Microalgal biomass and oil production in non-potable waters for potential application in biofuels and nutraceuticals

Thesis submitted under the Faculty of Science of the
Cochin University of Science and Technology
For the award of degree of

Doctor of Philosophy
in
Biotechnology

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September 2015

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Microalgal biomass and oil production in non-potable waters for potential application in biofuels and nutraceuticals**" is a *bona fide* record of the research carried out by **Ms Sabeela Beevi. U** (Reg No. 4348) under my guidance and supervision at the Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I declare that all suggestions made by the audience during pre-synopsis seminar and recommended by the Doctoral committee have been incorporated in the thesis. I also declare that this work or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associate ship or any other title or recognition

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Thiruvananthapuram
09 September 2015

DECLARATION

I hereby declare that the work presented in this thesis entitled ***"Microalgal biomass and oil production in non-potable waters for potential application in biofuels and nutraceuticals"*** is based on the original work done by me under the guidance of Dr Rajeev Kumar Sukumaran, Senior Scientist, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India and the thesis or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associateship or any other title or recognition.

Sabeela Beevi.U

Dedicated to my Family
and Teachers

Acknowledgements

I extend my heartfelt thanks to all the important people in my life who have been instrumental in making my dream come true

First of all I would like to give my sincere thanks to my supervisor Dr.Rajeev.K.Sukumaran who accepted me as PhD student without any hesitation. I am very much grateful to you Sir for your constant support, encouragement, advice, patiently guiding me in a right direction during the entire course of my PhD.

I extent my warm and sincere thanks to Prof.Ashok Pandey, Head of Biotechnology division, National institute for Interdisciplinary Science and Technology for his timely suggestions, persistent support and constant encouragement. He always viewed issues from multiple perspectives which helped me to work in different ways.

It is my privilege to place on record my gratitude to Dr.A.Ajayaghosh, Director, National Institute for Interdisciplinary Science and Technology for providing my necessary facilities in the Institute for my research work. I am very much grateful to Dr.Suresh Das, former Director of the Institute for his help and support.

I express my sincere gratitude to Dr.Sarita G Bhat, Professor, Department of Biotechnology, CUSAT and expert member of my Doctoral committee for her valuable suggestions and friendly approach which inspired me to improve my work.

I would like to thank Dr. RBN Prasad and his group (Department of Lipid Science and Technology, CSIR-Indian institute of Chemical technology Hyderabad) and the Agro Processing & Natural Products Division of CSIR- National Institute for Interdisciplinary Science and Technology, for extensive help in analysis and profiling of fatty acids of my samples.

I take this opportunity to thank Dr. K. Madhavan Namboothiri, Dr. Binod P, Dr. Ramesh N, Dr. Sindhu R, Dr. M. Arumugam, Er. Kiran Kumar M, Dr. Leena D, Dr. Anil Mathew and Mr. KM Prakash for their valuable suggestions, help and support throughout my study.

I am thankful to Mr.MK Chandran and his team at the Scanning Electron Microscopy section of CSIR-NIIST for their patience while taking images of my samples

My sincere thanks to MILMA Dairy, Thiruvananthapuram for providing us Pre ETP dairy effluents for my research work. It is my privilege to thank Kerala Forests and Wildlife Department for giving us the permission for collecting water samples from Silent Valley National Park, Kerala.

I would like to acknowledge financial support from UGC, Govt. of India, in the form of Research Fellowship for my PhD work.

I extend my gratitude to Dr. Sabu. A, Associate professor, Kannur University, whose constant words of motivation; support, encouragement and suggestion helped me to enter in to research life.

My sincere thanks to the Doctoral and Research committee of CUSAT, Academic Program Committee of CSIR-NIIST and the NIIST-CUSAT, Research Council for timely help during the entire course of my work and thesis submission. I also thank to the administrative and supporting staff of CUSAT and NIIST their help and support.

I am very much thankful to all the members of "Team Biotech" for creating a unique and healthy working environment. I am deeply thankful to Aravind Madhavan, Leya Thomas, Meena, Vani Sankar, Rajasree, Ayman, Mandavi, Deepthy Alex, Vidya and Ushasree for their constant motivation and support throughout my work. I express my gratitude towards Divya, Anusree, Nishanth, Karthik, Koyna, Kiran, Varsha, Arya Nandan, Kutti Raja and Preethi for their friendly help and moral support. I am also thankful to Vaisakhi, Aiswarya, Aswathi, Aswathi U, Sujitha, Reshma, Anand J, Resna, Sajitha, Silviya, Vini, Dilna, Dhanya, Sneha, Rahul, Remya, Likhitha, Susmitha, Merin, Dileep, Anand, Raja, Meera, Emrin, Soumya, Deepthi, Preethi, Salman, Prajeesh and Valan who helped me in one way or the other during the entire period of my work.

