REMEDIATION OF WASTEWATER AND BIOFUEL PRODUCTION USING MICROALGAE

Ph.D. THESIS

by

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DEPARTMENT OF POLYMER AND PROCESS ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE– 247 667 (INDIA)
SEPTEMBER, 2019

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A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

DOCTOR OF PHILOSOPHY

in

ENVIRONMENTAL ENGINEERING

by

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CANDIDATE'S DECLARATION

I hereby certify that the work presented in the thesis entitled "**REMEDIATION OF WASTEWATER AND BIOFUEL PRODUCTION USING MICROALGAE**" is my own work carried out during a period from January, 2014 to September, 2019 under the supervision of Dr. Uttam Kumar Ghosh, Associate Professor, Department of Polymer and Process Engineering, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted for the award of any other degree of this or any other Institute.

Dated: 15 **November, 2019**

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SUPERVISOR'S DECLARATION

This is to certify that the above mentioned work is carried out under my supervision.

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ACKNOWLEDGEMENTS

First and foremost, my greatest regards to the Almighty for bestowing upon me the courage to face the complexities of life and complete this thesis successfully.

I would like to express my heartfelt gratitude and thanks to my supervisor Dr. Uttam Kumar Ghosh, Associate Professor, Department of Polymer and Process Engineering, Indian Institute of Technology Roorkee, Saharanpur Campus for his proficient guidance, advice, and supervision throughout this investigation. I acknowledge his unfaltering patience, ever-lasting support and the belief in me. He provided me unflinching encouragement and support. His guidance helped me in all the time of research and writing of this thesis.

I would like to take this opportunity to put on record my gratitude to Y.S. Negi (Prof-In-Charge and internal expert) Department of Polymer and Process Engineering, IIT Roorkee for providing the various basic facilities for carrying out the present research work. I wish to express my sincere gratitude to the members of advisory committee namely Dr. Millie Pant (Chairman, DRC and SRC) and Prof. S.P. Singh (external expert), IIT Roorkee, Saharanpur Campus for their invaluable suggestions and unconditional support to accomplish this thesis. I am especially thankful to Prof. Amiya Kumar Ray, Prof. Pradeep Kumar and Prof. Indu Mehrotra for their valuable suggestions and moral support at every step of this thesis work. I am also thankful to Mr. S.P. Singh Pundir, Mr. Balveer Saini, Mr. Rakesh Arora, Mr. Nempal Singh and all other staff members of IIT Roorkee, Saharanpur Campus for their moral support and co-operation during my research work.

I am thankful to the Ministry of Human Resource Development (MHRD), Government of India for providing financial support to accomplish this research work.

I wish to thank my friends Dr. Manoj Kumar, Dr. Medalson Ronghang, Dr. Pooja Sharma, Dr. Surendra Pratap Singh, Dr. Alok Kumar Patel, Dr. S.K. Yadav, Dr. S.P. Yadav, Dr. Sachin Sharma, Dr. Shiv Shankar, Dr. Jiwan Singh, Mr. Surendra Singh, Dr. Pradeep Kumar, Mr. Sushil Kumar Verma, Dr. Kuldeep Bauddha, Dr. Uma Shankar, Dr. Santar Pal Singh, Dr. Umar Farooqui, Dr. Tushar Bhardwaj and Mr. Rohit Sharma for their moral support in this journey. I am also thankful to my lab mates and friends Mr. Amit, Mr. Jagdeep Kumar Nayak, Mr. Rinku, Mr. Uday Shankar, Mr. Sumit Singh, Mr. Gaurav Jain and Mr. Rahul Gautam for their unconditional help and support during achievement of this thesis. I wish to thank everybody with whom I have shared experiences in life and specially those who have played a significant role in my life.

I would like to express my sincere love and affection to my father Mr. Rambahadur and my late mother Mrs. Pushpa Devi.

I am extremely indebted to my wife Mrs. (Dr.) Snigdhendubala Pradhan for her utmost support, love, affection and being with me always specially whenever I felt low during my research work. I am also thankful for my beloved siblings, Mr. Mahesh Chandra, Mr. Bipin Kumar and Mrs. Ranjana Devi for being there with me at every moment of my life. My special thanks go to Mr. Benudhar Pradhan (father in-law), Mrs. Sampada Patra (mother in- law) and Mr. Satchidananda Pradhan (brother in-law) for their moral support during this research work. I am also thankful to my maternal sister Mrs. Vibha Devi's support at every moment of my life.

Last but not least, I would like to thank everybody who contributed to the successful realization of this thesis.

September, 2019 **Rajesh Chandra**

ABSTRACT

Increasing concentrations of carbon dioxide and other harmful gases into the environment from various sources like combustion of fossil fuels and thermal power plants, etc. are posing threat to the environment continuously. Fast growing population, urbanization and industrialization have led towards increase in energy demands across the world. Increasing energy demands have drawn attention of researchers towards the development of renewable energy sources. Fuels derived from biomass like biodiesel, bioethanol and biogas, etc. could be the possible substitutes of traditional energy resources in the near future. Increasing population, urbanization and industrialization are the major factors behind different types of environmental problems with generation of huge volume of wastewaters from municipal and industrial areas. Safe disposal of these wastewaters is a tedious task. Globally the consumption of water for domestic and industrial purposes is 450 billion m^3 $year^{-1}$, where domestic sector alone contributes to 315 billion m^3 $year^{-1}$ which is 70% of total water consumption. Usually wastewater discharges from industries have high organic loading, nutrients, toxic organics (xenobiotics), and heavy metals, etc. These pollutants have serious impact on human health, aquatic life and soil microbiology. However, inorganic and organic nutrients present in the wastewaters can be used as substrate for the of growth microorganisms. Biomass obtained from such micro-organisms can be utilized in the production of value added products like biodiesel, bioethanol, pharmaceuticals and proteins, etc. Open literature indicates that significant work has been accomplished to enhance microalgal biomass and lipid production. This thesis is focused on evaluating and demonstrating potential of microalgae for remediation of wastewaters and production of biodiesel. Four freshwater microalgae namely *Chlorella minutissima* (*C. minutissima*), *Scenedesmus abundans* (*S. abundans*), *Nostoc muscorum* (*N. muscorum*) and *Spirulina* sp. have been used for remediation of dairy, distillery and secondary treated wastewater contaminated with phenols. Batch experiments were performed in laboratory using conical flasks as photobioreactor. Among the four microalgae species *C. minutissima* was found the most efficient and promising microalga for production of biodiesel. Optimum temperature and light intensity observed for *C. minutissima* were 27 ± 2 ℃ and 9000 lux, respectively. At these conditions *C. minutissima* obtained highest biomass and lipid yield of 1840 and 405.36 mg/L, respectively when grown on modified CHU-13 medium. Remediation of distillery wastewater in combination with secondary treated domestic sewage was also performed using *C. minutissima*. Increase in biomass and lipid yield was observed with the addition of glucose in the cultivation medium. *C. minutissima*

achieved highest biomass and lipid yield of 5.23 ± 0.065 g/L and 976 mg/L, respectively, when cultivated on 25% distillery spent wash containing 20 g/L of glucose. Furthermore, remediation of secondary treated wastewater contaminated with different phenols (1,2-dihydroxy benzene, 2,4-dinitrophenol, 2,4-dichlorophenol and 2-chlorophenol) was also assessed using *C. minutissima*. Removal of phenols by *C. minutissima* and effect of different phenols on biomass and lipid yield of microalga were observed in presence and absence of additional organic carbon sources like glucose and glycerol. Highest removal of phenol (1,2-dihydroxy benzene) was achieved at 25 mg/L concentration in cultivation medium. Dairy wastewater remediation was also performed using different poly-microalgae cultures of *C. minutissima*, *S. abundans*, *N. muscorum* and *Spirulina* sp. Polyculture (*C. minutissima* + *N. muscorum* + *Spirulina* sp.) achieved 75.16, 61.37, 58.76, 84.48 and 84.58% removal of BOD, COD, total nitrogen (TN), total phosphorus (TP) and suspended solids (SS), respectively when cultivated on dairy wastewater diluted with 30% secondary treated domestic sewage at 12:12 h photoperiod along with biomass and lipid yield of 3.47 ± 0.07 g/L and 496.21 mg/L, respectively, in 10 days of cultivation period.

LIST OF PUBLICATIONS

Included in the thesis

Chandra, R. and Ghosh, U. K. (2019) Effects of various abiotic factors on biomass growth and lipid yield of *Chlorella minutissima* for sustainable biodiesel production. **Published in Journal of Environmental Science and Pollution Research (Springer)**. DOI: https://doi.org/10.1007/s11356-018-3696-1

Chandra, R. and Ghosh, U. K. (2019) Microalgal remediation of the mixture of distillery spent wash and secondary treated domestic sewage for biodiesel feedstock production using green microalga *Chlorella minutissima*. Submitted to **Journal of Environmental Science and Pollution Research (Springer)**. **Manuscript Number: ESPR-D-19-09996.**

Chandra, R. and Ghosh, U. K. (2019) Bio-remediation of secondary treated wastewater contaminated with phenols and biodiesel production using *Chlorella minutissima* and *Scenedesmus abundans*. Submitted to **Journal of Bioenergy Research (Springer). Manuscript Number: BERE-D-19-00499.**

Chandra, R. and Ghosh, U. K. (2019) Chloro-cyanophycean microalgae mixture in dairy wastewater remediation, nutrients removal and biodiesel production. Submitted to **Journal of Environmental Pollution (Elsevier). Manuscript Number: ENVPOL_2019_6535.**

Not included in the thesis

Amit, **Chandra**, **R.**, Ghosh, U. K., & Nayak, J. K. (2017). Phycoremediation potential of marine microalga Tetraselmis indica on secondary treated domestic sewage for nutrient removal and biodiesel production. **Environmental Science and Pollution Research**, *24*(26), 20868–20875. DOI: 10.1007/s11356-017-9734-6 .

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R. Chandra, U.K. Ghosh, Remediation of Pulp and Paper Mill Effluent and Bio-Diesel Production Using Mixed Microalgal Cultures "27th European Biomass Conference and Exhibition (EUBCE-2019)" held at Lisbon, Portugal. May 27-30, 2019.

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LIST OF ABBREVIATIONS

S. No	Abbreviation	Full form
1.	BOD	Biological oxygen demand
2.	COD	Chemical oxygen demand
3.	DSW	Distillery spent wash
4.	STDS	Secondary treated domestic sewage
5.	STWW	Secondary treated wastewater
6.	DWW	Dairy wastewater
7.	CN	Polyculture of *C. minutissima* + *N. muscorum*
8.	CNSS	Polyculture of *C. minutissima* + *N. muscorum* + *Spirulina* sp.
9.	SNSS	Polyculture of S. *abundans* + *N. muscorum* + *Spirulina* sp.
10.	d	Day
11.	D	No of double bond
12.	FAME	Fatty acid methyl esters
13.	GC-MS	Gas chromatography–mass spectrometry
14.	OS	Oxidative stability
15.	CN	Cetane number
16.	min	Minute

Chapter 1

Introduction and literature review

1.1 General

1.1.1 Importance of energy

Today researchers are doing remediation of wastewater using microalgae as a potential option as microalgae can treat wastewater, can use atmospheric CO_2 during photosynthesis, and production of microalgal biomass useful in the production of various value-added products and biofuel (Kebede-Westhead et al., 2004**,** Roesler et al. 2008)**.** Microalgae take nitrogen and phosphorus wastewater as nutrients and thus they help in the reduction of pollution load and from the problem of eutrophication caused by the discharge of wastewater into lakes, ponds, etc. containing nitrogen and phosphorus (Cai et al. 2013; Kumar and Anshumali 2019, Ruiz-Marin et al. 2010, Park et al. 2011, Bezbaruah et al. 2009, Pizarro et al. 2006, 2006; Woertz et al. 2009). Keeping this in view industrial (dairy, distillery, pulp and paper, textile industry, etc.) as well as municipal wastewaters are in use as a valuable nutritional source for microalgae cultivation, and it also does not need fresh water (Bezbaruah and Zhang 2009). Microalgae biomass growth and composition are influenced by nutrients availability as well as environmental conditions like pH, temperature, and light, etc. (Boelee et al.2013; de la Noüe et al. 1992; Hofmann et al. 1998). Microalgae can produce lipids, carbohydrates, proteins, and pigments, etc. (Fields et al. 2014; Spolaore et al. 2006).

Energy is an essential element for human welfare, economic development, social growth and quality of life. Rapid urbanization, industrialization and modernization are the major key factors behind the increase in energy demands in the last few decades. Some prominent issues like climate change, increasing costs of fossils fuels and high energy demands forced scientists to explore alternative sources of energy (Dowling 2013). Globally coal/peat (28.8%), oil (31.5%) and natural gas (21.3%) are the major energy sources (IEA, 2016). Limiting the use of these energy sources is a key priority for most of the countries because of pledges to reduce greenhouse gas emissions (Suranovic 2013).

Renewable energy derived from regenerative natural resources does not diminish over the time and emits fewer emission as compared to fossil fuels and imparts a great contribution in

energy conservation by decreasing dependence on fossil fuels (Demirbas et al. 2007, Jegannathan et al. 2009). Fuel cell technology, biomass energy, ocean energy, solar energy and geothermal energy are some sources of renewable energy that could be utilized to overcome energy shortage with lesser emission of greenhouse gases (GHGs). Renewable energy is an interesting area of research with a great opportunity in India. Many efforts have been made to derive the profits of renewable energy in those past two decades, such as decentralization of energy in villages and in semi-urban areas (Goyal et al. 2008). In addition, renewable energy is a source which can create many employment opportunities, accelerate economic development, decrease local air pollution, improve public health, and reduce carbon emissions. However, in India, renewable energy is in the booming stage for stakeholder and the government, industries, and the other private sectors have many technical challenges before could make an entry to the market. So, it becomes necessary to develop renewable energy sources to overcome shortfall in fossil fuels. Bioenergy is one of such sources (Junginger et al. 2008). Bioenergy could be generated by converting organic matters into an energy carrier such as liquid fuel, gas, electricity, and heat, etc. The sources of organic matter can be industrial or municipal waste streams, woodland residues, and energy crops, etc. (Fiorese et al. 2014, Sharma et al. 2012).

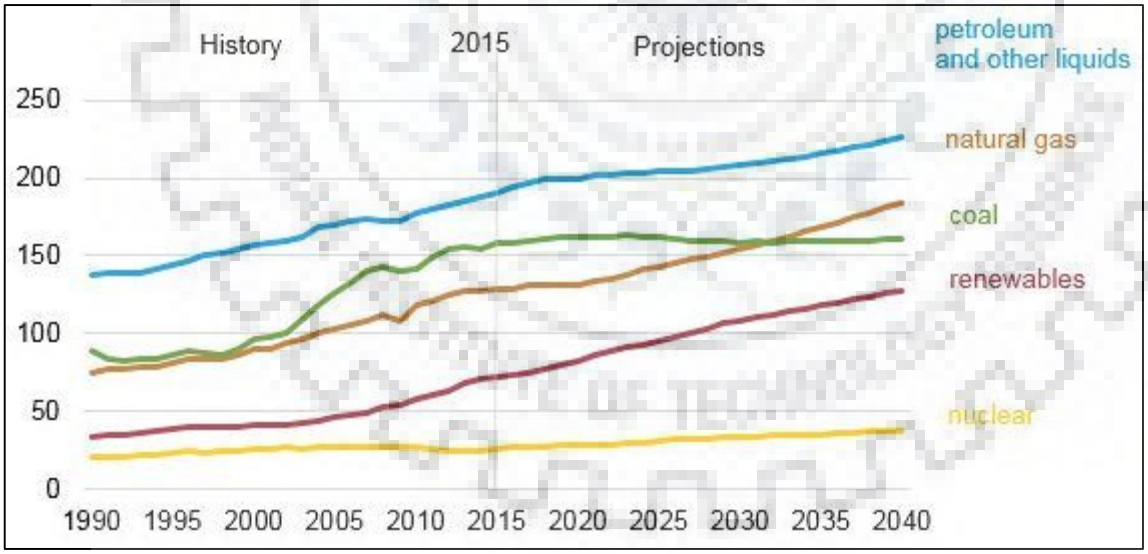

Figure 1.1 World energy consumption by energy source

Products like biofuels, fertilizers, nutraceuticals, other by-products, and solvents are being produced from microalgal biomass (Sharma et al. 2011, Chisti 2007a, Mehta et al. 2015).

In this thesis use of suspended microalgae cultures has been made in the remediation of wastewater and harvested biomass was used for biodiesel production.

1.1.2 Biofuel: An Overview

Most of the parts in the world this time are dependent on some traditional biomass, like fuelwood, animal dung and charcoal, etc. Renewable energy can be the dominant energy source for most of the world's population whose are financially below the poverty line and totally depends for household work (Tiwari et al. 2015). Many advanced technologies have been invented to extract biofuel in the form of solid, liquid and gas from the sources such as waste, biomass and wood. The focus in this thesis has been made on the production of biodiesel from lipids extracted from harvested microalgae biomass as liquid biofuels (biodiesel) is gaining prominence because of the rapid increase in its use for transportation.

1.1.3 Types of biofuels

Biofuels is the product derived from biomass which stored the energy. There is a lot of biomass sources for the production of bioenergy including food, agriculture etc. That includes food, wood process, fibre and waste residues from the industry like biomass-based crops, seasonal crops and agricultural residues from the agriculture; and forest residues sector can all be used to generate bioenergy like heat, electricity, combined heat and power, and other forms (Yogesh C. et al. 2011). Biofuels is a renewable energy because of its sources such as solar, biomass etc. Depends on the sources, biofuels can be classified in different types. The sources may be from agriculture residues or municipalities waste or food industries residues (Usmani et al. 2017).

Biofuels mainly are categorised in two groups mainly based on the process such as primary (unprocessed) and secondary (processed) biofuels:

- Primary biofuels, generally contain organic materials in its natural forms such as wood chips, pallets and firewood (as harvested). These sources are directly used for combustion, cooking fuel, electricity production in small and medium scale industrial application.
- Secondary biofuels are the processed form of sources in the form of ethanol, biodiesel, bio-oil solids, charcoal, biogas, hydrogen and synthesis gas, can be directly used in a wide range of application including automobile and other important industrial applications.

Fuels derived from starch, sugar, sunflower oil, soybean oil, animal fats and vegetable oil, etc. are called as first-generation biofuels. Major drawbacks of first generation biofuels are food security, sustainability, and less yield. To overcome these constraints second generation biofuels are introduced. The biomass feedstocks for second generation biofuels are the organic components of municipal solid wastes, non-edible oils of Karanj, Mahua, Calophyllum and Jatropha, etc. The lignocellulosic biomass like agricultural residues, forest biomass and bagasse, etc. also are being used in the production of second generation biofuels. However, commercialization is the major limitation of 2nd generation biofuels.

Then focus shifted towards third generation biofuels that derived from microalgae, bacteria, and yeast (Guldhe et al. 2014, Daramola et al. 2011). The fuels that are being produced from carbon captured from the environment using advanced technologies like petroleum hydro-processing constitute the fourth-generation biofuels (Chitsiga et al. 2016).

Biofuels can be produced in the form of biodiesel, bioethanol, biogas, and biohydrogen (Sekoai and Daramola 2015). At the industrial scale, only two attractive forms of biofuels (biodiesel and bioethanol) are being produced.

Wheat, maize (corn), sorghum and sugarcane are the most typical feedstocks have been utilized for the production of bioethanol (Onoji et al. 2017). Liquid biofuels are being used for the transportation sector and seen exponential growth in production. However, it is very low as compared to the total transport fuel consumption and total energy consumption worldwide.

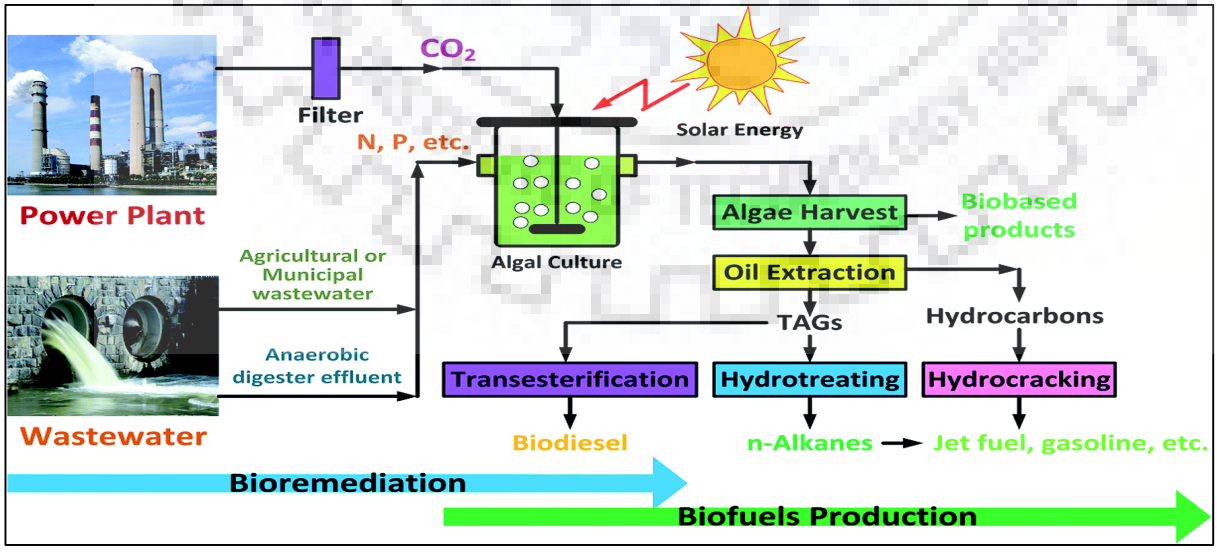

Figure 1.2 Process diagram of biodiesel production from microalgae cultivated on wastewater

Biodiesel is directly used to run compressed ignition engine, whereas bioethanol blended with gasoline is used in the spark-ignition engine. Currently, biodiesel has been popular as a non-traditional form of renewable and clean fuel. The scientific community showed interest due to its advantages such as biodegradability, non-toxicity, and lower emission of GHGs. Moreover, without any engine modification, it can be used in existing diesel vehicles. Biodiesel is free from aromatic and other chemical materials which are dangerous to the environment and mankind. The latest research on microalgal biofuels production showed new ways for sustainable development.

1.1.4 Microalgae as bioremediation agent

Wastewater handling, treatment and safe disposal have become one of the important issues worldwide due to increasing population, urbanization and change in modern lifestyle (Otter et al. 2019). It contains inorganic and organic nutrients with high biological oxygen demand (BOD) and chemical oxygen demand (COD) and is a threat to the environment if discharged without treatment (Sandhu et al. 2019). Phosphorus and nitrogen in wastewater cause a consequential effect on ecology and human health. Excessive eutrophication happens due to the nutrient enhancement of inland surface water (ponds and lakes) and therefore, an exasperation noticed in water supply and aesthetics (Glorian et al. 2018). Various types of nutrients and pollutants like pharmaceutical compounds, heavy metals, organic carbon, phosphate, nitrogen, dyes, volatile fatty acids, etc. are found in different concentrations in industrial and domestic wastewaters. Previous research works demonstrated that microalgae can grow in different wastewaters leading to simultaneously biomass production and wastewater treatment. Thus, microalgae may be the most promising feedstock for producing biodiesel to replace conventional diesel fuel.

1.1.5 Biodiesel production from microalgae

India needs more than 200 billion gallons of biodiesel annually to replace petroleum diesel required for transportation. Currently, jatropha is the most appropriate and emerging crop for biodiesel production (Aransiola et al. 2012). It needs 952 million acres of land area for cultivation of jatropha to fulfil above-said demand (Khan et al. 2008), which is more than 100% of all over in India. Therefore, non-edible or edible crops could not be the substitute for fossil fuels in the near future. This scenario will change if microalgae biomass is used as a raw material to produce biodiesel (Kwangdinata et al. 2014). Assuming biomass productivity of 1.535 kg/m^3-d and 30% average oil content on the basis of dry weight, it has been estimated that 98.4 m^3/ha

microalgae biodiesel yield may be obtained in a calendar year which requires only 2% of the geographical area of India (Chisti 2007a).

Microalgae are fast-growing microorganisms and it doubles within a day. They have 2 to 5 fold higher biomass productivity in comparison to traditional crops (Chisti 2007b). Microalgae may contain more than 80% lipids (oil) on the dry biomass weight basis (Chisti, 2007; Yogesh et al. 2011). Microalgae Hossain et al. (2008) reported 7 to 31% higher oil yields of microalgae as compared to palm oil.

1.1.6 Microalgae: A potential feedstock for biodiesel production

Being photosynthetic microorganisms having microalgae have chlorophyll-a as a primary pigment (Katsimpouras et al. 2017). Photosynthesis mechanism in microalgae is similar to other green plants, they are sunlight-driven cell factories that convert carbon dioxide to various types of renewable biofuels. Microalgae have certain advantages as a potential feedstock of biodiesel:

- Less area occupation for their cultivation in comparison to the other feedstocks.
- Fast growth rate and lesser doubling time.
- Can survive in different environments such as wastewater, marine water and fresh water, etc.
- High lipid content (10-80%) on the dry cell weight basis.
- Have higher photosynthesis efficiency (3-8%) than terrestrial plants (0.5%) (Lardon et al. 2009).
- Reduction in GHGs and sequestration of CO_2 produced from power plants, combustion of fossil fuels and other sources (Rodolfi et al. 2009a).
- Can be cultivated in both closed (photobioreactors) and open system (raceway ponds) round the year.
- Residual microalgae biomass can be used further for bioethanol or biogas production after lipid extraction.
- Microalgal biomass is a good source for the production of value-added products such as protein, fish feed and fertilizers, etc.
- No need of pesticides and herbicides for microalgae cultivation.

1.2 Literature review

1.2.1 About Microalgae

Algae are ubiquitous and versatile, unicellular to multicellular photosynthetic organisms (Chen et al. 2008) and exist in all the ecosystems present on earth. Different species of microalgae are living in various habitats and environmental conditions (fresh water and marine water, etc.). Microalgae are oxygenous and possess significant diversity in terms of cytological, morphological, molecular, and reproductive characteristics. Algae are divided into two (microalgae and macroalgae) groups based on their size and properties. Macroalgae are multicellular, large seaweeds and they are classified into three groups: brown-kelps (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta) (Fitzgerald et al. 2011). Microalgae can also grow on soil or vegetation (Aresta et al. 2005). Microalgae are eukaryotic or prokaryotic photosynthetic microorganisms which can survive in the hostile environment also due to their simple cellular structure (Mata et al. 1997). Prokaryotic microalgae (*Nostoc* sp., *Spirulina* sp., etc.) are devoid of membrane-bounded cell organelles. Eukaryotic microalgae are membrane-bounded organisms with advanced and modified functions which carry out all the cellular functions efficiently. It also resists foreign harmful molecules and proteins which protect membrane-bound cell organelles (Brennan et al. 2011).

Microalgae are classified into 10 taxonomic groups (1) cyanobacteria (blue-green algae), (2) yellow-green algae (Xanthophyceae), (3) green algae (Chlorophyceae), (4) brown algae (Phaeophyceae), (5) red algae (Rhodophyceae), (6) dinoflagellates (Dinophyceae), (7) diatoms (Bacillariophyceae), (8) Eustigmatophyceae, (9) Ulvophyceae, (10) Prasinophyceae (Van et al. 1997). The examples of eukaryotic and prokaryotic microorganisms are diatoms (Bacillariophyta) and green algae (Chlorophyta) and cyanobacteria (Cyanophyceae), respectively. Currently, microalgae are being explored for wastewater treatment, food production (both human and animals), pharmaceuticals and bioconversion of waste to bioenergy, etc. (Oliveira and Crispim, 2013).

Microalgae are the primitive plants (thallophytes) having no roots, leaves and stems. It has been reported that around 30000 out of more than 50000 existing microalgae species have been studied and analysed (Richmond, 2004). For biomass synthesis through photosynthesis microalgae utilize sunlight, water, and CO_2. Both carbon dioxide and solar energy are absorbed from the environment by chloroplasts present in the microalgae cell and convert them into

oxygen and adenosine triphosphate (ATP). This generated energy (ATP) is utilized in respiration to supply energy and development of the cell. The reaction of photosynthesis is as follows:

$$6\ CO_2 + 12\ H_2O + \text{Photons} \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O \quad (1.1)$$

Photosynthesis consists of light phase and dark phase reactions. Light phase reactions, supply energy molecules (ATP) and reducing molecules NADPH (nicotinamide adenine dinucleotide phosphate). In this phase, chlorophyll absorbs photons from sunlight have thylakoid membranes protein which used to push the electron stripping process of from water acts as an electron source. Firstly, electron crosses to quinone and pheophytin molecules and finally electron transport through electron transport chain (ETC) till the production of reduced NADPH. Meanwhile, a proton generates a chemiosmosis potential utilized by ATPase for ATP generation which was released during water photolysis. In the presence of ATP, CO_2 associated with ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and NADPH is produced during the light reaction period. This NADPH is utilized during the dark reaction to generate three-carbon sugars (glycerate-3-phosphate). The glycerate-3-phosphate is an intermediate product, which ultimately converts to the primary end product (carbohydrates) of photosynthesis and other organic products such as amino acids and lipids also synthesis during this process (Masojídek et al. 2013).

1.2.2 Microalgae composition

Microalgae constitute of proteins, carbohydrates and lipids at varying composition depending upon the type of species used. They may have a balanced composition of lipids, proteins and carbohydrates or can be rich in lipids or rich in proteins.

Carbohydrates

During nitrogen fixation by microalgae, carbohydrates are mainly produced which are further metabolized into carbon dioxide and water by photosynthesis process. Starch is the major reserve material in microalgae and different types of polysaccharides can be produced by different classes of microalgae. However, cellulose also occurs in the cell wall of microalgae. Starch containing amylase and amylopectin is produced as energy stock by green microalgae. Strains like *Chlorella* sp., *Tetraselmis* sp., *Chlamydomonas* sp., *Scenedesmus* sp. and *Dunaliella* sp. store more than 40% carbohydrates of its dry weight (John et al. 2011). The major microalgal carbohydrates are cellulose and starch which are being used in ethanol and sugars production.

Hence, it can be concluded that algae carbohydrates are a suitable source for bioethanol production (Harun et al. 2010).

Protein

Another important component of the microalgae cell wall is a protein whose content may be as high as 60% of algal biomass. Proteins containing essential amino acids are necessary for human beings as such amino acids cannot be synthesized by our body. In the past few decades, more than 75% of the annual microalgae biomass has been used by health and food market for the formulation of powders, tablets, and capsules. *Spirulina*, a filamentous blue-green alga is used worldwide as a food supplement in the form of tablets, flakes, or powder. Derivation of biofuel from proteins is not a feasible process because of the problems associated with the deamination of protein hydrolysate. However, reports are available on the extraction of biofuel from algal proteins using engineered bacteria.

Lipids

Lipids are classified as glycerophospholipids, fatty acids, sterol lipids, saccharolipids, glycerol lipids, polyketides and prenol lipids. It contains waxes, fat-soluble vitamins, phospholipids, triglycerides, diglycerides, monoglycerides, sterols and phospholipids as different biomolecules. Energy storage and cell signalling are the main molecular functions of lipid molecules. Triacylglycerol (TAGs) is a storage lipid that is made up of three fatty acid molecules bound to a glycerol backbone and is used in the synthesis of the biological parts of plant species. Lipid content of microalgal species varies species to species such as *Chlorella* sp., *Neochloris oleoabundans*, *Botryococcus braunii*, *Porphyridium cruentum*, *Nannochloropsis* sp., *Dunaliella tertiolecta*, *Chlorella emersonii*, accumulating lipid content up to 60 % on dry weight biomass basis (Maity et al. 2014).

1.2.3 Lipid biosynthesis in microalgae

Biological synthesis of lipid is a three-step process i.e., (1) fatty acids synthesis in plastids, (2) accumulation of glycerolipid in the cytoplasm of the cells, and (3) finally wrapping into the oil bodies. Microalgae in presence of photon energy, fix CO_2 in the form of sugar via photosynthesis process. Glycolysis is an oxygen-independent biological process that produces pyruvate along with two molecules of ATP. The end product of glycolysis i.e. pyruvate can be further metabolized into carbohydrates via gluconeogenesis process, to fatty acids by acetyl-

Coenzyme A, alanine (amino acid) and ethanol (Antonopoulou et al. 2020). Pyruvate is an important biological molecule and is formed in several other metabolic pathways. Decarboxylation of pyruvate occurs in the presence of pyruvate dehydrogenase (PDH) enzyme to form acetyl coenzyme A, which is an important precursor molecule to synthesize fatty acids (Fan et al. 2011). During the synthesis of fatty acids, malonyl-CoA is formed by the reaction of acetyl-CoA with bicarbonate in the presence of a multifunctional enzyme such as acetyl-CoA carboxylase (acetyl-CoA carboxylase). Malonyl-CoA is further converted into malonyl-acetyl carrier protein (malonyl-ACP) by malonyl transferase, which is an acyl carrier protein. The role of malonyl-ACP in fatty acid synthesis is the lengthening and desaturation of the carbon chains to obtain C16 and C18 fatty acids products. These fatty acids are present in the form of a synthetic membrane, organelle membranes and tri-acyl glycerol (TAG).

TAG is an important molecule for biodiesel production from microalgae and it is generated by enzyme catalysed sequential acylation of glycerol-3-phosphate (G3P) with three acyl-CoA molecules. The enzyme used for the reaction is acyltransferase which donates its acyl group to G3P. Lysophosphatidic acid (LPA) is also be obtained by the acylation reaction of G3P with glycerol-3-phosphate acyltransferase (GPAT). Further acylation of LPA gives phosphatidic acid (PA) in the presence of lysophosphatidic acid acyltransferase (LPAT) enzyme. phosphatidic acid phosphatase (PAP) enzyme catalyses the synthesis of diacylglycerol by removing a phosphate group from PA. Diacylglycerol acyltransferase (DGAT) enzyme catalyses the reaction of the formation of TAG form DAG, which is accumulated in the form of fat or oil in microalgal cells. During this process, DAG and PA are also obtained as substrate polar lipids (phosphatidylcholine and galactolipids).

Actually, carbon and energy are not only used to store by the accumulation of TAG but also helps to an important biological role in microalgal cells life cycle like light protection, the electron transport chain in the photosynthetic system and turnover membrane lipids. The algal lipid converts into biofuel in the form of TAG.

Selection of microalgae is a crucial step in biodiesel production. Our ecosystem is composed of various range of microalgae such as *Scenedesmus dimorphus*, *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *Chlorella minutissima*, *Schizochytrium sp*., *Chlorella sp*., *Nannochloris sp*., *Chlorella protothecoides*, *Dunaliella salina*, *Isochrysis galbana*, *Nannochloropsis oculate*, *Nitzschia sp*., *Crypthecodinium cohnii*, *Scenedesmus obliquus*, and *Skeletonema costatum*, etc., with lipid content in range of 40-60%. However, in some microalgal

strains, the low lipid content of less than 40% on dry weight biomass can also be seen. On the colour basis, the order of lipid content is green algae > yellow-green algae> red algae > blue-green algae (Baunillo et al. 2012; Damiani et al. 2010; Dote et al. 1994; Mata et al. 2010a; Minowa et al. 1995; Natrah et al. 2007; Pai and Lai, 2011; Rodolfi et al. 2009b; Deng et al. 2009).

1.2.4 Microalgae cultivation

Suitably designed setup for the cultivation of microalgae is very crucial to enhance the biomass yield of the selected microalgal strain. It is of great interest to know all the recent methodologies those are employed for the cultivation and harvesting of microalgae so that production can be maximized at very low cost. Optimization of experimental conditions along with physical and chemical properties of the culture medium is also important for achieving enhanced biomass production.

It has been reported that microalgae culture shows the ideal growth pattern: lag phase, log phase/exponential growth phase, stationary phase and lyse phase (Becker 1994). Microalgal cultivation generally employs both semi-continuous and continuous culture systems and follows a similar growth pattern. However, batch culture systems have limited supply of nutrient as no supplements can be added or removed during microalgae cultivation, whereas in continues system, fresh medium is added for microalgae cultivation and harvesting is done continuously. Continuous culture consists of three different types modes: cyclostat, chemostat and turbidostat. Cyclostat shows steady cyclical illumination and thus allowing microalgae cultivation under intermittent light illumination for 24 h. Chemostat signifies the process where culture removal is performed at a rate similar to the addition rate of the medium so that the volume remains constant. A turbidostat is an extended chemostat in which microalgal culture suspension is maintained at same turbidity. Fed-batch culture, also known as semi-continuous culture, where the medium is supplied intermittently or continuously and the biomass is removed periodically. Thus, the volume of culture is not constant in semi-continuous mode.

1.2.5 Microalgae culture conditions

Microalgae can be cultivated under different types of cultivation (heterotrophic, photoautotrophic, photoheterotrophic and mixotrophic) conditions. During photoautotrophic cultivation microalgae in the presence of sunlight absorb CO_2 from the environment to produce carbohydrates through photosynthesis. This cultivation condition is generally used for

microalgae growth and lipid content under this condition varies in between 5 to 68 %. This lipid content varies species to species of microalgae. Chiu et al. (2009a) have reported maximum lipid productivity of 179 mg/L-d of *Chlorella* sp. by supplying 2% CO_2 in the cultivation medium under photoautotrophic cultivation conditions

During heterotrophic cultivation, microalgae utilize organic carbon both as carbon and energy source in the dark cycle (Chojnack and Rocha, 2004). Such a cultivation condition is ideal for large scale microalgae production in a photobioreactor, as problems related to partial light exposure which leads to obstruction of high cell density is not observed (Huang et al. 2010). Some of microalgae strains showed higher biomass production as well as higher lipid content under heterotrophic cultivation conditions. For example, *Chlorella protothecoides* revealed 40% increment in lipid content when cultivation conditions were changed from phototrophic to heterotrophic (Xu et al. 2006). Under heterotrophic cultivation conditions, 20% more lipid productivity was observed as compared to phototrophic cultivation conditions.

When microalgal cell utilizes both the inorganic (CO_2) and organic carbon as carbon source and undergoes photosynthesis for growth then this cultivation condition is known as mixotrophic cultivation. This means that microalgae followed either heterotrophic or phototrophic conditions or both. CO_2 released in this process is captured and used again for phototrophic cultivation conditions (Mata et al. 2010b).

Photoheterotrophic cultivation conditions refer to the condition where microalgal cells need both light and organic carbon for its growth. The major difference between the photoheterotrophic and mixotrophic cultivation condition is energy source; under mixotrophic cultivation organic compounds are used as an energy source while phototrophic cultivation conditions use both light and organic compounds for microalgal growth.

1.2.6 Microalgae cultivation system

Open or closed ponds and photobioreactors are commonly used for remediation of wastewater and biodiesel production using microalgae.

Open system

Open pond cultivation includes raceway ponds, circular ponds, natural ponds, and inclined systems (cascade system). The open pond is used for the cultivation of microalgae in the open environment. It is mostly used to culture large scale production of microalgae/microalgal biomass (Grobbelaar 2012). To avoid loss of nutrients and media, these systems are generally

made up of concrete or clay coated with porcelain tiles or polyvinyl chloride. Depth of these systems is maintained in the range of 0.15 to 0.45 m to facilitate maximum penetration of sunlight (Murthy 2011). Paddlewheel and air pumps are used for mixing and circulation of growth medium. Cost of building an open pond is relatively less as compared to other systems and it is also simpler to build. They are very easy to operate because it requires control over nutrients and water loss only. However, among various types of open culture systems, raceways ponds are the most workable open system used for outdoor cultivation of microalgae. However, open pond system also has some drawbacks such as low mass transfer rate, insufficient mixing, low biomass productivity, water loss due to evaporation and difficulty in achieving microalgae monoculture due to the occurrence of contamination of bacteria, and other microalgae culture in the medium. It is also not suitable for frequently changing environmental conditions.

Closed system

Photo-bioreactors (PBR) are used as a closed system as they permit the exchange of light as well as energy but does not allow the exchange of any material with the surroundings. They are generally employed for bulk production of microalgae biomass. Photobioreactor generally allows the production of monoculture of microalgal species for the elongated duration. In comparison to an open system, photo-bioreactor has a low possibility of any contamination. Thus, PBR is a promising technique for achieving high microalgal biomass. Among different types of PBRs designed for higher productivity of microalgae flat panel, column and tubular PBRs are most commonly used (Ugwu et al. 2008). Pumping/stirring or airlift may be used for mixing in the PBRs. Mixing is also necessary for gas exchange (Brennan and Owende 2010). As compared to open ponds, PBRs do not require large space and can be easily accommodated in small areas to produce biomass. PBRs are usually more efficient and effective than open ponds culture system as they can efficiently utilize available light (natural or artificial) or combined light from different light sources. In photobioreactors, photoperiod can also be optimized by increasing exposure period to low light intensities due to variations in sunlight. Increased photoperiod and constant light intensity provided by the artificial light source improved yearly total oil yields up to 25–42% (Amaro et al. 2011).

1.2.7 Cultivation parameters

In order to achieve efficient microalgal growth, specific environmental conditions are necessary depending upon microalgae species. Factors affecting the growth of microalgae include biotic, abiotic, and operational factors. Biotic factors are fungi, viruses, bacteria, and

competition for abiotic matters with other microalgae species. Abiotic factors include nutrients (such as P, N, K), pH, salinity, light intensity, O_2, CO_2 and toxins. Operational factors refer to the experimental parameters such as stirring or mixing rate, depth and width of cultivation vessel, dilution rate, harvesting frequency and addition of bicarbonate.

Light

Sunlight is the fundamental source of energy for the photosynthesis in microalgae. Intensity and accessibility of light are some of the crucial factors affecting the growth of microalgae. There are two types of light sources used for microalgae cultivation such as natural and artificial light. The site location of photobioreactor (PBR) in respect of the availability of light affects biomass productivity. To minimize the capital cost, freely available sunlight is the most suitable option for the cultivation of microalgae. For example, *Chlorella pyrenoidosa* shows autotrophic and heterotrophic growth in daylight and in the dark, respectively which is a cost-effective technique as compared to only autotrophic growth which demands continuous supply of light (Chiu et al. 2009b).

Variation in light intensity on a daily or seasonal basis affects algal growth. In the autotrophic mode, sunlight is given in daytime only. However, to prevent the settling down of biomass generated during daylight hours, the biomass culture should be mixed unceasingly in the dark period. During the dark period to sustain cell growth, more than 25% of biomass are self-utilized (Chisti 2008). The culture growth in the mixotrophic mode requires light only when it changes into the autotrophic mode. The high-intensity light causes photo-inhibition effect on microalgae growth. Artificial light is provided in an indoor system for the cultivation of microalgae. Sunlight is harvested using light collectors and transferred through a bundle of fibres to decrease the use of artificial light (Huang et al. 2010). It has been demonstrated that light intensity can be different for different microalgal species. For example, 120 and 30 μmol m^{-2} s^{-1} light intensity was used for *Chlorella kessleri* and *Chlorella protothecoides,* respectively when concentrated wastewater used as growth medium (Li et al. 2012). Under the exposure of 100 μmol m^{-2} s^{-1} light intensity, *Nannochloropsis sp*. achieved the highest growth rate with maximum cell concentration for 18h/6h light/dark period. Change in algal cell composition occurs with changes in light intensity. The variation in illumination from 215 to 330 μmol m^{-2} s^{-1} increased the starch content of *Chlorella vulgaris* from 8.5 to 40% on a dry weight basis (Brányiková et al. 2011b). Presence of UV-b in the sunlight radiation affects the composition and lipid content of algal cells.

Temperature

Temperature is a crucial microalgae cultivation parameter which regulates metabolic and photosynthesis processes of the cell. It has been cited that the optimal microalgae growth can be achieved in the temperature range of 20-35 °C (Chisti 2008, Park et al. 2011). The efficiency of photosynthesis and specific growth rate of *C. sorokiniana* decrease under suboptimal temperature (20°C) and productivity was much lower than that of the culture under its optimal growth temperature (38°C) (Wijffels et al., 2012). Temperature also influences the composition and lipid content of microalgae (An et al. 2013; Sheng et al. 2011). Cultivation temperature can be easily maintained in a closed system for achieving optimum microalgal growth. However, in the open system, maintaining the optimal temperature for microalgal growth is not very easy due to diurnal or seasonal changes. In order to overcome this drawback of an open system, cooling and heating are necessary for summer and winter seasons, respectively. Studies also revealed that abrupt changes in temperature may cause declination in microalgal growth (Park et al. 2011). Temperature also governs the mass transfer of gas and solubility of micro or macronutrients in water. Only a few microalgae, like *Chlorella*, are thermotolerant species which grow at 42 °C.

Mixing

Another important parameter on which microalgal growth depends is mixing. Mixing can be performed in several ways such as using sparging, mechanical stirring or pumping (Kumar et al. 2010). The higher shear stress produced from strong mixing may immediately split the algal cells, therefore, it is not recommended for microalgae production as it decreases the biomass productivity (Grobbelaar et al. 2010). Proper mixing avoids sedimentation and aggregation of microalgal cells and facilitates uniform mass transfer for a higher uptake of CO_2 and nutrients. Mixing also favours uniform distribution of light and thus prevents collective shading of microalgal cells. Kunjapur and Eldridge (2010) have reported that during the photosynthesis effective mixing plays a crucial role and successfully removed excess oxygen from cultivation medium which reached up to 400% of air saturation value in closed PBRs. The joint effect of high light intensity and oxygen concentration causes photoinhibition which adversely affects microalgal growth. Temperature and pH stratification in the PBRs can be achieved by good mixing.

1.2.8 Nutrients for microalgae cultivation

Microalgae need several nutrients along with suitable environmental and operational parameters for its growth. The cultivation medium must contain macronutrients, micronutrients,

and other inorganic salts that are necessary for its growth. The demand of the constituents required in cultivation medium varies from species to species. Other than synthetic media municipal as well as industrial wastewaters can also be used as a cultivation medium for the growth of microalgae (Goyal 2006, Kumar et al. 2010b, Kong et al. 2010). Resulting compositions of microalgal strain depends largely upon the constituents used for the cultivation of microalgae (Demirbaş 2008).

Carbon

Carbon is an important microalgae growth factor and is consumed both organic as well as in inorganic forms. Algal cells may be classified into autotrophs or heterotrophs based on the carbon source they are consuming. Growth and accumulation of various metabolites are governed by the CO_2 concentration of the microalgae cultivation environment (Kim et al. 2004; Chiu et al. 2009c; Ota et al. 2009). The higher specific rate of growth and biomass productivity were observed in the presence of higher concentration of $NaHCO_3$ during microalgae cultivation (Yeh et al. 2010; Lin et al. 2012). Microalgae utilize organic carbon in the form of acetic acid, fructose, glucose and sucrose during heterotrophic and mixotrophic mode (Mayo and Noike, 1994; Yeh et al. 2012; Bhatnagar et al. 2011, Goyal 2006b).

Carbon presents about 50% of the dry weight of algal biomass (Becker 1994), which is mostly generated from inorganic carbonates or CO_2. Different pathways to capture and internalize CO_2 by algae are (Suryata et al. 2010; Wang et al. 2008) (i) through atmosphere (Hanagata et al. 1992) (ii) feeding on industrial flue gases (iii) by solubilizing carbonates ($NaHCO_3$ or Na_2CO_3) (Binaghi et al. 2003) and (iv) uptake of organic carbon sources (Patil et al. 2008).

Nitrogen

Microalgae utilize nitrate form of nitrogen usually. It has been observed that the microalgae grown under nitrogen stress conditions accumulated higher lipid content but decreased biomass growth (Wang et al. 2008; Sheng et al. 2011). Some of the microalgal strains use ammonium instead of nitrate which causes microalgal cells to multiply rapidly and grow fast (Kumar et al. 2010b).

Organic nitrogen is found in a variety of biological molecules such as protein, chlorophyll, peptide, enzyme genetic material (DNA and RNA) and energy transfer molecules (ADP, ATP). Organic nitrogen can also be generated from inorganic nitrogen like ammonia, ammonium, nitrite, nitric acid, nitrate, and nitrogen gas (Shankar and Nill 2015). Thus, microalgae have great contribution in the conversion of inorganic nitrogen into organic nitrogen

through assimilation. Moreover, cyanobacteria have the potential to convert atmospheric nitrogen into ammonia by the means of fixation (Shankar and Shikha 2017).

