Table 5.5 (b) Total lipid, Carbohydrate/Protein (C/P), Lipid/Carbohydrate (L/C) and Lipid/Protein (L/P) ratios derived from the FTIR spectra of cell biomass of mutant strain of *S. vacuolatus* under varying light irradiances (10-100 µmol m^{-2} s^{-1}). The data presented as mean±SD.

Light Intensity (µmol m^{-2} s^{-1})	Total lipid	Carbohydrate/Protein (C/P)	Lipid/Carbohydrate (L/C)	Lipid/Protein (L/P)
10	1.3±0.08	0.6±0.03	1.2±0.07	0.8±0.03
40	1.5±0.09	0.71±0.06	1.1±0.1	0.71±0.04
60	1.8±0.08	0.9±0.05	1.7±0.1	1.9±0.15
100	0.8±0.03	0.46±0.04	0.11±0.1	0.51±0.4

(B) Effect of spectral quality of light on the microalga *S. vacuolatus*

5.3.7 Effect of spectral quality of light on growth

The specific growth rate of WT and mutant strain of *S. vacuolatus* was observed in response to the different spectral quality of light over a period of 20 days. The specific growth rate of WT cells was achieved maximum under green light (0.065±0.003 d^{-1}) (Fig. 5.12), whereas the mutant strain found maximum specific growth rate under the orange light (0.089±0.004 d^{-1}). However, the growth rate was found lowest in both the WT (0.041±0.002 d^{-1}) and mutant strain (0.053±0.003 d^{-1}) under blue light. The statistical analysis Student 't' test showed a significant difference level between the result of WT and mutant strain. The other conditions of spectral light facilitated the growth of both the WT and mutant strain. However, under different spectral light irradiance, the overall growth of mutant cells was higher than the WT cells. These results showed better efficiency of light-harvesting in mutant strain compared to WT cells. The preference is shown for the different spectral quality of light by both mutant and WT cells that depend on their ability to use the same spectrum range in photosynthesis.

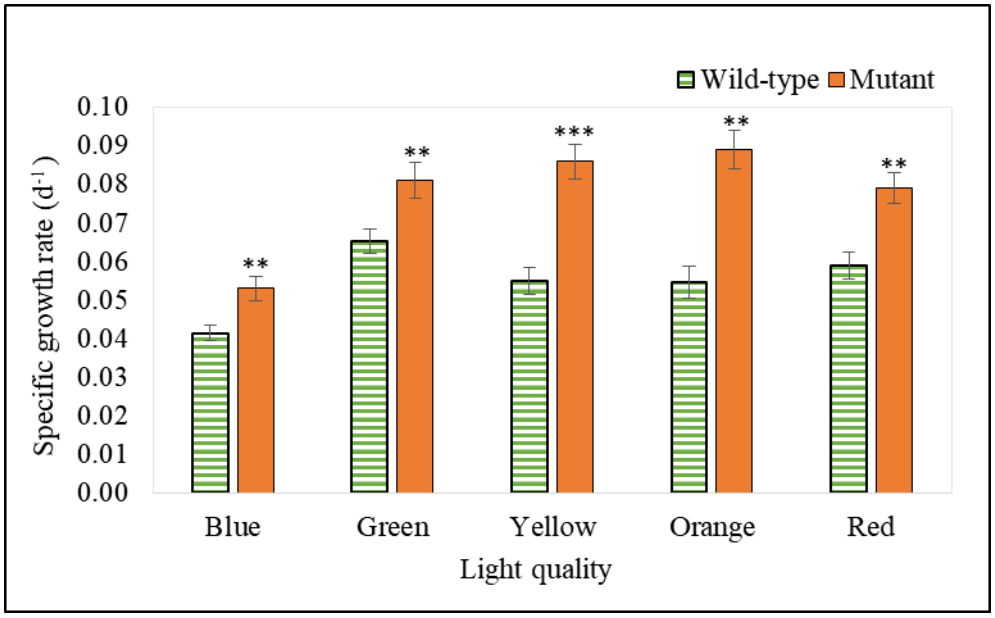

Fig. 5.12 Changes in specific growth rate (μ) of wild-type and mutant strain of *S. vacuolatus* exposed to the different spectral quality of light. Student't' test showed significance difference level at **p<0.01, ***p<0.001. Error bar showed the mean±SD.

5.3.8 Effect of spectral quality of light on biochemical constituents and pigment analysis

The effect of different light quality was measured on biochemical constituents of cells like protein and carbohydrate (Fig. 5.13 a, b). The results recorded higher protein content in the WT grown cells under green light (252±18.6 μg mL^{-1}) and followed by red light (246±16.6 μg mL^{-1}) (Fig. 5.13, a). Whereas, the highest protein content was observed in the mutant strain under orange light grown cells (310±22 μg mL^{-1}), followed by yellow light (301±20.8 μg mL^{-1}). Similarly, the results on carbohydrate content showed under the green light WT grown cells (178±15.3 μg mL^{-1}) maximum carbohydrate content, followed by red light (172±10.5 μg mL^{-1}) (Fig. 5.13, b). In the case of the mutant strain, the highest carbohydrate content was found under orange light grown cells (289±20.3 μg mL^{-1}), then yellow light (273±16.6 μg mL^{-1}). The results on total organic carbon (TOC) content showed the highest value in the green light grown cells of WT (0.701±0.04 mg L^{-1}) and red light grown cells (0.622±0.03 mg L^{-1}). Whereas mutant strain was achieved the highest TOC content in the orange light grown cells (1.06±0.08 mg L^{-1}), then yellow light grown cells (0.984±0.05 mg L^{-1}). On the other hand, the result showed that both the WT and mutant strain exhibited the lowest

TOC content under the blue light condition (0.278±0.02 and 0.472±0.021 mg L^{-1}, respectively) (Fig. 5.13, c).

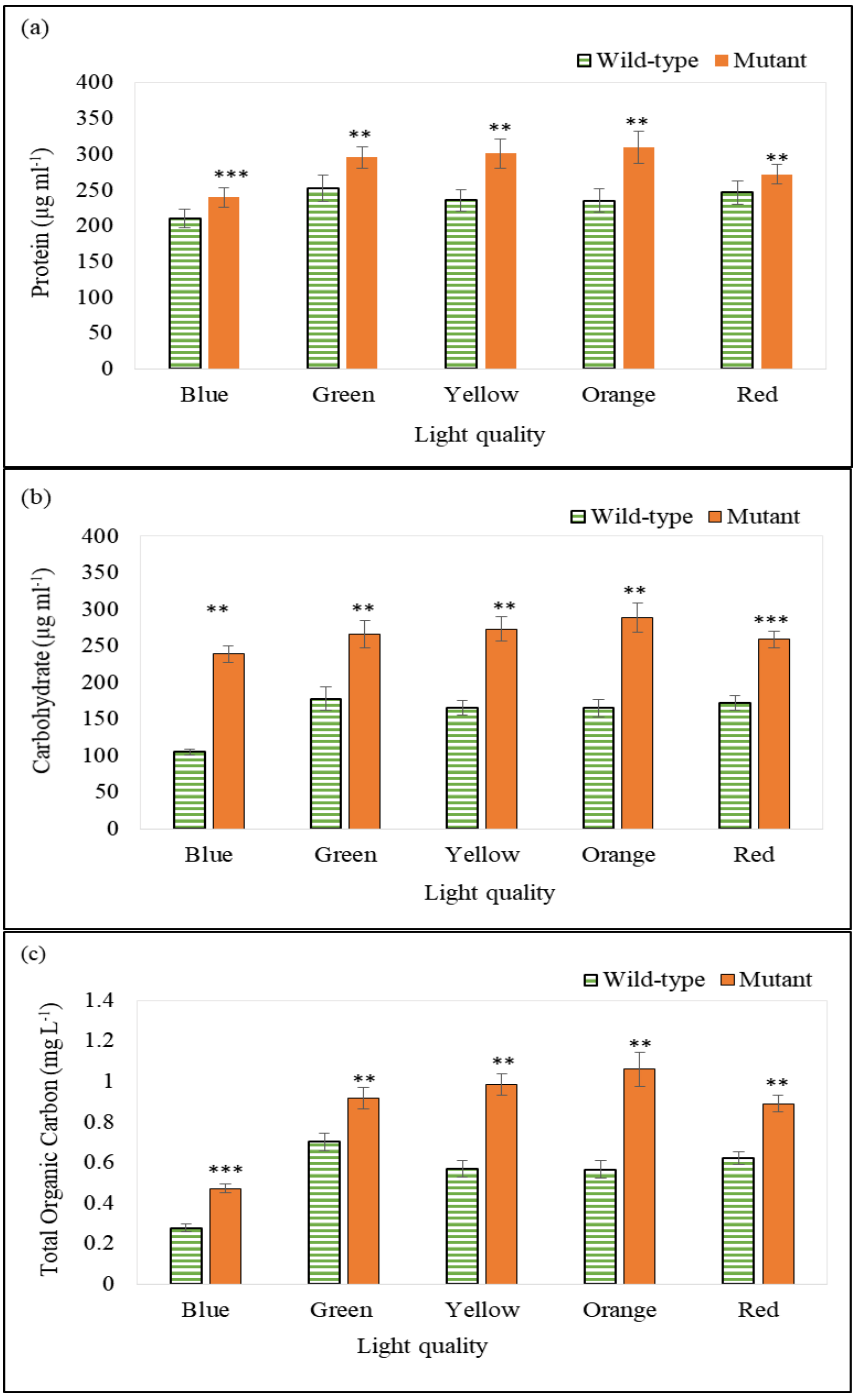

Fig. 5.13 Study of (a) protein (b) carbohydrate (c) Total organic carbon content of the wild-type and mutant strain of *S. vacuolatus* exposed to the different spectral quality of light. Student‘t’ test showed significance difference level at **p<0.01, ***p<0.001. Error bar showed the mean±SD.

The results of pigment analysis showed that maximum level of total chlorophyll in the WT grown cells of green light (22.02±1.5 µg mL^{-1}), then red light (18.7±1.0 µg mL^{-1}), whereas mutant strain was found maximum total chlorophyll under orange light (35.9±2.5 µg mL^{-1}) followed by yellow light (31.4±2.4 µg mL^{-1}) Fig. 5.14, (a, b). Similarly, carotenoid content in the WT was found to be maximum under green light grown cells (4.0±0.35 µg mL^{-1}), followed by the red light grown cells (3.0±0.20 µg mL^{-1}), whereas in the mutant strain was found maximum carotenoid level under orange light grown cells (5.1±0.21 µg mL^{-1}), then yellow light (4.6±0.13 µg mL^{-1}) Fig. 5.14 (a, b). However, the levels of the photosynthetic pigment in both WT and mutant strains were found to be the lowest in the blue light grown cells. The overall results revealed a significant variation (Student't' test) level of biochemical constituents in both the WT and mutant strains of *S. vacuolatus* under varying spectral quality light irradiances.

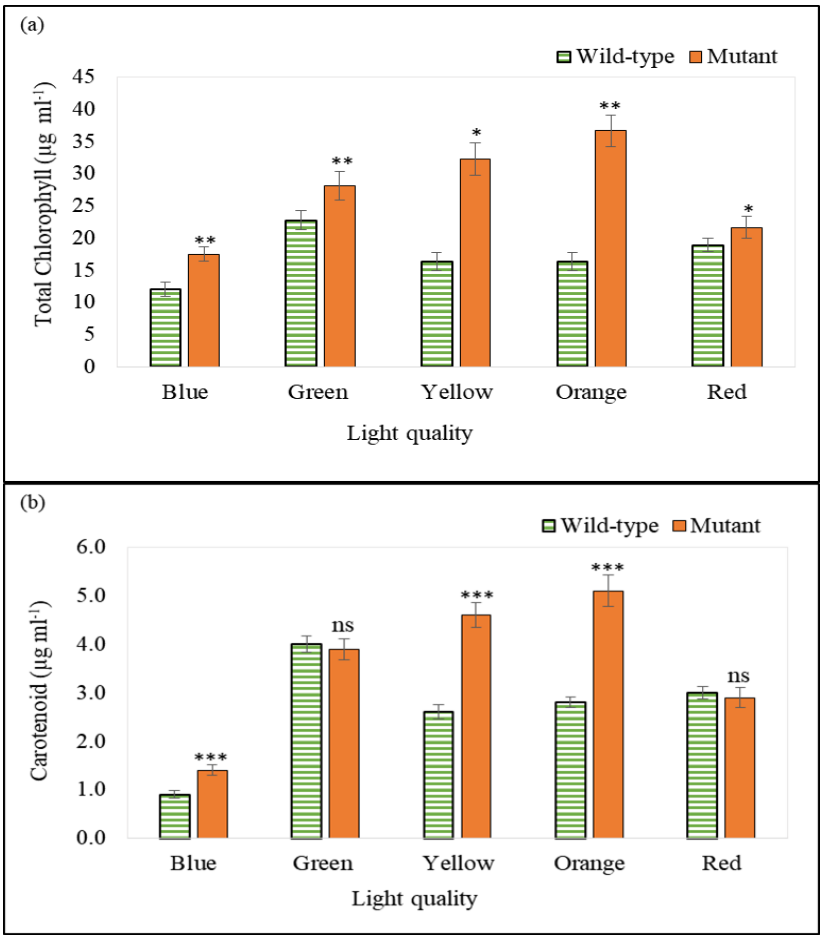

Fig. 5.14 Changes in the (a) Total Chlorophyll and (b) Carotenoid contents of wild-type and mutant strains exposed to the different spectral quality of light. Student't' test was applied to show significance level at *p<0.05, ** p<0.01, ***p<0.001, ns= non-significant. Error bar showed the mean±SD.

5.3.9 Flow cytometer analysis

In terms of Nile red fluorescent intensity, the flow cytometer study of neutral lipid content showed higher lipid content in green light-grown WT cells (40.2%), followed by red and orange light (34.2% and 29.2% respectively) (Fig. 5.15). On the other hand, it was observed that the lipid content in the mutant strain was the maximum in orange light (42.1%) (Fig. 5.16), followed by yellow light (32.9%) and green light (30.4%) respectively. Thus, the results of the flow cytometer analysis revealed that the lipid content in the WT and mutant strains were in commensuration with the best growth-supporting light irradiances for the respective strain; however, the total lipid content in the orange light was higher in the mutant strain than the WT green light grown cells.

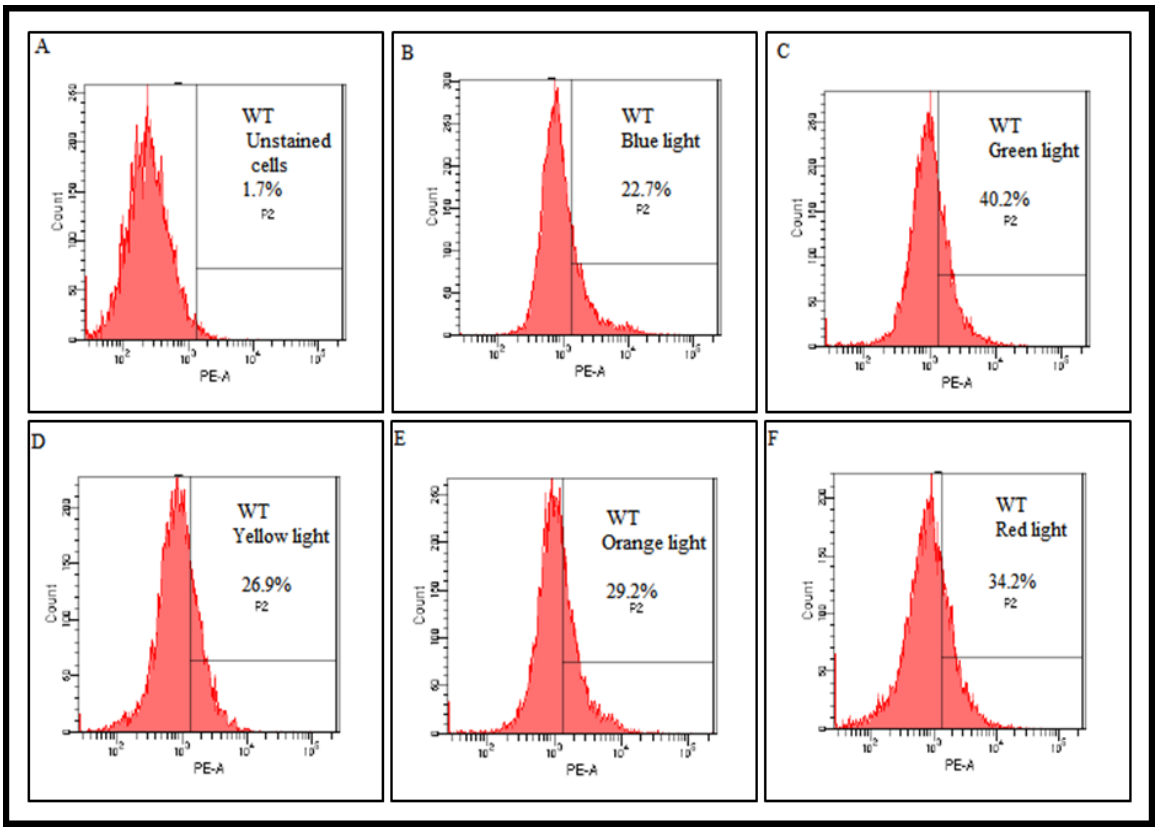

Fig. 5.15 Flow cytometer analysis of neutral lipid by using Nile red in the wild-type (WT) cells of *S. vacuolatus* exposed to the different spectral quality of light.

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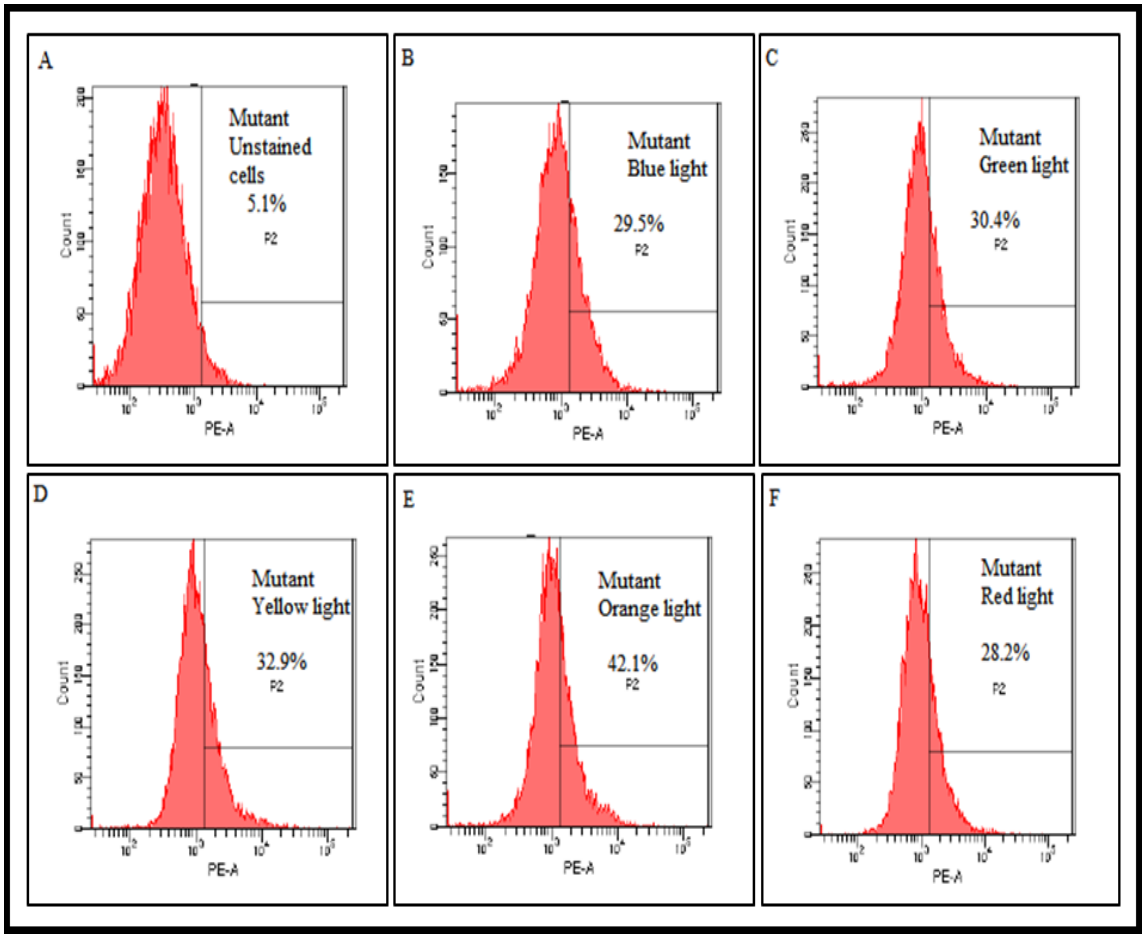

Fig. 5.16 Flow cytometer analysis of neutral lipid by using Nile red in the mutant strain of S. vacuolatus exposed to the different spectral quality of light

5.3.10 Effect of spectral quality of light on chlorophyll fluorescence induction kinetics (OJIP) and non-photochemical quenching (NPQ)

The OJIP curve of chlorophyll fluorescence indicated the primary photochemistry photosystem II. The main parameters of the primary photochemistry were calculated from the OJIP curve (Fig. 5.17, 5.18): the actual quantum yield $\Delta F/F_m$' (light-adapted state), maximum quantum yield F_v/F_0 (dark-adapted state), ABS/RC, RC/ABS, ET_0/RC and NPQ (Table 5.6 a, b).

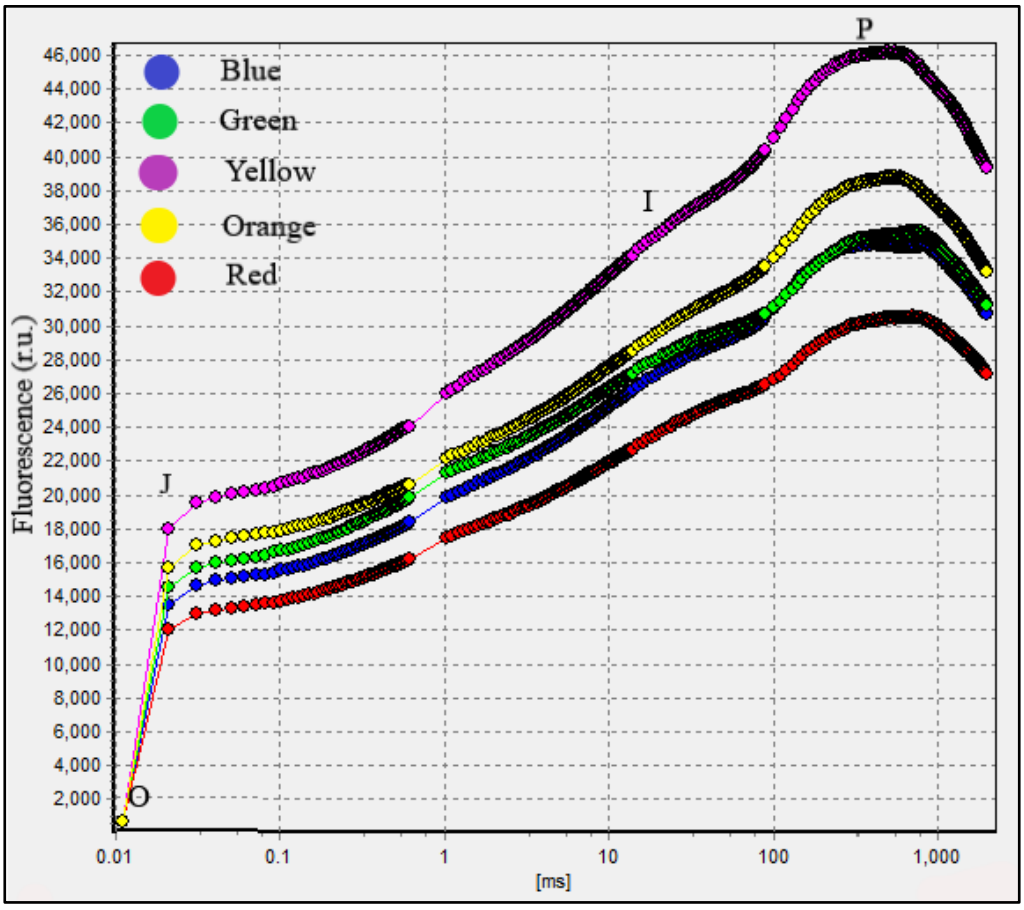

Fig. 5.17 Chlorophyll α fluorescence induction curve (OJIP) of wild-type cells of *S. vacuolatus* grown cells exposed to the different spectral quality of light. The O, J, I, P stand for O, (F_0) fluorescence intensity at 50 μs; J, (Fj) fluorescence intensity at J-step (at 2 ms); I, (Fi) fluorescence intensity at I step (at 60 ms); P, (Fm) maximal fluorescence intensity.

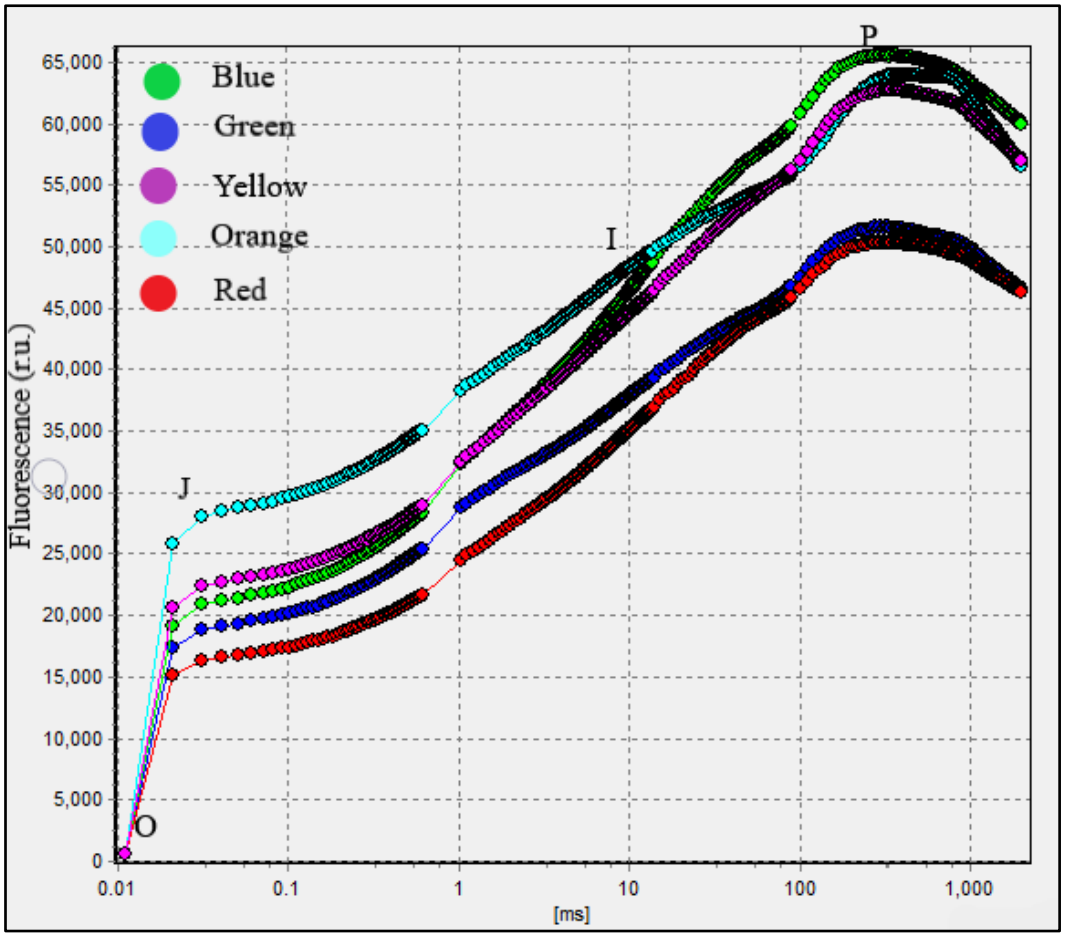

Fig. 5.18 Chlorophyll α fluorescence induction curve (OJIP) of mutant strain of *S. vacuolatus* grown cells exposed to the different spectral quality of light. The O, J, I, P stand for O, (F_0) fluorescence intensity at 50 μs; J, (Fj) fluorescence intensity at J-step (at 2 ms); I, (Fi) fluorescence intensity at I step (at 60 ms); P, (Fm) maximal fluorescence intensity.

The actual quantum yield ($\Delta F/F_m'$) range of PSII in the mutant strain was from 0.42±0.015 to 0.61±0.055 and that in the WT strain was between 0.42±0.55 and 0.49±0.035. The maximum quantum yield (F_v/F_0) of PSII in the blue light grown WT cells was 1.38±0.065 and that in the blue light-grown mutant cells was 2.15±0.13 as compared to other spectral quality of light. Thus, these findings suggested that the primary photochemistry of QA reduction was similar in both the WT and mutant strains. The ABS/RC denoted the absorption of energy flux per active reaction centre and showed the highest value in the WT grown cells of green light (2.54±0.17) and orange light grown cells of

mutant strain (2.17±0.11). Therefore, it can be deduced that the light energy absorption per reaction centre in both the WT and the mutant strain was high under green and orange light irradiance, respectively. Similarly, the value of ET_0/RC, denoting the efficiency of electron transport leading to a reduction of electron carriers beyond QA, was high in the WT grown cells of green light and mutant strain is grown cells of orange light (0.94±0.070 and 0.785±0.075, respectively).

The overall light-harvesting efficiency and electron transport per reaction centre in the WT and mutant strains were maximal under green and orange light conditions, respectively. Thus, the overall high energy absorption per reaction centre ABS/RC, leading to QA reduction was higher in the WT strain than the mutant. Since the level of photosynthetic pigments was higher in the mutant strain, the size active RC was smaller in the WT. Moreover, a lower F_m value and shorter duration required by the mutant to reach to F_m value than the WT strain, suggesting a more efficient electron transfer in the mutant strain on the reduction side of PSII, that minimizes the probability of closure of the PSII reaction centre in the mutant. Furthermore, a prolonged duration taken to reach the F_m value in the WT along with low quantum yield indicated a sluggish electron transfer process on the donor side of the PS II reaction centre. These results on NPQ indicated a high NPQ value in the WT cells grown under yellow light (0.207±0.012), followed by red light (0.206±0.008), and by blue light (0.205±0.011) grown cells Table 5.6 (a). On the other hand, the NPQ value was maximal in the mutant cells grown under green light (0.167±0.110), followed by blue light (0.162±0.011) and yellow light (0.147±0.010) Table 5.6 (b). However, the overall NPQ values in the mutant were significantly lower than those in the WT, thereby suggesting greater dissipation of absorbed light energy in the WT than the mutant strain of *S. vacuolatus.*

Table 5.6 (a) Study of changes in the OJIP parameters derived from chlorophyll fluorescence induction kinetics of the wild-type (WT) cells of *S. vacuolatus* grown under the different spectral quality of light. The data are presented as mean±SD.

Light quality	$\Delta F/F_m'$	Fv/F_0	ABS/RC	RC/ABS	ET_0/RC	NPQ
Blue	0.48±0.040	1.27±0.080	2.22±0.22	2.73±0.11	0.88±0.06	0.205±0.011
Green	0.49±0.035	1.38±0.065	2.54±0.17	2.47±0.12	0.94±0.07	0.192±0.010
Yellow	0.42±0.055	1.36±0.060	2.22±0.11	2.99±0.17	0.89±0.08	0.207±0.012
Orange	0.47±0.035	1.28±0.070	2.32±0.11	2.88±0.16	0.93±0.04	0.136±0.011
Red	0.45±0.040	1.30±0.036	2.24±0.09	1.56±0.07	0.71±0.05	0.206±0.008

Table 5.6 (b) Study of **c**hanges in the OJIP parameters derived from chlorophyll fluorescence induction kinetics of the mutant strain of *S. vacuolatus* grown under the different spectral quality of light. The data are presented as mean±SD.

Light quality	$\Delta F/F_m'$	F_v/F_0	ABS/RC	RC/ABS	ET_0/RC	NPQ
Blue	0.42±0.015	1.15±0.070	1.85±0.087	2.72±0.11	0.725±0.06	0.162±0.011
Green	0.59±0.055	1.74±0.18	1.59±0.075	4.08±0.37	0.716±0.069	0.167±0.110
Yellow	0.53±0.041	1.27±0.07	1.68±0.085	3.63±0.33	0.729±0.055	0.147±0.010
Orange	0.61±0.055	1.80±0.13	2.17±0.110	2.16±0.15	0.785±0.075	0.133±0.007
Red	0.60±0.055	2.10±0.14	1.57±0.090	4.49±0.42	0.727±0.040	0.125±0.012

5.3.11 FTIR analysis

The FTIR analysis of both WT and mutant strain of *S. vacuolatus* microalgal biomass grown under the varying spectral qualities of light showed changes in the IR spectrum (4000–400 cm^{-1}), indicating changes in the composition of the cell constituents. The IR spectra revealed changes in the absorption peak for carbohydrate (C-O-C) at 1250–950 cm^{-1}, amide I and amide II groups of proteins (1651 cm^{-1} and 1545 cm^{-1}, respectively) and lipids (1736–1712 cm^{-1}). The IR spectra cells absorption peak at 1740 cm^{-1} arises due to the carbonyl (C=O) group of lipid triglycerides (Mayers et al., 2013), while that at 1040 cm^{-1} indicated the polysaccharide content of the cells (Duygu et al., 2012). Each band is assigned to the specific molecular group based on the biochemical constituents

(protein, nucleic acid, and carbohydrate) and the associated functional groups in the biomass (Fig. 5.19, 5.20). Based on the FTIR spectra, the biochemical compositional changes in WT and mutant strain of S. vacuolatus cells were evaluated in terms of total lipid (2920/3011 cm^{-1}), Lipid/Carbohydrate (L/C) (1740/1040 cm^{-1}), Carbohydrate/Protein (C/P) (1040/1650 cm^{-1}) and Lipid/Protein (L/P) (1740/1650 cm^{-1}) ratio (Table 5.7). The results found that total lipid ratio showed maximum values in both the mutant (1.24±0.070) and WT strains (1.2±0.110) when grown under orange and green light irradiance. The L/C and L/P ratio in case of WT cells were found to be the highest in red light grown cells (1.0±0.072 and 1.4±0.070, respectively), followed by green light grown cells (0.82±0.061 and 0.52±0.026, respectively). On the other hand, the mutant showed the highest L/C and L/P ratio in the biomass grown under orange light (0.71±0.050 and 0.59±0.040, respectively), followed by red light condition (0.70±0.061), a lower value (L/P) (0.39±0.035) then blue light (0.5±0.030). The C/P ratio was observed maximum in the mutant strain under orange light condition (0.69±0.058), and WT showed the highest C/P ratio (1.4±0.100) under the red light. These results, together, suggested significant compositional alterations in the lipid, carbohydrate and protein content of both the strains in response to the spectral quality of light.

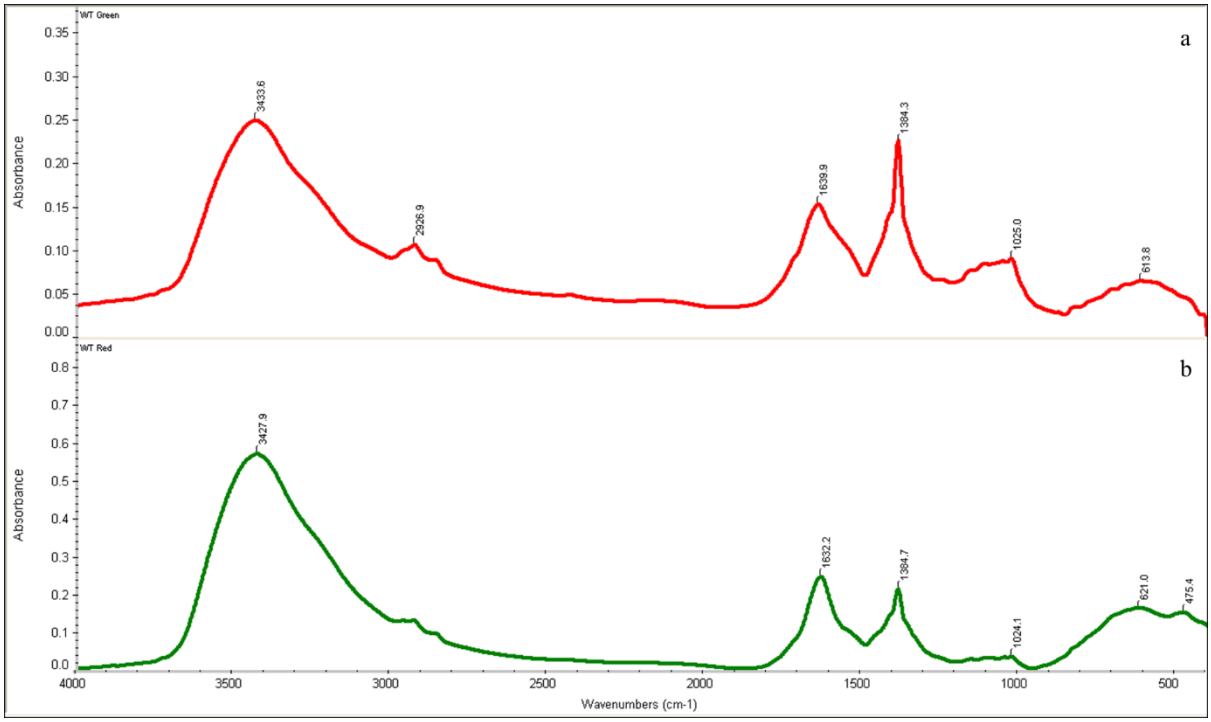

Fig. 5.19 FTIR spectra of the biomass of wild-type cells exposed to green (a) and red (b) spectral quality of light.

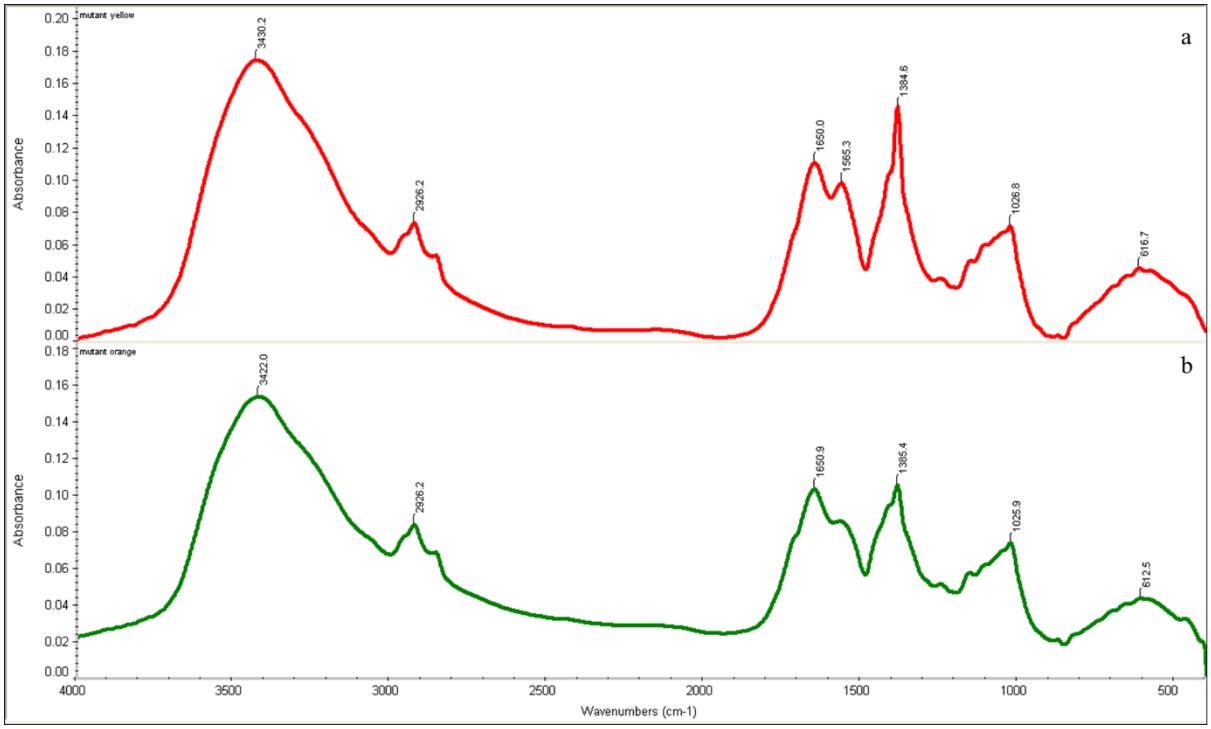

Fig. 5.20 FTIR spectra of the biomass of mutant strain exposed to yellow (a) and orange (b) spectral quality of light.

Table 5.7 FTIR spectra of cells biomass of both WT and mutant strain of *S. vacuolatus* cells grown under different spectral quality of light showing comparative study of cells constituents ratios such as Total lipid (2920/3011 cm^{-1}), Carbohydrate/Protein (C/P), (1040/1650 cm^{-1}), Lipid/Carbohydrate (L/C), (1740/1040 cm^{-1}), and Lipid/Protein (L/P), (1740/1650 cm^{-1}). The data are presented as mean±SD.

	Wild-type				**Mutant**			
Light Quality	**Total Lipid**	**C/P**	**L/C**	**L/P**	**Total Lipid**	**C/P**	**L/C**	**L/P**
Blue	1.0±0.095	0.61±0.040	0.6±0.062	0.42±0.020	0.60±0.03	0.53±0.047	0.69±0.030	0.5±0.030
Green	1.2±0.110	0.63±0.055	0.82±0.061	0.52±0.026	1.16±0.060	0.60±0.056	0.63±0.056	0.39±0.035
Yellow	1.1±0.080	0.53±0.047	0.42±0.045	0.21±0.020	1.20±0.065	0.58±0.045	0.50±0.030	0.27±0.025
Orange	1.1±0.110	0.51±0.036	0.61±0.058	0.36±0.035	1.24±0.070	0.69±0.058	0.71±0.050	0.59±0.040
Red	1.0±0.064	1.4±0.100	1.0±0.072	1.40±0.070	1.14±0.090	0.55±0.050	0.70±0.061	0.39±0.035

5.4 Discussion

Light is the driver of photosynthesis in microalgae and divided into the quality of light and intensity of light. Since microalgae are photosynthetic organisms, light quality (wavelength distribution) and light intensity were studied in order to determine their effects on growth, biomass and lipid content of microalgae (Atta et al., 2013; Hultberg et al., 2014; Teo et al., 2014). Current light intensity testing methods used fluorescent light with a wide distribution of wavelengths (Shu et al., 2012). Whereas light-emitting diodes (LEDs) are used more recently because they provide a small range of wavelength, are more effective and less heat dissipating for estimation of spectral quality of light (Schulze et al., 2014). The intracellular defence mechanism of the photosynthetic organisms enables them to overcome the high light stress-induced cellular oxidative damage (Snoeijs et al., 2011). Several studies have shown that stress-induced increase in the ROS level is accompanied by stimulation of antioxidative response of the cells (Yang et al., 2013; Yilancioglu et al., 2014). It has been also suggested that the level of ROS generation is coupled with the synthesis of lipid (Menon et al., 2013). Richardson et al. (1983) have reported that microalgae exhibit remarkable changes in their gross chemical composition, pigments and photosynthetic activity in response to varying light irradiances. The present study showed that DCMU tolerant mutant strain was relatively more tolerant to high light stress (60 µmol m^{-2} s^{-1}) as compared to WT (40 µmol m^{-2} s^{-1}). The high light intensity between 80-100 µmol m^{-2} s^{-1} caused a drastic decrease in the growth, protein and carbohydrate content of both the strains. FTIR analysis of cells grown under varying light intensities (10-100 µmol m^{-2} s^{-1}) also showed changes in the protein, carbohydrate and lipid content of both the strains as suggested earlier (Stehfest et al., 2005; Murdock & Wetzel, 2009). Lipid/Protein (1740/1650 cm^{-1}) ratio increased with an increase in the light intensity in both the WT and mutant strains, particularly prior to their respective growth saturating light intensities. A light intensity-dependent increase in L/P ratio might be attributed to an increase in the total lipid content (2920/3011 cm^{-1}) as indicated by the FTIR analysis. A comparison of protein, carbohydrate and lipid constituents of both the strains indicated that the level of all the cell constituents in the mutant strain was higher than the wild-type throughout the range of light irradiances. Further, flow cytometric analysis of cell biomass demonstrated light intensity dependent increase in the lipid content, which was higher in the mutant strain than the WT.

It is a well-established fact that photo-damage is proportional to the intensity of incident light, (Simionato et al., 2013; Murata et al., 2007). It has been reported that, under the oxidative condition, polyunsaturated fatty acids (PUFA) reacts with reactive oxygen species and cause extensive damage to cellular lipids (Ren et al., 2017). The intracellular level of Malondialdehyde (MDA) a byproduct of cellular lipid peroxidation indicated peroxidative damage to cellular lipid (Kumar et al., 2015). Results on the photobleaching of pigments, lipid peroxidation, loss of -SH groups and RNO bleaching indicated a greater level of ROS generation and oxidative stress experienced by the WT than mutant strain. Results on the effect of radical quenchers on RNO bleaching in both the strains showed a very prominent effect of histidine- a quencher of superoxide radical ($O_2^{\cdot -}$), followed by sodium formate – a quencher of hydroxyl radical ($^{\cdot}OH$) (Matheson et al., 1975) and sodium azide (Foote et al., 1984). A higher rate of superoxide dismutase (SOD) activity, carotenoid and glycollate production in the mutant strain indicated that mutant strain was better equipped with strong anti-oxidative defence system than the WT as suggested earlier (Singh & Kshatriya, 2002; Janknegt et al., 2007; Nishiyama et al., 2001). The intracellular SOD enzyme is known to catalyze the transformation of superoxide radical in H_2O_2 form (Ali et al., 2005). It has been also reported that enhanced SOD activity provides protection to the cell under the high light stress, the SOD activity remains lower at a low light intensity (Ali et al., 2005; Rossa et al., 2002). Apart from enzymatic antioxidative defences, thiol (-SH) groups also play a crucial role in the scavenging of ROS (Zeng et al., 2011). A higher level of the total -SH content in mutant strain than the WT under the high light irradiances indicated a mild photooxidative condition in the mutant strain. Similarly an excessive production of glycollate in the mutant strain than the WT might be associated with mitigation of photo-oxidative stress due to its ability to consume surplus oxygen and modulation of carbon flux from TCA cycle to fatty acid synthesis as suggested by Tang et al. (2016) and Jeennor et al. (2006). Thus, it could be possible that stress-induced activation of the antioxidative defence system and lipid accumulation in the algal system might be mediated by glycollate metabolism as suggested earlier (Shi et al., 2017; Yang et al., 2013; Han & Eley, 1973).

It has been suggested that processing of light energy and its conversation into biomass depends upon the photochemistry of PSII which is the most sensitive component of the electron transport chain (Roach & Krieger-Liszkay, 2014). Many

physiological and biochemical studied have led to the conclusion that photosynthetic organisms protect themselves from high light stress through down-regulation of photosystem II (Gururani et al., 2015; Takahashi & Badger, 2011). A lack of consistency and uniformity in the experimental results requires a thorough investigation of parameters related to photosynthetic attributes. The Chlorophyll induction kinetics (OJIP) parameters such as (F_v/F_m, ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS) (Stirbet & Govindjee, 2012) studied in *S. vacuolatus* indicated better photosynthetic performance and electron transport in the mutant strain than the WT even under high light intensity. The quantum yield (F_v/F_m), overall photosynthetic performance (RC/ABS) of the mutant strain, suggested better light utilization efficiency in the mutant strain than the WT. The captured photons in case of the WT were found to be largely dissipated in terms of heat (Muller et al., 2001) as evident from the higher value of NPQ in the WT as compared to mutant strain. A greater value of rETR (Malapascua et al., 2014) recorded higher photosynthesis saturating light intensity in the mutant strain that confirmed the hypothesis that mutant strain was metabolically well adapted to use the excess photons, which supports better light tolerance and photosynthetic efficiency in the mutant than the WT. Earlier findings have correlated the DCMU-tolerance in microalgae and cyanobacteria with the D1 protein of PS II (Jegerschold et al., 1990), which is involved in down-regulation of PS II electron transport (Vonshak & Novoplansky, 2008; Foyer et al., 2012). In view of the foregoing evidence, it was inferred that higher level of light intensity tolerance and lipid accumulation in the mutant strain than the WT could be attributed to its strong antioxidative defence strategy of the mutant strain as evident from high SOD activity, carotenoid and glycollate content.