I also thank my friends Vineetha, Sowmya, Janu, Arun, and Shama for their constant support and help. I express my thanks to Radha Chechi, Sasikala Chechi and Rajalakshmi Chechi for all the help and support extended to me.

Words cannot express my deep gratitude to my beloved family for their unconditional love, care, encouragement and support and prayers showed on me.

A number of friends, colleagues and well wishers were involved in my study directly or indirectly. I thank one and all for their support, help and encouragement throughout my work.

Above all I express my deepest gratitude to the Almighty whose blessings and strength helped me to complete my thesis.

Sabeela Beevi.U

List of Publications

SCI Journals

1. **Ummalyma SB,** Sukumaran RK, 2014, Cultivation of microalgae in dairy effluent for oil production and removal of organic pollution load, Bioresour. Technol. 165: 295-301 (**IF = 5.04**)

2. **Ummalyma SB**, Sukumaran RK, 2015, Cultivation of the fresh water microalga – *Chlorococcum* sp. RAP13 in sea water for producing oil suitable for biodiesel, J Appl. Phycol. 27: 141-147. (**IF = 2.492**)

Book Chapters

1. Sukumaran RK, Sankar V, Madhavan A, Sankar M, Satheesh V, Omar Idris AS, **Ummalyama SB**, Enzyme Technologies: Current and Emerging Technologies for Development of Novel Enzyme Catalysts, In : M Chandarsekaran (ed) Enzymes in Food and Beverage Processing, CRC Press, Ohio, USA (*In Press*)

Conference Papers/Presentations/Posters

1. **Sabeela Beevi U** & Sukumaran RK, Evaluation of fresh water micro algal isolates for growth and lipid production in seawater based medium, International Conference on Emerging Trends in Biotechnology, November 6-9, JNU, New Delhi, India (2014). **(Best Poster award)**

2. **Sabeela Beevi U** and Rajeev K Sukumaran. Oil production by micro alga grown on dairy waste water. International Conference on Advances in Biotechnology and Bioinformatics, November 25 - 27, Pune India (2013)

3. **Sabeela Beevi U**, Kuniparambil Rajasree, and Rajeev K Sukumaran. Heterotrophic growth and lipid production by Micro algae Chlorococcum sp.RAP-13 in industrial streams and sea water , In: Proceedings of 5th India –Korea Joint Workshop on Bioenergy, September 9-10, Trivandrum India (2013)

4. **Sabeela Beevi U**, Kuniparambil Rajasree, Rajeev K Sukumaran. *Chlorococcum* sp.RAP-13- a novel algal isolate from the Western Ghats with potential for heterotrophic growth and oil production on waste glycerol. ICIB & IX convention of the Biotech Research Society of India, November 21-23, Punjab (2012)

CONTENTS

Chapter1. Introduction and Review of literature

1.1.Microalgae

Microalgae are microscopic photosynthetic organisms that are found in both marine and freshwater ecosystems. Their photosynthetic mechanism is similar to land based plants, but due to a simple cellular structure, and being submerged in an aqueous environment where they have efficient access to water, CO_2 and other nutrients, they are generally more efficient in converting solar energy into biomass. They are unicellular or multi cellular organisms ranging in size from a few microns to hundred of microns that exist either individually or as chains or flocs, in both marine and fresh water environment**.** Algae are recognized as one of the oldest life-forms (Falkowski and Raven, 1997). Microalgae are primitive plants (Thallophytes), i.e. lacking roots, stems and leaves, have no sterile covering of cells around the reproductive cells and have chlorophyll A as their primary photosynthetic pigment (Lee, 1980). Microalgal structures are primarily for energy conversion without any development beyond cellular levels, and their simple development allows them to adapt to existing environmental conditions and thrive in the long term (Khan *et al,* 2009). The current systems of classification of microalgae are based on the following main criteria: kinds of pigments, chemical nature of storage products and cell wall constituents. Additional criteria taken into consideration is cytological and morphological characters that includes occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane (Tomaselli, 2004). There are two basic types of algal cells, prokaryotic and eukaryotic. Prokaryotic cells lack membrane-bounded organelles (plastids, mitochondria, nuclei, golgi bodies and flagella) and occur in the cyanobacteria and are more related to bacteria rather than algae (Lee, 2008). The remaining types are eukaryotic, and have organelles. Eukaryotic microalgae are further categorized into a variety of classes mainly defined by their pigmentation, life cycle and basic cellular structure (Khan *et al,* 2009). The most important classes are: green algae (Chlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta). It is estimated that more than 50,000 species exist, but only a limited number of around 30,000, have been studied and analyzed for various applications (Richmond. 2004).