Phosphorus

Phosphorus is a mandatory macronutrient for microalgae growth. It helps in the synthesis of functional and structural components of microalgal cells (Hu 2004). Phosphorus concentration in medium should be added in excess because it may precipitate in the form of phosphate after reacting with metals ions (Chisti, 2007). For balanced growth of microalgal cells, N/P ratio in the medium is very crucial and plays an important role in wastewater treatment (Leonardos and Geider 2004; Xin et al. 2010). Phosphorus is commonly provided in the form of orthophosphate (PO_4^{3-}) in the culture medium. $H_2PO_4^-$ and HPO_4^{2-} both are present in a synthetic medium and act as a buffer to maintain the pH of the medium within the range (Shikha et al. 2007). Microalgae cells stores a surplus amount of phosphorous in the polyphosphate granules form. Thus, these microalgal species can be used those granules during phosphate shortage conditions for cell growth (Larsdotter 2006). As a result, the unavailability of phosphates may affect the photosynthesis process of microalgae and lipid production.

Micronutrients

Micronutrients are the crucial nutrients which are required in minute amount for the growth of microalgae. They have an important role in the activation of cellular enzymes and of various growth factors during metabolic reactions. Different micronutrients such as Mg, Zn, Mo, Mn, S, Cu, Fe and Co are required in the synthetic medium for suitable microalgal growth. Calcium is necessary for cell membrane formation and cytoskeleton of the microalgal cell. Pro activation of acetyl–CoA is done by magnesium carboxylase which regulates the first performing step of microalgae lipid synthesis. Cell division, respiration and, nucleic acid and protein metabolism are regulated by zinc (Ajay and Rathore, 1991; Ting et al., 1991).

1.2.9 Microalgae harvesting

Harvesting is the process used to isolate microalgal biomass from the growth medium. This is a key step for a large-scale biodiesel production process because it cost 20–40% of total biodiesel generation expenditure cost (Mata et al. 2010). Microalgae cell suspension present in the diluted growth culture medium has density almost equal to water and due to the microscopic size (1-10 μm diameters), assortment of microalgal biomass from the medium is a tedious task. Many harvesting techniques like coagulation, electro-flotation, centrifugation, filtration, electrophoresis, ultra-sound and flocculation flotation have been employed to harvest

microalgae. In lab-scale experiments, centrifugation is the commonly used technique for harvesting and through it, 85–90% harvesting efficiency has been achieved within 2–5 minutes. However, it will be very expensive for large-scale algal culture by using centrifugal methods due to higher electricity use. The simple filtration also is not much effective for separation of microalgae. Fouling is the major issue during the filtration process which causes deposition of extracellular polymeric substances (present in microalgae culture media) on the surface of filters. Fouling causes replacement of backwashing and membrane, which increase the overall process cost. Besides those methods, microfiltration and ultra-filtration are some improved techniques for microalgae harvesting (Tang et al. 2010). Presently, flocculation is the most efficient harvesting method with capital and cost lower energy. Microalgae cells are negatively charged in the growth medium and repel each other due to electrostatic force. This helps microalgae cells to make a stable system. After the addition of flocculants into the microalgae culture medium, the surface charge is blocked so that microalgae cells are agglomerated and form stable flocs. The flocculants utilized must be low-cost, non-toxic, and effective in low application doses. A number of chemicals are identified to be utilized as flocculants for this purpose. Inorganic salts containing metal ions (Al^{+3} and Fe^{+3}) are generally used as flocculants. The high concentration of metal flocculants for biomass harvesting results in toxicity and also affects the biodiesel production process.

Currently, organic polymers such as chitosan, cationic and grafted starch are getting attention to replace inorganic flocculants. Harvesting efficiency of these flocculants is higher and they do not contaminate the biomass. Various researchers have investigated different flocculants such as chitosan (Xu et al. 2013), ferric salts (Godos et al. 2010), aluminium salts (Minayeva and Sirin 2012), and titanium tetrachloride (Zhao et al. 2012). In the flocculation process pH also plays a crucial role. Bosma et al. (2003) reported that ultrasound is also able to harvest microalgae. Matos et al. (2013) achieved 97% harvesting efficiency of marine *Nannochloropsis* sp. by using electro-coagulation. Bio-flocculation is also induced by utilizing flocculating microalgae to concentrate non-flocculating microalgae of interest (Salim et al. 2011), or by using microalgae-bacteria consortium to enhance flocculation of microalgae cells (Lee et al. 2013).

1.2.10 Extraction of lipid

After harvesting, extraction of oil from oven-dried algal biomass for biodiesel production is a challenging and expensive task. To extract oil from microalgae cells its disruption is necessary as oil is confined within the cell wall composed of proteins and carbohydrates. There

are numerous methods (physical, chemical and mechanical) have been cited in the open literature for oil extraction. The method used for oil extraction should be effective, fast, and scalable and at the same time, it should not cause any harm to the lipid cells. Mechanical pressing is the technique which is used for oil extraction of Karanja, mustard, and Jatropha. For scalable microalgal biodiesel production, the mechanical pressing method may be used, however, single-cell nature of microalgae and stiff cell wall of some microalgal strains is the main drawback of mechanical pressing (Cooney et al. 2011). The choice of best extraction technology greatly depends on the microalgae strain selected and shall be optimized.

Chemical method is the most common method used for oil extraction from microalgae. Here both, polar (ethanol, acetone, methanol, ethyl acetate) and non-polar (benzene, chloroform, toluene hexane, diethyl ether) solvents are used to extract oil from microalgae. Non-polar solvent helps in disrupting microalgal cell wall which has hydrophobic interaction between the natural and non-polar lipids. In the Folch method (Folch et al., 1953) chloroform and methanol are used in the ratio of 2:1; v/v to extract lipids. However, chloroform and methanol in the ratio of 1:2; v/v can be used for lipid extraction (Bligh and Dyer, 1959). According to Balasubramanian et al. (2013) lipids can be classified into 3 three groups e.g., neutral lipids, free fatty acids (FFA) and polar lipids. They also described that the lipid extraction from marine microalgae also depends on the factors like biomass drying, moisture content and solvent system used. Physical methods like ultrasonication, microwaves, osmotic shocks, autoclave, etc., are used to break the microalgae cell wall. Microalgae cell wall could be disrupted physically and extraction of lipid can be done using an organic solvent (Wijffels and Barbosa, 2010). Supercritical fluid extraction has the ability to extract nearly 100% of oil from biomass and it has the potential to replace conventional solvent extraction methods. The basic principle of this technique is that pressure and temperature of carbon dioxide (CO_2) gas is increased until it reaches above critical point or liquid–gas state. It is then mixed to microalgae biomass and acts as a solvent. Pressure and temperature (critical point) at which fluid liquefies vary from solvent to solvent. The main drawback of this process is high operational and infrastructure cost.

1.2.11 Transesterification

After oil (lipid) extraction from biomass, the microalgal crude oil is further processed for biodiesel production using transesterification reaction. Due to its highly viscous nature, there is a need to convert extracted crude oil into lower molecular weight compounds in the form of fatty acid methyl esters. In this process, extracted lipids (triglyceride) are converted into renewable, biodegradable, and non-toxic compounds. Lipid to alcohol ratio, type of catalyst, free fatty acids

(FFA), temperature and time are some of the crucial factors which can influence the efficiency of transesterification. Microalgae oil also contains phosphorus in the form of phospholipid. Phosphorus causes the formation of the emulsion during the refining step of the biodiesel process which affects production yield. Moreover, FFA are present in high proportion in microalgae lipids. So, degumming is necessary for removal of phosphorous content and to reduce FFA. Main drawbacks of conventional processes for microalgal biodiesel production are long conversion and extraction time, the requirement of the large quantity of chemical solvents and difficulty to maximize the biodiesel yield. The problems stated above can be solved using ultrasonic and microwave-assisted transesterification process. Due to rate enhancement, these techniques are very energy efficient with short reaction time (Lidstrom et al., 2001; Guerra, 2014). In comparison to the conventional process, these techniques are cost-effective and eliminate solvent extraction steps for biodiesel production. Additionally, direct esterification gives better results in laboratory-scale experiments (EHIMEN et al. 2009), but still has many challenges to scale up.

1.2.12 Biodiesel Quality

Suitability of microalgal biodiesel as an alternative to diesel fuel strongly depends on compliance with known standards such as EN 14214:2008, IS:15607 and ASTM D6751. Mostly the properties of biodiesel depend on fatty acid compositions. Typical fatty acids which present in biodiesel are palmitic, stearic, oleic, linoleic, and linolenic acid. The proportion of these fatty acids varies from feedstock to feedstock of microalgae. However, esters are rich in saturated fatty acids which have high viscosity, high probability of clogging the nozzle of the engine and high cloud point. On the other hand, esters rich in polyunsaturated fatty acids lead to better cloud point and poor oxidation stability. Miao et al. (2006) reported cold filter plugging point (CFPP), kinematic viscosity and density as, -13 °C, 4.43 cSt and 882 g/cm^3, respectively. Amin (2009) reported 0.864 g/cm^3 density, 5.2 cSt, kinematic viscosity, and -11°C CFPP was observed in microalgae oil biodiesel.

1.2.13 Wastewaters

Discharge of wastewater from municipal as well as from various industries such as sugar mill, distillery, pulp and paper mill, tannery, etc., can cause serious water pollution. Normally, microbes are used for wastewater treatment in stabilization pond or activated sludge facility after removal of suspended solids in primary treatment. In the current scenario, the left-over nutrient load may be managed by developing biological systems of algae. Wastewater is an alternative source of nutrients which replace expensive nutrients medium and contains both organic and

inorganic nutrients for the aquaculture of microalgae. Cultivation of algae in wastewater reduces cultivation cost as well as fresh water requirement in the cultivation medium (Wang et al. 2015). Actually, cultivation of microalgae depends upon proper quality and quantity of nutrients. Algae use both organic and inorganic nutrients which reduces the pollution load from wastewater with improved CO2 balance (Ji et al. 2013). Wastewaters usually contain nitrogen, phosphorus, Mg, Mn, Ca, Na, etc. which act as nutrients for microalgae culture.

Microalgae like *Chlorella* sp., *Scenedesmus* sp., S*pirulina* sp., *Tetraselmis* sp., etc. have been evaluated for wastewater treatment abilities. Currently, the scientific community is doing wastewater remediation using microalgae to get biodiesel from microalgal biomass (Hwang et al. 2016). Microalgae use organic carbon present in wastewater for their growth (Yang et al. 2014). Currently, wastewaters have become a potential source for microalgae cultivation and the production of high amounts of biomass (Gupta et al. 2019). Cultivation of microalgae on wastewaters can bring down the operating cost of microalgae cultivation. The main aim of growing microalgae on wastewaters is to assimilate and efficiently remove both inorganic and organic nutrients from wastewater.

1.3 Research objectives

1. Evaluation of the effects of various abiotic factors on biomass growth and lipid yield of microalga *Chlorella minutissima* for sustainable biodiesel production.
2. Remediation of distillery wastewater in combination with secondary treated domestic sewage using microalga *Chlorella minutissima* for biodiesel production.
3. Evaluation of bioremediation potential of microalgae *Chlorella minutissima* and *Scenedesmus abundans* cultivated on secondary treated wastewater contaminated with different phenols for biodiesel feedstock production.
4. To see the effectiveness of poly-microalgae cultures for remediation of dairy wastewater and the role of organic carbon source and photoperiod on the biomass and lipid yield of poly-microalgae cultures.

1.3.1 Organisation of the thesis

The thesis has been organised in six chapters as discussed below:

Chapter 1

This chapter briefs a review of the microalgae potential in the treatment of wastewater from different sources as well as their cultivation methods on wastewater. This chapter also

emphasizes how microalgae can compensate for increasing energy demands as well as their role in the production of biofuels and other value-added products. This chapter also discusses the role of different parameters required for microalgae cultivation, harvesting of microalgal biomass and extraction of lipid and production of biodiesel from microalgal biomass. It highlights the research problem, objectives and finally contributions. In short, it covers the thesis organization.

Chapter 2

In this chapter, the effects of different abiotic factors (nutrients, pH, temperature, light intensity and photoperiod) on the biomass growth and lipid yield of green microalga *Chlorella minutissima* (*C. minutissima*) have been explained. Different concentrations of nitrogen, phosphorus, glucose, iron, zinc, different values of pH, temperature, light intensity, and different photoperiods have been observed on the biomass growth and lipid yield of *C. minutissima* cultivated on modified CHU-13 medium. Initially, three cultivation media namely, Bold's basal medium (BBM), modified CHU-13 and blue-green-11 (BG-11) were used to culture *C. minutissima* in batch mode. Microalga cultivated on modified CHU-13 medium showed maximum biomass and lipid yield of 970 ± 0.21 and 356.63 ± 0.51 mg/L, respectively. To optimize biomass and lipid yield of microalga further, it was again cultivated on modified CHU-13 medium and variation of above mentioned abiotic factors was done. Fatty acid methyl ester profile of *Chlorella minutissima* was found to be mainly composed of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), gondoic acid (C20:1), and behenic acid (C22:0). Biodiesel obtained from the *C. minutissima* biomass was found suitable as per international standards.

Chapter 3

In this chapter remediation of distillery spent wash (DSW) diluted using secondary treated domestic sewage (STDS) using microalga *C. minutissima* in batch mode has been presented. Four (25, 50, 75 and 100%) concentrations of DSW in 250 mL conical flasks at different photoperiods (12:12, 16:8, 18:6, 20:4 and 24:0, light:dark) h at an illumination of 10000 lux and 25 °C were treated using microalga. Highest biomass yield of 5.23 g/L (lipid yield 976.46 mg/L) with biomass productivity of 254 mg/L-d was achieved with DSW25 + STDS75 + glucose (20 g/L) at a photoperiod of 20 h within a cultivation period of 20 days. It was observed that the *C. minutissima* removed 77.23% BOD, 66.15% COD, 79.73% TN, 44.74%TP, 69.83% TDS and 41.30% TS, respectively from DSW75+STDS25. The GC-MS analysis of biodiesel obtained

from microalgal biomass showed the presence of C14:0, C16:0, C18:0, C18:3, and C20:0 as major fatty acids.

Chapter 4

This chapter describes the bio-remediation potential of *Chlorella minutissima* and *Scenedesmus abundans* cultivated on secondary treated municipal wastewater contaminated with phenols (1,2-dihydroxy benzene or 2,4-dinitrophenol or 2,4-dichlorophenol or 2-chlorophenol). All the phenols present in the cultivation medium caused a reduction in the biomass and lipid yield of both strains. Supply of glucose or glycerol in the cultivation medium resulted in improved biomass growth of both strains. Biomass growth and lipid yield was decreased with increase in the concentration of phenols in the cultivation medium. *Chlorella minutissima* and *Scenedesmus abundans* removed 81 and 73% of phenol, respectively from the cultivation medium containing 25 mg/L of 1,2-dihydroxy benzene within a cultivation period of 8 days. Biodiesel properties of *C. minutissima* were within the limits set by international standards.

Chapter 5

This chapter describes remediation of dairy wastewater (DWW) diluted using secondary treated domestic sewage for biodiesel feedstock production using poly-microalgae cultures of four microalgae namely *Chlorella minutissima* (*C. minutissima*), *Scenedesmus abundans* (*S. abundans*), *Nostoc muscorum* (*N. muscorum*) and *Spirulina* sp. (SS). The poly-macroalgae cultures were prepared as *C. minutissima* + *N. muscorum* (CN), *C. minutissima* + *N. muscorum* + *Spirulina* sp. (CNSS) and *S. abundans* + *N. muscorum* + *Spirulina sp*. (SNSS). Poly-microalgae culture CNSS grown on 70% dairy wastewater (DWW) achieved 75.16, 61.37, 58.76, 84.48 and 84.58%, removal of biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), and suspended solids (SS), respectively. Fatty acid methyl ester (FAME) analysis showed the presence of C14:0 (myristic acid) C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid). This indicates that the lipids produced from poly-microalgae cultures were suitable for biodiesel production. Thus, poly-microalgae cultures could be more efficient than mono-microalgae cultures in the remediation of dairy wastewater and biodiesel feedstock production.

Chapter 6

This chapter concludes the thesis with the discussion on the results and contributions. The future scope of work has also been discussed in this chapter.

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Chapter 2

Effects of various abiotic factors on biomass growth and lipid yield of *Chlorella minutissima* for sustainable biodiesel production

Reproduced from the journal of Environmental Science and Pollution Research Vol. 26 (4), 2019

2.1 Introduction

Globally, high energy demands are pushing toward the fast depletion of fossil fuels. Rapid industrialization and transportation emissions are continuously contributing to global warming. Due to future fuel needs, 50% use of plant resources will increase by 2050 (Perlack et al. 2005). Currently, biofuels have become a viable option to compensate future energy needs. Previously biofuel production was mainly based on edible agri-resources like corn, sugarcane, soybeans and vegetable oil, etc., but it could not be propagated commercially because of criticism due to food supply issues and non-availability of arable lands worldwide (Patel et al. 2015; Searchinger et al. 2015). High demands of fossil fuels worldwide are compelling scientific community toward exploration and research on renewable energy production (Chisti 2007a). Development of new technologies for renewable energy production can replace the use of fossil fuels (Patel et al. 2014). Biodiesel gained popularity in recent years due to its renewable potential. Collectively, methyl esters of fatty acids are called biodiesel. Generally, biodiesel is produced by transesterification of fats/lipids in the presence of a suitable catalyst. Lipids can be obtained from animal tissues, plant seeds, and other biomass materials. (Chisti 2007b). Recently, microalgae have been extensively explored for biodiesel production. Microalgae can grow easily anywhere simply, like in ponds, photobioreactors, etc. in presence of light, carbon, nitrogen, and phosphorus source (Ji et al. 2014; Abomohra et al. 2017). Microalgae have 15–80% of lipid content and high growth rates (Liu et al. 2008; Smith et al. 2009). However, microalgal biodiesel industrial production is still facing many constraints due to the expensive downstream processes in biodiesel production. Sometimes, harvesting of microalgae involves 30–40% of the total capital cost of biodiesel production. So, the selection of microalgal strain, culture medium, and other growing conditions become important for the reasonable cost of biodiesel production. The high growth of microalgae can be achieved by

varying the chemical and physical parameters of the cultivation environment. Temperature, pH, light duration, and nutrients (N, P, and carbon source) greatly affect the growth and lipid synthesis in microalgae (Oh et al. 2009; Pandit et al. 2017). In spite of all these constraints, currently, microalgae are the potential and best-suited bio-resource for biodiesel generation (Weissman and Goebel 1987). Lipid content and biomass production can be increased by altering cultivation medium components and other growth conditions (Mandotra et al. 2014). This will help in the production of cost-effective industrial microalgal biofuel production up to an extent. Lipid content can be enhanced by altering culture media ingredients for a particular microalgal strain (Cheirsilp and Torpee 2012). Thus, by optimizing growth conditions, biomass, and lipid productivity, we may achieve economic biodiesel production (Mandotra et al. 2014).

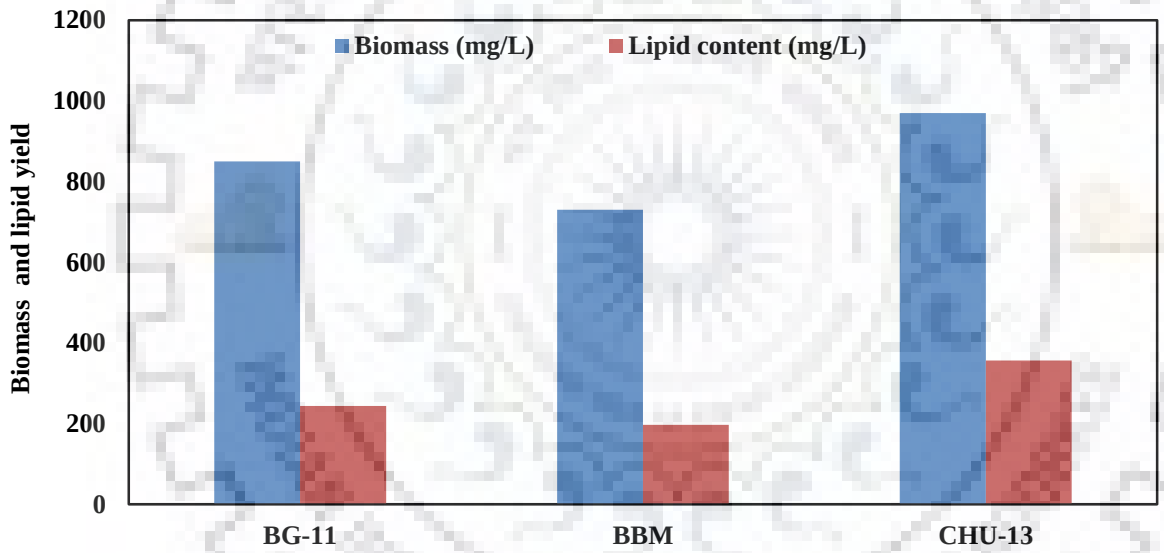

Figure 2.1 Biomass and lipid yield of *C. minutissima* obtained with three different cultivation media

2.2 Materials and methods

2.2.1 Microalgal culture

Culture of *C. minutissima* was procured from the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, India. The culture was maintained in BBM medium at 25 ± 1 °C under continuous illumination of 2500 lux.

2.2.2 Screening of most efficient growth medium

Initially, 5 mL samples of *C. minutissima* from maintained culture were inoculated in different 500 mL conical flasks having each of 300 mL of BG-11, modified CHU-13 and BBM medium at 25 ± 2 °C, 2500 lux under 12:12 h light/dark cycle for 18 days. The light was supplied using cool white fluorescent light tubes and light intensity was measured using digital lux meter. Compositions of three different media used in this study are presented in Table 2.1. In all the experiments, only one parameter was variable at a time and others were constant.

Table 2.1 Composition of three media used for the culture of C. *minutissima*

Components (mg/L)	Culture medium		
	Modified CHU-13	BG- 11	BBM
KNO_3	400	Nil	Nil
$NaNO_3$	Nil	1500	250
K_2HPO_4	80	40	75
KH_2PO_4	Nil	Nil	175
NaCl	Nil	Nil	25
Na_2CO_3	Nil	20	Nil
$MgSO_4{\cdot}7H_2O$	200	75	75
$CaCl_2{\cdot}2H_2O$	107	36	25
$C_{10}H_{16}N_2O_8$	Nil	1	50
$FeSO_4$	Nil	Nil	4.98
$C_6H_8FeNO_7$	Nil	6	Nil
$C_6H_5FeO_7$	20	Nil	Nil
$C_6H_8O_7$	100	6	Nil
$CoCl_2{\cdot}2H_2O$	0.02	Nil	Nil
H_3BO_3	5.72	2.86	11.42
$MnCl_2{\cdot}4H_2O$	3.62	1.81	1.44
$ZnSO_4{\cdot}7H_2O$	0.44	0.22	8.82
$CuSO_4{\cdot}5H_2O$	0.16	0.079	1.57
$Na_2MoO_4{\cdot}2H_2O$	0.084	0.39	Nil
$Co(NO_3)_2{\cdot}6H_2O$	Nil	0.049	0.49
MoO_3	Nil	Nil	0.79

Harvested microalgal biomass obtained from three different cultivation media was used for lipid extraction as per the protocol described by Folch et al. (1957). Highest microalgal biomass and lipid yield was obtained from the culture cultivated with modified CHU-13 medium. So, in further sets of experiments, modified CHU-13 medium was used for cultivation

of microalga. In all the experiments, only one parameter was variable at a time and others were constant.

2.2.3 Abiotic factors tested for maximum biomass and lipid yield of *C. minutissima*

Following nitrogen (KNO_3 as nitrogen source) concentrations of 0, 60,120, 180, 240, 300, and 360 mg/L, phosphorus (K_2HPO_4 as phosphorus source) concentrations of 10, 20 50, 100, 150, 250, and 350 mg/L, iron (FeSO4 as iron source) concentrations of 10, 20, 30, 40, 45, and 50 mg/L, zinc ($ZnSO_4$ as zinc source) concentrations of 20, 30, 40, 50, 100, and 200 mg/L and glucose concentrations of 3000, 5000,7000, 10,000, and 12,000 mg/L were used to see the effect on biomass and lipid yield of *C. minutissima*. To see the effect of pH it was tested for the values of 5, 6, 7, 8 and 9. Temperature and light intensity were varied in the range of 17 ± 2 to 33 ± 2 °C and 3500 to 10,000 lux, respectively, while different photoperiods (light/dark) cycles of 14:10, 16:08, 18:06 and 24:00 h were applied.

2.2.4 Biomass growth and dry cell weight determination

Biomass productivity (g/L-d) was measured gravimetrically on dry cell weight basis at an interval of 3 days. Microalgal suspensions of 10 mL each were centrifuged at 5000 rpm for 10 min, washed with double distilled water, and dried at 65 °C for 24 h until attainment of constant weight, cooled in a desiccator, and weighed. The biomass productivity was calculated as follows:

Biomass productivity (BP) = $(C_2-C_1)/(T_2-T_1)$ (2.1)

where, C_1 and C_2 are biomass concentrations (g/L) and T_1 and T_2 are initial and final sampling times.

2.2.5 Lipid extraction and estimation

Lipid extraction was performed according to the protocol described by Folch et al. (1957). In short, freeze-dried algal biomass was mixed with chloroform and methanol in the ratio of 2:1 (v/v) and the mixture was agitated for 20 min. The resulting mixture was centrifuged at 7000 rpm and 4 °C for 7 min to discard impurities from the supernatant. The supernatant was washed with 0.9% NaCl (0.2 volumes) and vortexed. After 5 min of 3000 rpm centrifugation, the upper phase was removed and the lower chloroform phase containing lipids

was evaporated under vacuum using a rotary evaporator in pre-weighed weighing bottles. The lipid productivity was calculated by the formula given by Griffiths and Harrison (2009).

Lipid Productivity (LP) = BP x lipid content (% dry cell weight basis) (2.2)

2.2.6 Transesterification of lipids

Extracted lipids were transesterified using 10% H_2SO_4 in the molar ratio of alcohol:lipid as 6:1 at 100 °C for 5 h with constant stirring (Mandotra et al. 2014). Direct transesterification was also performed by taking dry algal biomass and methanol in the ratio of 1:3 (w/v) at 100 °C under continuous stirring for 5 h in presence of H_2SO_4 (20% w/w of dry algal biomass) as catalyst (D'Oca et al. 2011). The remaining alcohol was removed through a rotatory evaporator and hexane was added to the mixture. The mixture was filtered and dried using anhydrous Na_2SO_4. Finally, hexane was removed using a rotatory evaporator to get fatty acid methyl esters (FAMEs).

2.2.7 Fatty acid methyl esters (FAMEs) and GC-MS analysis

GC-MS (Clarus 500, Perkin Elmer) analysis was done to see FAMEs profile according to the protocol described by Härtig (2008). Splitless injection mode at 250 °C was selected, and the injection volume was kept at 1 µL. Helium was used as a carrier gas. Initially, the column temperature was set at 50 °C for 1.5 min, later on, for 1 min, hold temperature was raised up to 180 °C (25 °C min^{-1}). A further increment in temperature of up to 220 °C (10 °C min^{-1}) was done and held for 1 min. The maximum increment in the temperature was made up to 250 °C (15 °C min^{-1}) and held for 3 min. Line of mass transfer and ion source were set at 250 and 200 °C, respectively. Scan mode (50–600 m/z) with electron ionization potential of 70 eV was used to detect FAME profile.

2.2.8 Attenuated total reflectance-fourier transform infrared and thermo-gravimetric analyses

Attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) spectra of biodiesel was obtained using Perkin Elmer FTIR Spectrum Two (USA) machine. The FTIR was equipped with an ATR sampling accessory. All spectra were collected at 20 (1 °C using an average of 32 scans and with a spectral resolution of 2/cm). Thermo-gravimetric analysis (TGA) was performed using Netzsch TG 209F3 TGA analyzer to measure the thermal

behaviour of biodiesel. Samples of 10 µL biodiesel were heated at a constant heating rate of 10 °C/min in an inert atmosphere using nitrogen gas. The temperature range was kept at 25 to 500 °C.

2.2.9 Statistical analysis

Data were analyzed by applying one-way analysis of variance (ANOVA) at a confidence level of 5%. Experiments were performed in triplicate.

2.3 Results and discussion

During screening for the most efficient growth medium for *C. minutissima*, modified CHU-13 medium resulted in a maximum biomass and lipid yield of 970 ± 0.21 and 356.63 ± 0.51 mg/L, respectively, followed by BG-11 medium which resulted in a biomass and lipid yield of 850 ± 0.12 and 243.65 ± 0.30 mg/L, respectively. The lowest biomass and lipid yields of 730 ± 0.42 and 196.83 ± 0.43 mg/L were achieved with BBM medium, respectively. Figure 2.1 depicts various values of biomass and lipid yield of *C. minutissima* obtained with three different cultivation media.

2.3.1 Effects of different abiotic factors on biomass and lipid yield of *C. minutissima*

Effect of nitrogen

Nitrogen is the basic element for the formation of proteins and nucleic acids in the cell. It constitutes 7–20% of cell dry weight. Nitrogen deficiency boosts protein translation and lipid synthesis (Ruangsomboon 2012). The highest biomass concentration of 1150.52 ± 0.44 mg/L, lipid yield of 579.86 ± 0.076 mg/L, the lipid content of 50.39 ± 0.018% and lipid productivity of 48.32 ± 0.0063 mg/L/day were obtained, respectively by cultivating microalga with nitrogen concentration of 300 mg/L Fig. 2.2(a,b).

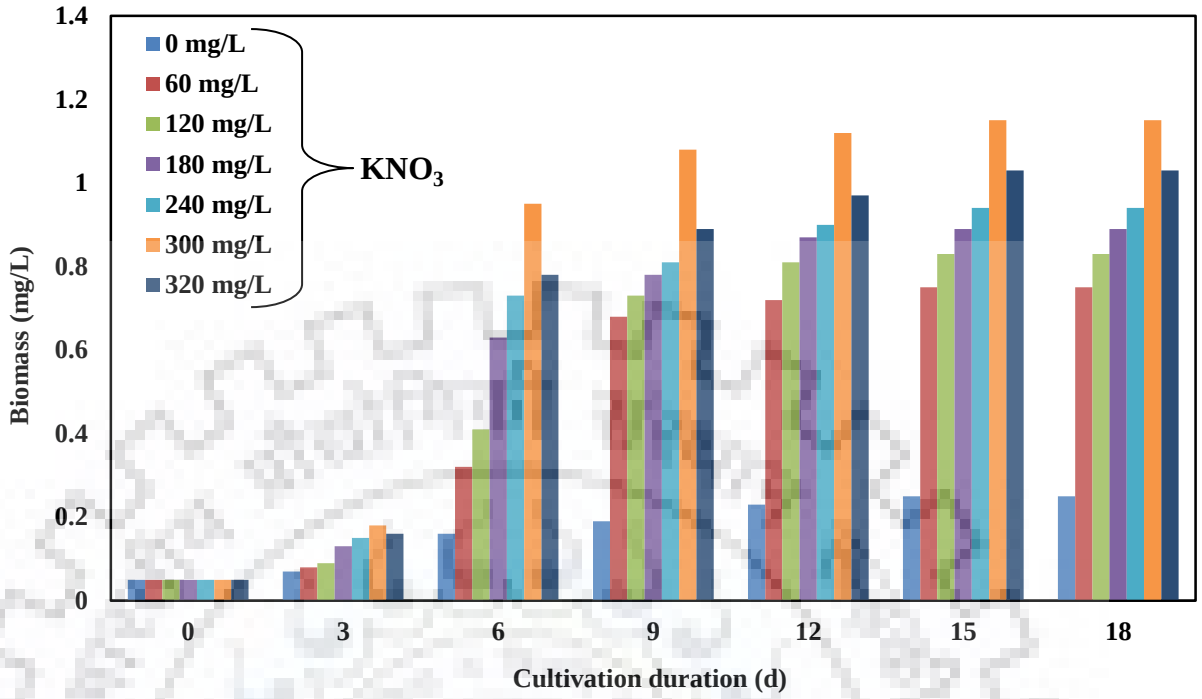

Figure 2.2(a) Growth pattern of *C. minutissima* under different concentrations of nitrogen using modified CHU-13 medium

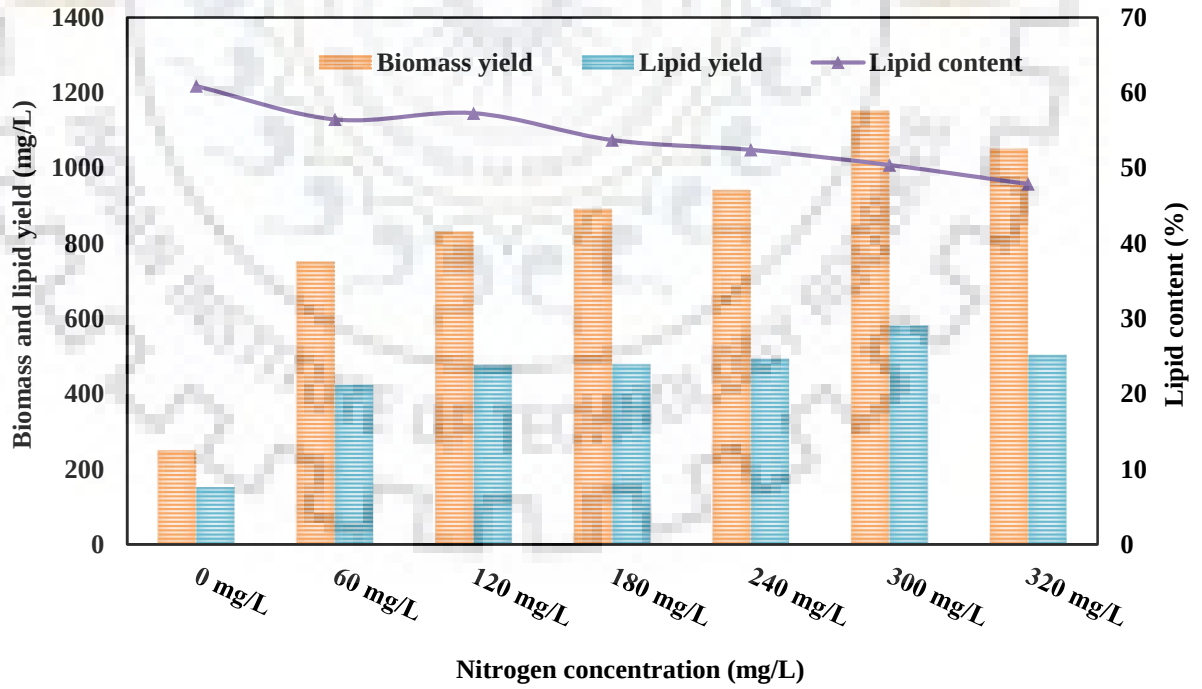

Figure 2.2(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of nitrogen using modified CHU-13 medium

Effect of phosphorus

Phosphorus is an important abiotic macronutrient for algae growth. Its requirement is necessary for protein synthesis, nucleic acids, and cellular framework development. Phosphorus deficiency can lead to chlorophyll death and poor protein content (Healey 1982). The effects of initial phosphorus concentration are shown in Fig. 2.3(a,b). Biomass and lipid yield of microalga increases with increasing phosphorus concentration from 10 to 350 mg/L. The highest biomass of 1185.71 ± 0.81 mg/L with lipid yield of 572.78 ± 0.45 mg/L, lipid content of 48.30 ± 0.023%, and lipid productivity of 44.81 ± 0.023 mg/L-d was achieved with 350 mg/L of phosphorus concentration while phosphorus concentration of 250 mg/L resulted in biomass yield of 1091 ± 0.64 mg/L with lipid yield of 537.79 ± 0.27 mg/L and lipid content of 49.31 ± 0.024%. Thus, higher concentration of phosphorus resulted in higher biomass but lower lipid content.

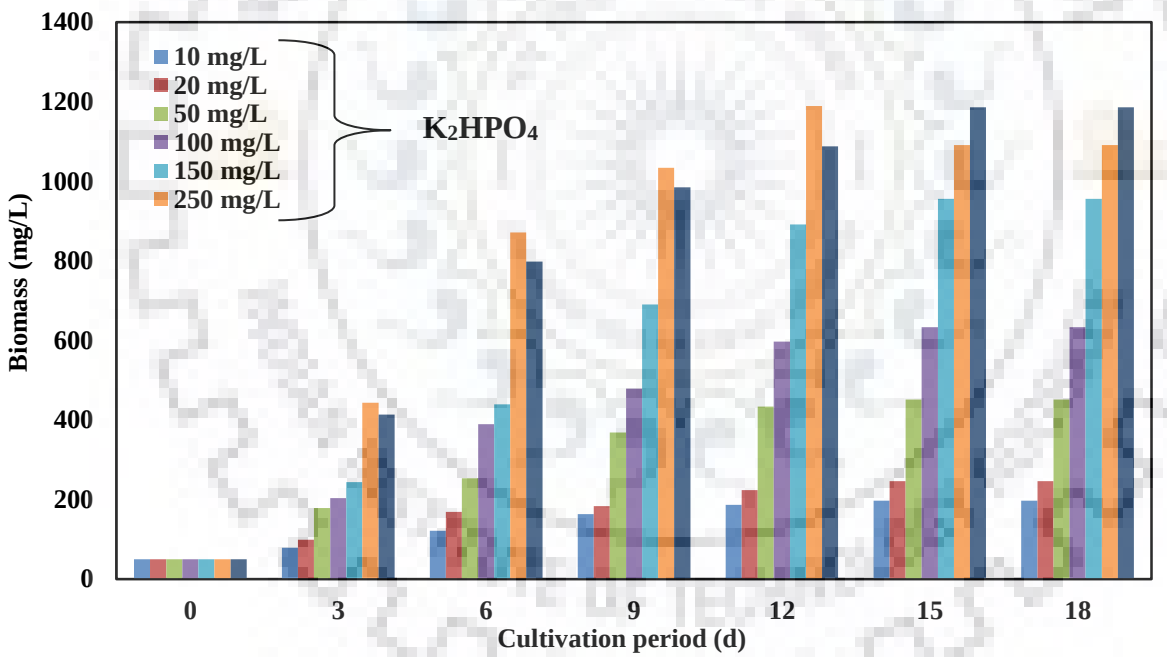

Figure 2.3(a) Growth pattern of *C. minutissima* under different concentrations of phosphorus using modified CHU-13 medium

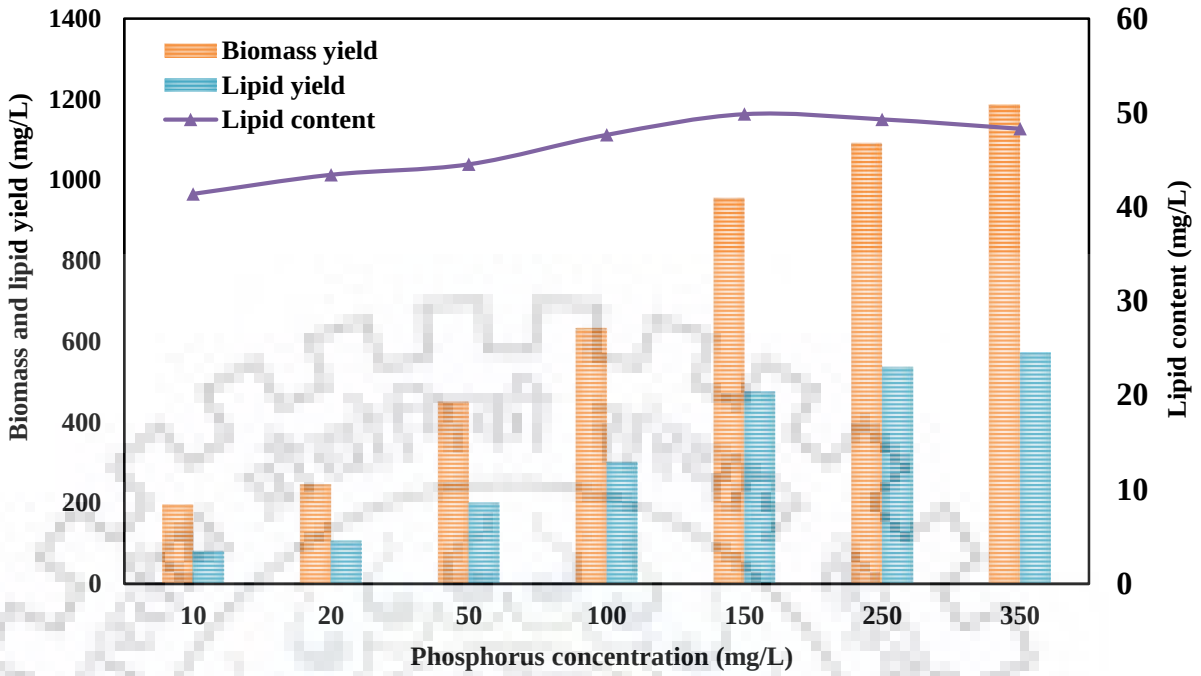

Figure 2.3(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of phosphorus using modified CHU-13 medium

This may be due to the inefficient utilization of higher concentrations of phosphorus by microalga. Ruangsomboon (2012) has demonstrated that the phosphorus concentration from 22 to 444 mg/L resulted in 7.3 times more biomass growth with a decrease in lipid content of *Botryococcus braunii* KMITL 2.

Effect of iron

Iron deficient medium can cause retarded microalgal growth. However, excess of iron can lead toward oxidative stress and physiological changes in microalgae (Hu 2004; Yeesang and Cheirsilp 2011). Various effects of iron concentration on *C. minutissima* biomass, lipid content, and lipid yield are depicted in Fig. 2.4(a, b). Biomass yield, lipid yield and lipid content of microalga increase with increasing iron concentration from 10 to 50 mg/L. The highest biomass of 988.68 ± 0.77 mg/L, lipid yield of 446.9 ± 0.42 mg/L, the lipid content of 45.2 ± 0.077%, and lipid productivity of 37.24 ± 0.035 mg/L-d were achieved with iron concentration of 50 mg/L. There was a very little difference in biomass yield (986.76 ± 0.33 mg/L), lipid yield (443.86 ± 0.62 mg/L), and lipid content of (44.98 ± 0.074%) microalga obtained with 45 mg/L of iron concentration in comparison with 50 mg/L iron concentration.

Maximum lipid content of 34.93 ± 1.89% and lipid yield of 0.08 g/L were reported with iron concentration of 27 mg/L in *Botryococcus braunii* KMITL 2 (Ruangsomboon 2012).

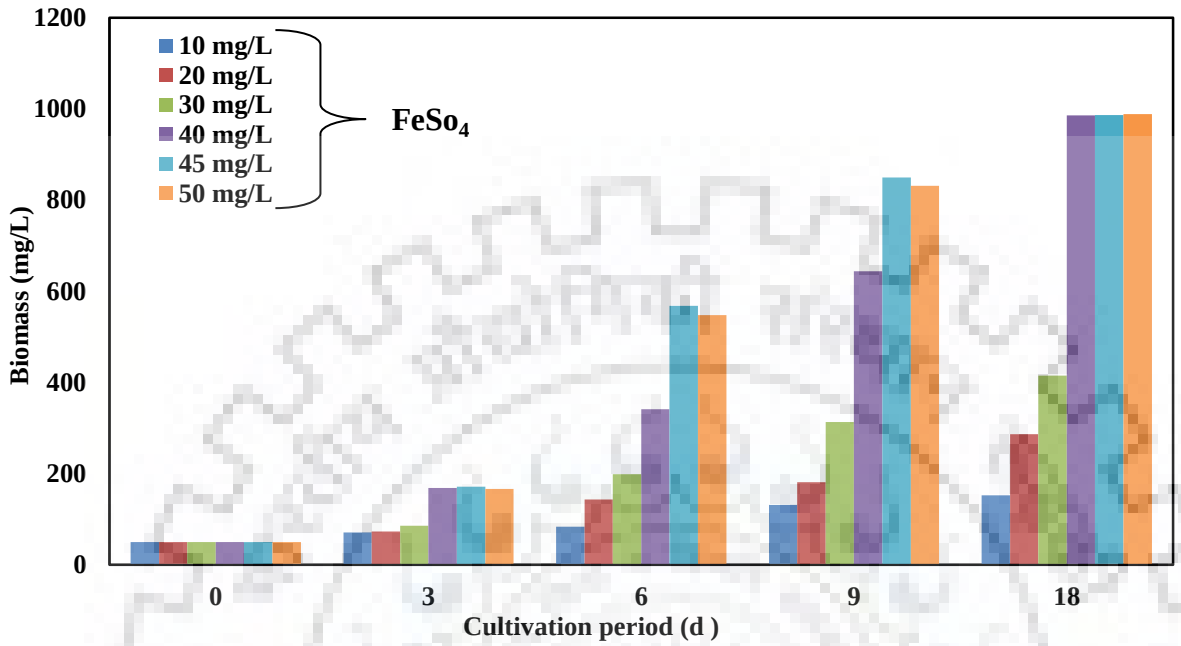

Figure 2.4(a) Growth pattern of *C. minutissima* under different concentrations of iron using modified CHU-13 medium

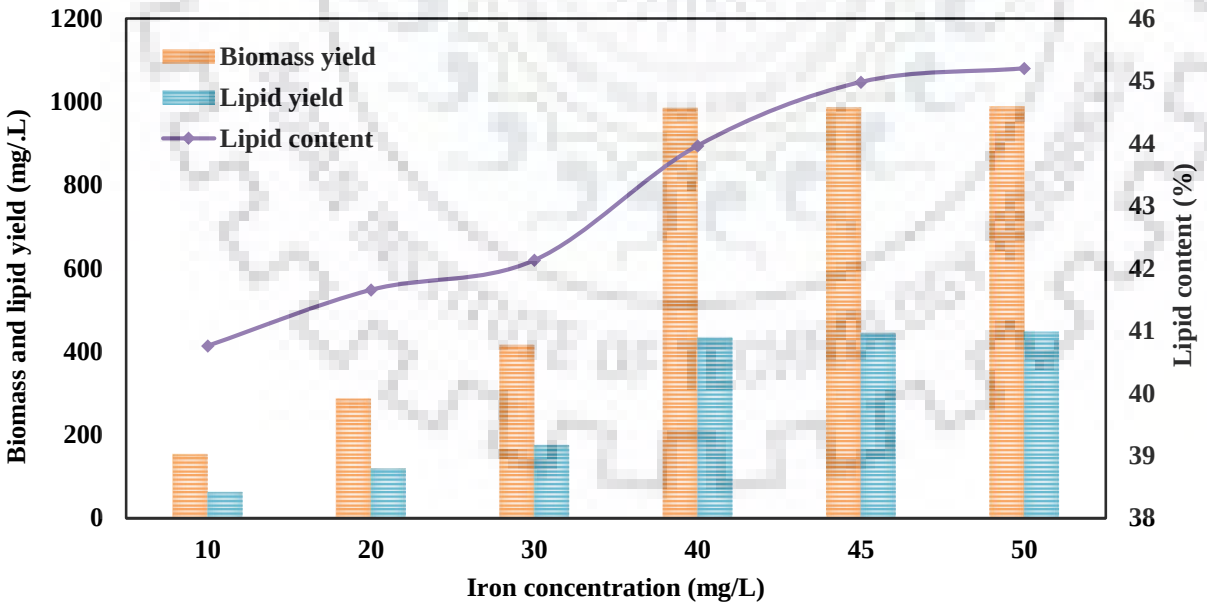

Figure 2.4(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of iron using modified CHU-13 medium

Effect of zinc

Zinc is an essential micronutrient for microalgal growth. Zinc deficient environment leads to retarded growth (Shrotri et al. 1981). The effects of initial zinc concentration are presented in Fig. 2.5(a, b). Biomass yield, lipid yield, and lipid content of microalga increase with increasing zinc concentration from 20 to 100 mg/L. The highest biomass of 1026.82 ± 0.51 mg/L, lipid yield of 437.04 ± 0.19 mg/L, the lipid content of 42.56 ± 0.0066%, and lipid productivity of 36.42 ± 0.016 mg/L-d were obtained by cultivation with the zinc concentration of 100 mg/L. Beyond this concentration (200 mg/L), decreased biomass yield, lipid yield, lipid content, and lipid productivity of 976.6 ± 0.77 mg/L, 368.19 ± 0.53 mg/L, 37.70 ± 0.057%, and 30.68 ± 0.044 mg/L-d were achieved, respectively. Travieso et al. (1999) have reported that *Chlorella vulgaris* showed 600 mg/L of zinc tolerance limit but microscopic observation of cells shows deformity in cell morphology.