Many reports are available on the spectral variations when the light passes through water columns (Kirk, 1994), depending upon the dissolved content in the water and living biota present on the surface (Larkum & Barrett, 1983). The microalgae growing in such water bodies at the different level of water column respond to variations in the spectral quality of light and tend to adapt themselves to such light regimes (Parlevliet & Moheimani, 2014). In several red algae, growth and photosynthesis are heavily dependent on light quality (Biebl, 1962; Leukart & Luning, 1994). The analysis of the spectrum of growth and photosynthesis of red alga

corresponded with the spectral distribution occurring in deep coastal seawater (Leukart & Luning, 1994). It has suggested that the response of the microalgae to light quality is species-specific and their survival is more dependent on the pigment composition (Li et al., 2019; Vadiveloo et al., 2015). Among various light spectra, red and blue light plays an important role in the photosynthesis and thus, influence the plant growth and metabolism (Manivannan et al., 2015). Earlier reports have shown that Chl-a, α-Carotene, and Lutein contents enhanced by white or green light and photosynthetic activity induced by blue and red light (Godinez-Ortega et al., 2008).

In the present investigation, quantitative changes in the biomass and photosynthetic pigments of WT and mutant strains of *S. vacuolatus* recorded in response to the varying spectral quality of light. The WT cells were grown well under the green light, whereas mutant strain showed better growth under the orange light condition. There was little growth in both the strains under the blue light regime. Other workers have also shown influences of different spectral irradiance on the photosynthetic efficiency, chlorophyll content and growth in both the photoautotrophic prokaryotes as well as eukaryotes (Wu, 2016). Kim et al. (2015) also reported that alga *Gracilaria tikvahiae* presented a slow growth rate under the blue light as compared to those grown under red and green light. Earlier workers have demonstrated that the red light has the potential of stimulating the lipid production in *Ettlia* due to an enhanced rate of photosynthesis (Yang & Weathers, 2015). However, some studies have found other colours are more favourable. Green light at high intensities was found to have the highest biomass and biomass productivity in *Spirulina platensis* (Ravelonandro et al., 2008). The orange light was found to produce the highest growth rate in *Chlorella vulgaris* as compared to red light (Mohsenpour et al., 2012).

This could be due to light absorption characteristics of alga, depending upon several other factors, especially the thallus morphology, thickness, composition of the photosynthetic system etc. The present investigation revealed that the overall growth and level of pigments, protein, carbohydrate and lipid contents were higher in the mutant strain than the WT during the growth under all the light conditions. It was found that almost all the cell constituents in both the strains were interestingly linked with overall growth performance of the strains in response to the spectral quality of light.

Previous studies demonstrated that metabolic response of the algal strains to spectral quality of light is dependent on the pigment composition and the efficient use of light (Mohsenpour et al., 2012; Seyfabadi et al., 2011). Therefore, it was interesting to study the changes in the photosynthetic attributes of *S. vacuolatus* strains, elicited in response to the varying spectral qualities of light. The OJIP induction kinetics of chlorophyll fluorescence was used to evaluate the photosynthetic characteristics of the PSII reaction centre of both WT and mutant strains grown under the different spectral qualities of light. Consequently, the overall quantum yield (QY) of the mutant strain was found to be higher than that of the WT, irrespective of the spectral quality of light irradiance used during the growth. The lower QY and longer duration required to reach the F_m value by the WT strain, irrespective of the spectral quality of light used during the growth, indicated a slower rate of electron transport on the acceptor side of PSII (Malapascua et al., 2014; Neubauer & Schreiber, 1987). However, the absorption of photon flux per reaction centre (ABS/RC) and the electron flux for reduction of QA (ET_0/RC) were found to be higher in the green light-grown WT than the orange light-grown mutant cells. A substantial increase in the ABS/RC indicated either an increase in the pigment antenna of reaction centre or small size of the PSII reaction centre as suggested previously (Stirbet & Govindjee, 2012; Nixon et al., 2010; Melis et al., 1998). Ronde et al. (2004) suggested that an increase in the ABS/RC value for transgenic soybean plants under heat stress was due to the loss of absorbed light energy exclusively via heat dissipation. The current results also revealed a higher value of non-photochemical quenching (NPQ) in the WT cells than in the mutant, indicating their greater dissipation of absorbed photo-energy in the form of heat. In agreement with the earlier observation, chlorophyll fluorescence induction kinetics of WT cells exhibited a sharp downward trend in the fluorescence after reaching the F_{max} value.

FTIR analysis of microalgal biomass of different spectral quality of light irradiances of WT and mutant strains, also supported the other variable changes the synthesis of cell constituents (Jebsen et al., 2012; Bartosova et al., 2015). Whereas, flow cytometer analysis of lipid content revealed that both WT and mutant strains showed a different response to the spectral quality of light. In

addition, under varying spectral qualities of light might also be associated with the capability of their respective photosynthetic machinery to utilize the specific range of spectral qualities of light (Baer et al., 2016; Manivannnan et al., 2015).

Therefore, it is concluded that WT and the mutant cells respond to the different spectral quality of light and light intensity. A variation in the cell composition and cell biomass in both the strains in response to light irradiance may not be solely attributed to absorption of particular light spectrum by pigments, but several other factors including conversion of light energy into biochemical energy and cellular adaptation such as photosynthetic efficiency, antioxidative system may also lead to changes in the metabolic process as suggested by other workers (Wu, 2016; Kumar et al., 2019). Taken together, it may be inferred that the selection of strains with better photosynthetic performance (DCMU-tolerant mutant strain of *S. vacuolatus*) can be exploited for enhanced production of value-added products such as β-carotene and lipids, using optimal spectral quality of light and light intensity.

5.5 Conclusion

- Light intensity tolerance, as well as spectral quality (i.e. green and orange), was maximum in terms of growth and cell constituents in the DCMU tolerant-mutant strain (60 µmol m^{-2} s^{-1}) than the WT (40 µmol m^{-2} s^{-1}).
- In addition, FTIR and flow cytometer analysis showed higher accumulation of lipid in the mutant strain under the influences of spectral quality of light as well a light intensity compared to WT.
- Photosynthetic parameters recorded from OJIP, NPQ and Light curve analysis, determined by PAM fluorometer under different light quality and light intensity, indicated better photosynthetic efficiency and cellular defence system in the mutant strain than the WT.
- Thus, a greater light-intensity-tolerance, better photosynthetic performance and lipid accumulation in the mutant strain might be attributed by its efficient cellular antioxidative defence system and changes in the photosynthetic attributes of PSII.
- Both WT and mutant strain of microalga *S. vacuolatus* respond to under varying spectral qualities of light and light intensity in terms of changes in growth, pigments and cell constituents.

Chapter- VI

EFFECT OF NITROGEN NUTRITION ON THE BIOCHEMICAL CONSTITUENTS IN THE *S. VACUOLATUS*

6.1 Introduction

The use of microalgae as next-generation biofuel approaches has received attention in recent years and it is being considered as an environment-friendly alternative source of energy. However, significant advances in the product yield are still required to improve the overall economics and sustainability of feedstocks (Work et al., 2013; Clarens et al., 2010; Pienkos & Darzins, 2009). Previous studies have reported enhanced accumulation of neutral lipids (particularly triacylglycerol) in microalgal cells, depending upon the availability of nitrogen and phosphorous nutrients (Converti et al., 2009). Many investigators have reported that the nitrogen-limiting growth condition of microalgae strongly influences the carbon metabolism of algal cells (Kim et al., 2016; Li et al., 2013). Now the nitrogen-limiting growth of microalgae is adopted as a key strategy to trigger the lipid accumulation in many algal species (De Farias et al., 2018; Xia et al., 2016; Mortensen & Gislerod, 2016). It has been reported that the nitrogen deprivation of microalgal cells can induce accumulation of carbohydrate and lipid up to a level of 60% of the dry weight of biomass (Chen et al., 2013), but it significantly lowers the biomass production (Berges et al., 2002). Though, nitrogen starvation condition causes a severe decline in the growth, photosynthetic activity and pigments content in many microalgal species such as *Chlamydomonas reinhardtii*, *Chlorella vulgaris, Dunaliella* sp., *Scenedesmus* sp., and cyanobacteria such as *Synechococcus* sp., *Rhodomonas* sp., *Botryococcus braunii*, and *Synechocystis* sp. (Chu et al., 2019; Choi et al., 2015; Sharma et al., 2012), but it is still treated as soft option to enhance the biofuel production. Recent research findings on different microalgae suggested that higher bio-oil production is achieved under nutrient limiting conditions, especially nitrogen limitation, but it adversely influences the biomass production of algae, which ultimately affects the overall lipid productivity (Arumugam et al., 2013; Pruvost et al., 2011). The nutrient deprivation technique may not be a beneficial technology for induced biofuel production in industries (Chen et al., 2015; Zhang et al., 2013) as the nitrogen deficiency impairs cellular protein synthesis, leading to poor enzyme activity and reduced biomass (Rehman & Anal, 2019; Eroglu & Melis, 2016). Gour et al. (2018) reported that the stress-induced increase the productivity of lipids per unit of biomass is accompanied by a simultaneous reduction in the productivity of biomass.

There is possibility that we can use a nitrogen source which does not negatively influence the cell metabolism and physiology of microalgae and induces the lipid accumulation without compromising the biomass production (Olofsson et al., 2014; Griffiths & Harrison, 2009) have suggested that nitrogen limiting condition as such favours the diversion of surplus energy and carbon pool from amino acid synthesis pathway to lipid synthesis in the absence of protein synthesis. The main cause of excessive accumulation of carbohydrate and lipid in the nitrogen-starved microalgal cells is perhaps due to the diversion of photosynthetically fixed carbon from the protein synthesis pathway to lipid and carbohydrate synthesis (Hu et al., 2008). Therefore, efforts should be made to establish a suitable cultivation condition which favours both higher productivity of biomass and increased the accumulation of lipids in order to achieve an effective and low-cost biofuel production technology (Cai et al., 2013). The selection of microalgal strain is another important strategy for the commercial production of biofuel under nitrogen limiting conditions (Griffiths & Harrison, 2009). Generally, many microalgae have a preference for varying nitrogen sources, but ammonium is a generally preferred by the majority of the microalgae as it is highly reduced form of inorganic nitrogen and requires low energy to get itself converted into amino acid. Few microalgae prefer nitrate-nitrogen for their better growth such as *Botryococcus braunii* and *Dunaliella tertiolecta* (Ruangsomboon et al., 2015; Chen et al., 2011). It has been reported that *Chlorella* sp. favours nitrate-nitrogen dependent growth rather than ammonium nitrogen, but it can also utilise the organic nitrogen sources such as yeast extract, glycine, peptone, and glutamate (Muthuraj et al., 2014; Li et al., 2013).

Effects of varying nitrogen sources on microalgal growth and lipid production have been reported on microalgal species such as *Porphyridium purpureum*, *Scenedesmus dimorphous, Tetraselmis suecia,* and *Thalassiosira pseudonana* (Arumugam et al., 2013; Li et al., 2013; Griffiths et al., 2012). Although nitrate is predominant nitrogen source in the environment, whereas the ammonium-a reduced form of nitrogen is commonly accepted as the preferred nitrogen source (Chaffin & Bridgeman, 2014; Muro-Pastor et al., 2005). Nitrogen is typically present in the wastewater in various forms such as dissolved inorganic nitrogen, i.e. ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), and dissolved organic nitrogen, i.e. urea ($CO(NH_2)_2$) and amino acids (Kim et al., 2016; Phillips et al., 2004). The nitrogen assimilatory pathway in microalgae is subject to strict regulation by the carbon, nitrogen nutrition or both carbon/nitrogen (C:N) ratio

(Flores et al., 2005). Microalgal sp. can be both photo-autotrophic and mixotrophic, mainly depends on their metabolic strategy of carbon utilization and nitrogen assimilatory system (Brennan & Owende, 2010). The microalgal strains capable of mixotrophic growth have the ability to utilize both inorganic and organic carbon sources for growth medium and usually exhibit higher growth rates as compared to photoautotrophic microalgae (Chapman et al., 2015; Johnson & Alric, 2013). There is a report about the mixotrophic growth of microalgae increases the biomass and induces accumulation of starch and lipid in the microalgal cells during nitrogen limiting condition (Moon et al., 2013).

Assimilation of inorganic nitrate nitrogen by algae into amino acid and protein takes place through the GS-GOGAT pathway (Hellebust & Ahmad, 1989). The most reduced inorganic nitrogen ammonium is incorporated into amino acid by utilizing the photosynthetically derived carbon skeleton (Temple et al., 1998). However, it has been reported that amino acids can be directly utilized by the algal cells to meet their cellular nitrogen requirement (Hellebust & Ahmad, 1989; Pinchetti et al., 1998). Earlier it has been shown that presence of some amino acids like tryptone or casamino acid in the seawater in conjunction with other carbon sources can support the better algal growth (Lewin et al., 1970). However, they suggested that glutamate is a good replacement for tryptone and casamaino acid for algal growth (Lewin et al., 1975). The transamination of glutamate can temporarily meet the cellular requirement of other amino acids in photosynthesizing cells (Hipkin et al., 1980) and spare surplus carbon in the form of skeleton alpha-ketoglutarate and ATP, mainly required for glutamate synthase activity in the nitrogen assimilatory pathway (Lu et al., 2005; Temple et al., 1998), can be made available for enhanced production of lipid. The future strategy of commercial production of lipid and biofuel should be focused on engineering a physiological state in the algal cells, which mimics the metabolic effects of nitrogen deprivation, without compromising the biomass yield and lipids productivity (Esteves-Ferreira et al., 2018; Kim et al., 2016). Thus, there is a need to manipulate the microalgal culture condition for enhanced biofuel production which can be a great advantage to the microalgae-based biofuel industry.

In the present investigation, the effect of different nitrogen sources on the growth, protein, carbohydrate and lipid production was evaluated to optimize the condition for increased production of biomass and lipid content in the wild-type and mutant strain of *Scenedesmus vacuolatus*. Further efforts are made to elucidate the role

of nitrogen assimilatory system and mixotrophic growth on the regulation of carbohydrate and lipid accumulation.

6.2 Materials and Methods

6.2.1 Experimental setup

In the present study, initially grown a monoclonal culture of both wild-type (WT) and mutant strain for inoculation in the experiment, the BG-11 medium (Stanier et al., 1971) (without nitrogen source) (pH 7.4±0.2) in an Erlenmeyer flask (500 mL) at 25±2 ℃ for 07 days, as provided by a white fluorescent tube light (10 watts/m^2) with 16/8 h light/dark cycle. The culture flasks agitated regularly to prevent the cells from settling.

The separate experiment was performed by microalga *S. vacuolatus* cells of WT and mutant grown in BG-11 medium supplemented with varying concentrations of (inorganic nitrogen) sodium nitrate (2.0-25.0 mM), ammonium chloride (0.1-2.0 mM) and (organic nitrogen) sodium glutamate (0.1-1.5 mM) compared with control (nitrogen starved media).

The microalgal growth under mixotrophic condition was observed in the presence of a varying concentration of carbon source (sodium acetate, 2-20 mM) and a fixed concentration of sodium nitrate (10.0 mM) (Carbon/Nitrogen) (0:10-20:10) ratio. The control was set by sodium nitrate-nitrogen (10.0 mM) and nitrogen starved cells.

The varying concentration of nitrogen sources was used in response to microalgal growth. All experimental culture flask inoculated from the exponential phase of both WT and mutant cells of approx. (0.1 OD) in each Erlenmeyer flask (500 mL), 25±2 ℃ temperature, and 16/8 h light/dark cycle. The experimental was observed in triplicate under similar conditions for 0-20 days. The culture flask was shaken five times a day for avoiding sticking and settling of cells.

6.2.2 Measurement of growth, biochemical constituents and pigment content

The turbidity of microalgal cell suspension (WT and mutant) was monitored periodically at 680 nm for 0-20 days by using a double beam UV- visible spectrophotometer (Shimadzu, Japan 1601). The specific growth rate (μ) was calculated as the procedure describes in the Materials and Methods of chapter III.

Effect of different nitrogen sources on the cellular biochemical constituents like protein, carbohydrate, pigments and total organic carbon (TOC) in *S. vacuolatus* grown cells of wild-type (WT) and mutant in BG-11 medium containing a varying concentration of sodium nitrate (2-25 mM), ammonium chloride (0.1-2 mM), glutamate (0.1-1.5 mM) and C/N ratio (0:10-20:10) were measured with compared to control.

6.2.2.1 Estimation of protein

The protein was estimated by the method of Lowry et al. (1951) modified by Herbert et al. (1971) as described in chapter III of Materials and Methods.

6.2.2.2 Estimation of carbohydrate

The carbohydrate content was quantified by the phenol-sulphuric acid method of Dubois et al. (1956) as described earlier in chapter III of Material and Methods.

6.2.2.3 Estimation of total organic carbon (TOC)

Total organic carbon (TOC) of microalgal cells was analysed as described by Grobler & Davies, (1979), the detail is shown in chapter III of Materials and Methods.

6.2.2.4 Estimation of total chlorophyll and carotenoid

The microalgal cells were homogenised and broken by repeated freezing and thawing before the measurement of cell constituents. For estimation of total chlorophyll and carotenoids, the procedure described earlier Material and Methods in chapter III.

6.2.3 Nitrate reductase (NR) assay

The Nitrate reductase (NR) activity measured in exponential phase grown cells of WT and mutant either in the presence of nitrate (10.0 mM), C:N ratio (12:10 mM) and glutamate (1.0 mM) as a nitrogen source. The cells were harvested by centrifugation (5000 rpm) and were suspended in BG11 medium (pH 7.5±0.2) devoid of any nitrogen

source for 12 h before the measurement of NR activity. After that, the cells suspended in phosphate buffer (20 mM, pH 7.5). Subsequently, the cells were broken by repeated freezing and thawing. After that, the activity initiated by adding a varying concentration of sodium nitrate (0-16 mM). The aliquots of sample withdrawn at regular interval of 2 h for estimation of nitrite formation. The nitrite formation estimated by the colorimetric method (Snell & Snell, 1959) and rate of NR activity expressed as n mole nitrite formed/mg protein/min.

6.2.4 Flow cytometer analysis

The lipid content (neutral lipid) of WT and mutant cells analysed by high-speed flow cytometer (BD FACS Influx^{TM} Becton Dickinson, San Jose, CA, USA). The procedure describes earlier in chapter III of Materials and Methods.

6.2.5 Fourier transform infrared (FTIR) analysis

The study of biochemical constituents including lipid of microalgal biomass of both WT and mutant cells by using FTIR, as a procedure, describes in chapter III of Materials and Methods.

6.2.6 Statistical analysis

The performed statistical analysis was described in detail chapter III of Materials and Methods.

6.3 Results

6.3.1 Effect of varying nitrogen sources on the growth

The specific growth rate μ (day^{-1}) of *S. vacuolatus* was monitored in response to varying concentration of sodium nitrate (2-25 mM), ammonium chloride (0.1-2 mM), glutamate (0.1-1.5 mM) and C/N (acetate/nitrate) ratio (0:10-20:10) over a period of 0-20 days in both wild-type (WT) and mutant strain (Fig. 6.1 a, b, c). The microalgal cultures devoid of nitrogen and organic carbon were taken as the control set.

The result in Fig. 6.1(a) shows the effect of varying concentration of sodium nitrate (0-25 mM) on the specific growth rate (μ) of WT (wild-type) and mutant strain. The results revealed that specific growth rate of the WT (0.125±0.010 d^{-1}) was maximum at 10.0 mM concentration of nitrate, whereas growth maxima in the mutant

strain (0.151±0.009 d^{-1}) was recorded as 15.0 mM concentration of sodium nitrate as compared to nitrogen starved cells of WT (0.06±0.002 d^{-1}) and mutant strain (0.081±0.004 d^{-1}) respectively. A further increase in the nitrate concentration beyond the optimum doses shows a decline in the specific growth rate in a concentration-dependent manner in both strains. As the result shows, the overall growth of the mutant strain was relatively higher than that of the WT by approximately 45% at their respective growth saturating concentrations. Similarly, the specific growth rate of *S. vacuolatus* in the presence varying concentration of ammonium chloride (0-2.0 mM) showed that specific growth rate of WT and mutant strain increased with increasing concentration of ammonium and reached to their respective growth maxima (0.081±0.0074 and 0.107±0.007 d^{-1} respectively) at 0.5 mM concentration of ammonium chloride (Fig. 6.1, b). Further increase in the ammonium chloride concentration from (0.5-2.0 mM) exhibited a fast decline in the specific growth rate of both WT and mutant strain. Complete inhibition of in the specific growth rate was observed at 2.0 mM concentration of ammonium chloride in both the strains. The overall result shows that the ammonium chloride dependent growth in the mutant strain was better than the WT.

The specific growth rate of both WT and mutant strain was recorded in the presence of a varying concentration of sodium glutamate (0-1.5 mM). The result (Fig. 6.1, c) showed specific growth rate of both WT (0.110±0.012 d^{-1}) and mutant strain (0.143±0.011 d^{-1}) increases up to 1.0 mM concentration of sodium glutamate. However, a further increase in glutamate concentration from 1.5 to 2.0 mM showed a concentration-dependent declining pattern in the specific growth rate of both the strains. The glutamate supported the growth of mutant strain was approximately 27% higher than the WT at 1.0 mM concentration of sodium glutamate. These values were higher than the nitrogen-deprived respective control sets for the WT (0.065±0.004 d^{-1}) and mutant strain (0.081±0.005 d^{-1}).

In order to see the effect of C:N ratio on the specific growth rate of WT and mutant strain, varying concentration of acetate (2-20 mM) and a fixed concentration of sodium nitrate (10.0 mM) were selected. Different C:N (sodium acetate (0-20 mM) / sodium nitrate (10 mM) ratio were used to record the growth response of WT and

mutant strain (Fig. 6.1 d). The result revealed an maximum increase in the specific growth rate of WT (0.214±0.018 d^{-1}) at 12:10 C:N ratio, whereas the mutant strain revealed maximum specific growth rate (0.27±0.008 d^{-1}) at 16:10 C:N ratio, when compared to sodium nitrate (10.0 mM) of WT (0.12±0.005 d^{-1}) and mutant strain (0.15±0.008 d^{-1}) respectively. Further, the increase in C:N ratio beyond their respective optimum C:N ratio showed a declining pattern in the growth of both the strains. The overall results suggested that both WT and mutant strain were capable of mixotrophic growth at an appropriate concentration of carbon and nitrogen. Further, results suggested that mutant strain showed better utilisation of carbon during mixotrophic growth than the WT.

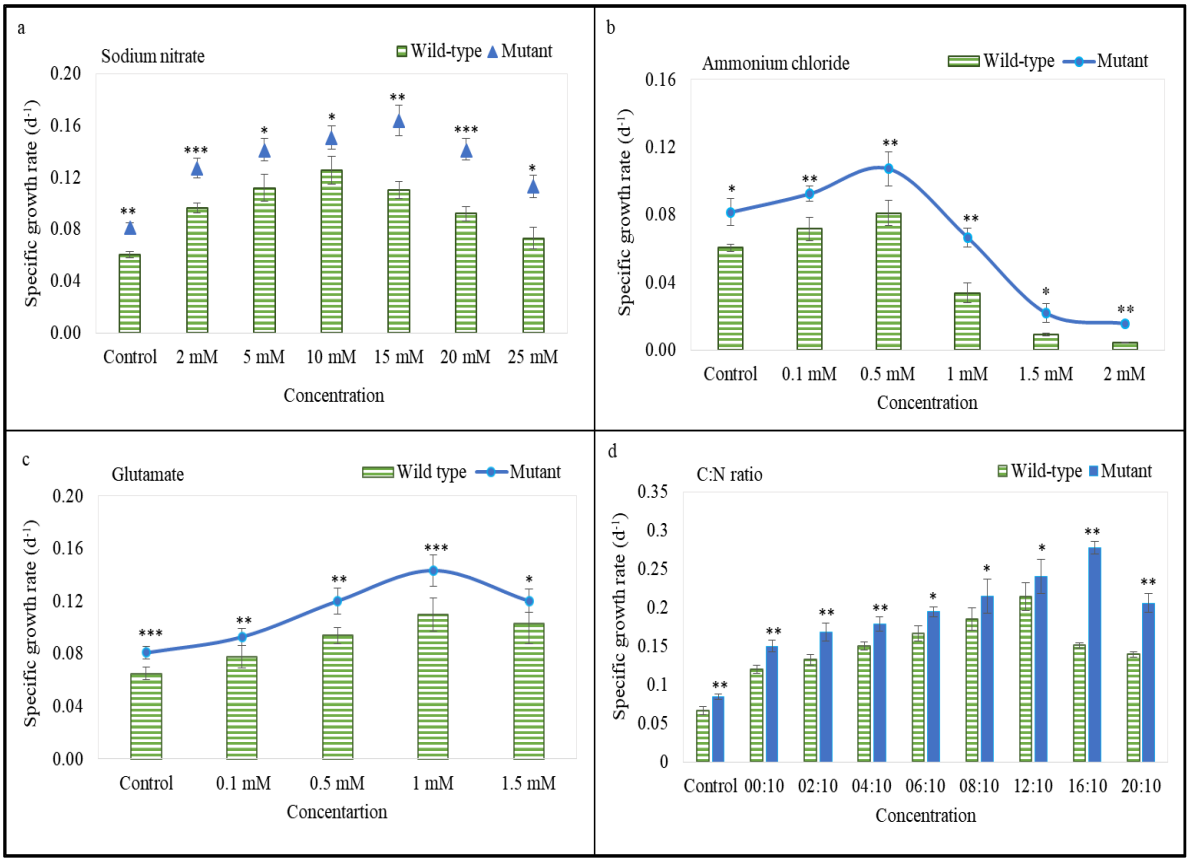

Fig. 6.1 Specific growth rate (μ) of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a varying concentration of (a) sodium nitrate (b) ammonium chloride (c) glutamate and (d) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

The performed statistical analysis paired-sample Student 't' test on the growth parameters showed a significant difference in the sodium nitrate, ammonium chloride, sodium glutamate and C:N ratio-dependent growth of WT and mutant strain when compared with their respective control sets (nitrogen depleted cells).

6.3.2 Effect of varying nitrogen sources on biochemical constituents

Biochemical constituents like protein, carbohydrates and total organic carbon (TOC) were measured on the microalgal cells of wild-type (WT) and mutant strain in the presence of varying concentrations of nitrate (2-25 mM), ammonium chloride (0.1-2 mM), glutamate (0.1-1.5 mM) and C:N ratio (2-20:0-10).

6.3.2.1 Effect of varying nitrogen sources on protein content

The results in Fig. 6.2 (a) depicts the effect of varying concentration of sodium nitrate (0-25 mM) on the protein content of WT and mutant strain. It was observed that the protein content of the WT (141±8.0 μg mL^{-1}) was maximum at 10.0 mM concentration of nitrate, whereas the maximum protein in the mutant strain (160±10.0 μg mL^{-1}) was recorded as 15.0 mM concentration of nitrate. These values were higher than the nitrogen-deprived respective control sets for the WT (42±4.0 μg mL^{-1}) and mutant strain (55±6.0 μg mL^{-1}). Further increase in the nitrate concentration beyond the optimum doses showed a concentration-dependent gradual decline in the protein content of both WT and mutant strain. As evident from the results on protein, mutant strain was higher protein content than the WT by approximately 11% at growth saturating concentration of nitrate. Further, results suggested that the mutant strain was able to assimilate higher concentration of nitrate than the WT. Similarly, the effect of varying concentration of ammonium chloride (0-2.0 mM) was recorded on the protein content in the WT and mutant strain Fig. 6.2 (b). The result showed that the protein content of WT (46±3.2 μg mL^{-1}) and mutant strain (66±5.3 μg mL^{-1}) increased with increasing concentration ammonium and reached to their respective maxima at 0.5 mM concentration of ammonium chloride when compared with their respective controls (devoid of nitrogen source) of WT (40.9±2.4 μg mL^{-1}) and mutant strain (56±3.7 μg mL^{-1}). Further increase in the ammonium chloride concentration from 0.5 to 2.0 mM exhibited a fast decline of the protein content of both the WT and mutant strain. Complete inhibition of protein content in both the strain was observed at 2.0 mM

concentration of ammonium chloride. The overall result showed that mutant strain was higher protein content than the WT in response to ammonium chloride.

The protein content of both WT and mutant strain was recorded in the presence of a varying concentration of sodium glutamate (0-1.5 mM). The result in Fig. 6.2 (c) found protein content of both WT (97±9.0 μg mL^{-1}) and mutant strain (120±6.0 μg mL^{-1}) increases up to 1.0 mM concentration of sodium glutamate when compared with nitrogen depleted respective control of WT (42±3.0 μg mL^{-1}) and mutant strain (54±4.0 μg mL^{-1}). However, an increase in the glutamate concentration beyond 1.0 mM showed a decrease in the protein content of both the strains. The glutamate supported the growth of mutant strain was found to be higher than the WT. Effect of different C:N ratio of (2-20 mM sodium acetate: 0-10 mM sodium nitrate) as shown in Fig. 6.2 (d) revealed an increase in the protein content of WT (206±13 μg mL^{-1}) at 12:10 C:N ratio, whereas the protein content in the mutant strain was maximum (250±12 μg mL^{-1}) at 16:10 C:N ratio, when compared with the nitrogen and carbon depleted control sets of both WT (44±4.0, 140±7.0 μg mL^{-1} respectively) and mutant strain (60±6.0, 161±10.0 μg mL^{-1} respectively). A further increase in the C:N ratio beyond their respective optimum C:N ratios showed a declining pattern in the protein content of both the strains. The overall results indicated higher protein content in the mutant strain than the WT, but both WT and mutant strains were capable of mixotrophic growth.

The performed statistical analysis paired-sample Student 't' test showed a significant difference ($p<0.05$) in the protein content of both WT and mutant strain as well as between the effects of varying nitrogen sources (sodium nitrate, ammonium chloride, sodium glutamate and C:N effect) as compared to control (N starved).

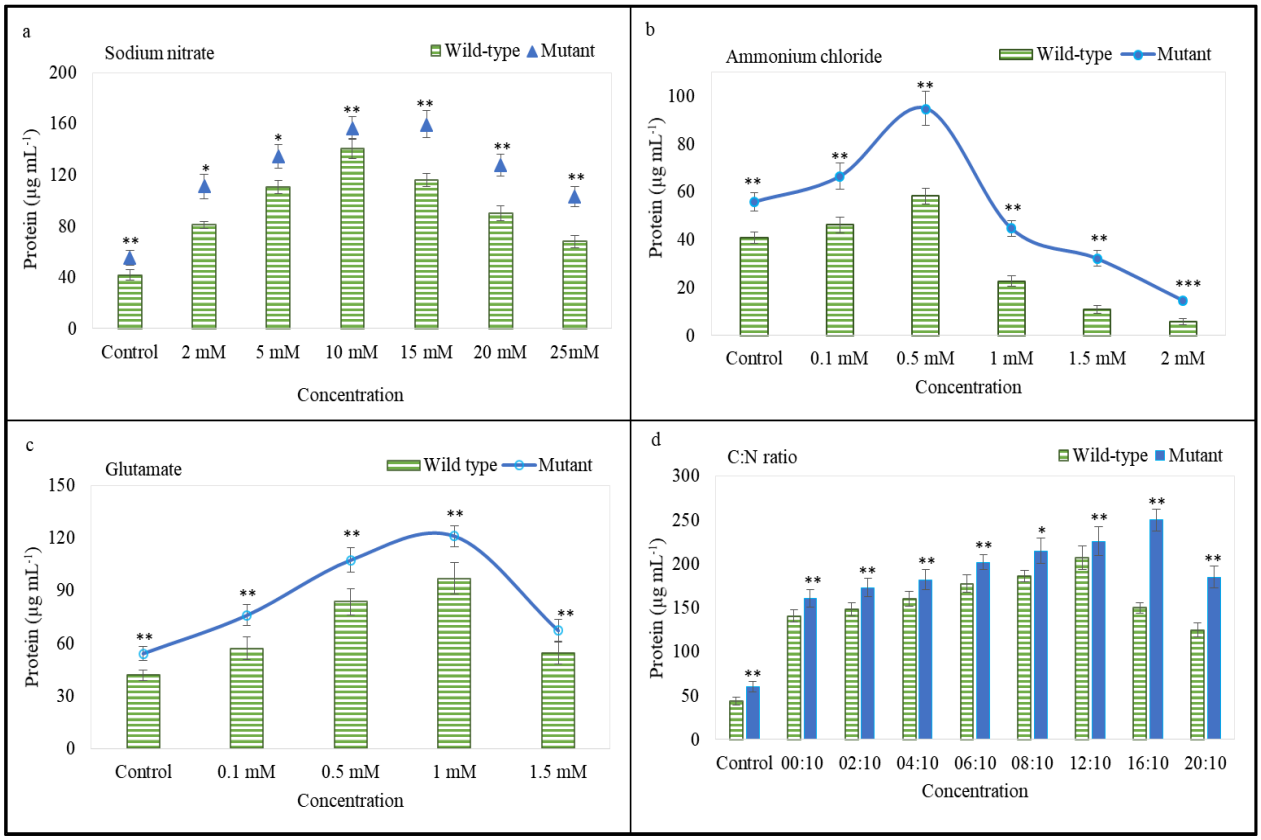

Fig. 6.2 Protein content of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a varying concentration of (a) sodium nitrate (b) ammonium chloride (c) glutamate and (d) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

6.3.2.2 Effect of varying nitrogen sources on the carbohydrate content

The result on carbohydrate content in both the WT and mutant strain in response to varying concentration of sodium nitrate (0-25 mM), as shown in Fig. 6.3 (a) revealed that level of carbohydrate in the WT (141±8.54 µg mL^{-1}) was maximum 10.0 mM concentration of nitrate, whereas maximum carbohydrate in the mutant strain (167±7.5 µg mL^{-1}) was observed at 15.0 mM concentration of nitrate, when compared with the control (respective nitrogen deprived cells) of both WT (49±3.5 µg mL^{-1}) and mutant strain (76±6.0 µg mL^{-1}). Further increase in nitrate concentration beyond the optimum doses showed a concentration-dependent gradual decline in the carbohydrate content of both the WT and mutant strain. Further, the results suggested that the mutant strain was able to assimilate higher concentration of nitrate than the WT.

Similarly, the effect of varying concentration of ammonium chloride (0-2.0 mM) was recorded on the carbohydrate content of WT and mutant strain Fig. 6.3 (b). The result showed that carbohydrate content in the WT (72±3.6 μg mL^{-1}) and mutant strain (108±9.3 μg mL^{-1}) increased in response to increasing ammonium concentration and reached to their respective maxima at 0.5 mM concentration of ammonium chloride. A further increase in the ammonium chloride concentration from 0.5 mM to 2.0 mM exhibited a fast decline in the carbohydrate content of both WT and mutant strain. The overall result showed that carbohydrate content in the mutant strain was better than the WT in response to ammonium chloride. These values were higher than the nitrogen-deprived respective control sets for the WT (53±2.5 μg mL^{-1}) and mutant strain (76±4.4 μg mL^{-1}).

The carbohydrate content in both the WT and mutant strain in the presence of a varying concentration of sodium glutamate (0-1.5 mM). The results on the effect of sodium glutamate as in Fig. 6.3 (c) showed a concentration-dependent increase in the carbohydrate content of both WT (127±9.6 μg mL^{-1}) and mutant strain (145±6.5 μg mL^{-1}) up to 1.0 mM concentration of sodium glutamate when compared with their respective controls (N-deprived cells) of WT (48±2.2 μg mL^{-1}) and mutant strain (65±5.0 μg mL^{-1}). However, a further increase in the glutamate concentration beyond 1.0 mM concentration showed a relative decline in the carbohydrate of both the strains. Effect of varying C:N ratio, using a varying concentration of acetate (2-20 mM) and a fixed concentration of sodium nitrate (10.0 mM), on the carbohydrate content in both WT and mutant strain, as shown in Fig. 6.3 (d), revealed an increase in the carbohydrate content of WT (220±12 μg mL^{-1}) at 12:10 C:N ratio, whereas mutant strain revealed the highest carbohydrate level (260±10 μg mL^{-1}) at 16:10 C:N ratio as compared to the carbohydrate content of nitrate of WT (145±6.0 μg mL^{-1}) and mutant strain (165±12.0 μg mL^{-1}). A further increase in the C:N ratio beyond their respective optimal C/N ratios showed a declining pattern in the carbohydrate content. The overall result suggested that maximum carbohydrate content in the mutant strain than the wild type in response to increasing doses of organic carbon. However, both WT and mutant strain were capable of mixotrophic growth at an appropriate concentration of carbon and nitrogen.

The carbohydrate content in both the WT and the mutant strain in response to the optimum concentration of varying nitrogen sources showed greater accumulation of carbohydrate in the presence of C/N nutrition (mixotrophic growth). The lowest level of carbohydrate in both WT and mutant strain was found in the presence of ammonium chloride. The statistical analysis paired-sample Student 't' test showed a significant difference ($p<0.05$) in the carbohydrate content of sodium nitrate, ammonium chloride, sodium glutamate and C:N grown microalgal cells of both WT and mutant strain when compared with the control.

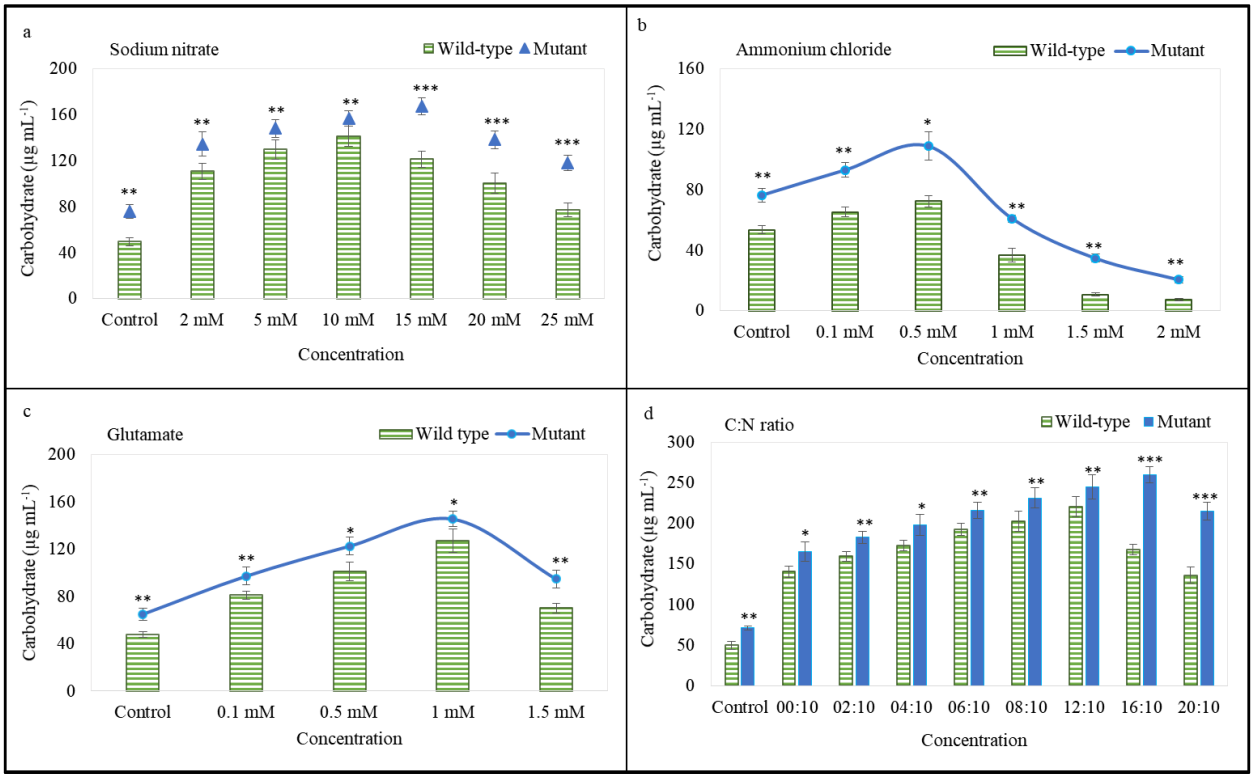

Fig. 6.3 Carbohydrate content of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of varying concentration of (a) sodium nitrate (b) ammonium chloride (c) glutamate and (d) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

6.3.2.3 Effect of varying nitrogen sources on pigment content

Effect of varying concentration of sodium nitrate (0-25 mM) was recorded on total chlorophyll and carotenoid content of both the WT and mutant strain of S. vacuolatus, as given in Fig. 6.4, 6.5 (a, b, c). The results showed that total chlorophyll and carotenoid content in the WT (0.83±0.041, 0.76±0.06, µg mL^{-1} respectively) was

maximum 10.0 mM concentration of nitrate, whereas maximum total chlorophyll and carotenoid content in the mutant strain (1.1±0.07, 0.68±0.66 μg mL^{-1} respectively) was recorded as 15.0 mM concentration of nitrate. These values were higher than the nitrogen-deprived respective control sets for the WT (0.28±0.02 μg mL^{-1}) and mutant strain (0.47±0.025). Although maximum carotenoid content (0.90±0.03 μg mL^{-1}) was recorded at 10.0 mM concentration of nitrate in the mutant strain Fig. 6.4, 6.5 (a). A further increase in the nitrate concentration beyond the optimum doses showed a concentration-dependent gradual decline in the total chlorophyll and carotenoid content of both WT and mutant strain.

Similarly, the result of total chlorophyll and carotenoid content in both the WT and mutant strain in response to varying concentration of ammonium chloride (0-2.0 mM), as depicted in Fig. 6.4 (b), 6.5 (b) showed that total chlorophyll of WT (0.23±0.025 μg mL^{-1}) and mutant strain (0.72±0.04 μg mL^{-1}) increased with increasing concentration of ammonium chloride and reached to their respective maxima at 0.5 mM concentration of ammonium chloride. These values were higher than the N-deprived respective control of both WT (0.31±0.02 μg mL^{-1}) and mutants strain (0.44±0.02 μg mL^{-1}). On the other hand, carotenoid contents in the WT (0.60±0.032 μg mL^{-1}) and mutant strain (0.76±0.03 μg mL^{-1}) were found to be maximum at 0.1 mM concentration of ammonium chloride as compared to their respective N-deprived control sets of WT (0.53±0.02 μg mL^{-1}) and mutant strain (0.68±0.037 μg mL^{-1}) (Fig. 6.5, b) However, a further increase in the ammonium chloride concentration from (0.1-2.0 mM) exhibited fast decline in the total carotenoid content of both the WT and mutant strain. The overall result shows that both total chlorophyll and carotenoid content of the mutant strain was better than the WT in response to ammonium chloride.

The total chlorophyll and carotenoid content of both WT and mutant strain were measured in response to varying concentration of sodium glutamate (0-1.5 mM). The result, as in Fig. 6.4 (c) and Fig. 6.5 (c) showed a concentration-dependent increase in the total chlorophyll and carotenoid content of both WT (0.78±0.07, 1.0±0.09 μg mL^{-1} respectively) and mutant strain (0.90±0.05, 1.2±0.11 μg mL^{-1} respectively) up to 1.0 mM concentration of sodium glutamate, when compared with their respective N-deprived controls of WT (0.26±0.03, 0.5±0.02 μg mL^{-1} respectively) and mutant strain (0.43±0.035, 0.71±0.04 μg mL^{-1} respectively). However, the overall result suggested

that total chlorophyll and carotenoid content in the mutant strain was higher than the WT in response to sodium glutamate.

Effect of C:N ratio on the total chlorophyll and carotenoid content of WT and mutant strain in the presence of a varying concentration of sodium acetate (2-20 mM) and a fixed concentration of sodium nitrate (10.0 mM) was observed. The results on different C:N ratio of (2-20:0-10 mM) showed that total chlorophyll and carotenoid content, as in Fig. 6.4 (d) and Fig. 6.5 (d), revealed maximum total chlorophyll and carotenoid content in the WT (5.2±0.5, 3.9±0.3 µg mL^{-1} respectively) at 12:10 C:N ratio, whereas the mutant strain revealed highest total chlorophyll and carotenoid at (6.8±0.70, 4.3±0.37 µg mL^{-1} respectively) at 16:10 C:N ratio, when compared with their respective nitrate control set of WT (0.84±0.035, 1.2±0.049 µg mL^{-1} respectively) and mutant strain (0.41±0.03, 0.71±0.038 respectively). Further, the increase in C:N ratio in both the WT and mutant strain showed a declining pattern with an increasing concentration of carbon. The results again indicated that both the WT and mutant strains of *S. vacuolatus* were capable of mixotrophic growth up to the appropriate C:N ratio.

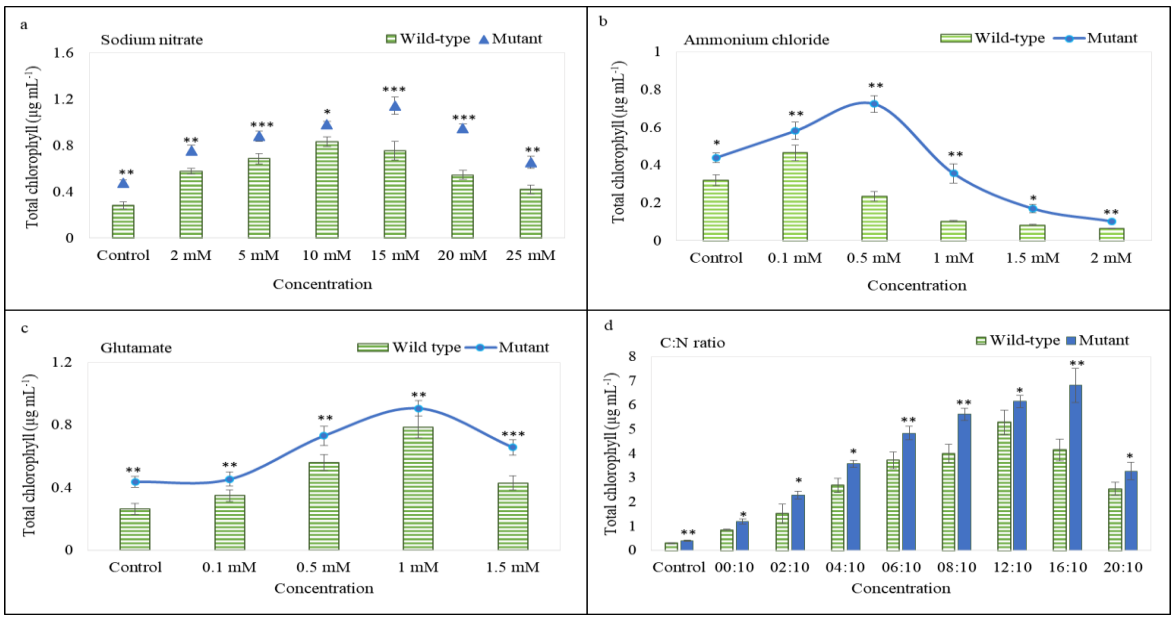

Fig. 6.4 Total chlorophyll of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a varying concentration of (a) sodium nitrate (b) ammonium chloride (c) glutamate and (d) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

The performed statistical analysis paired-sample Student 't' test at ($p<0.05$) level showed significant variation in the total chlorophyll and carotenoids content in both the strains grown in the presence of sodium nitrate, ammonium chloride, sodium glutamate and C:N ratio when compared with respective N-deprived control sets.