Recently, significant attention has been focused towards algal biofuels. The flexibility of algae to switch their nutritional mode based on substrate availability and light condition is

one of the inherent evolutionary advantages of the algae. However in the context of substrate, microalgae can be autotrophic, heterotrophic or mixotrophic. The autotrophic or phototrophic one requires only inorganic compounds such as CO_2, salts and a light energy source for growth. Phototrophic algae gain energy through light by fixing atmospheric CO_2. For autotrophic algae, photosynthesis is a key component of their survival, whereby they convert solar radiation and CO_2 absorbed by chloroplasts into adenosine tri phosphate (ATP) and O_2 the usable energy currency at cellular level, which is then used in respiration to produce energy to support growth (Zilinskas1976; Falkowski and Raven1997). Major limitations of phototrophic cultivation are the low biomass yield, requirement of cultivation systems with large surface area and shallow depth for better access of light. The heterotrophic algae are non photosynthetic and therefore requires an external source of organic compound as well as nutrients as an energy source. In the absence of light, the photosynthetic process gets suppressed and algae gain energy from alternative organic processes that convert sugar into lipids (Perez-Garcia *et al,* 2010). Advantages of heterotrophic system are the growth of algae can be significantly denser, greater yield, non-dependence on light. Such microalgae use organic molecules as primary energy and carbon source through heterotrophic nutritional mode and facilitate high biomass productivities which provide economical feasibility for large scale production (Behrens. 2005; Perez-Garcia *et al,* 2011). Main attraction of heterotrophic growth approach is the cost effectiveness, relative simplicity in operations and easy maintenance (Perez-Garcia *et al,* 2011). Some photosynthetic algae can also function under mixotrophic condition by combining both the autotrophic and heterotrophic mechanisms by assimilating available organic compounds as well as atmospheric CO_2 as carbon source (Lee, 1980).

Microalgae are looked upon as an alternative energy source because their oil production per unit area cultivation far exceeds the other oil crops such as soybean, coconut, corn and oil palm by as much as 2–3 orders of magnitude (Chisti.2007; Wijffles and Barbosa 2010). Furthermore, they do not compete for arable land and can be produced year-round in suitable climates. They also grow much faster than traditional crops (doubling time can be as fast as 24 hours) and are likely to recover more quickly from adverse effects (Becker, 1994; Masojídek *et al,* 2008). Oil content in microalgae can reach upto 80% by weight of dry cell mass (Metting, 1996; Spolaore *et al,* 2006). Oil levels ranging from 20–50% are quite common (Table 1.1). Oil productivity, that is the mass of oil produced per unit volume of the micro-algal broth per day, depends on the algal growth rate and the oil content of the

biomass. Microalgae with high oil productivities are desired for producing biodiesel (Chisti, 2007).

Table 1.1: Oil content of some microalgae (Chisti, 2007)

Microalga	Oil content (% DCW)*
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina N	20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