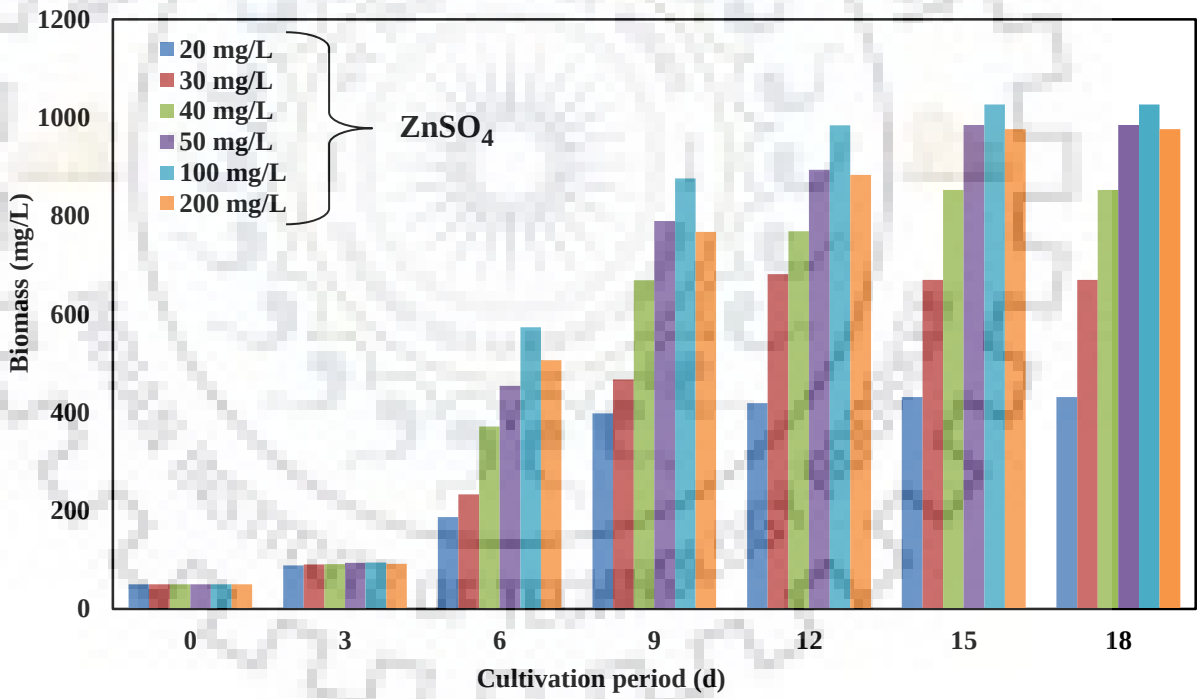

Figure 2.5(a) Growth pattern of *C. minutissima* under different concentrations of zinc using modified CHU-13 medium

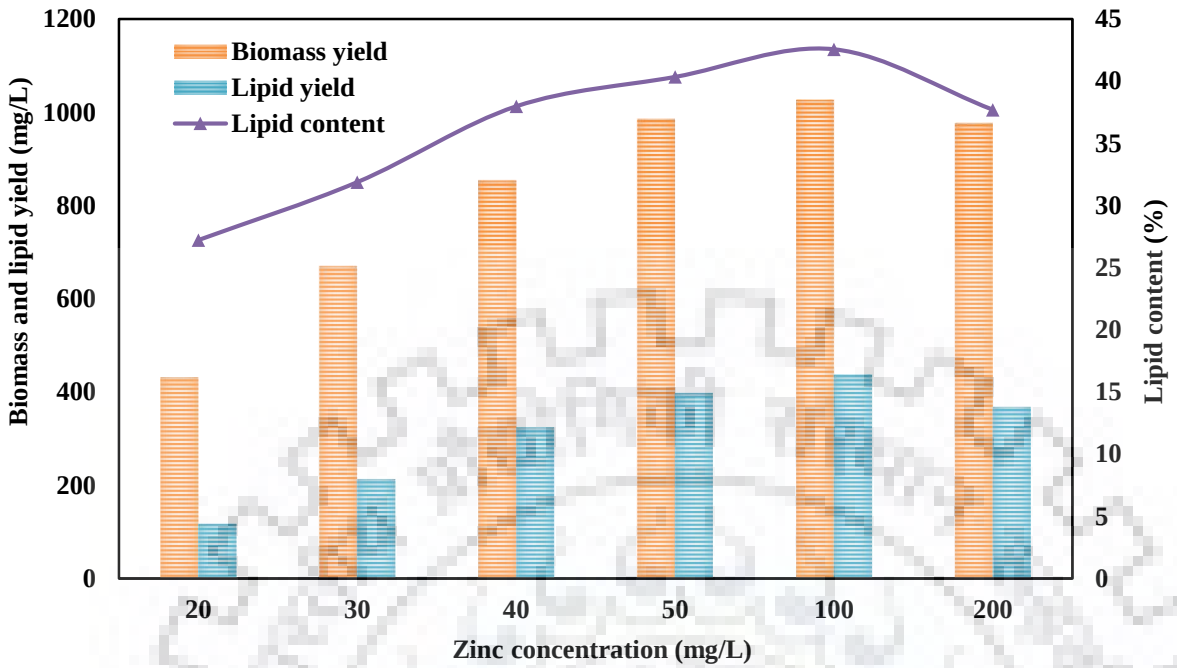

Figure 2.5(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of zinc using modified CHU-13 medium

Effect of pH

pH is one of the crucial parameters that control the microalgal biomass growth. However, it has been cumbersome to conclude a generalization for optimum pH. It depends on the selected species and its growth environment. Moreover, it can be derived on the basis of previous studies that in controlled pH environment improved microalgal growth can be achieved. Wang et al. 2010 reported improved biomass growth and lipid yield of *Chlorella vulgaris* at pH 7 to 8.5. Cho et al. (2015) reported that pH 7 is most suitable for *Chlorella vulgaris* in a study conducted by him. The effects of different pH values on biomass and lipid yield of *C. minutissima* are shown in Fig. 2.6(a, b). The highest biomass of 1023.17 ± 0.23 mg/L, lipid yield of 418.90 ± 0.73 mg/L, the lipid content of 40.94 ± 0.071%, and lipid productivity of 34.91 ± 0.061 mg/L-d were obtained at pH 8. Biomass and lipid yields of 1007.09 ± 0.13 and 382.91 ± 0.52 mg/L were achieved at pH 8.

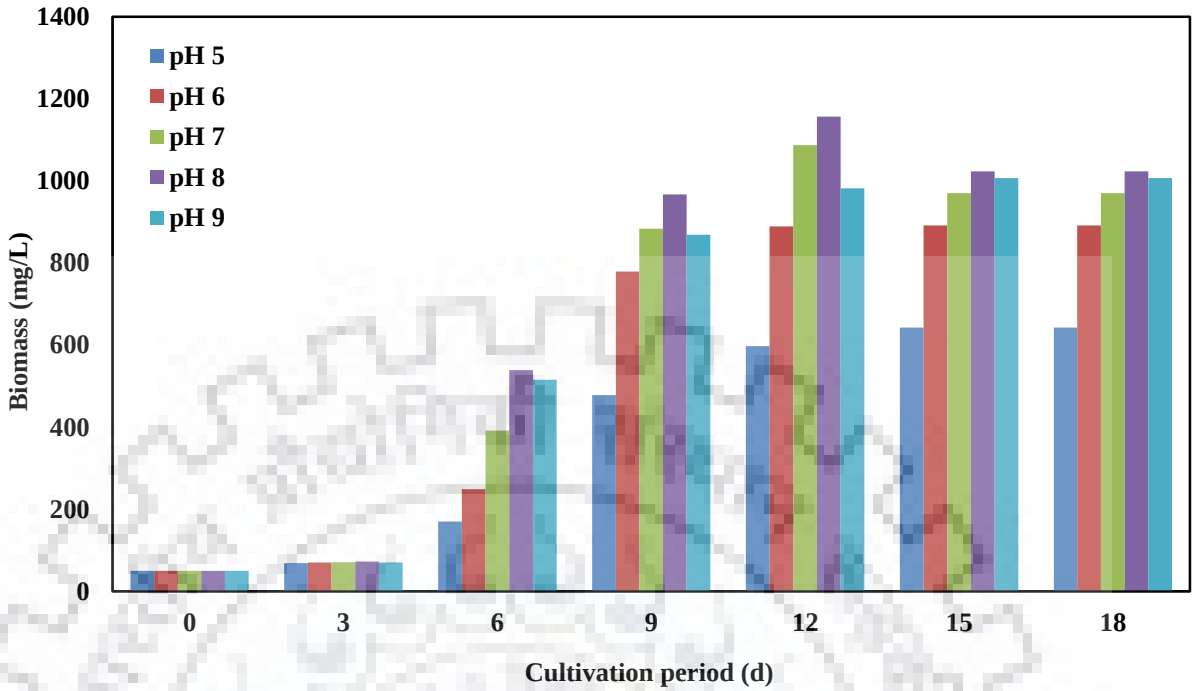

Figure 2.6(a) Growth pattern of *C. minutissima* under different pH using modified CHU-13 medium

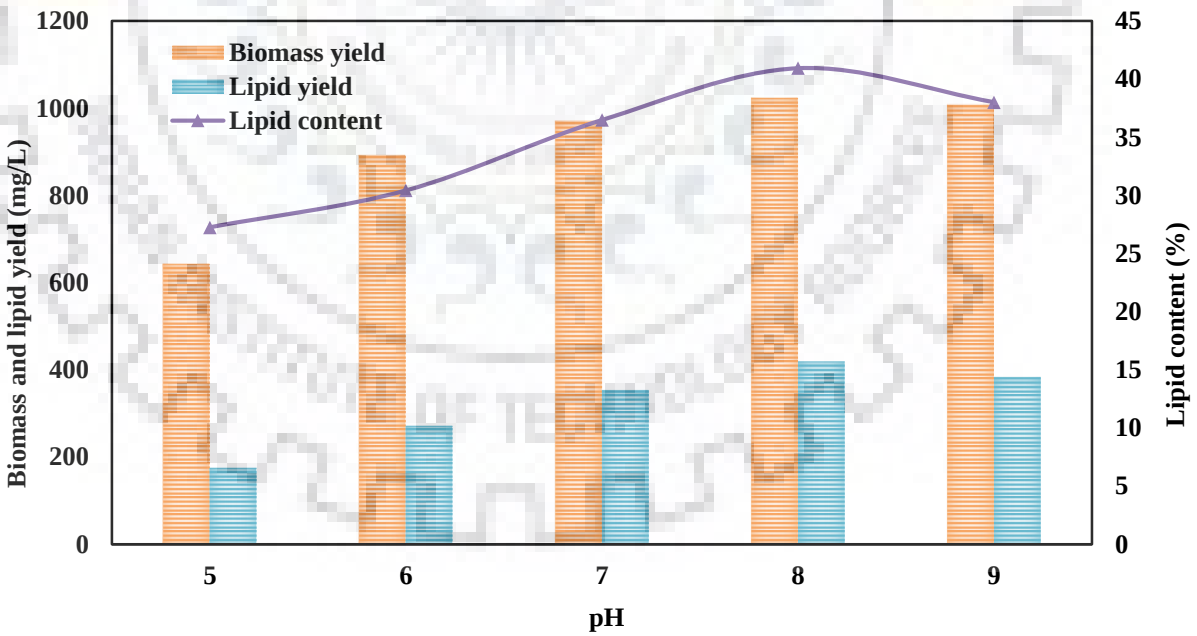

Figure 2.6(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different pH using modified CHU-13 medium

Effect of temperature

Temperature is the most important abiotic environmental factor that directly affects microalgal growth and metabolic activities of the cell. Its role in microalgae metabolism and lipid synthesis has been established by many authors (Sushchik et al. 2003; Amit et al. 2017). The effects of temperature variation on microalgal biomass and lipid yield are shown in Fig. 2.7(a, b). The highest biomass yield of 997.99 ± 0.064 mg/L, lipid yield of 378.32 ± 0.44 mg/L, the lipid content of 37.91 ± 0.041%, and lipid productivity of 31.52 ± 0.036 mg/L-d were obtained when microalgal was cultivated at 27 ± 2 °C.

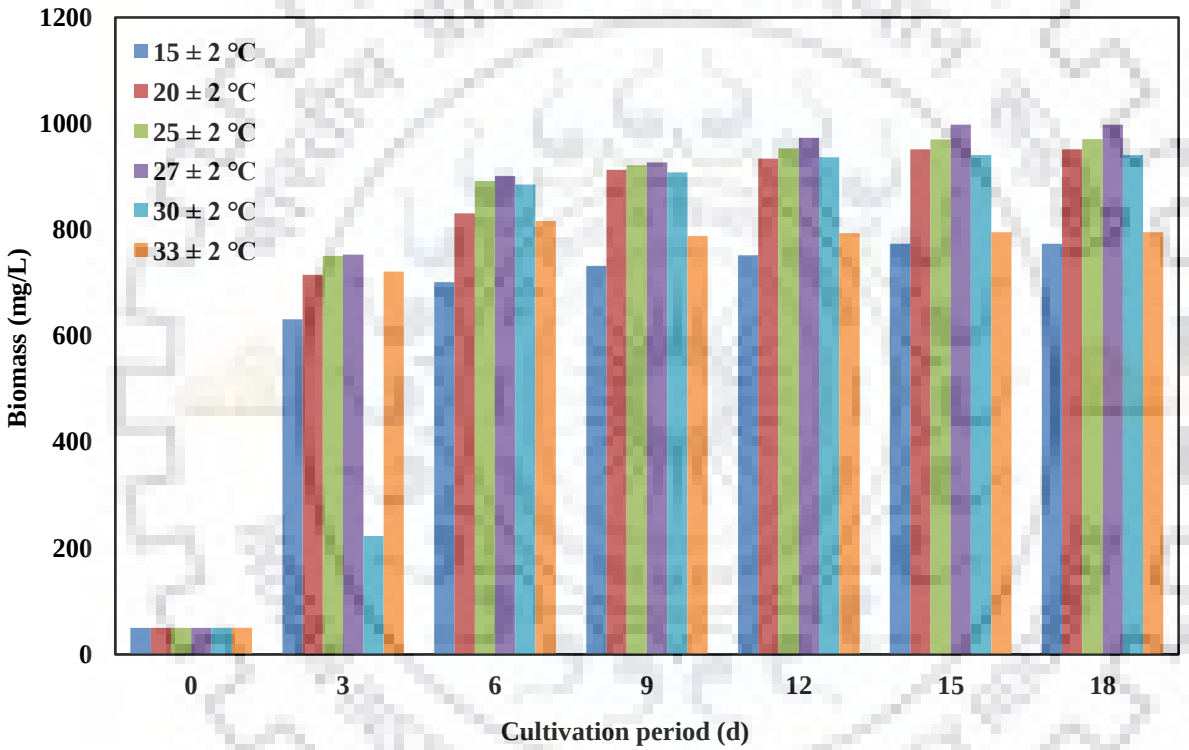

Figure 2.7(a) Growth pattern of *C. minutissima* under different temperatures using modified CHU-13 medium

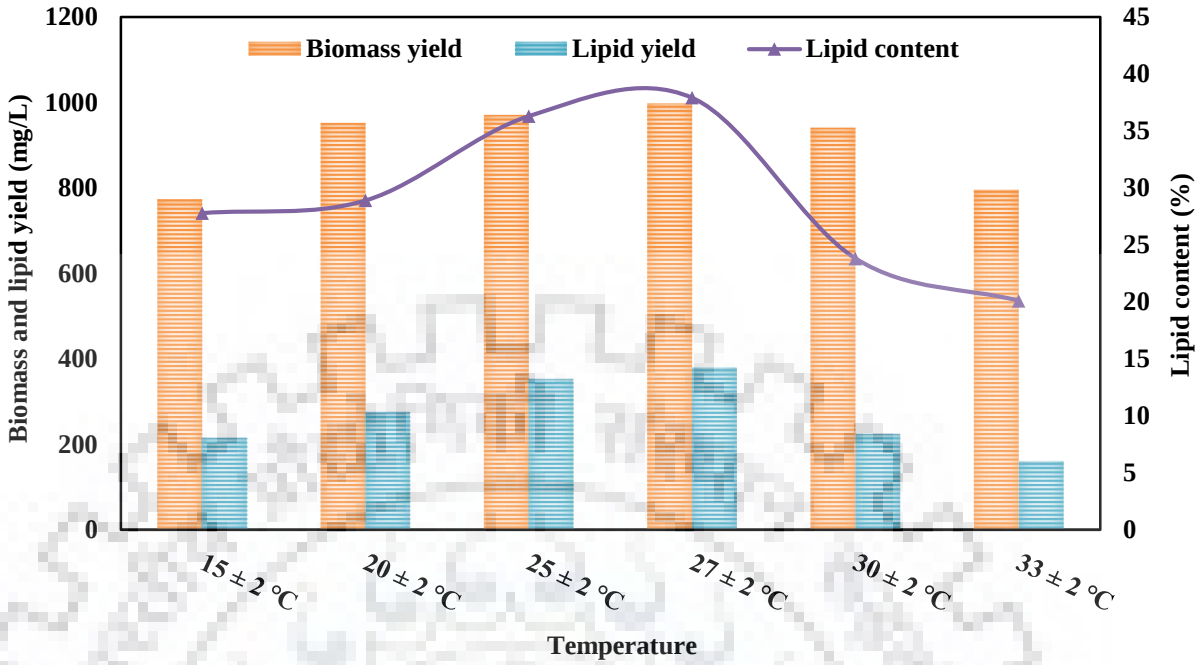

Figure 2.7(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different temperatures on modified CHU-13 medium

Effect of glucose

Altering cultivation conditions to improve the biomass and lipid yield of microalgae is well known (Borowitzka 1999). High biomass growth and lipid yield can be achieved by the heterotrophic cultivation of microalgae (Minowa et al. 1995; Miao and Wu 2006). Cheirsilp and Torpee (2012) reported that the *Chlorella* sp. and *Nannochloropsis* sp. high biomass and lipid yield glucose supplemented medium by using fed-batch cultivation system. Biomass yield of 2.01 g/L was achieved for *Phaeodactylum tricornutum* UTEX#640 with a glucose concentration of 5.0 g/L (Garcia et al. 2005). The effects of glucose concentration are shown in Fig. 2.8(a, b). Biomass and lipid yield of microalga increased with increasing glucose concentration up to 12,000 mg/L. The highest biomass of 1840.49 ± 0.62 mg/L, lipid yield of 405.36 ± 0.96 mg/L, the lipid content of 22.02 ± 0.060%, and lipid productivity of 33.78 ± 0.081 mg/L-d were obtained at glucose concentration of 12,000 mg/L.

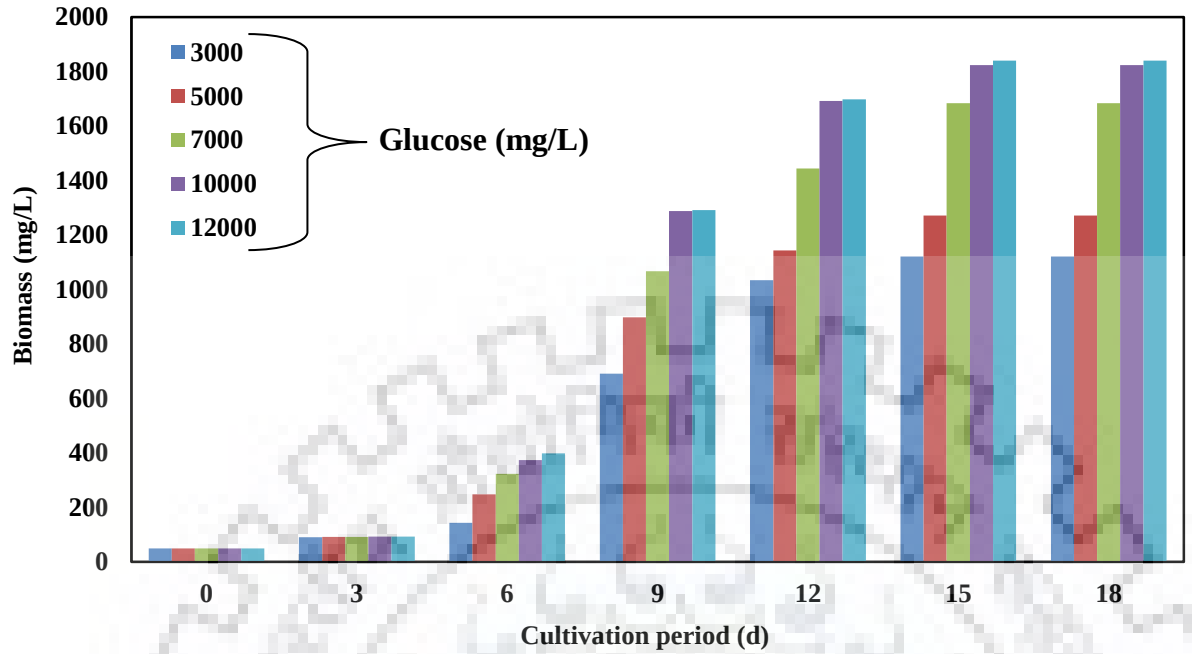

Figure 2.8(a) Growth pattern of *C. minutissima* under different concentrations of glucose cultivated on modified CHU-13 medium

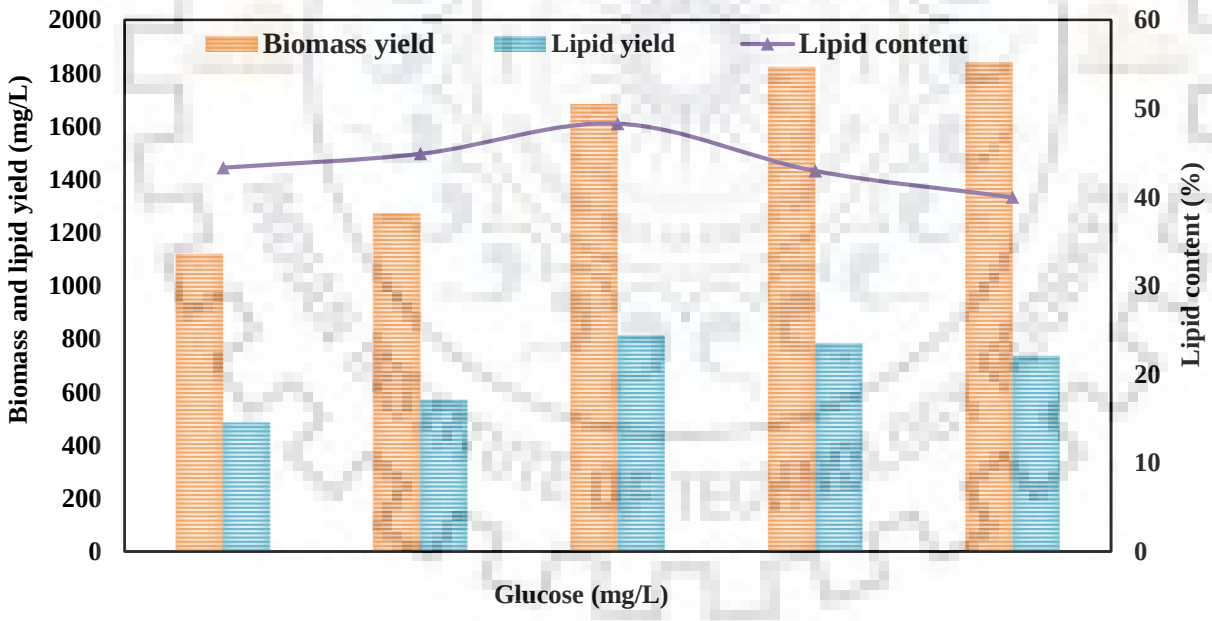

Figure 2.8(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of glucose cultivated on modified CHU-13 medium

Effect of photoperiod

The effects of various selected photoperiods (light/dark) cycles are shown in Fig. 2.9(a, b). *C. minutissima* accumulated a biomass yield of 1041.18 ± 0.17 mg/L, lipid yield of 382.38 ± 0.41 mg/L, the lipid content of 36.73 ± 0.042%, and lipid productivity of 31.86 ± 0.033 mg/L-d under a photoperiod of 24:00 hours. Matos et al. (2017) reported a maximum biomass growth of 1.25 g/L in *Nanochloropsis gadianta* with a photoperiod of 16:8 h. Ruangsomboon (2012) reported maximum biomass with a 24:00 h photoperiod in *Botryococcus braunii* KMITL 2.

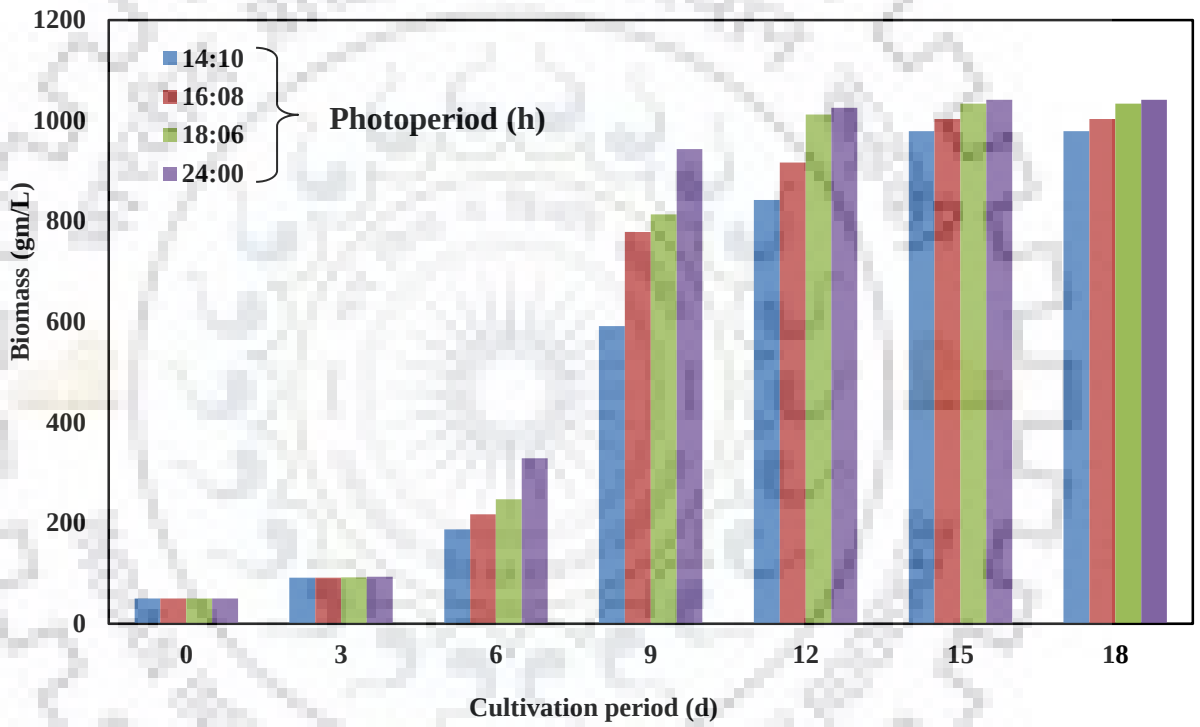

Figure 2.9(a) Growth pattern of *C. minutissima* under different photoperiods (L:D) h cultivated on modified CHU-13 medium

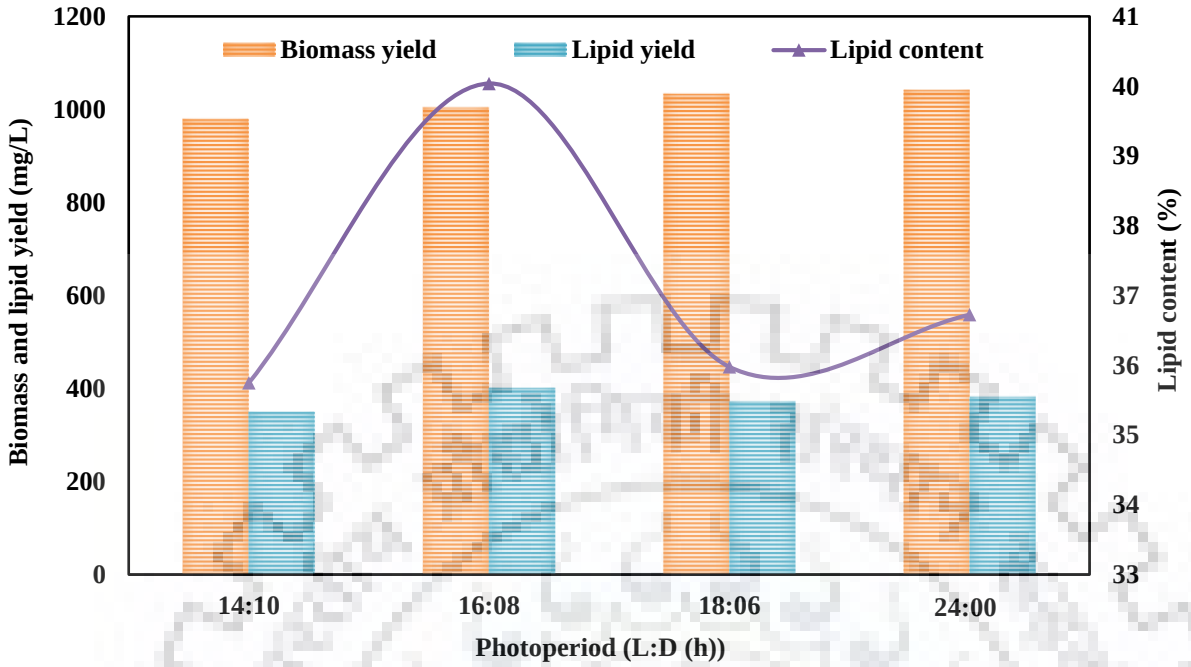

Figure 2.9(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different photoperiods cultivated on modified CHU-13 medium

Effect of light intensity

Light is one of the most crucial and mandatory abiotic factors which affect biomass growth and biosynthesis of many vital molecules in the cell. Ruangsomboon (2012) obtained a lipid yield of 0.45 g/L in *Botryococcus braunii* KMITL 2 at 538 μE/m^2-s. Kojima and Zhang (1999) reported that the optimized light intensities can enhance microalgal lipid yield. The effects of light intensity variation are shown in Fig. 2.10(a, b). The highest biomass yield of 1165 ± 0.26 mg/L, lipid yield of 491.73 ± 0.24 mg/L, the lipid content of 42.21 ± 0.015%, and lipid productivity of 40.97 ± 0.02 mg/L-d were obtained by cultivating microalga at light intensity of 9000 lux at 25 ± 2 °C. Decreased biomass and lipid yields of 1011.29 ± 0.50 and 387.04 ± 0.12 mg/L were obtained, respectively, at 10,000 lux at 27 ± 2 °C. These results are in compliance with work done by Cheirsilp and Torpee (2012) where the growth of marine *Chlorella* sp. increased up to 8000 lux light intensity, and beyond it, with 10,000 lux, biomass growth was decreased. This can be attributed to photo-inhibition. However, in *Botryococcus braunii*, maximum growth was reported under continuous irradiance (Dumrattana and Tansakul 2006).

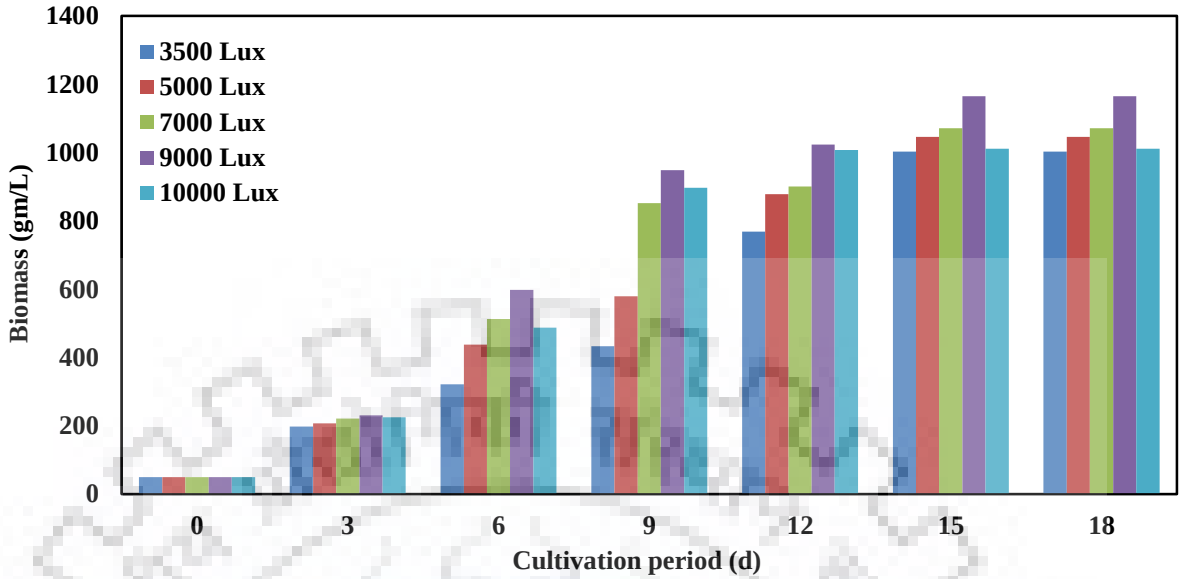

Figure 2.10(a) Growth pattern of *C. minutissima* under different light intensities cultivated on modified CHU-13 medium

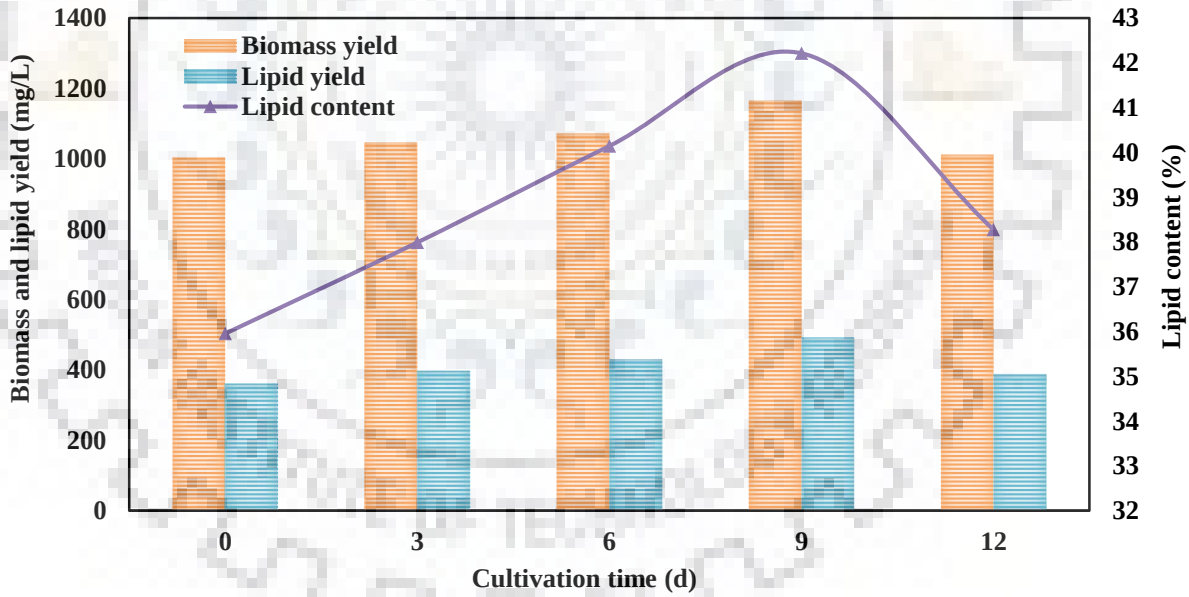

Figure 2.10(b) Biomass yield, lipid yield and lipid content of *C. minutissima* under different light intensities cultivated using modified CHU-13 medium

2.3.2 FAME profile and biodiesel characteristics

As shown in Fig. 2.11, the FAME profile of *C. minutissima* was found to be mainly composed of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), gondoic acid (C20:1), and behenic acid (C22:0) which account to 3, 21.78, 9.69, 3, 9.38, 24.23, 13.45, 10.33, 1 and 1%, respectively, of total fatty acids. Few important biodiesel properties are as follows:

Cetane number

Cetane number (CN) is the property of fuel that decides the ignition characteristics of fuel in terms of ignition and combustion. It affects the various parameters of engine performance such as noise, emissions of CO and stability (Suh and Lee 2016). Higher CN imparts the better ignition of biodiesel than the conventional diesel fuel ensuring better cold start behavior, smooth engine run and complete combustion leading to reduced gaseous and particulate emissions (Patel et al. 2017). Cetane number has both its lower and higher limits as lower cetane number of biodiesel causes difficulty of engine starting in cold environmental and generation of noise and pollution (emissions of hydrocarbons) without proper combustion of biodiesel while higher cetane number causes instant ignition without proper mixing of air that results in reduction of fuel efficiency.

Kinematic Viscosity

The property of viscosity of any fluid is just opposite to fluidity that repels the movement of fluid at intramolecular level. Kinematic viscosity (KV) is an important fuel property of biodiesel that defined by its ability to flow, speed and quality of injected spray in the combustion chamber of the engine (Jakeria et al. 2014). The fluidity of biodiesel hampers as its viscosity increases at low temperature. Viscosity increases with chain length of fatty acid or saturation of fatty acid, however, the viscosity of unsaturated fatty acid depends on number and nature of double bonds but less affected by position. KV of biodiesel is usually 10–15% higher than the conventional diesel fuels due its large molecular weight and structure(Atabani et al. 2012).

Viscosity affects almost all components of diesel engines as it affects the starting of the engine, injection quantity and quality, and mixing of fuel with air in the combustion chamber. Viscosity having higher limit causes performance related problems especially at low temperature while lower limit causes fine particles of fuel with high speed and low mass.

Density

Density (D) play crucial role to determine the fuel injection property as it is correlated with another parameter for engine performance such as cetane number and heating value (Wakil et al. 2015). It affects the pumping of fuel by its volume and not so by mass. It has been well documented that denser biodiesel has more energy than petroleum diesel (Patel et al. 2017). Density is limited to 860–900 kg m−3 at 15 °C in EN 14214 but there is no specification for density in the ASTM D6751. The density of the fuel is correlated with other properties such as HHV, viscosity, cetane number which depends on temperature, water content, and the presence of free fatty acid content in FAME.

Oxidative stability

Oxidative stability (OS) of biodiesel is an important yardstick to determine its self-life. Unsaturation and double bond in fatty acid chains are responsible for their interaction with oxygen when being exposed to air (Lanjekar and Deshmukh 2016). It has been well documented that the degree of unsaturation, location and number of double bond severely affect the rate of auto-oxidation (Kumar 2017). The multistep reaction of oxidative degradation is initiated with the generation of H atom from C which is adjacent to the double bond (Patel et al. 2017). Further, allylic hydroperoxides are formed with the reaction of oxygen after removal of H atom. This is followed by secondary oxidative products which are formed by isomerization and radical chain propagation reaction. Researchers have stated that oxidatively unstable biodiesel decreases the engine performance due to high viscosity, the formation of gums and deposition of sediments.

Table 2.2 presents the various biodiesel properties obtained in this study. Various physical properties of biodiesel like cetane number (CN), degree of unsaturation (DU), kinematic viscosity (kV), density (D), and oxidative stability (OS), which control the vehicular quality of biodiesel determined by empirical formulas (Francisco et al. 2010) as follows:

$$CN = 46.3+5458/SV-(0.255*IV) \quad (2.3)$$

$$DU\ (\%) = MUFA + (2*PUFA) \quad (2.4)$$

$$\ln(kV) = -12{:}503 + 2.496 * \ln(\Sigma M) - 0.178*\Sigma DB \quad (2.5)$$

$$Density = 0.8463 + 0.49/\Sigma M + 0{:}0118*\Sigma DB \quad (2.6)$$

$$OS = 117.9295 / (wt\%\ C18{:}2 + wt\%\ C18{:}3) + 2.5905 \quad (2.7)$$

Table 2.2 Biodiesel properties of *C. minutissima*

Physical properties of biodiesel	International standards		Present study
	ASTM-6751-02	EN14214	
Cetane number	47	51	53.35
Degree of unsaturation (%)	Not reported	Not reported	77.34
High heating value (MJ kg^{-1})	Not reported	Not reported	39
Kinematic viscosity ($mm^2\ s^{-1}$)	1.9–6.0	3.5–5.0	5.4
Density (g cm^{-3})	Not reported	0.86–0.90	0.92
Oxidative stability (h)	Not reported	≥ 6	9

where M is the molecular mass of each fatty acid component, DB is the number of double bonds, FC is the percentage of each fatty acid component, MUFA is the weight percentage of monounsaturated fatty acids, and PUFA is the weight percentage of polyunsaturated fatty acid. Degree of unsaturation (DU) is the sum of the molar masses of unsaturated fatty acids. DU is an important property that affects the OS of biodiesel (Francisco et al. 2010). The presence of polyunsaturated fatty acids ($PUFA_S$) in excess deteriorates the OS of the biodiesel as double and triple bonds carbon sites are prone toward free radical attacks. FAME profile of *C. minutissima* obtained shows 59.18% of saturated and monounsaturated fatty acids which is an indicator of good biodiesel quality. According to Gouveia and Oliveira (2009) and Pereira et al. (2013), linolenic acid (C18:3) and PUFAs with P4 double bonds are important for good quality biodiesel if they are present in the range of 12 and 1%, respectively, as per European standards EN14214. In the present study, the linolenic acid contributes 10.33% of the FAME, whereas, polyunsaturated FA with P4 double bond was completely absent. Cetane number (CN) is one of the important

fuel properties of biodiesel which is highly influenced by the fatty acid profile (Table 2). High cetane value is the indicator of better combustion, low nitrous oxide (NO_x) emission, less occurrence of knocking, and easier start-up of the engine (Knothe 2012; Predojević et al. 2012). Diesel fuel with large quantities of saturated and monounsaturated FAMEs have high value of CN.

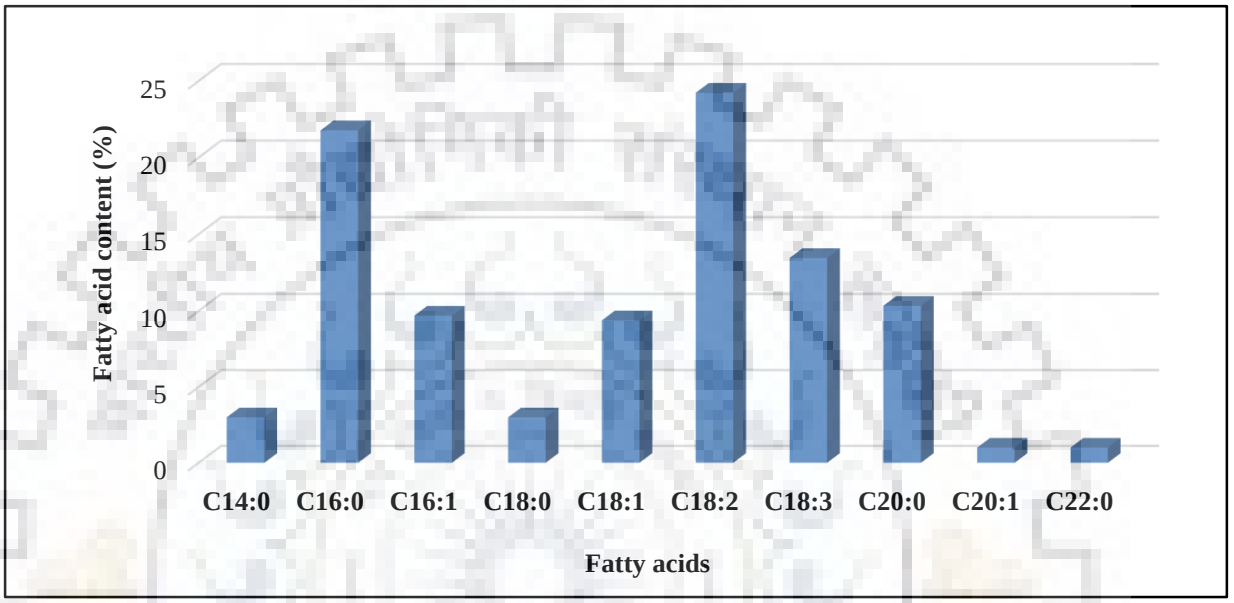

Figure 2.11 FAME profile of *C. minutissima*

2.3.3 Attenuated total Reflection-Fourier transform infrared spectroscopy and thermogravimetric analysis

The ATR-FTIR analysis showed biodiesel spectra of 4000–500 cm^{-1}, as shown in Fig. 2.12. In the spectrum biodiesel, the bands in the 2800–3000 cm^{-1} region are attributed to the symmetric CH_2 and asymmetric CH_3 and CH_2 stretching (Mahamuni and Adewuyi 2009). The characteristic bands for CO stretching of all samples are assigned in the 1800–1700 cm^{-1} region (Mahamuni and Adewuyi 2009). The major bands that allow structural distinction of FAMEs are allocated in the 1500– 1000 cm^{-1} region. The appearance of two major bands in the spectra of biodiesel at 1436 and 1198 cm^{-1} corresponding to CH_3 asymmetric bending and O–CH_3 stretching, respectively, are characteristic bands for FAMEs (Mahamuni and Adewuyi 2009). Figure 13 represents the TGA plots of biodiesel sample. The reduction in the mass of the biodiesel starts between 100 and 150 °C and continues to decrease

till the moment that all the biodiesel sample is vaporized.

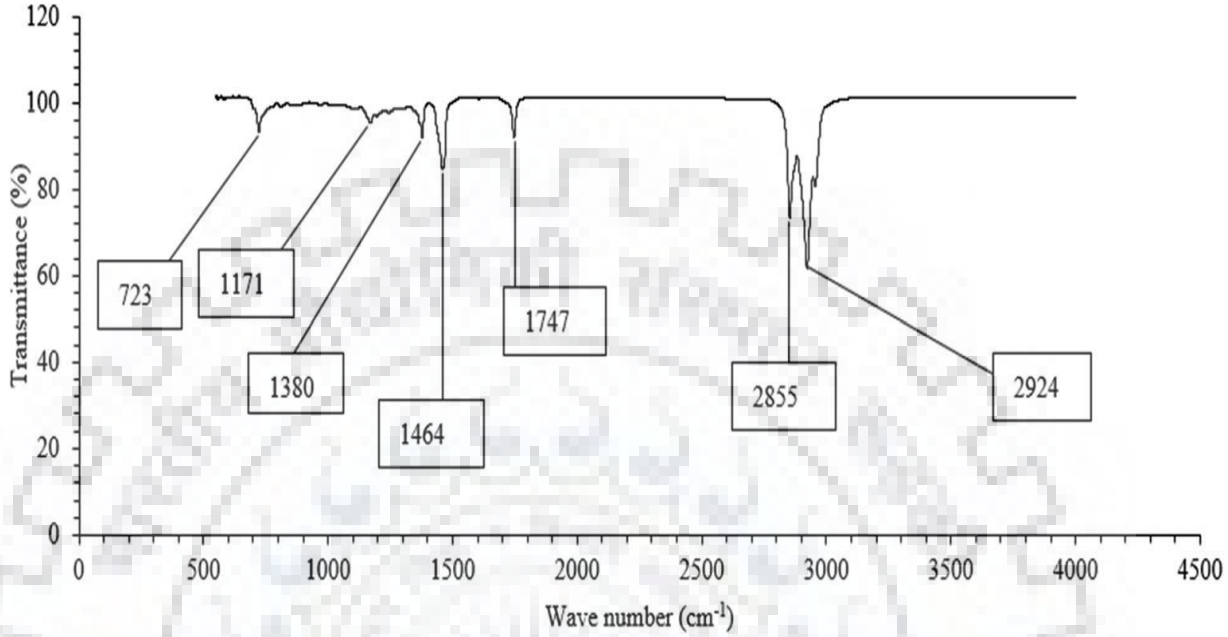

Figure 2.12 ATR-FTIR spectrum of biodiesel

It was assumed that the amount of biodiesel present in the sample is equal to the average weight percentage of over the range where weight starts to drop in between 100 and 150 °C. Beyond 200 °C, all the biodiesel is vaporized.