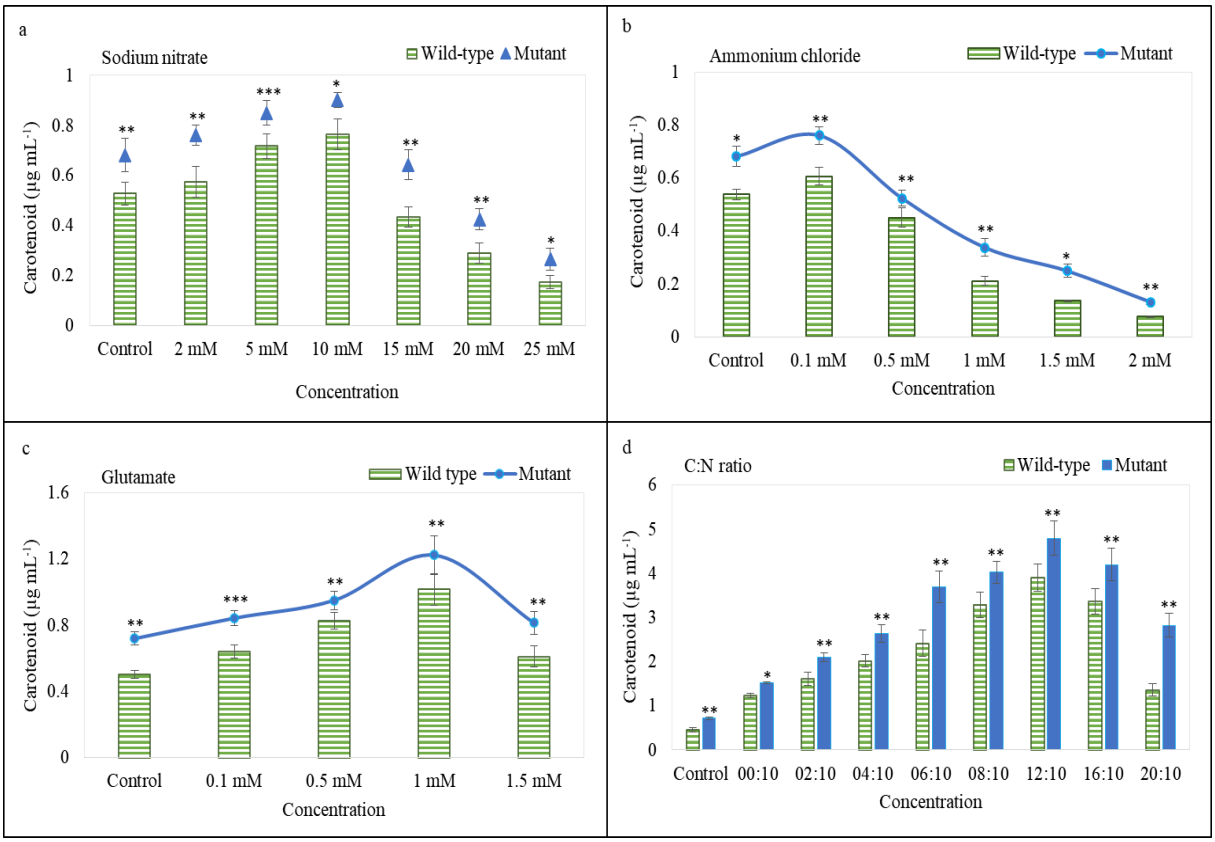

Fig. 6.5 Carotenoid content of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a varying concentration of (a) sodium nitrate (b) ammonium chloride (c) glutamate and (d) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

6.3.2.4 Effect of varying nitrogen sources on total organic carbon (TOC)

Effect of varying concentration of sodium nitrate (0-25 mM) on total organic carbon (TOC) in both WT and mutant strain is depicted in Fig. 6.6 (a). The results showed that TOC content of the WT (9.3±0.5 mg L^{-1}) was maximum at 10.0 mM concentration of nitrate, whereas the highest TOC in the mutant strain (12.6±1.2 mg L^{-1}) was recorded as 15.0 mM concentration of nitrate, when compared with the respective N-deprived control sets (3.1±0.32, 4.1±0.42 mg L^{-1} , respectively) of both WT and mutant strain.

A further increase in the nitrate concentration resulted into concentration-dependent decline in the TOC level of both the WT and mutant cells. These results suggested that the mutant strain was able to accumulate a greater amount of organic carbon than the WT in the presence of nitrate. Similarly, TOC content of both WT and mutant strain was recorded in the presence of a varying concentration of sodium glutamate (0-1.5 mM). The results in Fig. 6.6 (b) showed sodium glutamate concentration Dependent increase in the TOC content of both WT (10±1.5 mg L^{-1}) and mutant strain (12±0.6 mg L^{-1}) upto 1.0 mM concentration of sodium glutamate when compared with their respective nitrogen deprived control (3.0±0.4, 4.6±0.65 mg L^{-1} , respectively) of both WT and mutant strain. However, the mutant strain accumulated a higher amount of TOC than the WT in response to glutamate nitrogen.

The effect of C:N ratio on the TOC content of WT and mutant strain was studied in the presence of a varying concentration of sodium acetate (2-20 mM) and fixed concentration (10 mM) of sodium nitrate Fig. 6.6 (c). The result revealed C:N ratio-dependent increase in the TOC content of both the strains. The highest TOC content in the WT (17±1.0 mg L^{-1}) was recorded at 12:10 C:N ratio, whereas the mutant strain revealed the highest value of TOC (22±1.2 mg L^{-1}) at 16:10 C:N ratio when compared with respective control of WT (8.7±0.8 mg L^{-1}) and mutant strain (11±0.7 mg L^{-1}). However, a further rise in the C: N ratio showed a declining pattern in TOC value of both the strains. The overall result indicated that the mutant strain was better equipped for mixotrophic growth than the WT.

The statistical analysis paired-sample Student ‘t’ test at ($p<0.05$) level showed significant variation in the TOC content of both the WT and mutant strain is grown under varying nitrogen sources such as sodium nitrate, sodium glutamate, and C:N ratio when compared with the respective N-deprived control sets.

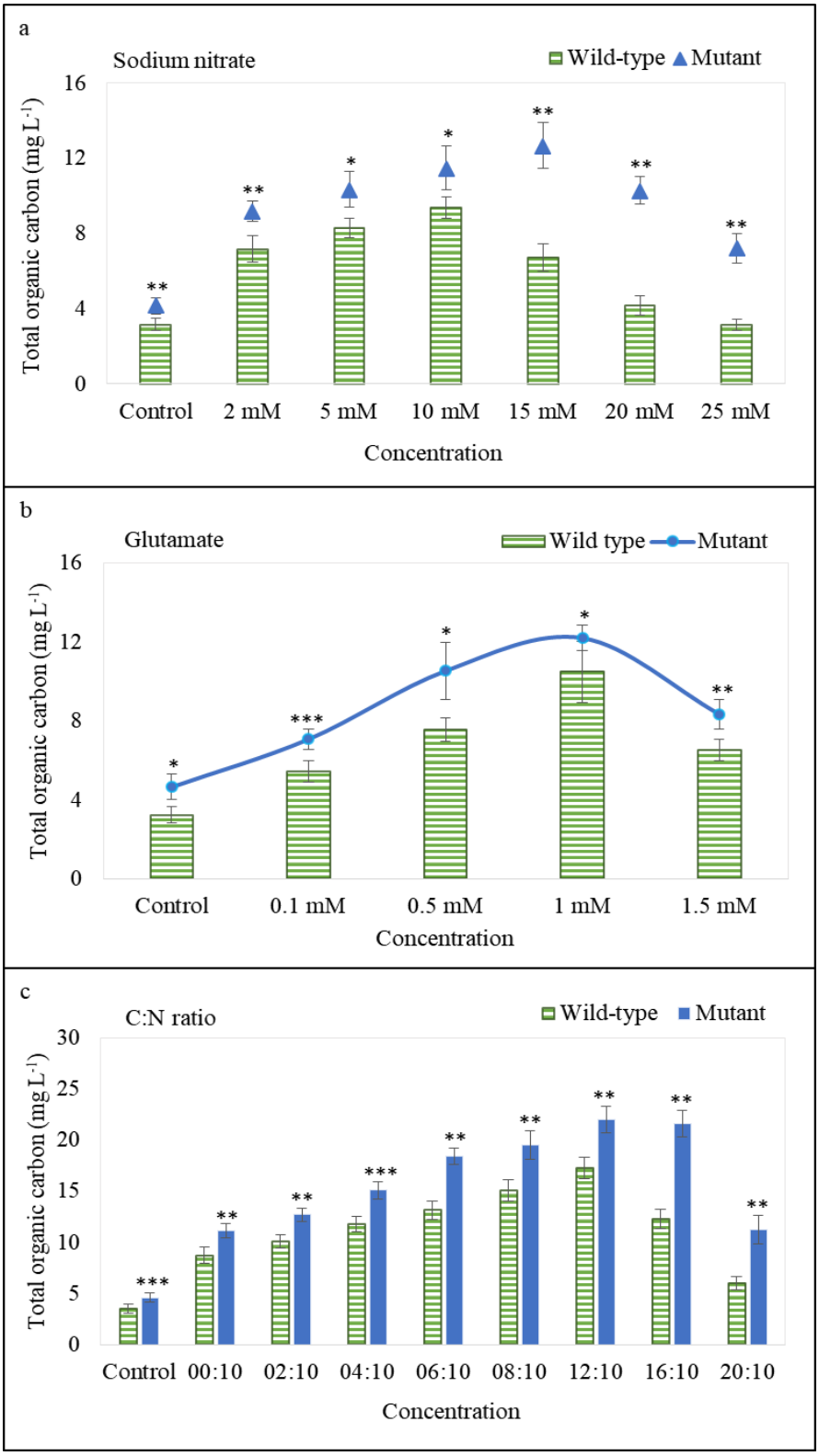

Fig. 6.6 Total organic carbon (TOC) of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a varying concentration of (a) sodium nitrate (b) glutamate and (c) C:N ratio. Student't' test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

6.3.3 Flow cytometer analysis

In flow cytometer analysis (mainly neutral lipid) of selected population events (10,000 cells) of WT and mutant strain of *S. vacuolatus*, the presence of lipid was recorded using the Nile red dye fluorescence and the results are expressed in terms of percentage of lipid. The flow cytogram showed the fluorescence intensity of Nile red dye stained cells of WT and mutant strain grown in the presence of varying concentration of nitrogen sources such as sodium nitrate (2-20 mM), ammonium chloride (0.1-2 mM), sodium glutamate (0.1-1.5 mM) and C:N (2-20:0-10) ratio. The WT and mutant cells of *S. vacuolatus* grown in the absence of exogenous nitrogen source were treated as control.

The results of flow cytometer (neutral lipid) on the effect of varying concentration (concentration) of sodium nitrate (2-25 mM) nitrogen as shown in Fig. 6.7 (a, b), revealed the highest lipid content in the WT (15.05%) cells grown at 5.0 mM concentration of nitrate, whereas the mutant strain exhibited the highest lipid content (20.30%) at 10 mM concentration of nitrate (Fig. 6.7, b). Beyond their respective optimum doses of nitrate nitrogen, the lipid content declined in both the strains. However, the WT and mutant cells grown in the absence of exogenous nitrogen source showed higher lipid content in both the WT and mutant strain (26.05% & 30.10%, respectively), when compared with their corresponding nitrogen supplemented cells.

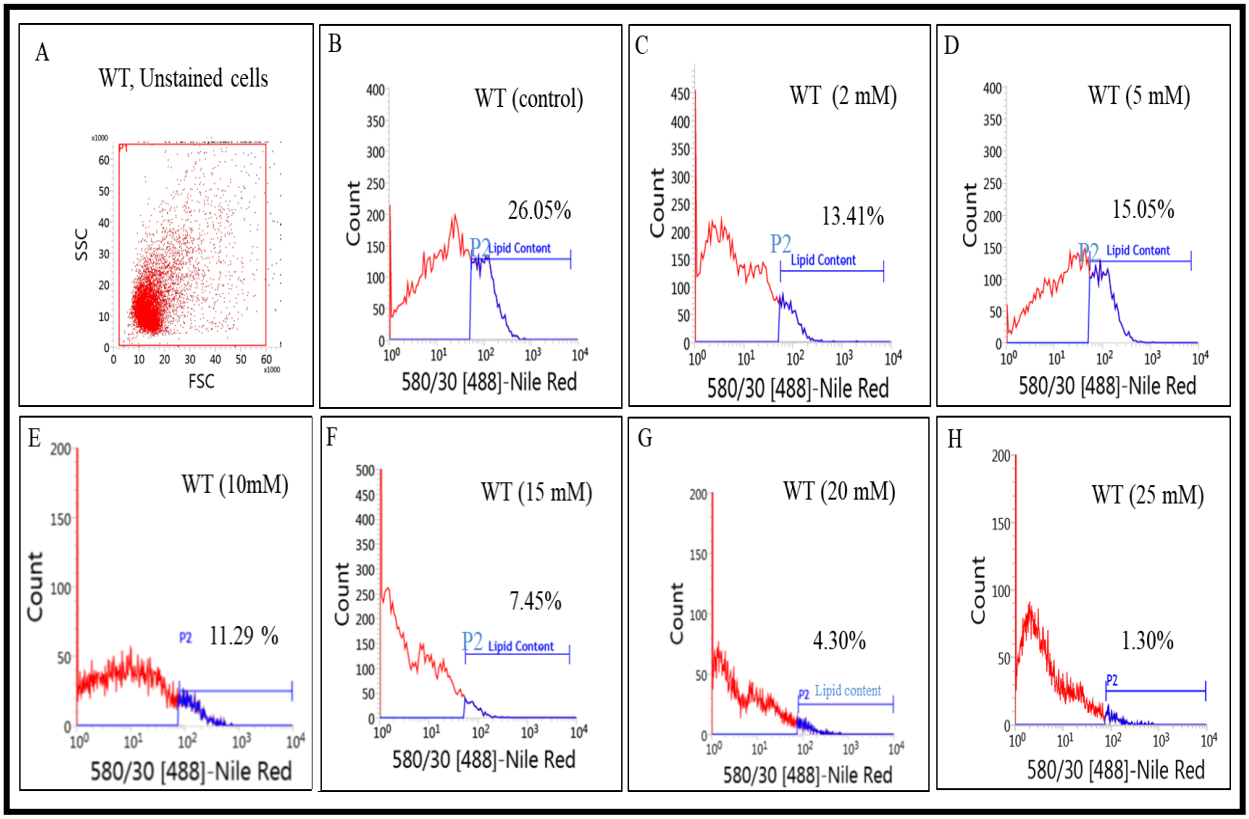

Fig. 6.7 (a) Flow cytometer analysis of neutral lipid content of wild-type cells of *S. vacuolatus* in the presence of a varying concentration of sodium nitrate (2-25 mM).

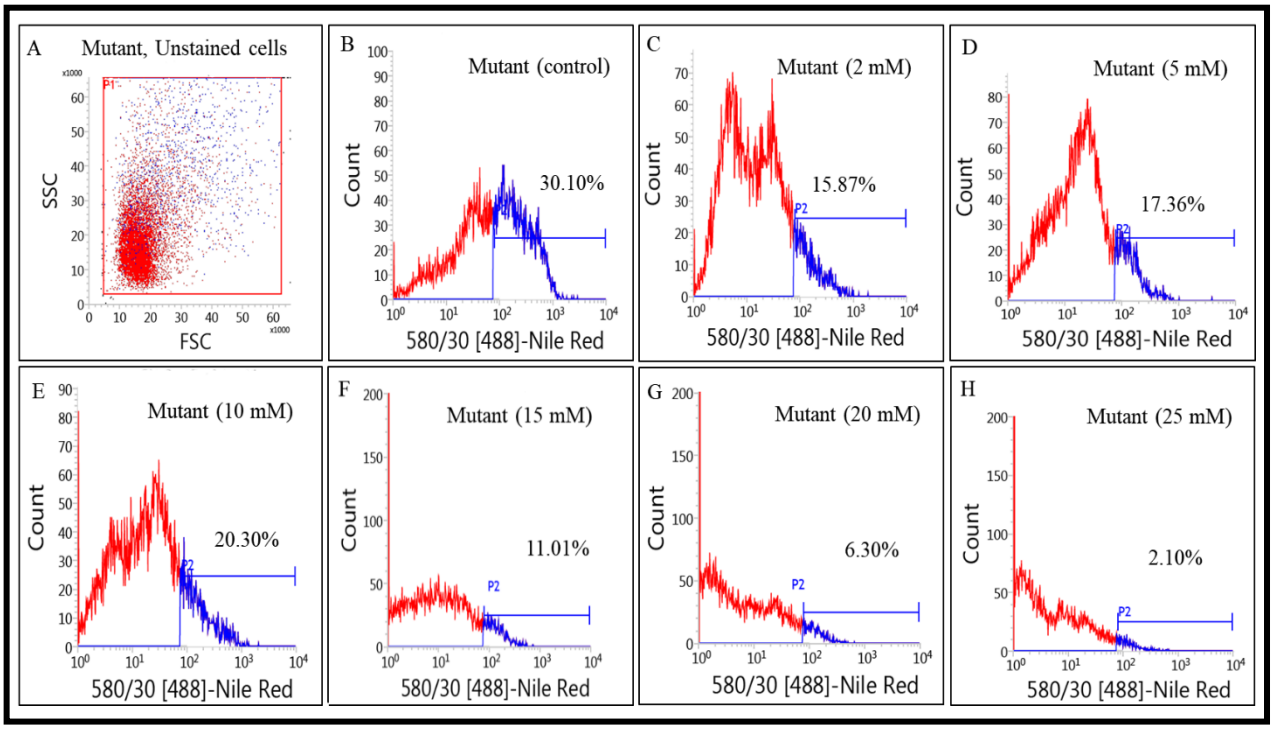

Fig. 6.7 (b) Flow cytometer analysis of neutral lipid content of mutant strain of *S. vacuolatus* in the presence of a varying concentration of sodium nitrate (2-25 mM).

Similarly, lipid content in both the WT and mutant strain was recorded in response to varying concentration of ammonium chloride (0-2.0 mM). The flow cytometer analysis of WT and mutant strain (Fig. 6.8 a, b) showed the highest lipid content in both the WT (8.78%) and mutant (10.10%) cells grown at 0.1 mM concentration of ammonium chloride. A higher concentration of ammonium chloride (0.5-2.0 mM) exhibited a fast decline in the lipid content of both the WT and mutant cells. Overall results showed higher lipid content in the mutant strain than the WT in response to ammonium chloride concentration.

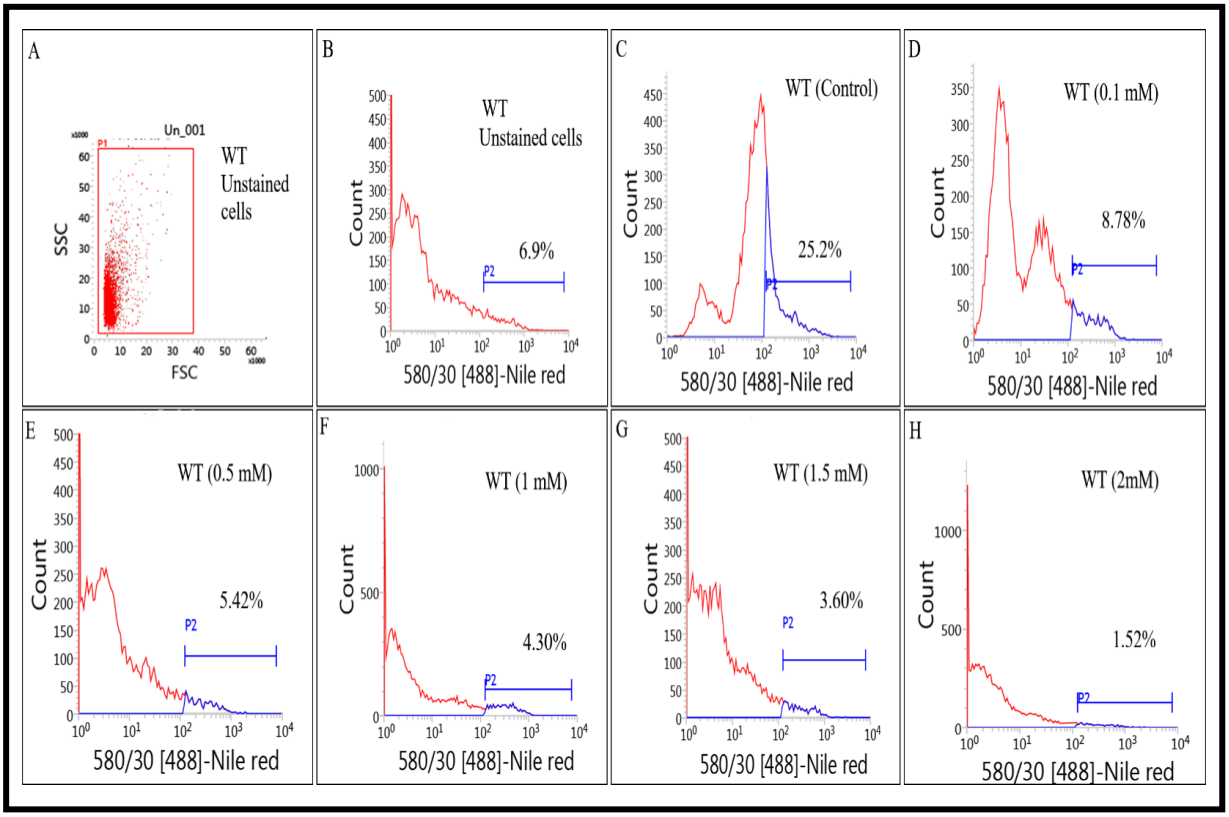

Fig. 6.8 (a) Flow cytometer analysis of neutral lipid content of wild-type cells of *S. vacuolatus* in the presence of a varying concentration of ammonium chloride (0.1-2 mM).

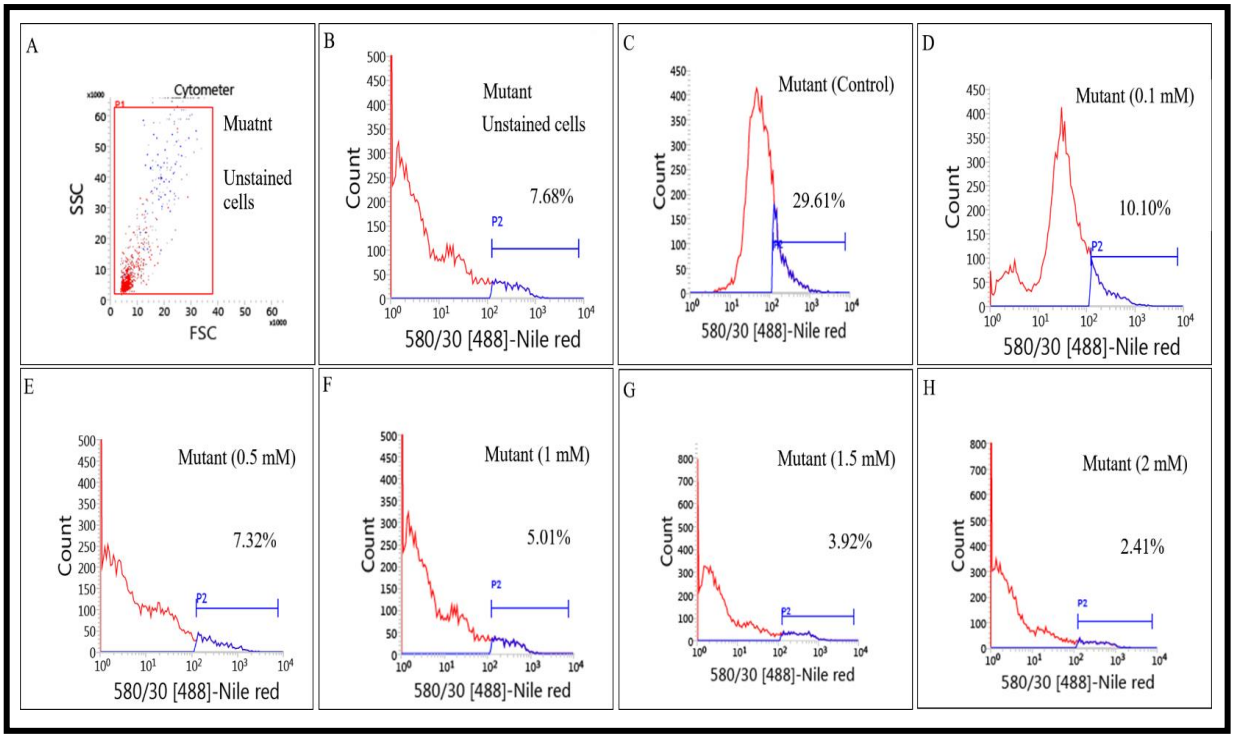

Fig. 6.8 (b) Flow cytometer analysis of neutral lipid content of mutant strain of *S. vacuolatus* in the presence of a varying concentration of ammonium chloride (0.1-2 mM).

The lipid content in both the WT and mutant cells were grown in the presence of a varying concentration of sodium glutamate (0-1.5 mM) was measured using the Nile red fluorescence. The results showed lipid content of both WT (42.01%) and mutant strain (46.0%) increases up to 1.0 mM concentration of sodium glutamate (Fig. 6.9 a, b) when compared with their respective N-deprived control sets (26.05%, 30.61% respectively) of both WT and mutant strain. However, a further increase in the glutamate concentration beyond 1.0 mM concentration of glutamate resulted in a relative decline in the lipid content of both the strains. The overall results indicated higher lipid content in the mutant strain than the WT in response to glutamate concentration.

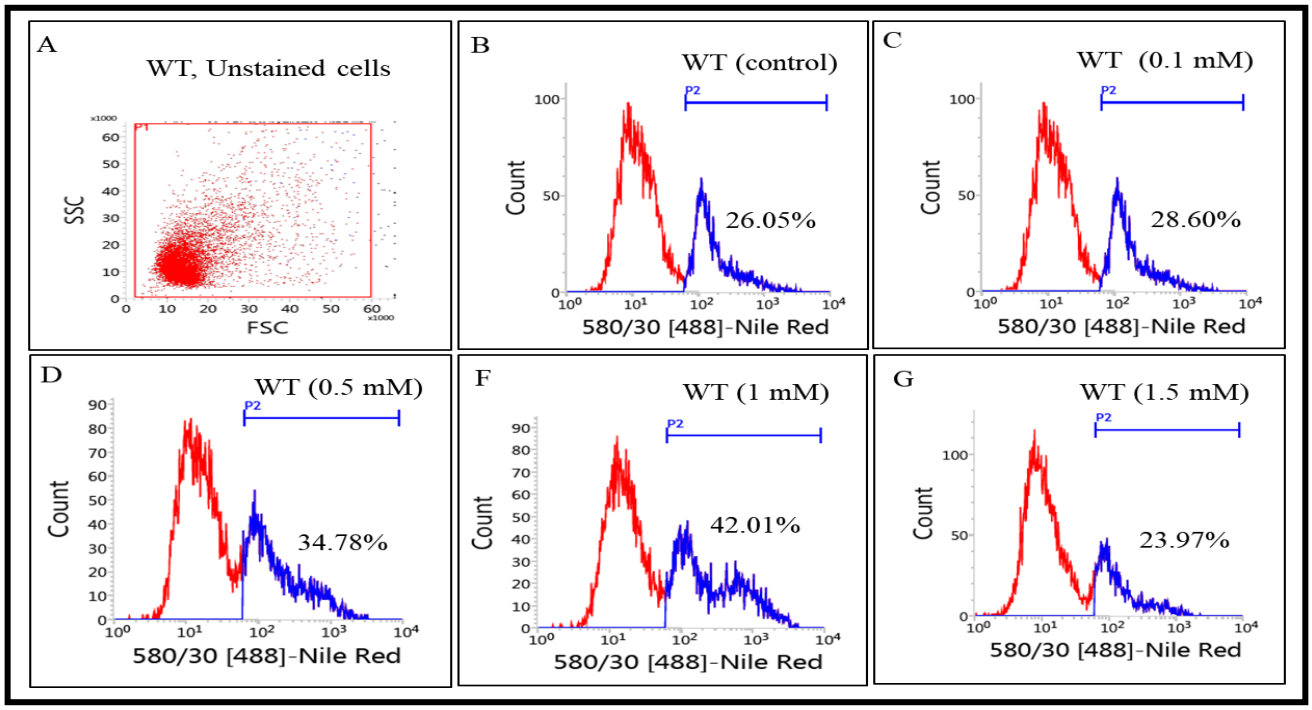

Fig. 6.9 (a) Flow cytometer analysis of neutral lipid content of wild-type cells of *S. vacuolatus* in the presence of a varying concentration of sodium glutamate (0.1-1.5 mM).

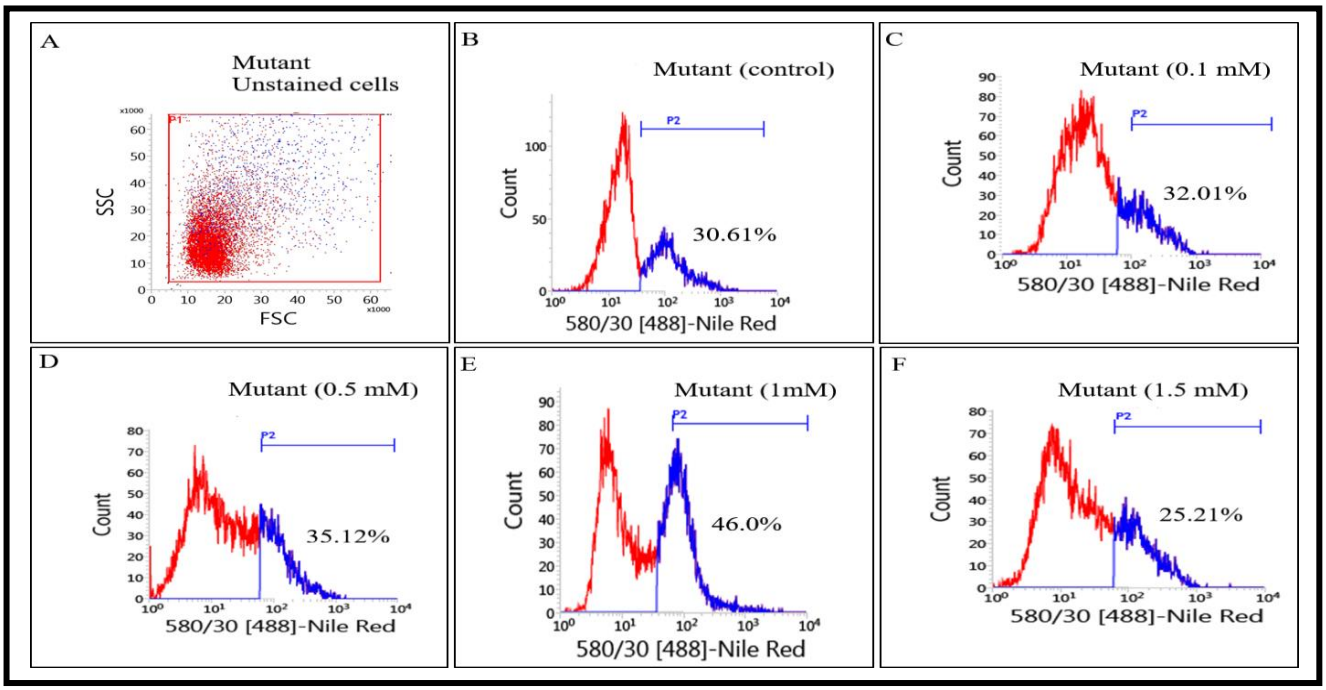

Fig. 6.9 (b) Flow cytometer analysis of neutral lipid content of wild-type cells of *S. vacuolatus* in the presence of a varying concentration of sodium glutamate (0.1-1.5 mM).

The effect of different C:N (sodium acetate, 2-20 mM and nitrate, 10 mM) ratio on the lipid content of both the WT and mutant strain (Fig. 6.10 a, b) revealed a higher lipid content in the WT (14.06%) and mutant strain (26.41%) at 4:10 C:N ratio. A further increase in the C:N ratio depicted a declining pattern in the lipid content with increasing C:N ratio. Whereas lipid content in the WT and mutant cells supplemented with 10 mM nitrate (without carbon) was 11.29% and 19.61%. Taken together results suggested that organic carbon supplemented mixotrophic growth could support the enhance lipid synthesis. Further, the mutant strain exhibited better utilization of organic carbon as compared to WT cells.

The data related to flow cytometry analysis of lipid content revealed maximum lipid content in both the strains of *S. vacuolatus* grown in the presence of glutamate nitrogen as compared to respective nitrate nitrogen, ammonium chloride and C:N ratio grown cells. These results indicated that it is not the carbon nutrient alone which determines the lipid accumulation in the microalgae. There might be an interactive role of both carbon and nitrogen nutrition regulating the carbon partitioning in the microalgal cells in favour of enhanced lipid synthesis.

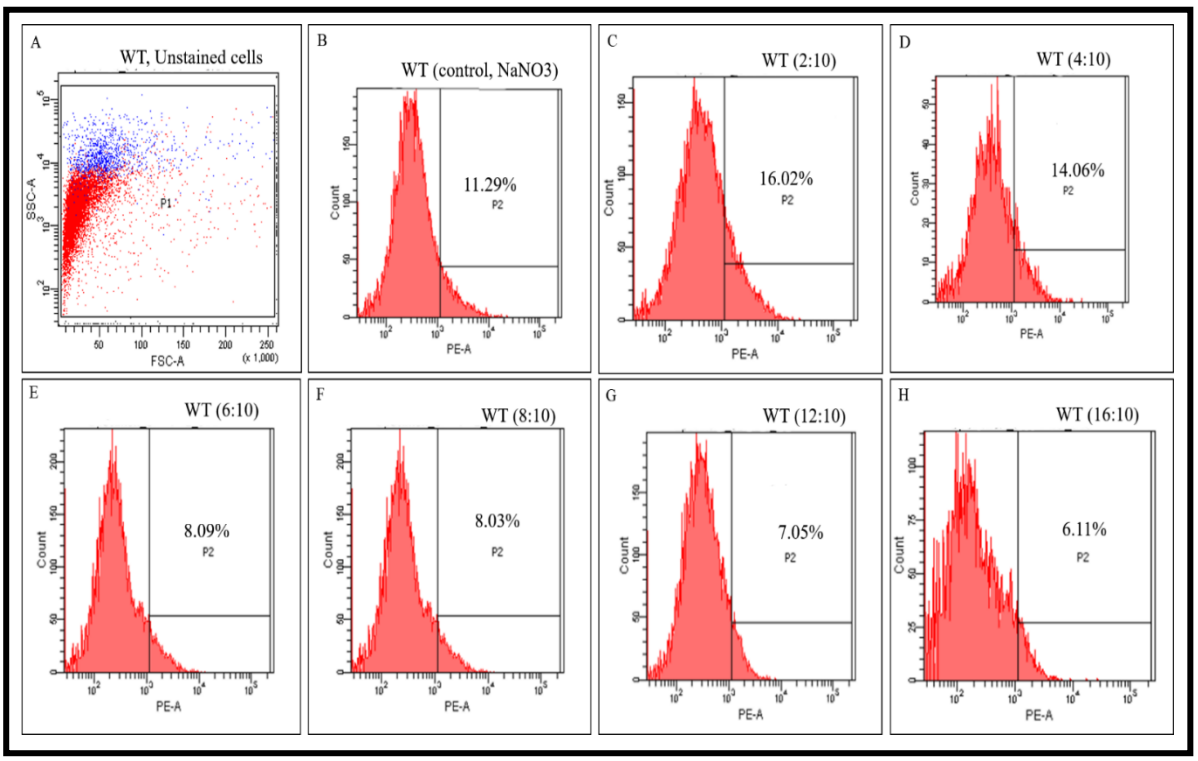

Fig. 6.10 (a) Flow cytometer analysis of neutral lipid content of wild-type cells of *S. vacuolatus* in the presence of a varying concentration of C:N ratio (2:10-16:10 mM).

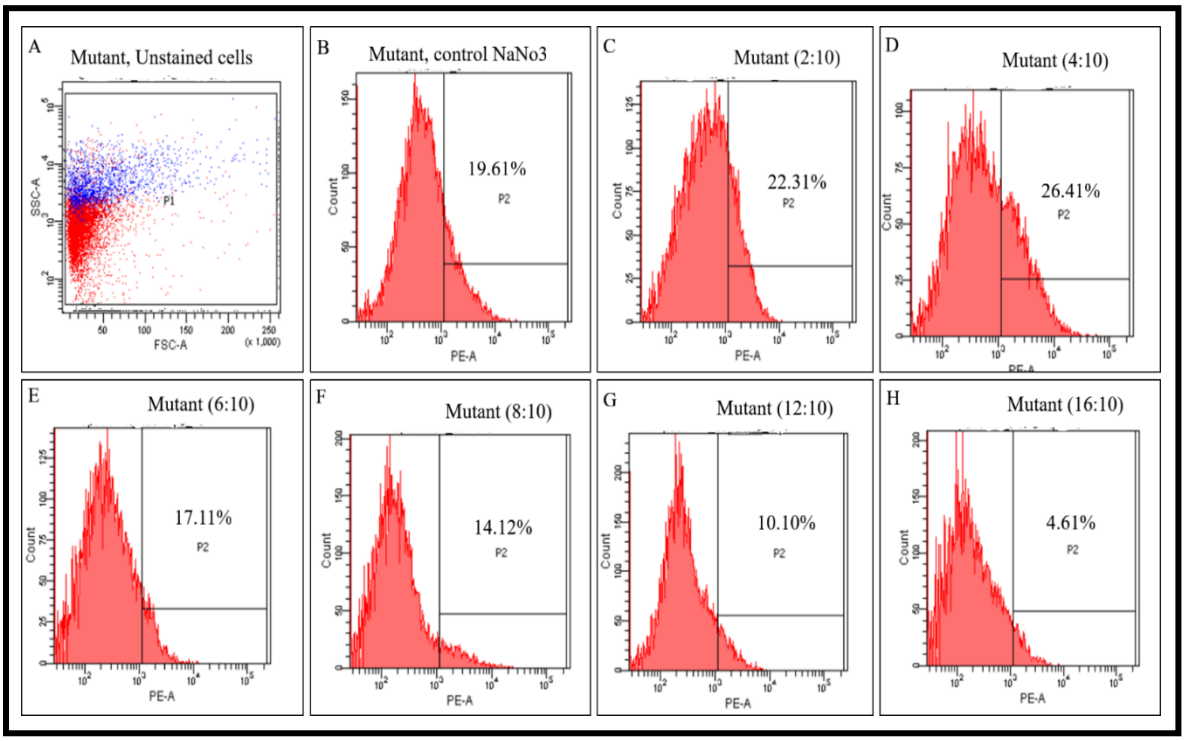

Fig. 6.10 (b) Flow cytometer analysis of neutral lipid content of mutant strain of *S. vacuolatus* in the presence of a varying concentration of C:N ratio (2:10-16:10 mM).

6.3.4 Nitrate reductase assay of the *S. vacuolatus* cells grown under varying nitrogen nutrition

Nitrate reductase (NR) activity in *S. vacuolatus* was taken as a metabolic indicator of nitrogen assimilatory function in the microalgal cells. The microalgal cells, grown separately in the presence of a fixed concentration of nitrate (10.0 mM) or glutamate (1.0 mM) and C:N ratio (12:10 mM) were assayed for NR activity (Fig. 6.11 a, b, c). The results on NR activity showed the maximum rate of enzyme activity in the mixotrophically grown (C+N) cells of WT (2.3±0.34 µM/mg protein/min.) and mutant strain (4.6±0.35 µM/mg protein/min) with rate saturation of NR activity at 16.0 mM concentration of nitrate. The NR activity in the nitrate grown both the WT and mutant strain (1.32±0.105, 2.2±0.21 µM/mg protein/min, respectively) also exhibited the highest enzyme activity at 16.0 mM concentration of nitrate (Fig. 6.11 a, c). However, the rate of NR enzyme activity was higher in the mutant strain than the WT. The glutamate grown cells of both the WT and mutant strain exhibited a maximum rate of NR activity (0.82±0.04, 0.96±0.03 µM/mg protein/min, respectively) at 8.0 mM

concentration of glutamate (Fig. 6.11 b). However, the overall rate of NR enzyme activity in the C+N grown cells was found to be much higher than that in the glutamate and nitrate nitrogen grown cells. Overall results on NR activity and its rate saturating concentrations under different nitrogen nutrition indicated that nitrate assimilatory system in the glutamate grown cells was highly suppressed.

These results suggested that unlike the C+N and nitrate-grown cells, the NR activity- an indicator of nitrate assimilatory system was highly suppressed in the glutamate grown cells. The statistical significant variation level demonstrated by paired sample Student't' test ($p<0.05$) in different concentration of nitrate.

6.3.5 FTIR analysis

FTIR fingerprint is one of the most effective tools to study the changes of biochemical constituents of microalgal biomass grown in the presence of a varying concentration of sodium nitrate (2-25 mM), ammonium chloride (0.1-2 mM), glutamate (0.1-15 mM), and C:N (2-20:0-10) effect. The FTIR spectrum reflects the distribution of macromolecules in the microalgae (Fig. 6.12 a, b). According to Giordano et al. (2001), the total lipid is indicated by the absorption band around 1740 cm^{-1}. The amide I and amide II bands of protein occur around 1660-1540 cm^{-1}. However, the signature spectrum of carbohydrate region occurs around 1200-900 cm^{-1} and the absorption peaks around 1040-1050 cm^{-1} are related to the sugar monomers. The FTIR peak around 2925-2930 cm^{-1}, represents the methylene group and peak at 2850 cm^{-1} wavenumber shows the methyl and methylene groups, indicating the saturation and unsaturation of lipids. The results of the IR spectrum of nitrogen starved WT cells showed a more pronounced peak at 2826 cm^{-1}, followed by glutamate and ammonium grown cells. On the contrary, the absorption peaks in the same IR region of the spectrum of mutant cells were observed in the presence of all the nitrogen sources, when compared with the nitrogen starved cells. However, the total lipid content in the microalgal cells as indicated by IR absorption peak at approximately 1740 cm^{-1} was prominent in both the WT and mutant strain only when both the strains are grown without nitrogen source or with grown with glutamate and ammonium chloride (Table 6.1).

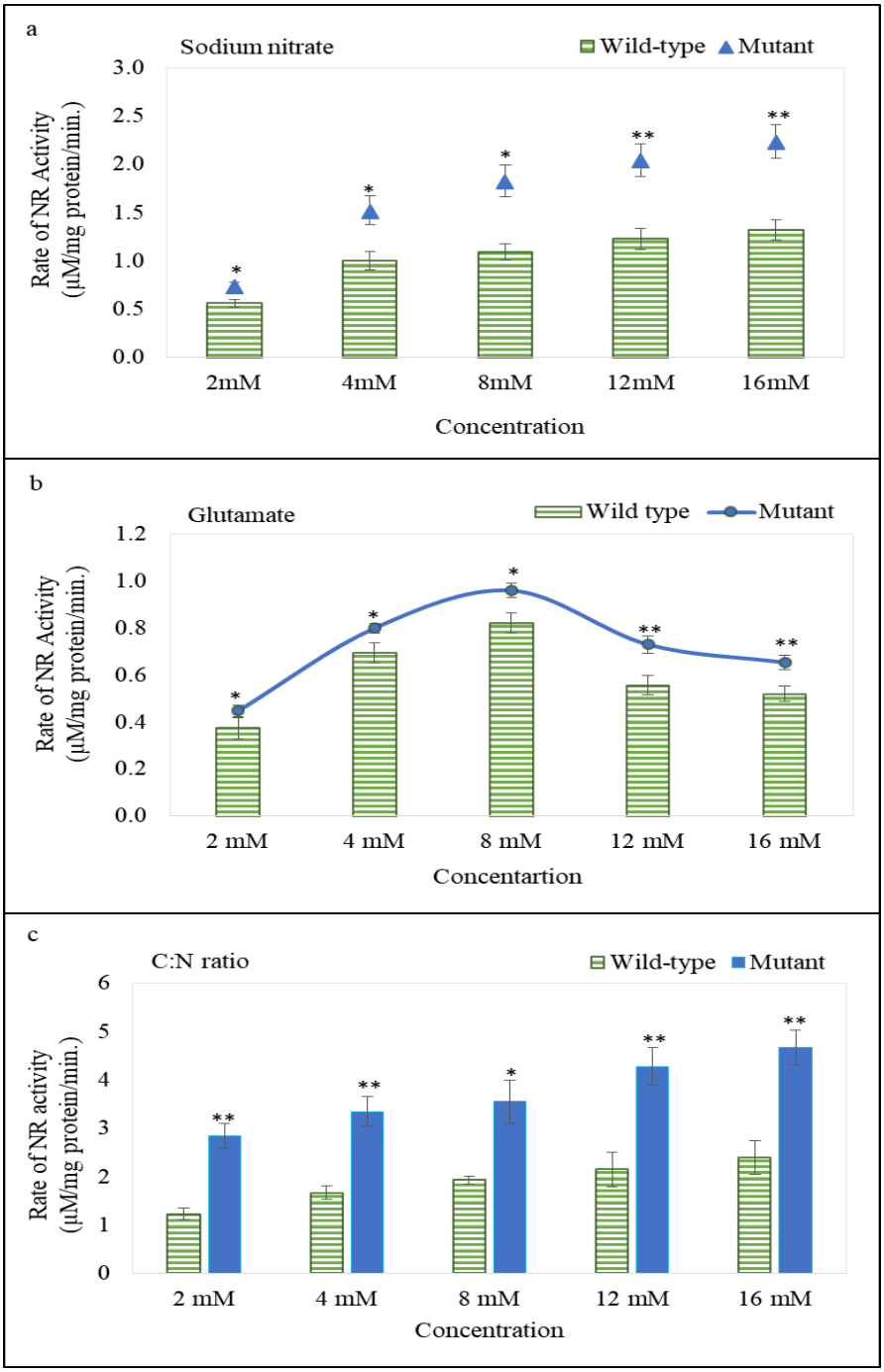

Fig. 6.11 Nitrate reductase (NR) activity of wild-type (WT) and mutant strain of *S. vacuolatus* cells grown in the presence of a fixed concentration of sodium nitrate (10 mM) (a), glutamate (1.0 mM) (b) and C/N ratio (12:10) (c). The experiment was conducted in the presence of a varying concentration of sodium nitrate (2-16 mM) in all nitrogen sources. Student‘t’ test showed significant difference level at **p<0.01, ***p<0.001 between WT and mutant strain. Error bar showed the mean±SD.

The absorption peak 1652-1650 cm^{-1}, belonging to protein I amide band, were found to be present in both WT and mutant strain under various nitrogen supplemented conditions. However, the amide II (wavenumber at 1545 cm^{-1}) band was found to be absent in the WT cells under the nitrogen starved and ammonium chloride supplemented condition, indicating reduced synthesis of protein (Table 6.1). The FTIR absorption range between 1000-1100 cm^{-1}, representing polysaccharides of microalgal cells were found to be more prominent under nitrogen starved and glutamate supplemented conditions in both the WT and mutant strain when compared with corresponding IR spectra of both the strain under nitrate, C:N supplemented conditions. These results suggested that polysaccharide accumulation was enhanced under N-deprived and glutamate supplemented condition in both the strains. The peak around 1000-1100 cm^{-1} associated with polysaccharides or nucleic acid (1026-30 cm^{-1}) were also observed in both the strains under all nitrogen nutrition except glutamate. This may not be solely due to polysaccharides present in the cells, but it may be associated with nucleic acids and phosphodiester bonds (Stehfest et al., 2005). Overall FTIR result suggested that there was changes in the macromolecule of both the strains, depending upon the physiological condition of nitrogen nutrition. The result further suggested that lipid content was greatly enhanced in both the strain under nitrogen starved and glutamate supplemented conditions.