*Dry cell weight

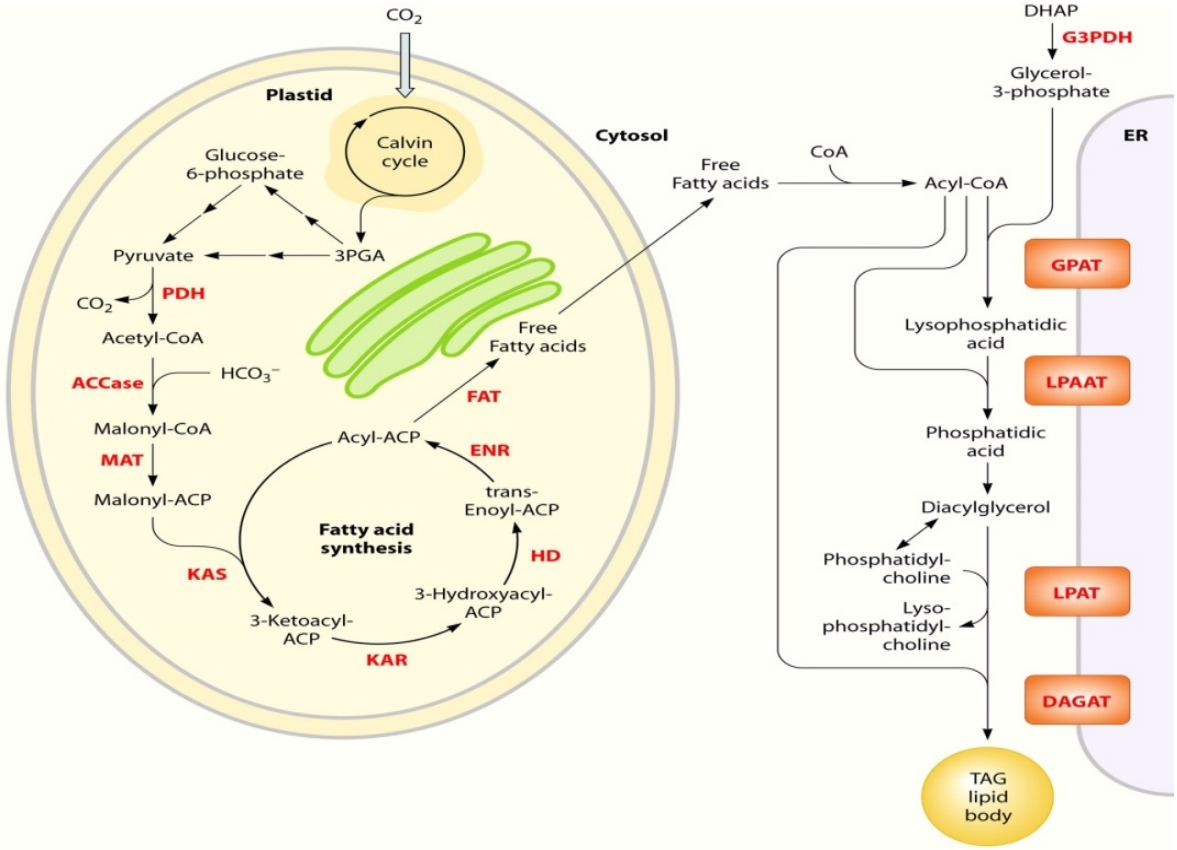
1.2 Biosynthesis of lipids/fatty acids in microalgae

It is recognized that both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate, etc.) can be utilized by microalgae for lipid production. The components and contents of lipids in microalgal cells vary from species to species. The lipids are divided into neutral lipids (e.g., triglycerides, cholesterol) and polar lipids (e.g., phospholipids, galactolipids) (Huang *et al,* 2010). Triacylglycerols (TAGs) are repositories of fatty acids, the most concentrated form of energy available in eukaryotic cells (Durrett *et al,* 2008). Many microalgae are known to be capable of accumulating large amounts of TAGs, particularly under nutrient starved conditions (Hu *et al*, 2008), which are increasingly being discussed as a source of renewable alternatives to petroleum fuels (Chisti, 2007; Hu *et al,* 2008: Wijffles and Barbosa, 2010). Recent years have seen major advances in our understanding of lipid metabolism and its regulation in plants, particularly in the model plant Arabidopsis (Wallis

and Browse 2010). However, the biochemical pathways, the enzymes and the regulatory factors involved in TAG accumulation in microalgae are still poorly defined (Miller *et al,* 2010). Synthesis of lipids in microalgae consist of three steps in three compartments of the cell (a) the formation of acetyl coenzyme A (acetyl-CoA) in the chloroplast (b) the elongation and desaturation of carbon chain of fatty acids in the cytoplasm and (c) the biosynthesis of triglycerides in the membrane of endoplasmic reticulum.