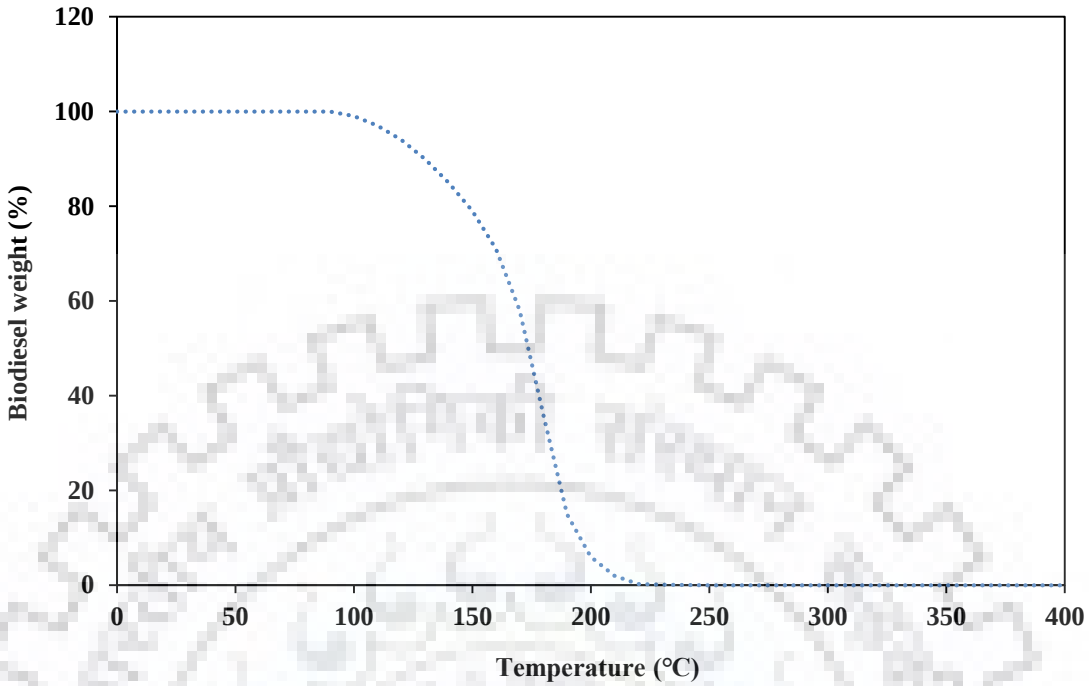

Figure 2.13 TGA plot of biodiesel

The loss in mass of biodiesel in the range of 100–150 °C correlates to the mass percentage of biodiesel present in the sample.

2.4 Conclusions

The results of the present work indicate that microalga *C. minutissima* is a good renewable bio-resource for biodiesel production. It showed a significant increase in biomass and lipid yields by varying different abiotic factors when cultivated with modified CHU-13 medium. The biodiesel quality of *C. minutissima* met the criteria of ASTM-6751-02 and European biodiesel standard EN14214.

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Chapter 3

Microalgal remediation of the mixture of distillery spent wash and secondary treated domestic sewage for biodiesel feedstock production using green microalga *Chlorella minutissima*

Results of this chapter have been submitted for publication

3.1 Introduction

Different chemical industries like fertilizers, refineries, pulp and paper, etc., are the major polluting sources of surface water all over the world (Pradhan et al, 2018). Effluents from these industries are rich in nitrate, phosphate and other nutrients which cause eutrophication of water bodies (Krishnamoorthy et al. 2019). Hence, the remediation of industrial effluents before discharge into water bodies is essential. Most of the conventional treatment methods are energy-intensive and expensive as well. Distillery effluents contain high amounts of phosphorus, organic matter with the offensive smell and high pH (Pant and Adholeya 2007; Bux and Chisti 2016; Tsolcha et al. 2017; Gupta et al. 2019). Thus, treatment of such effluents must be done before their disposal into the water streams. Distillery industry generates large volumes of wastewater during ethanol production. Distillery wastewaters have high chemical oxygen demand (COD) values in the order of 20000 mg/L, and they also contain sufficient amounts of nitrate, ammonia, and phosphate, etc. along with few organic compounds (Nayak et al. 2018). Distillery wastewaters are also rich in inorganic pollutants as they have a strong odour, brown colour and low pH. These properties of distillery effluents make them a potential threat to the environment unless properly treated (Kaushik and Thakur 2013). There are many (physical and chemical) remediation technologies including bioremediation (using micro-organisms) for distillery wastewater into practice (Grace Pavithra et al. 2019). These practices include microbial (anaerobic/aerobic) as well as physico-chemical techniques in practice as a feasible technology for remediation of DSW and protection from environmental pollution (Patel et al. 2017; Goswami et al. 2017; Pradhan et al. 2019). Distillery wastes are rich in nutrients and this could be used in the production of value-added substances during the treatment process. In most of the cases spent wash in anaerobic digesters is used to produce methane gas to reduce the organic load of it. Currently, researchers are focusing on alternative methods to treat industrial effluents. These methods are usually involving microorganisms (bacteria, fungi and

microalgae, etc.) which feed upon the organic matter/chemical compounds for their growth present in distillery wastewater. Microalgal wastewater remediation is a green approach as well as microalgal biomass is a good bioresource for production of bioenergy and other value-added substances (Gupta et al. 2017). Microalgae are capable enough to adopt different nutritive modes (mixotrophic or heterotrophic) during less availability of light (Liu et al. 2018). Microalgae can assimilate organic load/pollutants and can store them as carbohydrates, proteins, lipids while inorganic pollutants as nutrients for its growth and multiplication (Kharayat 2012). Currently, microalgae have emerged as one of the potential bio-resource for remediation of wastewater and industrial effluents in the last few years (Anbarasan et al. 2018). Being photosynthetic in nature microalgae can synthesize carbohydrates for their nutrition as well as they can utilize nitrogen and phosphorus from wastewater when grown on it. Additionally, they are fast-growing and can synthesize lipid intracellularly. Thus, microalgae play a dual role during their use in wastewater remediation as lipid can be transesterified to biodiesel (Amit et al. 2017; Chandra et al. 2019). Microalgal treatment of wastewater is purely natural and low-cost system and it has no harm to the environment (Naveed et al. 2018). This chapter is aimed at the treatment of distillery wastewater diluted with secondary treated domestic sewage (STDS) as well as biodiesel production using green microalga *Chlorella minutissima* (*C. minutissima*).

3.2 Materials and methods

3.2.1 Microalgae culture

Culture of *C. minutissima* was purchased from Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India. It was maintained in BG-11 medium (medium composition is the same as described in chapter 2) in 250 ml flasks with a working volume of 150 mL with continuous illumination of 2500 lux at 25 ± 1°C. All the reagents used were of analytical grade. Experiments were performed in triplicate.

3.2.2 Sampling of distillery spent wash and analysis

Distillery spent wash (DSW) was sampled from the Pilkhani Distillery and Chemical Works, Private Limited, Pilkhani, Saharanpur, Uttar Pradesh, India. The secondary treated domestic sewage (STDS) was sampled from the municipal sewage treatment plant, Saharanpur, Uttar Pradesh, India. Both the samples were stored at 4 °C in the laboratory. The physicochemical properties like biochemical oxygen demand (BOD), COD, total phosphorus (TP), total nitrogen (TN), total

dissolved solids (TDS), total suspended solids (TSS) and total solids (TS) of the distillery wastewater were measured as per standard methods described by APHA (2005) (Table 3.1). pH was measured using a digital multimeter (HI 3512, Hanna Instruments). Samples were prepared by diluting DSW and STDS in the ratios of 100:0, 75:25, 50:50 and 25:75 (v/v) and were denoted as DSW100 + STDS0, DSW75 + STDS25, DSW50 + STDS50, DSW25 + STDS75, respectively.

Table 3.1 Characteristics of DSW, STDS and mixtures of DSW + STDS

Parameters (mg/L) *	Distillery spent wash (DSW)	STDS	DSW75 + STDS25	DSW50 + STDS50	DSW25 + STDS75
pH	3.8 ± 1.5	7 ± 0.5	6.5 ± 1	7.3 ± 1	7.3 ± 1
COD	(70000 – 80000) ± 35	355 ± 7	58215 ± 12	36750 ± 10	18950 ± 7
BOD	26487 ± 8	23 ± 1	23547 ± 5	18679 ± 3	12356 ± 3
TDS	38500 ± 12	600 ± 5	28000 ± 10	19800 ± 8	9800 ± 6
TSS	7300 ± 8	430 ± 5	5250 ± 7	3700 ± 5	1975 ± 3
TS	45000 ± 5	945 ± 8	32350 ± 15	23500 ± 10	11780 ± 7
Total nitrogen	1235 ± 5	53 ± 0.8	1045 ± 1.5	947 ± 1.3	785 ± 1.3
Total Phosphorous	13795 ± 3	6 ± 0.5	10869 ± 1.5	6745 ± 1	3565 ± 1

*pH is unitless

3.3 Experimental

3.3.1 Acclimation of *C. minutissima* in DSW

To acclimatize *C. minutissima* in DSW it was cultivated on different (3, 5, 7, 10, 15, 20 and 25%) concentrations of DSW diluted using STDS. Aliquots of 5 ml from the maintained culture of *C. minutissima* were transferred into conical flasks (250 mL) having 100 mL of 3% DSW diluted with STDS under a continuous light intensity of 2500 lux, at 25 ± 1℃ for 8 days in an orbital incubator shaker at 130 rpm. After 8 days samples were collected and centrifuged at 3000 rpm for 2 min. The supernatant was discarded and cell pellets were washed twice with double-distilled water. Obtained cell pellets were again cultivated on 5% DSW and the same procedure was repeated as described above before further cultivation of microalgae cell pellets on 7, 10, 15, 20 and 25% concentrations of DSW. Thus, *C. minutissima* biomass obtained from 25% DSW was used for the treatment of DSW diluted using STDS.

3.3.2 Remediation of DSW by acclimated *C. minutissima*

Cell pellets obtained from 25% DSW were used for remediation of four (25, 50, 75 and 100%) concentrations of DSW prepared by diluting it with STDS. Conical flasks of 250 mL containing 150 mL of (DSW100 + STDS0 or DSW75 + STDS25 or DSW50 + STDS50 or DSW25 + STDS75) were inoculated with acclimated microalgae cells. Flasks were kept under the illumination of 10000 lux using white cool fluorescent at 25 ± 1 ℃ at 12:12 h light and dark period. Small aquarium pumps were used to supply ambient air in flasks. All the experiments were performed in triplicate.

3.3.3 Microalgae biomass growth

Samples of 5 mL from each flask were taken out at an interval of 2 days. Glass microfiber filter paper (GFC, Whatman) was used to filter samples and were oven-dried at 105 ℃ for 24 h and cooled to room temperature in desiccators before measurement of dry weight. Biomass productivity (B_P) was calculated using the following equation (Gani 2016).

$$B_P = (FDB_2\text{-}FDB_1) / (T_2\text{-}T_1) \quad (3.1)$$

where, FDB_2 = weight of final dry biomass, FDB_1 = weight of initial dry biomass, T_1= initial sampling time and T_2 = final sampling time.

3.3.4 Total lipid extraction and estimation

Lipid extraction and estimation from harvested microalgal biomass was done using previously reported method in Chapter 2 (Rajesh and Ghosh, 2018) following the method described by Folch et al. (1957).

3.3.5 Transesterification and GC-MS analysis

Transesterification of lipid was performed according to the method of Mandotra et al. 2014 as reported in Chapter 2. GC-MS analysis was performed according to the method of Härtig, 2008 as described in Chapter 2.

3.3.6 Biodiesel properties

Biodiesel physical properties like cetane number (CN), degree of unsaturation (DU), kinematic viscosity (kV), density (D), and oxidative stability (OS), were estimated as per protocol reported by (Patel et al. 2017).

3.3.7 Statistical analysis

Average of three replications and their standard deviation for biomass yield and lipid content was calculated. Analysis of variance (ANOVA) at 95% confidence level (p-value < 0.05) was performed. Lipid yield was determined as the average value of lipid content multiplied by the average biomass yield so, it is reported as a single value without standard deviation.

3.4 Results and discussion

3.4.1 Microalgae growth pattern

Effluents from distilleries contain (nitrogen, phosphorus and organic carbon, etc.) in sufficient amount. Usually, these nutrients are mandatory for biomass growth of microalgae when cultivated on different types of growth media. Wastewaters from distilleries have low pH, and high concentrations of nitrogen, phosphorus and other nutrients. Thus, cultivation of microalgae is not feasible in raw DSW. In the present study remediation of four (DSW100 + STDS0, DSW75 + STDS25, DSW50 + STDS50, DSW25 + STDS75) concentrations of DSW have been performed using microalga *C. minutissima*. It is obvious from (Fig. 3.1) that sample DSW 25 + STDS 75 is most suitable for *C. minutissima* biomass growth. Biomass growth of *C. minutissima* on mixture DSW25 + STDS75 started from the beginning of the cultivation. However, a small lag phase of 4 days was observed before it entered into log phase from 4^{th} day of cultivation and lasted upto 20^{th} day. After 20^{th} day of cultivation, microalgae entered into stationary phase. In the case of *C. minutissima* cultivated on DSW50 + STDS50, the lag phase of 8 days was observed after which microalgae entered into log phase which lasted upto 20^{th} day. No significant biomass growth was observed for *C. minutissima* cultivated on DSW75 + STDS25 and DSW100 + STDS0 within the experimental period of 22 days. In the present work, *C. minutissima* obtained biomass yield of 0.77 and 0.38 g/L, respectively, when cultivated on DSW25 + STDS75 and DSW50 + STDS50 cultivation medium at a photoperiod of 12:12 within 20 days of cultivation (Fig. 3.1).

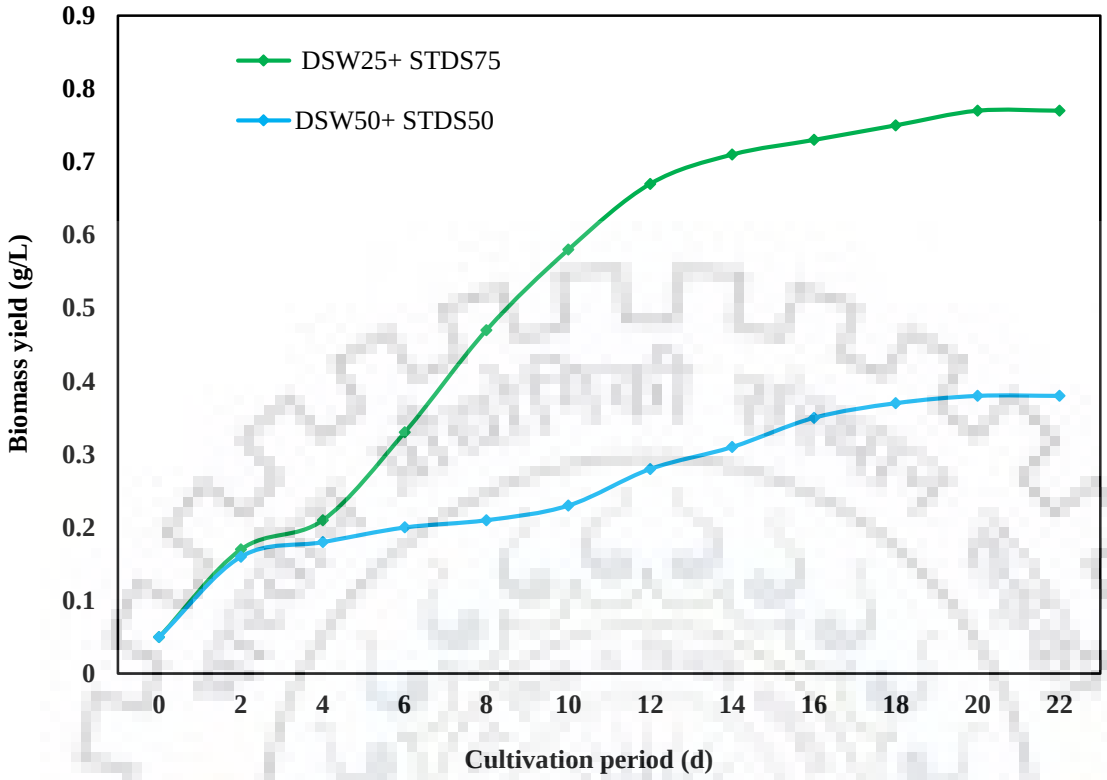

Figure 3.1 Biomass growth curve of *C. minutissima* cultivated on (DSW50 + STDS50 and DSW25 + STDS75)

Retarded biomass growth was observed for *C. minutissima* cultivated on DSW75 + STDS25 and no growth was seen for *C. minutissima* grown on DSW100 + STDS00. Chiranjeevi and Venkata Mohan. (2017) reported biomass productivity of 5.3 g/L-d for microalgae grown on anaerobically digested distillery effluent in mixotrophic conditions. It has been reported that microalga *Chlorella vulgaris* attained biomass yield of 2.6 g/L when cultivated on food waste hydrolysate Zeng et al. (2018). Treatment of diluted distillery effluent was successfully achieved by *Chlorella vulgaris* (Raposo et al. 2010). Krishnamoorthy et al. (2019) have reported biomass productivity (0.09-0.094 g/L-d) for *Spirulina* sp. cultivated on anaerobically digested distillery wastewater. Microalga *Chlorella sorokiniana* CY-1 has shown biomass yield of 2.12 g/L with 11.21% of lipid content when cultivated on acid-heat pretreated 30% (v/v) palm oil mill effluent (Kamyab et al. 2017). Under optimized conditions, *Chlorella vulgaris* attained 1.39 g/L dry cell weight and biomass productivity of 0.13 g/L-d cultivated on growth medium containing 5 g/L glucose (Gupta et al. 2016). It is well noted that the microalgae are the most dominating candidates among other microflora present in the polluted aquatic environment. Thus, it can be concluded that the microalgae have evolved natural

selective resistance for environmental pollutants present in polluted systems (Al-Hasan et al. 2001). Data for lipid productivity, biomass productivity, and lipid content for various microalgae strains are recorded in Table 3.2.

Table 3.2 Lipid productivity, biomass productivity and lipid content of different microalgal species

Microalgae	Cultivation medium	Biomass productivity (g/L-d)	Lipid productivity (mg/L-d)	Lipid content (%)	References
Scenedesmus sp.	Artificial wastewater	126.54	12.8	16.2	(Voltolina et al. 1999)
Nannochloropsis sp.	Outdoor green wall panels	0.17–0.51	20.0–56.0	60.9–76.5	(Bondioli et al. 2012)
Botryococcus braunii	Secondary effluent	345.6	62	17.85	(Órpez et al. 2009)
Scenedesmus sp. LX1	Secondary effluent	9.2	8	31–33	(Xin et al. 2010)
Mixture of *Chlorella* sp., *Micractinium* sp., *Actinastrum* sp.	Dairy waste water	59	29	17	(Woertz I. et al. 2009)
Nannochloropsis oculate	Pulp and paper industry wastewater	0.37–0.48	22.7–29.7	84–142	(Polishchuk et al. 2015)
Chlorella vulgaris	Artificial wastewater	--	147	42	(Feng et al. 2011)
Chlorella minutissima	Mix of distillery wastewater + secondary treated domestic sewage	0.254	48.82	18.67	Current research

As distillery effluents are highly rich in organic matter, inorganic nutrients, heavy metals and other toxic compounds like meladonin might have retarded the growth of *C. minutissima* in higher concentrations of DSW. The high growth with DSW25+STDS75 was due to the optimum concentration of nutrients available in medium.

3.4.2 Effect of photoperiod on biomass growth and lipid yield of *C. minutissima*

To monitor the effects of light exposure on biomass growth and lipid yield of *C. minutissima*, photoperiods of 16:8, 18:6, 20:4 and 24:0 h (light: dark) were evaluated. Light exposure is one of the major factors which directly affects the physiology of microalgae. In the present study *C. minutissima* achieved highest biomass yield of 2.95 ± 0.052 g/L and biomass productivity of

140 mg/L-d cultivated on DSW25 + SWW75 at 24:0 h photoperiod, while biomass yield of 0.98 ± 0.021 g/L and biomass productivity of 41.5 mg/L-d were achieved at 16:8 h photoperiod. Various authors have reported that availability of light is responsible for the synthesis of lipids, pigments, carbohydrates, and proteins in the microalgae cells (Meseck et al. 2005; Jacob-Lopes et al. 2009; Seyfabadi et al. 2011; Krzemińska et al. 2014). In this study also increase in light exposure increased biomass growth of *C. minutissima*. Data on the effects of different photoperiods on the biomass and lipid yield of *C. minutissima* are presented in Table 3.3.

Table 3.3 Effect of photoperiod on biomass and lipid yield of *C. minutissima*

Photoperiod (light:dark, h)	**Biomass yield (g/L)**	**Biomass Productivity (mg/L-d)***	**Lipid yield (mg/L)**	**Lipid Productivity (mg/L-d)***	**Lipid content (%)***
16:8	0.98 ± 0.021	41.5	66.49 ± 0.070	3.32	6.78
18:6	1.25 ± 0.052	55	116.62 ± 0.086	5.83	9.33
20:4	1.89 + 0.040	87	191.28 ± 0.062	9.56	10.12
24:0	2.95 ± 0.052	140	295.96 ± 0.043	14.78	10.00

***** *Calculation of biomass productivity was done on the basis of the average value of biomass yield similarly lipid productivity and lipid content were calculated on the basis of the average value of lipid yield. So, values of biomass productivity, lipid productivity and lipid content are reported as a single value without standard deviation.*

It has been reported that the light exposure of 12 h resulted in accumulation of 0.80 g/L of dry biomass in *Scenedesmus obliquus* (Mata et al. 2012). It is interesting that biomass growth of *C. minutissima* was slow during light exposure of 16 and 18 h. However, biomass yield of 1.89 ± 0.040 and 2.95 ± 0.052 g/L was achieved at 20 and 24 h light exposure. It shows that 24:00 h is the most suitable photoperiod in the present study. Amini Khoeyi and his co-workers (2012) reported biomass yield of 2.05 ± 0.1 g/L at 16:8 h photoperiod when *Chlorella vulgaris* was cultivated under axenic environment. Microalgae *Neochloris conjuncta, Neochloris terrestris, Neochloris texensis, Botryococcus braunii and Scenedesmus obliquus* showed more biomass productivities of 0.155, 0.150, 0.098, 0.089 and 0.037 g/L-day, respectively, at 24:0 h photoperiod in comparison to 0.034, 0.050, 0.0125, 0.117 and 0.114 g/L-day biomass productivities, respectively, at 12:12 h photoperiod (Krzemińska et al. 2014).

3.4.3 Effect of glucose supply on biomass growth and lipid yield of *C. minutissima*

Various authors have reported that the biomass growth and lipid content of algae is can be changed by varying cultivation conditions like temperature, aeration, and nutrients concentration, etc. (Prathima Devi et al. 2012; Kong et al. 2012; Mujtaba et al. 2015) Thus, heterotrophic microalgae cultivation has been beneficial in wastewater treatment, nutrients removal and environmental improvement. Nutrients and other organic compounds present in the wastewater can serve as a nutrient medium for the growth of algae (Rier and Stevenson 2002; Cerón García et al. 2005; Pahl et al. 2010). In present work different dosages (5,10,15 and 20 g/L) of glucose as organic carbon source was mixed separately in cultivation medium at 24:0 h photoperiod. Values of biomass yield, biomass productivity, lipid yield, lipid productivity and lipid content have been summarized for different dosages of glucose supply in cultivation medium (Table 3.4).

Table 3.4 Effect of glucose on biomass and lipid yield of *C. minutissima*

Glucose (g/L) at 24:00 h	Biomass yield (g/L)	Biomass Productivity (mg/L-d)	Lipid yield (mg/L)	Lipid Productivity (mg/L-d)	Lipid content (%)
5	3.12 ± 0.088	148.5	445.48 ± 0.061	22.27	14.27
10	4.02 ± 0.062	193.5	610.80 ± 0.056	30.54	15.19
15	4.55 ± 0.048	220	761.44 ± 0.049	38.07	16.73
20	5.23 ± 0.039	254	976.46 ± 0.059	48.82	18.67

Cultivation of *Scenedesmus acuminatus* on bio-sludge of paper industry resulted in biomass yield of 10.2 ± 2.2 g/L (with digested bio-sludge) and 10.8 ± 1.2 g/L (without digested bio-sludge), respectively (Tao et al. 2017). Highest biomass yield of 5.23 ± 0.039 g/L with 254 mg/L-d of biomass productivity was observed when 20 g/L of glucose was supplied to the cultivation medium. It is noteworthy that the biomass growth increased with increasing concentration of glucose concentration and no feedstock inhibition was reported till 20 g/L of glucose. Increased biomass productivity of 1.35 g/L-d was reported by Cerón García et al. (2005) when microalgae *Phaeodactylum tricornutum* UTEX-640 was cultivated on the seawater with the addition of glycerol. Heredia-Arroyo et al. (2011) observed an increase in cell density of *chlorella vulgaris* grown mixotrophically on modified optimized culture medium. This can be attributed to the fact that higher carbon concentration stimulates microalgae growth due to rapid consumption of glucose with the

help of ATP synthesized during photochemical reactions (Yamane et al. 2011). Increased biomass yield of 3.7 g/L (7.7 times more than in autotrophic conditions) was observed for *Chlorella saccharophila* when grown on cultivation medium containing (20 g/L glucose + 1 g/L bacteriological peptone) in a 3 L stirred tank bioreactor (Isleten-Hosoglu et al. 2012).

3.4.4 Remediation of DSW and nutrients utilization

The physico-chemical parameters of DSW25 + SWW75 only were estimated after the treatment as it was found the most suitable medium for biomass growth of *C. minutissima*. Removal of pollution load as BOD, COD, TDS, TSS, TS, TN and TP were calculated at an interval of three days. Figure 2 represents the removal of BOD, COD, TDS, TSS, TS, TN and TP from DSW25 + SWW75 within a period of 22 days. Wastewater is being utilized as a cultivation medium for microalgae and on the other side, microalgae remove various nutrients from wastewater for their growth and thus they help in conservation of fresh water as well as reduction of BOD, COD, TDS, TN and TP from wastewater. Microalgae can adopt different nutrition modes as per exiting cultivation environment. Thus, microalgae can assimilate inorganic and organic substrates present in wastewater as nutrients during their cultivation either by autotrophic or by mixotrophic nutrition mode (Li et al. 2011). Use of wastewater for microalgae farming as growth medium has been beneficial in the remediation of wastewater as microalgae are capable in the removal of organic load (COD, BOD, nitrogen, phosphorus and heavy metals) and other nutrients (Órpez et al. 2009; Gupta et al. 2019). However, the remediation potential of microalgae varies species to species and as per conditions available in the cultivation environment. It was observed that the *C. minutissima* removed 77.23% and 66.15% of BOD and COD, respectively from DSW75+STDS25. Reduction of 69.83% in TDS and 41.30% in TSS, respectively were seen Fig 3.2. Major nutrients for microalgae growth are nitrogen and phosphorus (Li et al. 2014; Baglieri et al. 2016). The requirement of nitrogen and phosphorus is essential during the synthesis of nucleic acids (RNA and DNA). Except this nitrogen and phosphorus are an integral part of proteins, carbohydrates, synthesis as well as of various intermediates. The removal of TN and TP by *C. minutissima* in this study is presented in Fig 3.3. About 79% TN and 44.74% TP removal was achieved by *C. minutissima* were achieved on the 20th day of cultivation. Cultivation of *Chlorella pyrenoidosa* removed 77.8% COD, 88.8% nitrogen and 70.3% phosphorus from soybean processing wastewater (Hongyang et al. 2011). Usually, variations in nutrients removal and growth of microorganisms depend on the compositions and concentrations of effluents used for cultivation.

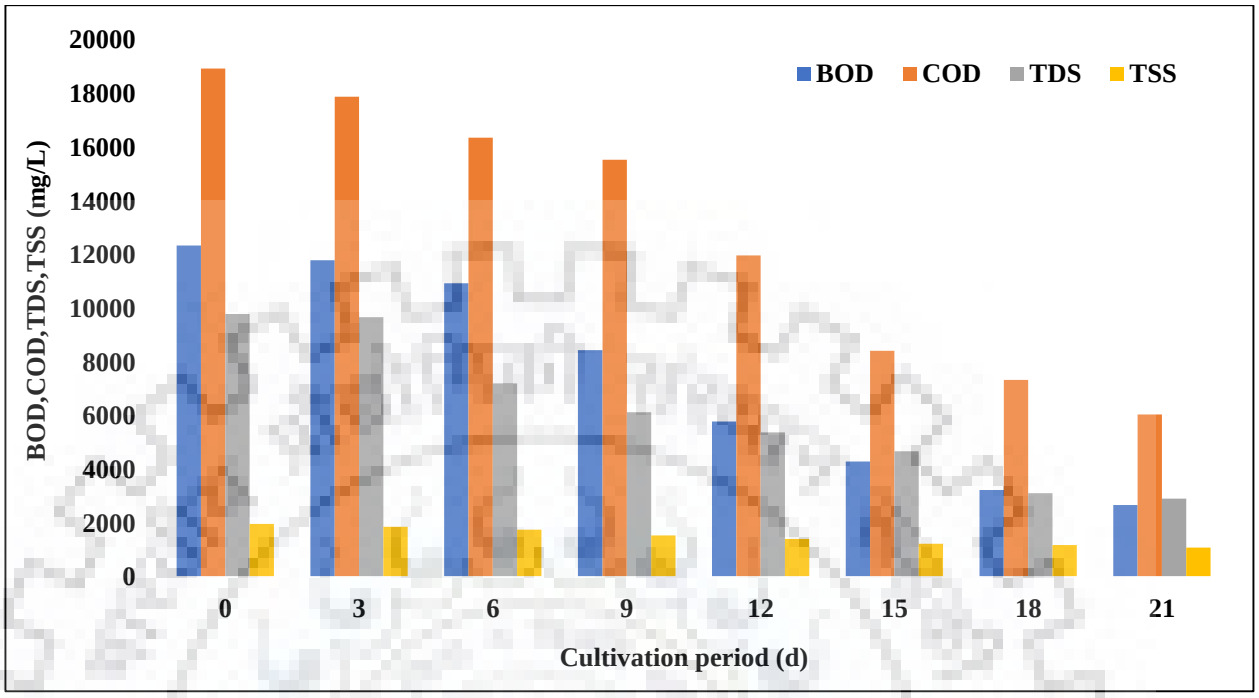

Figure 3.2 Removal of BOD, COD, TDS and TSS by *C. minutissima* from DSW25+STDS75

It has been reported by (Ling et al. 2014) that the combination of *Rhodosporidium toruloides* and *Chlorella pyrenoidosa* removed soluble COD (95.34 ± 0.07%), TN (51.18 ± 2.17%) and TP (89.29 ± 4.91%) after 5 days of cultivation on a mixture (1:1 v/v) of distillery effluent and domestic sewage. Travieso et al. (2006) reported COD and BOD removal up to 54% and 74%, respectively, from anaerobically pre-treated distillery effluent using laboratory scale stabilization pond system with a hydraulic retention time of 30 days. Wang et al. (2010) have shown phosphorus and COD removal in the range of 80-90% and 50-80%, respectively, by cultivating *Chlorella* sp. on different wastewaters. About 7–20% of the total dry cell weight of microalgae is constituted by nitrogen. It is an extremely important inorganic element required for various metabolic activities of microalgae cells. Nitrogen is an essential element for the synthesis of nucleic acids and energy compounds such as adenosine triphosphate in living organisms.

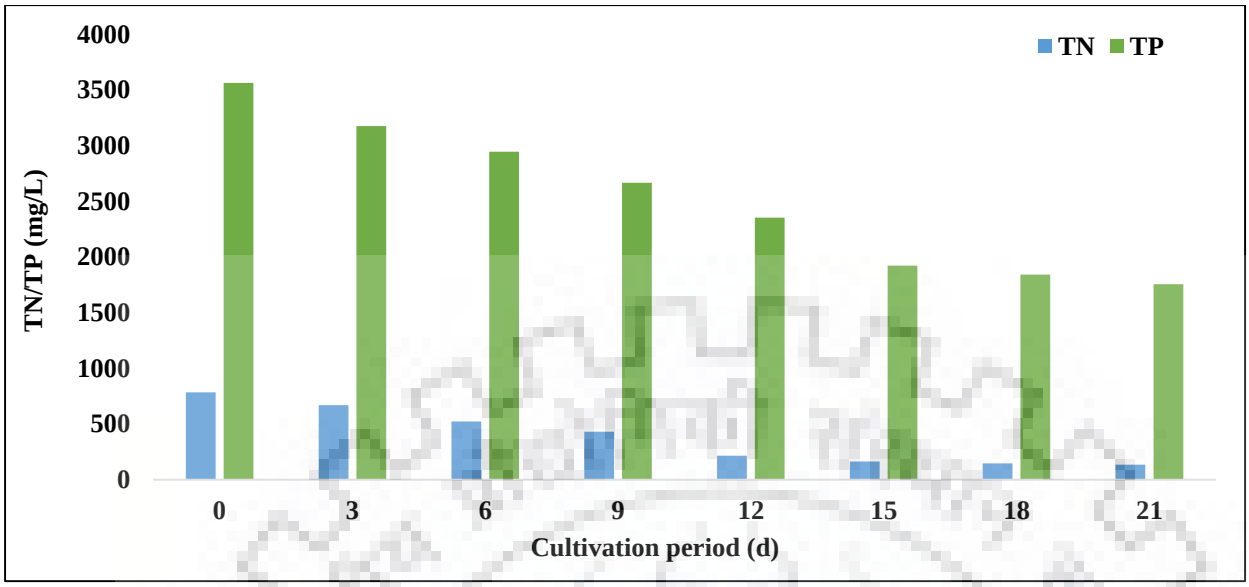

Figure 3.3 Removal of TN and TP by *C. minutissima* from DSW25+STDS75

Microalgae can utilize both organic and inorganic forms of nitrogen but nitrogen in the form of ammonium is most readily consumed by microalgae as ammonium nitrogen does not require reductase enzymes for its assimilation. Presence of high concentrations of phosphorus can result in higher microalgal biomass production (Chowdhary et al. 2018). It is necessary for cell multiplication and it is also an integral part of nucleic acids (Ling et al. 2016). Usually, the amount of phosphorus is higher than nitrogen in the distillery effluent. This may be due to the use of chemicals having phosphorus in the production unit. It is reported in the open literature that the phosphorous removal rates have been lower than those observed for nitrogen removal in most of the studies. This can also be attributed to higher phosphorus content as compared to nitrogen in microalgae.

3.4.5 Fatty acid profile and biodiesel characteristics

The biodiesel properties were estimated as per empirical equations described by Francisco et al. (2010) as given in chapter 2 of this thesis. The oleaginous microalga *C. minutissima* is capable to produce triacylglycerol (TAG). These TAG's may contain carbon chain from C_{16} to C_{18} when grown under autotrophic, mixotrophic and heterotrophic conditions. TAG's produced by *C. minutissima* cultivated on DSW25+STDS75 and on DSW25+STDS75 +glucose were transmethylated into FAME's. The corresponding fatty acid profiles are depicted in Fig 4. Seven types of fatty acids including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were detected. The fatty acid profile of *C. minutissima* cultivated on

DSW25+STDS75 shown the presence of mainly C14:0 (2.46%), C16:0 (13.3%), C16:1 (3.35%), C18:0 (28.43%), C18:1 (30.98%), C18:2 (6.33%), and C18:3 (11.45%), and included other (3.25%) while supplementation of glucose to DSW25 + STDS75 showed the presence of mainly C14:0 (1.38%), C16:0 (15.61%), C16:1 (1.98%), C18:0 (3.7%), C18:1 (18.63%), C18:2 (29.54%), and C18:3 (25.25%), and included other (3.31%) (Fig 3.4). It can be observed that saturated fatty acid content is lower in biodiesel when glucose was added to the cultivation medium.

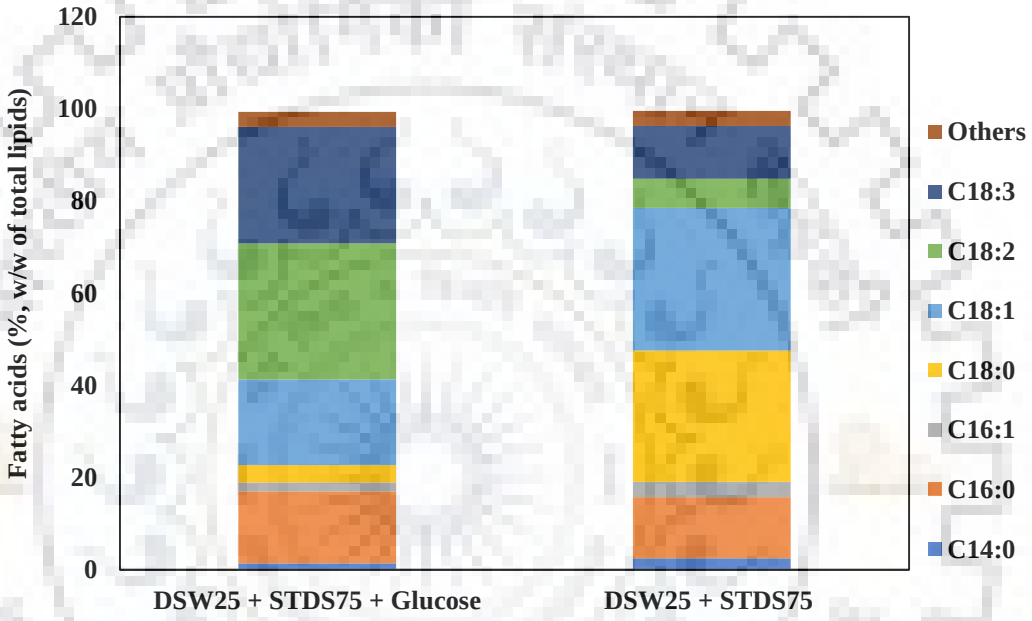

Figure 3.4 Percentage of fatty acids present in biodiesel obtained from *C. minutissima*

Characteristics of biodiesel as fuel are much influenced by its constituent fatty acid methyl esters. It has been reported that the cetane number, the heat of combustion and viscosity, etc. increases with increase in the length of the carbon chain and decrease with unsaturation (Patel et al. 2017). Unsaturated fatty acids make biodiesel more susceptible to autooxidation. Lower-grade biodiesel quality can be improved by blending biodiesel having higher saturated fatty acids content. The FAME profile showed that the majority of the fatty acids were MUFA (monounsaturated fatty acids). Excess presence of PUFA (specifically $C_{18:2}$) plays an important role for cell integrity during lag phase, while SFA (specifically $C_{18:0}$) and MUFA (specifically $C_{18:1}$). These fatty acids are mainly synthesized during the early stationary phase of growth. In the later phase of growth $C_{18:0}$ is changed to $C_{18:1}$ by addition of a double bond (Hassan et al. 1993). It results in poor oxidative stability and thus the requirement of high quantities of oxygen, NADH, NADPH, and a substrate that prevents

formation and accumulation of reactive oxygen species under stress conditions such as nitrogen limitation (Meesters et al. 1996). The chain length of the fatty acids, including the number and position of the double bonds, are responsible for the physiochemical properties of biodiesel. International standards such as ASTM 6751-3 (USA) and EN 14214 (Europe) have described ideal values for different properties of biodiesel. Long chain saturation factor (LCSF) is one of the most important properties of biodiesel of microbial origin. This includes fatty acids where all the carbons of the fatty acid chains are totally saturated with H atoms and this is directly correlated to the cetane number (CN), cold filter plugging point (CFPP), and the viscosity of biodiesel. High the value of LCSF greater will be the value of CN value. Biodiesel with high CN generates reduced nitrogen oxides emissions (He 2016).

Table 3.5 Biodiesel properties

Physical properties	**International standards**		**Present study**	
	ASTM D6751	**EN 14214**	***C. minutissima* + glucose**	***C. minutissima***
Degree of unsaturation (% wt.)	76.81	87.04	130.190	69.890
Saponification value (mg KOH)	NA	NA	195.822	195.506
Iodine value (g I_2 100 g^{-1})	NA	120	141.341	74.005
Cetane number	47	51	42.370	57.566
Long chain saturated factor (%)	5.97	5.75	3.411	15.545
Cold filter plugging point (℃)	≤ 5/ ≤ -20	3.93	-5.761	32.361
Oxidation stability (h)	≥ 6	9.21	4.743	9.223
Higher heating value (MJ Kg^{-1})	NA	NA	37.784	38.047
Kinematic viscosity (mm^2 s^{-1})	1.9-6.0	3.5-5.0	3.257	3.765
Density (g cm^{-3})	0.86-0.9	0.87	0.849	0.842

Sum of the molar mass of unsaturated fatty acids present in biodiesel is called as degree of unsaturation (DU). It is correlated to the oxidative stability (OS) of biodiesel (Francisco et al., 2010). Excess presence of PUFAS deteriorates the OS of the biodiesel as double and triple bond carbon sites are prone to free radical attacks. Contrary to PUFAS, presence of saturated and monounsaturated fatty acids is good for biodiesel as they improve OS and cold flow properties of biodiesel (Arias-Peñarands et al. 2013; Chen et al. 2012; Demirbas, 2009; Whalen et al. 2013).

FAME profile of *C. minutissima* shows the presence of 59.29% saturated and monounsaturated fatty acids which is an indicator of good biodiesel quality. As per European standards EN14214 linolenic acid (C18:3) content in the range of 12% and PUFAs with P4 double bonds are important for good quality biodiesel if they are present in the range of 12% and 1%, respectively (Gouveia and Oliveira 2009; Pereira et al. 2013). In the present study, the linolenic acid contributes 10.33 % of the FAME, whereas, polyunsaturated FA with P4 double bonds are completely absent. Cetane number (CN) is one of the important properties of biodiesel and is highly influenced by the fatty acid methyl ester profile. combustion, low nitrous oxide (NOx) emission, less occurrence of knocking and easier start-up of the engine (Arias-Peñarands et al. 2013; Knothe, 2012; Predojevic´ et al. 2012). Diesel fuel with large quantities of saturated and monounsaturated FAMEs have a high value of CN.

3.5 Conclusions

It is evident from this study that effluents like DSW having high organic loading and high pH and other toxic compounds can be treated after dilution with secondary treated sewage using microalgae. Biomass can be enhanced by optimizing light exposure and supply of external carbon sources like glucose in the cultivation medium. Biodiesel quality was also as per international standards for biodiesel. Thus, it may be concluded that microalgal remediation of effluents with high organic content is possible after its proper dilution with treated wastewater.

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Chapter 4

Bio-remediation of secondary treated wastewater contaminated with phenols and biodiesel production using microalgae *Chlorella minutissima* and *Scenedesmus abundans*

Results of this chapter have been submitted for publication

4.1 Introduction

Microalgae are one of the major components of aquatic ecosystems as they are primary producers and due to their ability of photosynthesis they can assimilate CO_2 (Gupta et al. 2016). In comparison to terrestrial plants, they are able to fix more CO_2 per unit area of solar radiation received (Xiong et al. 2018). A variety of various value-added products like biofuels, nutraceuticals, pharmaceuticals, etc. can be obtained from microalgae cultivation (Vashi et al. 2018). Thus, in recent years researchers have explored the possibility of conversion of microalgae biomass to obtain biofuels as well as other value-added products (Vulsteke et al. 2017). In this study, we focused on the development of new strategies for biomass production by investigating the effects of phenols as toxic chemical stress on biomass conversion. Phenols are oxidizing organic compounds and are produced during various industrial processes, thus become a part of effluent streams (Al-Dahhan et al. 2018). It has been found that phenols occur naturally in water in the range of about 0.01 to 2.0 μg/L (Wang et al. 2007). Usually, phenols are carcinogenic as well as persistent in nature (Goswami et al. 2019). They have a tendency of bioaccumulation in the environment thus their presence affects the food chain and food web at various trophic levels. Individuals at high trophic levels are more prone towards bioaccumulation (Xiong et al. 2018). Phenol has high solubility and chemical stability in water and is considered one of the most important organic pollutants in the aquatic environment (Bajaj et al. 2008; Raj et al. 2014) and has been categorized in the list of priority pollutants by the US Environmental Protection Agency (Solé and Matamoros 2016). They are able to produce phenoxy radicals commonly known as reactive oxygen species (ROS). ROS are strong oxidizing agents which trigger oxidative damage to aquatic organisms such as of peroxidation of lipids in cell membranes (Bajaj et al. 2008; Xiong et al. 2017a, b). However, aerobic organisms have the ability to release antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase catalase, and glutathione-S-transferase to deal with oxidative stress (Cho et al. 2015). Many studies carried out in the past showed that some microalgae strains like *Chlamydomonas debaryana*, *Chlorella luteoviridis*, *Desmodesmus intermedius*, *Hindakia tetrachotoma*, and *Parachlorella kessleri* are able to adopt themselves in hostile aquatic environment and showed enhanced biomass production and increased antioxidant

enzyme activities (Surkatti and Al-Zuhair 2018). Phenol is a possible substrate for *Chlorella* sp. but high concentrations of phenol is also toxic to microalgae (Wang et al. 2016). From this information it can be concluded that microalgae may respond to dissolved chemical-exposures through enhanced antioxidant defenses and that these responses may persist beyond the period of chemical exposure. In this chapter effects of different phenols on biomass growth, lipid yield and remediation potential of *Chlorella minutissima* (*C. minutissima*) and *Scenedesmus. abundans* (*S. abundans*) in aqueous environment in presence or absence of glucose or glycerol has been evaluated.

4.2 Materials and methods

4.2.1 Microalgae strains

Culture of *C. minutissima* was procured from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India while *S. abundans* was procured from National Chemical Laboratory, Pune, Maharashtra, India. All the cultures were maintained in BG-11 medium (composition same as described in chapter 2) under continuous illumination of 2500 lux in 250 mL conical flasks at 25 ± 1℃. All the reagents were procured from Hi Media Laboratories, Mumbai, India. All the experiments were performed in triplicate.

4.2.2 Wastewater sampling and analysis

Secondary treated wastewater (STWW) was collected from sewage treatment plant situated in Saharanpur, Uttar Pradesh India, and analyzed in the laboratory. The values estimated for biological oxygen demand (BOD), chemical oxygen demand (COD), nitrate-nitrogen (NO_3^--N), phosphate phosphorus (PO_4^{3-}-P) and total organic carbon (TOC) were 63 ± 0.8, 130 ± 1.5, 3.5 ± 0.002, 9.5 ± 0.003 and 135 ± 1.8 mg/L, respectively. Except TOC, all the other parameters of STWW were estimated as per standard protocols described in APHA (1995). Estimation of TOC was performed using TOC analyzer (CPH, Schimazdu).

4.2.3 Batch experiments

All the phenols were purchased from Sigma-Aldrich, Mumbai, India. Batch experiments were performed in 250 mL Erlenmeyer flasks containing 150 mL secondary treated wastewater (STWW) with different concentrations (25, 50, 75 and 100 mg/L) of four phenols (1,2-dihydroxy benzene, 2,4-dinitrophenol, 2,4-dichlorophenol and 2-chlorophenol) inoculated with 10 mL of microalgal suspension. All the flasks were kept in a shaking incubator (150 rpm and 25 ± 1 °C)

under white fluorescent light illumination (light/dark period, 18/6 h) of 10000 lux for 8 days. All the experiments were performed in triplicate.

4.2.4 Microalgal wastewater remediation

In this set of experiments both the microalgae were cultivated on medium (STWW + glucose or glycerol) or on medium (STWW + phenol + 20 g/L of glucose or glycerol) as per the conditions described above under head batch experiments.

4.2.5 Biomass growth

Biomass growth was measured gravimetrically. Aliquot of 10 mL microalgal suspension was withdrawn daily, filtered through a pre-weighed glass microfiber filter paper (Whatman) and dried at 105 °C for 24 h. After cooling to room temperature, the filter paper with algal biomass was weighed again and the dry cell weight (DCW) was calculated.