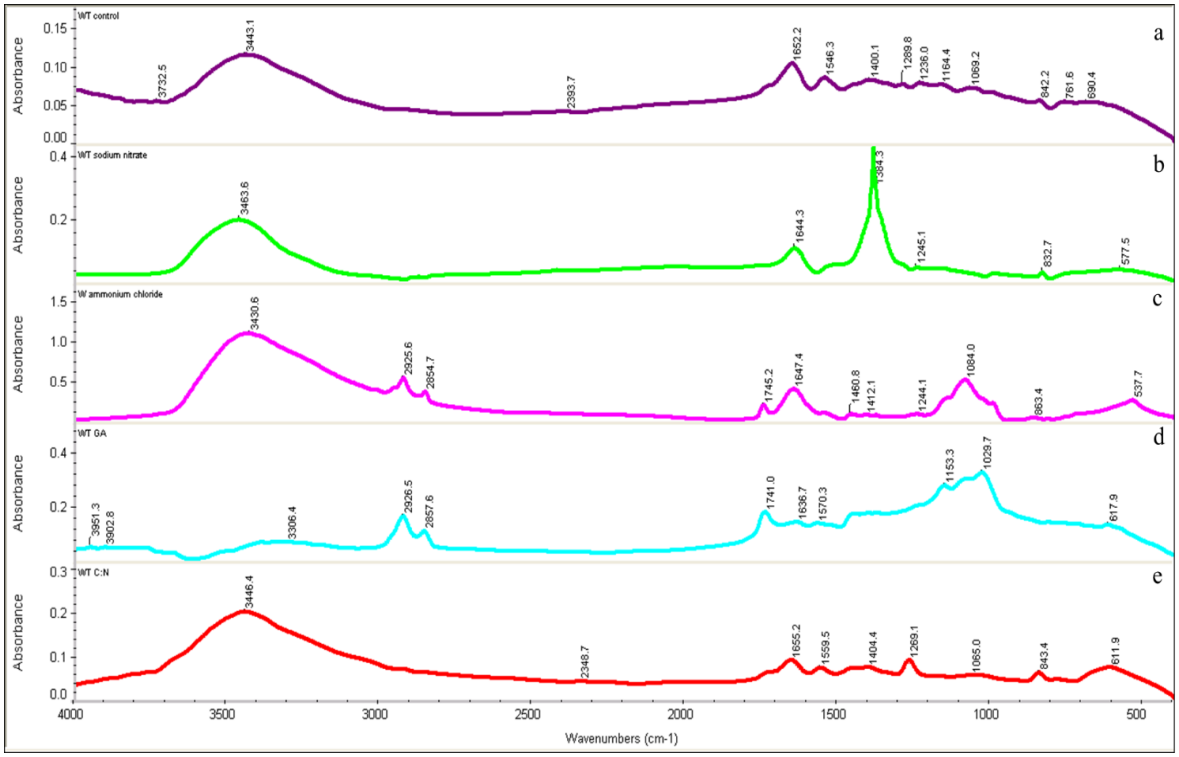

Fig. 6.12 (a) FTIR analysis of dry biomass of wild-type cells of *S. vacuolatus* in the presence of nitrogen starved (a) sodium nitrate (10.0 mM) (b) ammonium chloride (0.5 mM) (c) glutamate (1.0 mM) (d) and C:N ratio (12:10 mM).

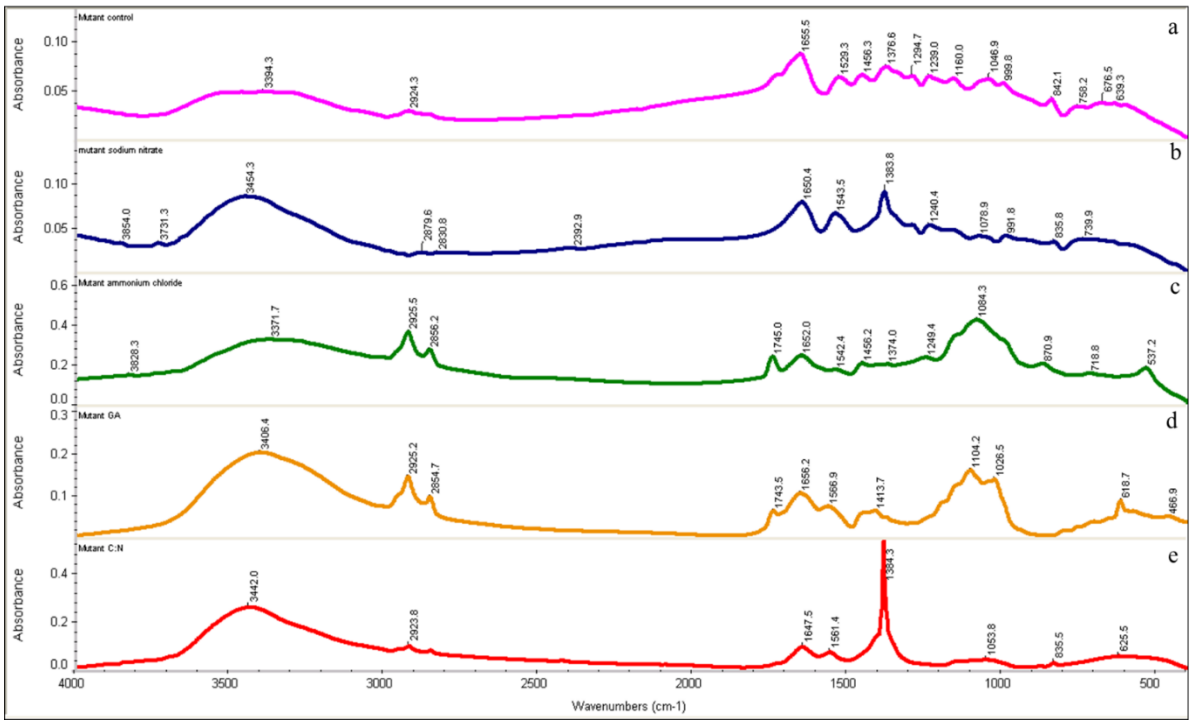

Fig. 6.12 (b) FTIR analysis of dry biomass of mutant strain of *S. vacuolatus* in the presence of nitrogen starved (a) sodium nitrate (15.0 mM) (b) ammonium chloride (0.5 mM) (c) glutamate (1.0 mM) (d) and C:N ratio (16:10 mM).

Table 6.1 FTIR spectra absorption peak (wavenumber) of wild-type and mutant strain of *S. vacuolatus* grown cells in the presence of varying nitrogen sources showed changes in the biochemical constituents.

Wavenumber (absorbance peak) showing changes in biochemical constituents of wild-type and mutant strain of *S. vacuolatus* under varying nitrogen sources

	Wild-type					Mutant					
S.N.	**Nitrogen starved cells**	**Sodium nitrate (10.0 mM)**	**Ammonium chloride (0.5 mM)**	**Sodium glutamate (1.0mM)**	**C:N ratio (12:10)**	**Nitrogen starved cells**	**Sodium nitrate (15.0 mM)**	**Ammonium chloride (0.5 mM)**	**Sodium glutamate (1.0mM)**	**C:N ratio (16:10)**	**Band assignment**
1	3732	3463	3430	3306	3446	3402	3454	3371	3406	3422	Water *v*(O-H) stretching Protein *v*(N-H) stretching
2	2926 2857	-	2925 2854	2926 2857	-	2925 2854	2930 2879	2925 2856	2925 2854	2923 2853	Lipid – carbohydrate, *vas*(CH_2) and *vs*(CH_2) stretching
3	1741	-	1745	1741	-	1743	-	1745	1743	-	fatty acids *v*(C=O) stretching of esters
4	1656	1644	1647	1636	1655	1656	1660	1652	1666	1647	Protein amide I band (C=O) stretching
5	-	-	-	-	-	-	1543	1542	1556	1561	Protein amide II band, *δ*(N-H) bending and *v*(C-N) stretching
6	1455 1372	1384	1421	1384	1404	1376	1383	1456 1374	1413	1384	Protein *δs*(CH_2) and *δs*(CH_3) bending of methyl carboxylic acid *vs*(C-O) of COO- groups of carboxylates Lipid *δs*($N(CH_3)_3$) bending of methyl
7	1158 1099	-	1084	-	1065	1159 1099	1078	1084	1104 1026	1053	Carbohydrate *v*(C-O-C) of polysaccharides, Nucleic acid, *vs*(>P=O) stretching of phosphodiesters

A semi-quantitative analysis of macromolecules in terms of Lipid/Carbohydrate (L/C) 1740/1040 cm^{-1}, Carbohydrate/Protein (C/P) 1040/1650 cm^{-1}, Lipid/Protein (L/P) 1740/1650 cm^{-1} ratios indicated compositional changes in the biochemical constituents of both the strains (Dean et al., 2010). The results also revealed that total lipid (2920/3011 cm^{-1}) in WT (1.7±0.08) and mutant strain (2.8±1.2) were found to be higher under nitrogen starved condition. However, the addition of 10.0 mM of nitrate, 0.1 mM ammonium chloride, 12:10 C:N ratio resulted in reduced content of total lipid. On the contrary, glutamate grown WT (2.67±0.10) and mutant strain (3.30±1.9) showed that 30-50% increase in the total lipid content (Table 6.2 a, b), which were higher than the lipid content present in the corresponding nitrogen starved cells of each strain. The lipid/protein (L/P) ratio in nitrogen grown WT and mutant strain (5.9±3.1) declined under varying nitrogen nutrition except for glutamate grown cells. This results again indicated higher lipid accumulation in both WT and mutant strain under nitrogen starved and glutamate supplemented conditions.

Similarly, the lipid/carbohydrate (L/C) and carbohydrate/protein (C/P) ratio also responded to varying nitrogen sources in both the strain. The L/C ratio in the WT was higher under C:N and nitrate supplemented condition. Addition of glutamate to both WT and mutant strains significantly improved the L/C ratio, which was comparable to that obtained under nitrogen starved condition (Table 6.2 a, b). Finally, it was deduced that lipid accumulation in both WT and mutant strain were greatly accentuated under nitrogen starved or glutamate supplemented condition.

Table 6.2 (a) FTIR analysis of cells constituents such as total lipid (2920/3011 cm^{-1}), lipid/carbohydrate (L/C) (1740/1040 cm^{-1}), carbohydrate/amide I (C/P), (1040/1650 cm^{-1}) and lipid/protein (L/P), (1740/1650 cm^{-1}) ratios in wild-type (WT) of *S. vacuolatus* cells grown under different nitrogen sources. Data depicted as mean±SD.

Treatment	Total lipid (2920/3011cm^{-1})	Lipid/Carbohydrate (L/C), (1740/1040 cm^{-1})	Carbohydrate/Amide I (C/P), (1040/1650 cm^{-1})	Lipid/Protein (L/P), (1740/1650 cm^{-1})
Control	1.7±0.08	0.44±0.022	1.3±0.11	1.6±0.12
Nitrate (10.0 mM)	0.6±0.035	0.58±0.024	0.2±0.10	0.52±0.022
Ammonium Chloride (0.1mM)	0.4±0.031	0.39±0.012	0.2±0.008	0.48±0.026
Glutamate (1.0mM)	2.67±0.10	0.69±0.031	2.1±0.15	1.2±0.071
C/N ratio (12:10 mM)	0.9±0.041	1.2±0.058	0.55±0.025	0.79±0.064

Table 6.2 (b) FTIR analysis of cells constituents such as total lipid (2920/3011 cm^{-1}), lipid/carbohydrate (L/C) (1740/1040 cm^{-1}), carbohydrate/amide I (C/P), (1040/1650 cm^{-1}) and lipid/protein (L/P), (1740/1650 cm^{-1}) ratios in mutant strain of *S. vacuolatus* cells grown under different nitrogen sources. Data depicted as mean±SD.

Treatment	Total lipid (2920/3011cm^{-1})	Lipid/Carbohydrate (L/C), (1740/1040 cm^{-1})	Carbohydrate/Amide I (C/P), (1040/1650 cm^{-1})	Lipid/Protein (L/P), (1740/1650 cm^{-1})
Control	2.8±1.2	0.9±0.085	1.6±1.1	1.7±0.13
Nitrate (10.0 mM)	1.0±0.076	1.0±0.076	0.48±0.02	0.51±0.33
Ammonium Chloride (0.1mM)	0.5±0.021	0.25±0.016	0.25±0.013	0.59±0.071
Glutamate (1.0mM)	3.30±1.9	4.4±1.6	1.3±0.10	5.9±3.1
C/N ratio (16:10 mM)	1.1±0.085	0.88±0.021	0.75±0.048	0.82±0.041

6.4 Discussion

The present study based on the effect of varying nitrogen sources on the growth, biochemical constituents and lipid production in the wild-type (WT) and mutant strain of *S. vacuolatus*. The wild-type (WT) and mutant strain of *S. vacuolatus* were grown in the presence of varying concentrations of sodium nitrate, ammonium chloride, sodium glutamate and different carbon: nitrogen (C/N) ratio. The present study demonstrated that low concentration of all nitrogen sources were more effective in enhancing the growth and biochemical constituents in both WT and mutant strain. Further, the result showed that growth and biochemical constitutes in the mutant strain were better in the presence of all the nitrogen sources as compared to WT. However, the best growth of both WT and mutant strain was observed in the C+N and nitrate supplemented condition. It is possible that the mode and type of nitrogen nutrition may influence carbohydrate and protein synthesis (De Farias et al., 2018; Chen et al., 2013). The utilization of nitrogen sources like sodium nitrate, ammonium chloride, and amino acids is directly involved in the regulation of nitrogen assimilation. Chi et al. (2015); Li et al. (2008); and Dortch et al. (1982) have reported that nitrate and urea are better nitrogen sources for microalgal growth whereas amino acids are also potential nitrogen source for microalgal growth. Antia et al. (1991) have shown that the nitrogen nutrition determines the cell constituents and carbon allocation pattern of planktonic marine diatom *Chatocer muellerii*, when grown by using ammonium and nitrate as nitrogen source. The growth response of microalgal cells to ammonium chloride was suppressed at higher concentrations (Corey et al., 2013; Solomon & Gilbert, 2008). The inhibitory effect of ammonium chloride on the microalgal growth is attributed to the fact that the surplus transfer of ammonium to the microalgal cells, which blocks the formation of ATP in the chloroplast and inhibits the photosynthesis (Ramanna et al., 2014). The mixotrophic growth of several microalgae has been reported by using various organic carbon source as a medium for microalgal growth such as sodium citrate, sodium acetate (Qiao & Wang, 2009), fructose, sucrose (Gao et al., 2010), and glucose (Lin & Wu, 2015). The results showed a higher level of TOC and carbohydrate accumulation in the C+N grown cells, unlike the nitrate, ammonium, glutamate supplemented and nitrogen starved cells. Other studies show that nitrogen as a limiting nutrient is an important factor for growth and lipid production (Jaiswal & Sharma, 2016). The nitrogen limiting condition is described to be one of the most important key regulators for triggering lipid synthesis in microalgal

cells (Wu et al., 2013). In order to achieve the enhanced lipid production, a modulated physiological condition is required which mimics the nitrogen deprivation condition and favourably influences the production of cell biomass and lipid (Vitova et al., 2015). A reduction in the cellular protein is described to have an immense impact on cell growth, pigment content and biomass production (Costa et al., 2001).

Recently it has been reported that intracellular accumulation of lipid essentially requires the higher activity of acetyl-CoA carboxylase along with enhanced activities of other enzymes involved in the conversion of carbohydrate to lipid **(**Bellou et al., 2014). The growth response of each microalgal species depends on its ability to take up amino acid from the medium and the regulation of the nitrogen assimilation activity (Dao et al., 2014). Hellebust & Ahmad, (1989), reported microalgae *Stichococcus bacillaris*, utilize various sources of amino acid as nitrogen for higher growth. In another study, *Chlorella* sp., have more versatile use of amino acids for growth than other microalgae (Sauer et al., 1983). In the present investigation, glutamate was used as a nitrogen source to support growth and synthesis of biochemical constituents in both WT and mutant strain. Earlier reports have shown that carbohydrate accumulation is better under nitrogen deprived microalgal cells, whereas protein content declines (Bellou et al., 2014; Zhu et al., 2014). Earlier it has been suggested that when cells have reached to stationary growth phase and the cells are subjected to growth-limiting condition, more carbon is incorporated into carbohydrates and lipids (Janssen et al., 2018). The FTIR spectra of both WT and mutant strain grown under varying nitrogen nutrition showed changes in IR peaks at 2964 cm^{-1}, 2924 cm^{-1}, 1740 cm^{-1}, 1650 cm^{-1} and 1040 cm^{-1}, assigned to lipids, fatty acids, protein and carbohydrates (Murdock & Wetzel, 2009). Andrus et al. (2006) has observed changes in the lipid profile and membrane damage in the cells are indicated by enhanced CH_3/CH_2 ratio of methylene and methyl peak. The compositional changes in the microalgal cells was semi-quantitatively assessed in terms of total lipid (2920/3011cm^{-1}), lipid/carbohydrate (L/C) (1740/1040 cm^{-1}) lipid/protein (1740/1650 cm^{-1}) also supported by the other workers (Jiang et al., 2012; Dean et al., 2010). The cells grown under different nitrogen nutrition revealed higher L/C ratio in the glutamate grown than nitrate and C+N grown WT and mutant cells. Similarly, the L/P ratio also showed the same trend, which was higher in the glutamate grown cells of both WT and mutant cells when compared with nitrate, ammonium, C+N supplemented conditions. Accumulation of higher lipid content in the mutant

strain than the WT was subsequently confirmed by flow cytometry analysis of lipid content in the microalgal cells. The results showed (46.0%) lipid in the mutant than the WT (42.01%) when grown under glutamate supplemented condition. These values were higher than the lipid percentage in the mutant (30.61%) and WT (26.05%) strain observed under the nitrogen starved condition.

In the present study, the nitrate reductase (NR) activity in the cells grown under varying nitrogen nutrition was taken as an indicator to assess the status of the nitrogen assimilatory system (Flynn et al., 1993). Based on the results, reduced functioning of the nitrogen assimilatory system in the glutamate grown and nitrogen starved cells of both the strains was correlated with higher accumulation of lipid. The common factor in the nitrogen starved and glutamate cells have apparently reduced the functioning of the nitrogen assimilatory system. Thus, overall results indicated that the carbon partitioning in favour of lipid synthesis might be dependent on the functioning of nitrogen assimilatory, not on the total organic carbon status of the system, as suggested by the earlier workers (Cullimore & Sims, 1981). There is a greater possibility that glutamate-dependent reduced functioning of the nitrogen assimilatory system in the microalgal cells might be able to divert the carbon pool and metabolic energy from amino acid synthesis to lipid synthesis (Turpin et al., 1991; Syrett et al., 1986). The glutamate nutrition of the *S. vacuolatus* cells was similar to that of nitrogen deprivation, which reduced the demand for carbon skeleton required for amino acid synthesis pathway and diverted it to lipid synthesis pathway (Moussa et al., 2017; Clayton et al., 1986). Since nitrogen nutrition is directly involved in the regulation of nitrogen assimilation (Johnson et al., 2017; Jiang et al., 2012), it may be concluded that reduced functioning of the nitrogen assimilatory system triggers the lipid accumulation, not the carbon status of the cells (Esteves-Ferreira et al., 2018).

The overall finding depicted that mutant strain showed better growth, biochemical constituents and lipid content as compared to WT. Other workers have also suggested that nitrogen deprived condition can enhance the lipid content (Vadivel et al., 2019; Gour et al., 2018; Costa et al., 2017; Xia et al., 2016). In the present study, it was concluded that the effect of glutamate nitrogen triggers the lipid synthesis, which is comparable to lipid content in the nitrogen starved microalgal cells.

6.5 Conclusion

- The present study found that glutamate- as organic nitrogen, was found to be a better nitrogen source for lipid accumulation in the microalgae than the nitrate or acetate + nitrate (C/N) and nitrogen starved cells in both WT and mutant strain.
- The result evidence that lipid content increases in the nitrogen starved cells when compared to nitrate, ammonium chloride, C/N grown cells expect glutamate grown cells in both WT and mutant strain.
- The optimum growth level, biochemical constituents and lipid content achieved in glutamate grown cells at (1.0 mM), sodium nitrate (10.0 mM, 15.0 mM) and C+N grown cells at 12:10 mM, 16:10 mM respectively in both WT and mutant strain.
- The ammonium chloride was found toxic for microalgal growth in both WT and mutant strain.
- Carbon status of the cells generally promoted the biomass production as in case C+N grown cells, irrespective of its impact on the synthesis of the lipid. These findings were supported by FTIR analysis and flow cytometer data on the lipid content.
- A corollary of the results indicated that reduced functioning of the nitrogen assimilatory system, whether by glutamate or by nitrogen deprivation triggers the lipid synthesis in glutamate grown cells.
- The overall result demonstrated that DCMU-tolerant mutant strain found higher growth, biochemical constituents and lipid content compared to wild-type (WT). These result depicted that alteration in the physiology of the microalgal cells enhanced the potential for biomass and lipid production as a sustainable and economical way.

Chapter- VII

EFFECT OF WASTEWATER AS NUTRIENT SOURCE ON THE BIOMASS AND BIOFUEL PRODUCTION IN *S. VACUOLATUS*

7.1 Introduction

Agro-industry generates huge amounts of liquid and solid wastes, which emerge from processing operations and residues (Gupta et al., 2019). The composition and quantity of agro-industrial wastes depend on the nature and source of raw materials, nature of the products, operations and processing steps (Rajagopal et al., 2013). Due to high nutrient content in the agro-industrial wastes, they have a high potential to cause severe pollution problems, if not managed or treated properly. Solid wastes from many agro-food industries are a big hazard to the environment and require appropriate management approach (Leiva-Candia et al., 2014). World over, environmental regulatory authorities are setting stringent criteria for disposal of wastes from industries. Due to strict regulations, there is a need to treat and reuse these wastes quickly (Ramos et al., 2019). With the fast pace of development of molecular technique, sustainable biotechnology, substantial research is now devoted to coping with the problem of industrial wastes and their ever-increasing complexity (Fernandez et al., 2019; Koutinas et al., 2014).

Bioremediation is a technology that exploits the metabolic potential of microorganisms to clean up the contaminated sites i.e. wastewater, ground or surface waters, soils and sediments etc. (Das et al., 2019). However, the term bioremediation encompasses both microbial remediation and phytoremediation of environmental contaminants. Microbial remediation, in turn, includes the use of bacteria, fungi and algae for remediation purposes (Luo et al., 2016). In this context, wastewater is used as a solid waste nutrient source to cultivate microalgae can be one of the best biological treatments (Chen et al., 2012). This method could greatly reduce the cost of the synthetic nutrient medium as the nutrients for algal growth are derived from the wastewater (Alam & Wang, 2019). Besides, the technology of using microalgae to treat wastewater is based on the principle of natural ecosystems, which may not cause secondary pollution (Logan & Visvanathan, 2019; Zamani et al., 2012). Microalgae are preferred for treating the wastewaters due to their ability to take up nutrients and convert them into biomass (Chinnasamy et al., 2010). Nutrients from the wastewater are assimilated for growth, thus reducing the minerals and nutrients wastewater in which ultimately reduces the chemical oxygen demand (COD) and biological oxygen demand (BOD) (Angeles et al., 2019). The small size of microalgae provides a larger surface area, which increases the nutrient uptake rate from the wastewater (Javed et al., 2019). The application of microalgae for wastewater treatment is also known as

phycoremediation. Phycoremediation enables industries to address two important issues: the high use of chemicals and high energy costs associated with treating the wastewater. In the wastewater treatment, microalgae involve removal of nutrients, heavy metal ions, pathogens and the reduction in the BOD via the oxygen produced by photosynthesis in microalgae (Molinuevo-Salces et al., 2019; Prajapati et al., 2013). Unlike terrestrial plants, microalgae do not require a proper system for water and nutrient uptake; instead, they depend on their large surface area for the uptake of water, nutrients and carbon dioxide (CO_2) (Dominic et al., 2009; Udaiyappan et al., 2017).

The microalgal species are found to be quite versatile in the removal of nutrients and pollutants (Hadiyanto et al., 2013; Bich et al., 1999). The microalgal species used for the treatment of wastewater include *Chlorella* sp. *Arthrospira platensis, Anabaena sp., Scenedesmus obliquus, Oscillatoria* sp. *and Nostoc* sp. (Gupta et al., 2019; Udaiyappan et al., 2017). Microalgae offer the most attractive advantage for treating the wastewaters include their ability to several types of wastewaters. Microalgae also use the organic matter available in the wastewater during their mixotrophic and heterotrophic growth. During the autotrophic growth, they consume only carbon dioxide as a carbon source instead of organic carbon present in the wastewaters (Zhou & Smith, 2002). In recent years, microalgae have been used for the treatment of industrial wastewater (Sun et al., 2013), municipal wastewater (Caporgno et al., 2015) and agricultural wastewater (Abou-Shanab et al., 2013). Sun et al. (2013) cultivated *Chlorella pyrenoidosa* by using Riboflavin (B2) manufacturing effluent and obtained maximum biomass productivity of 1.25 g/L and COD, total nitrogen, total phosphorus removal up to 89.2%, 64.5%, 82.2%, respectively within 8-days of their cultivation. Caporgno et al. (2015) cultivated *Chlorella kessleri* by using urban wastewater containing nitrogen and phosphorus concentrations to the level of 139 and 5.8 mg/L, respectively. The maximum removal efficiency of nitrogen and phosphorus and maximise the productivity of biomass was found to be around 96%, 99% and 2.70 g/L, respectively, after 11-day batch cultivations. Abou-Shanab et al. (2013) observed that *Chlamydomonas mexicana* could remove 62% nitrogen, 28% phosphorus and 29% inorganic carbon from piggery wastewater.

Soybean foods, such as tofu, soy yoghurt, soy sauce, soy cheese, soymilk, soybean sprouts, and soy protein, are widely used in Asia. Soybean foods are also consumed in many countries because of their delicious taste as well as their health

benefits (Li et al., 2013). Therefore, it is reasonable to assume that an increasing amount of soybean will be required to meet the market for various soy food products. Soybean processing industries generate about 7–10 tons of wastewater with a chemical oxygen demand (COD) value of 10-20 gL^{-1} per ton of processed soybeans (Yu et al., 2002). A large amount of wastewater produced by soybean processing waste industries water mainly contains monosaccharide, oligosaccharides, vitamins, organic acids, amino acids, lipids, whey protein, isoflavone, saponin, P, Ca, Fe, and other nutrients (Barbosa et al., 2006; Tang & Ma, 2009). It is considered that microalgae are not only capable of removing pollutants, but also produce value-added products including lipid- a vital source of biodiesel (Perez-Garcia et al., 2011; Bhatnagar et al., 2010). Since soybean processing wastewater contains a significant amount of usable nutrients without toxic and hazardous substances which may inhibit the growth of microalgae. However, cultivation of oleaginous microalgae in such nutrient-rich medium may reduce the cost of algae cultivation and biodiesel production (Hongyang et al., 2011).

The poultry industry is one of the largest and fastest-growing agro-based industries in the world. Wastes from the poultry industry include a mixture of excreta (manure), bedding material or litter (e.g. wood shavings or straw), waste feed, broken eggs and feathers (Oliveira et al., 2018; Rawat et al., 2016). The litter and manure component of the solid waste has a high nutritional value and is occasionally used as organic fertiliser, for recycling of nutrients such as nitrogen, phosphorus and potassium. The composition of litter and manure predominantly contains organic carbons smaller amounts of nitrogen (N) and phosphorus (P) and trace levels of chlorine (Cl), calcium (Ca), magnesium (Mg), sodium (Na), manganese (Mn), iron (Fe), copper (Cu) and zinc (Zn) (Han et al., 2019; Bustillo-Lecompte & Mehrvar, 2015). Poultry feed contains significant concentrations of organic nitrogen due to the presence of high levels of protein and amino acids. The nitrogen in fresh manure is typically 60–80% in an organic form such as urea and protein (Chastain et al., 2010; Bolan et al., 2010). Depending on environmental conditions a large percentage of this organic nitrogen (40–90%) is converted to ammonia within a year (Thyagarajan et al., 2013; Mallin & Cahoon, 2003). The poultry industry is currently facing a number of environmental problems. One of the major problems is the accumulation of a large amount of nitrogen-rich wastes, especially manure and litter, generated by intensive production. (Singh et al., 2011; Markou et al., 2016; Kelleher et al., 2002). Most of the poultry waste (manure and litter) finds its way into water bodies either due to rain cleaning operation. However, pollution

and nuisance problems due to poultry waste can do not favour agronomic utilisation (Rahman & Muyeed, 2010). Such properties of wastes have consequently led to a number of studies demonstrating its use as algal growth medium (Miah et al., 2016; Kebede-Westhead et al., 2006; Chinnasamy et al., 2010). Mahadevaswamy & Venkataraman, (1986) studied integrated aerobic digestion and algal cultivation system using *Spirulina platensis* for the treatment of poultry waste for the production of biogas and algal biomass. An aqueous extract of poultry litter has been reported to support up to 180% higher algae biomass when compared to that growth on a synthetic medium (Bhatnagar et al., 2011).

Wastewater treatment is the major global challenges for the sustainable development of our society as it is increasingly contaminating potable water sources. Microalgae offer various attractive advantages over current wastewater treatment systems. The benefits of phycoremediation include low energy requirements, little sludge formation and low greenhouse gas emission (Molinuevo-Salces et al., 2019; Gupta & Bux, 2019). The quick stress adaptation ability of microalgae enables them to thrive even under extreme environmental conditions and it offers an advantage to microalgal in the treatment of these wastewaters (Rincon et al., 2014). The removal of nutrients through consumption by microalgae and the oxygen produced by the microalgae creates a suitable environment for other microorganisms to grow during the treatment of wastewater, thereby reducing the need for aeration and the associated costs (Fernandez et al., 2019). In general, the cost reductions achieved by using microalgae as a bioremediation agent of wastewaters are as follows (Udaiyappan et al., 2017):

• Operation costs are reduced because the nutrients in the wastewaters support microalgal growth.

• The photosynthesis carried out by microalgae requires only sunlight and CO_2.

• Aeration cost is reduced because microalgae produce oxygen which can be used by aerobic bacteria for further mineralization waste materials.

• Microalgal biomass is suitable for obtaining various value-added products.

Thus, the present study aims to develop an efficient approach to investigate the growth, nutrient removal, lipid and biodiesel production in the (wild-type) and mutant strain of *S. vacuolatus* when used for the treatment of poultry and soybean wastewater. The results on wastewater treatment were compared with the corresponding results obtained from the algal growth on BG-11 and bore well water (tap water).

7.2 Materials and Methods

7.2.1 Cultivation of microalgal inoculum

For the experiment, microalgae *S. vacuolatus* strain of wild-type (WT) and mutant, a monoclonal culture were grown in the Erlenmeyer flask (250 mL) in the presence of BG-11 medium (Stanier et al., 1971) (pH 7.5±0.2). The culture flasks were maintained under the white fluorescent tube light (10 watts/m^{-2}) with a light/dark cycle of 16/8 h for 10 days under 25±2 ℃. The culture flasks were shaken five times a day to avoid the settling of the microalgal cells.

7.2.2 Preparation of leachates of solid waste for algal growth

The solid waste of poultry manure was collected from a Rudauli poultry farm (Rajajipuram, Lucknow, U.P.) whereas soybean waste collected from S.B. Foods Product, Panki, Kanpur, U.P. The collected solid wastes of these two agro-industries were suspended in water (250 mg L^{-1}) and left for 2 hours. Thereafter, leachates of solid-waste were filtered by using Whatman filter paper no. 42. The leachates hereby termed as wastewater were autoclaved at 121℃ for 20 min to remove microorganisms. The leachates (wastewater) was stored at 4℃ for further experiments.

Further, Physico-chemical characterisation of wastewater sample was done and in the detail was discussed under the subheading 7.2.9 Physico-chemical characteristics of soybean and poultry wastewater. The sterilized poultry and soybean wastewater of different dilution was used for optimum microalgal growth. Both wastewaters of different strength (10, 20, 30, 40, 50, 60, 80, 100% respectively) were used for microalgal growth and results were compared with tap water (TW) (borewell water) grown cells. The growth of both wild-type and mutant strain in the wastewater was optimised with respect to select the strength of wastewater, pH and temperature.

7.2.3 Experimental setup

In order to evaluate growth efficiency of algal strains on the poultry wastewater (PWW), soybean wastewater (SWW) and BG-11 medium, tap water (TW) (bore well

water) was used as control. The optimum strength of different wastewater supporting maximum growth of wild-type (WT) and mutant strain was selected for further studies.

The BG-11 grown cells (exponential phase) of both WT and mutant strain were washed twice with deionized water (DW) and the flasks containing different growth media were inoculated with inoculum approximately (0.1 OD) and cultured for 0-20 days. The flasks were kept under the white fluorescent tube light (10 watts/m^2) with marinating 16/8 h light/dark condition, 25±2℃, pH 7.8±2. The culture flask was shaken five times a day for avoiding sticking and settling of the cells.

7.2.4 Estimation of biomass content

The 50 mL of *S. vacuolatus* grown in the cells suspension tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) was withdrawn and they were centrifuged. The pellet was washed thrice with DW then dried in an oven at 50°C overnight to determine the weight of biomass (in gram per litre) throughout the growth period (20 days). Biomass productivity (P) was calculated as described by Issarapayup et al. (2009):

Biomass Productivity (P) ($g\ L^{-1}\ d^{-1}$) = $(W_1-W_0) \div (t_1-t_0)$

Where W_1 and W_0 are dry biomass ($g\ L^{-1}$) and t_1, t_0 denoting time respectively.

7.2.5 Estimation of biochemical constituents and pigment content

The biochemical constituents (protein, carbohydrate, total organic carbon (TOC) and pigment content) were determined in *S. vacuolatus* WT and mutant strain cells grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) were determined. Before the analysis, the microalgal cells were washed thrice with deionised water (DW) and the original volume was maintained with DW.

7.2.5.1 Estimation of protein

The protein content was estimated by the method of Lowry et al. (1951) modified by Herbert et al. (1971) as described earlier in the Materials and Methods of chapter III.

7.2.5.2 Estimation of carbohydrate

The carbohydrate content was measured by the phenol-sulphuric acid method of Dubois et al. (1956) as described in the Material and Methods of chapter III.

7.2.5.3 Estimation of total organic carbon (TOC)

Total organic carbon (TOC) was measured by the Walkley-black method as described by Grobler & Davies, (1979), as the procedure described in the Materials and Methods of chapter III.

7.2.5.4 Estimation of pigment content

The microalgal cells were grown on tap water (TW), BG-11, poultry wastewater (PWW), and soybean wastewater (SWW) were homogenised and broken by repeated freezing and thawing before the measurement of pigments. For estimation of total chlorophyll and carotenoids, the procedure has been described earlier in the Material and Methods, in chapter III.

7.2.6 Lipid extraction by gravimetric method

The lipid was extracted by the modified Bligh & Dyer, (1959) protocol. The microalgal cells were grown for 20 days on the tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) of 500 mL were centrifuged for 5 min at 5000 rpm. The supernatant was removed and the pellet was washed thrice with DW. The cells residue was allowed to dry overnight in a vacuum oven at 80°C and then dried biomass was powdered by using a mortar and pestle. The powdered dried biomass was weighed and then washed twice with Phosphate buffer saline (PBS) of pH 7.0±2. Thereafter, 0.5 mL PBS was added to the dried biomass along with add 1 mL glass beads. The suspension was vortexed for 10 min (stop vortex every one min. and maintained cooling by keeping it in the cold water). Further, 0.4% (v/v) phosphoric acid and sulfuric acid were added in the ratio of 2:0.75. The mixture was incubated at 50°C for 5 min. Thereafter, the solution was centrifuged at 2000 rpm for 5 min and the precipitate was removed. The chloroform: methanol (2:1 v/v) solution was added in the residual biomass. The mixture was vortexed for 5 min and left for 24 h at room temperature. After 24 h, 1mL distilled water and 2 mL chloroform: methanol (2:1 v/v) solution were added and again the mixture was vortexed for 1 min and then centrifuged at 4000 rpm for 5 min. The bottom organic phase layer was withdrawn to another flacon tube (50 mL) and washed with 5% NaCl solution (1:1 v/v) to remove the impurities. The organic phase (lipid) was evaporated to dryness at room temperature and the weight of lipid residue was

recorded. The lipid content was calculated gravimetrically and expressed as percent (%) lipid.

Lipid content (%) = weight of lipid/weight of algal biomass ×100

Lipid productivity (LP) was calculated as described by Dhup et al., (2017):

Lipid productivity (LP) (g L^{-1} d^{-1}) = L_{lipid} ×DCW/t

Where L_{lipid} is the lipid content in percentage (%), DCW is the dry cell weight (g/L) and t is the number of cultivation days.

7.2.7 Flow cytometer analysis

The lipid content in the microalgal cells grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) was as described earlier in the Materials and Methods of chapter III.

7.2.8 Transesterification process

The transesterification process of lipid extracted from the algal cells grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) was carried out by the method of Hossain et al. (2008). 300 µL lipid extract (oil) was mixed with 2 mL hexane and the mixture was vortexed for 5 min. Thereafter, the solution was poured in a mixture (0.2 mL) of catalyst NaOH and methanol (2 N) and left for 3 h on a continuous rotating shaker (200 rpm). Then, the upper organic layer was separated from biodiesel and lower pigment containing glycerine layer. The Biodiesel was separated with the help of funnel and washed with double distilled water and subsequently with sodium sulphate to remove the impurities. Further, the layer was evaporated at 50 ℃ and weight of biodiesel was calculated gravimetrically in terms of percent (%) of biodiesel as given below:

Biodiesel of microalgae (%) = weight of oil (g)/weight of the sample (g) ×100

7.2.9 Physico-chemical characteristics of soybean and poultry wastewater

The study of physico-chemical characteristics of was carried out in terms of water quality parameters like pH, BOD, COD, nitrate, nitrite, ammonium, phosphate, alkalinity, hardness, total dissolved solids (TDS), total suspended solids (TSS) etc. by

the standard protocol described by Maiti, (2004). After required dilution of wastewaters centrifuged at 5000 rpm for 10 min and the supernatant was filtered through 0.45 mm filter paper. The filtrate was analysed for nutrient removal efficiency (%) by using the following equation:

$$\textbf{Nutrient removal } (\%) = N_0\text{-}N_t / N_0 \times 100$$

Where N_0 (mg L^{-1}) is the mean value of initial concentration and N_t (mg L^{-1}) mean value of final concentration respectively.

7.2.10 Biomass settling and recovery

Biomass settling and biomass recovery was analysed was conducted at the end of the cultivation period (i.e. 20^{th} day) in 500 mL glass cylinders under static hydraulic conditions for 30 min (Su et al., 2011). Optical density at 680 nm of wavelength was measured for liquid samples collected at the centre of each cylinder after 5, 20 and 30 minutes. The efficiency (η) of microalgal biomass recovery was estimated according to the following equation:

$$\textbf{Biomass recovery efficiency } (\eta) = OD\ 680^0 - OD\ 680^t\ /\ OD\ 680^0\ \%$$

Where, OD 680^0 (absorbance) at an initial time and OD 680^t (absorbance) at the end time (Vandamme et al., 2012).

7.2.11 Fourier transform infrared (FTIR) analysis

Semi-quantitative analysis of cell constituents in the microalgal biomass characteristics of lipid and biodiesel extracted from both WT and mutant cells were grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) was carried out by FTIR spectrum. The detailed procedure for FTIR analysis is described earlier in chapter III- Materials and Methods. The changes in biomass and lipid were monitored in terms of ratios of constituents and functional group.

7.2.12 LC-MS/MS (Liquid chromatography-mass spectroscopy) analysis

The amino acid, polysaccharides and fatty acid of microalgal biomass grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) were analysed by using LC-MS/MS (Liquid chromatography-mass spectrometry) Aquity UPLC (Waters) and coupled with electrospray ionisation (ESI) mass spectrometry (API 4000 triple quadrupole) (AB Sciex).

Sample preparation and quantification of amino acids, polysaccharides and fatty acids

1 mg of microalgal biomass was extracted in 1 mL of 0.1N HCL in Eppendorf tube. The mixture was vortexed for 2 mins and sonicated for 30 min. at 25℃. After sonication, the samples were centrifuged at 10,000 rpm for 5 min. The extract was filtered by using 0.2 µm PVDF syringe filter. The sample (10 µL) injected to the LC-MS/MS system for amino acid analysis.

For polysaccharides analysis, 1 mg of microalgal biomass was extracted in 1 mL of MilliQ/ACN/Propanol (5:2:3) in eppendrof tube and it was vortexed for 2 min and sonicated for 30 min at 50^{o}C. After sonication, the samples were centrifuged at 10,000 rpm for 5 mins. The extract was filtered by 0.2 µm PVDF syringe filter and 10 µL sample was injected to the LC-MS/MS system for polysaccharides analysis.

For fatty acid analysis, 10 mg microalgal biomass was extracted in 1 mL Chloroform and Methanol (2:1) solution. The solution was vortexed for 2 mins and sonicated for 60 min at 50^{o}C. After sonication, the sample were centrifuged at 10,000 rpm for 5 min. The extract was filtered by 0.2µm PVDF syringe filter and 10 µL sample was injected to the LC-MS/MS system.

The analysis was performed on LC-MS/MS Aquity UPLC (Waters) and coupled with electrospray ionisation (ESI) mass spectrometry (API 4000 triple quadrupole) (AB Sciex). The separation of amino acid, polysaccharides and fatty acid were performed using UPLC BEH C-18 (1.7µm × 2.1 × 50 mm) column with the isocratic flow of 0.250 mL/min. For the amino acid and polysaccharide analysis, a solvent system of 0.1% formic acid and acetonitrile (90:10, 1:99 respectively) were used. The mass spectrometer was operated in positive ion mode. Whereas as separation of fatty acid was performed by using a solvent system of 0.1% formic acid and methanol (5:95) and mass spectrometer was operated in negative ion mode.

7.2.13 GC-MS (Gas chromatography-mass spectroscopy) analysis

The fatty acid profile of microalgal biodiesel of WT and mutant strain was grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) were analysed by using GC-MS (gas chromatography-mass spectroscopy). The analysis was performed by using Trace GC Ultra TSQ Quantum XLS Mass spectrometer (Thermo,

USA) equipped with Elite 5MS capillary column (30 m X 0.25mm), 0.25µm film thickness of stationary phase, 5% phenyl and 95% dimethyl polysiloxane). This was operated in splitless injection mode with an injector temperature of 250 °C. Helium was used as a carrier gas with a flow rate of 1.1 mL min^{-1}. The GC oven temperature was programmed as follows- 65 °C (hold for 2 min), increased to 230 °C at a rate of 6 °C min^{-1}, and finally reached to 290 °C (hold for 20 min) at a rate of 10 °C min^{-1}. Total run time was 55 min. The ion source and interface temperature were set at 220 °C and 300 °C, respectively. The samples were derivatized by silylation method by using BSTFA+1%TMCS as a derivatizing agent. The mass spectrometer was operated at an electron energy of 70eV. 1µL of the sample was injected into the chromatographic system and analysed in full scan mode of mass in the range of m/z 50-600. The unknown peak of the mass spectrum was compared with the spectrum of the known fatty acid database.

7.2.14 Statistical analysis

The performed statistical analysis was described earlier in chapter III of Materials and methods.

7.3 Results

7.3.1 Effect of different concentration of wastewater (soybean and poultry) on the microalgal growth

Both wild-type (WT) and mutant cells were grown at different dilution (10-100% v/v) of soybean wastewater (SWW) and poultry wastewater (PWW) (Fig. 7.1, 7.2). The growth of both WT (Fig. 7.1) and mutant strains (Fig. 7.2) at a lower concentrations of soybean wastewater (SWW) and poultry wastewater (PWW) increased in a time-dependent manner up to 16 days as compared to respective control BG-11 and tap water (TW) grown cells and thereafter cells reached to stationary phase. At higher concentrations (60-100%), both soybean wastewater and poultry wastewater were found to be toxic for WT strain. In the case of WT, maximum growth was observed at 30% dilution of soybean wastewater and 40% dilution of poultry leachates (wastewater). There was an approximately two-fold increase in the growth of WT (90%, 113% respectively) at optimum dilution of both SWW and PWW. A further increase in the strength of wastewater beyond the respective optimum doses of the wastewater the growth of WT strain was inhibited. A complete inhibition of growth of WT was observed at 100% concentration of both soybean and poultry wastewater. In

case of mutant strain growing in soybean and poultry wastewater, a dose-dependent increase in the growth was observed up to 40% of soybean wastewater and 60% dilution of poultry wastewater, as compared to the control (TW) grown cells. At respective optimum doses of soybean and poultry wastewater growth of mutant was enhanced by 130% and 175%, respectively as compared to control (100%). A further increase in the optimum doses (40% and 60% respectively) in the soybean and poultry wastewater resulted in a sharp decline in the growth of mutant strain. The growth of mutant strain was completely arrested at 80% dilution of both wastewater. These results showed that poultry wastewater was is a better source of nutrient than the soybean wastewater for both WT and mutant strain. Further, the mutant strain was found to be more responsive and better tolerant to the soybean and poultry wastewater as evident from overall increase in the growth supported by SWW and PWW.

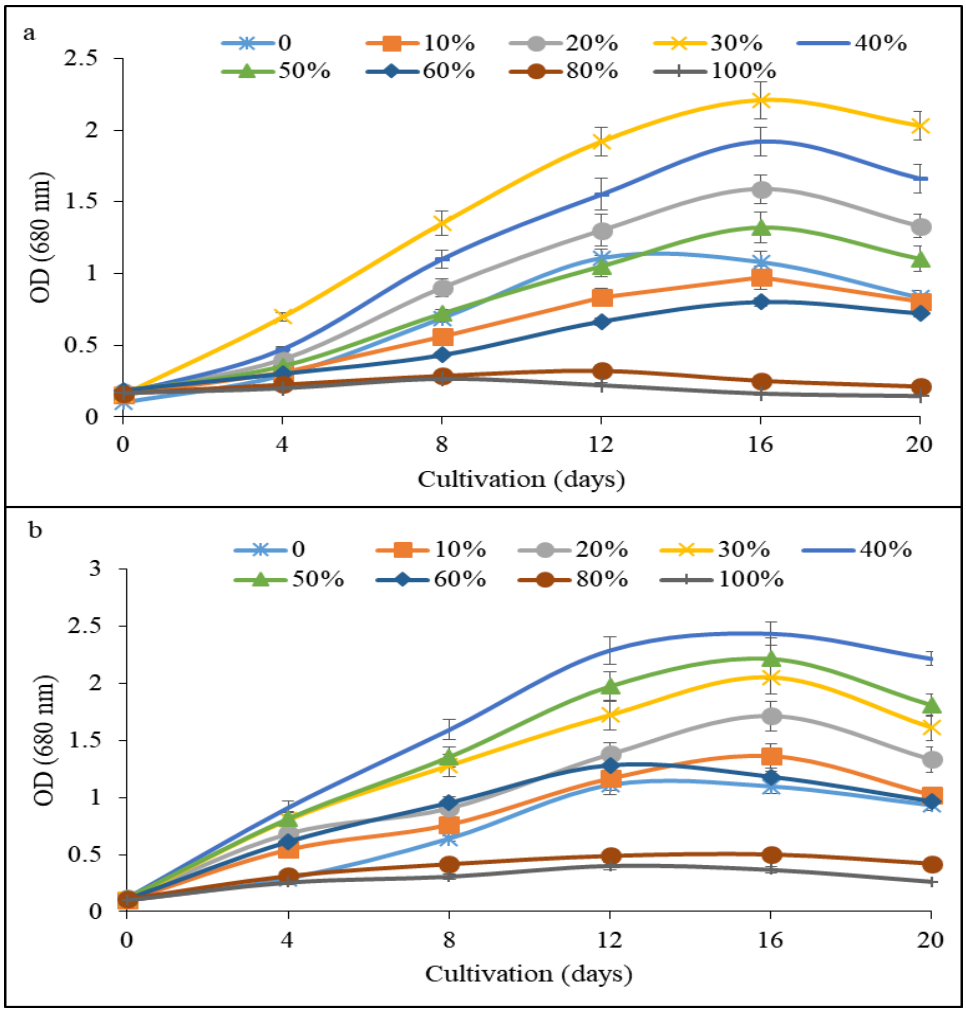

Fig. 7.1 Optimisation of growth of wild-type of microalgae *S. vacuolatus* at different concentration of wastewater (a) Soybean wastewater (SWW) (b) Poultry wastewater (PWW) compared to control (Tap water (TW)). Data depicted as mean±SD.