The metabolic flux route on the utilization of carbon dioxide and glucose for the formation of acetyl-CoA in microalgae is described by Yang *et al* (2000). Microalgae can fix CO_2 into sugars using energy from the sun. The fixed sugars are further processed to produce acetyl-CoA, and more than one pathway may contribute to maintain the acetyl-CoA pool. Acetyl-CoA provided by photosynthesis serves as the precursor for fatty acid synthesis in the chloroplast. Similar to biosynthesis of fatty acids in higher plants, the building blocks for TAG and membrane lipids, occurs in the chloroplast (Sirevag and Levine, 1972), and is catalyzed by two large, evolutionarily conserved enzymes: acetyl-CoA carboxylase (ACCase) and type-2 fatty acid synthase (Riekhof, 2005; Moellering *et al* 2009; Riekhof and Benning, 2009). The resulting fatty acids can be used directly in the chloroplast to sequentially acylate glycerol-3-phosphate (G-3-P) by chloroplast-resident acyl transferases to produce lysophosphatidic acid (LysoPA) and phosphatidic acid (PA). The PA and its dephosphorylated product diacylglycerol (DAG) generated in the chloroplast serve primarily as precursors for structural lipids of the photosynthetic membrane system (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). Alternatively, fatty acids can be exported into the cytosol by fatty acyl transferases. Free fatty acids linked with acetyl- CoA are used to sequentially acylate G-3-P in the ER by ER-resident acyl transferase isoforms (Fig 1.1). Further modifications of fatty acids like chain elongation and desaturation happens in the membrane of the ER. The resultant PA can be dephosphorylated to produce DAG that can be used to synthesize both membrane lipids and stored form of TAG (Browse and Somerville, 1991; Ohlrogge and Browse 1995). Due to the stringent substrate specificity of LysoPA acyltransferases present in the chloroplast and ER, lipids made by the chloroplast or ER pathway are characterized by the presence of a 16- or 18-carbon fatty acid at the sn-2 position of glycerol backbone, respectively (Giroud *et al,* 1988; Frentzen, 1998). The biosynthesis of TAG has long been known to occur in microsomal membranes on the basis of subcellular fractionation studies (Wilgram and Kennedy 1963; Stymne and Stobart. 1987). Synthesised TAG in the ER being deposited in ER-derived lipid droplets in the cytosol (Martin and Parton, 2006) called lipid bodies.

Figure 1.1: Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis



Adapted from Radakovits et al, 2010

1.3. Cultivation of microalgae

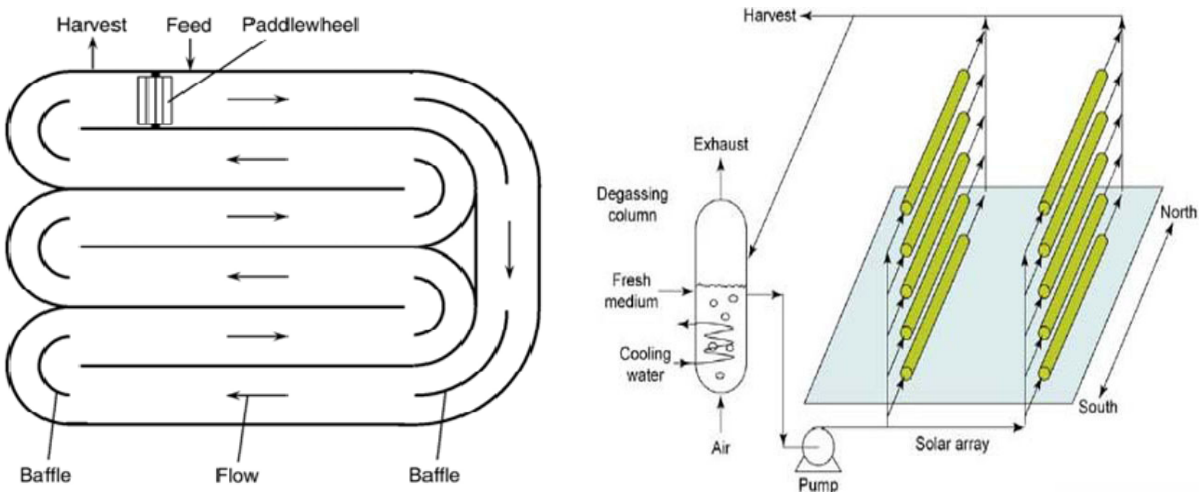
For the mass cultivation of microalgae there are two most commonly used techniques. These are open raceway pond system and closed photobioreactor systems. The open pond system is less favorable due to limitations in controlling contaminations from predators and other micro algal species while the photobioreactors provide an easy system of controlling nutrients for growth, cultivation parameters such as temperature, dissolved CO_2 and pH and prevent contaminations (Ugwu *et al,* 2008). For unialgal cultivation, photobiotreactors are preferred. Nevertheless, photobioreactors have a high initial cost and are very specific to the physiology of microalgae strain being cultivated. Microalgal production facility is an important factor to be considered for the optimum production of a specific microalgal species.