4.2.6 Lipid extraction and fatty acid methyl ester (FAME) analysis

The modified method by Bligh and Dyer (Bligh and Dyer,1959) was used for the total lipid extraction from the dried microalgal biomass. Algal broth (50 mL) was centrifuged for 5 min at 3000 rpm and the supernatant was discarded. Cell pellets obtained after centrifugation was washed twice using double distilled water and was kept in an oven at 104 ℃ overnight. The dried microalgal biomass was sonicated for 5 min at 20 kHz and after addition of 10 mL of chloroform:methanol (2:1, v/v), this mixture was allowed to stir for 30 min. The mixture was filtered using sintered glass funnel containing 5 mL of 0.034% $MgCl_2$ and centrifuged at 3000 rpm for 5 min. The aqueous upper layer was allowed to aspirate and the residual organic phase was washed with 1 mL of 2N KCl:methanol (4:1, v/v) and followed by addition of 5 mL of artificial upper phase (chloroform:methanol:water; 3:48:47, v/v/v) until the phase boundary becomes clear. For internal standards nonadecanoic acid (0.1µg/mL) was used. The chloroform layer at bottom was transferred to the test tube, and the lipid yield was determined gravimetrically. Fatty acid methyl ester analysis was performed using GC-MS (Agilent, Santa Clara, CA, USA) having DB-5MS capillary column (30m x 0.25m x 0.25µm). Splitless injection mode was used to inject samples at (1µL at 250 ℃) using helium as a carrier gas. FAME's were eluted using an oven temperature program of 5 min for 100 °C, then a gradient from 100 to 240 °C at 4 °C min^{-1} and then 240 °C for 20 min. FAME's were identified by comparison of retention times to known standards.

4.2.7 Measurement of residual phenol concentration

Residual phenol concentration was measured according to the 4-aminoantipyrene method for estimation of phenolic compounds (APHA 1995) in aqueous medium. The photodegradation of different phenols was monitored by adding the same concentrations of phenols without microalgae cells.

4.2.8 Effects of glucose, glycerol on biomass growth and removal of phenols

The effects of organic carbon source (glucose or glycerol) on the phenol removal efficiency of *C. minutissima* and *S. abundans* were studied by adding 20 g/L of glucose or glycerol in cultivation medium.

4.3 Results and discussion

4.3.1 Microalgae cultivation on wastewater

C. minutissima cultivated on STWW, STWW + 20 g/L glucose and STWW + 20 g/L glycerol obtained biomass yield of 0.83 ± 0.03, 1.93 ± 0.03 and 1.40 ± 0.02 g/L, respectively Fig. 4.1(a). Under similar conditions, *S. abundans* obtained biomass yield of 0.75 ± 0.02, 1.73 ± 0.015 and 1.25 ± 0.015 g/L, respectively Fig. 4.1(b).

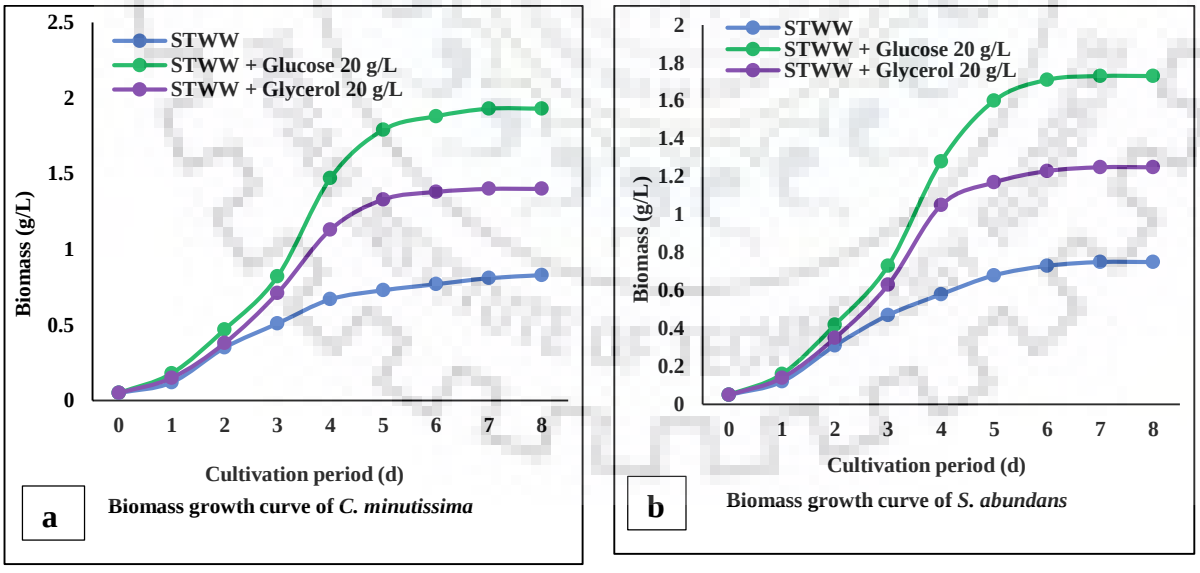

Figure 4.1 Biomass growth curves of (a) *C. minutissima* (b) *S. abundans* cultivated on STWW, STWW+ glucose and STWW+ glycerol

C. minutissima attained lipid yield of 143.59 ± 0.44, 373.45 ± 0.094 and 262.92 ± 0.37 mg/L, when cultivated on STWW, STWW + 20 g/L glucose and STWW + 20 g/L glycerol,

respectively. Under similar conditions, *S. abundans* attained lipid yield of 123.6 ± 0.049, 316.7 ± 0.06 and 217.25 ± 0.05 mg/L, respectively. It is noteworthy that wastewater supplemented with glucose achieved higher biomass growth and lipid yield in comparison to wastewater supplemented with glycerol. This can be attributed to that the microalgae can utilize glucose (being 6-carbon sugar glucose can easily be breakdown by microalgae) directly for energy needs after absorption inside the cell.

4.3.2 Effects of phenols on microalgae growth

Growth of *C. minutissima* and *S. abundans* cultivated on STWW + different phenols were assessed. It was observed that both the microalgae achieved the highest biomass growth at lowest concentration of each phenol (25 mg/L) present in STWW. The decrease in biomass growth of both the strains was observed with increasing concentration (25-100 mg/L) of phenols. Biomass growth curves of *C. minutissima* and *S. abundans* cultivated on STWW containing different phenols are presented in Fig. 4.2(a-h). *C. minutissima* obtained biomass yield of 0.51 ± 0.008, 0.43 ± 0.004, 0.38 ± 0.005 and 0.33 ± 0.004 g/L when grown on STWW containing 25, 50, 75 and 100 mg/L of 1,2-dihydroxy benzene (catechol), respectively while under similar conditions *S. abundans* obtained biomass yield of 0.45 ± 0.006, 0.38 ± 0.006, 0.33 ± 0.005 and 0.25 ± 0.005 g/L, respectively. The lowest biomass yield for *C. minutissima* (0. 2 ± 0.016 g/L) and *S. abundans* (0.15 ± 0.008 g/L) was observed with 100 mg/L of 2-chlorophenol in STWW, whereas, the highest biomass yield by both the strains was achieved when grown on STWW+ 1,2-dihydroxy benzene. Phenols are known for the production of phenoxy radicals (reactive oxygen species, ROS). These are oxidizing in nature and inside the cell, they can interfere with cellular activities resulting in poor cellular metabolism. ROS can also damage nucleic acids and cell organelles (nucleus, nucleolus, Golgi bodies and mitochondria, etc.) when their concentration is high inside the cell. It has been reported that ROS caused lipid peroxidation and increase in the concentration of malondialdehyde when cultivated on levofloxacin containing medium (Baldiris-Navarro et al. 2018). Exposure of microalgae to phenol derivatives has also witnessed oxygen evolution and photosystem II activity inhibition (Al-Dahhan et al. 2018). During phenolic stress, microalgae are able to survive by enhancing photosynthetic pigments, carotenoid, biochemical characteristics and antioxidant enzymes which help species adapt to and protect against the stress induced by the toxicants (Duan et al. 2018). In present work the sequence of biomass growth observed for both the microalgae with different phenols was found in following order 1,2-dihydroxy benzene > 2,4-dinitrophenol > 2,4-dichlorophenol > 2-chlorophenol.

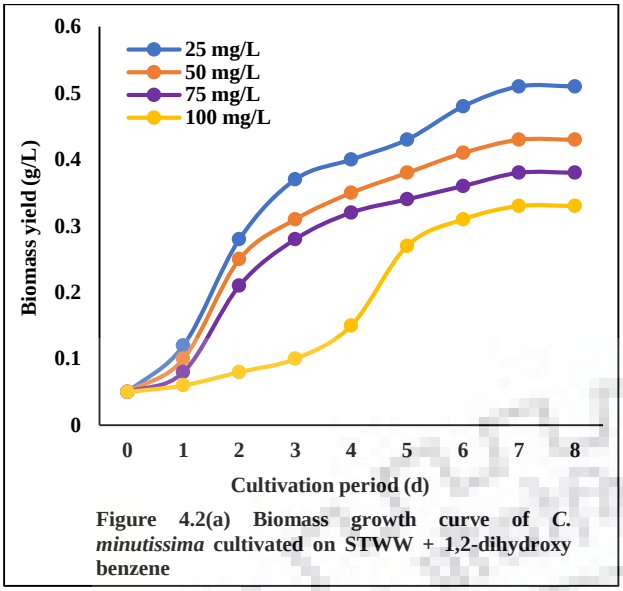

Figure 4.2(a) Biomass growth curve of *C. minutissima* cultivated on STWW + 1,2-dihydroxy benzene

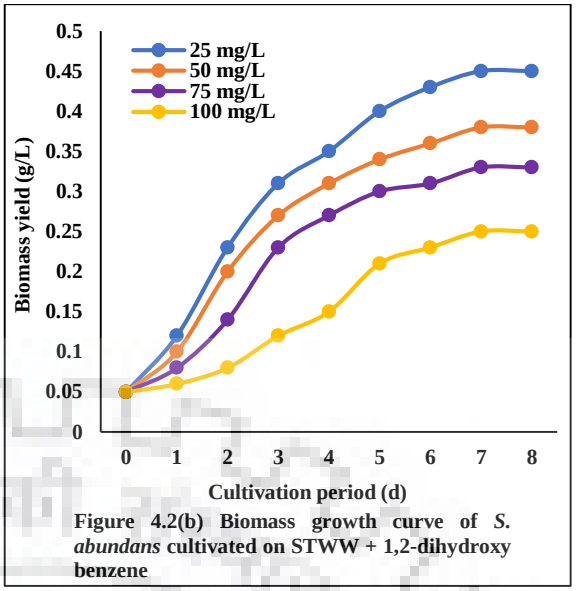

Figure 4.2(b) Biomass growth curve of *S. abundans* cultivated on STWW + 1,2-dihydroxy benzene

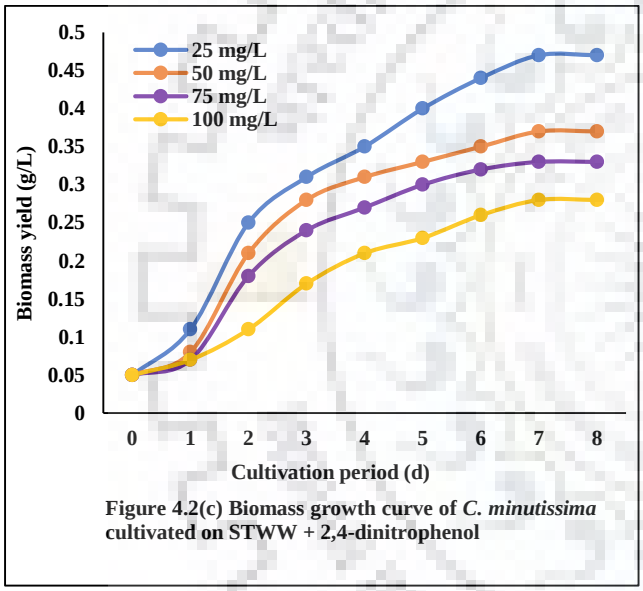

Figure 4.2(c) Biomass growth curve of *C. minutissima* cultivated on STWW + 2,4-dinitrophenol

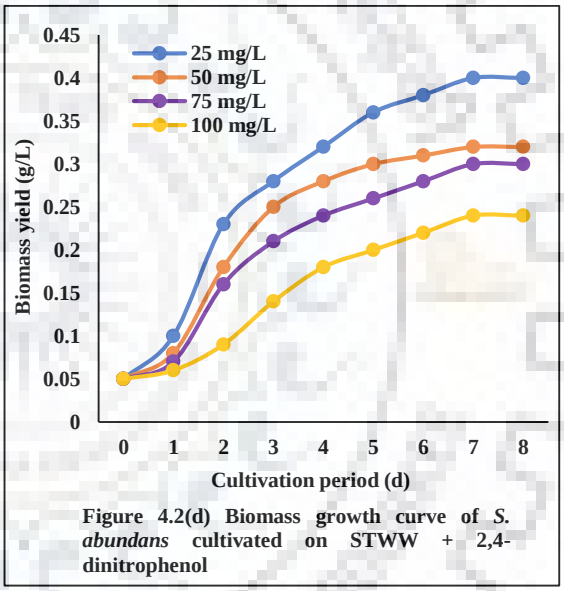

Figure 4.2(d) Biomass growth curve of *S. abundans* cultivated on STWW + 2,4-dinitrophenol

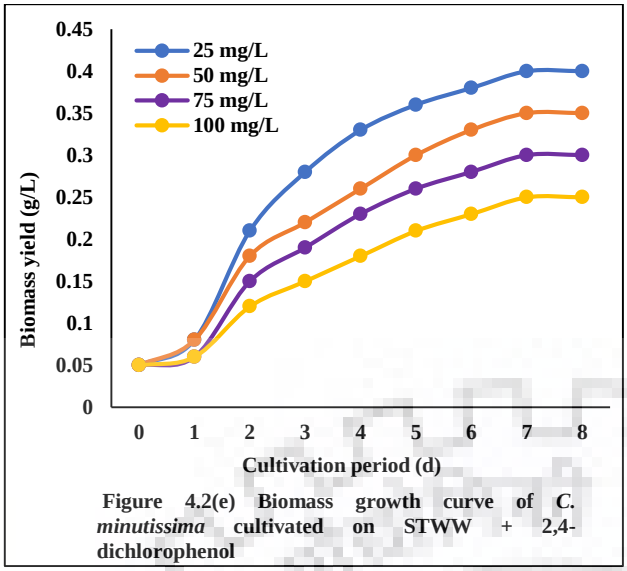

Figure 4.2(e) Biomass growth curve of *C. minutissima* cultivated on STWW + 2,4-dichlorophenol

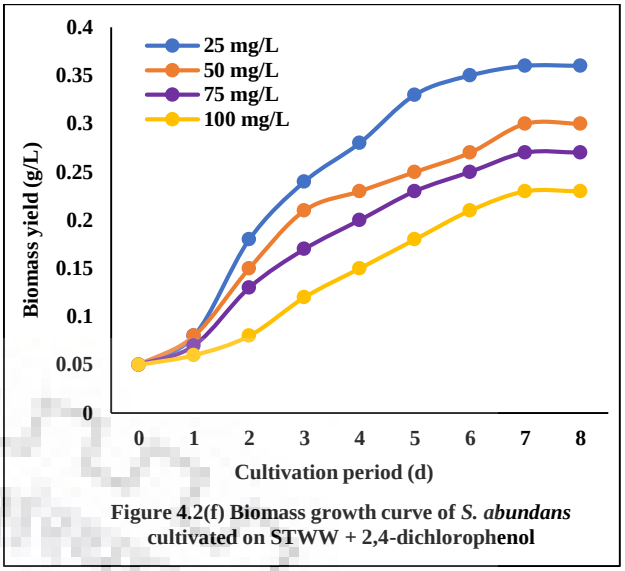

Figure 4.2(f) Biomass growth curve of *S. abundans* cultivated on STWW + 2,4-dichlorophenol

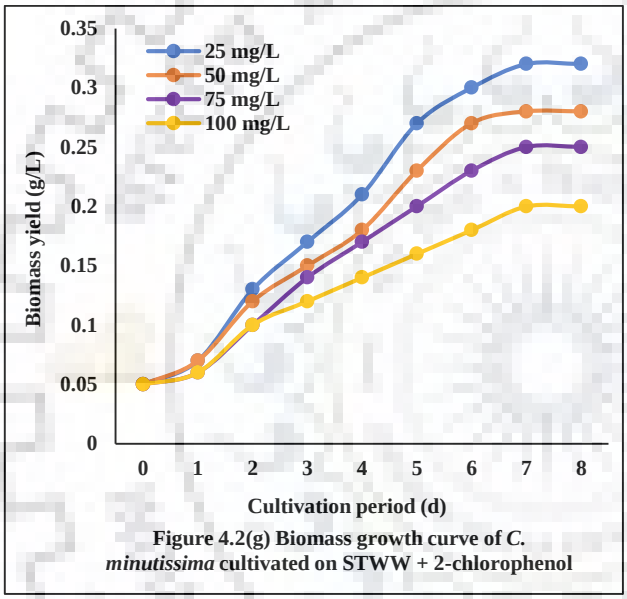

Figure 4.2(g) Biomass growth curve of *C. minutissima* cultivated on STWW + 2-chlorophenol

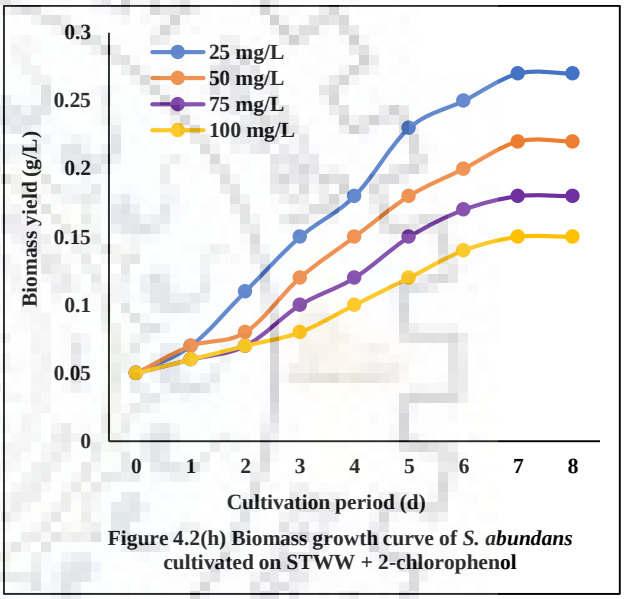

Figure 4.2(h) Biomass growth curve of *S. abundans* cultivated on STWW + 2-chlorophenol

Figure 4.2 (a-h) Biomass growth curves of *C. minutissima* and *S. abundans* cultivated on STWW spiked with different phenols

It was observed that the increase in the concentration of different phenols from 25 to 100 mg/L decreased the microalgal biomass yield. Data for biomass yield achieved by both the microalgae cultivated on STWW amended with different phenols are recorded in Table 4.1. The highest biomass yield attained by *C. minutissima* and *S. abundans* at the highest concentration of phenol (100 mg/L of 1,2-dihydroxy benzene + STWW) was 0.33 ± 0.004 and 0.25 ± 0.005 g/L, respectively.

Table 4.1 Biomass yield (g/L) of *C. minutissima* and S. *abundans* cultivated on STWW + phenols

	Biomass yield (g/L)			
Phenol concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
Phenol name	***C. minutissima***			
1, 2-dihydroxy benzene	0.51 ± 0.008	0.43 ± 0.004	0.38 ± 0.005	0.33 ± 0.004
2,4 dinitro phenol	0.47 ± 0.007	0.37 ± 0.005	0.33 ± 0.003	0.28 ± 0.005
2,4 di chlorophenol	0.40 ± 0.006	0.35 ± 0.003	0.30 ± 0.004	0.25 ± 0.003
2-chlorophenol	0.32 ± 0.005	0.28 ± 0.003	0.25 ± 0.003	0.20 ± 0.004
	S. abundans			
1, 2-dihydroxy benzene	0.45 ± 0.006	0.38 ± 0.006	0.33 ± 0.005	0.25 ± 0.005
2,4 dinitro phenol	0.40 ± 0.005	0.32 ± 0.004	0.30 ± 0.004	0.24 ± 0.005
2,4 di chlorophenol	0.36 ± 0.005	0.30 ± 0.003	0.27 ± 0.004	0.23 ± 0.003
2-chlorophenol	0.27 ± 0.006	0.22 ± 0.005	0.18 ± 0.006	0.15 ± 0.006

It is noteworthy that the lipid yield of both strains was affected by the presence of phenols in the cultivation medium. Lipid yield of *C. minutissima* and *S. abundans* are presented in Table 4.2.

Table 4.2 Lipid yield of *C. minutissima* and *S. abundans* cultivated on STWW having different concentrations of phenols

	Lipid yield (mg/L) of *C. minutissima*			
Phenol name/Concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
1, 2-dihydroxy benzene	67.47 ± 0.45	52.80 ± 0.35	45.45 ± 0.36	30.95 ± 0.33
2,4 dinitro phenol	60.06 ± 0.45	42.22 ± 0.33	34.52 ± 0.38	25.67 ± 0.25
2,4 di chlorophenol	49.04 ± 0.38	38.12 ± 0.32	30.15 ± 0.32	21.95 ± 0.23
2-chlorophenol	37.98 ± 0.41	28.00 ± 0.35	24.45 ± 0.35	17.06 ± 0.15
	Lipid yield (mg/L) of *S. abundans*			
1, 2-dihydroxy benzene	56.83 ± 0.41	44 ± 0.36	35.87 ± 0.31	23.77 ± 0.28
2,4 dinitro phenol	49.36 ± 0.31	34.52 ± 0.38	31.77 ± 0.33	22.06 ± 0.26
2,4 di chlorophenol	42.58 ± 0.35	31.11 ± 0.32	26.13 ± 0.25	20.70 ± 0.25
2-chlorophenol	30.21 ± 0.20	22.30 ± 0.25	15.96 ± 0.31	13.17 ± 0.28

The decrease in the lipid yield of both the strains was observed with increase in the concentration of phenols. Highest lipid yield of 67.47 ± 0.45 and 56.83 ± 0.41 mg/L was achieved by *C. minutissima* and *S. abundans* when cultivated on (STWW + 25 g/L of 1,2-dihydroxy benzene), respectively. *C. minutissima* accumulated lipid yield of 102.1 ± 0.13 mg/L, 289.5 ± 0.008 mg/L and 193.2 ± 0.007 mg/L, respectively when cultivated on STWW. In similar conditions *S. abundans* gained lipid yield of 88.87 ± 0.10 mg/L, 248 ± 0.21 mg/L and 161.87 0.15 mg/L, respectively. *C. minutissima* obtained lipid yield of 53.29 ± 0.008 mg/L and 31.02 ±

0.007 mg/L when cultivated on 50 and 100 mg/L of catechol concentration in STWW, respectively.

4.3.3 Effect of addition of glucose/glycerol in cultivation medium on microalgae growth

Addition of 20 g/L of glucose or glycerol in cultivation medium (STWW + 20 g/L of glucose or glycerol + Phenol) resulted in improved biomass growth of both the strains as depicted in Fig. 4.3 (a-p) and Table 4.3. *C. minutissima* and *S. abundans* resulted in biomass yield of 2.15 and 1.75 g/L, respectively, when cultivated on (STWW + 25 mg/L 1,2-dihydroxy benzene + 20 g/L glucose) and (STWW + 25 mg/L 1,2-dihydroxy benzene + 20 g/L glycerol), respectively. Under similar conditions, *S. abundans* obtained biomass yield of 1.90 ± 0.06 and 1.60 ± 0.05 g/L, respectively. This can be attributed to the fact that during phenolic stress microalgae used organic carbon present in the growth medium when photosynthesis was poor due to phenol toxicity which resulted in increased biomass production of microalgae. However, the addition of glucose caused more biomass production as compared to glycerol for both the microalgae. It was observed that the highest biomass growth for both microalgae strains was achieved in 1,2-dihydroxy benzene containing medium while the lowest growth was observed in 2-chlorophenol containing medium. It may be due to the more toxic nature of chlorophenols than non-chlorophenols.

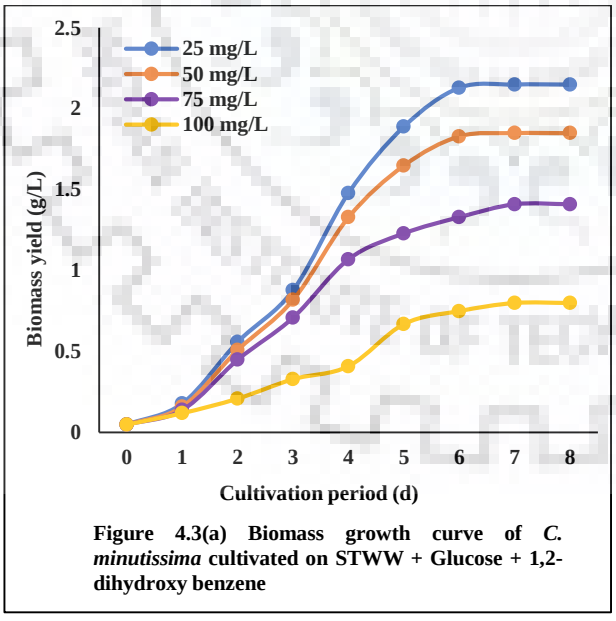

Figure 4.3(a) Biomass growth curve of *C. minutissima* cultivated on STWW + Glucose + 1,2-dihydroxy benzene

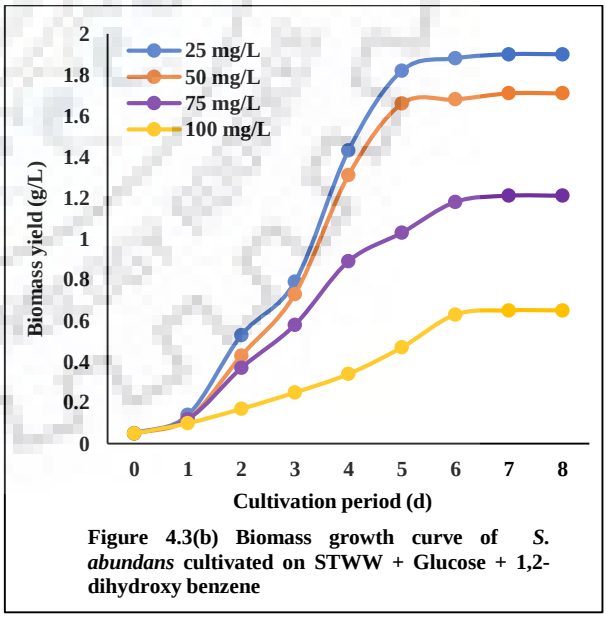

Figure 4.3(b) Biomass growth curve of *S. abundans* cultivated on STWW + Glucose + 1,2-dihydroxy benzene

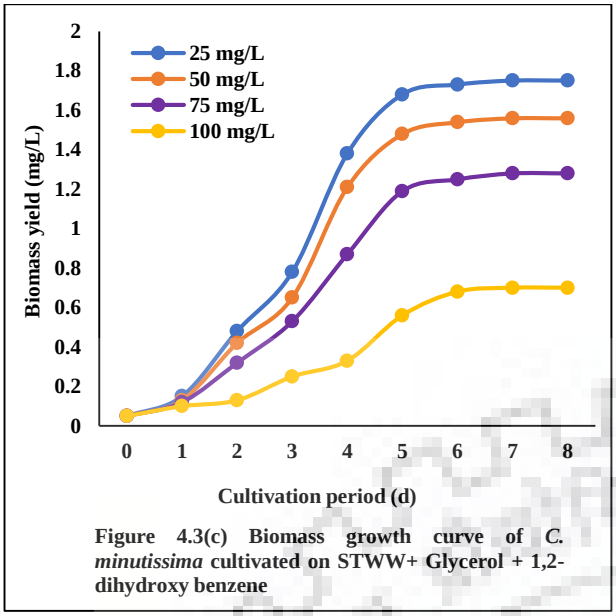

Figure 4.3(c) Biomass growth curve of *C. minutissima* cultivated on STWW+ Glycerol + 1,2-dihydroxy benzene

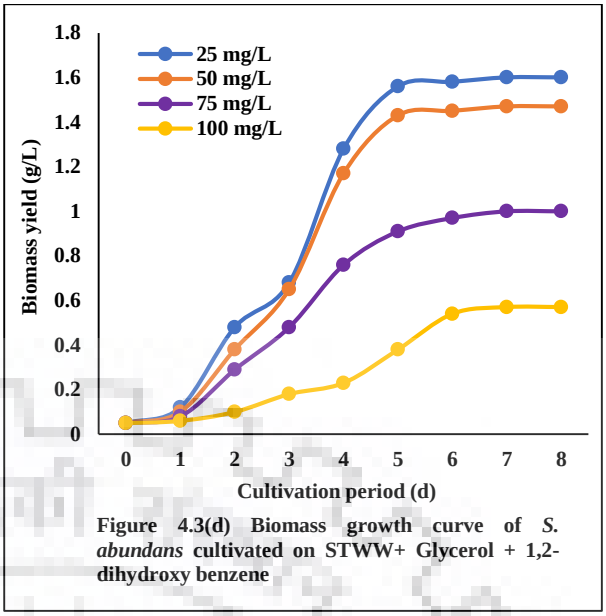

Figure 4.3(d) Biomass growth curve of *S. abundans* cultivated on STWW+ Glycerol + 1,2-dihydroxy benzene

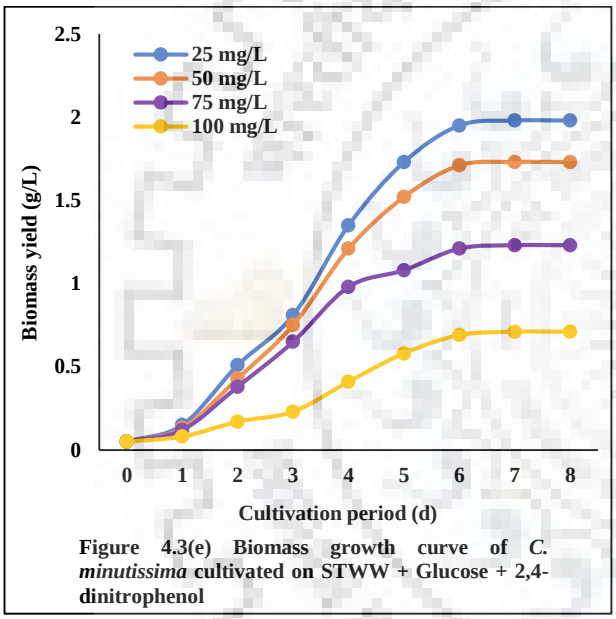

Figure 4.3(e) Biomass growth curve of *C. minutissima* cultivated on STWW + Glucose + 2,4-dinitrophenol

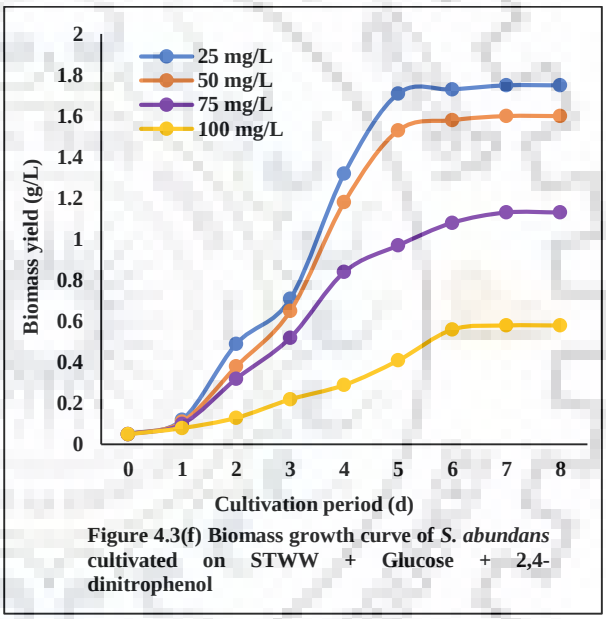

Figure 4.3(f) Biomass growth curve of *S. abundans* cultivated on STWW + Glucose + 2,4-dinitrophenol

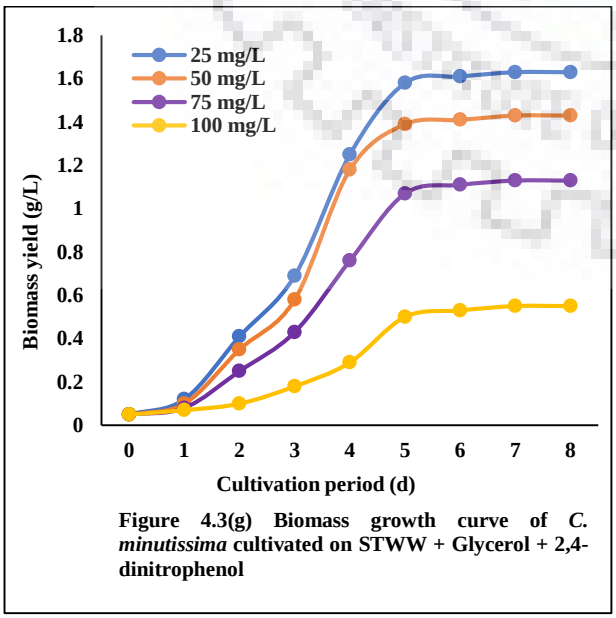

Figure 4.3(g) Biomass growth curve of *C. minutissima* cultivated on STWW + Glycerol + 2,4-dinitrophenol

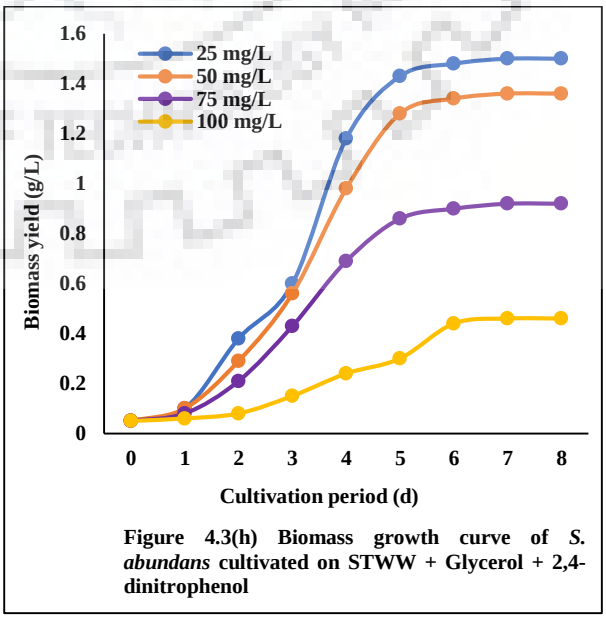

Figure 4.3(h) Biomass growth curve of *S. abundans* cultivated on STWW + Glycerol + 2,4-dinitrophenol

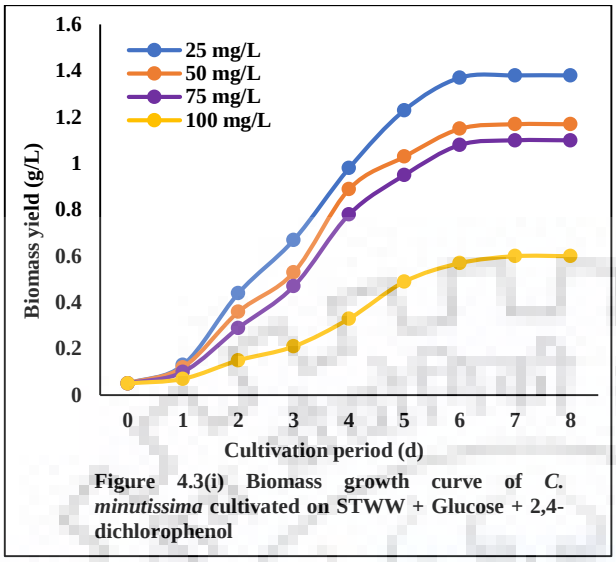

Figure 4.3(i) Biomass growth curve of *C. minutissima* cultivated on STWW + Glucose + 2,4-dichlorophenol

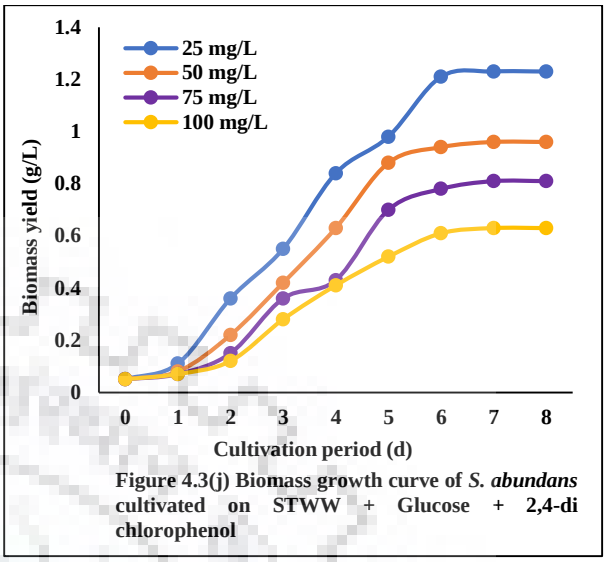

Figure 4.3(j) Biomass growth curve of *S. abundans* cultivated on STWW + Glucose + 2,4-di chlorophenol

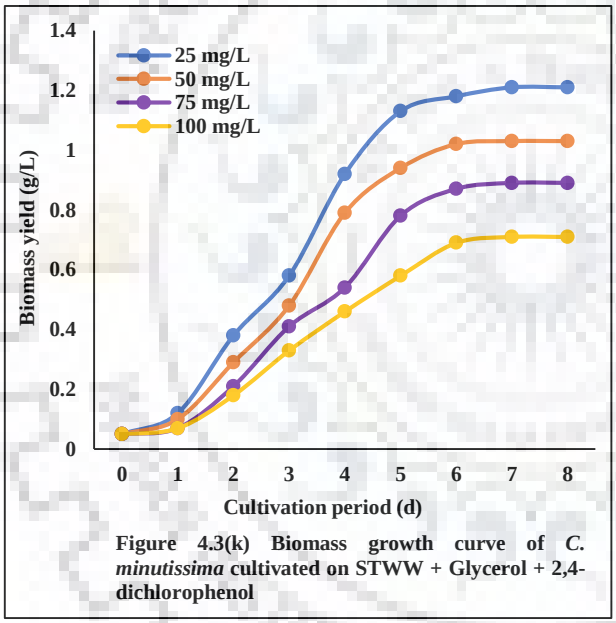

Figure 4.3(k) Biomass growth curve of *C. minutissima* cultivated on STWW + Glycerol + 2,4-dichlorophenol

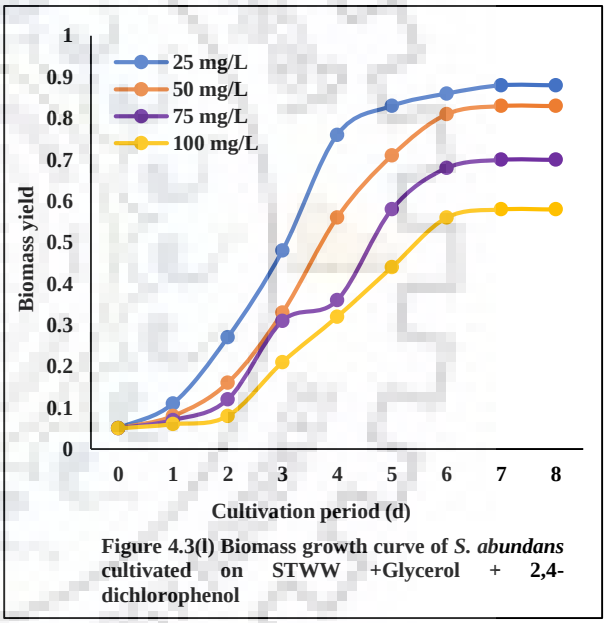

Figure 4.3(l) Biomass growth curve of *S. abundans* cultivated on STWW +Glycerol + 2,4-dichlorophenol

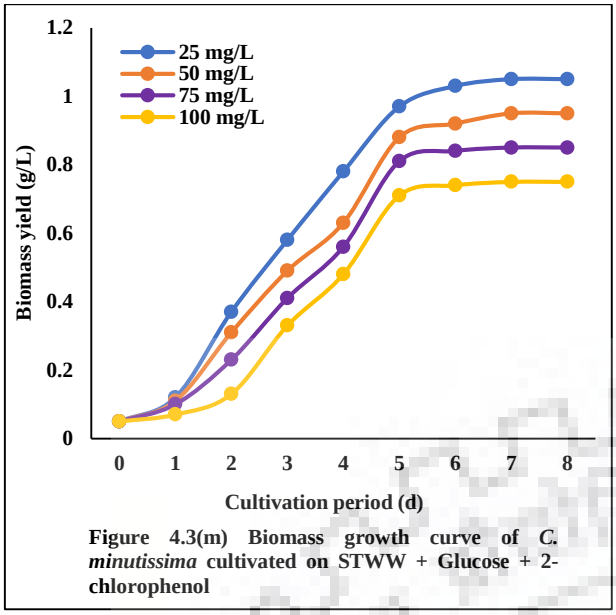

Figure 4.3(m) Biomass growth curve of *C. minutissima* cultivated on STWW + Glucose + 2-chlorophenol

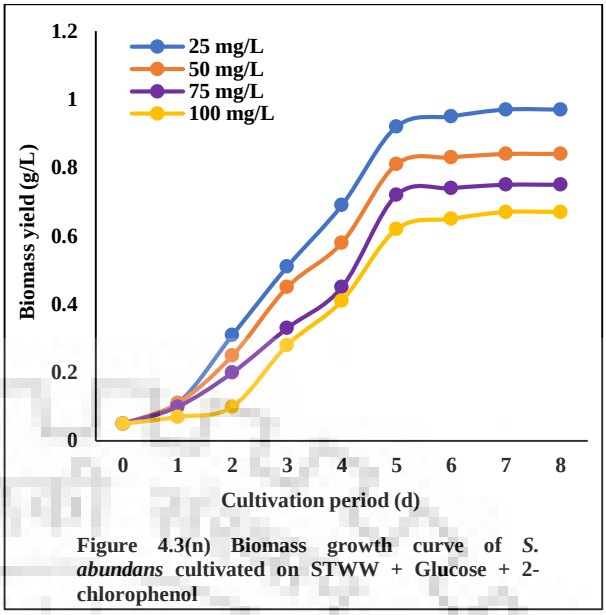

Figure 4.3(n) Biomass growth curve of *S. abundans* cultivated on STWW + Glucose + 2-chlorophenol

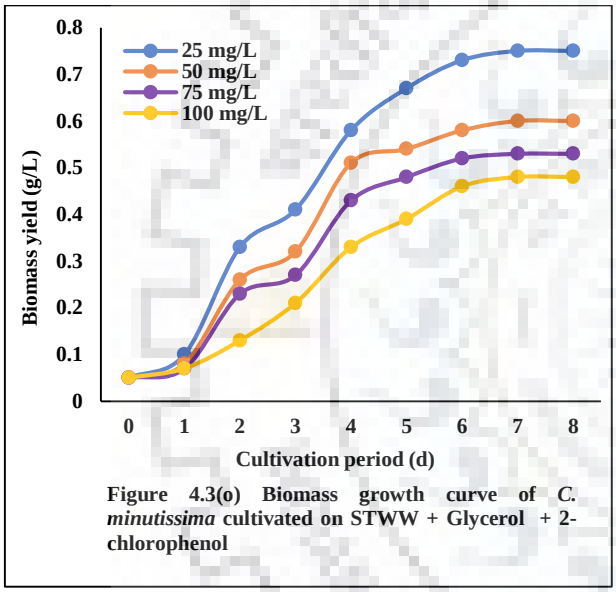

Figure 4.3(o) Biomass growth curve of *C. minutissima* cultivated on STWW + Glycerol + 2-chlorophenol

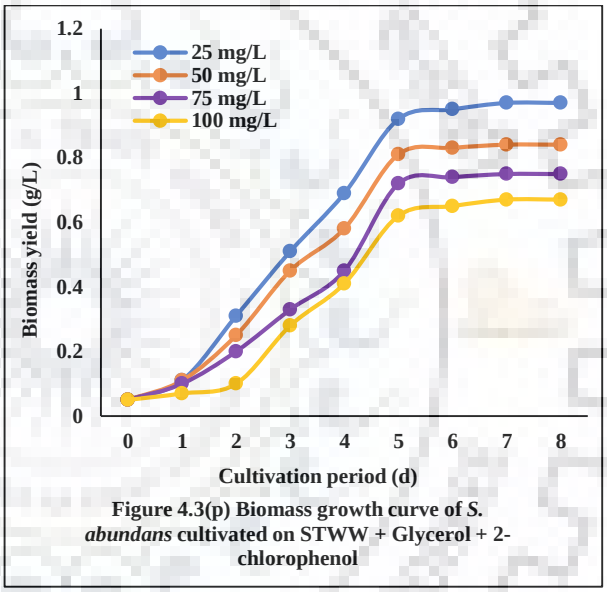

Figure 4.3(p) Biomass growth curve of *S. abundans* cultivated on STWW + Glycerol + 2-chlorophenol

Figure 4.3 Biomass growth curves of *C. minutissima* and *S. abundans* cultivated on (STWW + different phenols + glucose or glycerol)

Variation in nutrients, light regime, temperature, etc. has been beneficial usually for improvement in biomass growth of microalgae (Mishra and Kumar 2017). It has been observed that the addition of external carbon source in microalgae cultivation during stress or adverse conditions can help in the sustenance of microalgae and can easily acclimatize to changes in cultivation environment (Liu et al. 2010).

Table 4.3 Biomass yield (g/L) of *C. minutissima* and *S. abundans* in different concentrations of phenols with STWW and glucose or glycerol

	Biomass yield (g/L)			
Phenol concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
Phenol name	***C. minutissima* + 20 g/L Glucose**			
1, 2-dihydroxy benzene + 20 g/L Glucose	2.15 ± 0.06	1.85 ± 0.03	1.41 ± 0.04	0.80 ± 0.03
2,4 dinitro phenol + 20 g/L Glucose	1.98 ± 0.05	1.73 ± 0.02	1.23 ± 0.03	0.70 ± 0.02
2,4 di chlorophenol + 20 g/L Glucose	1.38 ± 0.05	1.17 ± 0.03	1.10 ± 0.03	0.60 ± 0.02
2-chlorophenol + 20 g/L Glucose	1.05 ± 0.03	0.95 ± 0.02	0.85 ± 0.02	0.75 ± 0.02
	***C. minutissima* + 20 g/L Glycerol**			
1, 2-dihydroxy benzene + 20 g/L Glycerol	1.75 ± 0.04	1.56 ± 0.05	1.28 ± 0.03	0.70 ± 0.03
2,4 dinitro phenol + 20 g/L Glycerol	1.63 ± 0.03	1.43 ± 0.03	1.13 ± 0.02	0.55 ± 0.02
2,4 di chlorophenol + 20 g/L Glycerol	1.21 ± 0.05	1.03 ± 0.02	0.89 ± 0.03	0.71 ± 0.02
2-chlorophenol + 20 g/L Glycerol	0.75 ± 0.02	0.60 ± 0.02	0.53 ± 0.03	0.48 ± 0.02
	***S. abundans* + 20 g/L Glucose**			
1, 2-dihydroxy benzene + 20 g/L Glucose	1.90 ± 0.06	1.71 ± 0.05	1.21 ± 0.06	0.65 ± 0.015
2,4 dinitro phenol + 20 g/L Glucose	1.75 ± 0.05	1.60 ± 0.06	1.13 ± 0.05	0.58 ± 0.012
2,4 di chlorophenol + 20 g/L Glucose	1.23 ± 0.03	0.96 ± 0.05	0.81 ± 0.02	0.63 ± 0.010
2-chlorophenol + 20 g/L Glucose	0.97 ± 0.03	0.84 ± 0.03	0.75 ± 0.02	0.67 ± 0.005
	***S. abundans* + 20 g/L Glycerol**			
1, 2-dihydroxy benzene + 20 g/L Glycerol	1.60 ± 0.05	1.47 ± 0.03	1.00 ± 0.05	0.57 ± 0.016
2,4 dinitro phenol + 20 g/L Glycerol	1.50 ± 0.04	1.36 ± 0.02	0.92 ± 0.05	0.46 ± 0.015
2,4 di chlorophenol + 20 g/L Glycerol	0.88 ± 0.02	0.83 ± 0.02	0.70 ± 0.06	0.58 ± 0.008
2-chlorophenol + 20 g/L Glycerol	0.63 ± 0.04	0.50 ± 0.02	0.40 ± 0.02	0.35 ± 0.007

Lipid synthesis in microalgae depends upon various factors like temperature, period of light exposure and nutrients availability, etc. (Yu et al. 2013). Lipid accumulation in microalgae in response to toxicant stress has been regarded as a detoxification mechanism by absorbing lipophilic xenobiotics to reduce their bioavailability (Yang et al. 2017). Lipid yield for *C. minutissima* and *S. abundans* cultivated on different media has been recorded in Table 4.4.