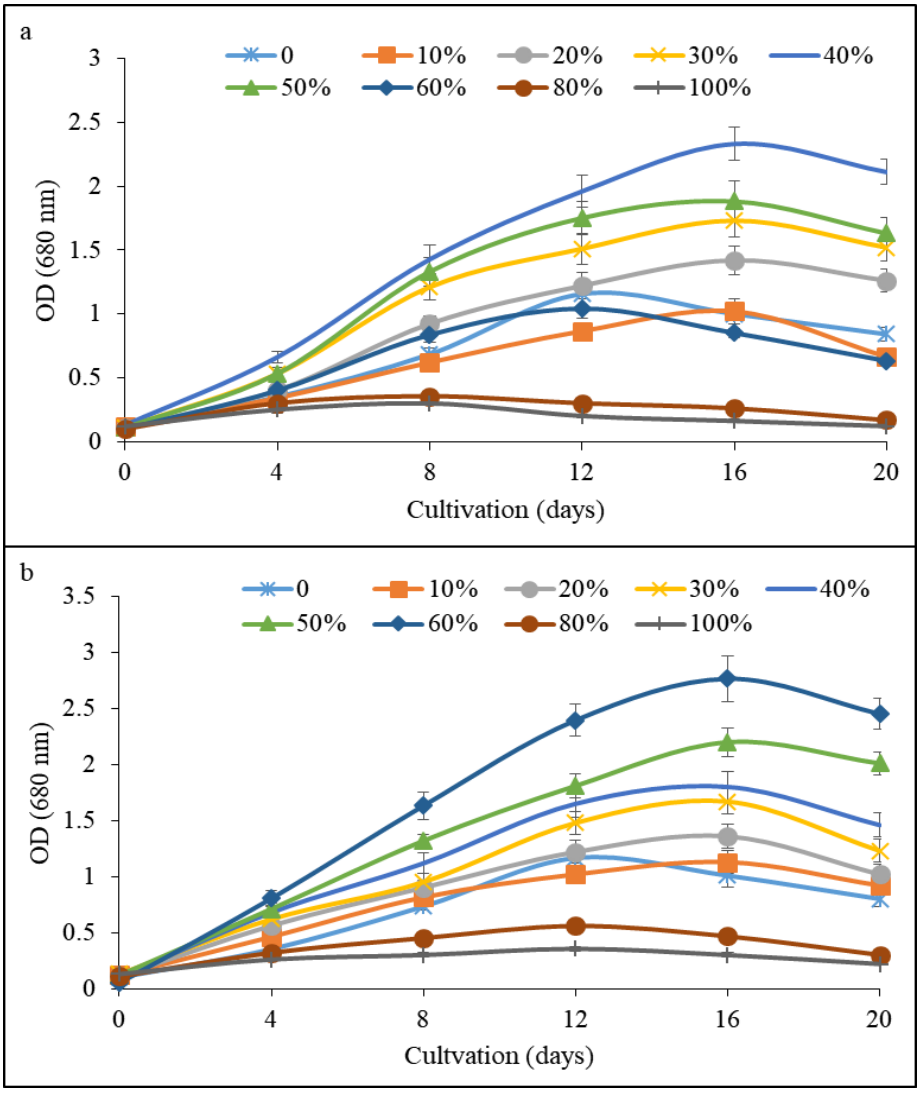

Fig. 7.2 Optimisation of growth of mutant strain of microalgae *S. vacuolatus* at different concentration of wastewater (a) Soybean wastewater (SWW) (b) Poultry wastewater (PWW) compared to control (Tap water (TW)). Data depicted as mean±SD

7.3.2 Effect of soybean and poultry wastewater on biomass, biochemical constituents and pigment content

The dry biomass (g/L) of both WT and mutant strain were measured in response to the optimum strength of soybean and poultry wastewater. The results were compared with the biomass obtained during their growth in the presence of tape water (TW) and BG-11 medium (Fig. 7.3). The result showed that the biomass of both WT and mutant strain increased upto 16 days in the presence of soybean and poultry wastewater. Both WT and mutant strain showed maximum biomass growth in the presence of poultry

wastewater, followed by soybean wastewater, BG-11 and TW. The lowest biomass was recorded in the presence of TW. Both soybean wastewater and poultry wastewater proved to be a better source of nutrient for biomass production than BG-11 and TW.

Biomass productivity in the WT cells grown on poultry wastewater (0.14±0.005 g L^{-1} d^{-1}) was marginally higher than the soybean wastewater grown WT cells (0.12±0.008 g L^{-1} d^{-1}), whereas the biomass productivity in mutant strain was better in poultry wastewater (0.16±0.012 g L^{-1} d^{-1}) than soybean wastewater (0.13±0.01) grown cells (Table 7.1). Based on the result it could be concluded that soybean wastewater and poultry wastewater rich in the organic source can be utilised in a better way by both WT and mutant strain.

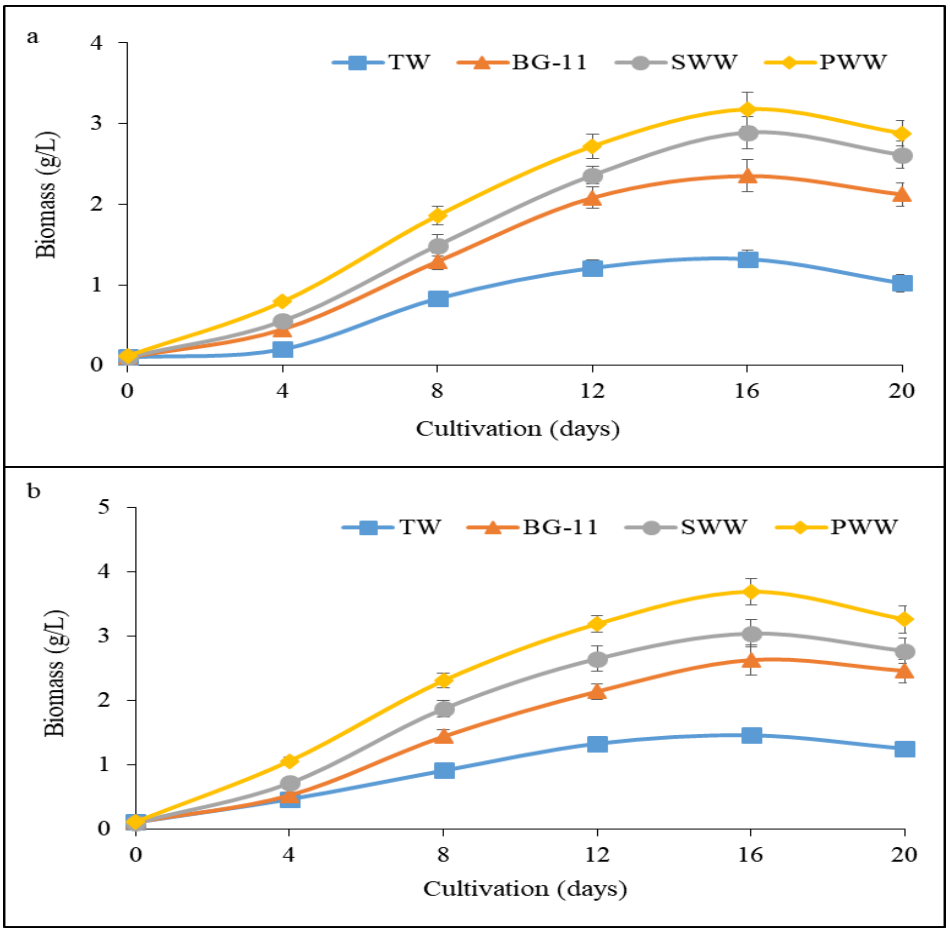

Fig. 7.3 Biomass of microalgae *S. vacuolatus* of wild-type (a) and mutant strain (b) in the presence of tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW) of cultivation days. Data depicted as mean±SD.

Result on total organic carbon (TOC) content in the presence of respective optimum strength of soybean wastewater and poultry wastewater exhibited that TOC

content of mutant strain than the WT. In the soybean wastewater, the TOC value in both WT (4.7± 0.75 mg L^{-1}) and mutant strain (6.5±0.39 mg L^{-1}) exhibited about 6-9 fold greater than the TOC value in the WT (0.69±0.03 mg L^{-1}) and mutant (1.0±0.07 mg L^{-1}) grown on TW. Maximum TOC content in both WT and mutant strain grown on poultry wastewater (7.5±0.8, 9.8±0.5 mg L^{-1} respectively) was found to be higher than that obtained in the BG-11 grown WT (3.2±0.2 mg L^{-1}) and mutant cells (4.6±0.3 mg L^{-1}). These results indicated a significant increase in TOC content of both the strains when grown on the soybean and poultry wastewaters (Fig. 7.4). However, poultry wastewater was found to be a better source of organic carbon than soybean wastewater. Besides, mutant strain exhibited better growth and TOC accumulation in the presence of SWW and PWW than the WT strain.

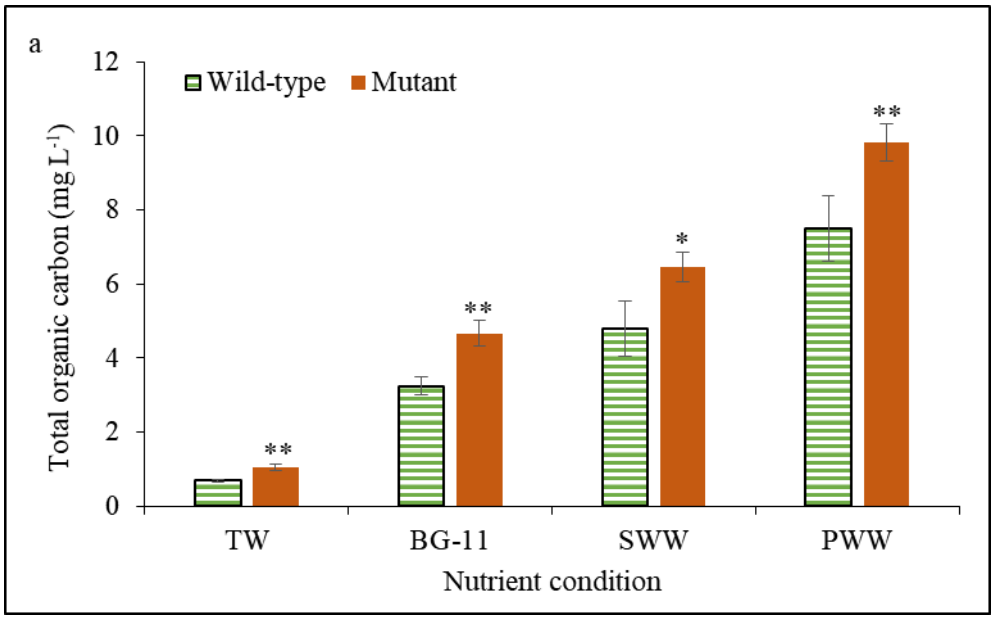

Fig. 7.4 Total organic carbon content of microalgae *S. vacuolatus* of wild-type and mutant strain in the presence of tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW). Student't' test showing the significant difference level ($p<0.05$) between WT and mutant strain. Data depicted as mean±SD.

Similar results were obtained for the carbohydrate and protein content when both the strains were grown in the presence of soybean and poultry wastewater. Both the carbohydrate and protein content were enhanced in the poultry wastewater grown cells of WT (160±13, 176±12 µg mL^{-1} respectively) and mutant strain (182±14, 206±14 µg mL^{-1} respectively) and lowest carbohydrate and protein content was observed in both WT (97±4.5, 102±8.5 µg mL^{-1} respectively) and mutant strain (122±4.5, 123±7.5

µg mL^{-1} respectively) grown on TW (Fig. 7.5 a, b). Both soybean and poultry wastewater supported the synthesis of carbohydrate and protein in both the strains better than the BG-11 and TW. These results suggested that accumulation of carbohydrate and protein by mutant strain in response to soybean and poultry wastewater was significantly higher than that in WT.

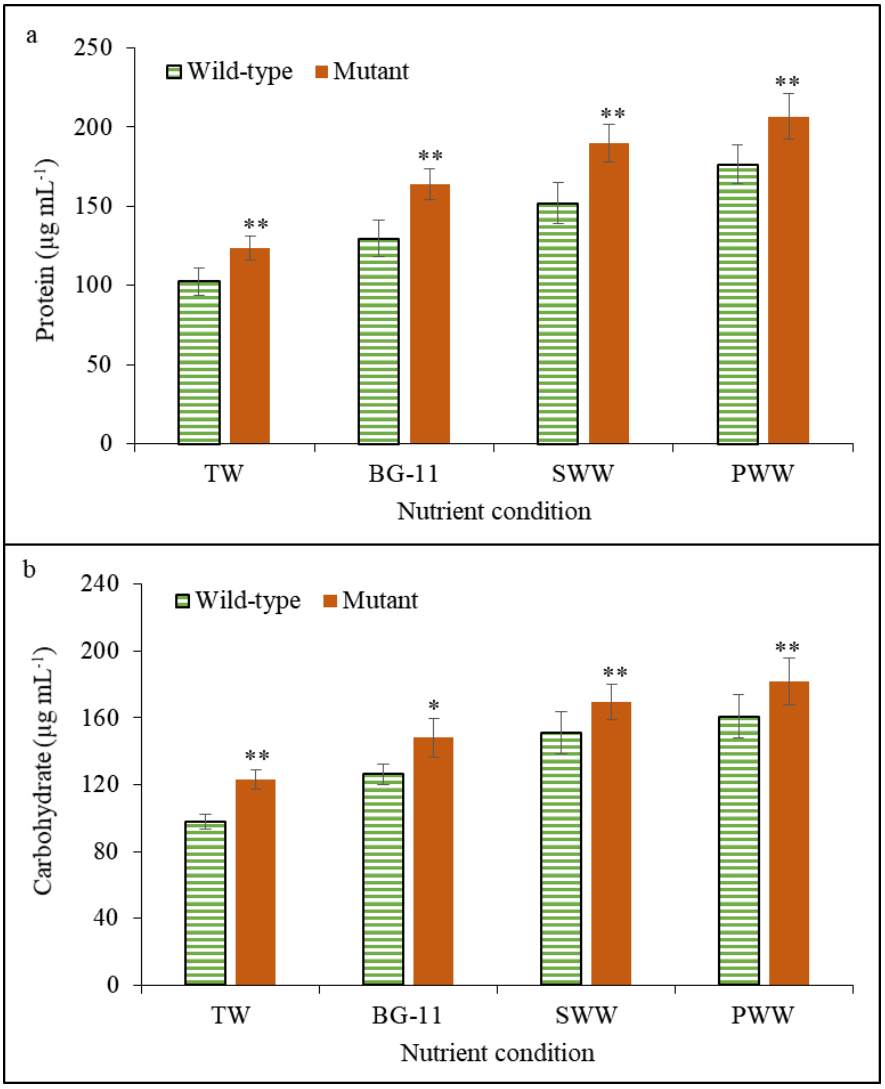

Fig. 7.5 Protein (a) and Carbohydrate (b) content of microalgae *S. vacuolatus* of wild-type and mutant strain in the presence of tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW). Student't' test showing the significant difference level ($p<0.05$) between WT and mutant strain. Data depicted as mean±SD.

In the soybean grown cells of WT (7.3±0.5 µg mL^{-1}) and mutant strain (12.2±0.70 µg mL^{-1}) were showed about 3 fold higher chlorophyll content when compared with chlorophyll content in the respective strain grown on tap water (2.1±0.18, 3.2±0.2 µg mL^{-1} respectively). However, poultry wastewater supported the

better synthesis of chlorophyll in both WT (10.2±1.0 µg mL^{-1}) and mutant strain (14.7±0.6 µg mL^{-1}), followed by SWW, BG-11 and TW (Fig. 7.6 a, b). Similar results were obtained on carotenoid content of both WT and mutant strain. The synthesis of carotenoid was found to be the highest in both WT (3.8±0.35 µg mL^{-1}) and mutant strain (5.5±0.6 µg mL^{-1}) when grown on the poultry wastewater supplemented condition followed by SWW, BG-11 and TW. However, the pigments were always significantly higher in the mutant strain than the WT under different nutritional conditions.

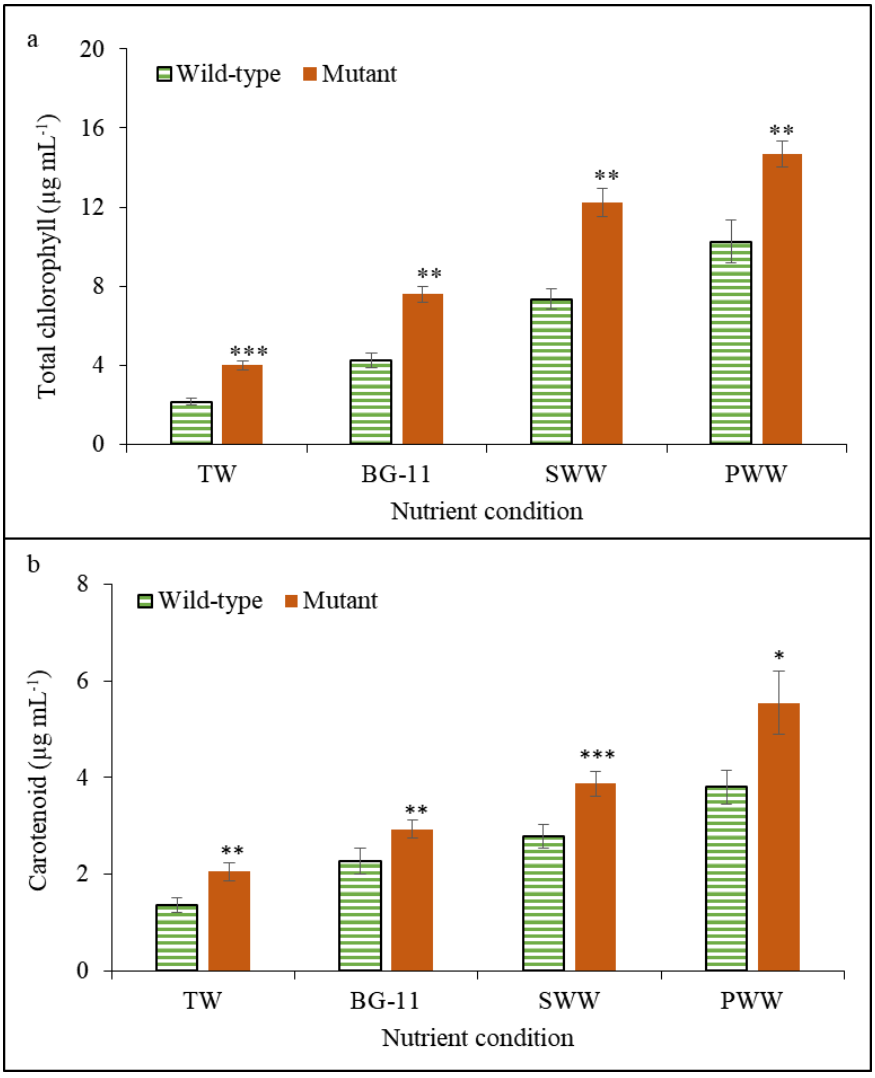

Fig. 7.6 Estimation of total chlorophyll (a) and carotenoid (b) content of microalgae *S. vacuolatus* of wild-type and mutant strain in the presence of tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW). Student't' test showing the significant difference level ($p<0.05$) between WT and mutant strain. Data depicted as mean±SD.

7.3.3 Estimation of the lipid content and biodiesel

The lipid content was higher in the mutant cells than the WT under both poultry and soybean wastewater supplemented conditions. The lipid productivity in the PWW grown mutant strain (30.0%) higher than the PWW grown WT cells (25.0%). The biodiesel in the PWW wastewater grown mutant strain (13.7%) was also found to be higher than that the WT (10.8%) cells when grown on the PWW. However, the maximum lipid productivity and biodiesel was recorded in both the strain in the order of PWW, followed by SWW, BG-11 and TW (Table 7.1).

Table 7.1 Different parameters values of Biomass productivity, lipid content (%), lipid productivity, and biodiesel (%) of microalgae *S. vacuolatus* of wild-type and mutant strain at different nutrient conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW). Data depicted as mean±SD.

	Wild-type				Mutant			
	TW	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Biomass productivity (g L^{-1} d^{-1})	0.046± 0.003	0.10±0.007	0.12±0.008	0.14±0.005	0.057± 0.001	0.11±0.006	0.13±0.01	0.16±0.012
Lipid content, (%)	7.0	17.2	23.6	25.0	8.0	23.1	25.2	30.0
Lipid productivity (g L^{-1} d^{-1})	0.011± 0.007	0.098± 0.005	0.17±0.012	0.2±0.015	0.015± 0.008	0.16±0.010	0.21±0.015	0.33±0.011
Biodiesel (%)	4.7	7.8	8.6	10.8	5.1	9.6	10.6	13.7

7.3.4 Flow cytometer analysis

The flow cytometry analysis of lipid content measured by using Nile red (Fig. 7.7 a, b) showed maximum lipid content in the poultry wastewater grown mutant strain (28.01%) which was higher than the poultry wastewater (25.32%) grown WT cells. Similarly, the second-highest lipid content was observed in the soybean wastewater grown WT (20.01%) and mutant (23.70%) cells, which were higher than the corresponding value of lipid content of the WT (7.71%) and mutant (11.61%) grown on TW. Thus it was concluded that synthesis of lipid in both the strains was maximally supported by PWW, followed by SWW, BG-11 and TW. However, under all the nutritional conditions the overall lipid content in the mutant was found to be higher than the WT.

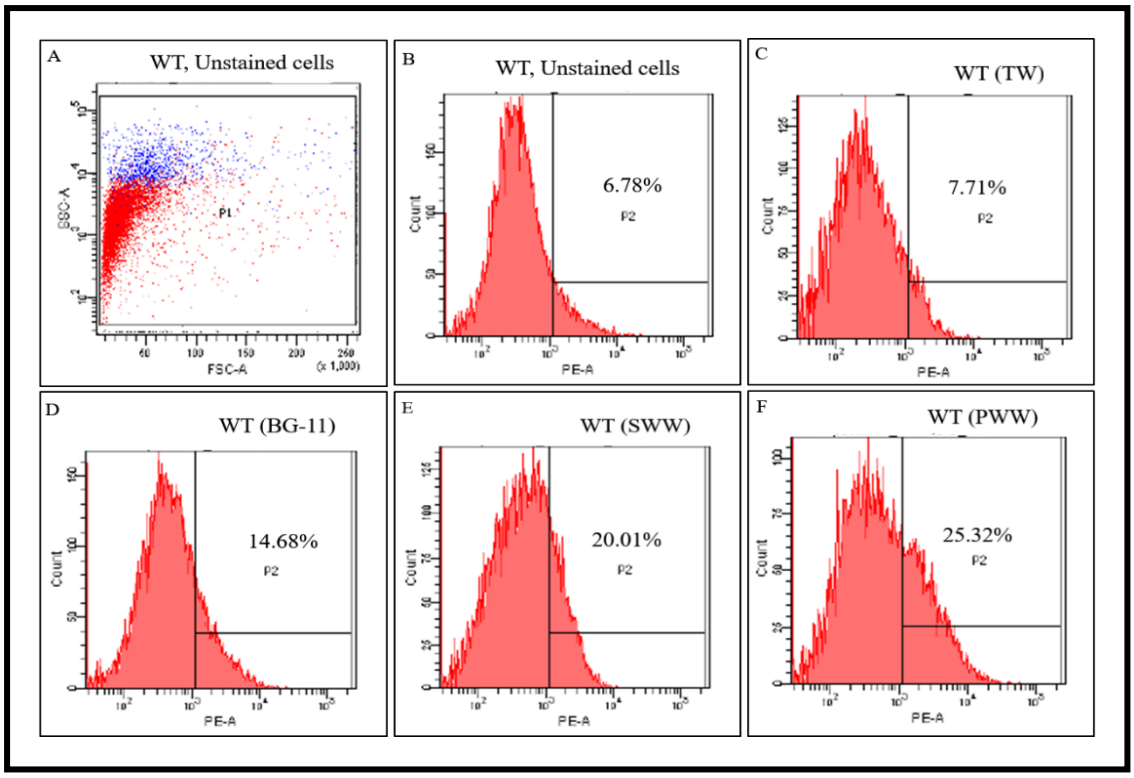

Fig. 7.7 (a) Flow cytometer analysis of lipid content of microalgae *S. vacuolatus* of wild-type cells under varying nutrition conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW) by using Nile red.

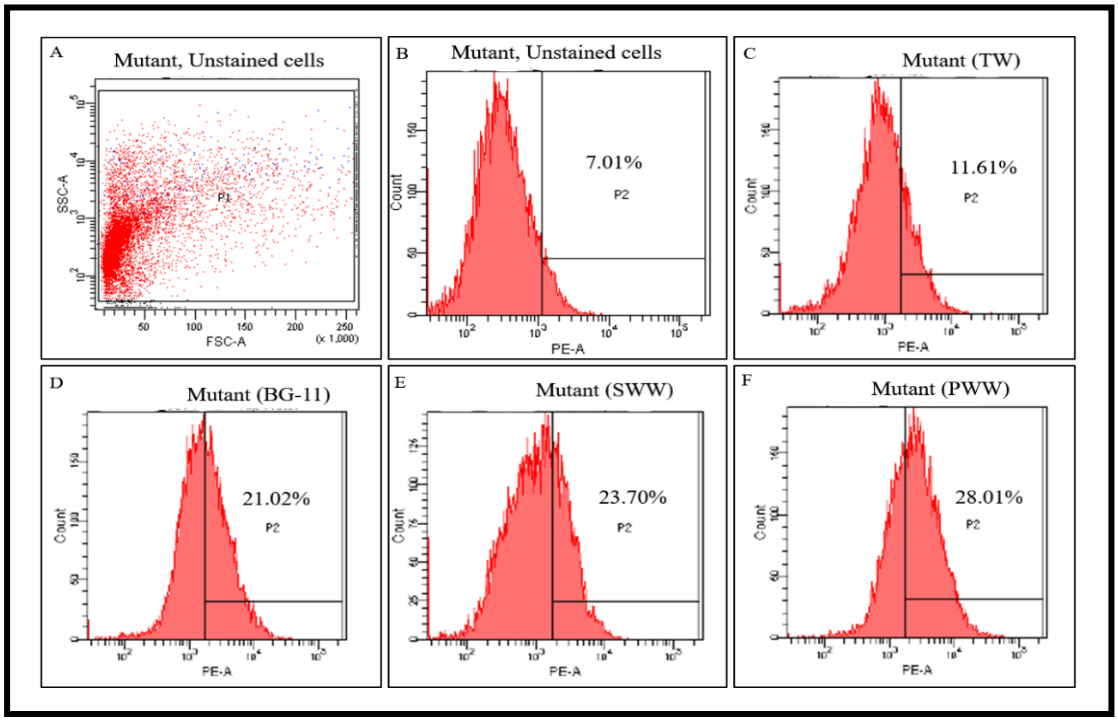

Fig. 7.7 (b) Flow cytometer analysis of lipid content of microalgae *S. vacuolatus* of mutant strain cells under varying nutrition conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW) by using Nile red.

7.3.5 Biomass settling and recovery

Biomass settling and recovery efficiency (η) value was evaluated at different temperatures (10, 25, 50 ℃) in both WT and mutant cells grown on under poultry and soybean wastewater for 20 days. (Fig. 7.8) Result showed higher biomass recovery efficiency in the soybean wastewater grown WT cells than the mutant under all the temperature condition. However, settling efficiency of both strains was found to be maximum at 10℃, followed by 25 & 50℃. The settling efficiency of both the strain increased with increase in the temperature form 10 & 15℃, in case of PWW grown cells of both strains.

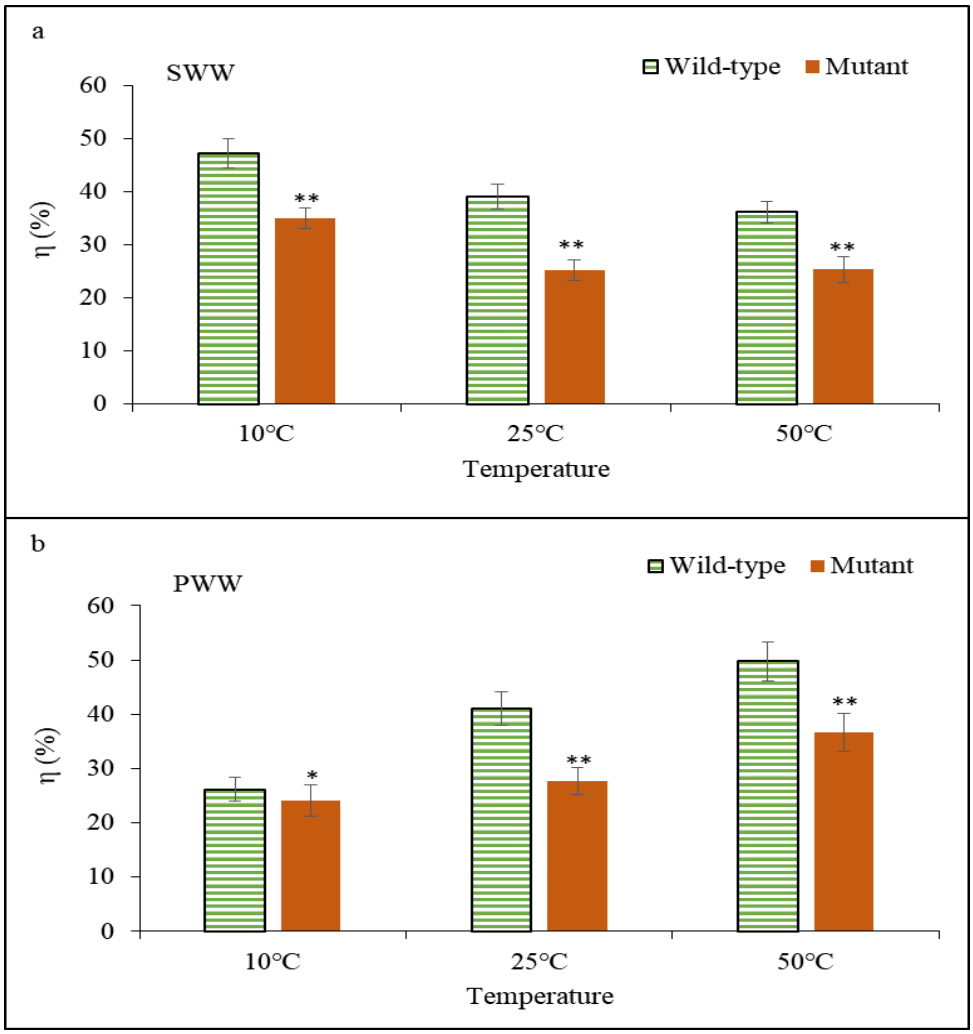

Fig. 7.8 Biomass recovery efficiency (η) evaluated at different temperature (10-50℃) after 20 days of growth (a) soybean wastewater (SWW) (b) poultry wastewater (PWW) under the different temperature of wild-type and mutant strain. Student‘t’ test showing the significant difference level ($p<0.05$) between wild-type and mutant strain. Data depicted as mean±SD.

The WT cells exhibited higher settling ability than the mutant strain. A comparison of WT and mutant cells with respect to others differential settling rate, it was concluded that faster settling of WT might be dependent on the larger size of the WT cells as compared to the smaller size in the mutant exhibiting low settling efficiency. The response of the poultry wastewater grown both WT and mutant cells at higher temperature 50℃ might be interpreted in terms of higher lipid content and fluidity of lipid in the mutant strain resulting into clump formation and faster settling in the mutant cells.

Biomass settling and recovery efficiency (η) of both WT and mutant strain grown in soybean wastewater under different pH conditions (4.0, 7.0, and 10.0) was measured (Fig. 7.9).

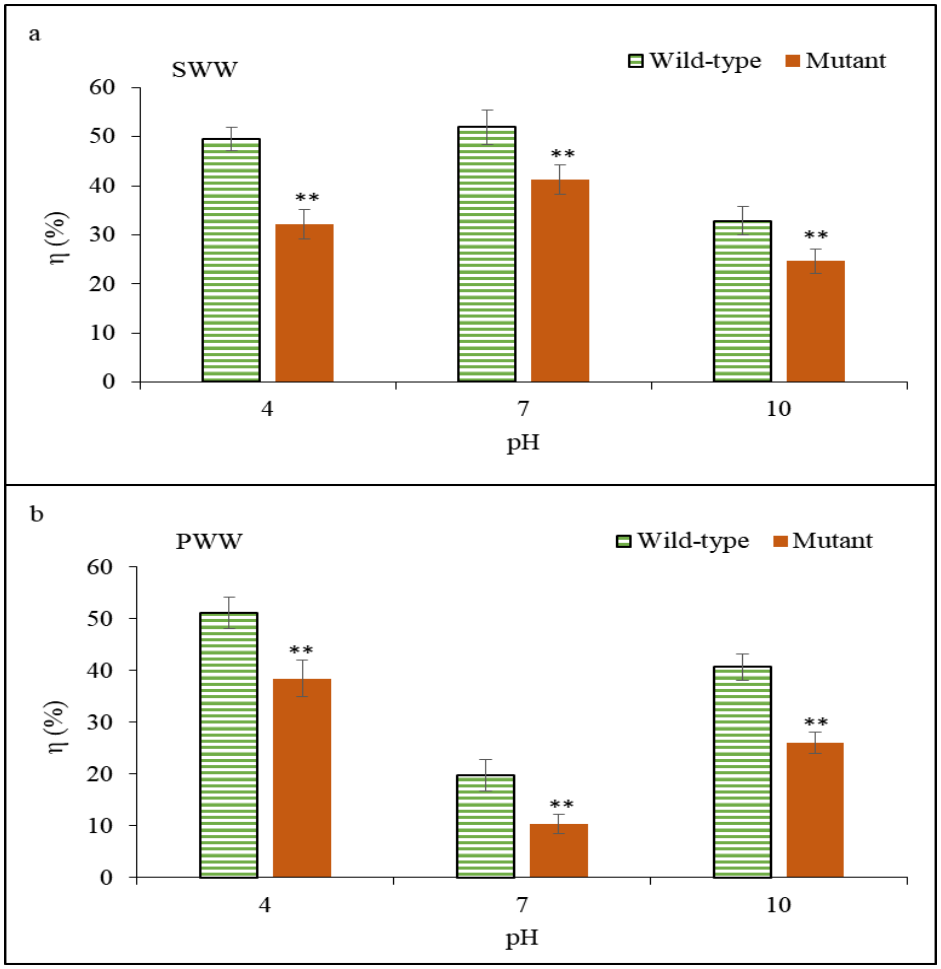

Fig. 7.9 Biomass recovery efficiency (η) under different pH conditions (4, 7,10) after 20 days of growth (a) soybean wastewater (SWW) (b) poultry wastewater (PWW) under varying pH condition of wild-type and mutant strain. Student‘t’ test showing the significant difference level ($p<0.05$) between wild-type and mutant strain. Data depicted as mean±SD.

The result initially showed higher settling rate at pH 4.0 & 7.0 in both WT and mutant strain. However the settling efficiency of both WT and mutant strain at pH 10.0 declined, it was perhaps due to slower growth and different cells size of both the strains. In the case of poultry wastewater grown WT and mutant cells, the biomass recovery efficiency was the highest at pH 4.0, followed by pH 10.0 and 7.0. In both the strain the biomass recovery efficiency was lowest at pH 7.0. The results showing minimum biomass recovery efficiency at pH 7.0 and the highest biomass recovery efficiency at pH 10.0 might be interpreted in terms of adverse pH condition leading to cell clumping formation. However, the results revealed that biomass settling efficiency in the WT strain was higher than that mutant strain.

7.3.6 Physico-chemical characteristics of soybean and poultry wastewater

7.3.6.1 Microalgal treatment of soybean wastewater (SWW)

Maximum growth supporting concentration of soybean wastewater (SWW) (30%, v/v) for WT and (40%, v/v) for mutant was taken for growth. The water quality parameters were measured on 0, 7^{th} and 15^{th} days in terms of pH, BOD, COD, nitrate, nitrite, ammonium, phosphate, alkalinity, hardness, total dissolved solid (TDS), total suspended solid (TSS) etc. (Table 7.2). The two separate sets of soybean wastewater were taken for the growth of WT (30%, v/v) and mutant strain (40%, v/v). The time-dependent measurement of water quality parameters showed a change in the initial odour of soybean wastewater from light pungent smell to algal smell after 15 days of growth. The colour of the soybean wastewater also changed from mustard-yellow brown to light brown after algal growth for 15 days (Table 7.2). The result on changes in pH on the soybean wastewater showed a time-dependent gradual increase in the pH from 7.2 to 9.7 in both the cases. The total dissolved solid (TDS) and total suspended solid (TSS) in case of both WT and mutant strain inoculated wastewater declined by 50-60% after 15 days of growth. Further, the result showed that ammonium, nitrate and nitrite content depleted faster in both SWW and PWW after 15 days of growth of both WT and mutant strain. There was about approximately 89% reduction in ammonium content whereas nitrate and nitrite content declined by approximately 30-60% after 15 days of growth. However, nitrate and nitrite removal by mutant strain was substantially higher than the WT. BOD and COD content of both SWW and PWW after 15 days of

growth of WT and mutant strain declined by about 80-89% and 60-65%, respectively. Similarly, the removal of phosphate content by the WT and mutant strain from soybean wastewater after 15 days of growth showed 60-70% decrease in the phosphate content. Similarly, the hardness, alkalinity and chloride content were substantially reduced by WT and mutant strain to the extent of 70-80% in both SWW and PWW (Fig. 7.10, a). However, removal of sulphate by both the strains was approximately 40-45% which was less than the removal of phosphate from the wastewater. The overall results indicated that the removal of different nutrients and improvement in the water quality was better in case of the mutant strain than the WT.

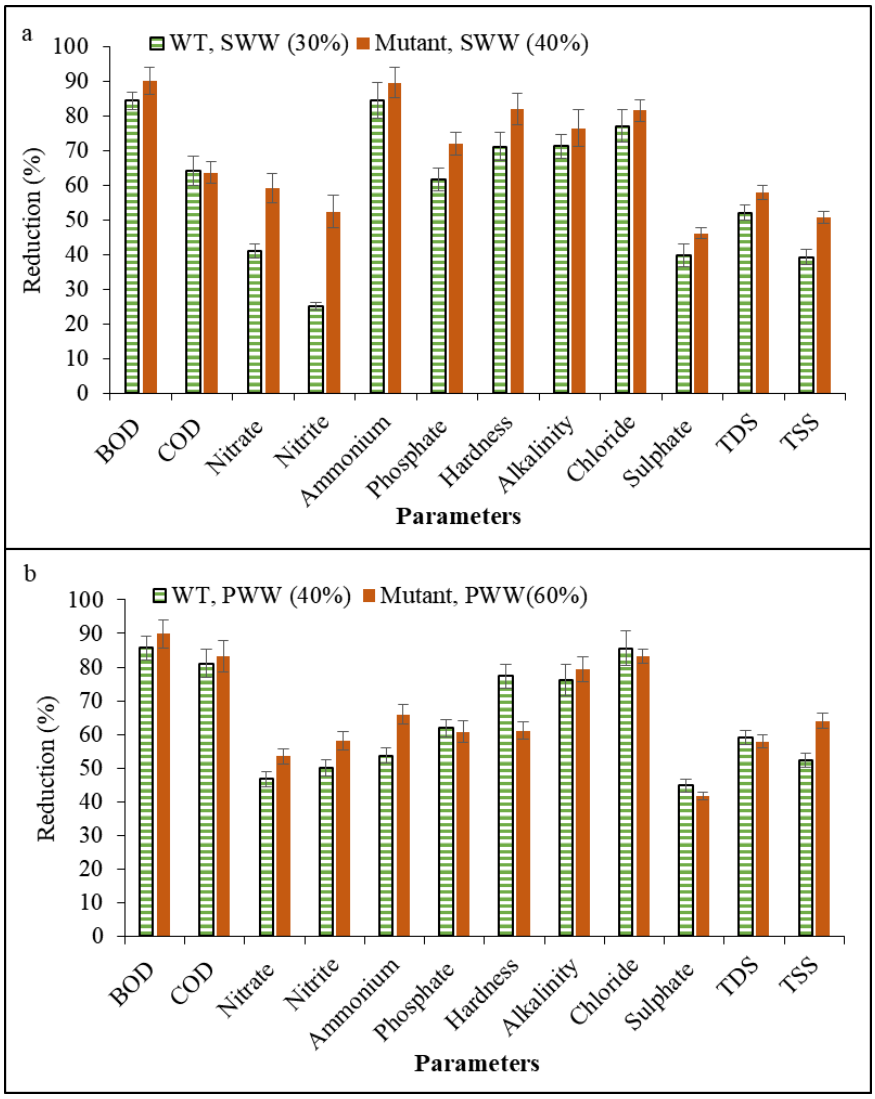

Fig. 7.10 Reduction (%) of pollutant load of soybean wastewater (SWW) (a) and poultry wastewater (PWW) (b) of microalgae *S. vacuolatus* of wild-type (SWW 30% and PWW 40%) and mutant strain (SWW 40% and PWW60%). Data depicted as mean±SD.

7.3.6.2 Microalgal treatment of poultry wastewater (PWW)

Treatment of poultry wastewater (PWW) by the WT (40% v/v) and mutant strain (60% v/v) was carried out separately and water quality parameters recorded on 0, 7^{th}, and 15^{th} days after inoculation. The odour of the effluent changed from pungent to algal smell and colour changed from dark brown to light brown during the poultry wastewater treatment by both WT and mutant strain (Table 7.3). The pH of the poultry wastewater gradually increased in a time-dependent manner from 7.2-10.0 pH after 15 days of treatment, TDS and TSS were reduced by approximately 60% in the poultry wastewater by the both the WT and mutant strain after 15 days of treatment, when compared with corresponding value of parameters on zero-day (Fig. 7.10, b). The removal of ammonium, nitrate and nitrite from SWW and PWW both WT and mutant strain were found in the range of 50-70% as compared to respective parameters on zero-day. However, the value of BOD and COD of the poultry wastewater declined by approximately 80-90%. The hardness, alkalinity and chloride were also reduced to the extent of 60-80% by both the strains. The removal of phosphate from the wastewater by both the strain was approximately in the range of 55-60%, which was higher than the removal of sulphate 40-45%. The overall performance of the mutant strain in the removal of nutrients and improvement in the water quality was found to be better in mutant strain than the WT except for chloride, sulphate and phosphate.

Table 7.2 Physico-chemical characteristics of soybean wastewater (SWW) during *S. vacuolatus* cultivation (0, 7th, and 15th days) of wild-type (30% v/v dilution) and mutant strain (40% v/v dilution). Data depicted as mean ± SD.

Wild-type, soybean wastewater -30%				**Mutant, soybean wastewater -40%**			**EPA 1996**
Wastewater parameters	**0th**	**7th**	**15th**	**0th**	**7th**	**15th**	**Inland Surface water**
Odour	Extremely pungent	Light pungent	Light algal smell	Extremely pungent	Light pungent	Light algal smell	-
Colour	Mustard yellow-brown	Light brown	Light brown	Mustard yellow-brown	Light brown	Light brown	-
pH	7.2±0.2	8.0±0.2	9.7±0.2	7.2±0.2	8.2±0.2	9.7±0.2	5.5-9.0
BOD (mg/L)	560±42	260±12	88±4.5	600±26	206±8.8	60±2.1	30
COD (mg/L)	6400±235	4110±210	2300±150	7000±361	4500±210	2550±122	250
Nitrate (mg/L)	22±1.0	18±7.6	13±1.3	22±1.1	17±11	9±0.6	10
Nitrite (mg/L)	20±1.5	17±8.6	15±0.16	21±1.6	16±11	10±0.01	-
Ammonium (mg/L)	0.9±0.05	0.22±0.01	0.14±0.01	0.96±0.036	0.2±0.01	0.1±0.008	5.0
Phosphate (mg/L)	180±06	120±4.6	69±3.5	200±15	126±07	56±1.3	5.0
Hardness (mg/L)	4.5±2.8	2.9±0.16	1.3±1.1	6.0±02	4.1±2.2	1.1±0.01	200
Alkalinity (mg/L)	7080±321	5540±354	2036±177	7780±336	3840±205	1840±102	-
Chloride (mg/L)	1309±96	749±214	300±26	1349±125	509±33	249±14	300
Sulphate (mg/L)	123±6.6	96±6.6	74±4.2	126±06	94±6.6	68±3.2	1000
Total dissolved solid (TDS) (mg/L)	2100±155	1470±86	1008±76	2150±114	1558±86	908±52	500
Total suspended solid (TSS) (mg/L)	130±86	104±4.8	79±4.6	138±08	106±5.2	68±4.6	100

Table 7.3 Physico-chemical characteristics of poultry wastewater (PWW) during *S. vacuolatus* cultivation (0, 7th, and 15th days) of wild-type (40% v/v dilution) and mutant strain (60% v/v dilution). Data depicted as mean ± SD.

Wild-type, poultry wastewater-40%			**Mutant, poultry wastewater-40%**			**EPA 1996**
Wastewater parameters	**0th**	**7th**	**15th**	**0th**	**7th**	**15th**	**Inland Surface water**
Odour	Pungent	Light pungent	Algal smell	Pungent	Light pungent	Algal smell	-
Colour	Dark brownish-grey	Light brown	Light brown	Dark brownish-grey	Light brown	Light brown	-
pH	7.2±0.2	8.8±0.2	10±0.2	7.8±0.2	8.5±0.2	9.8±0.2	55-9.0
BOD (mg/L)	716±22	370±12	102±5.0	870±6.3	303±12	88±5.6	30
COD (mg/L)	5500±302	2700±106	1040±88	6000±468	3220±122	1002±63	250
Nitrate (mg/L)	15±11	10±5.0	8.1±63.6	17±2.0	11±0.6	8.0±6.6	10
Nitrite (mg/L)	30±12	22±1.6	15±0.1	31±3.0	20±1.6	13±1.2	-
Ammonia (mg/L)	0.561±0.02	0.355±0.02	0.26±0.32	0.561±0.03	0.24±0.2	0.191±0.01	5
Phosphate (mg/L)	186±11	113±6.0	71±0.56	209±12	121±6.3	82±4.6	10
Hardness (mg/L)	8.1±2.3	3.96±1.6	1.84±0.13	9.0±0.5	5.4±3.3	3.5±1.2	200
Alkalinity (mg/L)	4053±163	2621±187	965±8.2	5143±365	3380±251	1060±76	-
Chloride (mg/L)	2209±165	1009±66	319±22	2329±112	1109±86	389±22	300
Sulphate (mg/L)	68±2.6	50±26	38±4.0	73±6.6	62±5.1	43±4.0	1000
Total dissolved solid (TDS) (mg/L)	2500±156	1410±86	1022±88	2800±176	1908±110	1180±76	500
Total suspended solid (TSS) (mg/L)	180±7.2	128±09	86±9.1	200±11	100±5.6	72±4.3	100

7.3.7 FTIR analysis

7.3.7.1 Biomass analysis

FTIR spectra of the biomass of both WT and mutant strains were grown in the presence of tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) were recorded in the range of 4000-400 cm^{-1} (Table 7.4). The IR spectrum recorded between 2850-2930 cm^{-1} denotes the methyl and methylene group of lipid and carbohydrate. The IR absorbance at 1740 cm^{-1} is related to stretching of esters bond of fatty acids, whereas IR absorbance at 1650 & 1550 cm^{-1} is assigned to be amide I and amide II bands of protein. The spectral region 1300-1450 cm^{-1} indicates the bending of methyl and carboxylic groups related to protein and lipid whereas IR absorbance 1040-1150 cm^{-1}, represents the presence of polysaccharide carbohydrate. When the both WT and mutant strain were grown in the presence of tape water (TW), amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) peaks were highly suppressed. The lipid content was found to be reduced as indicated by absorbance by 1380 to 1420 cm^{-1}. However, moderate accumulation of carbohydrate in both the strain was evident from the absorbance peak 1040-1120 cm^{-1}. The FTIR spectra of mutant and WT cell biomass grown in poultry wastewater showed a prominent peak at 1650 & 1545 cm^{-1} due to enhanced synthesis of protein content. The carbohydrate accumulation was also higher as indicated by IR absorption between 1050-1056 cm^{-1} wavenumbers (Table 7.4). The IR peak 2927 & 2855 cm^{-1} were more pronounced in poultry wastewater grown cells of mutant than that in the WT. Similarly, the IR peak related to lipid at 1385 cm^{-1} in the poultry wastewater grown mutant cells was distinguishable, as compared to the corresponding peak in the poultry wastewater grown WT cells. The IR spectrum of WT and mutant strain grown in soybean wastewater exhibited almost similar IR spectra. The carbohydrate accumulation in both the strain as indicated by the spectrum between 1040-1100 cm^{-1}, were reduced when compared with corresponding IR absorption in both strains TW grown cells. The soybean grown WT and mutant cells exhibited prominent IR absorption at 1383 cm^{-1}, indicating greater accumulation of carboxylate polymers such as lipid. However, protein content in both the strains was substantially suppressed as evident from the IR peak for amide I and amide II of protein at 1645 & 1550 cm^{-1}, respectively. The overall results indicated less accumulation of protein as compared to the lipid and carbohydrate in soybean wastewater grown cells biomass both mutant and WT (Fig. 7.11 and 7.12).