Table 4.4 Lipid yield of *C. minutissima* and *S. abundans* cultivated on STWW + phenol + glucose or glycerol)

	Lipid yield (mg/L) of *C. minutissima* and *S. abundans*			
Phenol concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
Phenol name	***C. minutissima* + 20 g/L Glucose**			
1, 2-dihydroxy benzene + 20 g/L Glucose	320.14 ± 0.12	239.95 ± 0.12	176.67 ± 0.12	79.28 ± 0.08
2,4-dinitrophenol + 20 g/L Glucose	274.63 ± 0.13	208.12 ± 0.10	138.13 ± 0.10	66.95 ± 0.10
2,4-dichlorophenol + 20 g/L Glucose	177.33 ± 0.12	134.20 ± 0.13	117.37 ± 0.12	54.42 ± 0.08
2-chlorophenol + 20 g/L Glucose	129.26 ± 0.10	99.47 ± 0.10	86.11 ± 0.08	67.20 ± 0.08
	***C. minutissima* + 20 g/L Glycerol**			
1, 2-dihydroxy benzene + 20 g/L Glycerol	243.78 ± 0.15	196.25 ± 0.10	154.62 ± 0.14	67.41 ± 0.06
2,4-dinitrophenol + 20 g/L Glycerol	215.32 ± 0.13	169.60 ± 0.13	122.61 ± 0.12	51.43 ± 0.05
2,4-dichlorophenol + 20 g/L Glycerol	151.98 ± 0.10	115.46 ± 0.08	91.85 ± 0.08	63.55 ± 0.05
2-chlorophenol + 20 g/L Glycerol	90.60 ± 0.08	60.78 ± 0.07	58.19 ± 0.08	42.00 ± 0.04
	***S. abundans* + 20 g/L Glucose**			
1, 2-dihydroxy benzene + 20 g/L Glucose	248.52 ± 0.14	208.45 ± 0.10	136 ± 0.08	65.33 ± 0.05
2,4-dinitrophenol + 20 g/L Glucose	225.05 ± 0.12	183.20 ± 0.12	124.87 ± 0.07	56.43 ± 0.05
2,4-dichlorophenol + 20 g/L Glucose	154.242 ± 0.12	103.30 ± 0.08	85.94 ± 0.05	59.41 ± 0.06
2-chlorophenol + 20 g/L Glucose	114.363 ± 0.10	90.05 ± 0.08	68.55 ± 0.06	63.32 ± 0.05
	***S. abundans* + 20 g/L Glycerol**			
1, 2-dihydroxy benzene + 20 g/L Glycerol	204.96 ± 0.12	174.49 ± 0.10	110.8 ± 0.10	59.74 ± 0.07
2,4-dinitrophenol + 20 g/L Glycerol	182.85 ± 0.10	149.33 ± 0.08	99.08 ± 0.08	43.15 ± 0.05
2,4-dichlorophenol + 20 g/L Glycerol	107.008 ± 0.08	85.24 ± 0.06	70 ± 0.05	53.42 ± 0.07
2-chlorophenol + 20 g/L Glycerol	70.749 ± 0.07	51.90 ± 0.05	36 ± 0.05	31.96 ± 0.06

4.3.4 Removal of phenols

Many authors have reported that the microalgae are able to remove phenols from aqueous environment successfully (Pinto et al.; Portnoy et al. 2011; Xiong et al. 2017b; Al-Dahhan et al. 2018). Removal of phenols and other pollution parameters was estimated at the end of the experiment after 8 days. Data for the removal of phenols are presented in Table 4.5(a and b). It can be observed that the removal of phenols was decreased with increasing concentration of phenols in STWW. However, the addition of glucose or glycerol in STWW resulted in enhanced removal of phenols by both microalgae strains Table 4.5(b). Werner and Pawlitz (1978) have shown that green microalgae (*Euglena gracilis, Chlorella pyrenoidosa, and Scenedesmus obliquus*) are effective in phenol degradation.

Table 4.5(a) Removal of phenols in absence of glucose or glycerol

	Removal by *C. minutissima* (%)			
Phenol name/Concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
1,2-dihydroxy benzene	27.76 ± 0.03	23.63 ± 0.03	10.83 ± 0.012	2.81 ± 0.005
2,4-dinitro phenol	26.82 ± 0.04	23.42 ± 0.02	10.28 ± 0.010	2.73 ± 0.003
2,4-dichlorophenol	25.76 ± 0.03	22.87 ± 0.02	9.96 ± 0.008	2.31 ± 0.004
2-chlorophenol	25.48 ± 0.02	20.31 ± 0.03	9.45 ± 0.007	1.90 ± 0.003
	Removal by *S. abundans* (%)			
1,2-dihydroxy benzene	26.68 ± 0.03	23.34 ± 0.03	10.16 ± 0.02	2.68 ± 0.003
2,4-dinitro phenol	25.92 ± 0.02	23.18 ± 0.02	10.05 ± 0.02	2.37 ± 0.003
2,4-dichlorophenol	25.16 ± 0.02	22.46 ± 0.03	9.90 ± 0.02	2.07 ± 0.002
2-chlorophenol	24.94 ± 0.02	20.18 ± 0.02	9.76 ± 0.03	1.74 ± 0.003

Chlorella vulgaris and *Chlorella* VT-1 (Scragg, 2006), and *Chlorella pyrenoidosa* has also been effective in phenol degradation and phenol is a possible substrate (at low concentration) for *Chlorella* sp. but high concentrations of phenol are also toxic to microalgae (Das et al. 2011).

Table 4.5(b) Removal of phenols in presence of glucose or glycerol

Phenol concentration	**25 mg/L**	**50 mg/L**	**75 mg/L**	**100 mg/L**
Phenol name	**Removal (%) by *C. minutissima* + 20 g/L Glucose**			
1, 2-dihydroxy benzene + 20 g/L Glucose	81 ± 0.03	63.47 ± 0.03	27.14 ± 0.018	12.67 ± 0.008
2,4-dinitro phenol + 20 g/L Glucose	77.31 ± 0.03	60.97 ± 0.03	22.83 ± 0.015	10.64 ± 0.006
2,4-dichlorophenol + 20 g/L Glucose	75.26 ± 0.02	58.26 ± 0.025	20.72 ± 0.012	9.54 ± 0.005
2-chlorophenol + 20 g/L Glucose	67.65 ± 0.03	56.89 ± 0.025	17.45 ± 0.012	9 ± 0.005
	Removal (%) by *C. minutissima* + 20 g/L Glycerol			
1, 2-dihydroxy benzene + 20 g/L Glycerol	78.26 ± 0.03	60.73 ± 0.04	22.78 ± 0.03	10.38 ± 0.005
2,4-dinitro phenol + 20 g/L Glycerol	75.45 ± 0.03	57.51 ± 0.03	20.33 ± 0.05	9.57 ± 0.004
2,4-dichlorophenol + 20 g/L Glycerol	72.37 ± 0.02	52.76 ± 0.03	18.28 ± 0.03	8.41 ± 0.003
2-chlorophenol + 20 g/L Glycerol	70.47 ± 0.02	49.82 ± 0.02	16.81 ± 0.02	7.54 ± 0.003
	Removal (%) by *S. abundans* + 20 g/L Glucose			
1, 2-dihydroxy benzene + 20 g/L Glucose	73.86 ± 0.05	58.78 ± 0.03	20.67 ± 0.03	9.23 ± 0.002
2,4-dinitro phenol + 20 g/L Glucose	70.45 ± 0.04	53.21 ± 0.03	18.71 ± 0.02	8.96 ± 0.003
2,4-di chlorophenol + 20 g/L Glucose	67.26 ± 0.03	50.67 ± 0.02	15.48 ± 0.02	7.27 ± 0.003
2-chlorophenol + 20 g/L Glucose	65.39 ± 0.03	47.91 ± 0.03	12.44 ± 0.03	6.78 ± 0.002
	Removal (%) by *S. abundans* + 20 g/L Glycerol			
1, 2-dihydroxy benzene + 20 g/L Glycerol	72.48 ± 0.03	55.23 ± 0.02	18.54 ± 0.015	8.40 ± 0.010
2,4-dinitro phenol + 20 g/L Glycerol	68.51 ± 0.03	50.84 ± 0.03	16.42 ± 0.012	7.86 ± 0.005
2,4-di chlorophenol + 20 g/L Glycerol	62.83 ± 0.02	45.23 ± 0.03	12.17 ± 0.015	6.95 ± 0.003
2-chlorophenol + 20 g/L Glycerol	60.54 ± 0.03	42.66 ± 0.02	10.56 ± 0.012	6.27 ± 0.003

Highest removal of phenols was achieved at the lowest concentration (25 mg/L) of phenol in all the mediums. *C. minutissima* and *S. abundans* achieved a maximum of 27.76 ± 0.03 and 26.68 ± 0.03% phenol (1,2-dihydroxy benzene) removal from cultivation medium containing 25 mg/L of 1,2-dihydroxy benzene, respectively, in absence of glucose or glycerol. However, removal of 1,2-dihydroxy benzene by *C. minutissima* increased to 81 ± 0.03 and 78.26 ± 0.03%

when glucose and glycerol, respectively, were added to the cultivation medium. Under similar conditions, *S. abundans* removed 73.86 ± 0.05 and 72.48 ± 0.03% of phenol (1,2-dihydroxy benzene), respectively.

4.3.5 Removal of pollution load

Removals of BOD, COD, TOC, NO_3^- and PO_4^{3-} were estimated for *C. minutissima* and *S. abundans* cultivated on (STWW + Glucose + 1,2-dihydroxy benzene (25 mg/L) and (STWW + Glucose) and depicted in Fig. 4.11. *C. minutissima* removed 86.46 ± 0.10% BOD, 72.92 ± 0.08% COD, 85.14 ± 0.05% NO_3^-, 91% ± 0.05 PO_4^{3-} and 91 ± 0.03% TOC from the cultivation medium (STWW + Glucose) while under similar conditions *S. abundans* removed 84 ± 0.10% BOD, 72.20 ± 0.15% COD, 78.85 ± 0.05% NO_3^-, 87.47 ± 0.03% PO_4^{3-} and 90.78 ± 0.16% TOC. It is interesting that the presence of phenol in the cultivation medium caused a reduction in removal of pollution load. *C. minutissima* removed 53.96 ± 0.12% BOD, 46 ± 0.15% COD, 77 ± 0.08% NO_3^-, 68 ± 0.05% PO_4^{3-} and 67 ± 0.14% TOC from the cultivation medium (STWW + Glucose + 1,2-dihydroxy benzene (25 mg/L)) while under similar conditions *S. abundans* removed 53.07 ± 0.12% BOD, 45.37 ± 0.15% COD, 71.06 ± 0.07% NO_3^-, 63.02 ± 0.05% PO_4^{3-} and 66.17 ± 0.16% TOC. Reduction in pollution load removal in the presence of phenols possibly occurred due to the toxic nature of phenols which hinders cell metabolism.

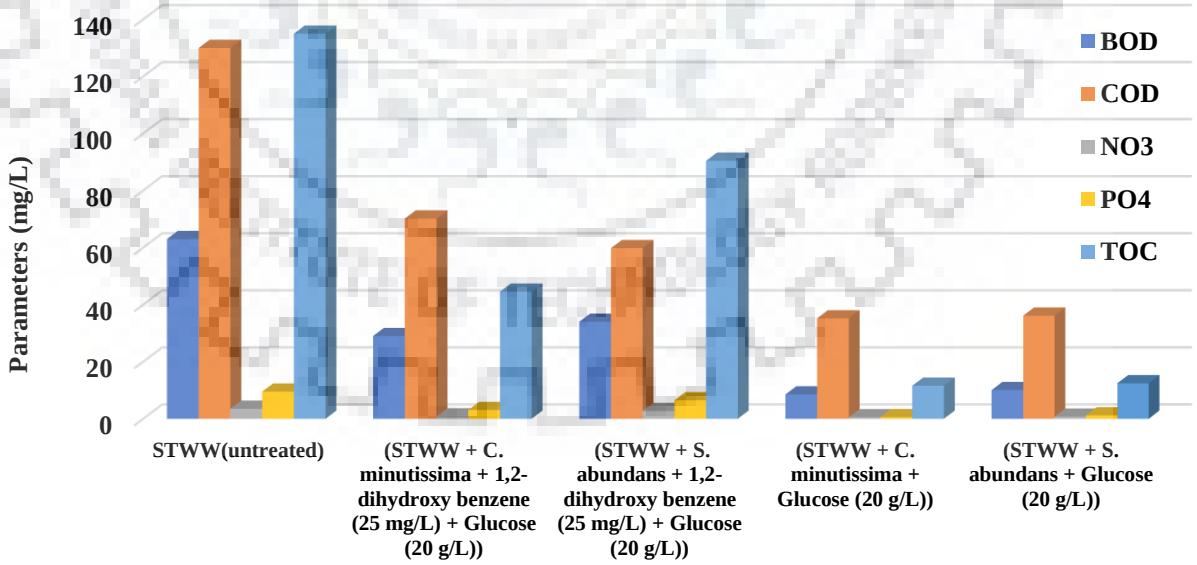

Figure 4.4 Removal of pollution load by *C. minutissima* and *S. abundans* from STWW in presence and absence of phenol (1,2-dihydroxy benzene)

4.3.6 Biodiesel properties

The biodiesel properties were estimated as per empirical equations described by Francisco et al. (2010) as given in chapter 2 of this thesis. Biodiesel properties of *C. minutissima* cultivated on (STWW + Glucose + 1,2-dihydroxy benzene (all 4 concentrations)) were analyzed using GC-MS. It was observed that the increasing concentration of phenols resulted in decrease of saturated fatty acids. As recorded in Table 4.6, with increased phenol (1,2-dihydroxy benzene) concentration, higher levels of FAMEs, specifically C18:0, were decreased. Percentage (44.19%) of C18:0 was decreased to 28.36% when the concentration of 1,2-dihydroxy benzene was increased from 25 to 100 mg/L in the cultivation medium.

Table 4.6 Fatty acid profile of *C. minutissima* cultivated on (STWW + 1,2-dihydroxy benzene + 20 g/L Glucose)

	Phenol (1,2-dihydroxy benzene) concentration (mg/L)			
FAMEs	**25**	**50**	**75**	**100**
C14:0	4.2	3.35	0.3	0.18
C16:0	31.47	28.26	23.46	22.12
C16:1	2.2	2.25	2.28	2.18
C17:0	0.98	0.18	0.08	0.05
C18:0	44.19	38.79	32.24	28.36
C18:1	12.01	22.32	36.36	42.28
C18:2	2.12	2.23	2.53	1.96
C18:3	0.78	1.12	1.21	1.35
Others	2.01	1.43	1.23	1.6

However, an increase in unsaturated fatty acid (C18:1) content was maximum (42.28%) in the medium containing 100 mg/L of 1,2-dihydroxy benzene. Previously it has been reported that the increase in unsaturated fatty acid (C18:3) mainly occurs under oxidative stress (Patel et al. 2015a). The present study indicates that phenol-induced stress is responsible for the increased share of unsaturated fatty acids. It also suggests that increased unsaturated fatty acid level is an important factor for stress acclimation and adaptation of microalgae. Liu et al. (2010) have suggested microalgae prone towards oxidative stress protects themselves by the desaturation of fatty acids present in the lipid membrane. Thus, this can be concluded that the phenol stress similarly can affect fatty acid desaturase expression. The biodiesel properties determined from the FAME profile Table (4.7). Cetane number (CN) is an important biodiesel parameter because

it is related to nitrous oxide emissions, engine performance, and combustion efficiency of diesel fuel (Gautam et al. 2013; Patel et al. 2015b).

Table 4.7 Biodiesel properties of *C. minutissima* cultivated on (STWW + 1,2-dihydroxy benzene + 20 g/L Glucose)

Parameters	**Phenol (1,2-dihydroxy) concentration (mg/L) in cultivation medium**			
	25	**50**	**75**	**100**
Saturated fatty acids (%)	80.84	70.58	56.08	50.71
Monounsaturated fatty acids (%)	14.21	24.57	38.64	44.46
Polyunsaturated fatty acids (%)	2.9	3.35	3.74	3.31
Degree of unsaturation (% wt.)	20.01	31.27	46.12	51.08
Saponification value (mg KOH)	202.5	202.57	200.19	199.93
Iodine value (g I_2 100 g^{-1})	18.97	29.42	42.86	47.44
Cetane number	68.98	66.62	63.91	62.92
Long chain saturation factor	25.24	22.22	18.46	16.39
Cold filter plugging point	62.82	53.33	41.53	35.02
Oxidative stability	43.25	37.79	34.12	38.21
Higher heating value (MJ Kg^{-1})	38.71	38.94	38.96	38.97
Kinematic viscosity (mm^2 s^{-1})	4.12	4.12	4.117	4.09
Density (g cm^{-3})	0.849	0.855	0.856	0.857

Therefore, the biodiesel property standard, ASTM D 6751 recommends a CN of at least 47 for diesel used as an engine fuel (Katiyar et al. 2017). The IV value is related to the degree of unsaturation of the fatty acids and the cold flow properties of diesel. The European standard, EN14214, recommends a maximum value of 120 (Guldhe et al. 2017).

4.4 Conclusions

The output of the present work shows that STWW contaminated with phenols can be efficiently treated using microalga *C. minutissima.* Biomass growth and lipid yield can be enhanced by the addition of glucose in the cultivation medium. Phenols at lower concentrations (25 mg/L) can be removed efficiently from the aqueous environment using microalgae cultivation. Removal of various pollution load (BOD, COD, NO_3^-, $PO_4^{3-}$and TOC) from the medium (STWW +glucose) in the presence and absence of phenols was efficiently done by *C. minutissima* and *S. abundans*. Biodiesel quality estimated for *C. minutissima* cultivated on (STWW + 1,2-dihydroxy benzene + 20 g/L Glucose) is in accordance with international standards like ASTM D6751 and European standard, EN14214.

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Chapter 5

Remediation of dairy wastewater and biodiesel feedstock production using microalgae poly-cultures

Results of this chapter have been submitted for the publication

5.1 Introduction

Milk is an essential commodity of daily needs. A country like India, where most of the population earn their livelihood from livestock and agriculture has become a major place for dairy industries. India has ranked one in milk production worldwide. Milk is collected from villages and then it is processed further for production of different milk products in dairy industries. Dairy effluents are among the listed source of water pollution (Kwangdinata et al. 2014; Al-Hussieny et al. 2017). However, dairy wastewater (DWW) is one of the most explored suitable nutrient medium used in microalgae cultivation as it contains sufficient phosphorus and nitrogen (Kothari et al. 2013; Lu et al. 2015; Choi 2016). Usually, physico-chemical treatment of DWW is expensive because in these technologies high energy input is needed (Lu et al. 2015). Various biological methods involving bacteria, fungi, etc., have been applied by various researchers for the treatment of DWW (Kothari et al. 2012; Ummalyma and Sukumaran 2014; Ding et al. 2015). Microalgae have been explored widely worldwide for bioenergy feedstock production due to their fast growth and high lipid content (Chokshi et al. 2016; Shoener et al. 2019). Microalgae have an advantage over other microorganisms involved in remediation process of different effluents, as they can utilize carbon dioxide from the environment (Chiu et al. 2015; Hoh et al. 2016; Gupta et al. 2017). Simultaneously microalgae are able to generate triglycerides rich biomass as a third-generation feedstock suitable for bioenergy production (Pancha et al. 2015; Chandra et al. 2019). Consortium of microalgae-bacteria and individual microalgae like *Chlorella pyrenoidosa*, *Chlorella vulgaris, Chlamydomonas sp. and Scenedesmus obliqus*, etc., have been demonstrated by various researchers by growing them on wastewaters from different sources like municipal, industrial and synthetic wastewater to produce biodiesel and wastewater remediation (Abdel-Raouf et al. 2012; Gao et al. 2015; Hena et al. 2015; Gonçalves et al. 2017; Paranjape et al. 2016). Significant lipid content of microalgae makes them suitable for biodiesel production as well as they do not create food security issues as in the case of food crops (García et al. 2018). Nutrients cost and high energy input are still the major constraints

for commercial use of microalgae in bioenergy production (Pires et al. 2013). Many authors have told that microalgae Poly-microalgae cultures are able to increase cultivation system sustainability as they can compensate cultivation loss of one of the microalgae species during cultivation (Pires et al. 2013; Sarkar et al. 2006; Baglieri et al. 2016; Jafari et al. 2018). Microalgae remediation successfully has been implied to treat landfill leachate and piggery wastewater (Chang et al. 2019; Nair et al. 2019; Leite et al. 2019). Thus, poly-microalgae cultures can help in improved remediation of wastewater and higher microalgae biomass production. Still, intensive studies are needed to develop promising poly-microalgae cultures for efficient wastewater remediation and sustainable biodiesel production. In the present work four microalgae strains namely *C. minutissima*, *S. abundans*, *N. muscorum*, *Spirulina* sp. and their poly-cultures were cultivated on DWW in batch mode to compare biomass and lipid yields of poly-microalgae cultures with mono-microalgae cultures.

5.2 Materials and methods

5.2.1 Microalgae strains and consortia

Culture of *C. minutissima* was procured from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India while *S. abundans*, *N. muscorum* and *Spirulina* sp. were procured from National Chemical Laboratory, Pune, Maharashtra, India. All the cultures were maintained in BG-11 medium (composition same as described in chapter 2 of this thesis) under continuous illumination of 2500 lux in 250 mL conical flasks at 25 ± 1°C. All the reagents were procured from Hi Media Laboratories, Mumbai, India. All the experiments were performed in triplicate.

5.2.2. Characterization and pretreatment of raw DWW

Collection of DWW was done at SMC foods, private, limited, Nanauta, Saharanpur, Uttar Pradesh, India, while secondary treated wastewater (STWW) was collected from local sewage treatment plant located at Saharanpur, Uttar Pradesh, India. Samples were filtered and stored in the laboratory at 4°C. The three (40:60, 70:30 and 100:0, DWW:STWW, v/v) concentrations of DWW were prepared by mixing it with STWW. The filtered samples were autoclaved for 20 min at 121 °C. Characteristics of the autoclaved DWW diluted with STWW are recorded in Table 5.1.

Table 5.1 Characteristics of different concentrations of autoclaved DWW

Parameters (mg/L)	DWW		
	100%	70%	40%
pH*	9.15 ± 0.2	8.3 ± 0.1	7.6 ± 0.2
BOD	2178 ± 10	1953 ± 7	862 ± 5
COD	2843 ± 13	2581 ± 11	1467 ± 10
Total nitrogen	105 ± 3	97 ± 2	45 ± 1.5
Total phosphorus	36 ± 3	31 ± 2	14 ± 1
Suspended solids	1586 ± 18	571 ± 15	378 ± 12

*pH is unitless.

BOD, COD and suspended solids (SS)were measured as per standard protocols described in (APHA, 2005). TN and TP were measured using a spectrophotometer, Shimadzu 1800.

5.2.3 Microalgae cultivation and optimization of microalgae growth on DWW

Mono-microalgae cultures of *C. minutissima, S. abundans, N. muscorum* and *Spirulina* sp. and their poly-cultures as *C. minutissima* + *N. muscorum* (CN), *C. minutissima* + *N. muscorum* + *Spirulina* sp. (CNSS) and *S. abundans* + *N. muscorum* + *Spirulina* sp. (SNSS) were cultivated on three (40, 70 and 100%) concentrations of DWW diluted using STWW in 500 mL conical flasks with working volume of 300 mL at 27 ± 1 °C, 7.5 ± 0.3 pH at (12:12, light:dark) h photoperiod for 12 days. Poly-microalgae cultures were prepared by mixing 3 mL of each constituent monoculture. All the cultures were exposed to light intensity of 10000 lux using cool white fluorescent tubes. Aliquots of 5 mL of mono-microalgae culture and poly-microalgae cultures were used as inoculum.

5.2.4 Determination of biomass growth

Samples of 5 mL from each flask were withdrawn and filtered through pre-weighed Whatman GF/C glass fibre filter paper. Filter papers with microalgae biomass were rinsed using double distilled water and oven-dried at 80 °C till attainment of constant weight to determine dry microalgae biomass weight. Biomass yield and biomass productivity were calculated as follows:

$$Y = DCW/V_L \quad (5.1)$$

$$BP = (DCW_f - DCW_i/T) \quad (5.2)$$

where, Y, DCW_f, DCWi, V_L, BP and T are biomass yield (g/L), dry cell weight final (g), dry cell weight initial (g), sample volume (L), biomass productivity (g/L-d) and cultivation period (d), respectively.

5.2.5 Determination of total lipid concentration (g/L), lipid content (%), and lipid productivity (mg/L-d)

Dried microalgae biomass was converted into fine powder form using a mortar and pestle. Lipids were extracted using chloroform and methanol in the ratio of (2:1, v/v) overnight at 25 °C with constant shaking and the resultant slurry was filtered through 0.22 μm Whatman GF/C glass fibre filter paper. The filtrate, containing lipids was transferred to pre-weighed weighing bottles. The weighing bottles were dried under vacuum and were weighed again to estimate the total lipid concentration (g/L). The lipid content (%) was computed using the equation as given below:

$$LC = \frac{L_W}{DCW} \quad (5.3)$$

where LC is total lipid content (%) and L_w is the weight of extracted lipids and DCW is dry cell weight of microalgae biomass (g).

Lipid productivity was calculated using the following equation:

$$LP = \frac{LC \times BP}{100} \quad (5.4)$$

where, LP, LC and BP are lipid productivity (mg/L-d), lipid content (%) and biomass productivity (g/L-d), respectively.

5.2.6 Fatty acid methyl ester (FAME) analysis

Extracted lipids were transferred to a round bottom flask having toluene, and 1% sulphuric acid and methanol as (1:2, v/v). The reaction temperature was set at 50 °C overnight with constant stirring using a magnetic stirrer with a hot plate. Extraction of FAMEs was done using hexane. To explore FAME profile GC-MS study of FAME was performed as reported by (Patel et al. 2015). GC–MS (Agilent, Santa Clara, CA, USA) equipped with DB-5MS capillary column (30 m × 0.25 mm ID and 0.25 μm film thickness) was used. Split-less injection mode (1 μl at 250 °C) was set and helium was used as carrier gas (1 mL min^{-1}). The column temperature was kept at 50 °C for 1.5 min and then it was elevated (25 °C min^{-1}) to 180 °C and held for 1 min. After that, the temperature was raised to 220 °C at a rate of 10 °C min^{-1} and was held for 1 min. Finally, the temperature of the

column was increased (15 °C min^{-1}) to 250 °C and kept constant for 3 min. Temperature values of 250 °C and 200 °C were set for mass transfer line and ion source, respectively. The electron ionization potential of 70 eV and 50–600 m/z scan mode were set to detect FAMEs.

5.2.7 Pollution load removal

Samples of 5 mL at every two days were collected and at 3000 rpm were centrifuged for 10 min. Supernatant was filtered through pre-weighed Whatman GF/C glass fibre filter paper. The pollution load removal (%) was calculated as follows:

$$Removal\ (\%) = \frac{P_i - P_f}{P_i} \times 100 \quad (5.5)$$

where, P_i and P_f are initial and final values (mg/L) of a particular parameter at the sampling time, respectively.

5.3 Results and discussion

5.3.1 Biomass growth of mono and poly-microalgae cultures

It is evident from Fig. 5.1(a and b), the mono and poly-microalgae cultures cultivated on different concentrations of DWW, maximum biomass growth by all the microalgae cultures was observed cultivated on 70% DWW. Microalgae cultures cultivated on 40 and 100% DWW, failed to achieve higher biomass growth in comparison to 70% DWW. The possible reason behind this fact may be the low availability of nutrients in 40% DWW and excess availability of nutrients in 100% DWW. In such cultivation environment microalgae cells could not adopt properly and resulted in lower biomass growth. Biomass growth profiles for different microalgae cultures are illustrated in Fig. 5.1(a and b).

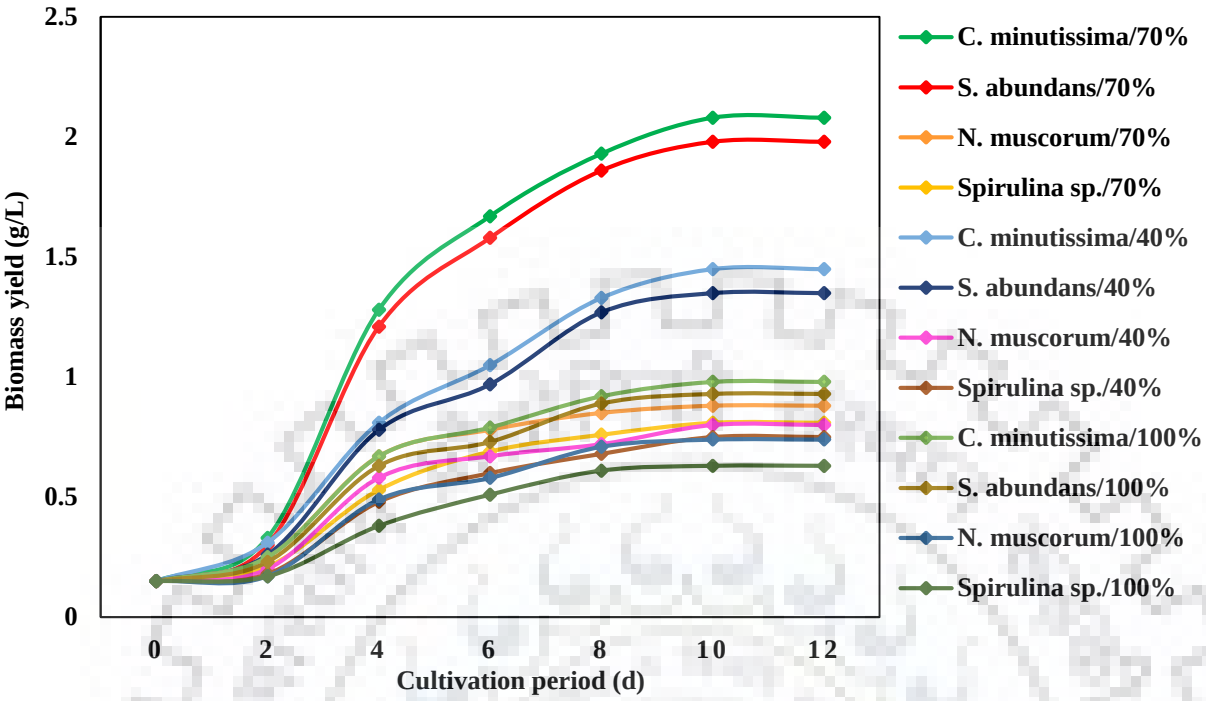

Fig. 5.1a Biomass growth curve of mono-microalgae cultures grown on different concentrations of DWW

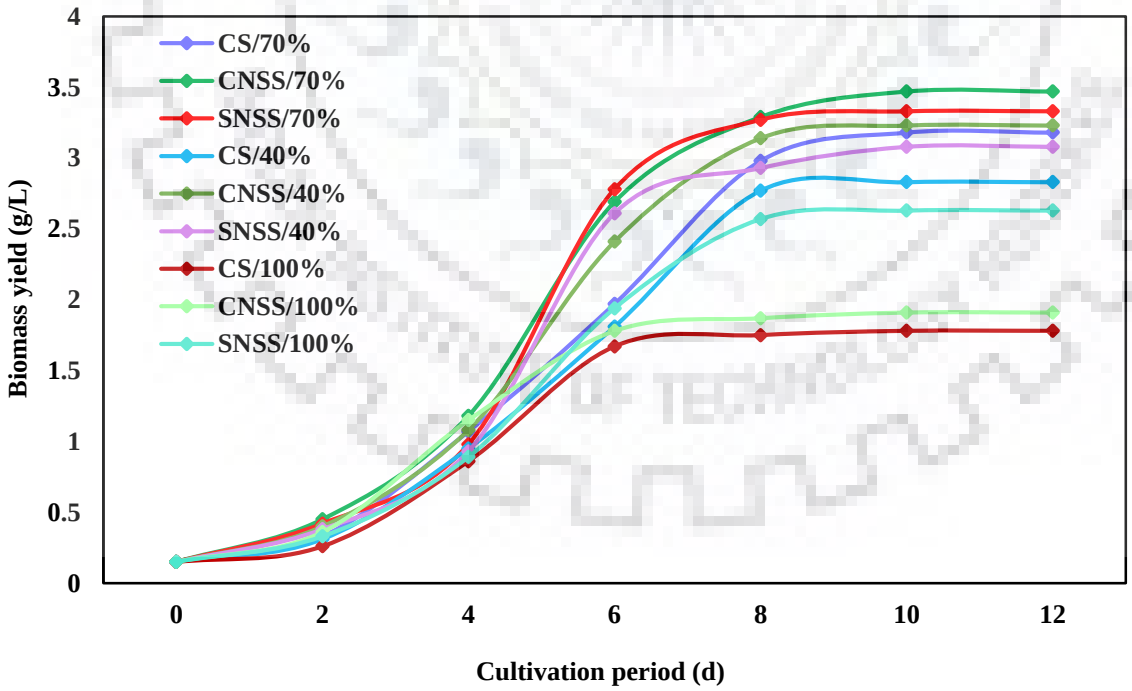

Fig. 5.1b Biomass growth curve of poly-microalgae cultures grown on different concentrations of DWW

It has been observed that the biomass growth started with a lag phase of 2 days for all the microalgae cultures. After that, all the cultures entered into exponential growth phase which lasted up to 10^{th} d of cultivation. Among all the poly-microalgae cultures the highest biomass yield (3.47 ± 0.07 g/L) was observed with CNSS followed by SNSS and CS for all concentrations of DWW while in case of mono-microalgae cultures *C. minutissima* attained highest biomass (2.56 ± 0.10 g/L) followed by *S. abundans*, *N. muscorum* and *Spirulina* sp. The biomass yield of microalgae depends on how fast cellular growth takes place. Microalgae with high biomass yield usually have shown high biomass productivity. In this work, all the microalgae cultures cultivated on 70% DWW achieved the highest biomass yield, biomass productivity and lipid yield. Biomass yield. lipid yield and biomass productivity of all the poly-microalgae cultures were higher than mono-microalgae cultures. Poly-microalgae cultures CNSS achieved the highest biomass productivity of 332 mg/L-d with lipid yield of 496.32 ± mg/L and lipid productivity of 49.63 mg/L-d. In the case of mono-microalgae cultures, *C. minutissima* attained the highest biomass productivity of 241 mg/L-d with lipid yield of 291.87 ± 0.071 mg/L, and lipid productivity 29.18 mg/L-d. It is interesting to note that poly-microalgae cultures CNSS and SNSS achieved higher biomass yield than poly-microalgae culture CS. It may be due to fast nitrogen assimilation by cyanobacteria (*N. muscorum* and *Spirulina* sp.) as cyanobacteria are exceptionally capable in nitrogen fixation. Poly-microalgae cultures CNSS and SNSS have cyanobacteria in association with them. Thus, these poly-microalgae cultures might have consumed nitrogen from the cultivation medium at a faster rate fixed by cyanobacteria along with other nutrients. In the case of poly-microalgae culture CS, which do not have association with cyanobacteria could not show higher biomass and lipid yield in comparison to poly-microalgae cultures. It can be attributed to that the two different microalgae strains of the same group might have shown competition to utilize substrates from cultivation medium for their metabolic activities. This competition might be one of the possible reason for less biomass growth of poly-microalgae culture CS. This competition may be one of the reasons for slow cellular biomass growth of poly-microalgae culture CS. Microalgae store acyl glycerides during the photosynthesis and utilize it during respiration to derive energy for cellular activities like cell division, etc. (D'Alessandro and Antoniosi Filho 2016). It is well established that the nitrogen limitation in microalgae cultivation increases lipid synthesis but reduced biomass growth and thus lower lipid productivity (Converti et al. 2009). Data for biomass yield, lipid yield, biomass productivity, lipid productivity, and lipid content of all the microalgae cultures used in this work are presented in Table 5.2.

Table 5.2. Biomass and lipid yield of poly and mono microalgae cultures cultivated on different concentrations of DWW

Microalgae culture (% DWW)	Biomass yield (g/L)	Biomass productivity (mg/L-d) *	Lipid yield (mg/L) *	Lipid productivity (mg/L-d) *	Lipid content (%)
CNSS (40% DWW)	3.23 ± 0.06	308	435.41 + 0.028	43.54	13.48
CNSS (70% DWW)	3.47 ± 0.07	332	496.32 + 0.065	49.63	14.30
CNSS (100% DWW)	1.91 ± 0.03	176	240.32 + 0.029	24.03	12.58
SNSS (40% DWW)	3.08 ± 0.06	293	391.31 + 0.045	35.79	11.62
SNSS (70% DWW)	3.33 ± 0.05	318	461.29 + 0.094	46.12	13.85
SNSS (100% DWW)	1.87 ± 0.04	172	230.20 + 0.041	23.02	12.31
CS (40% DWW)	2.83 ± 0.03	268	362.23 + 0.073	36.22	12.79
CS (70% DWW)	3.18 ± 0.14	303	419.79 + 0.049	41.97	13.20
CS (100% DWW)	1.78 ± 0.03	163	208.37 + 0.038	20.83	11.70
C. minutissima (40% DWW)	2.21 ± 0.06	206	232.23 + 0.016	23.22	10.50
C. minutissima (70% DWW)	2.56 ± 0.10	241	291.87 + 0.071	29.18	11.40
C. minutissima (100% DWW)	1.23 ± 0.04	108	116.85 + 0.055	11.68	9.50
S. abundans (40% DWW)	2.08 ± 0.12	193	208.16 + 0.033	20.81	10
S. abundans (70% DWW)	2.45 ± 0.08	230	269.49 + 0.11	26.97	11
S. abundans (100% DWW)	1.15 ± 0.08	100	107.55 + 0.075	10.75	9.35
N. muscorum (40% DWW)	0.8 ± 0.06	65	50.11 + 0.11	5.01	6.26
N. muscorum (70% DWW)	0.88 ± 0.08	73	64.14 + 0.032	6.41	7.28
N. muscorum (100% DWW)	0.74 ± 0.06	59	51.15 + 0.01	5.11	6.91
Spirulina sp. (40% DWW)	0.75 ± 0.08	60	39.83 + 0.051	3.98	5.31
Spirulina sp. (70% DWW)	0.81 ± 0.07	66	56.34 + 0.038	5.63	6.95
Spirulina sp. (100% DWW)	0.63 ± 0.06	48	40.97 + 0.021	4.09	6.50

** Estimation of biomass productivity was done on the basis of the average value of biomass yield divided by cultivation period, similarly, lipid productivity was estimated on the basis of the average value of lipid yield divided by the cultivation period and lipid content was estimated on the basis of the average value of lipid yield divided by dry cell weight. So, values of biomass productivity, lipid productivity and lipid content are reported as a single value without standard deviation.*

Shen et al. (2008) reported similar results for the cultivation of *Botrycoccus* sp. on dairy wastewater. Growth of microalgae is directly proportional to nutrients profile of cultivation medium

and other operating parameters. Qin et al. (2016) reported biomass yield (5.41 g/L) and lipid productivity (150.6 mg/L-d), respectively in consortia of *Chlorella* sp., *Scenedesmus* sp. and *Chlorella zofingiensis* cultivated on DWW in a bubble column reactor. These results are in good agreement with the present work. Bhatnagar et al. (2011) reported biomass yield of 342 mg/L for consortia of *Chlorella globosa*, *C. minutissima*, and *Scenedesmus bijuga* cultivated on poultry litter extract. Kothari et al. (2012) achieved biomass yield and lipid productivity of 6.8 g/L and 3.5 mg/L-d, respectively, when *Chlorella pyrenoidosa* was cultivated on dairy effluent. Data presented in Table 5.3a shows that all the poly-microalgae cultures achieved higher biomass and lipid yield in comparison to mono-microalgae cultures. It was observed that poly-microalgae cultures for wastewater remediation have been more efficient because of nutrients removal for different microalgae species have different metabolic processes and the system become versatile toward different environmental conditions (Johnson and Admassu 2013; Pancha et al. 2015). It became feasible due to that the different individuals of consortium can adopt a symbiotic mode of action which make poly-microalgae cultures more potent for nutrients removal and resulting in more biomass production and lipid synthesis, etc. (Renuka et al. 2013).

5.3.2. Effect of photoperiod on biomass and lipid yield of microalgae cultures

In general, microalgae cells in mass cultures are subject to light availability as light is absorbed and reflected back by microalgae cells. Light period duration is extremely important for photoautotrophic growth of microalgae. During photosynthesis, light energy stimulates the synthesis of sugars to derive nutrition for cellular machinery to support various metabolic activities and biomass growth of microalgae. Sugars are synthesized by assimilation of inorganic carbon, usually obtained from ambient CO_2, into organic carbon. Upto a certain limit of light intensity, biomass growth depends on light irradiance but at light saturation point excess light irradiance does not affect photosynthesis of microalgae. It has been observed that exposure to higher light irradiance can cease vital metabolic activities of microalgae cells as higher light irradiance can cause an increase in temperature of the system. This usually happens due to the damage of photosynthetic receptor system of microalgae cells and no photosynthesis takes place (Formaggio et al. 2001; Perrine et al. 2012; Ota et al. 2015). *Nanochloropsis salina* obtained biomass of 0.75 g/L biomass yield when the culture was exposed to 24:0 h light/dark cycle and decrease in biomass (from 0.70 to 0.52 g/L), when the light period was reduced from 18:06 to 12:12 h light/dark cycle, respectively (Sirisuk et al. 2018). In the current study to see the effect of photoperiods (16:08, 18:06 and 24:00 h, light:dark) on

biomass and lipid yield, all the microalgae cultures were cultivated on 70% DWW and obtained data have been recorded in Table 5.3(a and b). It is obvious from Fig. 5.2(a and b) that the photoperiod (18:06) h is most suitable for maximum biomass growth and lipid production in all the microalgae cultures.

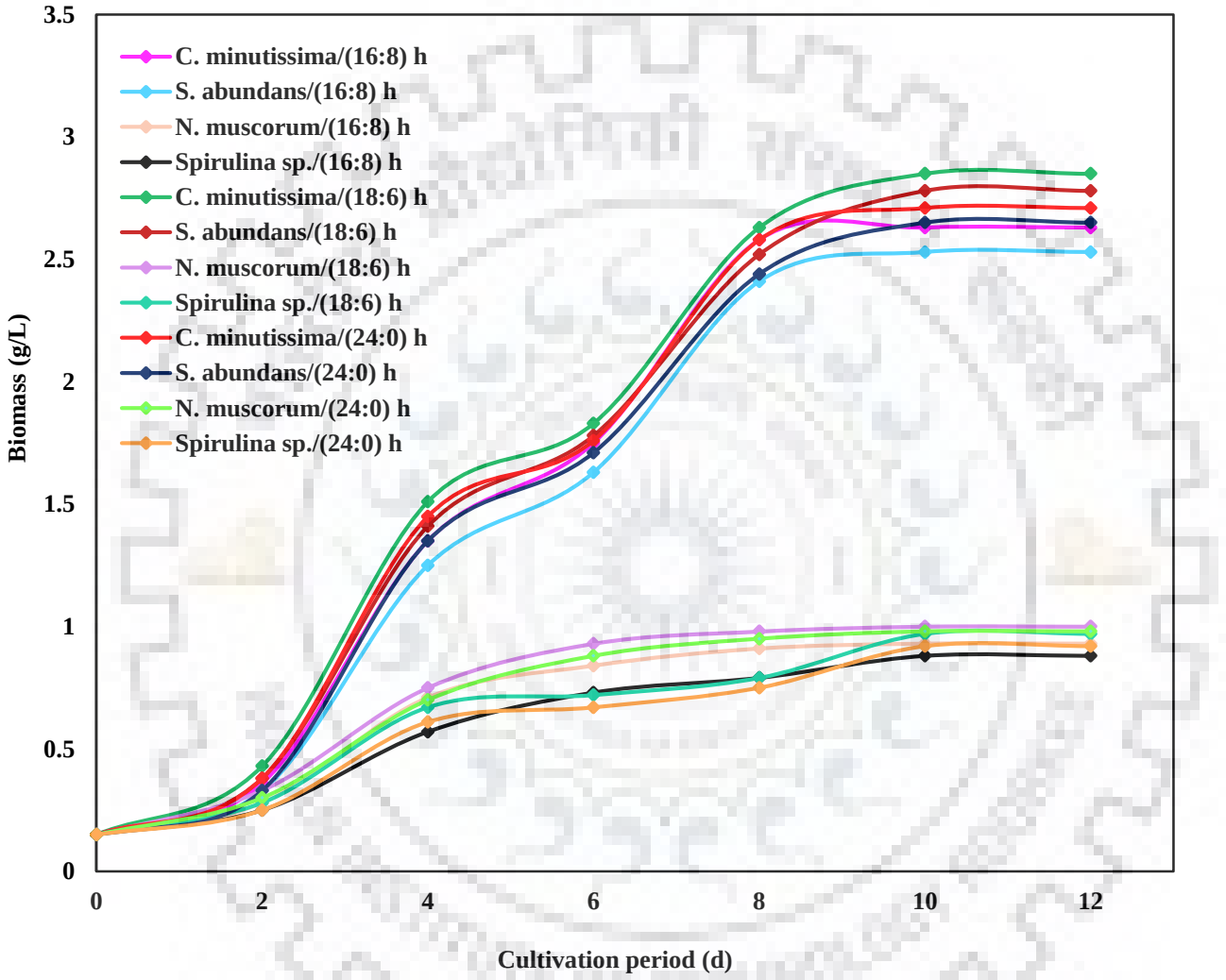

Figure 5.2a Biomass growth of mono- microalgae cultures cultivated on 70% DWW at different photoperiods

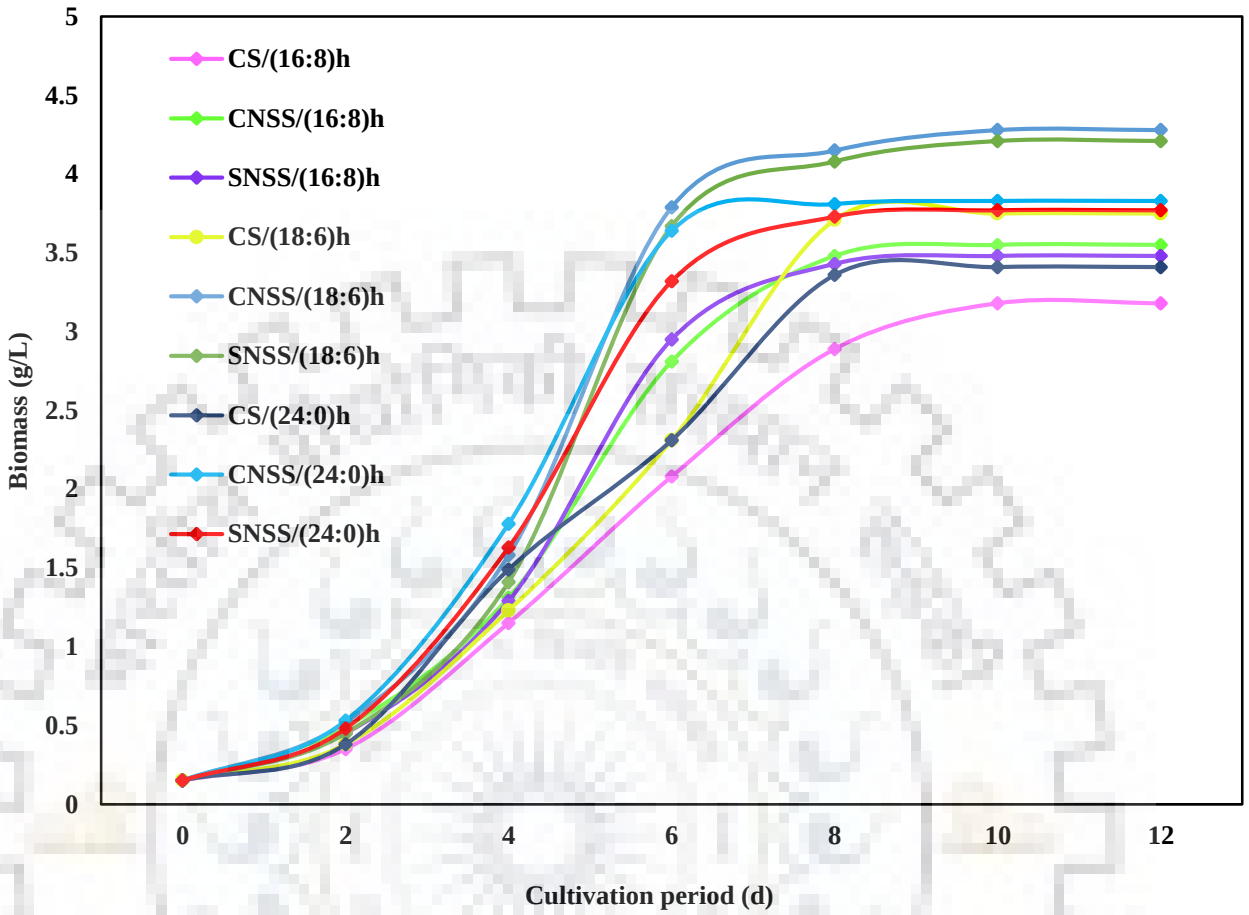

Figure 5.2b Biomass growth of poly-microalgae cultures cultivated on 70% DWW at different photoperiods

Poly-microalgae culture CNSS achieved maximum biomass yield of 4.28 ± 0.07, 3.83 ± 0.05 and 3.55 ± 0.05 g/L with lipid yield of 741.35 ± 0.049, 612.18 ± 0.032 and 582.86 ± 0.061 mg/L at 18:06, 24:00 and 16:08 h photoperiod, respectively on the 10th day of cultivation. Values of biomass and lipid yield for all the microalgae cultures are presented in Table 5.3(a and b) for different photoperiods.