Table 7.4 FTIR spectra absorption peak (wavenumber) of dry biomass of wild-type and mutant strain of *S. vacuolatus* showed changes in biochemical constituents under varying nutrition conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW).

Wavenumber (absorbance peak) showed changes in biochemical constituents of wild-type and mutant strain of *S. vacuolatus* under varying nutrition conditions									
	Wild-type				**Mutant**				
S. No.	**Tap water (TW)**	**BG-11**	**Soybean wastewater (SWW)**	**Poultry wastewater(PWW)**	**Tap water (TW)**	**BG-11**	**Soybean wastewater (SWW)**	**Poultry wastewater (PWW)**	**Band assignment**
1	3434	3432	3448	3433	3433	3438	3449	3429	Water *ν*(O-H) stretching Protein *ν*(N-H) stretching
2	2935 2850	2821	2848	2927	2818	2926	2929	2925 2855	Lipid – carbohydrate, *vas*(CH_2) and *vs*(CH_2) stretching
3	-	-	-	-	-	-	-	-	fatty acids *ν*(C=O) stretching of esters
4	1656	1651	1644	1652	1651	1649	1646	1652	Protein amide I band (C=O) stretching
5	1548	1555	1547	1548	1554	1543	1552	1546	Protein amide II band, *δ*(N-H) bending and *ν*(C-N) stretching
6	1446 1384	1409 1313	1383	1411	1413	1407	1383	1451 1385	Protein *δs*(CH_2) and *δs*(CH_3) bending of methyl carboxylic acid *vs*(C-O) of COO- groups of carboxylates Lipid *δs*($N(CH_3)_3$) bending of methyl
7	1059	1060	1097	1051	1060	1081	1101	1063	Carbohydrate *ν*(C-O-C) of polysaccharides, Nucleic acid, *vs*(>P=O) stretching of phosphodiesters

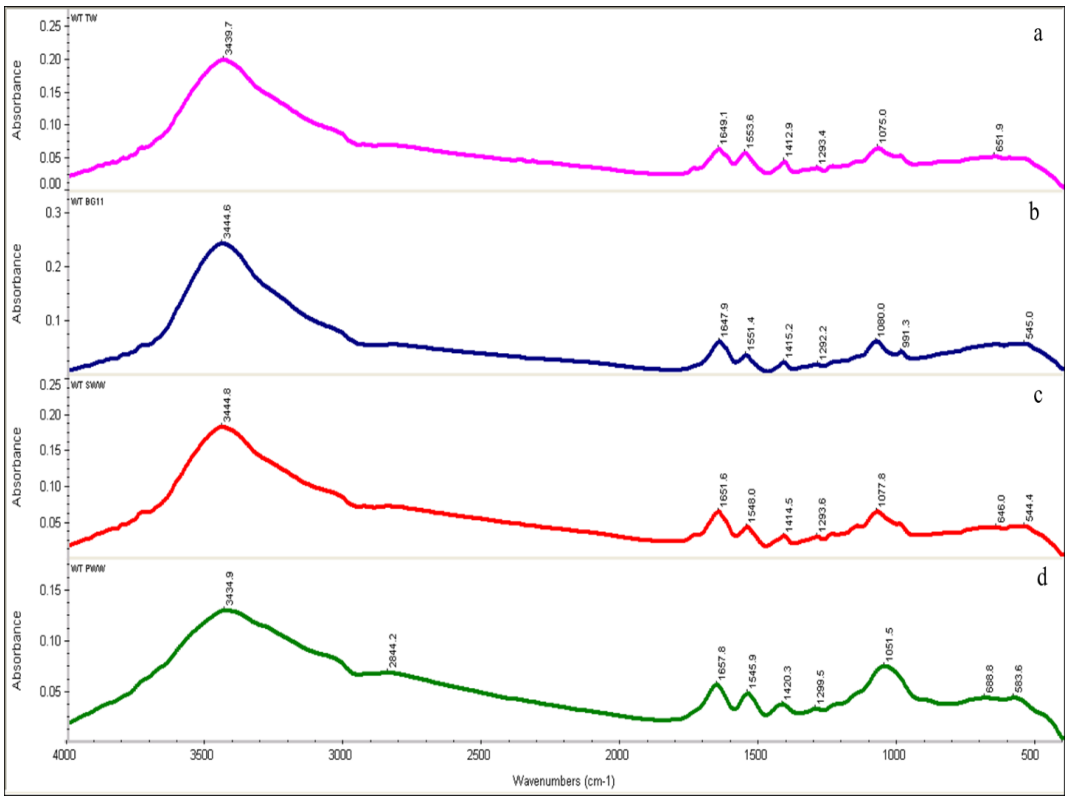

Fig 7.11 FTIR spectra of dry biomass of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions TW (a) BG-11 (b) SWW (c) and PWW (d).

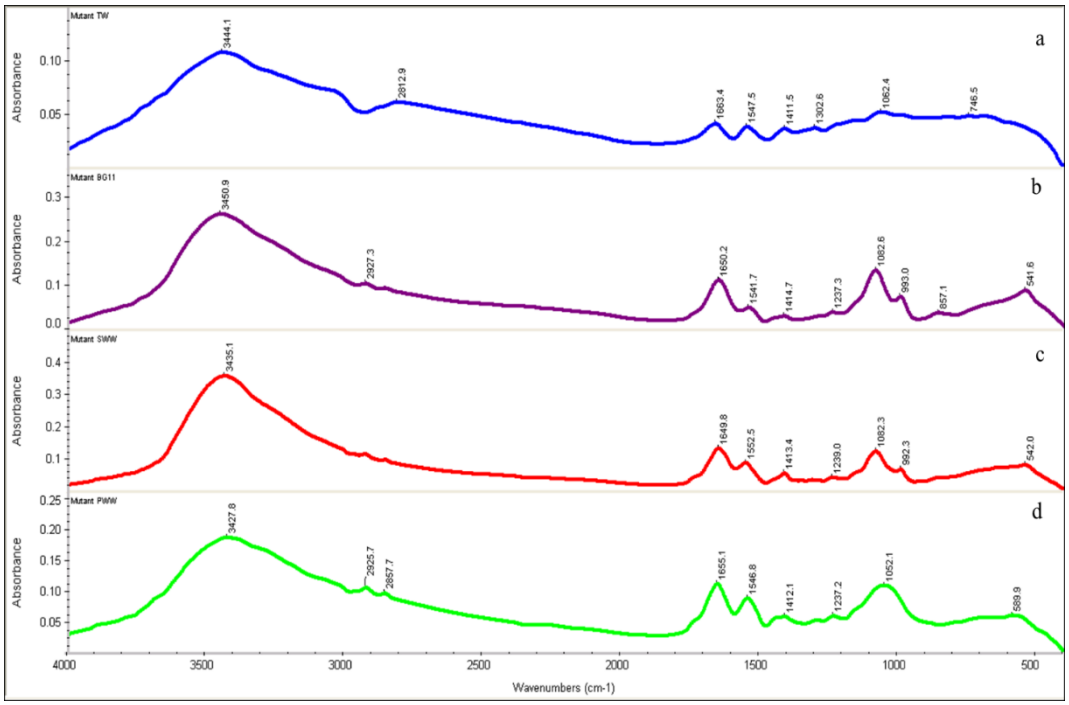

Fig 7.12 FTIR spectra of dry biomass of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions TW (a) BG-11 (b) SWW (c) and PWW (d).

The IR absorbance spectra was used to calculate the ratio of amide I/amide II (1650/1545 cm^{-1}), Lipid/Protein (L/P) (1740/1650 cm^{-1}), Lipid/Carbohydrate (L/C) (1740/1040 cm^{-1}) and Carbohydrate/Amide I (C/P) (1040/1650 cm^{-1}) ratios in both WT and mutant strains grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW). The results in Table 7.5 related to amide I/amide II ratio increased in the mutant strain in the increasing order of tap water (TW), BG-11, SWW and PWW. In the tap water grown mutant strain, amide I/amide II ratio (1.0±0.008) was lowest, followed by BG-11(1.75±0.011), SWW (2.0±0.15) and PWW (2.4±0.2) in the increasing order. On the other hand, the IR spectrum in the WT strain showed the highest ratio amide I/amide II in BG-11 (1.8±0.016) and lowest in the tap water (0.8±0.02) grown cells. The amide I/amide II ratio grown in the WT grown on soybean and poultry wastewater were found to be the same (2.0±0.012, 2.1±0.01 respectively). The Protein/Lipid (1650/1740 cm^{-1}) ratios was the lowest in the WT strain grown on tap water (1.2±0.01) and was found to be maximum in poultry wastewater grown cells (2.1±0.12), but Lipid/Protein (1740/1650 cm^{-1}) ratio in the mutant strain was found to be lowest in the tap water (1.5±0.011) and the highest ratio in the BG-11 grown cells (3.0±0.21). The Lipid/Carbohydrate (1740/1040 cm^{-1}) ratio in both WT and mutant strain was found to be lowest in the tap water (1.0±0.007, 1.0±0.08, respectively) and the highest L/C (1040/1740 cm^{-1}) ratio in the poultry wastewater grown cells (1.9±0.13, 2.2±0.10, respectively). More or less similar results were obtained in the case of Carbohydrate/Amide I (1040/1650 cm^{-1}) ratio, the lowest C/P (1040/1650 cm^{-1}) ratio was obtained in both WT and mutant strain grown on the tap water (0.7±0.05, 0.8±0.006, respectively) The ratio of C/P (1040/1650 cm^{-1}) in both the strain grown in soybean and poultry wastewater was found to be in the higher range of (1.3 to 1.5 respectively). Based on these results it was concluded that different nutritional media for growth of WT and mutant strain showed variation in the synthesis of different macromolecules such as protein, carbohydrate and lipid. However, among different cells constituents, higher synthesis of carbohydrate and lipid in both WT and mutant were supported by the SWW and PWW. The mutant was able to utilize the wastewaters as in a better manner than the WT for the synthesis of various cell constituents

Table 7.5 A comparative study of changes in biochemical constituents of biomass of *S. vacuolatus* strain of wild-type and mutant under different nutrient conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW) by analysis of FTIR spectra absorption ratios Amide I/Amide II (1650/1545 cm^{-1}), Lipid/Protein (1740/1650 cm^{-1}), Lipid/Carbohydrate (1740/1040 cm^{-1}) and Carbohydrate/Amide I (1040/1650 cm^{-1}). Data depicted as mean±SD.

Wild-type					**Mutant**			
FTIR spectra ratio	**TWW**	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Amide I/Amide II (1650/1545 cm^{-1})	0.8±0.02	1.8±0.016	2.0±0.012	2.1±0.01	1.0±0.008	1.75±0.011	2.0±0.15	2.4±0.2
Lipid/Protein (1740/1650 cm^{-1})	1.2±0.01	1.5±0.012	1.9±0.018	2.1±0.12	1.5±0.011	3.0±0.21	2.5±0.2	2.9±0.22
Lipid/Carbohydrate (1740/1040 cm^{-1})	1.0±0.007	1.3±0.012	1.5±0.11	1.9±0.13	1.0±0.08	1.5±0.08	1.9±0.11	2.2±0.10
Carbohydrate/Amide I (1040/1650 cm^{-1})	0.7±0.05	0.9±0.042	1.4±0.08	1.5±0.11	0.8±0.006	1.0±0.061	1.5±0.11	1.3±0.1

7.3.7.2 Lipid analysis

Total lipid was extracted from both WT and mutant cells grown on tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW). The extracted lipid content was taken for measurement of IR spectra (4000-400 cm^{-1}) (Fig. 7.13 and 7.14) The FTIR result showed that total lipid content (2920/3011 cm^{-1}) was higher in the tap water grown mutant strain (0.41±0.02) an the WT (0.3±0.015). A sharp peak at 1460 & 1373 cm^{-1} was relatively sharp in the tap water grown mutant strain. The IR peak at 1160 & 1090 cm^{-1} was apparently very high in the WT than mutant strain indicating high lipopolysaccharides and phospholipid content in the WT than the mutant. The peak at 1740 cm^{-1}, denoting the ester bond of fatty acids was very prominent in the mutant strain than the WT. The IR absorbance ratio of (3011/2920 cm^{-1}) representing unsaturation/saturation ratio of fatty acids in both WT and mutant

strain indicated the almost a similar degree of unsaturation/saturation of fatty acids. The IR spectra of the biomass of WT (0.41±0.023) and mutant strain (0.5±0.03) grown in BG-11 medium showed a different pattern of results. The total lipid (1740 cm^{-1}) was found to be relatively higher in the mutant strain than the WT when grown on the BG-11. The IR absorbance at 1082, 1161 & 1219 cm^{-1} in the BG-11 grown WT cells as compared with the corresponding IR peak in the mutant strain showed indicated higher proportion lipopolysaccharides in the WT. The degree of unsaturation/saturation (3011/2920 cm^{-1}) ratio of lipid in the poultry wastewater grown WT (0.68±0.041) and mutant (0.86±0.04) showed a higher amount of unsaturated fatty acid in the mutant. WT and mutant strain was grown on soybean wastewater (0.56±0.032, 0.63±0.04, respectively) exhibited similar results (Table 7.6). The FTIR spectra of extracted lipid of mutant strain showed higher lipid amount of triglycerides content than the WT as evident from the higher IR absorption between 1730-1740 cm^{-1}. The carbohydrate and phosphate content associated with lipid in the mutant strain grown in the presence of soybean wastewater were found to be relatively higher than as compared to the poultry wastewater grown mutant cells. A very prominent peak at 1646 cm^{-1} wavenumber suggested that the mutant strain grown on soybean wastewater possess a higher amount of lipoproteins when poultry wastewater grown mutant cells. A prominent at 1650 cm^{-1} protein peak in the soybean wastewater grown WT cells indicated the presence of lipoproteins when compared with poultry wastewater grown WT cells. The overall FTIR spectrum indicated the presence of lipoproteins and lipopolysaccharides with different level of unsaturation/saturation fatty acids in both the strains. The overall result suggested that the total quantity and type of lipid in the mutant and WT was dependent depending upon the sources of nutrition. However, the overall lipid content was higher in the mutant strain compared to WT, particularly in the poultry wastewater grown cells.

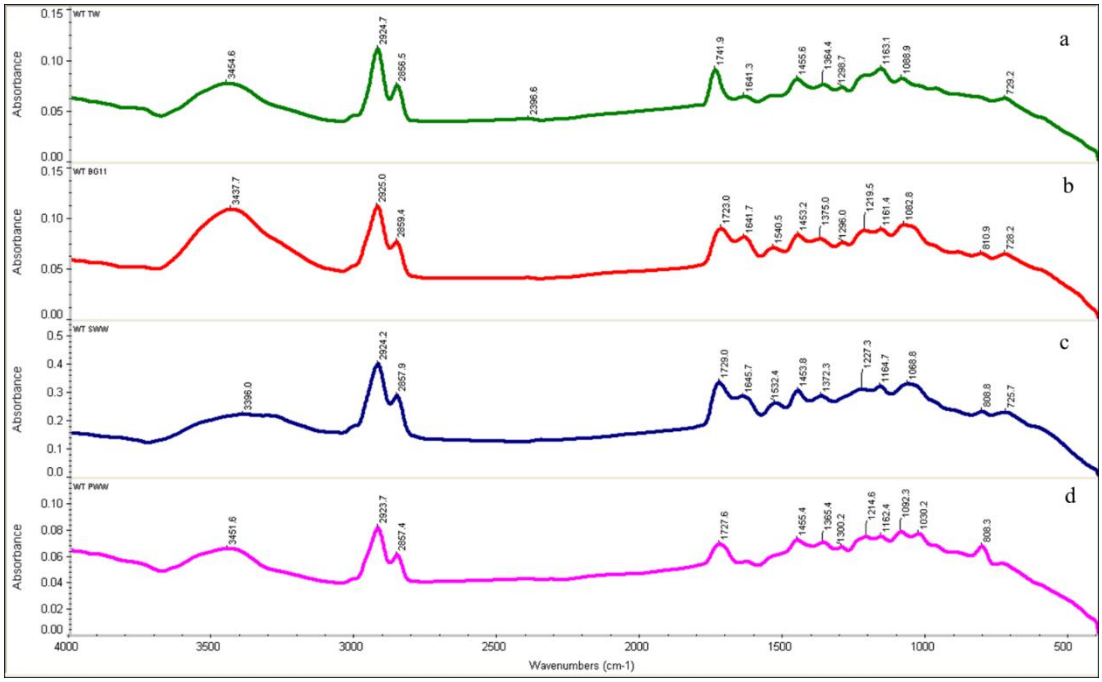

Fig 7.13 FTIR spectra of extracted lipid of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions TW (a) BG-11 (b) SWW (c) and PWW (d).

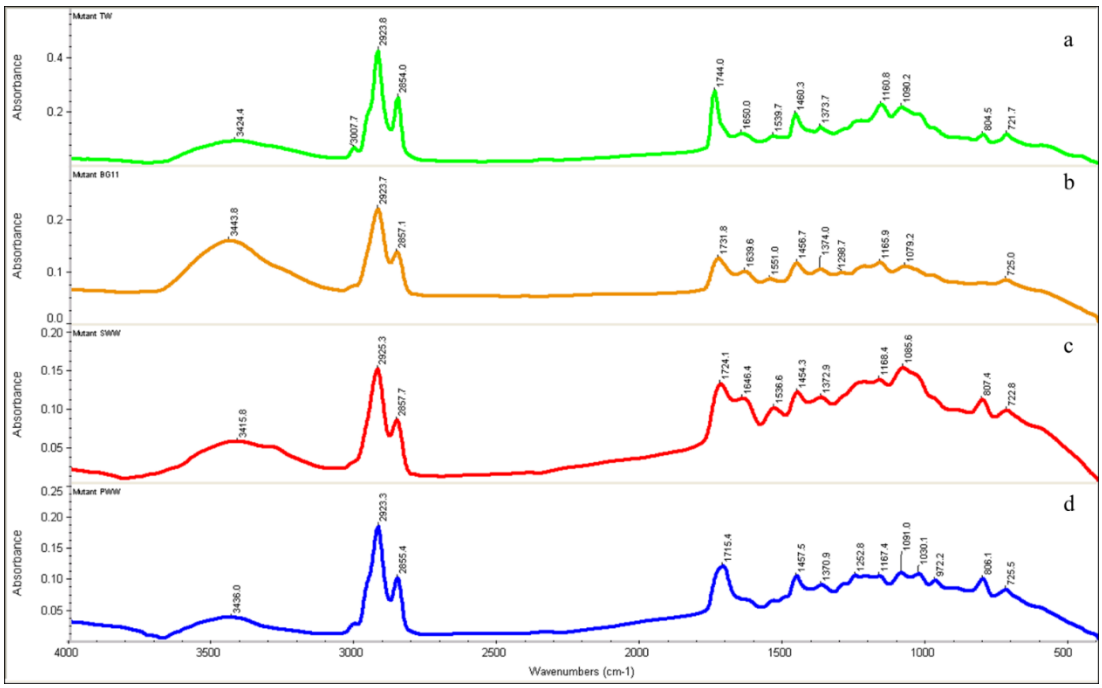

Fig 7.14 FTIR spectra of extracted lipid of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions TW (a) BG-11 (b) SWW (c) and PWW (d).

Table 7.6 Study of changes in lipid profile of *S. vacuolatus* strain of wild-type and mutant under different nutrient conditions tap water (TW), BG-11, soybean wastewater (SWW), poultry wastewater (PWW) by analysis of FTIR spectra absorption ratio total lipid (2920/3011 cm^{-1}) and unsaturation/saturation (3011/2920 cm^{-1}). Data depicted as mean±SD.

	Wild-type				Mutant			
FTIR spectra ratio	**TW**	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Total Lipid (2920/3011 cm^{-1})	0.4±0.02	2.0±0.07	2.2±0.2	2.8±0.22	0.6±0.02	2.3±0.2	4.3±0.26	6.1±0.32
Unsaturation/ saturation (3011/2920 cm^{-1})	0.3±0.015	0.41±0.023	0.56±0.032	0.68±0.041	0.41±0.02	0.5±0.03	0.63±0.04	0.86±0.04

7.3.8 LC-MS/MS analysis

7.3.8.1 Polysaccharides analysis

Carbohydrate (mono, di and polysaccharides) concentration was analysed by using LC-MS/MS in both WT and mutant strain grown under various nutritional conditions such as tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) (Fig. 7.15, 7.16) The results in WT and mutant strain grown in tap water showed maximum level of simple sugars like sucrose (213.6, 254.3 µg/mg respectively), followed by maltose (108.5, 169.4 µg/mg, respectively), and mannose (39.7, 28.9 µg/mg, respectively). The amount of dextrose, galactose and fructose were found to be in the range of (24.3-40 µg/mg respectively) in the tap water grown WT and mutant strain. Similarly, all the major carbohydrate in the WT and mutant strain grown under BG-11 medium were found to be in the range (28-89 µg/mg). However, the quantity of mannose in both the WT and mutant strain in the BG-11 was higher (71.8, 89.1 µg/mg, respectively) than the other saccharides, but maltose was present at the lower level (28.3, 45.7 µg/mg, respectively). Further, the result showed that WT and mutant strain grown in the presence of soybean and poultry wastewater showed the highest level of mannose followed by fructose, galactose and maltose. These results indicated that disaccharides were present in higher quantity in soybean and poultry

wastewater grown cells of both the strains than the polysaccharides and monosaccharides. Except for dextrose, mannose was the only abundant polysaccharides present in the soybean and poultry wastewater grown cells of WT (173.9, 66.5 µg/mg, respectively) and mutant strain (173.6, 110.7 µg/mg, respectively) (Table 7.7). The overall sugar content in the soybean wastewater grown WT and mutant cells was relatively higher than the poultry wastewater grown cells. Thus results revealed that soybean wastewater promotes the accumulation of carbohydrate in both the strain. The mutant strain grown on soybean wastewater was accumulated a higher level of carbohydrate when compared with poultry wastewater grown cells.

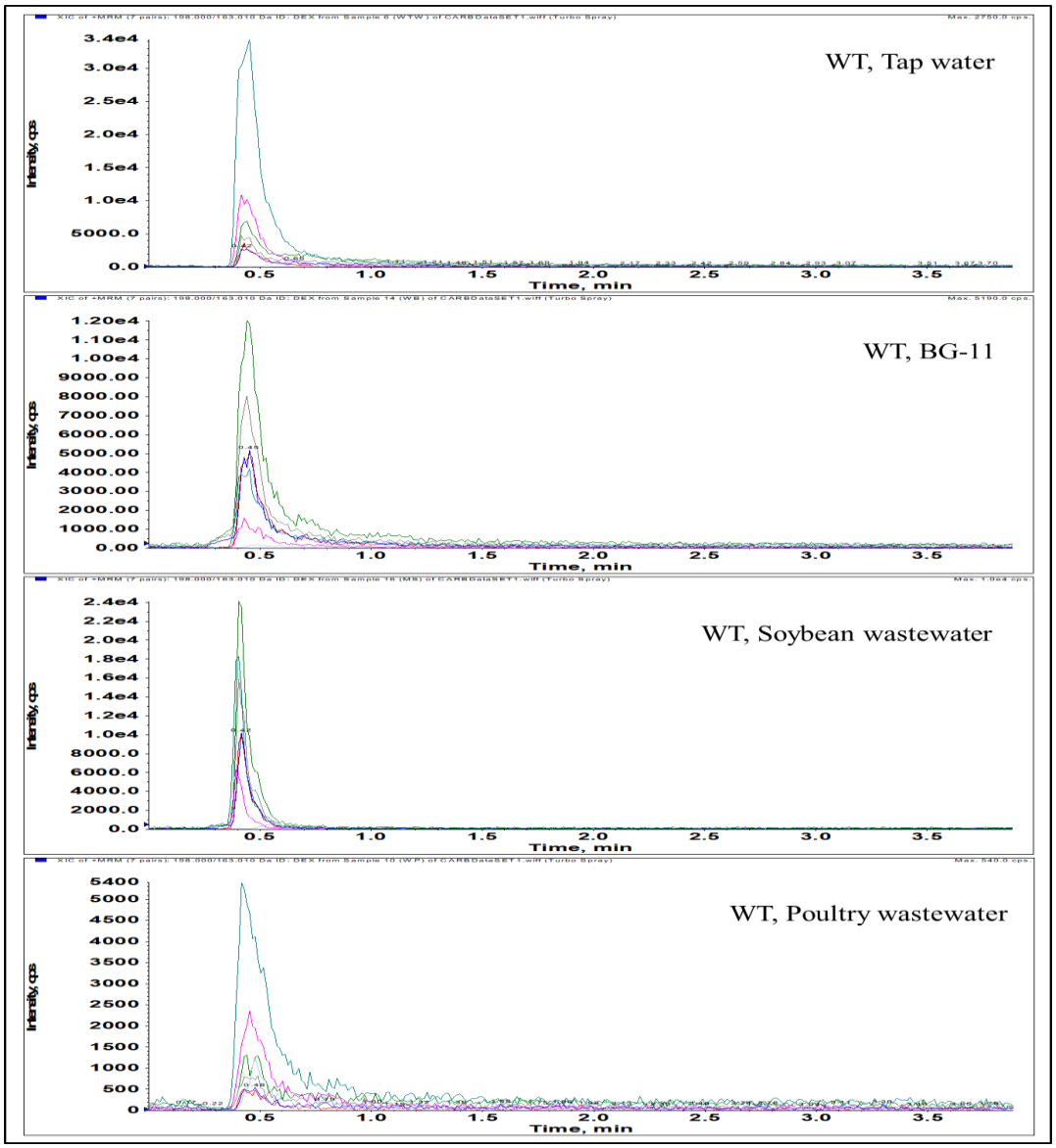

Fig. 7.15 LC-MS/MS chromatogram of polysaccharides of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

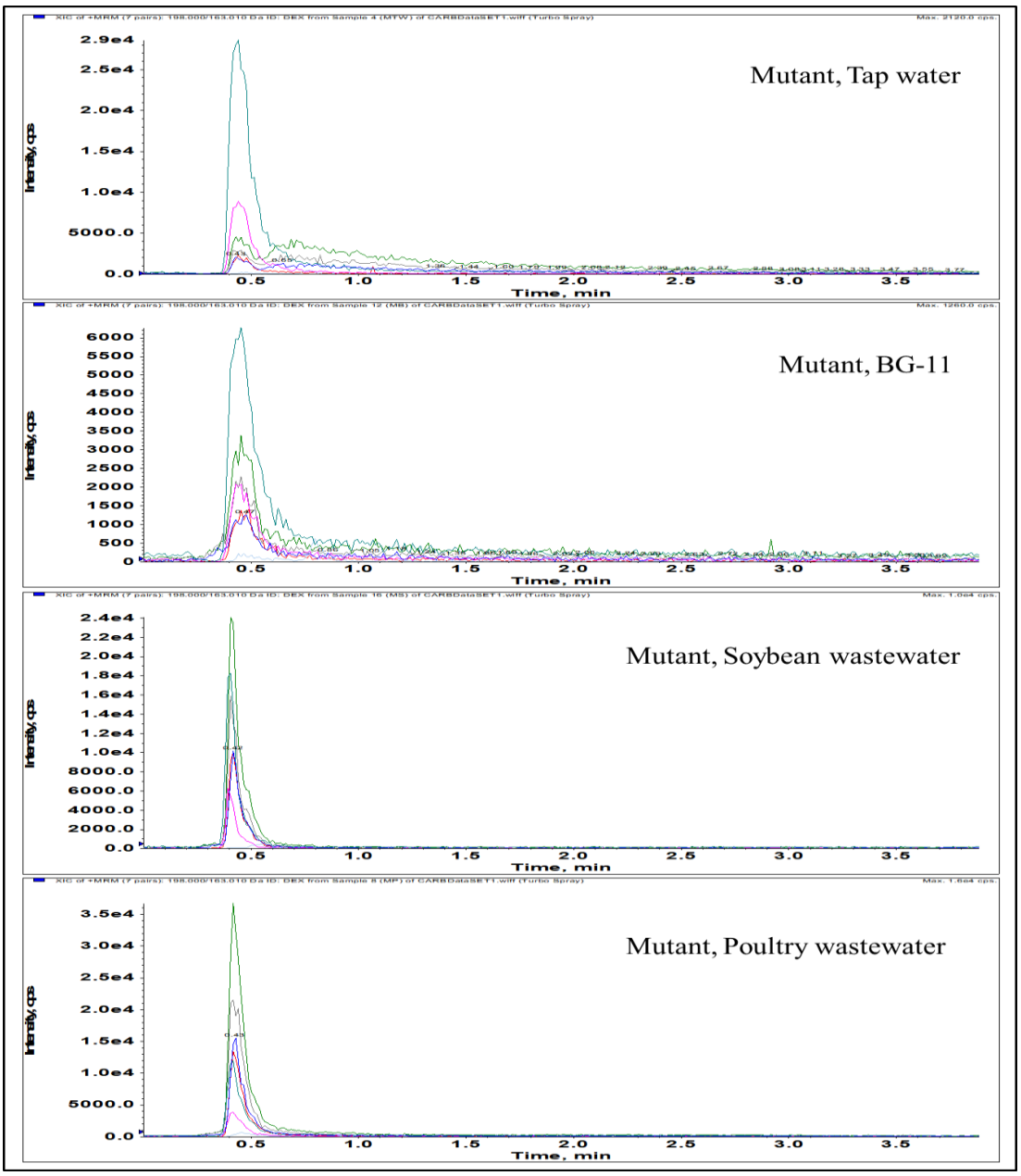

Fig. 7.16 LC-MS/MS chromatogram of polysaccharides of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

Table 7.7 Identification of polysaccharides by using LC-MS/MS analysis of microalgal biomass of tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) of wild-type and mutant strain of *S. vacuolatus.*

	Wild-type				Mutant			
Polysaccharides (µg/mg)	**TW**	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Dextrose	26.24	48.20	101.80	40.83	12.61	52.73	129.32	83.45
Mannose	39.77	71.80	173.90	66.54	28.93	89.11	173.69	110.71
Galactose	27.42	48.79	108.74	40.83	22.73	53.34	124.86	85.50
Fructose	24.79	48.59	108.76	51.20	36.50	58.70	126.75	91.10
Maltose	108.50	28.33	68.34	58.40	169.48	45.73	53.68	54.67
Sucrose	213.60	40.24	73.42	61.84	254.39	67.00	81.03	67.00

7.3.8.2 Amino acid analysis

Identification of amino acid extracted from WT and mutant strain cells grown under the various nutritional condition such as tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) were analysed by using LC-MS (Fig. 7.17, 7.18). The results on amino acid content in both WT and mutant strain grown on soybean and poultry wastewater was compared with BG-11 and tape water grown cells (Table 7.8). The result showed that alanine content in both WT and mutant strain (2387.8, 3611.1 µg/mg, respectively) grown on soybean wastewater was found to be highest as compared to the BG-11 (311.2, 441.7 µg/mg, respectively) and tap water (145.3, 120.7 µg/mg, respectively) grown cells. The poultry wastewater grown WT and mutant strain accumulated more alanine content (539.5, 576.9 µg/mg, respectively) than the BG-11 and tap water grown cells but lesser than that recorded for soybean wastewater grown cells. The synthesis of serine amino acid in both WT and mutant strains grown on the soybean wastewater were found to be higher (50.7, 34.76 µg/mg, respectively), whereas poultry wastewater grown mutant strain accumulated higher serine content (65.03 µg/mg) as compared to WT cells (23.04 µg/mg). However, the serine amino acid contents in the mutant strain was substantially higher than the WT under different nutritional conditions. Further the result on proline content in soybean and poultry wastewater grown mutant strain

(1039.3, 342.9 µg/mg, respectively) was higher than the WT strain grown in the soybean and poultry wastewater (618.1, 163.3 µg/mg, respectively). Similarly, the threonine content in the soybean wastewater grown WT and mutant strain was (213.3, 337.1 µg/mg, respectively) was higher than the poultry wastewater grown WT and mutant strain (28.5, 118.9 µg/mg, respectively). Most of the amino acid like aspartic acid, isoleucine, glutamic acid and methionine also exhibited several fold increase in their content when both WT and mutant strain were grown in the presence of soybean wastewater. The accumulation of these amino acids in both WT and mutant strain grown on the presence of poultry wastewater was found to be relatively lesser than their corresponding level under other nutritional conditions. Similar observation were made for glycine, leucine, phenylalanine which indicated higher accumulation of these amino acids in the soybean wastewater grown WT and mutant strain as compared to poultry wastewater grown cells. These results indicated that the accumulation of amino acid in both the strains was promoted by soybean and poultry wastewater as compared to the BG-11 and tap water. Accumulation of alanine, proline, tyrosine and arginine was promoted by BG-11 medium in both the strains as compared to tap water, but their contents were higher in both the strains grown on soybean wastewater and poultry wastewater. The overall results indicated that soybean and poultry wastewater were a good nitrogen source for amino acid synthesis. The overall amino acid content in soybean wastewater grown mutant strain was higher than the soybean wastewater grown WT cells. The accumulation of amino acid in poultry wastewater grown WT and mutant strain was relatively less than that in the soybean wastewater grown cells.

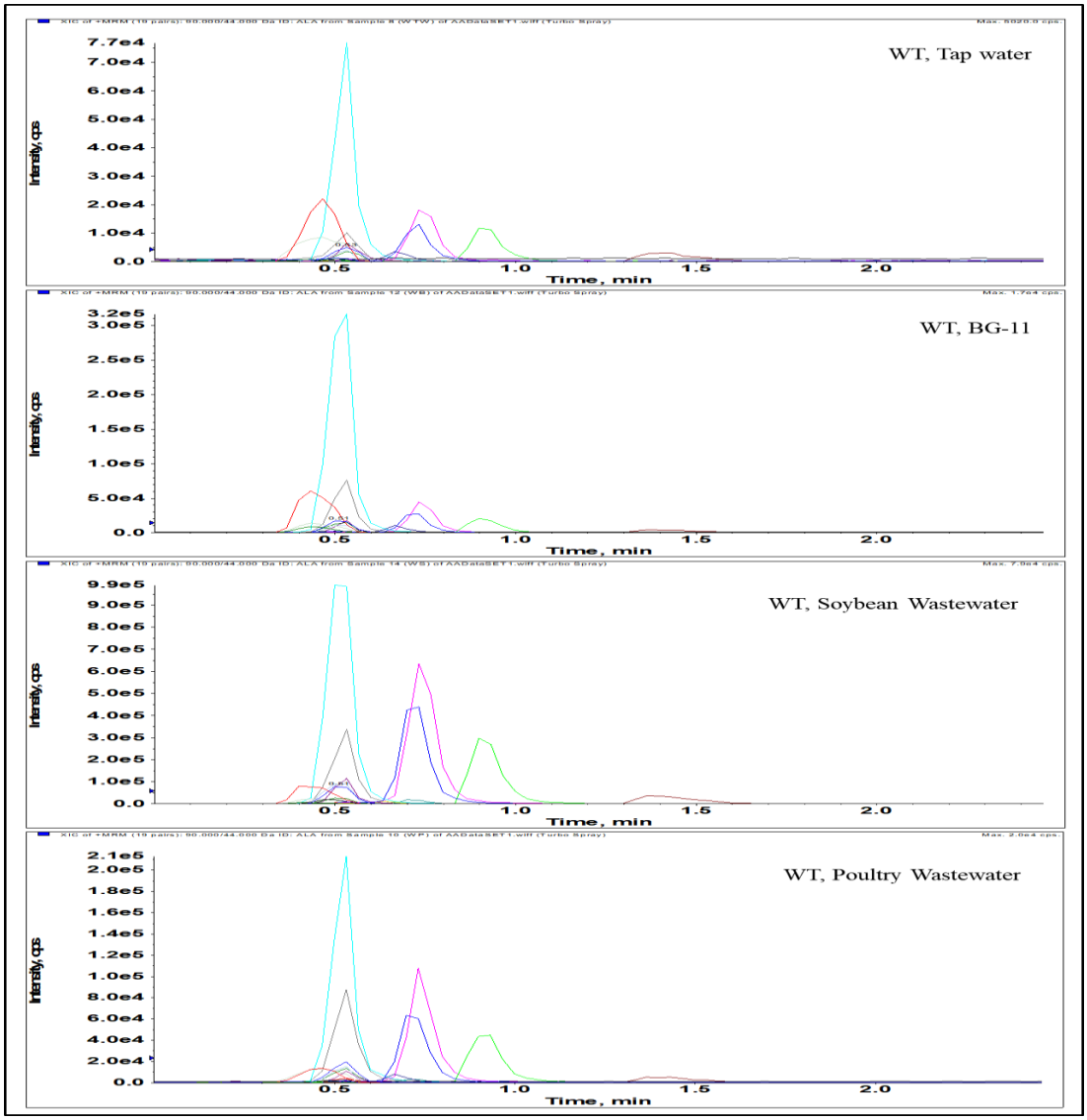

Fig. 7.17 LC-MS/MS chromatogram of amino acid of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

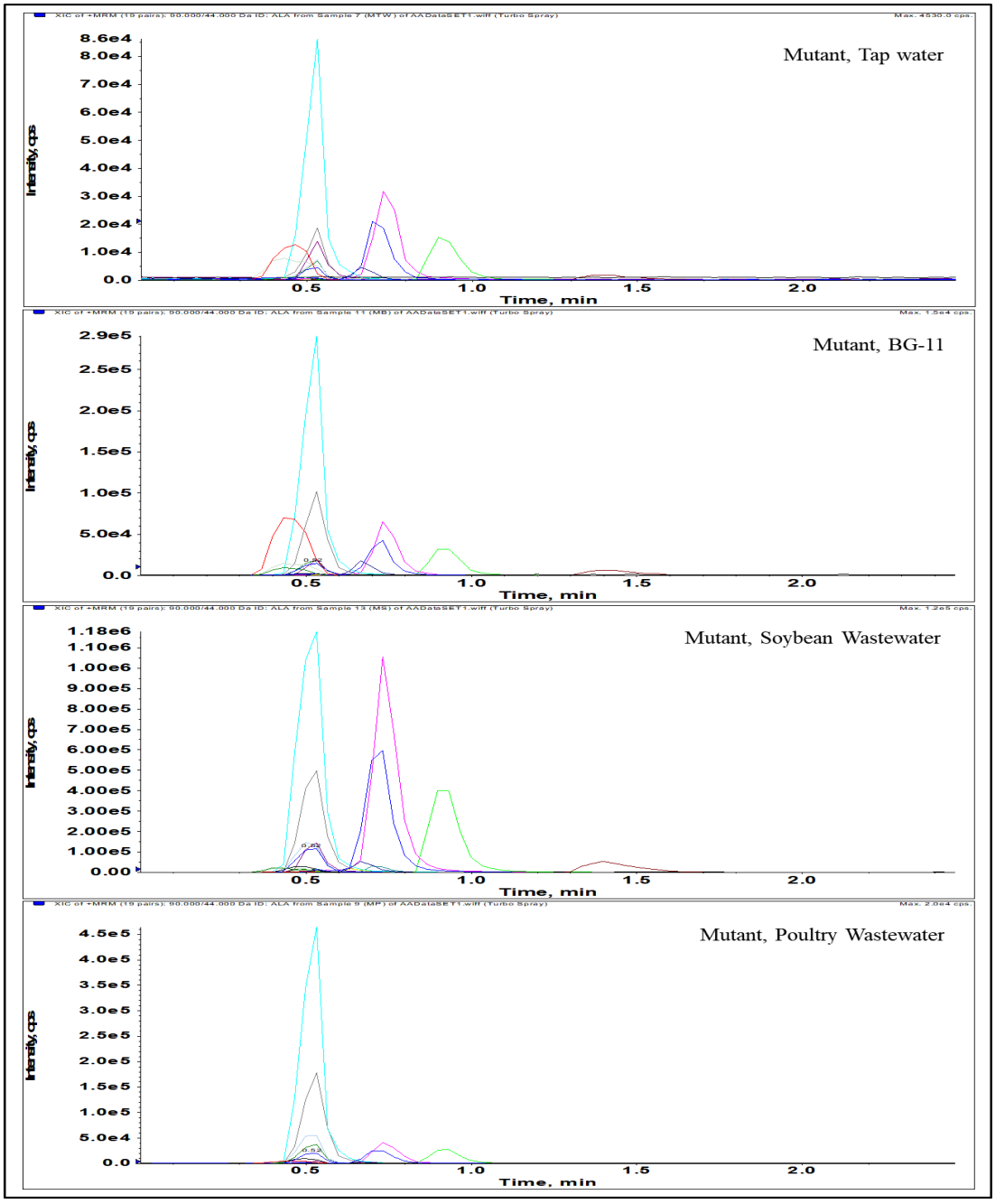

Fig. 7.18 LC-MS/MS chromatogram of amino acid of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

Table 7.8 Identification of amino acid by using LC-MS/MS analysis of microalgal biomass of tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) of wild-type and mutant strain of *S. vacuolatus.*

	Wild-type				Mutant			
Amino acid (µg/mg)	**TW**	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Alanine	145.30	311.22	2387.82	539.53	120.73	441.77	3611.11	576.92
Aminobutyric Acid	0.77	2.10	27.96	16.36	1.34	1.24	36.25	4.39
Serine	6.01	26.05	50.70	23.04	9.97	32.06	34.76	65.03
Proline	21.69	135.83	618.11	163.39	32.80	188.58	1039.37	342.91
Threonine	7.46	24.27	213.31	28.51	12.42	30.89	337.10	118.95
Isoleucine	14.71	33.00	517.14	76.71	24.86	49.29	790.00	34.43
Norleucine	36.96	69.19	1011.34	164.46	68.62	97.35	1531.19	109.64
Aspartic Acid	17.13	39.66	289.66	27.46	38.82	47.00	410.46	11.48
Glutamic Acid	154.90	769.61	2671.57	450.49	171.57	832.35	3205.88	1034.31
Methionine	6.13	30.28	421.90	29.51	8.32	31.56	624.20	22.98
Histidine	40.56	55.73	92.36	57.28	38.13	67.60	99.59	20.38
Phenylalanine	48.43	88.89	1235.29	200.65	66.67	144.44	1790.85	120.92
Tyrosine	35.15	104.70	4.29	78.95	42.29	160.15	541.35	30.64
Tryptophan	34.24	50.68	405.64	57.49	20.91	73.05	554.47	12.84
Dopa	1.78	5.45	12.29	1.48	1.07	9.09	41.86	4.27
Glycine	25.42	69.38	675.84	45.63	10.35	71.77	1028.71	383.97
Leucine	11.11	25.16	429.74	62.91	18.30	38.56	591.50	79.08
Arginine	133.88	393.44	568.31	92.90	186.07	487.25	36.52	37.43
Ornithine	9.14	72.76	47.13	9.21	10.46	81.49	187.36	4.24

7.3.8.3 Fatty acid analysis

The fatty acid profile of both WT and mutant strain grown under various nutritional conditions such as tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) was analysed by LC-MS (Fig. 7.19, 7.20). The result showed that synthesis of decanoic acid (10:0) was found to be highest in poultry wastewater grown WT and mutant strain (206.6 & 248.8 µg/mg, respectively) followed by BG-11 grown cells (156.6 & 163.8 µg/mg, respectively) (Table 7.9). The soybean wastewater grown

cells of both the WT and mutant strain exhibited higher decanoic acid content in the mutant strain (154.4 µg/mg) than the WT (60.8 µg/mg). The palmitoleic acid (16:1) content in poultry wastewater grown WT and mutant strain were found to be the highest (3.8 & 5.0 µg/mg respectively) followed by BG-11 (0.78 & 0.82 µg/mg respectively) and soybean wastewater grown cells (0.99 & 0.57 µg/mg respectively). Octadecanoic acid (18:0) was found to be maximum in soybean and poultry wastewater grown cells mutant (266.8 & 111.6 µg/mg, respectively), which registered several fold increase as compared to the soybean and poultry wastewater grown of WT (21.5, 108.5 µg/mg, respectively). Accumulation of oleic acid (cis 18:1) and elaidic acid (trans 18:1) content were found higher in the soybean and poultry wastewater grown mutant strain when compared with the corresponding fatty acid content in the WT cells grown in the presence of soybean and poultry wastewater. The unsaturated fatty acid linoleic acid (18:2) and linolenic acid (18:3) were found to be maximum in poultry wastewater grown cells of both the WT (73.3 & 1320.1 µg/mg, respectively) and mutant strain (84.6 & 2869.5 µg/mg, respectively) and their quantity was comparable with the corresponding fatty acid content of both the WT (64.2 & 1652.9 µg/mg, respectively) and mutant strain (66.2 & 2501.1 µg/mg, respectively) grown in BG-11 medium. The arachidonic acid (20:4) fatty acid was found to be higher in the mutant strain than the WT when grown under different nutritional conditions, the maximum quantity of arachidonic acid (20:4) was in the mutant strain was grown on poultry wastewater (39.1 µg/mg) followed by soybean wastewater (24.9 µg/mg). Docosanoic acid (22:0) was maximum in the poultry wastewater grown WT and mutant strain (418.1 & 471.7 µg/mg, respectively) followed by BG-11(117.6 & 108.6 µg/mg, respectively). The foregoing results depicted that most of the essential fatty acid content were higher in both the WT and mutant strain when grown in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and TW (Table 7.9). However, the overall level of fatty acids was found to be higher in the mutant strain than the WT under different nutritional conditions.

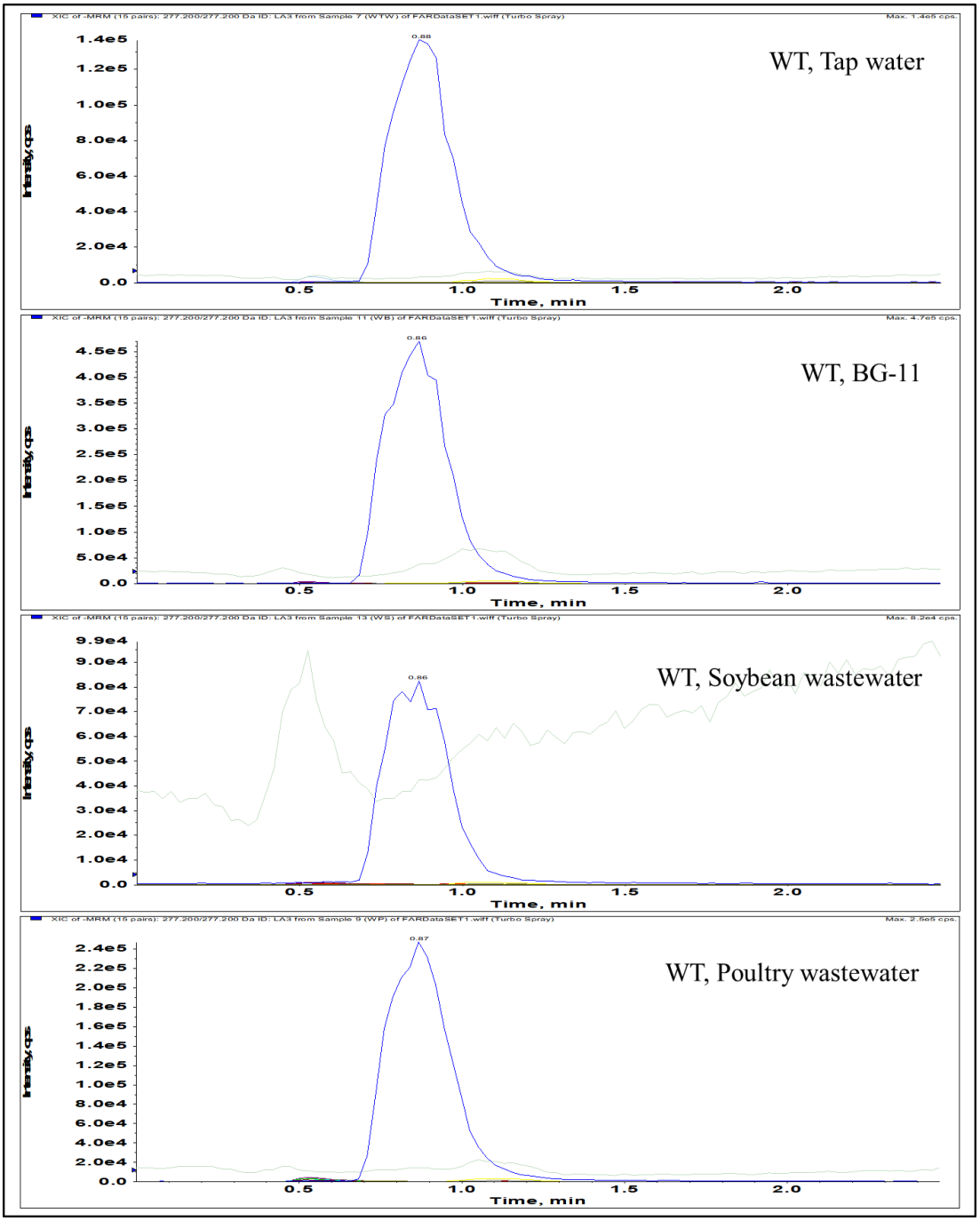

Fig. 7.19 LC-MS/MS chromatogram of fatty acids of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

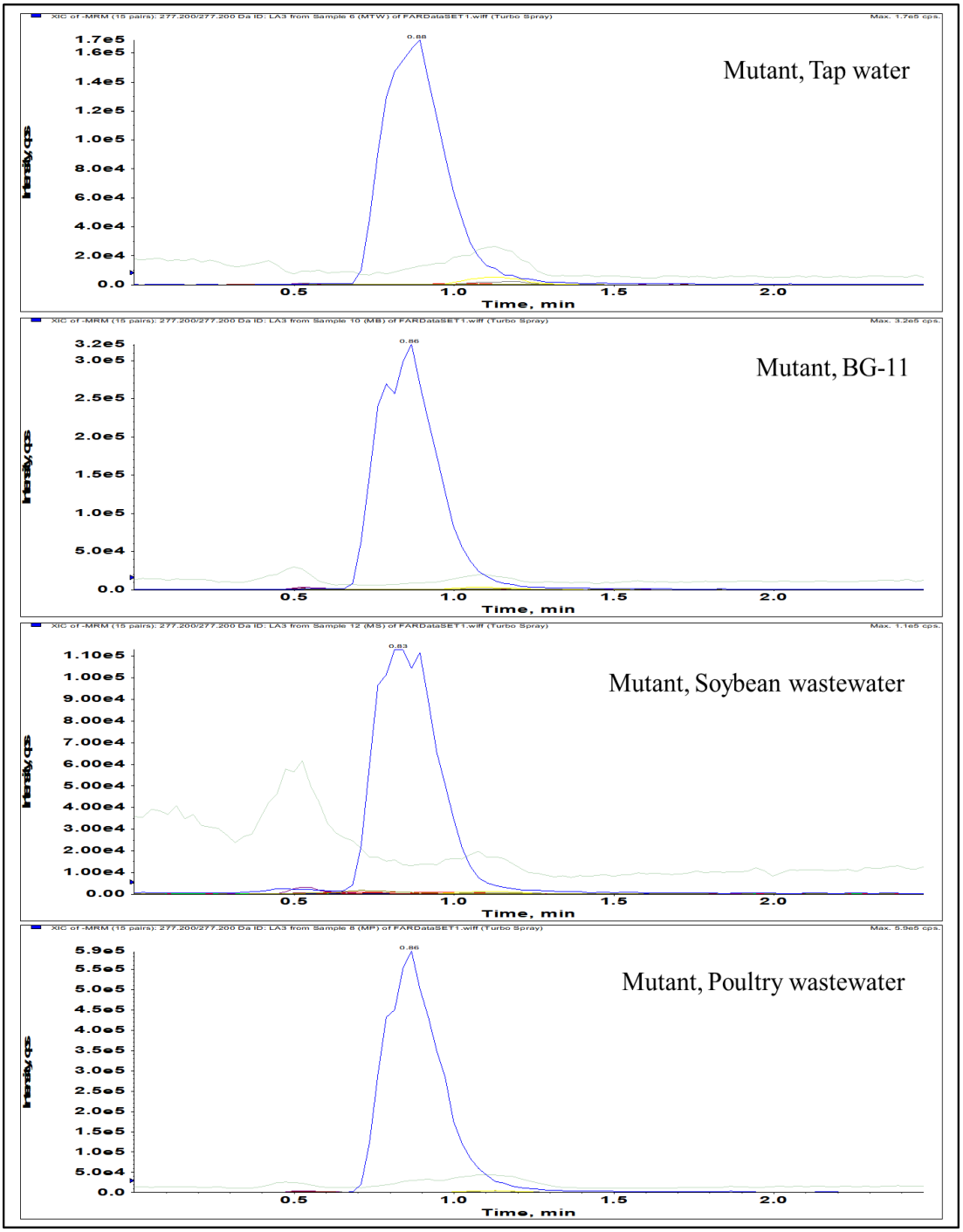

Fig. 7.20 LC-MS/MS chromatogram of fatty acids of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

Table 7.9 Identification of fatty acid by using LC-MS/MS analysis of microalgal biomass of tap water (TW), BG-11, soybean wastewater (SWW) and poultry wastewater (PWW) of wild-type and mutant strain of *S. vacuolatus.*

	Wild-type				Mutant			
Fatty acid (µg/mg)	**TW**	**BG-11**	**SWW**	**PWW**	**TW**	**BG-11**	**SWW**	**PWW**
Hexanoic acid (6:0)	0.99	5.68	6.19	6.69	2.97	9.15	7.30	5.20
Decanoic acid (10:0)	30.36	156.63	60.84	206.63	51.81	163.86	154.22	248.80
Palmitoleic acid (16:1)	0.15	0.78	0.99	3.87	0.27	0.82	0.57	5.03
Octadecanoic acid (18:0)	6.85	47.26	21.51	108.58	7.74	52.84	266.80	111.69
Oleic acid (cis 18:1)	19.84	8.19	1.20	13.31	7.69	5.39	2.97	14.65
Elaidic acid (trans 18:1)	24.39	81.74	14.55	86.16	52.64	98.50	25.25	125.82
Linoleic acid (18:2)	8.70	64.16	39.25	73.38	26.25	66.21	33.96	84.64
Linolenic acid (18:3)	719.37	1652.98	450.59	1320.16	909.09	2501.17	636.36	2869.57
Eicosanoic acid (20:0)	5.79	18.48	55.89	71.70	27.41	83.93	38.48	82.23
Arachidonic acid (20:4)	3.65	12.82	7.02	8.38	17.38	20.98	24.89	39.16
Docosanoic acid (22:0)	22.73	117.65	12.27	418.12	63.43	180.60	54.69	471.70
Docosahexaenoic acid (22:6)	0.57	6.95	18.50	21.09	9.53	7.46	28.94	18.40

7.3.9 GC-MS analysis

The biodiesel extracted from both the wild-type (WT) and mutant cells were analysed by GC-MS. The methylated fatty acids were compared with the known standard on the basis of area curve and retention time (RT) percentage area was calculated to denote the concentration of different methylated esters of fatty acids (Fig. 7.21, 7.22). The chromatogram of biodiesel derived from WT and mutant grown on the tap water (TW) revealed the absence of certain low molecular fatty acid in the mutant strain such as

methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl arachidate (C20:0).On the other hand, the results showed the presence of methyl eicosatrienoate (C20:3) in the mutant strain, but it was found to be absent in WT (Table 7.10). Most of the other fatty acids were found to be more or less the same in the biodiesel of both the strain. The biodiesel obtained from WT and mutant strain grown on BG-11 medium showed the high percentage of long-chain methyl nervonate (C24:1) and docosahexaenoate (C22:6) in the mutant strain (5.47%), which was higher than that in the WT (3.28%). The concentration of methyl behenate (C22:0) and methyl eicosapentaenoate (20:5), methyl arachidate (C20:0) was in the mutant strain was more pronounced as compared to the WT (Table 7.11). The overall results indicated presence of long-chain fatty acids in the BG-11 grown mutant strain as compared to WT cells.

The biodiesel obtained from WT and mutant strain grown in the presence of soybean wastewater revealed a higher percentage of methyl behenate (C22:0) and methyl eicosapentaenoate (20:5) in the mutant strain (1.39%) than the WT (0.87%). The percentage of methyl nervonate (C24:1) was lower in the biodiesel of mutant strain (3.93%) than the WT (5.12%). However, the low molecular weight fatty acids were found to be absent in the SWW grown cells of both the strain (Table 7.12). The biodiesel obtained from both WT and mutant strains grown on the poultry wastewater revealed that a higher percentage of nervonate (C24:1) and docosahexaenoate (C22:6), both long-chain fatty acids. The presence of methyl palmitate (C16:0) was not detectable in the biodiesel of both the strains grown on poultry wastewater. The concentration of long chain fatty acids like methyl behenate (C22:0) and methyl eicosapentaenoate (20:5), methyl lignocerate (C24:0) and methyl linoleate (C18:2) were found to be higher in the biodiesel of mutant strain than the WT.

The overall results indicated that WT and mutant cells grown on SWW and PWW in the different wastewaters exhibited relatively less quantity of saturated fatty acid like methyl palmitate (C16:0), and methyl lignocerate (C24:0). The long-chain fatty acids methyl behenate (C22:0) and methyl eicosapentaenoate (20:5) were the most predominant in the biodiesel of both the strains grown in the presence of different wastewaters. The prominent unsaturated fatty acids like methyl linoleate (C18:2), linolenate (C18:3) and methyl arachidate (C20:0) were present in the biodiesel obtained from the WT and mutant cells were grown on the soybean and poultry wastewater (Table 7.13).

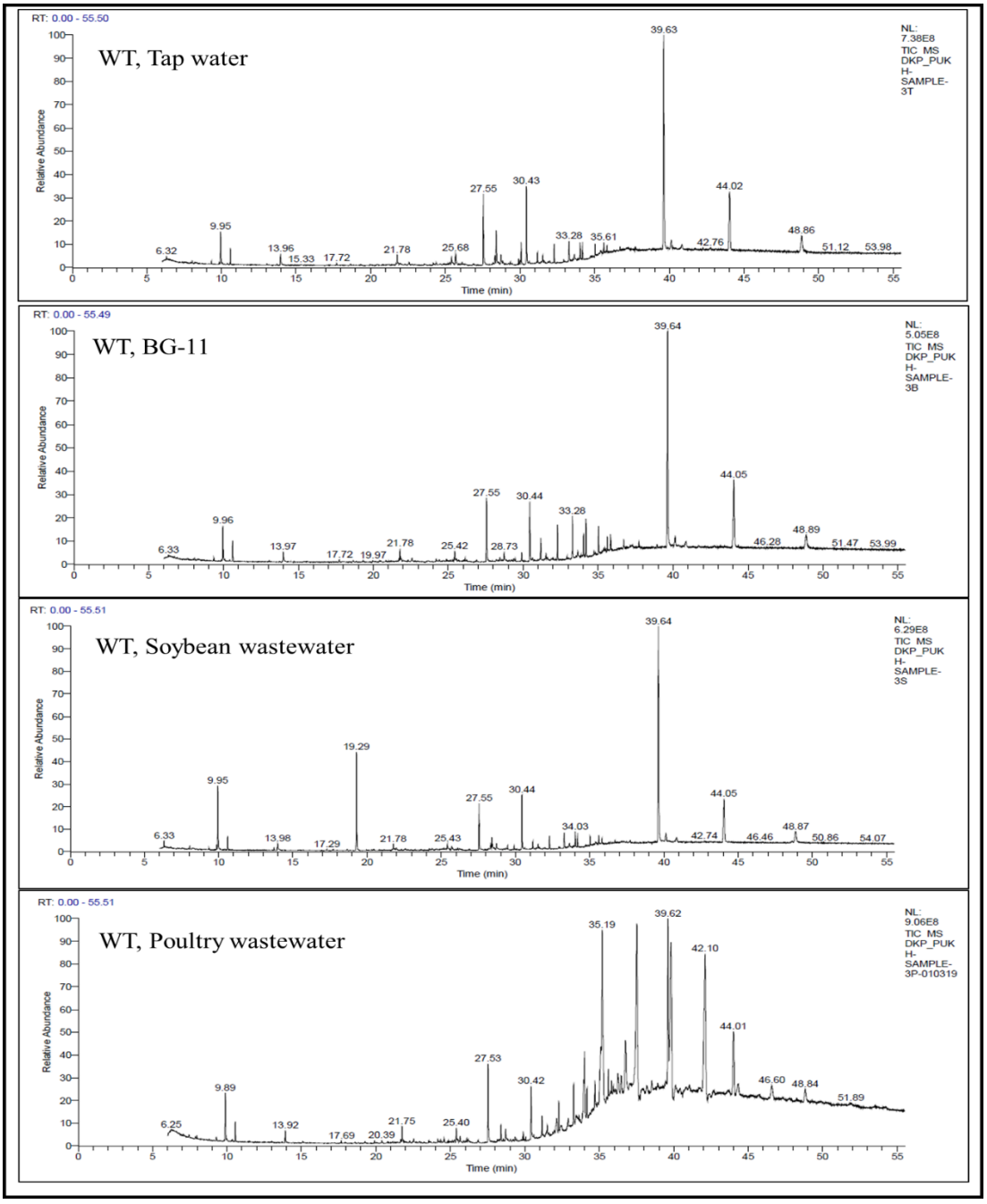

Fig. 7.21 GC-MS chromatogram of biodiesel of microalgae *S. vacuolatus* of wild-type under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

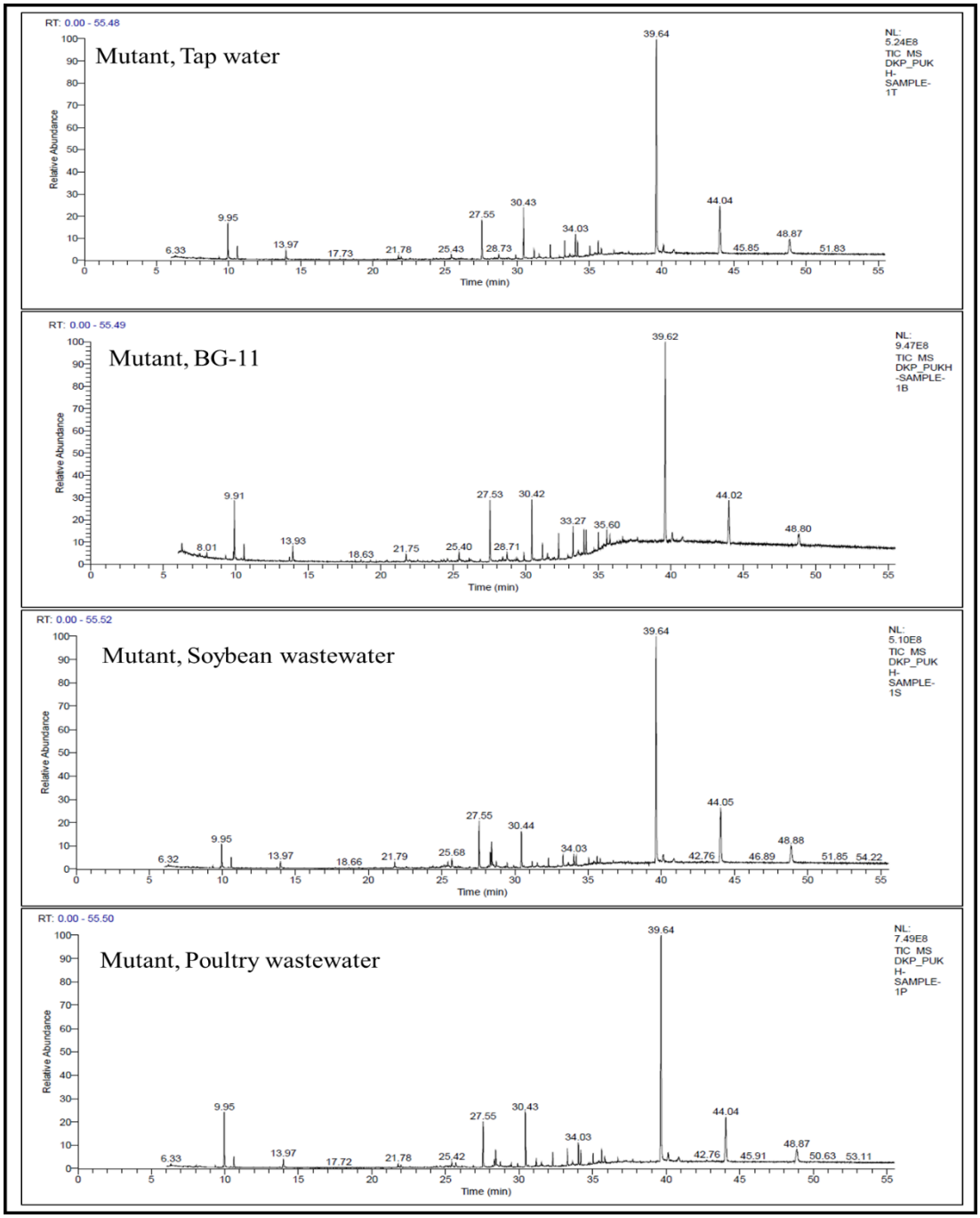

Fig. 7.22 GC-MS chromatogram of biodiesel of microalgae *S. vacuolatus* of mutant strain under varying nutrition conditions tap water, BG-11, soybean and poultry wastewater.

Table 7.10 GC-MS analysis of biodiesel of microalgae *S. vacuolatus* of wild-type and mutant strain under nutrition condition of tap water (TW).

Wild-type - Tape water (TW)					
S. No.	**Compound name**	**Carbon atom of fatty acid: number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.32	$C_7H_{14}O_2$	0.33
2.	Methyl palmitoleate	(C16:1)	18.65	$C_{17}H_{32}O_2$	0.22
3.	Methyl stearate	(C18:0)	20.43	$C_{19}H_{38}O_2$	0.23
4.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	1.2
5.	Methyl linolenate	(C18:3)	22.57	$C_{19}H_{32}O_2$	0.40
6.	Methyl eicosadienoate	(C20:2)	23.64	$C_{21}H_{38}O_2$	0.18
7.	Methyl arachidonate	(C20:4)	24.64	$C_{21}H_{34}O_2$	0.17
8.	Methtyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.68	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	1.4
9.	Methyl erucate	(C22:1)	26.12	$C_{23}H_{44}O_2$	0.25
10.	Methyl lignocerate	(C24:0)	29.36	$C_{25}H_{50}O_2$	0.36
11.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.43	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	6.10
Mutant - Tape water (TW)					
S. No.	**Compound name**	**Carbon atom of fatty acid: number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.32	$C_7H_{14}O_2$	0.24
2.	Methyl stearate	(C18:0)	20.47	$C_{19}H_{38}O_2$	0.12
3.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	0.54
4.	Methyl linolenate	(C18:3)	22.58	$C_{19}H_{32}O_2$	0.24
5.	Methyl eicosatrienoate	(C20:3)	24.7	$C_{21}H_{36}O_2$	0.17
6.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.42	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	0.98
7.	Methyl erucate	(C22:1)	26.13	$C_{23}H_{44}O_2$	0.18
8.	Methyl lignocerate	(C24:0)	29.36	$C_{25}H_{50}O_2$	0.25
9.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.43	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	5.43

Table 7.11 GC-MS analysis of biodiesel of microalgae *S. vacuolatus* of wild-type and mutant strain under nutrition condition of BG-11.

Wild-type - BG-11					
S. No.	**Compound name**	**Carbon atom of fatty acid:number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.32	$C_7H_{14}O_2$	0.29
2.	Methyl palmitoleate	(C16:1)	18.66	$C_{17}H_{32}O_2$	0.23
3.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	1.24
4.	Methyl linolenate	(C18:3)	22.57	$C_{19}H_{32}O_2$	0.55
5.	Methyl eicosadienoate	(C20:2)	23.66	$C_{21}H_{38}O_2$	02.0
6.	Methyl arachidonate	(C20:4)	24.39	$C_{21}H_{34}O_2$	0.29
7.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.51	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	0.55
8.	Methyl erucate	(C22:1)	26.12	$C_{23}H_{44}O_2$	0.27
9.	Methyl lignocerate	(C24:0)	29.47	$C_{25}H_{50}O_2$	0.31
10.	Methyl nervonate Methyl docosahexaenoate Methyl stearate	(C24:1) (C22:6) (C18:0)	30.44	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$ $C_{19}H_{38}O_2$	3.28
Mutant - BG-11					
S. No.	**Compound name**	**Carbon atom of fatty acid:number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.28	$C_7H_{14}O_2$	0.85
2.	Methyl palmitoleate	(C16:1)	18.63	$C_{17}H_{32}O_2$	0.23
3.	Methyl linoleate	(C18:2)	21.75	$C_{19}H_{34}O_2$	1.0
4.	Methyl linoleate	(C18:2)	21.97	$C_{19}H_{34}O_2$	0.45
5.	Methyl linolenate	(C18:3)	22.54	$C_{19}H_{32}O_2$	0.32
6.	Methyl eicosadienoate	(C20:2)	23.56	$C_{21}H_{38}O_2$	0.24
7.	Methyl arachidonate	(C20:4)	24.60	$C_{21}H_{34}O_2$	0.41
8.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.40	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	1.05
9.	Methyl erucate	(C22:1)	26.87	$C_{23}H_{44}O_2$	0.36
10.	Methyl lignocerate	(C24:0)	29.88	$C_{25}H_{50}O_2$	0.89
11.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.42	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	5.47

Table 7.12 GC-MS analysis of biodiesel of microalgae *S. vacuolatus* of wild-type and mutant strain under nutrition condition of soybean wastewater (SWW).

Wild-type - Soybean wastewater (SWW)					
S. No.	**Compound name**	**Carbon atom of fatty acid: number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.33	$C_7H_{14}O_2$	0.70
2.	Methyl palmitoleate	(C16:1)	18.66	$C_{17}H_{32}O_2$	0.16
3.	Methyl stearate	(C18:0)	20.48	$C_{19}H_{38}O_2$	0.16
4.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	0.74
5.	Methyl linolenate	(C18:3)	22.58	$C_{19}H_{32}O_2$	0.23
6.	Methyl arachidonate	(C20:4)	24.66	$C_{21}H_{34}O_2$	0.21
7.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.43	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	0.87
8.	Methyl erucate	(C22:1)	26.21	$C_{23}H_{44}O_2$	0.18
9.	Methyl lignocerate	(C24:0)	29.47	$C_{25}H_{50}O_2$	0.50
10.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.43	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	5.12
Mutant – Soybean wastewater (SWW)					
S. No.	**Compound name**	**Carbon atom of fatty acid: number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.32	$C_7H_{14}O_2$	0.28
2.	Methyl palmitoleate	(C16:1)	18.6	$C_{17}H_{32}O_2$	0.12
3.	Methyl stearate	(C18:0)	20.47	$C_{19}H_{38}O_2$	0.13
4.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	0.73
5.	Methyl linolenate	(C18:3)	22.05	$C_{19}H_{32}O_2$	0.13
6.	Methyl eicosadienoate	(C20:2)	23.60	$C_{21}H_{38}O_2$	0.12
7.	Methyl arachidonate	(C20:4)	24.38	$C_{21}H_{34}O_2$	0.26
8.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.43	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	1.39
9.	Methyl erucate	(C22:1)	26.12	$C_{23}H_{44}O_2$	0.21
10.	Methyl lignocerate	(C24:0)	29.47	$C_{25}H_{50}O_2$	0.58
11.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.44	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	3.93

Table 7.13 GC-MS analysis of biodiesel of microalgae *S. vacuolatus* of wild-type and mutant strain under nutrition condition of poultry wastewater (PWW).

Wild-type - Poultry wastewater (PWW)					
S. No.	**Compound name**	**Carbon atom of fatty acid:number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.16	$C_7H_{14}O_2$	0.26
2.	Methyl stearate	(C18:0)	20.39	$C_{19}H_{38}O_2$	0.14
3.	Methyl linoleate	(C18:2)	21.75	$C_{19}H_{34}O_2$	0.55
4.	Methyl linolenate	(C18:3)	22.55	$C_{19}H_{32}O_2$	0.26
5.	Methyl arachidonate	(C20:4)	24.57	$C_{21}H_{34}O_2$	0.25
6.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.40	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	0.52
7.	Methyl erucate	(C22:1)	26.19	$C_{23}H_{44}O_2$	0.16
8.	Methyl lignocerate	(C24:0)	29.88	$C_{25}H_{50}O_2$	0.31
9.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.42	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	5.47
Mutant – Poultry wastewater (PWW)					
S. No.	**Compound name**	**Carbon atom of fatty acid:number of double bond**	**Retention time (RT)**	**Molecular Formula**	**Area%**
1.	Methyl hexanoate	(C6:0)	6.33	$C_7H_{14}O_2$	0.33
2.	Methyl stearate	(C18:0)	20.48	$C_{19}H_{38}O_2$	0.11
3.	Methyl linoleate	(C18:2)	21.78	$C_{19}H_{34}O_2$	0.59
4.	Methyl linolenate	(C18:3)	22.58	$C_{19}H_{32}O_2$	0.14
5.	Methyl eicosadienoate	(C20:2)	23.59	$C_{21}H_{38}O_2$	0.16
6.	Methyl arachidonate	(C20:4)	24.64	$C_{21}H_{34}O_2$	0.31
7.	Methyl behenate Methyl eicosapentaenoate	(C22:0) (C20:5)	25.42	$C_{23}H_{46}O_2$ $C_{21}H_{32}O_2$	0.95
8.	Methyl erucate	(C22:1)	26.89	$C_{23}H_{44}O_2$	0.20
9.	Methyl lignocerate	(C24:0)	29.47	$C_{25}H_{50}O_2$	0.45
10.	Methyl nervonate Methyl docosahexaenoate	(C24:1) (C22:6)	30.43	$C_{25}H_{48}O_2$ $C_{23}H_{34}O_2$	5.87

Table 7.14 GC-MS analysis of biodiesel of microalgae *S. vacuolatus* of wild-type and mutant strain identified fatty acid in varying nutrition conditions.

S. No.	Fatty acids	Carbon atom:double bond	Type of fatty acid
1.	Methyl hexanoate	(C6:0)	Saturated
2.	Methyl palmitate	(C16:0)	Saturated
3.	Methyl stearate	(C18:0)	Saturated
4.	Methtyl behenate	(C22:0)	Saturated
5.	Methyl lignocerate	(C24:0)	Saturated
6.	Methyl palmitoleate	(C16:1)	Unsaturated
7.	Methyl linoleate	(C18:2)	Unsaturated
8.	Methyl linolenate	(C18:3)	Unsaturated
9.	Methyl eicosadienoate	(C20:2)	Unsaturated
10.	Methyl eicosatrienoate	(C20:3)	Unsaturated
11.	Methyl arachidonate	(C20:4)	Unsaturated
12.	Methyl eicosapentaenoate	(C20:5)	Unsaturated
13.	Methyl erucate	(C22:1)	Unsaturated
14.	Methyl docosahexaenoate	(C22:6)	Unsaturated
15.	Methyl nervonate	(C24:1)	Unsaturated

7.4 Discussion

Treatment of wastewater for removal of pollutant load and utilisation of resulting biomass for biofuel production is a sustainable approach to solve many environmental problems (Sukla et al., 2019; Abinandan et al., 2018). Soybean and poultry wastewater were used for removal of nutrient by using wild-type and mutant strain of *S. vacuolatus*. The growth profile of microalga *S. vacuolatus* (WT and mutant strain) will depend on the nutrient present in the growth media and culture condition. In the present investigation, the results showed that higher biomass and biochemical constituents in soybean and poultry wastewater grown cells compared to BG-11 and tap water grown cells of both WT and mutant strain. The result depicted that utilisation of wastewater nutrients by the mutant strain was higher than the WT. Markou et al. (2016) reported that *C. vulgaris* produce maximum biomass content when the cells were grown on different dilution of poultry litter. In the present investigation, microalgal growth and

biochemical constituents were found to be maximum in both the strain grown on poultry wastewater when compared with corresponding use of soybean wastewater. The tap water grown cells exhibited the lowest biomass and biochemical constituents. However, BG-11 grown cells of both the strain showed higher biomass and biochemical constituents than the tap water, but much lower than that shown by soybean and poultry wastewater. It has been reported earlier that microalgal species are capable of mixotrophic growth in the presence of organic carbon (Bhatnagar et al., 2011). It has also been observed they are capable of simultaneously photoautotrophic and mixotrophic growth.

The results showed that higher biomass productivity, lipid productivity and lipid content (Bligh & Dyer method and Flow cytometry) of WT and mutant strain were found maximum in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and tap water. Other previous investigation showed that *Chlorella pyrenoidosa* grown in the presence of soybean processing wastewater produced maximum biomass 640 mg L^{-1} d^{-1} and lipid productivity (240 mg L^{-1} d^{-1}) and lipid content 37% (Su et al., 2011). Yu et al. (2018) reported higher lipid content (43%) and (7.9 g/L) dry biomass when oleaginous yeast *Trichosporon fermentans* grown in the refined soybean oil wastewater. Another study by Singh et al. (2011) found that maximum biomass productivity (76 mg L^{-1} d^{-1}) in *S. bijuga* in the presence of anaerobically digested poultry litter effluent. The biomass and lipid productivity in different species of microalgae depends upon the type of wastewater used and culture conditions (Gupta & Bux, 2019; Li et al., 2008; Xin et al., 2010).

The harvesting of algal biomass or settling of biomass are another hurdle in the mass cultivation of microalgae (Prajapati et al., 2013). The result of biomass settling and recovery efficiencies (η) were higher in WT as compared to mutant strain under varying temperature and pH conditions. The settling and recovery efficiency of WT (50-60%) was higher than mutant strain (35-40%), perhaps, due to differences in the cell size of both the strains. Several studies demonstrated that the flocculation of microalgae biomass can be naturally induced by increasing the pH of the growth medium (Vandamme et al., 2012). This phenomenon is commonly known as 'autoflocculation'. Vandamme et al. (2012) reported microalgal biomass recovery efficiencies (75%) for *Chlorella* cultivated in an artificial medium at pH 11.0 and at further pH 12.0 a higher value (96%). The present investigation exhibited that

temperature is another factor which determines the settling ability of cells. A similar study by Iasimone et al., (2018) reported that 52-72% biomass recovery efficiency was improved with changes in the light intensity (20 µmol m^{-2} s^{-1}) and other cultivation conditions of microalgae.

The successful investigation of wastewater treatment and biofuel production on a large scale has been reported for several microalgae (Das et al., 2019; Pragya et al., 2013). For higher growth and lipid production, microalgae consume nutrients and minerals which ultimately reduces the pollution load in wastewater (Ray et al., 2019; Gouveia & Oliveira, 2009). The main nutrients in many wastewater effluents include nitrogen and phosphorus one of the major causes of eutrophication in the water bodies (Wang & Lan, 2011). The remediation of wastewater by using microalgae have been used because of their capability to remove the several nutrients like nitrogen, phosphorus, and reduce BOD, and COD (Gupta et al., 2019; Duenas et al., 2003). In the present investigation, soybean and poultry wastewaters were taken to generate the microalgal biomass used for biodiesel production. The results demonstrated that nutrient load was efficiently reduced form soybean and poultry wastewaters when microalgae *S. vacuolatus* strains (WT and mutant strain) were grown in at appropriate dilution of wastewater. The overall nutrient removal efficiency 40-90% was achieved by both WT and mutant strain when grown exclusively on the soybean and poultry wastewater grown cells of both WT and mutant strain. The results of previous studies have also shown that reduction in nitrate content (57.89%), total dissolved solid (38.2%), phosphate content (34.59%), BOD and COD (77.89%, 79.12%), and favour the poultry wastewater during the growth of *Chlorella vulgaris* (Murugesan et al., 2010). Another study by Mandal & Mallick, (2011) reported the complete removal of nitrate and nitrite (100%), ammonium (97%), orthophosphate (92-98%) and TOC (14-52%) after 21 days of growth of microalga *S. obliquus* on different concentration of poultry litter wastewater. In the present study, the nutrient load of soybean wastewater was removed faster than the poultry wastewater by both the strains. Earlier reports have shown that *Chlorella, Ankistrodesmus* and *Scenedesmus* species successfully used for the wastewater treatment of olive oil, mill wastewaters and paper industry (Narro, 1987; Pinto et al., 2002). According to Rincon et al. (2014), *Chlorella protothecoides, Chlorella vulgaris, Nannochloropsis* sp., and *Neochloris oleabundans* have been identified as potential candidates for biomass and biodiesel production due to their high

lipid content and lipid productivity. However, overall findings suggested that the both WT and mutant strain exploit their capability of mixotrophic growth and thereby reduce the pollution load, as evident from their higher biomass yield in the presence of wastewater not in the BG-11 medium.

The FTIR analysis of microalgal biomass and lipid content of both WT and mutant cells were grown on tap water, BG-11, soybean and poultry wastewater. The triglycerides (TGAs) is the main component of lipids in the mutant. Lipoprotein and phospholipid are the major components in the lipid of WT cells (Grace et al., 2020; Wagner et al., 2010). The compositional changes in biomass and lipid content of both the strain analysed by the lipid/protein (P/L), lipid/carbohydrate (C/L), carbohydrate/amide I (C/P), total lipid and unsaturation/saturation ratios (Mahapatra et al., 2013; Dean et al., 2010) showed that such changes are associated with the nutritional conditions. The maximum lipid content was found in poultry wastewater grown cells of both WT and mutant strain, followed by soybean wastewater, BG-11 and tap water.

Further LC-MS (liquid chromatography-mass spectrometry) analysis of microalgae *S. vacuolatus* of both the strains (WT and mutant strain) grown on soybean and poultry wastewater, BG-11 and tap water. The result showed that amino acid content, carbohydrate and lipid in the cells again depends upon the nutrients condition. The result showed a higher accumulation of carbohydrate in both strains grown on soybean wastewater compared to poultry wastewater. The mannose was the most predominant sugar followed by fructose, galactose and maltose in the presence of soybean and poultry wastewater of both strains. The result of amino acids demonstrated that soybean wastewater was a good source of nitrogen nutrition than the poultry wastewater, particularly for amino acid synthesis. The accumulation of amino acid in both the strains was promoted by soybean and poultry wastewater as compared to the BG-11 and tap water. Further, the results depicted that most of the essential fatty acid content was higher in both the WT and mutant strain when grown in the presence of poultry wastewater followed by soybean wastewater, BG-11 and tap water. The overall results revealed that unsaturated fatty acid content was higher in both the strains, dominated by mainly linolenic acid (C18:3) and elaidic acid (C18:1). However, the overall level of lipid and fatty acid in the mutant strain was found to be higher than the

WT under different nutritional conditions. These results supported the findings of Lopez et al. (2017) and Yang et al. (2017).

Methyl esters of fatty acid were analysed in the biodiesel of soybean and poultry wastewater, BG-11 and tap water grown cells by using GC-MS. Biodiesel is mainly composed of methyl esters of long-chain fatty acids (mainly C12-C18 groups) derived from triglycerides (Yen et al., 2013). In the present study, results revealed the presence of monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA). Several studies have suggested that higher percentages of palmitic (C16:0), stearic (18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) fatty acids were more suitable for biodiesel (Knothe, 2009). The relative degree of unsaturation and saturation of fatty acids in biodiesel feedstock influences the biodiesel properties (Gour et al., 2016). It was observed that the fatty acid composition changed considerably in the biodiesel extracted from both WT and mutant strain depending upon nutritional conditions of the growth. The fatty acid profile of microalgal lipids is vital because the quality of biodiesel is highly dependent on the composition of the constituent fatty acids, which strongly influences the combustion efficiency and heating power of engines (Singh & Gu, 2010, Talebi et al., 2013). The palmitic acid is positively correlated with cetane number and negativity correlated with oleic acid and alpha-linolenic (Brennan & Owende, 2010; Gopinath et al., 2010).

Chavan et al. (2018) reported that oleic acid (65.83%), palmitic acid (5.81%) fatty acid were maximum in the biodiesel of *S. vacuolatus.* Several species-rich in docosahexaenoic acid (C22:6), a functional ingredient showing great benefits to people's health (Tang et al., 2011; Zhao et al., 2011), as in the *S. vacuolatus* cells observed in higher concentration. Therefore, for good quality biofuel production by using microalgae as a feedstock the ratio of saturated and unsaturated fatty acid is very important. (Hoekman et al., 2012). Undoubtedly, both the strains (WT and mutant strain) of *S. vacuolatus* are suitable for phycoremediation of wastewater particularly, the mutant strain was more efficient than the WT.

7.5 Conclusion

- In the present investigation, soybean and poultry wastewater were used for algal growth, using WT and mutant strain of *S. vacuolatus*.
- With respect to biomass, poultry wastewater performed better than soybean wastewater, followed by BG-11 and tap water.
- The maximum nutrient removal was recorded in the poultry wastewater followed by soybean wastewater by the mutant strain (40%, 60% respectively) than the WT (30%, 40% respectively).
- The biomass, biochemical constituents, lipid content were found to be better in the mutant than WT strain when grown under different nutritional conditions.
- Biomass recovery and settling efficiency (η) were higher in WT cells compared to mutant strain, perhaps due to its larger cell size.
- FTIR analysis and GC-MS analysis supported the results of the lipid content of both WT and mutant strain were found to be better in the poultry wastewater followed by soybean wastewater grown cells.
- FAMEs analysis of transesterification algal oil of both WT and mutant strain under different nutrition conditions (soybean and poultry wastewater, BG-11 and tap water) showed long-chain fatty acid which are most applicable methyl esters, desirable for good quality biodiesel.

Chapter- VIII

GENERAL DISCUSSION

Microalgae have colonized almost all the habitats of the earth about three and a half billion years ago ranging from oceans to glaciers, from salt lakes to freshwater, soils, rocks and trees (Alam et al., 2014). These photosynthetic organisms, which use light as an energy source to fix inorganic carbon (CO_2), play a key role in the functioning of many ecosystems and shape the food chain and participating the environment by CO_2 sequestration (Riebesell et al., 2007). Algae show higher growth and biomass productivity far superior to that of any terrestrial plants. They show the ability to store large quantities of lipids, which placed them as a potential source of so-called third-generation biofuel (Mata et al., 2010). Today, the use of fossil fuels as a major source of energy is widely disputed due to its effect on climate change and the decline in global oil resources (Hook & Tang, 2013). Thus, these microalgae are drawing increasing attention of scientist in the field of bioenergy (Chisti, 2007; Wijffels & Barbosa, 2010). There has been a considerable increase in the demand for energy all over the world in the last few decades (De Bhowmick et al., 2019). Microalgal biofuels have emerged as an alternative source of renewable energy, as microalgal cells produce carbohydrates (30-60%) and lipids (20-60%) that can be used for the production of bioethanol and biodiesel. In addition, biofuels based on microalgae (biodiesel) are non-toxic, easily biodegradable, emit low sulfur content as compared to petroleum (Rawat et al., 2013; Khan et al., 2018). A new focus on microalgal as biorefinery has been motivated due to increasing interest in microalgae as a renewable and sustainable feedstock for biofuel production and as a possible source of sustainable bioproducts of microalgae. There is a need to enhance the biomass production technologies by genetic manipulation of existing strains (Khan et al., 2018).

Every year, agro-industries produce vast quantities of waste. If this waste is released in the environment without proper disposal procedure, it can cause severe pollution in the environment and adversely affects human and animal health (Miksch et al., 2015). Most of the agro-industrial waste is dumped untreated or disposed of in an unplanned landfill. These untreated wastes create different problems with climate change by increasing greenhouse gases (Bos & Hamelinck, 2014). The waste sources of agro-industries are distillery, legumes, potatoes, soya, and wheat flour, dairy and poultry industry etc. As per the composition of these agro-industrial waste, and their nutritional perspective, they are categorized into different types of agro-industrial waste (Graminha et al., 2008). The wastewaters or leachates originating from agro-based industries have been reported to be useful for the cultivation of microalgae species (Huo

et al., 2012; Qin et al., 2014; Gupta & Pawar, 2018; Lu et al., 2015). In fact, every microalgae species responds differently to different kinds of wastewaters based on the different composition of C:N:P and source of wastewater. At present, many microalgal species like *Chlorella vulgaris, Chlorella pyrenoidosa, Chlorella sorokiniana, Scenedesmus obliquus, Scenedesmus abundans, Neochloris oleoabundans* have been efficiently used for their growth on agro-industrial wastewater (Huo et al., 2012; Qin et al., 2014; Tan et al., 2014; Olguin et al., 2015; Gupta & Pawar, 2018). However, Algae can play a dual role in wastewater treatment as well as in the utilisation of wastewater as a nutrient source and pollutant removal agents. Further, microalgal biomass after remediation of wastewater can be integrated to produce biodiesel by extracting lipid content of biomass and also it can clean up the wastewater.

Apart from wastewater treatment, many environmental stress conditions also affect microalgal growth such as light, temperature, pH, salinity and nutrition sources. Therefore, optimizing the environmental conditions that favour the growth of microalgae would help the potential of microalgae feedstock for sustainable production of food to fuels in years to come (Chen et al., 2016). As a consequence, lipids or carbohydrates generally start to accumulate in microalgae cells under physical or chemical stress condition, such as extremes light, temperature, pH, CO_2, accessibility of nutrients, salinity and levels of metals (Chen et al., 2013b, Rodriguez Couto, 2008). This high intensity stresses often result in growth inhibition, lower productivity of biomass and a higher risk of contamination (Hu et al., 2008). In order to improve the economic feasibility of using microalgae for the production of biofuels, greater knowledge is required as to how fast accumulation of lipids and carbohydrates in microalgae cells with higher biomass can be regulated by manipulating circumstances of environmental stress during cultivation (Chen et al., 2017).

In the present investigation, we studied the effect of environmental stress condition (such as light, nitrogen sources and wastewater) on the growth, biochemical constituents and lipid content of *Scenedesmus vacuolatus*. Further, genetic modification of the *S. vacuolatus* was achieved through chemical mutagenesis was applied to isolate the mutant strain exhibiting DCMU (75 µM) tolerance. Mutagenesis is an effective tool to select mutant strains of microalgae, which are capable of synthesizing higher lipid content (Larkum et al., 2012; Stephens et al., 2010). The ethyl methanesulfonate (EMS) was used as a mutagen to isolate the mutant strain which is not specifically dependent on the DNA repair or initial DNA damage,

ti can create a stable mutation in DNA such as DCMU- a herbicide-tolerant mutant (David et al., 2017; Phillips, 1994). In the present study DCMU-tolerant mutant, exhibiting better growth characteristics compared to wild-type (WT). The mutant strain showed about 2 fold greater DCMU-tolerance than the WT.

However, the optimum pH (7.8) and temperature (25 ℃) were the same for both the WT and mutant strain as reported by other workers (Rai & Rajashekhar, 2014; Pawlita-Posmyk et al., 2018). The result of flow cytometer analysis of neutral lipid by using Nile red demonstrated higher lipid content in the mutant strain as compared to WT. The mutant strain was further evaluated with respect to its photosynthetic characteristics by measuring the fast chlorophyll fluorescence induction parameters (OJIP curve) in the dark-adapted cells and was compared with WT, using the PAM fluorimeter. The results revealed that the photosynthetic performance of the mutant strain was better than the WT in the presence and absence of DCMU. It is a well-known fact that herbicide -DCMU blocks the electron transfer between Q_A and Q_B and it binds to D1 protein at the Q_B site (Antonacci et al., 2018; Trebst, 2007; Astier et al., 1984). Further, it has also been suggested that herbicide binding in the photosynthetic organisms, not only inhibits forward electron transfer to PQ, but it also leads to impairment in the recombination between $P680^+$Pheo radical pair and formation of a chlorophyll triplet as well as singlet oxygen ($^1O_2$) (Rutherford & Krieger-Liszkay, 2001; Krieger-Liszkay, 2005). A higher degree of DCMU-tolerance in the mutant strain of *S. vacuolatus* might be associated with changes in the DCMU binding 33- kDa D1 protein and its turnover in the photosynthetic apparatus (Matto et al., 2018; Astier et al., 1984; Erickson et al., 1984). In many photosynthetic organisms, the degree of DCMU induced damage to PSII under various environmental stresses has been correlated with a decrease in the level of the D1 protein (Przibilla et al., 1991; Roncel et al., 2007).

The transient chlorophyll fluorescence induction kinetics (OJIP) was used to evaluate the alteration in the photosynthetic parameters (F_v/F_m, F_v/F_o, ET_0/RC, TR_0/RC, ABS/RC, and PI_{ABS}). Besides, non-photochemical quenching (NPQ) (heat dissipation) and light curve (LC) analysis of both WT and mutant

strain were carried out to assess the photochemical efficiency of PSII (Zaghdoudi et al., 2011; Stirbet & Govindjee, 2012). The result showed change in F_v/F_m, F_v/F_o values, suggesting higher yields photochemical efficiency of PS II in the mutant strain. The other photosynthetic parameters of the mutant strain explain the ability of the cells to maintain a higher level of photosynthetic activity and to utilize the absorbed energy in a more efficient way in comparison to wild-type (Strasser et al., 2000). As evident from the OJIP curves, it was observed that a shorter time was taken by the mutant strain than the WT to reach the fluorescence maxima (F_m), indicating an efficient electron transfer in the mutant strain on the reducing side of PSII, which might be due to reduced probability of closure of PSII reaction centre (Strasser & Govindjee, 1992). The present results showed a decrease in all these photosynthetic parameters (ABS/RC, TR_0/RC, ET_0/RC) in the DCMU treated WT and mutant cells as compared control (without DCMU). However, the effect of DCMU was more pronounced on ABS/RC, TR_0/RC, and ET_0/RC in the WT than the mutant strain. Wang et al. (2010) have also reported that stress factor decreased the number of active reaction centres and activity of the water-splitting complex. The result on ET_0/RC, ABS/RC suggested a reduction in energy flux parameters for electron transport, trapping of electrons, absorption of photons per active reaction center in PS II are reduced in the presence of DCMU as suggested earlier (Lu & Vonshak, 2002; Kumar et al., 2015). The performance index PI_{ABS} value in the mutant strain, under both the absence and presence of DCMU, suggested better light utilisation efficiency of the mutant strain than the WT. Taken together, it was inferred that DCMU-tolerant mutant was endowed with better photosynthetic efficiency resulting in greater biomass and lipid synthesis.