Table 5.3a Biomass and lipid yield of poly-microalgae cultures cultivated on 70% DWW at different photoperiods

Photoperiod (h)	CNSS					SNSS					CS				
	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)
16:08	3.55 ± 0.05	340	582.86 + 0.061	58.28	16.41	3.48 ± 0.04	333	559.31 + 0.050	55.93	16.07	3.18 ± 0.05	303	548.59 + 0.041	54.85	17.25
18:06	4.28 ± 0.07	413	741.35 + 0.049	74.13	17.32	4.21 ± 0.06	406	617.62 + 0.037	61.76	14.67	3.75 ± 0.04	360	461.32 + 0.053	46.13	12.30
24:00	3.83 ± 0.05	368	612.18 + 0.032	61.21	15.98	3.77 ± 0.05	364	580.92 + 0.032	58.09	15.40	3.41 ± 0.05	326	521.78 + 0.050	52.17	15.30

Table 5.3b Biomass and lipid yield of mono-microalgae cultures cultivated on 70% DWW at different photoperiods

Photoperiod (h)	*C. minutissima*					*S. abundans*					*N. muscorum*					*Spirulina* sp.				
	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass content (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)
16:08	2.63 ± 0.04	248	322.58 + 0.053	32.26	12.26	2.53 ± 0.04	238	280.28 + 0.026	28	11	0.93 ± 0.03	78	85.12 + 0.026	8.51	9.15	0.88 ± 0.04	70	68.15 + 0.032	6.82	7.75
18:06	2.85 ± 0.06	270	356.21 + 0.043	35.62	12.49	2.78 ± 0.05	263	309.16 + 0.031	30.91	11	1 ± 0.05	85	99.7 + 0.041	9.97	9.97	0.97 ± 0.05	84	74.06 + 0.033	7.41	7.64
24:00	2.71 ± 0.05	256	333.24 + 0.26	33.32	12.29	2.65 ± 0.04	250	318.15 + 0.11	31.81	12	0.98 ± 0.04	83	95.97 + 0.024	9.597	9.79	0.92 ± 0.03	78	68.3 + 0.064	6.83	7.43

Chandra et al. (2019) reported biomass and lipid yield of 1041.18 ± 0.17 and 382.38 ± 0.41 mg/L, respectively, with *C. minutissima* cultivated on modified CHU-13 medium at 18:06 h photoperiod. However, Ruangsomboon (2012) observed four times more biomass at 24:00 h as compared to 12:12 h photoperiod in case of microalga *Botrycoccucs* KMITL 2 cultivated on *chlorella* medium. Here maximum biomass productivity of 413 mg/L-d was observed with poly-microalgae culture CNSS followed by 270 mg/L-d by mono-microalgae culture *C. minutissima* at 18:06 h photoperiod. Microalgae *Aphanothece* sp. showed higher productivity and maximum cell density at (12:12, dark:light) h. in respect of other light and dark cycles of 0:24, 2:22, 4:20, 6:18, 8:16, 10:14, 12:12, 14:10, 16:8, 18:6, 20:4, 22:2, and 24:0 (Jacob-Lopes et al. 2009). In an experiment Lee et al. (2016) have shown that photoperiod (12:6 h dark:light) followed by 12:12 h photoperiod resulted in the production of dry cell weight (282.6 mg/L) and lipid productivity (71.4 mg/L-d) with a consortium of microalgae and bacteria. Sirisuk et al. (2018) observed highest lipid content (58.1% w/w) with *Isochrysis galbana* exposed to continuous illumination in comparison to 48.2 and 35.5% (w/w) at 18:06 and 12:12 h light/dark cycles, respectively. Thus, the effect of photoperiod on biomass growth and lipid yield varies according to the cultivation environment and microalgae species.

5.3.3 Effect of glucose and glycerol on biomass and lipid yield of microalgae cultures

To see the effect of external carbon sources (glucose and glycerol) on biomass and lipid yield of microalgae cultures 4,7 and 10 g/L glucose or glycerol were mixed in separate flasks with 70% DWW at 18:06 h photoperiod. It is well established that many times heterotrophic cultivation has been beneficial in the achievement of improved microalgae biomass (Alkhamis and Qin 2013; Minowa et al. 1995; Miao and Wu 2006). Data collected from these experiments showed that mixing of glucose or glycerol in cultivation medium resulted in enhanced biomass growth and lipid yield. However, maximum biomass and lipid yields were obtained with 10 g/L of glucose for all the microalgae cultures. Effects of glucose or glycerol supply on biomass growth of mono and poly-microalgae are depicted in Fig. 5.3(a-d). Poly-microalgae culture CNSS achieved maximum biomass yield of 5.76 ± 0.06, 4.55 ± 0.05 and 4.31 ± 0.05 g/L with lipid yield of 1152.37 ± 0.065, 929.16 ±0.040 and 869.82 ± 0.057 mg/L when cultivated on 70% DWW having 10, 7 and 4 g/L of glucose, respectively. In case of mono-microalgae cultures, *C. minutissima* cultivated on 70% DWW supplied with 10, 7 and 4 g/L of glucose maximum biomass yields of 3.55 ± 0.08, 3.18 ± 0.06 and 2.88 ± 0.04 g/L with lipid yields of 428.16, ± 0.032, 392.36 ± 0.032 and 387.90 ± 0.020 mg/L, respectively were noted. Data for biomass and lipid

yield of mono and poly- microalgae cultures obtained with different dosages of glucose and glycerol are presented in Table 5.4(a and b).

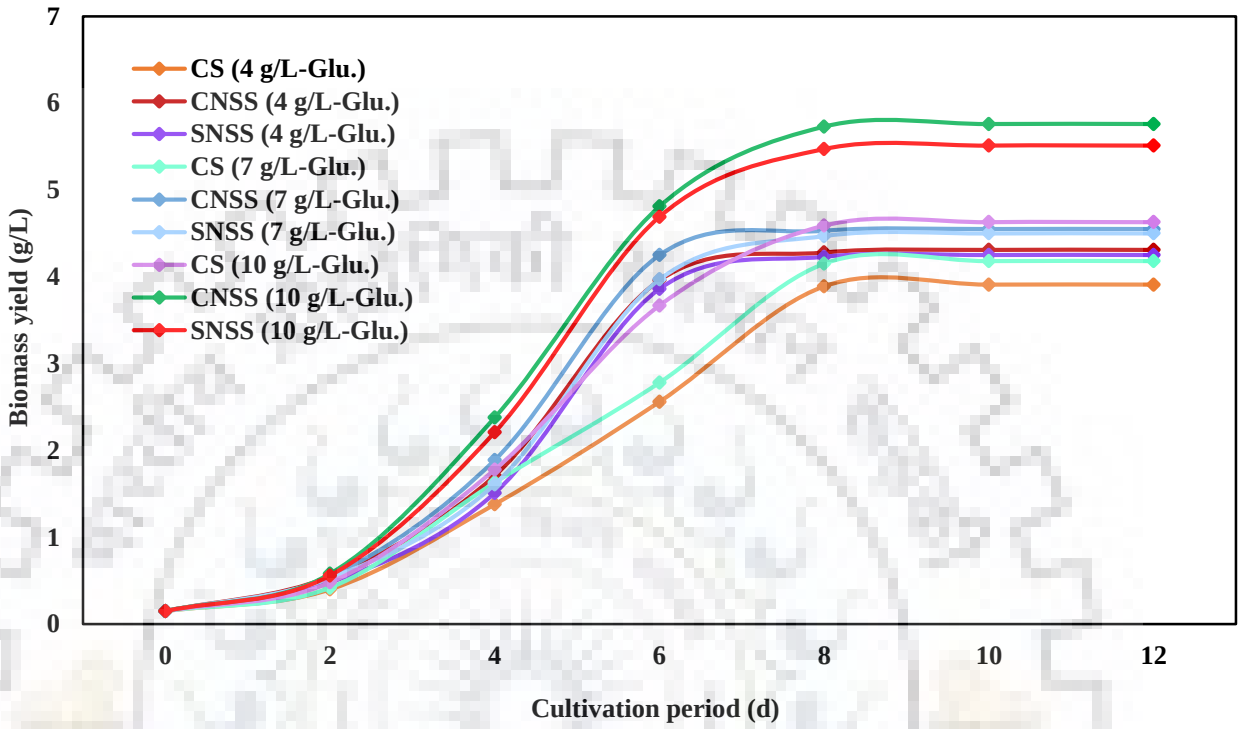

Figure 5.3(a) Biomass growth curves of poly-microalgae cultures at different concentrations of glucose

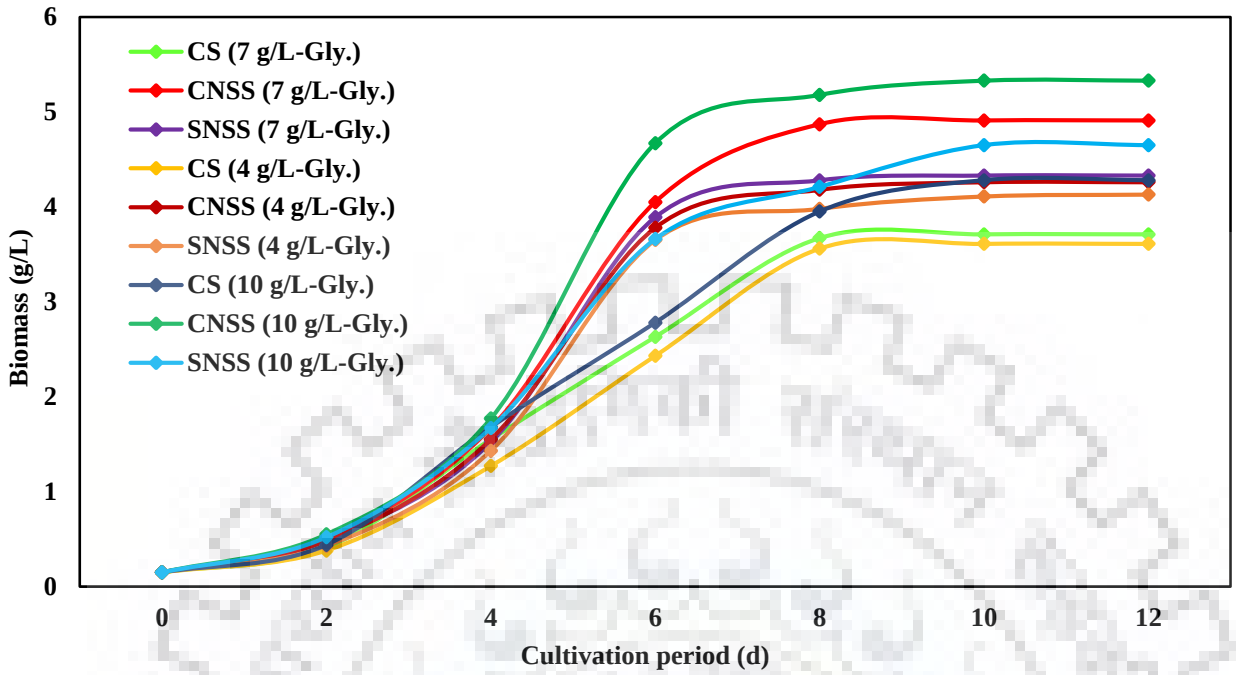

Figure 5.3(b) Biomass growth curves of poly-microalgae cultures at different concentrations of glycerol

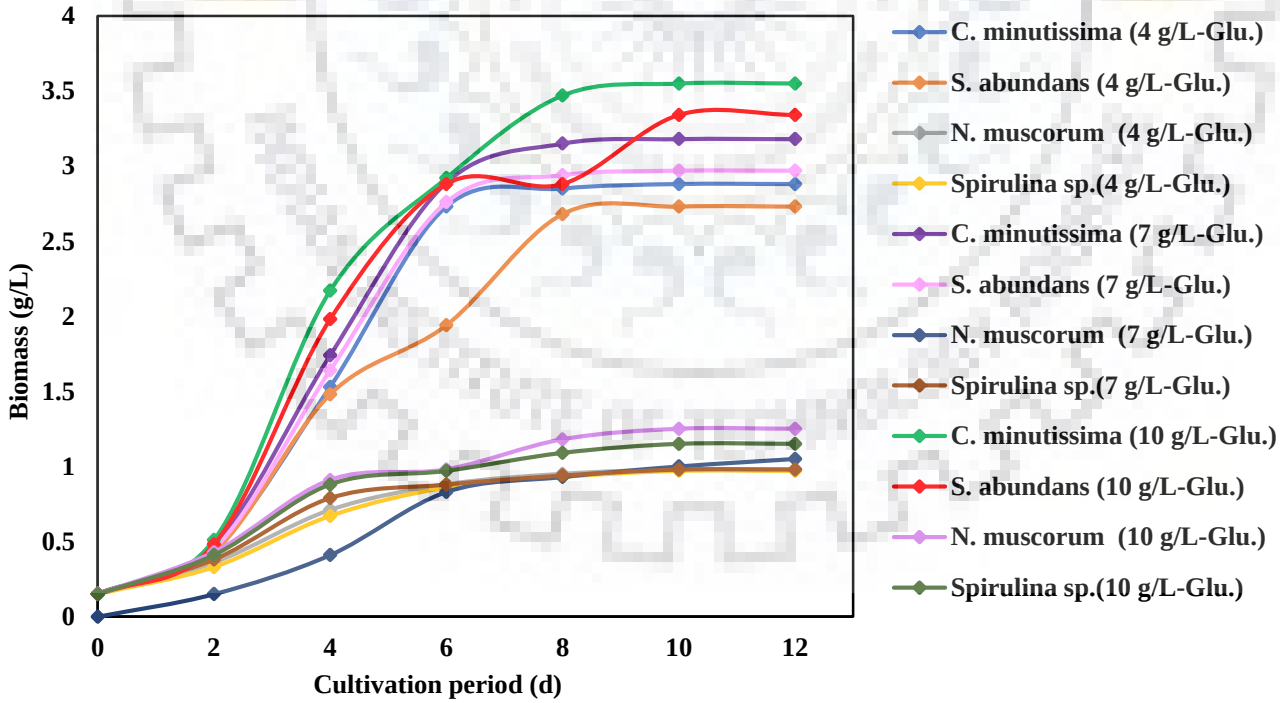

Figure 5.3(c) Biomass growth curves of mono-microalgae cultures at different concentrations of glucose

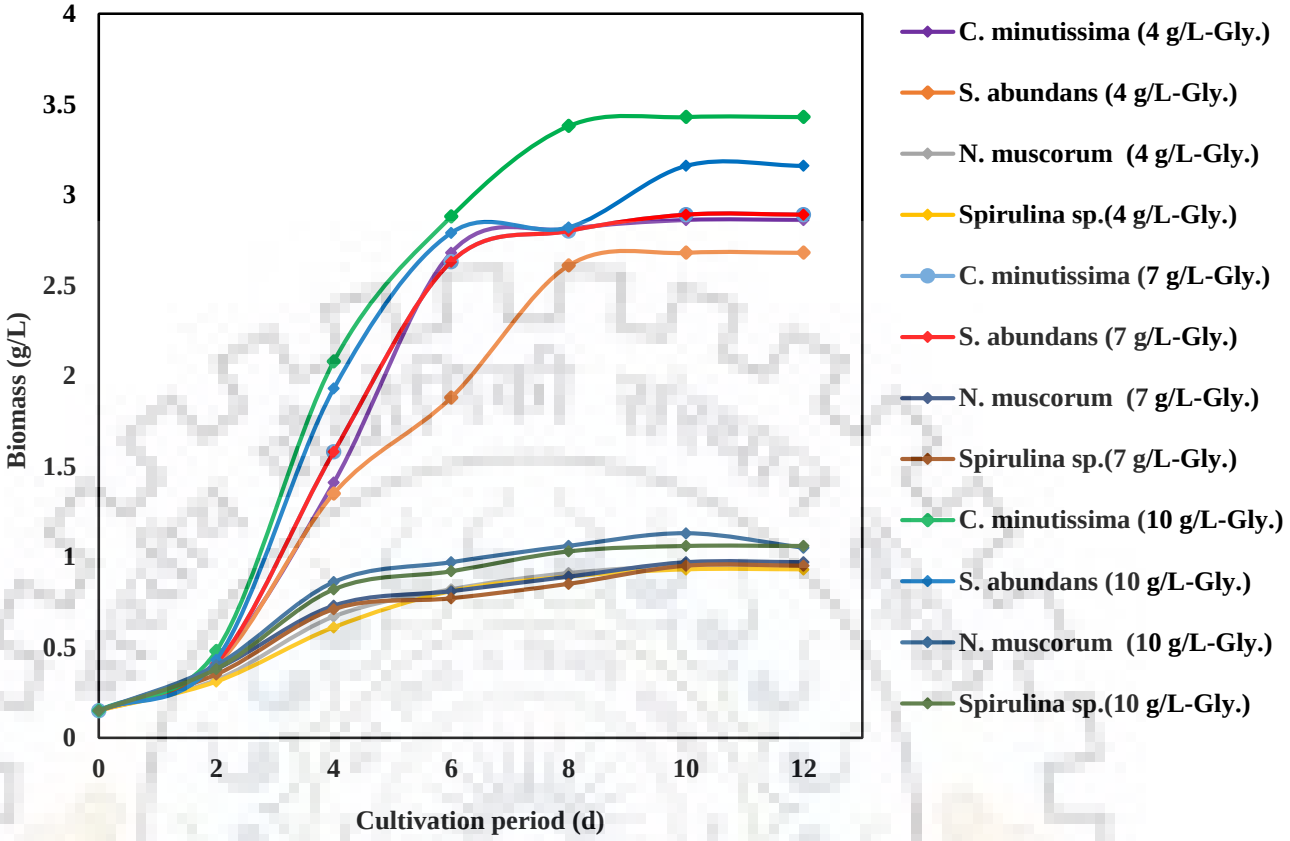

Figure 5.3(d) Biomass growth curves of mono-microalgae cultures at different concentrations of glycerol

Table 5.4(a) Effect of glucose and glycerol supply on biomass and lipid yield of mono-microalgae cultures cultivated on 70% DWW

Carbon source (g/L)	*C. minutissima*					*S. abundans*					*N. muscorum*					*Spirulina* sp.				
	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)
Glucose (4)	2.88 ± 0.04	273	387.90 ± 0.020	38.79	13.46	2.73 ± 0.04	258	306.80 ± 0.030	30.68	11.23	0.98 ± 0.05	0.083	87.89 ± 0.064	8.78	8.96	0.97 ± 0.04	0.082	68.53 ± 0.040	6.85	7.06
Glucose (7)	3.18 ± 0.06	303	392.36 ± 0.032	39.23	12.33	2.97 ± 0.05	282	368.84 ± 0.026	36.88	12.41	1.05 ± 0.06	0.09	104.53 ± 0.047	10.45	9.95	1.08 ± 0.05	0.093	79.32 ± 0.037	7.93	7.34
Glucose (10)	3.55 ± 0.08	340	428.16 ± 0.032	42.81	12.06	3.34 ± 0.06	319	389.76 ± 0.041	38.97	11.66	1.25 ± 0.07	0.11	106.30 ± 0.040	10.63	8.50	1.15 ± 0.03	0.1	85.51 ± 0.061	8.55	7.43
Glycerol (4)	2.74 ± 0.05	259	374.10 ± 0.085	37.41	13.65	2.62 ± 0.05	247	296.1 ± 0.029	29.60	11.30	0.93 ± 0.05	0.078	85.78 ± 0.036	8.57	9.22	0.9 ± 0.04	0.075	66.56 ± 0.038	6.65	7.39
Glycerol (7)	2.96 ± 0.06	281	387.23 ± 0.067	38.72	13.08	2.83 ± 0.06	268	348.38 ± 0.037	34.83	12.31	1 ± 0.06	0.085	101.7 ± 0.081	10.17	10.17	0.98 ± 0.05	0.083	78.18 ± 0.057	7.81	7.97
Glycerol (10)	3.41 ± 0.07	326	420.25 ± 0.020	42.02	12.32	3.24 ± 0.05	309	366.17 ± 0.037	36.61	11.30	1.18 ± 0.08	0.103	105.19 ± 0.043	10.51	8.91	1.06 ± 0.03	0.091	83.96 ± 0.016	8.39	7.92

Table 5.4(b) Effect of glucose and glycerol supply on biomass and lipid yield of poly-microalgae cultures cultivated on 70% DWW

Carbon source (g/L)	CNSS					SNSS					CS				
	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)	Biomass yield (g/L)	Biomass productivity (mg/L-d)	Lipid yield (mg/L)	Lipid productivity (mg/L-d)	Lipid content (%)
Glucose (4)	4.31 ± 0.05	416	869.82 ± 0.057	86.98	20.18	4.25 ± 0.04	0.410	632.44 ± 0.033	63.24	14.88	3.91 ± 0.04	0.376	620.91 ± 0.032	62.09	15.88
Glucose (7)	4.55 ± 0.05	440	929.16 ± 0.040	92.91	20.42	4.50 ± 0.04	0.435	742.53 ± 0.040	74.25	16.50	4.18 ± 0.05	0.403	733.23 ± 0.049	73.32	17.54
Glucose (10)	5.76 ± 0.06	561	1152.37 ± 0.065	115.23	20.00	5.51 ± 0.04	0.536	962.65 ± 0.044	96.26	17.47	4.63 ± 0.04	0.448	807.91 ± 0.024	80.79	17.44
Glycerol (4)	4.26 ± 0.05	411	786.46 ± 0.049	78.64	18.46	4.13 ± 0.04	0.398	588.94 ± 0.028	58.89	14.26	3.61 ± 0.05	0.346	579.45 ± 0.032	57.94	16.05
Glycerol (7)	4.91 ± 0.04	476	828.88 ± 0.057	82.88	16.88	4.33 ± 0.04	0.418	701.85 ± 0.032	70.18	16.20	3.71 ± 0.05	0.356	691.23 ± 0.049	69.12	18.63
Glycerol (10)	5.33 ± 0.05	518	1032.95 ± 0.024	103.27	19.38	4.65 ± 0.04	0.450	922.13 ± 0.024	92.21	19.83	4.28 ± 0.05	0.4130	783.67 ± 0.016	78.36	18.31

Heterotrophic cultivation of microalgae on dairy effluent resulted in increased lipid content when waste glycerol was supplied in dairy effluent. (Ummalyma and Sukumaran 2014). Supply of glucose (5.0 g/L) in piggery wastewater resulted in biomass yield of 2.01 g/L in *Phaeodactylum tricornutum* UTEX #640 (Garcia et al. 2005). Heterotrophic cultivation of *Chlorella sorokiniana* achieved 2 times improved biomass than in autotrophic conditions (Kim et al. 2013). Crude glycerol supply in cultivation medium resulted in two times more biomass productivity (446.50 ± 1.50 mg/L-d) and four times lipid productivity (165.15 ± 0.55 mg/L-d) in *Chlorella* sp. in comparison to Bold's Basal medium (Katiyar et al. 2017). Thus, heterotrophic cultivation of microalgae often has been beneficial for improved biomass growth and lipid production.

5.3.4 Pollution load removal from DWW

Removal of pollution load and nutrients is a basic criterion for assessment of microalgae cultivation in wastewater. Microalgae help in nutrients removal from wastewater as they utilize nitrogen, phosphorus and organic matter for their growth (Jones and Harrison 2014; Katiyar et al. 2019). These nutrients are metabolized by microalgae for the synthesis of nucleic acids and other important compounds required in different metabolic activities (Jafari et al. 2018; Mousavi et al. 2018). During cellular growth, microalgae utilize carbon, nitrogen and phosphorous and thus help in the reduction of capital cost as in case of mechanical treatment (Bux and Chisti 2016; Fito and Alemu 2019). Microalgae remediation of wastewater needs a limited mechanical operation, resulting in reduced treatment cost in comparison to traditional treatment technologies where high energy is required during aeration, etc. (Razzak et al. 2013). In the present work, efficient removals of BOD, COD, TN, TP and SS were achieved in all the concentrations of DWW by all the microalgae cultures. However, poly-microalgae cultures appeared to be more potent in remediation of DWW than mono-microalgae cultures. Figures 5.4(a-d) and 5.5(a-c) show pollution load removal by mono and poly-microalgae cultures cultivated on 70% DWW.

5.3.5. Pollution load removal by mono-microalgae cultures

It was observed that *C. minutissima* and *S. abundans* efficiently removed the pollution load from 70% DWW. About 70% BOD, 56% COD, 53% TN, 80% TP and 70% SS reductions were observed with *C. minutissima* and *S. abundans* in 10 days of cultivation period. However, *N. muscorum* and *Spirulina* sp. do not show significant removal of pollution load as can be seen from

Fig. 5.4 (c and d). They both removed about 38% BOD, 24% COD, 14% TN, 75% TP and 70% SS in 10 days. Kothari et al. (2012)

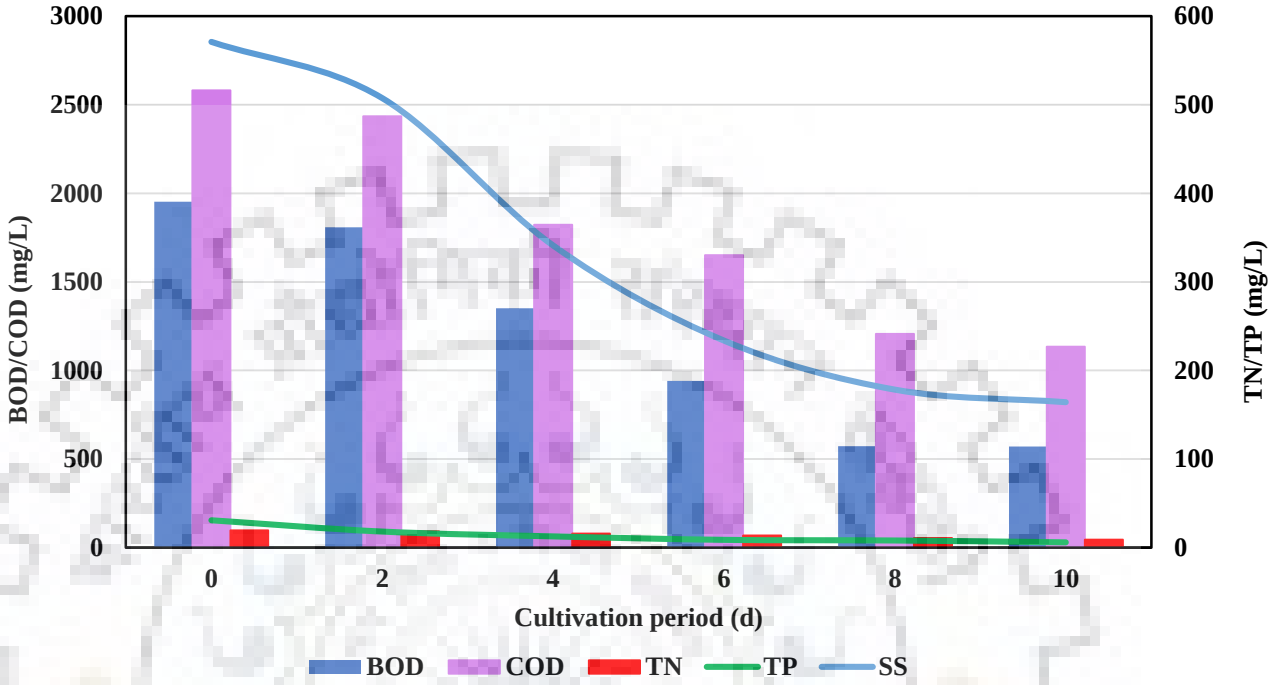

Figure 5.4(a) Pollution load removal by *C. minutissima* from 70% DWW

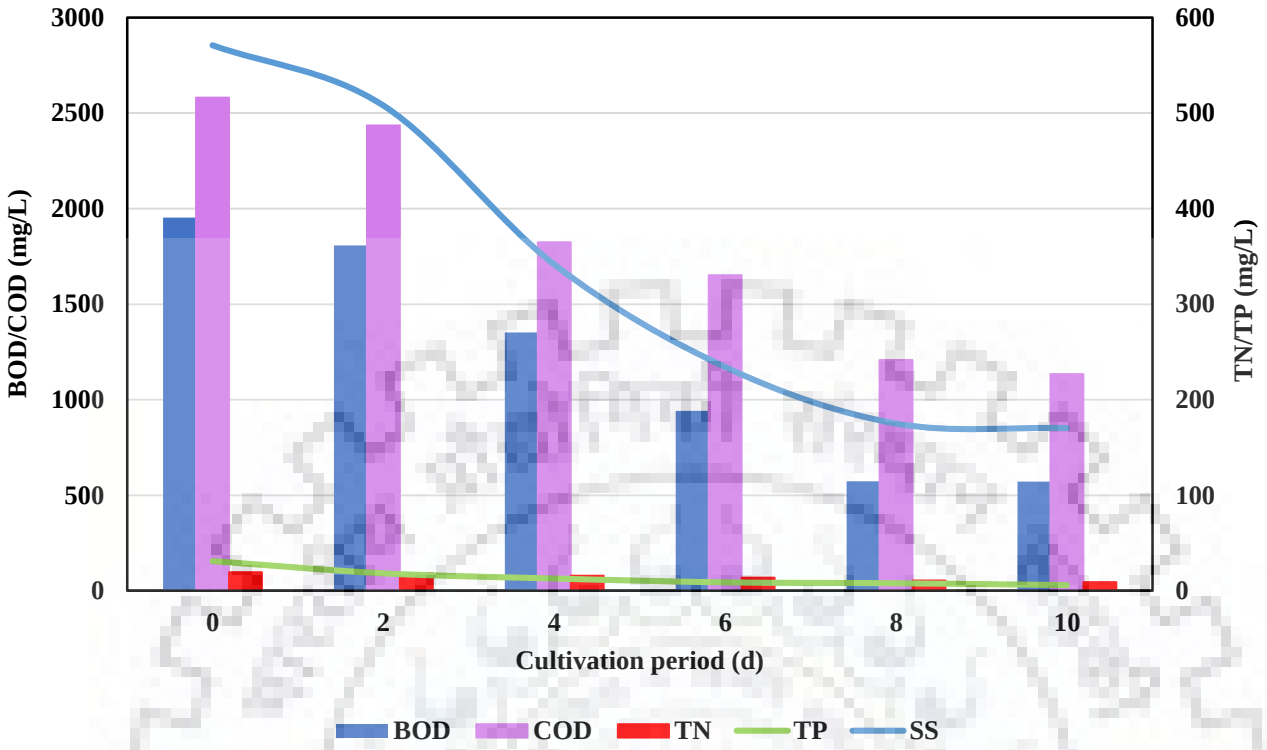

Figure 5.4(b) Pollution load removal by *S. abundans* from 70% DWW

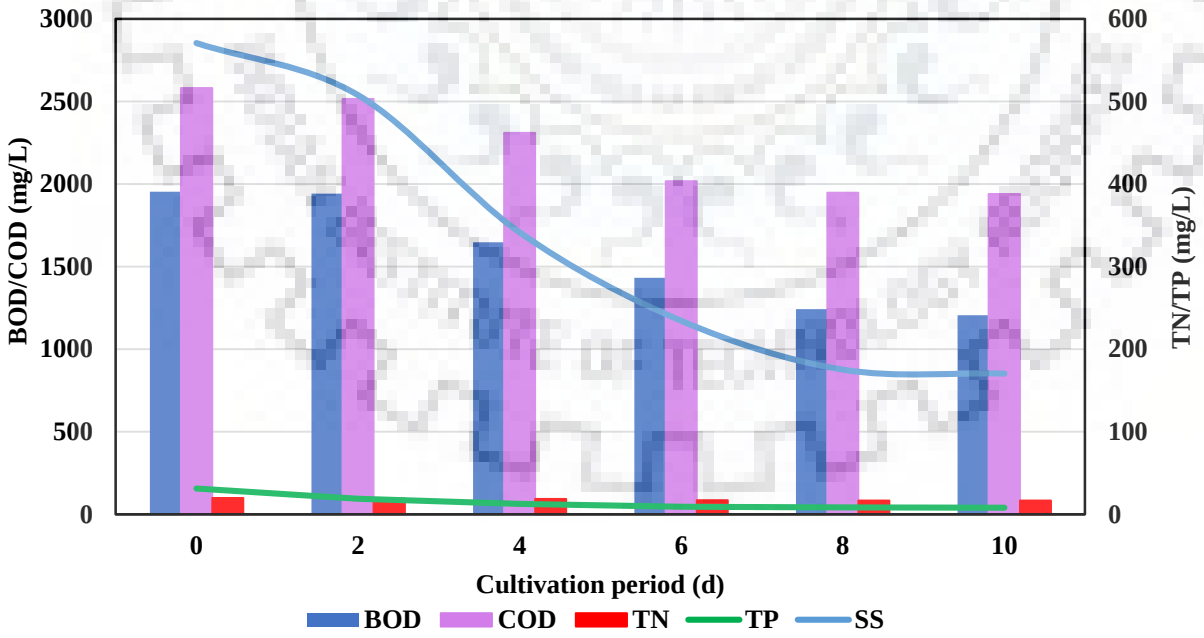

Figure 5.4(c) Pollution load removal by *N. muscorum* from 70% DWW

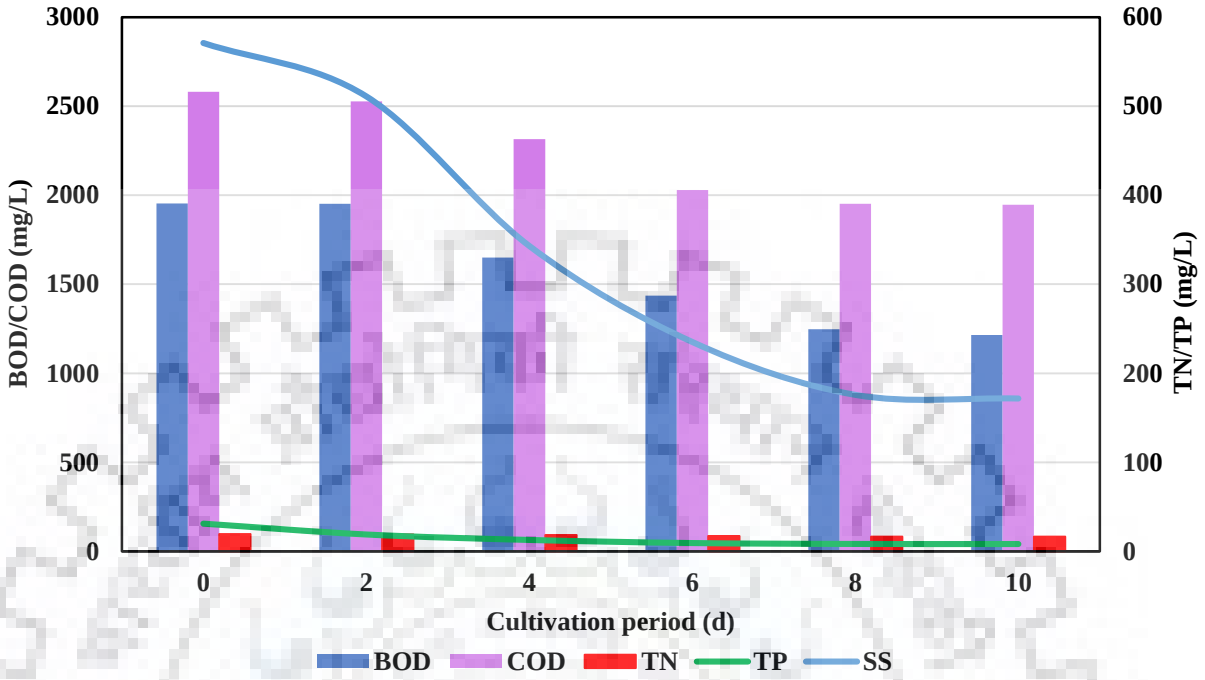

Figure 5.4(d) Pollution load removal by *Spirulina* sp. from 70% DWW

reported 60% nitrate and 87% phosphate removal with *Chlorella pyrenoidosa* from DWW. Consortia of *Chlorella* sp., *Chlamydomonas* sp. and *Scenedesmus* sp. with naturally existing micro flora (bacteria) in municipal wastewater at light intensity of 120 μE/m^2-s, removed TKN 69%, TP 59%, PO_4^{3-}P 73%, COD 84% and BOD_5 85% from municipal wastewater (Fito and Alemu 2019). Another strain of microalgae *Chlorella sorokiniana* removed 46.5 mg/L nitrate and 13.3 mg/L phosphate within 4 days when cultivated on modified BG-11 medium (Kim et al. 2013). Vishwakarma et al. (2019) reported that *Monoraphidium minutum* removed 67.1% phosphorus in 8 days from dairy effluent.

5.3.6 Pollution load removal by poly-microalgae cultures

It is obvious from the nutrients removal profile Fig. 5.5 (a-c) of different poly-microalgae cultures CNSS, SNSS and CS, that they all efficiently removed BOD, COD, TN, TP and SS in the range of 73-75, 59-61, 56-59, 83-84 and 82-84%, respectively from 70% DWW within 10 days of cultivation period. However, highest removal of BOD (75.16%) COD (61.37%) TN (58.76%), TP (84.48%) and SS (84.58%), respectively, was observed with poly-microalgae culture CNSS. Chinnasamy et al. (2010) reported that native microalgae consortium removed 96% nutrients from

carpet mill wastewater. Kothari et al. (2013) reported 90.74 and 70%, nitrate and phosphate removal, respectively by *Chlamydomonas* sp. when cultivated on dairy effluent. In case of poly-microalgae culture SNSS 74.55% BOD, 60.36% COD, 58.31% TN, 83.96% TP and 83.36% SS, respectively removal was observed in 10 days, while Poly-microalgae culture CS in similar conditions removed about 73.73, 59.66, 56.31, 83.29 and 82.83% BOD, COD, TN, TP and SS, respectively. Choi (2016) reported 85.61%, 80.62%, 29.10%, 85.47%, and 65.96% removal of BOD, COD, SS, TN and TP, respectively, in 10 days from DWW using *Chlorella vulgaris*.

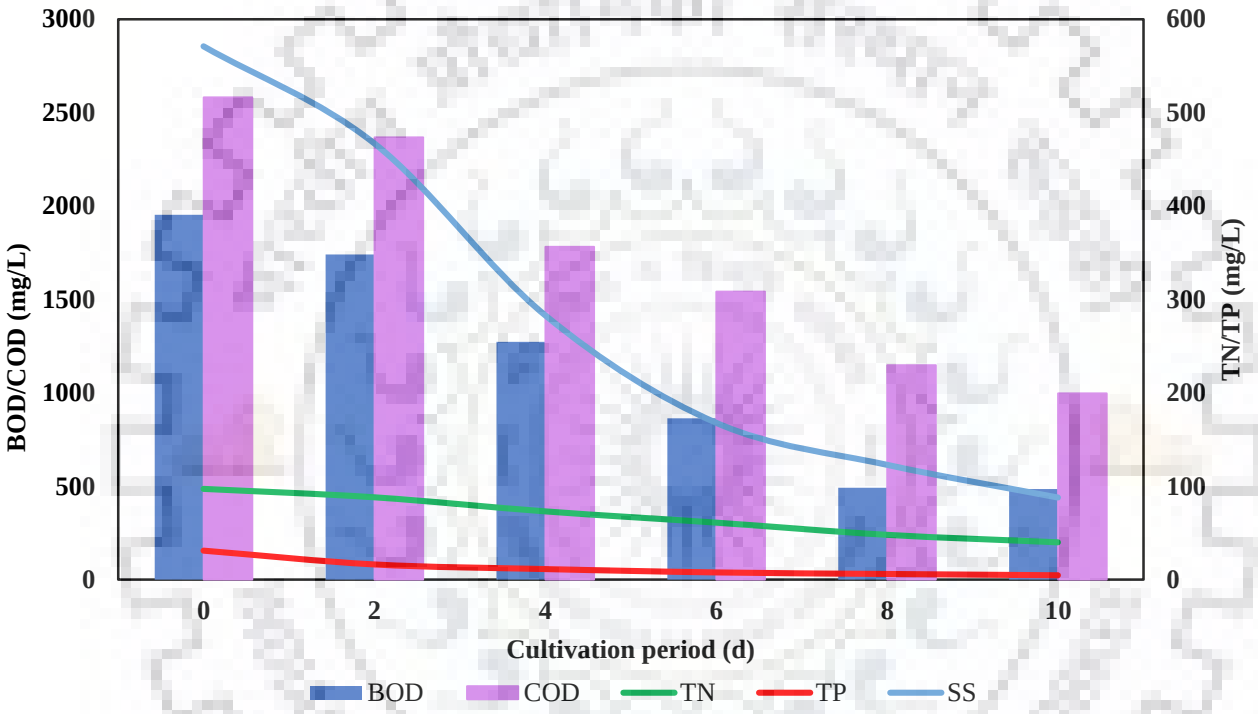

Figure 5.5(a) Pollution load removal by CNSS from 70% DWW

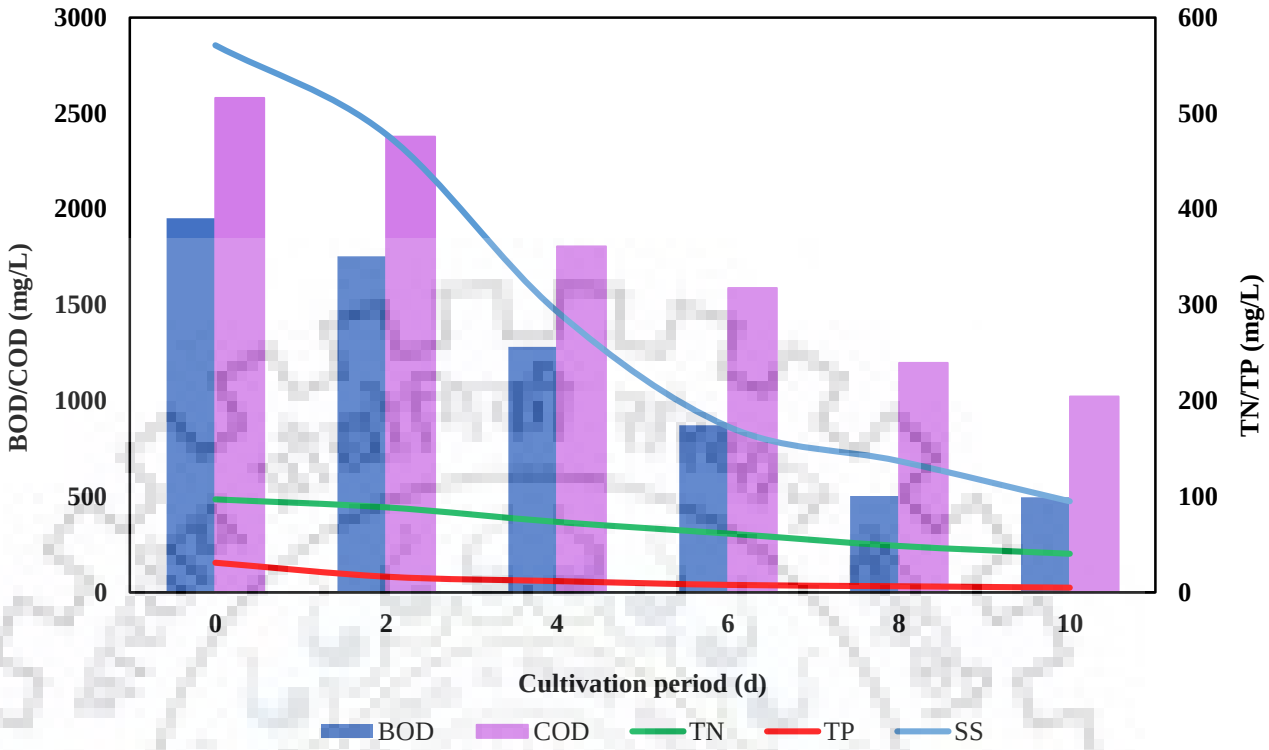

Figure 5.5(b) Pollution load removal by SNSS from 70% DWW

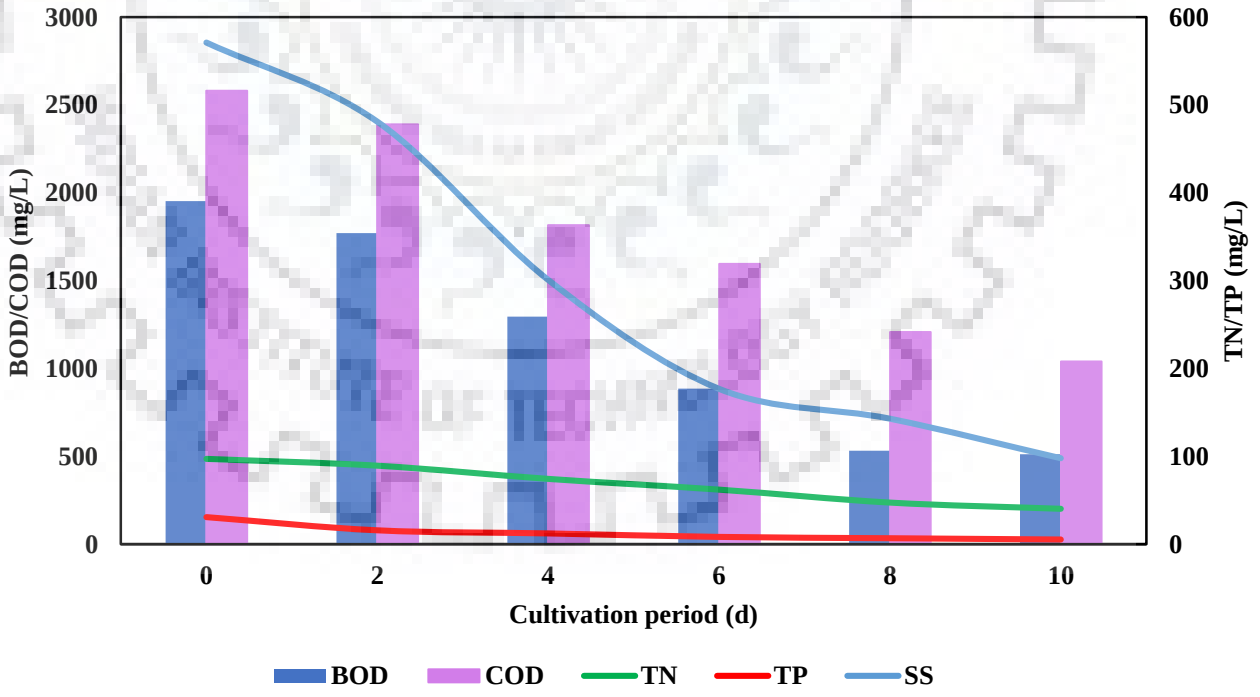

Figure 5.5(c) Pollution load removal by CS from 70% DWW

All the poly-microalgae cultures used in this work, were almost equally efficient in TN and TP removal. Poly-microalgae cultures CNSS, SNSS and CS removed about 58.76, 58.31 and 56.31% TN, respectively in 10 days. TN removal by CNSS and SNSS was slightly higher than CS. This may be due to nitrogen fixation by cyanobacteria (*N. muscorum* and *Spirulina* sp.) present in these two poly-microalgae cultures. Removal of TP was observed in the range of 83-84%. The above-mentioned results are in accordance with the results reported by (Patel et al. 2012; Qin et al. 2016; El-Sheekh et al. 2019).