The effect of varying light intensity and spectral quality of light on microalgal growth of WT and mutant strain of *S. vacuolatus* demonstrated that mutant strain was relatively more tolerant to high light stress (60 µmol m^{-2} s^{-1}) as compared to WT (40 µmol m^{-2} s^{-1}). The result showed that high light intensity between 80-100 µmol m^{-2} s^{-1} caused a drastic decrease in the growth, protein and carbohydrate content of both the strains. Several studies have shown that stress-induced increase in the ROS level is accompanied by stimulation of antioxidative response of the cells. It has been also suggested that the level of ROS generation is also coupled with the synthesis of lipid (Menon et al., 2013). The intracellular defence mechanism of the photosynthetic

organisms enables them to overcome the high light stress-induced cellular oxidative damage (Snoeijs et al., 2012).

FTIR analysis of cells grown under varying light intensities (10-100 µmol m^{-2} s^{-1}) also showed changes in the protein, carbohydrate and lipid content. Lipid/Protein (1740/1650 cm^{-1}) ratio increased with an increase in the light intensity in both the WT and mutant strains, particularly prior to their respective growth saturating light intensities. A light intensity-dependent increase in L/P ratio might be attributed to an increase in the total lipid content (2920/3011 cm^{-1}) as indicated by the FTIR analysis. A comparison of protein, carbohydrate and lipid constituents of both the strains indicated that the level of all the cell constituents in the mutant strain was higher than the WT throughout the range of light irradiances. Further, flow cytometric analysis of cell biomass demonstrated light intensity-dependent increase in the lipid content, which was higher in the mutant strain than the WT.

The result of RNO bleaching, -SH group, glycollate, lipid peroxidation, and SOD activity on light intensity showed a well-established fact that photo-damage is proportional to the intensity of incident light, (Simionato et al., 2013; Murata et al., 2007). The intracellular level of Malondialdehyde (MDA) a byproduct of cellular lipid peroxidation indicated peroxidative damage to cellular lipid (Kumar et al., 2015). Results on the photobleaching of pigments, lipid peroxidation, loss of -SH groups and RNO bleaching indicated a greater level of ROS generation and oxidative stress experienced by the WT than mutant strain. Results on the effect of radical quenchers on RNO bleaching in both the strains showed a very prominent effect of histidine- a quencher of superoxide radical ($O_2^{\cdot -}$), followed by sodium formate – a quencher of hydroxyl radical ($^{\cdot}OH$) (Matheson et al., 1975) and sodium azide (Foote et al., 1984). A higher rate of superoxide dismutase (SOD) activity, carotenoid and glycollate production in the mutant strain indicated that mutant strain was better equipped with strong anti-oxidative defence system than the WT as suggested earlier (Singh & Kshatriya, 2002; Janknegt et al., 2007; Nishiyama et al., 2001).

Apart from enzymatic antioxidative defences, thiol (-SH) groups also play a crucial role in the scavenging of ROS (Zeng et al., 2011). A higher level of the total -SH content in mutant strain than the WT under the high light irradiances indicated a mild photooxidative condition in the mutant strain. Similarly, an excessive production

of glycollate in the mutant strain than the WT might be associated with mitigation of photo-oxidative stress due to its ability of consuming surplus oxygen and modulation of carbon flux from TCA cycle to fatty acid synthesis as suggested by Tang et al. (2016) and Jeennor et al. (2006). Thus, it could be possible that stress-induced activation of the antioxidative defence system and lipid accumulation in the mutant strain might be mediated by glycollate metabolism as suggested earlier (Shi et al., 2017; Yang et al., 2013; Han & Eley, 1973).

It has been suggested that processing of light energy and its conversation into biomass depends upon the photochemistry of PSII which is the most sensitive component of the electron transport chain (Roach & Krieger-Liszkay, 2014). Many physiological and biochemical studied have led to the conclusion that photosynthetic organisms protect themselves from high light stress through down-regulation of photosystem II (Gururani et al., 2015; Takahashi & Badger, 2011). The photosynthetic parameters (F_v/F_m, ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS) (Stirbet & Govindjee, 2012) studied in *S. vacuolatus* indicated better photosynthetic performance and electron transport in the mutant strain than the WT even under high light intensity. The quantum yield (F_v/F_m), overall photosynthetic performance (RC/ABS) of the mutant strain, suggested better light utilization efficiency in the mutant strain than the WT. The captured photons in case of the WT were found to be largely dissipated in terms of heat (Muller et al., 2001) as evident from the higher value of NPQ in the WT as compared to mutant strain. A higher value of rETR (Malapascua et al., 2014) with higher photosynthesis saturating light intensity in the mutant strain supported the hypothesis that mutant strain was metabolically well adapted to handle the excess photons due to its altered photosynthetic system, which supports better light tolerance and photosynthetic efficiency in the mutant than the WT. In view of the foregoing evidence, it was inferred that higher level of light intensity tolerance and lipid accumulation in the mutant strain than the WT could be attributed to its strong antioxidative defence strategy of the mutant strain as evident from high SOD activity, carotenoid and glycollate content.

In the present investigation, quantitative changes in the photosynthetic pigments and biomass of both WT and mutant strains of *S. vacuolatus* observed in response to the spectral quality of light. WT cells are grown well under the green light, whereas

mutant strain showed better growth under the orange light condition. There was little growth in both the strains under the blue light regime. Other workers have shown that influences of different spectral irradiance on the photosynthetic efficiency, chlorophyll content and growth in both the photoautotrophic prokaryotes as well as eukaryotes (Wu, 2016). Kim et al. (2015) reported that alga *Gracilaria tikvahiae* showed slow growth rate under the blue light as compared to those grown under red and green light. It suggested that the response of the microalgae to light quality is species-specific and their survival is more dependent on the pigment composition (Li et al., 2019; Vadiveloo et al., 2015). Among various light spectra, red and blue light plays an important role in the photosynthesis and thus, influence the plant growth and metabolism (Manivannan et al., 2015).

The result revealed that the overall growth and level of pigments, protein, carbohydrate and lipid contents were higher in the mutant strain than the WT during the growth under all the spectral quality of light conditions. It was noticed that almost all the cell constituents in both the strains were interestingly linked with overall growth performance of the strains in response to the spectral quality of light. The OJIP induction kinetics of chlorophyll fluorescence (Antal et al., 2018; Stirbet & Govindjee, 2011; Strasser et al., 1995) was used to evaluate the photosynthetic characteristics of PSII reaction centre of both WT and mutant strains grown under the different spectral qualities of light. Consequently, the overall quantum yield (QY) of the mutant strain was found to be higher than that of the WT, irrespective of the spectral quality of light irradiance used during the growth. The lower QY and longer duration required to reach the F_m value by the WT strain, irrespective of the spectral quality of light used during the growth, indicated a slower rate of electron transport on the acceptor side of PSII (Malapascua et al., 2014; Neubauer & Schreiber, 1987). However, the absorption of photon flux per reaction centre (ABS/RC) and the electron flux for reduction of QA (ET_0/RC) were found to be higher in the green light-grown WT than the orange light-grown mutant cells. The current results also revealed a higher value of non-photochemical quenching (NPQ) in the WT cells than in the mutant, indicating their greater dissipation of absorbed photo-energy in the form of heat. In agreement with the earlier observation, chlorophyll

fluorescence induction kinetics of WT cells exhibited a sharp downward trend in the fluorescence after reaching the F_{max} value.

The FTIR analysis of microalgal biomass of WT and mutant strains, grown under the different spectral quality of light irradiances, also confirmed the variable synthesis of cell constituents (Dean et al., 2010; Jebsen et al., 2012; Bartosova et al., 2015). The flow cytometric analysis of neutral lipid revealed that the lipid content increased in both WT (green and red light) and mutant strains (orange and yellow light) in response to the spectral quality of light. Interestingly, a differential response of both WT and mutant strains in terms of changes in cell constituents, including lipid, under various spectral qualities of light might also be associated with the capability of their respective photosynthetic machinery to utilize the specific range of spectral qualities of light (Baer et al., 2016; Manivannnan et al., 2015).

Hence, these results suggested that WT and the mutant cells respond differentially to the different spectral quality of light. A variation in the cell biomass and composition in both the strains in response to spectral quality of light may not be solely attributed to absorption of particular light spectrum by pigments, but several other factors including conversion of light energy into biochemical energy in a particular spectral range of light may also lead to the process as suggested by other workers (Wu, 2016; Kumar et al., 2019). Taken together, it may be inferred that the selection of strains with better photosynthetic performance (DCMU-tolerant mutant strain) can be exploited for enhanced production of value-added products such as β-carotene and lipids, using optimal spectral quality of light.

The growth profile of microalga *S. vacuolatus* (WT and mutant strain) will depend on the nutrient present in the growth media and culture condition. Here, we studied the effect of soybean and poultry wastewater, BG-11 and tap water (TW) on the growth, cell constituents and biofuel production in both WT and mutant strain. Treatment of wastewater for the removal of pollutant load and utilisation of resulting biomass for biofuel production is considered a sustainable approach to solve many environmental problems. In this study, results showed that higher biomass and biochemical constituents in both the strains when grown on soybean and poultry wastewater grown cells as compared to that grown on BG-11 and tap water. The growth

and biochemical constituents were maximum in both the strain grown on poultry wastewater when compared with soybean wastewater. The tap water grown cells exhibited the lowest biomass and biochemical constituents. However, BG-11 grown cells of both the strain showed higher biomass and biochemical constituents than the tap water grown cells, but much lower than grown on soybean and poultry wastewater. However, the overall result depicted that utilisation of wastewater nutrients by the mutant strain was higher than the WT. A study by Markou et al. (2016) reported that *C. vulgaris* produced maximum biomass content when the cells were grown on different dilution of poultry litter. It has been reported earlier that microalgal species are capable of better mixotrophic growth in the presence of organic carbon.

Further, the results showed that higher biomass productivity, lipid productivity and lipid content (Bligh & Dyer and flow cytometry) of WT and mutant strain were found in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and tap water. Other previous investigation showed that *Chlorella pyrenoidosa* grown in the presence of soybean processing wastewater produced maximum biomass 640 mg L^{-1} d^{-1} and lipid productivity (240 mg L^{-1} d^{-1}) and lipid content (37%) (Su et al., 2011). Another study by Yu et al. (2018) reported higher lipid content (43%) and dry biomass (7.9 g/L) when oleaginous yeast *Trichosporon fermentans* was grown on the refined soybean oil wastewater. Another research by Singh et al. (2011) observed maximum biomass productivity (76 mg L^{-1} d^{-1}) in *S. bijuga* in the presence of anaerobically digested poultry litter effluent. Several studies have shown that biomass and lipid productivity in different species of microalgae depends upon the type of wastewater used and culture conditions (Li et al., 2008).

The harvesting of algal biomass or settling of biomass is another hurdle in the mass cultivation of microalgae. The result of biomass settling and recovery efficiencies (η) were higher in WT as compared to mutant strain under varying temperature and pH conditions. The settling and recovery efficiency of WT (50-60%) was higher than mutant strain (35-40%), perhaps, due to differences in the cell size of both the strains. Several studies demonstrated that the flocculation of microalgae biomass can be naturally induced by increasing the pH of the growth medium. Vandamme et al. (2012) reported microalgal biomass recovery efficiencies for *Chlorella* cultivated in an artificial medium at pH 11.0 (75%) and pH 12.0 (96%). The present investigation exhibited that temperature is another factor which determines the settling ability of cells. A similar study by Iasimone et al. (2018) reported that 52-72% biomass recovery

was further improved with changes in the light intensity (20 µmol m^{-2} s^{-1}) and other cultivation conditions of microalgae.

Wild-type and mutant strain of *S. vacuolatus* were grown in the presence of varying nitrogen sources such as sodium nitrate, ammonium chloride, sodium glutamate and different carbon: nitrogen (C/N) ratio. The result demonstrated that lower concentration of nitrogen sources were more effective in enhancing the growth and biochemical constituents in both WT and mutant strain. Further, it was observed that growth and biochemical constitutes in the mutant strain were better in the presence of all the nitrogen sources as compared to WT. However, the best growth of both WT and mutant strain was observed in under mixotrophic growth the C+N (Carbon: Nitrogen) and sodium nitrate supplemented conditions. The result demonstrated that the utilization of nitrogen sources such as sodium nitrate, ammonium chloride, and amino acids (glutamate) are directly involved in the regulation of nitrogen assimilation. Chi et al. (2015); Li et al. (2008); Dortch et al. (1982) have reported that nitrate and urea are the better nitrogen sources for microalgal growth, whereas amino acids are also potential nitrogen source for microalgal growth.

Other studies have shown that nitrogen as a limiting nutrient is an important factor for growth and lipid production (Jaiswal & Sharma, 2016). The nitrogen limiting condition is described to be one of the most important key regulators for triggering lipid synthesis in microalgal cells (Wu et al., 2013). In order to achieve the enhanced lipid production, a modulated physiological condition maybe is required which mimics the nitrogen deprivation condition and favourably influences the production of cell biomass and lipid content (Vitova et al., 2015). A reduction in the cellular protein under nitrogen limiting condition is described to have an immense impact on algal growth, pigment content and biomass production (Costa et al., 2001). Earlier reports have shown that carbohydrate accumulation by the algal cells is better under nitrogen deprived condition but the protein content declines (Bellou et al., 2014; Zhu et al., 2014).

The mixotrophic growth of several microalgae has been reported by using varying organic carbon source as a nutrient medium for microalgal growth such as sodium citrate, sodium acetate (Qiao & Wang, 2009), fructose, sucrose (Gao et al., 2009), and glucose (Lin & Wu, 2015). The results showed that a higher level of TOC

and carbohydrate accumulation in the C+N grown cells, compared to nitrate, ammonium, glutamate supplemented and nitrogen starved cells.

The growth response of each microalgal species depends on its ability to take up amino acid from the medium and the regulation of the nitrogen assimilation activity (Dao et al., 2014). Hellebust & Ahmad, (1989), reported microalgae *Stichococcus bacillaris,* utilize various amino acids as nitrogen for higher growth. In the present investigation, glutamate nitrogen was used as a nutrient medium to support growth and synthesis of biochemical constituents in both WT and mutant strain. It was observed that the glutamate nutrition of the *S. vacuolatus* cells was similar to that of nitrogen deprivation, which reduced the demand for carbon skeleton required for amino acid synthesis pathway and diverted it to lipid synthesis pathway (Moussa et al., 2017; Clayton et al., 1986). Since nitrogen deprivation and glutamate nutrition are directly involved in the regulation of nitrogen assimilation (Johnson et al., 2017; Jiang et al., 2012) as evident from reduced nitrogen reactivity, it may be concluded that reduced functioning of the nitrogen assimilatory system triggers the lipid accumulation, not the carbon status of the cells (Esteves-Ferreira et al., 2018).

The FTIR spectra of both WT and mutant strain grown under varying nitrogen nutrition showed changes in IR peaks at 2964 cm^{-1}, 2924 $cm^{-1,}$ 1740 cm^{-1} 1650 cm^{-1} and 1040 cm^{-1}, assigned to lipids, fatty acids, protein and carbohydrates (Murdock & Wetzel, 2009). The compositional changes in the microalgal cells was semi-quantitatively assessed in terms of total lipid (2920/3011cm^{-1}), lipid/carbohydrate (L/C) (1740/1040 cm^{-1}) lipid/protein (1740/1650 cm^{-1}) ratio (Jiang et al., 2012; Dean et al., 2010). The cells grown under different nitrogen nutrition revealed higher L/C ratio in the glutamate grown than nitrate and C+N grown WT and mutant cells. Similarly, the L/P ratio also showed the same trend, which was higher in the glutamate grown cells of both WT and mutant cells when compared with nitrate, ammonium, C:N supplemented conditions. Accumulation of higher total lipid content (1740 cm^{-1}) in the mutant strain than the WT was subsequently confirmed by flow cytometry analysis of neutral lipid in the microalgal cells. The results showed lipid in the mutant strain (46.0%) than the WT (42.01%) when grown under glutamate supplemented condition. The glutamate supported lipid synthesis in both the mutant and WT strain were higher than obtained under the nitrogen starved condition (30.61% and 26.05%, respectively).

The successful investigation of wastewater treatment and biofuel production on a large scale has been reported for several microalgae (Abomohra et al., 2018; Ajayan et al., 2015; Gupta et al., 2016; Lim et al., 2010). For higher growth and biomass, microalgae consume nutrients and minerals which ultimately reduces the nutrient load in the wastewater (Sukla et al., 2019). The main nutrients in many wastewater effluents are nitrogen and phosphorus one of the major causes of eutrophication in the water bodies (Boelee et al., 2011; Hernandez et al., 2006) The remediation of wastewater by using microalgae have been used because of their capability to remove the several nutrients like nitrogen, phosphorus, and reduce BOD, and COD (Gupta et al., 2019; Duenas et al., 2003). In the present investigation, soybean and poultry wastewaters were taken to generate the microalgal biomass used for biodiesel production. The results demonstrated that nutrient load was efficiently reduced form soybean and poultry wastewaters when both (WT and mutant strain) were grown in the wastewater at appropriate dilution. The overall nutrient removal efficiency 40-90% was achieved by both WT and mutant strain when grown exclusively on the soybean and poultry wastewater. The previous reports have shown substantial reduction in the nitrate content (57.89%), total dissolved solids (38.2%), phosphate content (34.59%), BOD and COD (77.89%, 79.12%), favour the poultry wastewater during the growth of *Chlorella vulgaris* (Murugesan et al., 2011). Another study by Mandal & Mallick, (2011) reported the complete removal of nitrate and nitrite (100%), ammonium (97%), orthophosphate (92-98%) and TOC (14-52%) after 21 days of growth of microalga *S. obliquus* on different concentration of poultry litter wastewater. The removal of ammonia nitrogen (99.5%) and COD (75%) from poultry manure wastewater by using anaerobic process were achieved (Li et al., 2016). In the present study, the nutrient load of soybean wastewater was removed faster than the poultry wastewater by both the strains. Earlier reports have shown that *Chlorella, Ankistrodesmus* and *Scenedesmus* species successfully used for the wastewater treatment of olive oil, mill wastewaters and paper industry (Narro, 1987; Pinto et al., 2002). According to Rincon et al. (2014), *Chlorella protothecoides, Chlorella vulgaris, Nannochloropsis* sp., and *Neochloris oleabundans* have been identified as potential candidates for biomass and biodiesel production due to their high lipid content and high lipid productivity. Oliveira et al. (2018) reported that bioremediation of poultry wastewater by microalga *S. obliquus* reduced ammonium, phosphate and COD in the poultry wastewater. However, overall findings suggested that the both WT and mutant strain cab be exploited for their

capability of mixotrophic growth and thereby reduce the pollution load, as evident from their higher biomass yield in the presence of wastewaters, as compared to BG-11 medium and tap water.

The FTIR analysis of microalgal biomass and lipid content of both WT and mutant cells were grown on tap water, BG-11, soybean and poultry wastewater. The triglycerides (TGAs) is the main component of lipids in the mutant. Lipoprotein and phospholipid are the major components in the lipid of WT cells. The compositional changes in biomass and lipid content of both the strain analysed by the protein/lipid (P/L), carbohydrate/lipid (C/L), carbohydrate/amide (C/P), total lipid and unsaturation/saturation ratios (Mahapatra et al., 2013) The maximum lipid content was found in poultry wastewater grown cells of both WT and mutant strain, followed by soybean wastewater, BG-11 and tap water.

Further LC-MS (liquid chromatography-mass spectrometry) analysis of microalgae *S. vacuolatus* of both the strains (WT and mutant strain) grown on soybean and poultry wastewater, BG-11 and tap water. The result showed that amino acid content, carbohydrate and lipid in the cells depend upon the nutrient conditions showed a higher accumulation of carbohydrate in both strains grown on soybean wastewater compared to poultry wastewater. The mannose was the most predominant sugar in both strains followed by fructose, galactose and maltose in the presence of soybean and poultry wastewater. The result of amino acids demonstrated that soybean wastewater was a good source of nitrogen nutrition than the poultry wastewater, particularly for amino acid synthesis. Further, the results depicted that most of the essential fatty acid content was higher in both the WT and mutant strain when grown in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and tap water. The overall results revealed that unsaturated fatty acid content was higher in both the strains, dominated by mainly linolenic acid (C18:3) and elaidic acid (C18:1). However, the overall level of lipid and fatty acid in the mutant strain was found to be higher than the WT.

Methyl esters of fatty acid were analysed in the biodiesel of soybean and poultry wastewater, BG-11 and tap water grown cells by using GC-MS. Biodiesel is mainly composed of methyl esters of long-chain fatty acids (mainly C12-C18 groups) derived from triglycerides (Yen et al., 2013). In the present study, results revealed the presence of monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA)

and saturated fatty acid (SFA). Several studies have suggested that higher percentages of palmitic (C16:0), stearic (18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) fatty acids were more suitable for biodiesel (Knothe, 2009). The relative degree of unsaturation and saturation of fatty acids in biodiesel feedstock influences the biodiesel properties (Gour et al., 2016).

It was observed that the fatty acid composition changed considerably in the biodiesel extracted from both WT and mutant strain depending upon nutritional conditions of the growth. The fatty acid profile of microalgal lipids is vital because the quality of biodiesel is highly dependent on the composition of fatty acids, which strongly influences the combustion efficiency and heating power of engines (Singh & Gu, 2010, Talebi et al., 2013). The palmitic acid is positively correlated with cetane number and negativity correlated with oleic acid and alpha-linolenic (Gopinath et al., 2010). Chavan et al. (2018) reported that oleic acid (65.83%), palmitic acid (5.81%) fatty acid were maximum in the biodiesel of *S. vacuolatus.* Several species-rich in docosahexaenoic acid (C22:6), a functional ingredient showing health benefits to people (Tang et al., 2011; Zhao et al., 2011), as observed in the *S. vacuolatus* at higher concentration. Therefore, the proper ratio of saturated and unsaturated fatty acid is very important in the microalgae, if used as a biodiesel feedstock.

The overall finding showed that DCMU-tolerant mutant strain has higher overall attributes such as growth, biochemical constituents, and biofuel production compared to WT. Improved photosynthetic efficiency of the mutant strain as revealed by the result of chlorophyll induction kinetics (OJIP) and non-photochemical quenching (NPQ) and light curve (LC) analysis make it better strain than the WT for biomass and lipid production. The result of FTIR and flow cytometer on biochemical constituents and lipid content established the fact that mutant was better than WT for using it as feedstock for biofuel production.

SUMMARY

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CONCLUSION

Algae have developed broad tolerance to environmental stress conditions including high nutrient levels. This advantage has led to the wide use of the algae in bioremediation of wastes, resulting in treated waters as well as the production of useful biomass (El-Din, 2019; Olguin, 2003). The algal biomass serves as feedstock for several valuable products, including food, feed, fertiliser, pharmaceutical and biofuel production. Nutrient removal by algae is economical, sustainable, simple and beneficial for the environment (Filippino et al., 2015). Integration of wastewater treatment and biomass production may lead to economic savings as well as the opportunity to avoid costly wastewater treatment method and production of high-value algal biomass (Gupta et al., 2019). Microalgae have been found to be promising in the removal of nutrients primarily nitrogen and phosphorus, BOD, and COD (Aslan & Kapdan, 2006; Lebeau & Robert, 2003). Further, significant progress in the field of cultivation of microalgae coupled with treatment of wastewater has resulted in the improvement in the production of algal biomass and biofuel production (Salama et al., 2017).

The present investigation was an attempt to study the ability of wild-type and mutant strain of *S. vacuolatus* to withstand the various environmental stresses. The overall stress tolerance ability was exploited for wastewater treatment, biomass production and improvement in the accumulation of lipid- a source of biofuel. The first objective of the work was to isolate a DCMU-tolerant mutant strain of microalga *S. vacuolatus*, which has the potential to sequester more carbon due to as a modification in the photosynthetic apparatus of the alga. The growth of microalgae under harsh environmental conditions such as limited nitrogen nutrition and high light intensity and different spectral quality of light was also optimized with respect to photosynthetic efficiency, biomass and lipid content. Further, agro-industrial wastewater was used as a nutrient source for growth, while remediation of wastewater was carried out to reduce the pollutant load.

The isolated algae from Leh, Ladakh, India was identified by 18S rRNA analysis as *S. vacuolatus* (MH459062). Further, for the improvement of algal strain for production of higher biomass and lipid content was achieved by improvement in the photosynthetic apparatus of *S. vacuolatus* by random chemical mutagenesis by using EMS (ethyl methanesulfonate) as a chemical mutagen. According to Larkum et al. (2012) and Stephens et al. (2010), mutagenesis is an effective tool to select mutant strains of microalgae, which are capable of synthesizing higher lipid

content. The selection of mutant strain after mutagenesis of *S. vacuolatus* was done by screening the mutant population, using a sub-lethal concentration of DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea) a herbicide (also known as diuron, direx, karmex). DCMU is known to inhibit the photosynthetic electron transport by blocking the electron transfer at the level of QB site of photosystem II (Manandhar-Shrestha et al., 2009; Huber & Edwards, 1975). The comparative study of wild-type (WT) and mutant strain demonstrated that about 2 fold greater DCMU-tolerance in the mutant strain as compared to wild-type (WT).

The mutant strain was further evaluated in terms of its photosynthetic characteristics by measuring the fast chlorophyll fluorescence induction parameters (OJIP curve) and was compared with the WT. The results revealed that the photosynthetic performance of the mutant strain was better than the WT in the presence as well as the absence of DCMU. A higher degree of DCMU-tolerance in the mutant strain of *S. vacuolatus* might be associated with changes in the DCMU binding 33-kDa D1 protein and turn over in the photosynthetic apparatus (Matto et al., 2018; Astier et al., 1984; Erickson et al., 1984). Further, a high non-photochemical quenching (NPQ) value in the mutant strain than the WT might be the main reason behind high light intensity tolerance of the mutant strain as evident from the value of rETR (Relative Electron Transport Rate) measured under varying photon flux density.

Further, the analysis of the morphology of both WT and mutant strain by Scanning electron microscope (SEM) and flow cytometer, revealed that mutant strain has smaller cell size, in comparison to WT cells. However, optimum pH (7.8) and temperature (25℃) condition both the WT and mutant strain were the same. The analysis of lipid content by flow cytometer during different phases of growth of both WT and mutant strain showed higher lipid content during the stationary phase of both the strains when compared with exponential and lag phase of growth.

Another study based on the effect of light quality and light intensity on the photosynthetic efficiency and cells constituents of microalgae *S. vacuolatus*. The result showed that mutant strain was relatively more tolerant to high light stress (60 µmol m^{-2} s^{-1}) as compared to WT (40 µmol m^{-2} s^{-1}). The high light intensity between 80-100

µmol m^{-2} s^{-1} caused a drastic decrease in the growth, protein and carbohydrate content of both the strains. FTIR analysis of cells grown under varying light intensities (10-100 µmol m^{-2} s^{-1}) also showed changes in the protein, carbohydrate and lipid content of both the strains. FTIR and flow cytometric analysis of cell biomass demonstrated light intensity-dependent decrease in protein and carbohydrate, but lipid content was increased. However, the cell constituents including lipid was higher in the mutant strain (60 µmol m^{-2} s^{-1}) than the WT (40 µmol m^{-2} s^{-1}). Results on the photobleaching of pigments, lipid peroxidation, loss of -SH groups and RNO bleaching indicated a greater level of ROS generation in the WT than the mutant.

The photosynthetic parameters (F_v/F_m, ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS) (Stirbet & Govindjee, 2012) studied indicated better photosynthetic performance and electron transport in the mutant strain than the WT even under the high light intensity. The quantum yield (F_v/F_m) and PIabs or RC/ABS of the mutant strain, suggested better light utilization efficiency in the mutant strain than the WT. The captured photons in case of the mutant were found to be largely dissipated in terms of heat as evident from the higher value of NPQ. Whereas, a higher value of rETR with higher photosynthesis saturating light intensity in the mutant strain supported the hypothesis that mutant was metabolically well adapted to handle the excess photons due to its altered photosynthetic system, which supports better light tolerance and photosynthetic efficiency in the mutant than the WT.

Whereas the result of light quality demonstrated that WT cells are grown well under the green light, whereas mutant strain showed better growth under the orange light condition. There was little growth in both the strains under the blue light regime. FTIR analysis of WT and mutant strains confirmed the variable synthesis of cell constituents. The flow cytometric analysis of neutral lipids in both the strains revealed increased in the lipid of WT under green and red light, whereas mutant strains exhibited maximum lipid under orange and yellow light. Interestingly, a differential response of both WT and mutant strains exhibited changes in the cell constituents, including lipid, in response to varying spectral qualities of light as well as light intensity, strain-specific characteristics of the photosynthetic machinery.

The OJIP induction kinetics of chlorophyll fluorescence was used to evaluate the photosynthetic characteristics of the PSII reaction centre of both WT and mutant strain under the different spectral qualities of light. The overall quantum yield (QY) of the mutant strain was found to be higher than that of the WT, irrespective of the spectral quality of light irradiance used during the growth. The current results also revealed a higher value of non-photochemical quenching (NPQ) in the WT cells than the mutant, indicating greater dissipation of absorbed photo-energy by WT in the form of heat. The highest use efficiency of mutant strain was better than WT, resulting into its higher biomass and lipid contents.

Further study on the effect of varying nitrogen sources (sodium nitrate, ammonium chloride, sodium glutamate and different carbon: nitrogen (C/N) ratio) on the growth, biochemical constituents, lipid production in the wild-type (WT) and mutant strain of *S. vacuolatus* demonstrated nitrogen limiting condition was more effective in enhancing the lipid production but biomass content was reduced in both WT and mutant strain. Further, the result suggested that growth and biochemical constitutes in the mutant strain were better in the presence of all the nitrogen sources as compared to WT. However, the best growth of both WT and mutant strain was observed under the mixotrophic (carbon: nitrogen) and nitrate supplemented conditions but the lipid content was reduced. On the contrary lipid content was highest in the glutamate supplemented and nitrogen starved conditions. It is possible that the mode and type of nitrogen nutrition may influence carbohydrate and protein synthesis (De Farias et al., 2018; Chen et al., 2013). The nitrogen limiting condition is described to be one of the most important key regulators for triggering lipid synthesis in microalgal cells (Wu et al., 2013).

In the present investigation, glutamate was used as a nitrogen source to support growth and synthesis of biochemical constituents including lipid in both WT and mutant strain. Based on the results, reduced functioning of the nitrogen assimilatory system in the glutamate grown and nitrogen starved cells of both the strains was found to be the main reason for the higher accumulation of lipid as evident from the flow cytometry analysis in both the strains. The overall findings suggested that mutant strain showed better growth, biochemical constituents and lipid content as compared to WT.

The microalgal strains of *S. vacuolatus* were used for the treatment of soybean and poultry wastewaters. The results on both WT and mutant strain grown on the soybean and poultry wastewater were compared with bore well water (tap water) and synthetic medium (BG-11). The results exhibited higher biomass and biochemical constituents in soybean and poultry wastewater grown cells of both WT and mutant strain, when compared with BG-11 and bore well (tap) water grown cells. However, microalgal growth and biochemical constituents in both the WT and mutant strain grown on poultry wastewater was maximum when compared with the corresponding value of both the strains grown on soybean wastewater. The tap water grown cells exhibited the lowest biomass and biochemical constituents. However, BG-11 grown cells of both the strain showed higher biomass and biochemical constituents than the tap water, but much lower than that shown by soybean and poultry wastewater. The result also indicated that utilisation of wastewater nutrients by the mutant strain was better than the WT.

FTIR analysis of microalgal biomass and lipid content of both WT and mutant cells were grown on tap water, BG-11, soybean and poultry wastewater showed that triglycerides (TGAs) were the dominant component of lipids in the mutant strain. Whereas lipoprotein and phospholipid were the major components of lipid in WT cells. The harvesting of algal biomass or settling of biomass is another hurdle in the mass cultivation of microalgae. The result of biomass settling and recovery efficiencies (η) were found to be higher in WT (50-60%) than the mutant strain (35-40%) under varying temperature and pH conditions, perhaps, due to differences in the cell size of both the strains. The present investigation suggested that temperature can be one important factor to determine the settling ability of cells.

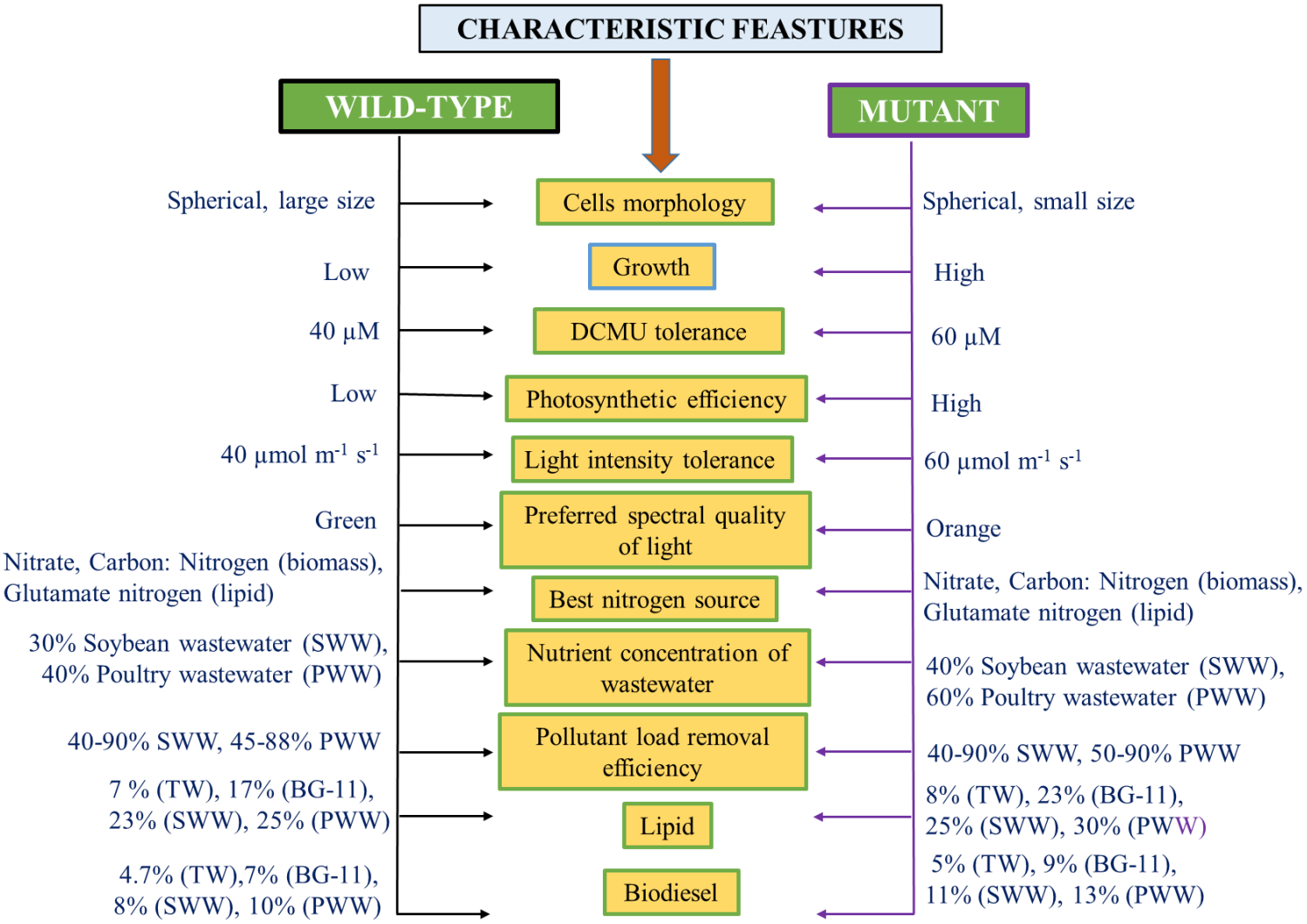
In the present investigation, soybean and poultry wastewaters grown microalgal biomass was used for biodiesel production and remediation of wastewater. The results demonstrated that nutrient load was efficiently reduced form soybean and poultry wastewaters when both the strains (WT and mutant strain) were grown at appropriate dilution of wastewater. The overall nutrient removal efficiency of both WT and mutant strain was in the range of 40-90% when grown exclusively on the soybean and poultry wastewater. However, removal of the nutrient load of soybean wastewater was faster than the poultry wastewater in the presence of both the strains.

Further LC-MS (liquid chromatography-mass spectrometry) analysis of microalgae *S. vacuolatus* of both the strains (WT and mutant strain) grown on soybean and poultry wastewater, BG-11 and tap water showed higher accumulation of carbohydrate by both strains grown on soybean wastewater when compared with that in the poultry wastewater grown cells. The mannose was the most predominant sugar followed by fructose, galactose and maltose in the presence of soybean and poultry wastewater of both strains. The result of amino acid accumulation demonstrated that soybean wastewater was a good source of nitrogen nutrition than the poultry wastewater, particularly for amino acid synthesis. The accumulation of amino acid in both the strains was promoted by soybean and poultry wastewater as compared to the BG-11 and tap water. Further, the results depicted that most of the essential fatty acid content was higher in both the WT and mutant strain when grown in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and tap water. The overall results also revealed that unsaturated fatty acid content was higher in both the strains, mainly dominated by mainly linolenic acid (C18:3) and elaidic acid (C18:1). However, the overall level of lipid and fatty acid in the mutant strain was found to be better than the WT under different nutritional conditions.

Methyl esters of fatty acid were analysed in the biodiesel of soybean and poultry wastewater, BG-11 and tap water grown cells by using GC-MS. The higher biodiesel concentration was obtained from the poultry wastewater grown cells, followed by soybean wastewater, BG-11 and tap water grown cells. The results revealed the presence of monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA). The biodiesel from the microalgal irrespective of source of nutrition showed mainly methyl esters of hexanoate (C6:0), palmitic (C16:0), palmitoleate (C16:1), stearate (C18:0), linoleate (C18:2), linolenate (C18:3), behenate (C22:0), erucate (C22:1), lignocerate (C24:0), nervonate (C24:1) and docosahexaenoic (C22:6).

Hence, foregoing results revealed that the unique characteristics of the DCMU-tolerant mutant strain of *S. vacuolatus* was its higher light intensity tolerance, higher photosynthetic performance and better nutrient removal efficiency as compared to WT strain. The biomass productivity, lipid and biofuel production capability of the mutant strain was better than the WT. These attributes of DCMU-tolerant mutant strain make it an efficient tool to develop a sustainable, eco-friendly technology to integrate the phycoremediation of wastewater and biofuel production at a very reduced cost.

Flow chart showing characteristic features of wild-type and mutant strain of *S. vacuolatus*



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PUBLICATIONS

RESEARCH PAPERS

- Gupta, N., Khare, P., & Singh, D. P. (2019). Nitrogen-dependent metabolic regulation of lipid production in microalga *Scenedesmus vacuolatus*. *Ecotoxicology and environmental safety*, *174*, 706-713.
- Gupta, N., Khare, P., & Singh, D. P. (2019). Effect of spectral quality of light on growth and cell constituents of the wild-type (WT) and DCMU-tolerant strain of microalga *Scenedesmus vacuolatus*. *Energy, Ecology and Environment*, *4*(4), 175-188.

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- Gupta, N., & Singh, D. P. (2016). Low-cost Production of Algal Biofuel from Wastewater and Technological Limitations. In: *Emerging Energy Alternatives for Sustainable Environment*, (Eds.). Singh, D.P., Kothari R., & Tyagi, V.V. CRC Press, Technology & Engineering, pp 522.

CONFERENCES

- Attended a workshop on "Hands-on-Training on SEM, FTIR, FPLC and Ion Chromatography" organised by University Science Instrumentation Center, B.B. Ambedkar University, Lucknow (U.P.), during 18th - 20th February 2015.
- Participated an International Conference on Strategies for Environmental Protection and Management (ICSEPM-2016) mini-symposium on Environmental Biotechnology, Biorefinery and Solid Waste Management (BRSI), organised by Jawaharlal Nehru University, New Delhi, 11-13 December 2016, with a paper entitled " Microalgae: A step towards the sustainability of the renewable energy" by Neha Gupta and D.P. Singh.
- Attended a National symposium on "IPRs in Agricultural Research" organised by B.B. Ambedkar University, Lucknow, (U.P.) and U.P. Council of Agricultural Research, Lucknow (U.P.) on 30-31 August 2017.

- Attended a workshop on "Socio-environmental Dimensions of Rejuvenating River Gomti" on 23rd April 2018 organised by Department of Environmental Science, BBAU, Lucknow in association with Lokbharti.
- Presented a paper entitled "Potential application of microalga *Chlorococcum humicola* for treatment of wastewater: A sustainable approach for biomass and biofuel production", Neha Gupta and D.P. Singh, held at 58th Annual Conference of Association of Microbiologists of India (AMI-2017) and International Symposium on Microbes for Sustainable Development: Scope & Application (MSDSA-2017) organised by B.B. Ambedkar University, Lucknow (U.P.), during 16-19th November 2017.
- Attended one-day seminar on "Environmental Sustainability: Present Scenario and Future Aspects" organised by Department of Environmental science, B.B. Ambedkar University, Lucknow, U.P. on 10th January 2019.
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Nitrogen-dependent metabolic regulation of lipid production in microalga *Scenedesmus vacuolatus*

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ABSTRACT

Microalga *Scenedesmus vacuolatus* exhibited maximum growth, protein and carbohydrate contents at 10.0 mM concentration of nitrate, 1.0 mM of glutamate nitrogen and at C/N ratio (12 mM acetate + 10 mM nitrate). However, these cell constituents showed the highest values in the C + N grown cells, but the lipid content was found to be the highest glutamate grown cells. FTIR analysis of Lipid/Carbohydrate and Lipid/Protein ratio and flow cytometric analysis of neutral lipids revealed higher lipid content in the glutamate grown cells than in the nitrogen starved, nitrate and C + N grown cells. The nitrate reductase activity was the highest in the C + N grown cells and the lowest activity was found in the glutamate grown cells. A corollary of these results suggested that suppression of nitrogen assimilatory system, whether by glutamate or by nitrogen deprivation, was the most suitable physiological condition for enhanced lipid synthesis and biofuel production in microalgal cells.

1. Introduction

Microalgae have recently started gaining recognition as a potential source of third generation biofuel due to their low nutrient requirement, rapid biomass production and relatively high oil content (Amin, 2009). In comparison to plant crops, microalgae commonly double in size every 24 h and they have a more efficient photosynthetic system to produce biomass (Chisti, 2007). The higher algal bio-oil yield in microalgae depends on the overall biomass productivity and lipid content. Many microalgal strains have been reported to show higher oil yield, on being induced for enhanced lipid accumulation (Faried et al., 2017; Pittman et al., 2011). Previous studies have reported enhanced accumulation of neutral lipids (particularly triacylglycerol) in microalgal cells, depending upon the availability of nitrogen and phosphorous nutrients (Converti et al., 2009). Many investigators have reported that nitrogen-limiting growth condition of microalgae strongly influences the carbon metabolism of algal cells (Kim et al., 2016; Tao et al., 2013) and is adopted as key strategy to trigger the lipid accumulation in many algal species (Silva et al., 2018; Xia et al., 2016; Mortensen and Gislerod, 2016). Though the nitrogen deprivation of microalgal cells can induce accumulation of carbohydrate and lipid up to a level of 60% the dry weight of biomass (Chen et al., 2013), it significantly lowers the biomass production (Berges et al., 2002). Hu et al. (2008) suggested that the main cause of excessive accumulation of carbohydrate and lipid in the nitrogen-starved microalgal cells was due to the diversion of photosynthetically fixed carbon from the protein synthesis pathway to lipid and carbohydrate synthesis. Olofsson et al. (2014) also reported that the nitrogen limiting condition favoured the diversion of surplus energy and carbon pool from an amino acid synthesis pathway to lipid synthesis. But the application of nutrient deprivation such as nitrogen starvation technique may not be beneficial technology for biofuel production in industries (Zhang et al., 2013; Chen et al., 2015) as the nitrogen deficiency impairs cellular protein synthesis, leading to poor enzyme activity and reduced biomass (Eroglu and Melis, 2016). However, it has been observed that stress-induced rise in lipid productivity per unit biomass is accompanied by a simultaneous decrease in biomass productivity (Kumar et al., 2018; Flynn et al., 1993). Thus, efforts should be made to determine cultivation conditions which favoured both higher biomass productivity as well as enhanced lipid accumulation for achieving an efficient and low-cost biofuel production technology (Griffiths and Harrison, 2009).

Assimilation of inorganic nitrate nitrogen by algae into amino acid and protein takes place through the GS-GOGAT pathway (Hellebust and Ahmad, 2013). The most reduced inorganic nitrogen ammonium is incorporated into amino acid by utilizing the photosynthetically derived carbon skeleton (Temple et al., 1998). However, it has been reported that amino acids can be directly utilized by the algal cells to meet their cellular nitrogen requirement (Hellebust and Ahmad, 2013;

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ORIGINAL ARTICLE

Effect of spectral quality of light on growth and cell constituents of the wild-type (WT) and DCMU-tolerant strain of microalga *Scenedesmus vacuolatus*

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Abstract Wild-type (WT) and DCMU-tolerant mutant strain of microalga *Scenedesmus vacuolatus* were used to study the effect of different spectral quality of light on the growth and biochemical constituents. Results showed that overall growth, cell constituents and total organic carbon were higher in the mutant strain than the WT under all types of light irradiances. However, green and orange light conditions were found to be optimum for both WT and mutant strain, respectively. FTIR analysis of biomass of WT and the mutant strain showed the minor difference in the lipid/carbohydrate (0.82 ± 0.061, 0.71 ± 0.05, respectively) and lipid/protein (0.52 ± 0.026, 0.59 ± 0.040, respectively) ratios due to changes in the spectral quality of light. Flow cytometer analysis of WT and mutant cells, grown under green and orange light, respectively, exhibited maximum lipid content, but the lipid content in the mutant strain was higher than the WT. The PAM fluorimeter parameters $\Delta F/F_m'$, F_v/F_o ABS/RC, RC/ABS and ET_0/RC derived from chlorophyll fluorescence induction (OJIP) curve of WT and the mutant strain showed better photosynthetic performance (RC/ABS) in the green light-grown WT and orange light-grown mutant strain. However, a reduced level of growth, cell constituents and photosynthetic yield in the WT than the mutant strain might be attributable to the higher value of NPQ, indicating greater loss of absorbed photo-energy in the WT cells. Thus, efficient utilization of spectral quality of light irradiance by the microalga was strain-specific, depending upon the photosynthetic attributes of individual strain as evident from the comparison of photosynthetic properties of both wild-type and DCMU-tolerant mutant strain.

Keywords DCMU-tolerant microalga · Spectral quality of light · Biochemical constituents · Flow cytometer · FTIR analysis · Chlorophyll fluorescence induction kinetics

Abbreviations

F_0	Fluorescence intensity at 50 μs
F_v	Variable fluorescence
F_m	Maximum chlorophyll fluorescence in the dark-adapted state
F_m'	Maximum chlorophyll fluorescence in the light-adapted state
V_j	Variable fluorescence at point *J*
M_0	Initial slope of induction curve
RC	Reaction centre
$\Delta F/F_m'$	Actual quantum yield of PSII photochemistry
F_v/F_0	Maximum quantum yield of PSII photochemistry
ET_0/RC	Trapping of energy flux per reaction centre for electron transport
ABS/RC	Absorption of energy flux per reaction centre
RC/ABS	Performance index on absorption basis
NPQ	Non-photochemical quenching
QY	Quantum yield
LED	Light-emitting diode
PAR	Photosynthetically active radiation

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