5.3.7 Fatty acid methyl ester profile and biodiesel properties

The biodiesel properties were estimated as per empirical equations described by Francisco et al. (2010) as given in chapter 2 of this thesis. Biodiesel quality is affected by several factors, such as fatty acids composition of the feedstock, production process, refining process, and post-production parameters (Patel et al., 2015). The higher percentage of saturated fatty acids in biodiesel is an indicator of its good quality because of its contribution to oxidation resistance and stability in the presence of light and oxygen. Usually, biodiesel contains methyl esters of palmitic, stearic, oleic, linoleic, and linolenic acid (Knothe 2008; Qin et al. 2016; Chandra et al. 2019). Higher the presence of saturated fatty acids greater will be the oxidation stability (Patel et al. 2015). Figure 5.6(a and b) shows fatty acid methyl ester (FAME) profiles of poly and mono-microalgae cultures used in this work. GC-MS analyses of poly-microalgae cultures showed the presence of mainly C16:0 (palmitic), C16:1 (palmitoleic), C18:0 (stearic acid), C18:2 (linoleic), and C18:3 (linolenic). This biodiesel profile is in accordance with the biodiesel profiles reported by (Chinnasamy et al. 2010; Kothari et al. 2013; Qin et al. 2016) for different microalgae consortia.

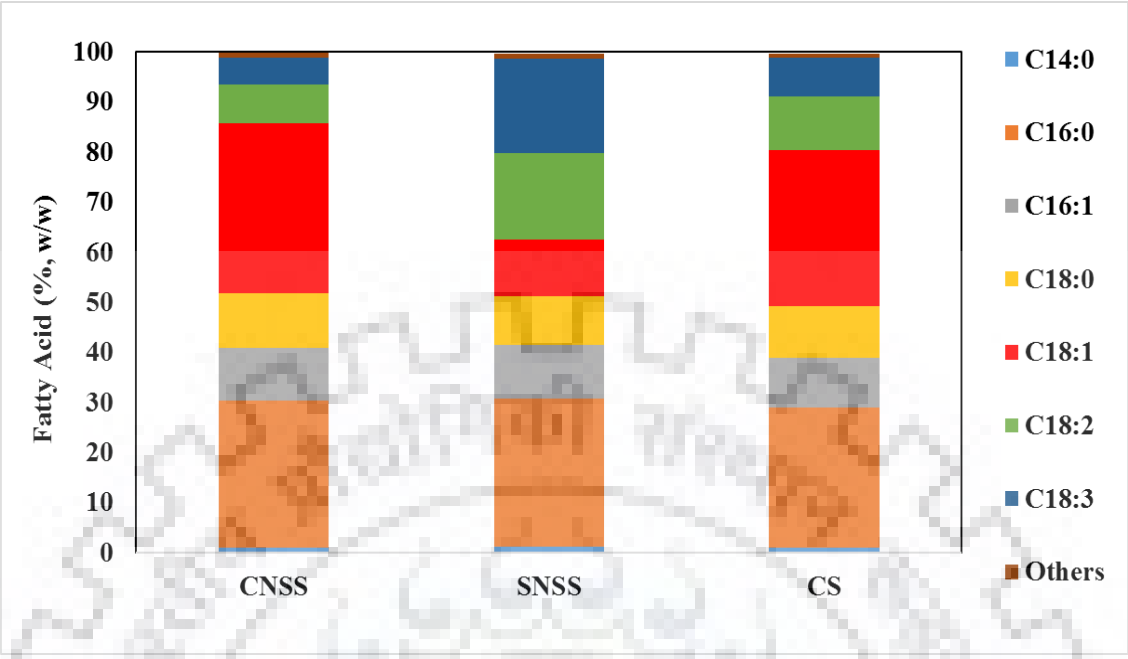

Figure 5.6(a) Fatty acid profile of different poly-microalgae cultures cultivated on 70% DWW

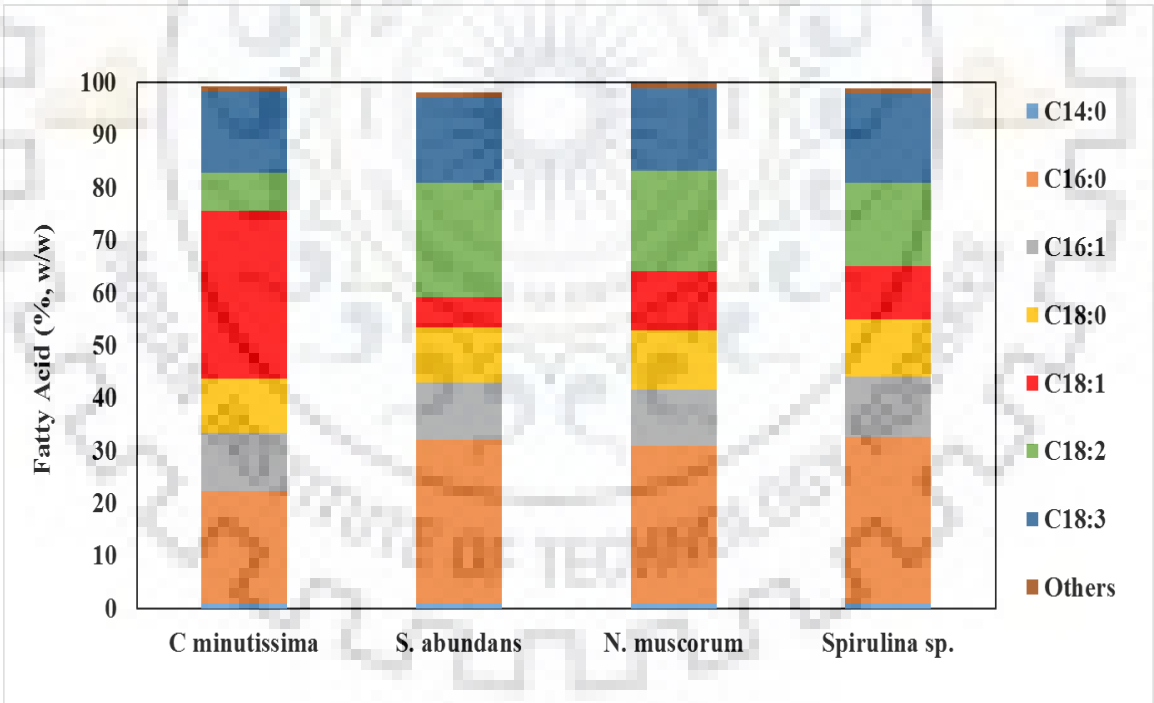

Figure 5.6(b) Fatty acid profile of different mono-microalgae cultures cultivated on 70% DWW

Poly-microalgae cultures CNSS, SNSS and CS, showed the presence of 41.19, 40.19, and 39.27% of saturated fatty acid content, respectively. Presence of more unsaturated fatty acids downgrades

the quality of biodiesel as it affects ignition quality of fuel (Qin et al. 2016). Biodiesel properties of poly and mono-microalgae cultures are presented in Table 5(a and b).

Table 5.5(a) Biodiesel properties of poly-microalgae cultures cultivated on 70% DWW

Physical properties	**International standards**		**Present work**		
	ASTM D6751	**EN14214**	**CNSS**	**SNSS**	**CS**
Degree of unsaturation (% wt.)	76.81	87.04	70.89	94.70	77.86
Saponification value (mg KOH)	NA	NA	204.81	205.04	204.23
Iodine value (g I_2 100 g^{-1})	NA	120	70.01	104.16	78.33
Cetane number	47	51	57.19	49.48	55.39
Long chain saturated factor (%)	5.97	5.75	8.38	7.73	7.93
Cold filter plugging point (℃)	≤ 5/ ≤ -20	3.93	9.87	7.82	8.45
Oxidation stability (h)	≥ 6	9.21	11.52	5.84	9.00
Higher heating value (MJ Kg^{-1})	NA	NA	38.95	38.75	38.88
Kinematic viscosity (mm^2 s^{-1})	1.9-6.0	3.5-5.0	3.76	3.47	3.70
Density (g cm^{-3})	0.86-0.9	0.87	0.86	0.86	0.86

It is mentioned in the open literature that different cultivation environments impart a significant role in the composition of fatty acids (Pandit et al. 2017; Chandra et al. 2019). Biodiesel obtained from all the microalgae cultures is in accordance with prescribed limits set by international standards. Cetane number of biodiesel from all the cultures was measured in between 47-49 which is in compliance with standard values of 47 and 51 as prescribed in ASTM D6751 and EN14214.

Table 5.5(b) Biodiesel properties of mono-microalgae cultures cultivated on 70% DWW

Physical properties	International standards		Present work			
	ASTM D6751	**EN 14214**	***C. minutissima***	***S. abundans***	***N. muscorum***	***Spirulina* sp.**
Degree of unsaturation (% wt.)	76.81	87.04	88.68	93.05	91.63	87.47
Saponification value (mg KOH)	NA	NA	202.44	202.47	205.47	204.15
Iodine value (g I_2 100 g^{-1})	NA	120	95.46	100.36	98.52	95.94
Cetane number	47	51	51.78	50.67	50.69	51.44
Long chain saturated factor (%)	5.97	5.75	7.20	8.30	8.59	8.58
Cold filter plugging point (°C)	≤ 5/ ≤ -20	3.93	6.16	9.60	10.51	10.49
Oxidation stability (h)	≥ 6	7.76	5.68	5.98	6.18	7.76
Higher heating value (MJ Kg^{-1})	NA	38.69	38.19	38.87	38.49	38.69
Kinematic viscosity (mm^2 s^{-1})	1.9-6.0	3.59	3.41	3.53	3.47	3.59
Density (g cm^{-3})	0.86-0.9	0.86	0.85	0.86	0.85	0.86

However, monoculture *C. minutissima* showed the highest cetane number of 49 of among all the microalgae cultures. It is also noteworthy that the oxidation stabilities of biodiesel from all the microalgae cultures were slightly lower than the prescribed value of 5 or more by ASTM D6751. Hence, to produce high quality biodiesel microalgae poly-cultures shall be promoted for cultivation in suitable conditions. In general, unsaturation in fatty acids enhances the operability of biodiesel in a cold environment due to a low CFPP; whereas their saturation prevents auto-oxidation of the fuel and improves the shelf life of biodiesel (Knothe and Razon 2017). It is quite difficult to find a feedstock that meets all criteria for biodiesel; therefore, blending different biodiesels to achieve the optimum ratio of saturated to unsaturated fatty acids could be an effective solution (Suh and Lee 2016; Mahmudul et al. 2017).

5.4 Conclusions

As reflected in this work, microalgae poly-microalgae cultures may be more favourable for biodiesel feedstock production as well as DWW remediation. It is noteworthy that each poly-

microalgae culture efficiently removed pollution load from DWW with significant microalgae biomass yield (5.76 g/L). The FAME content of mono and poly-microalgae cultures indicated that biodiesel quality is in in the limit as prescribed by ASTM D6751 and EN14214. This work gives an idea about efficient remediation of DWW using poly-microalgae cultures as well as the production of biodiesel feedstock. However, still extensive research work is required to conclude superiority of poly-microalgae cultures over mono-microalgae cultures for biodiesel production and wastewater remediation.

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Chapter 6

Summary, conclusions and future scopes

6.1 Summary and conclusions

This thesis demonstrated the potential of four microalgae (*Chlorella minutissima*, *Scenedesmus abundans*, *Nostoc muscorum* and *Spirulina* sp.) for remediation of wastewaters and biodiesel production under indoor cultivation conditions. Among the four microalgae species used in this work, *Chlorella minutissima* was found the most suitable microalgae species for wastewater remediation and biodiesel production. However, impoved wastewater remediation and biodiesel production was observed when microalgae polycultures were used. Best results were observed with polycultre of *C.minutssima + N. muscorum + Spirulina* sp. (CNSS). Thus, *Chlorella minutissima* has been preferred in the experimental works carried out to accomplish this thesis. Microalgae biomass harvesting was done using centrifugation. Harvested biomass was dried in hot air oven or vacuum oven. To extract lipid from dry microalgal biomass Folch method or modified Bligh and Dyer method was applied. Biodiesel properties were analyzed according to ASTM D6751 and EN14214 standards.

Specific conclusions are as follows:

- Microalga *C. minutissima* emerged as the most suitable species for wastewater remediation and biodiesel production among four microalgae studied.
- The maximum biomass and lipid yield of 1840.49 and 579.86 mg/L, respectively was achieved by *C. minutissima* cultivated on modified CHU-13 medium having 12 g of glucose and 0.3 g of nitrogen.
- Biodiesel profile of *C. minutissima* cultivated on modified CHU-13 medium showed presence of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), gondoic acid (C20:1), and behenic acid (C22:0).
- *C. minutissima* cultivated on DSW25 + STDS75 + 20 g/L glucose at 24 h photoperiod achieved biomass yield of 5.23 g/L (lipid yield 976.46 mg/L) over a cultivation period of 20 days.

- *C. minutissima* removed 77.23% BOD, 66.15% COD, 69.83% TDS, 41.30% TSS, 79% TN and 44.74%, respectively from DSW75+STDS25 in a cultivation period of 20 days.
- The fatty acid profile of *C. minutissima* cultivated on DSW25 + STDS75 shown the presence of mainly C14:0 (2.46%), C16:0 (13.3%), C16:1 (3.35%), C18:0 (28.43%), C18:1 (30.98%), C18:2 (6.33%), and C18:3 (11.45%).
- *C. minutissima* cultivated on DSW25+STDS75 supplied with glucose showed enhanced biomass and lipid yield, however, quality of bioidsel degraded due to the presence of more unsaturated fatty acids.
- *C. minutissima* obtained 0.51 and 0.33 g/L of biomass yield, respectively at 25 and 100 mg/L of 1,2-dihydroxy benzene concentration in STWW. Under similar conditions, *S. abundans* obtained 0.45 and 0.25 g/L of biomass yield, respectively. This indicates that presence of phenol in wastewater has adverse effect on biomass and lipid yield of both the microalgae.
- *C. minutissima* cultivated on STWW + 1,2 di-hydroxy benzene + 20 g/L of glucose achieved 2.15 g/L of biomass yield (320.14 mg/L lipid yield) at 25 mg/L of phenol (1,2 di-hydroxy benzene). This biomass yield was reduced to 0.80 g/L (79.28 mg/L lipid yield) when phenol concentration was raised to 100 mg/L in the cultivation medium. Similar observations were recorded for *S. abundans* also.
- Addition of glucose in the cultivation medium improved removal of phenols for both *C. minutissima* and *S. abundans*. The maximum phenol (1,2 di-hydroxy benzene) removal of 81% was achieved by *C. minutissima* at concentration of 25 mg/L in STWW + 20 g/L glucose while in absence of glucose the removal was 27.76%. Similar trend was observed with *S.* also.
- *C. minutissima* removed 86.46% BOD, 72.92% COD, 85.14% NO_3^-, 91% PO_4^{3-} and 91% TOC from the cultivation medium (STWW + Glucose) while under similar conditions *S. abundans* removed 84% BOD, 72.20% COD, 78.85% NO_3^-, 87.47% PO_4^{3-} and 90.78% TOC.
- In the presence of phenols in the cultivation medium reduced pollution load removal was achieved by *C. minutissima* as 53.96% BOD, 46% COD, 77% NO_3^-, 68% PO_4^{3-} and 67% TOC from the cultivation medium (STWW + Glucose + 1,2-dihydroxy benzene (25 mg/L)) while under similar conditions *S. abundans* removed 53.07% BOD, 45.37% COD, 71.06% NO_3^-, 63.02% PO_4^{3-} and 66.17% TOC.

- Increase in the phenol (1,2-dihydroxy benzene) concentration caused a decrease in the, higher levels of FAMEs, specifically C18:0, was decreased.
- Biodiesel obtained from *C. minutissima* biomass cultivated on STWW having different concentrations of phenols showed the presence of C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3 and biodiesel quality is found within the limits prescribed by internal standards.
- The maximum biomass yield of 3.41 g/L along with lipid yield of 420.25 mg/L was achieved by *C. minutissima* when grown on 70% DWW supplied with 10 g/L of glucose. Under similar conditions, *S. abundans* achieved lipid yield of 3.24 g/L along with lipid yield of 366.17 mg/L.
- The highest biomass yield of 5.76 g/L along with lipid yield of 1152.37 mg/L was achieved by polyculture CNSS grown on 70% DWW + 10 g/L glucose indicating superiorty of polycultures over monocultures.
- Microalgae polycultures are found more efficient for DWW remediation and they achieved higher biomass and lipid yield in comparison to mono-microalgae cultures.
- Properties of biodiesel obtained from *Chlorella minutissima* and *Scenedesmus abundans* cultivated on 70% DWW were found in accordance with international standards.
- Pollution load removal by mono-microalgae cultures (*C. minutissima* and *S. abundans*) cultivated on 70% DWW removed about 70% BOD, 56% COD, 53% TN, 80% TP and 70% within 10 days of cultivation period. However, under similar conditions *N. muscorum* and *Spirulina* sp. could not show significant removal of pollution load, they both could remove about 38% BOD, 24% COD, 14% TN, 75% TP and 70% SS.
- Poly-microalgae cultures (CNSS, SNSS and CS) efficiently removed BOD, COD, TN, TP and SS in the range of 73-75, 59-61, 56-59, 83-84 and 82-84%, respectively from 70% DWW within 10 days of cultivation period. However, highest removal of BOD (75.16%), COD (61.37%), TN (58.76%), TP (84.48%) and SS (84.58%), respectively, was achieved by poly-microalgae culture CNSS.
- GC-MS analysis of poly-microalgae cultures showed the presence of mainly C16:0 (palmitic), C16:1 (palmitoleic), C18:0 (stearic acid), C18:2 (linoleic), and C18:3 (linolenic acid) while mono-microalgae cultures shown the presence of fatty acids… complete the sentence. Biodiesel obtained from superiorty.

- Biodiesel properties of mono and poly-microalgae cultures were found in accordance with international standards.

6.2 Future scope

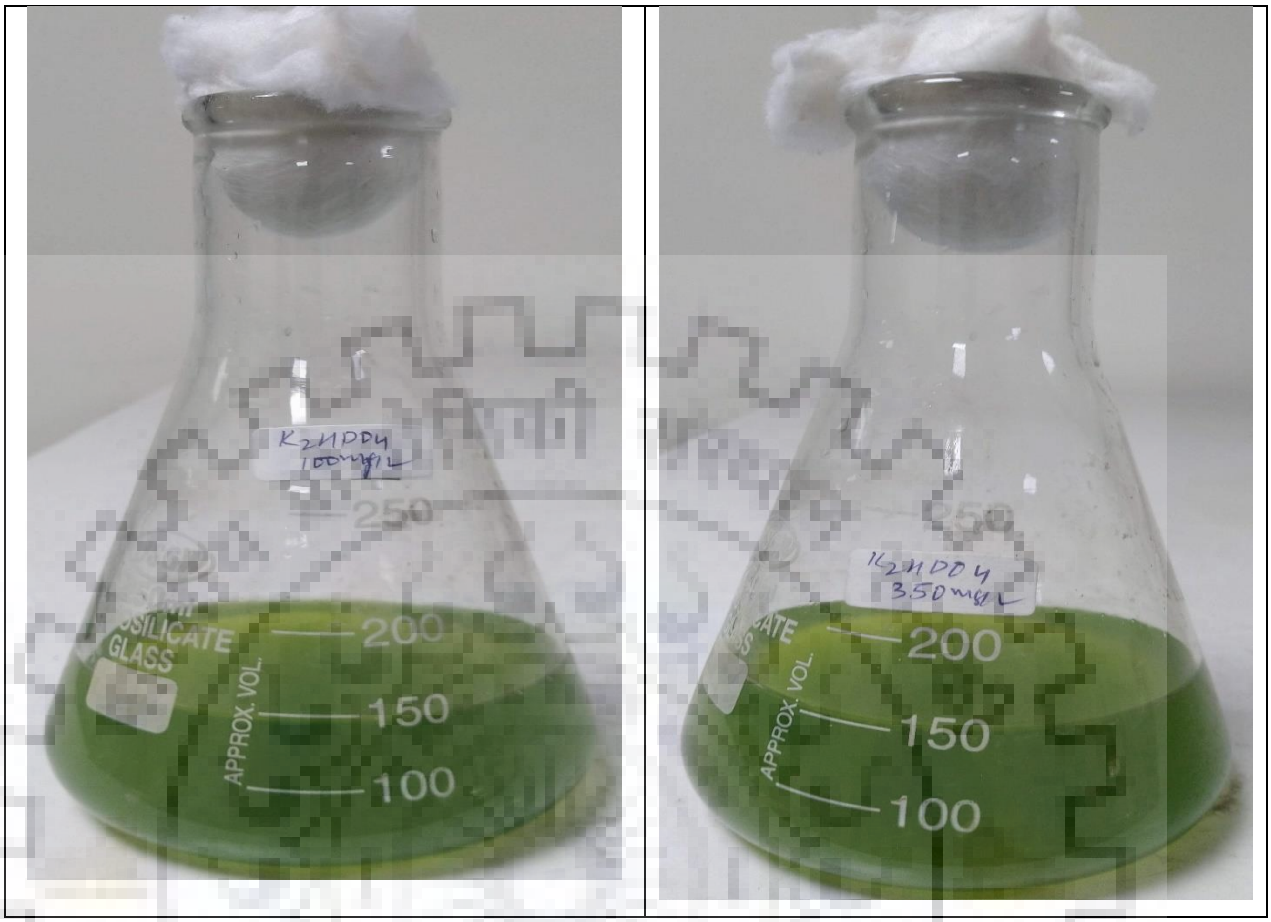
Considering the future trends, the present work has the following scopes:

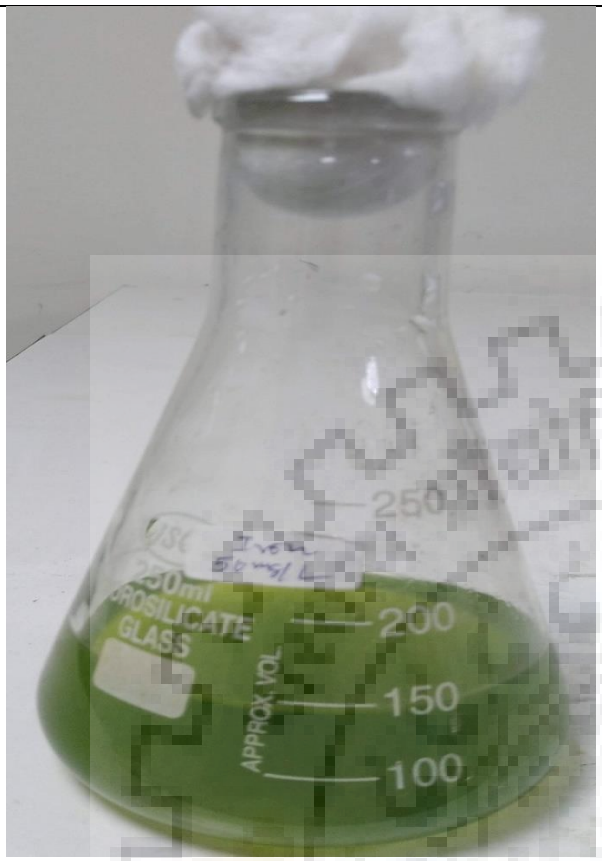
- Scale-up for large scale cultivation of microalgae in raceway ponds as well as in photobioreactors in outdoor conditions.
- Use of wild and genetically modified microalgae species for higher biomass and lipid yield.
- Use of poly-cultures which may involve microalgae and bacteria for enhanced remediation of wastewater and higher biomass production.
- Development of efficient and less energy-intensive microalgae harvesting technologies.
- Production of value-added products using glycerol generated during biodiesel production.

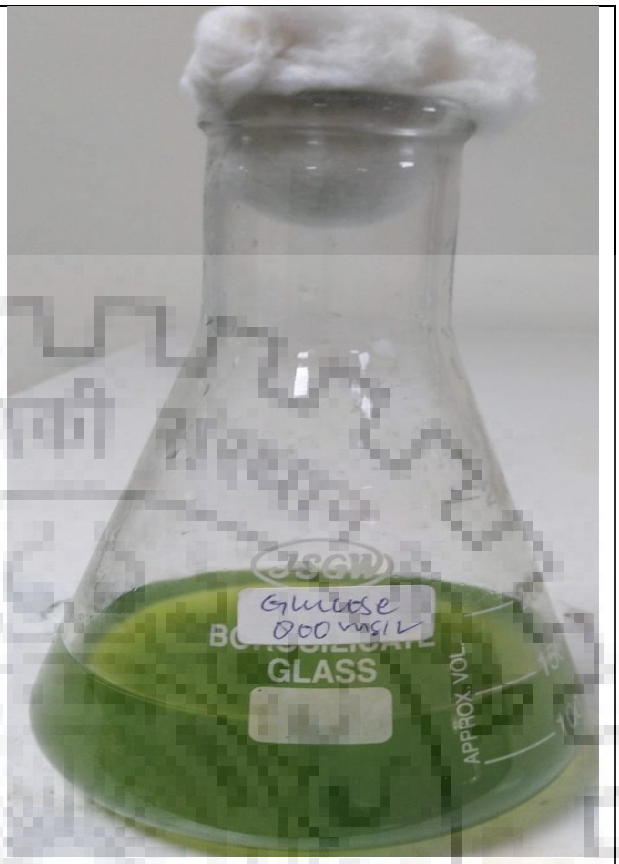
Annexure-I

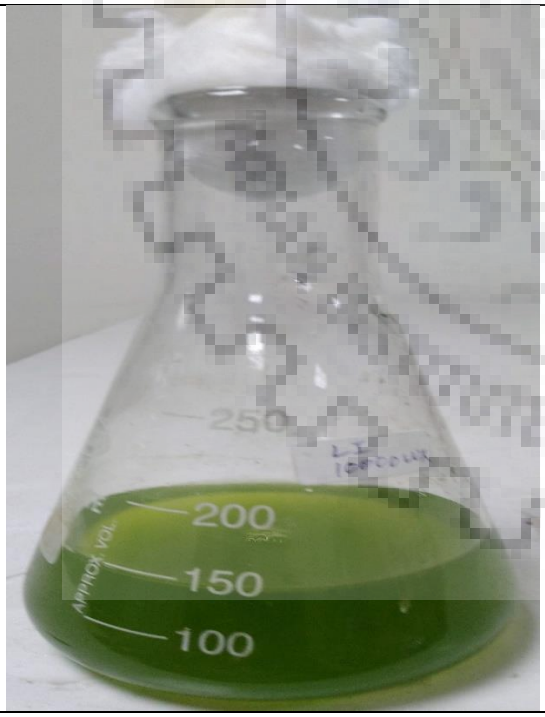
Pictures of sampling of secondary treated wastewater at Uflow Anaerobic Sludge Blanket Reactor (38 MLD), Saharanpur, Uttar Pradesh, India

Pictures of microalga *C. minutissima* growth at different conditions as described in Chapter 2 of the thesis

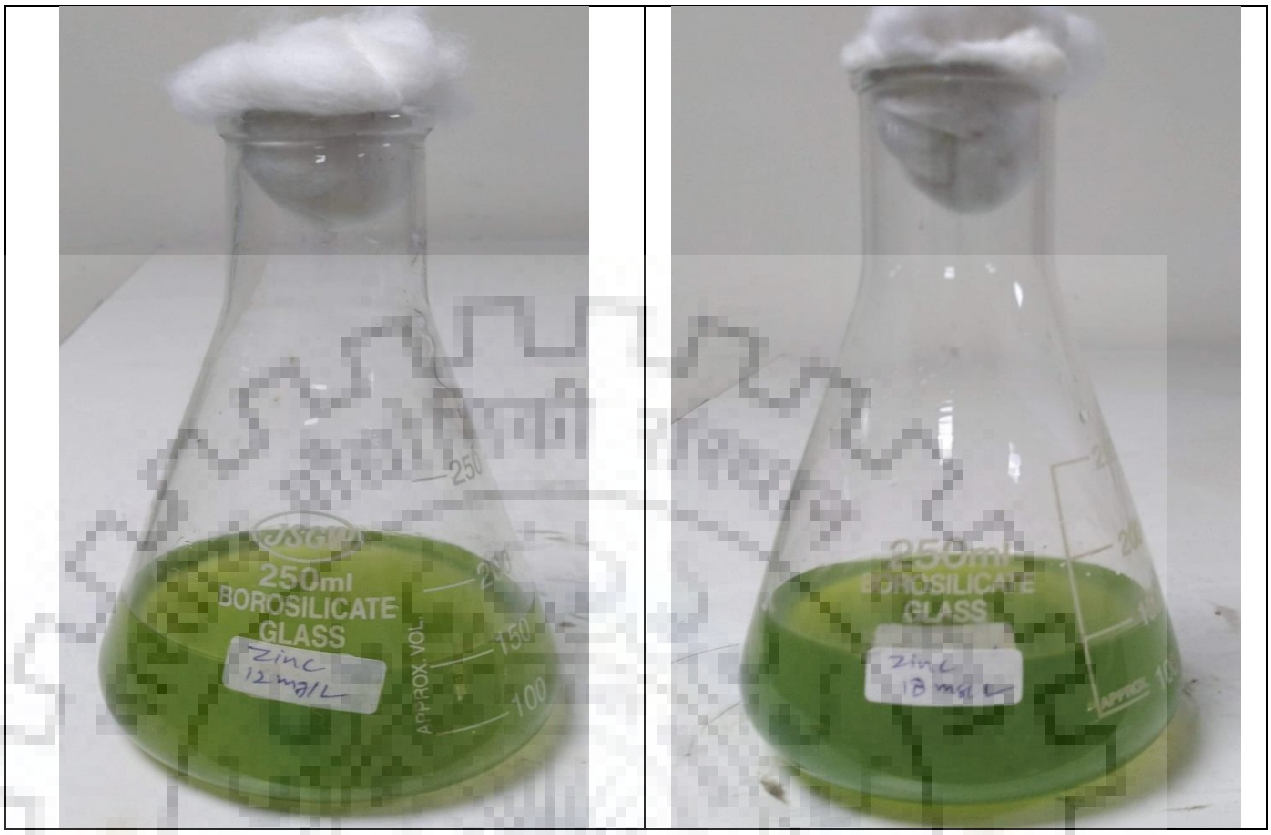

K2HPO4
100mg/L
250
200
150
100
APPROX. VOL.
K2HPO4
350mg/L
200
150
100
APPROX. VOL.


250
250ml
BOROSILICATE
GLASS
APPROX. VOL.
200
150
100

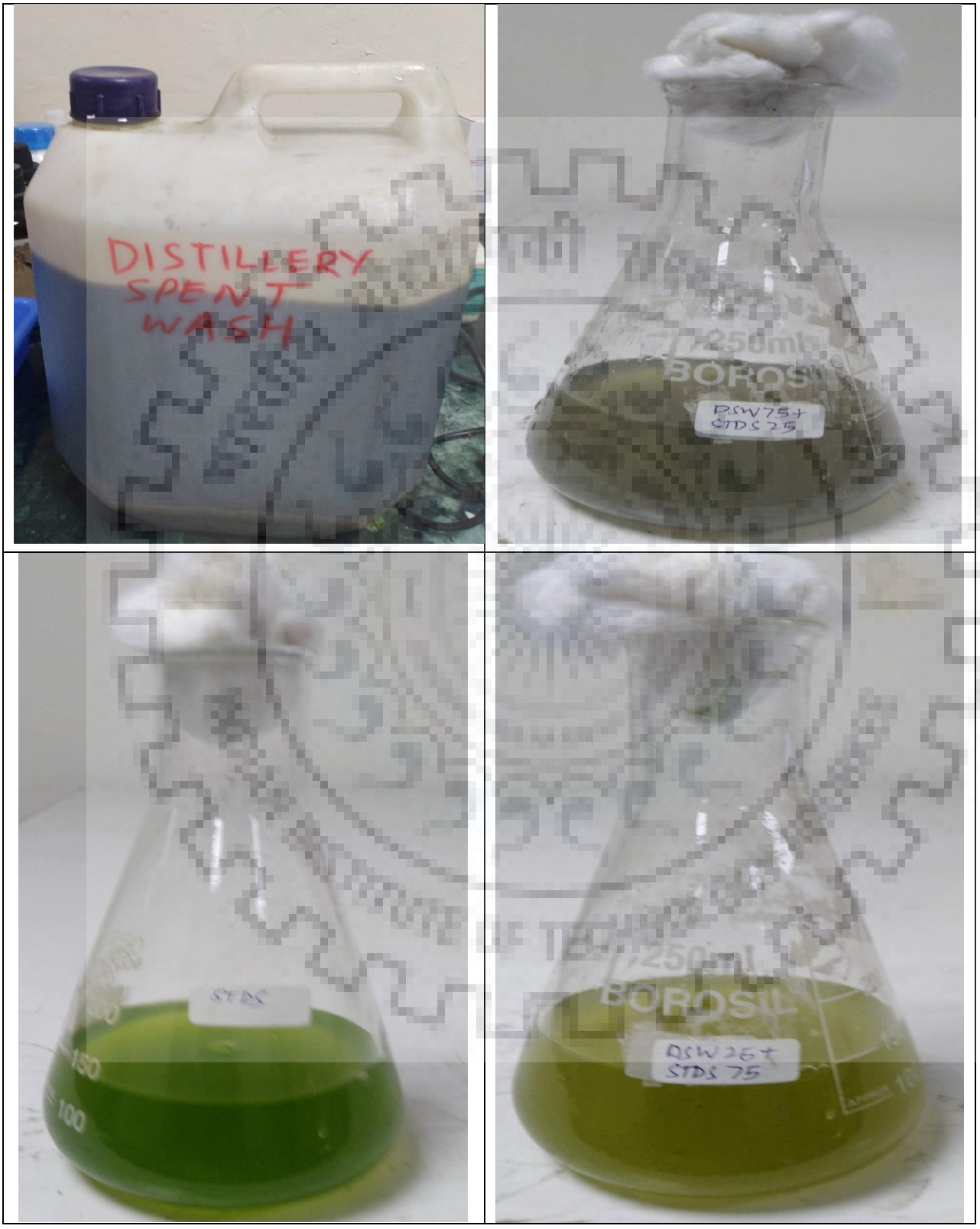

JSGW
Glucose
000 mg/L
GLASS
APPROX. VOL.


250
200
150
100


250
150
100

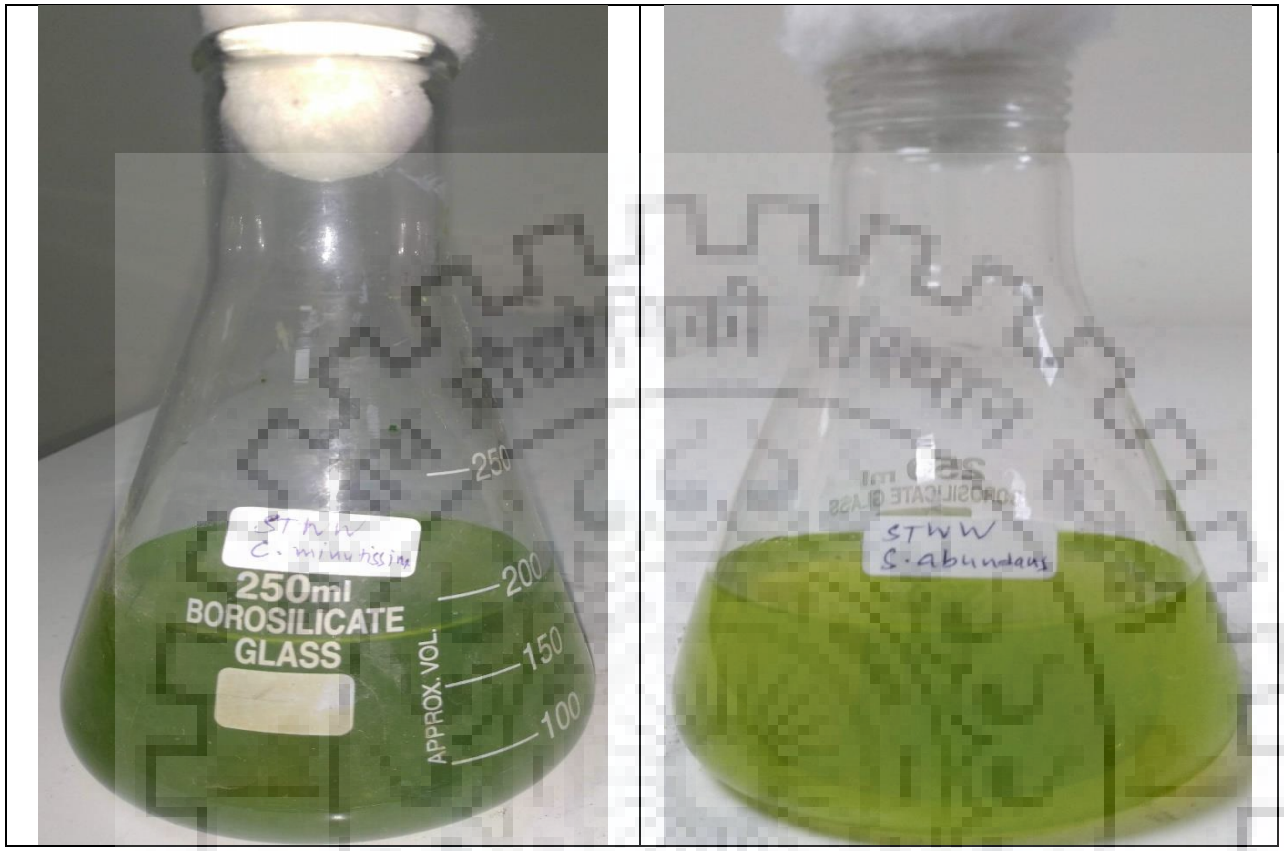

250ml
BOROSILICATE
GLASS
Zinc
12mg/L
250ml
BOROSILICATE
GLASS
Zinc
18mg/L

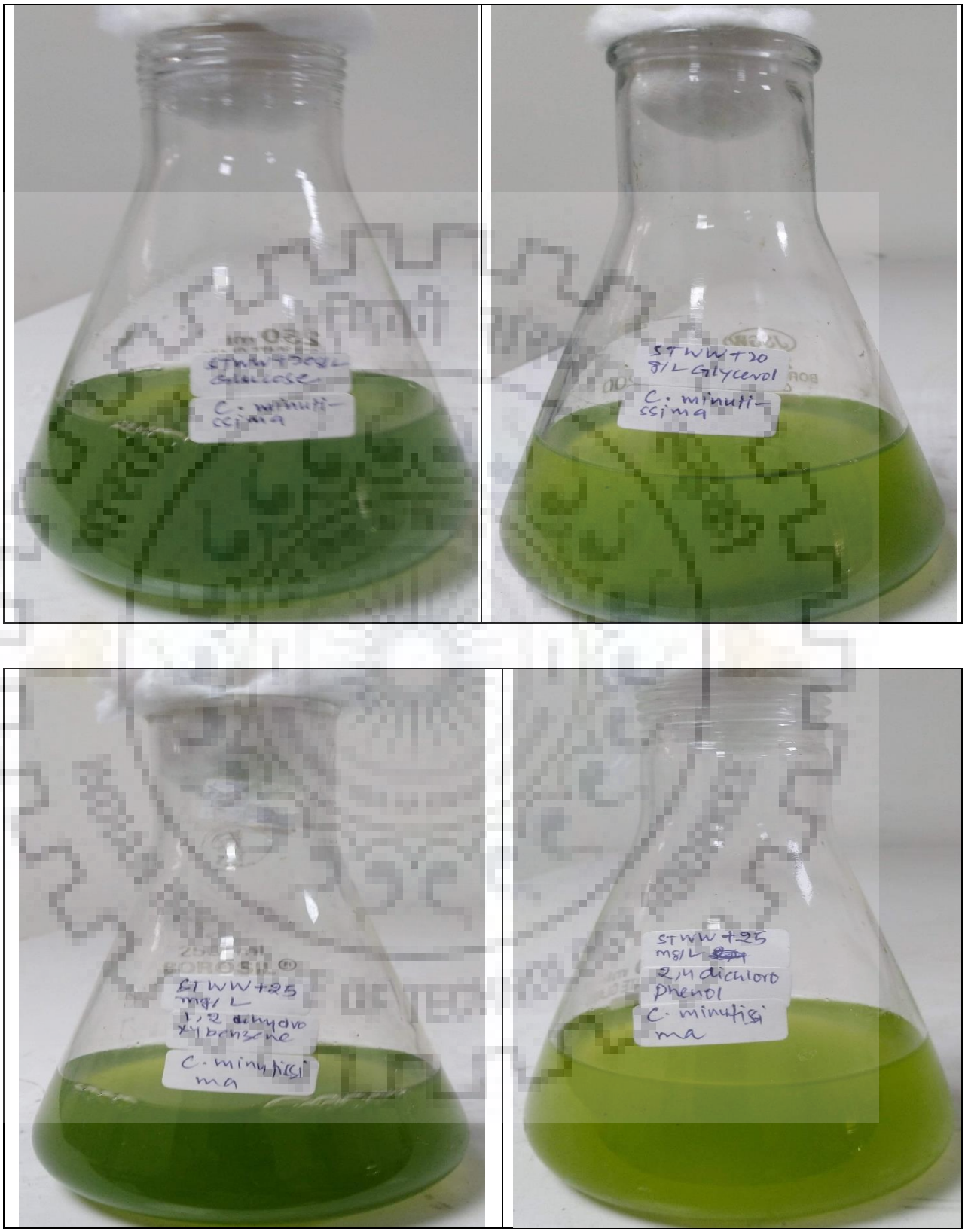
Pictures of *C. minutissima* growth during treatment of distillery spent wash as described in Chapter 3 of the thesis

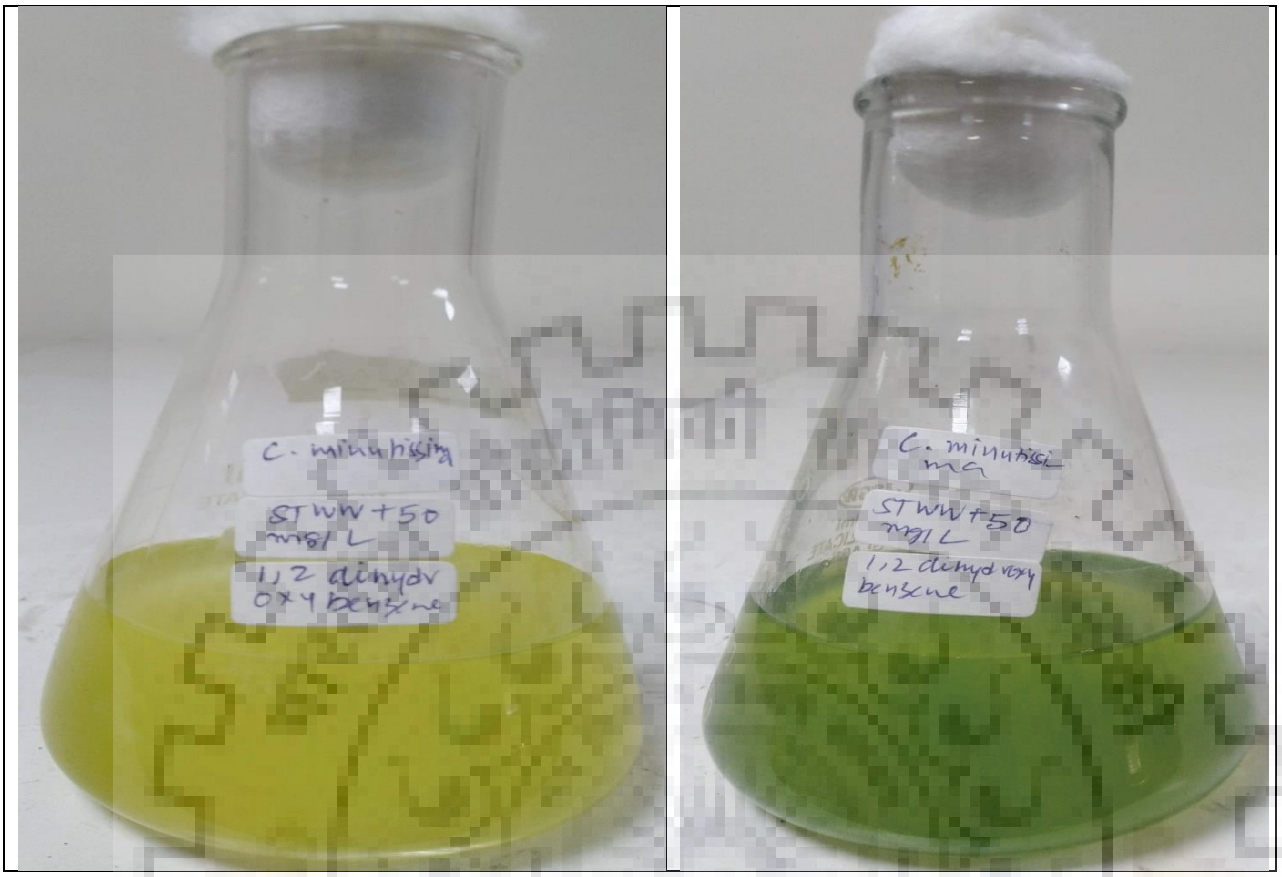



200
150
100
APPROX
150
100
250
150
100
APPROX
250
150
100
APPROX. VOL

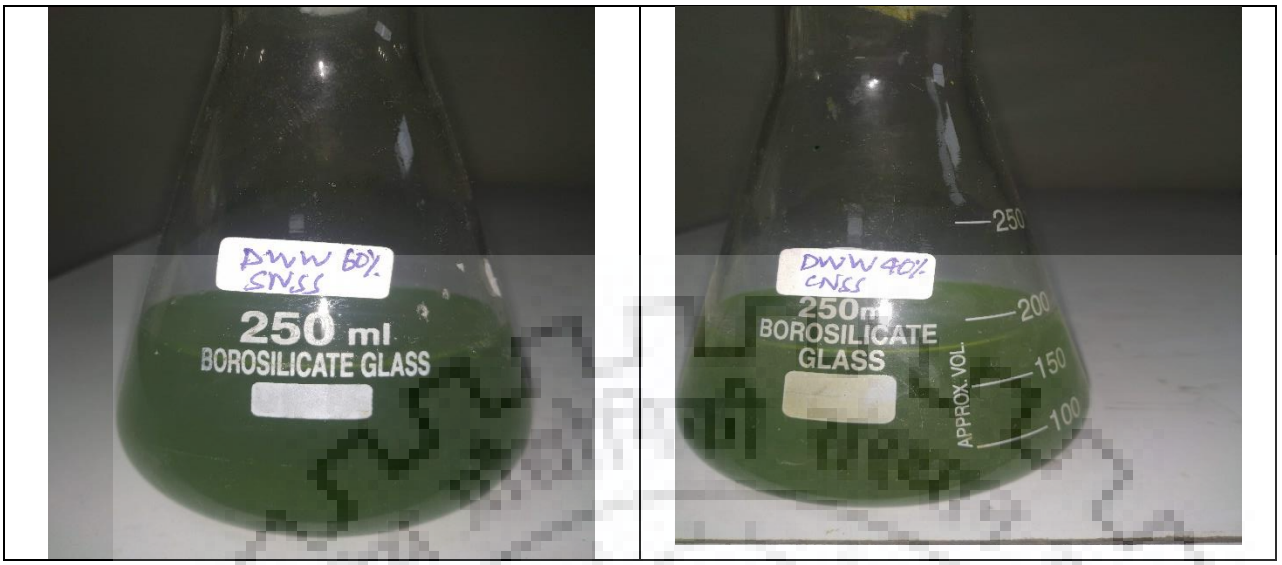
Pictures of *C. minutissima* and *S. abundans* growth on wastewater contaminated with different phenols as described in Chapter 4 of the thesis




ST WW+20
g/L Glycerol
C. minuti-
ssima
ST WW+25
mg/L
1,2 dihydro
xybenzene
C. minutissi
ma
ST WW+25
mg/L
2,4 dichloro
phenol
C. minutissi
ma


C. minutissima
STWW+50 mg/L
1,2 dihydroxy benzene
C. minutissima
STWW+50 mg/L
1,2 dihydroxy benzene

Pictures of mono and poly-microalgae cultures grown on dairy wastewater as described in Chapter 5 of the thesis



Picture of Biodiesel