1.3.1 Open pond

Open ponds are the oldest and simplest systems for mass cultivation of microalgae. In this system, the shallow pond is usually about one-foot deep, and algae are cultured under conditions identical to their natural environment. Open pond comes in many different forms and shapes each having their own advantages and drawbacks. The types of ponds that are currently used in research and industry include raceway ponds, shallow big ponds, circular ponds, tanks and closed ponds. Among these, the raceway ponds are commonly employed for micro-algal cultivations. A raceway pond is made of a closed loop recirculation channel that is typically about 0.3 m deep. Mixing and circulation are produced by a paddlewheel (Fig. 2A). Flow is guided around bends by baffles placed in the flow channel. Raceway channels are built in concrete or compacted earth, and may be lined with white plastic in order to get more photosynthetically active radiations (PAR). During daylight, the culture is fed continuously in front of the paddlewheel where the flow begins. Broth is harvested behind the paddlewheel, on completion of the circulation loop. The paddlewheel operates all the time to prevent sedimentation. Raceway ponds for mass culture of microalgae have been used since the 1950s. The largest raceway-based biomass production facility occupies an area of 440,000 m^2 and is used to produce cyanobacterial biomass for food applications (Spolaore *et al,* 2006). The location in which the pond is situated is a critical factor in determining the type of pond selected, algal strain and amount of light for photosynthesis. Due to the lack of control involved with open systems, the pond becomes a function of the local climatic conditions, thus the location of the pond significantly contributes to the success of the cultivation (Masojidek and Torzillo, 2008). Although open ponds cost less to build and operate than photobioreactors, this culture system has its intrinsic disadvantages. Because they are open-air systems, they often experience a lot of water loss due to evaporation. Thus, open ponds do not allow microalgae to use carbon dioxide as efficiently, and biomass production is limited (Chisti, 2007). Biomass productivity is also limited by contamination with unwanted algal species as well as organisms that feed on algae (protozoans). In addition, optimal culture conditions are difficult to maintain in open ponds, and recovering the biomass from such a dilute culture is expensive (Chisti, 2007; Harun *et al,* 2010). Several studies have assessed the possibility of microalgal cultivation using the open ponds system. *Dunaliella salina* is one of the most successful species that have been used for production of carotenoids which the alga produces to defend itself against the intense of climatic conditions in open pond system (Tafreshi and Shariati, 2006) Hase *et al,* (2000) achieved stable photosynthetic

efficiency of *Chlorella sp*. and *Chlorophyta* sp. in raceway system. Moreover, Blanco *et al,* (2007) used *Muriellopsis sp*. to produce lutein rich cells in the open tank agitated with a paddlewheel.

Figure 1.2: Cultivation systems for microalgae



A: Open raceway cultivation **B**: Photobioreactor

Adapted from Chisti (2007)

1.3.2. Photobioreactors

Major limitations of open ponds are contamination and evaporation of water. These problems may be overcome by the use of photobioreactors. These systems are made of transparent materials and are generally placed outdoors for illumination by natural light. The cultivation vessels have a large surface area-to-volume ratio. Higher productivity is achieved in the photobioreactor due to the controlled environment in the reactor. Biomass productivity is the most important indicator for success of technology behind a bioreactor. Basically, photobioreactor comes in a different range of designs: tubular and plate-types. The most widely used photobioreactor is a tubular design, which has a number of clear transparent tubes, usually aligned with the sun's rays (Fig.2B). The tubes are generally less than 10 cm in diameter to maximize sunlight penetration. The culture medium is circulated through a pump to the tubes, where it is exposed to light for photosynthesis, and then back to a reservoir. A portion of the algae is usually harvested after it passes through the solar collection tubes, making continuous algal culture possible. The tubing can be arranged in various

configurations and the appropriateness of the configuration depends on the specifications of the system. Common configurations include straight line and coiled tubing (Ugwu *et al,* 2008). For high value products, the tubes are coiled spirals known as a helical-tubular photobioreactor. These systems sometimes require artificial illumination, which adds to production costs, so this technology is not suited for biodiesel production. In order to maintain high turbulent flow, a mechanical pump or an airlift pump is employed in the reactor, which prevents the algal biomass from settling (Chisti, 2007). The geometry of the reactor is also an important factor, as tubular reactors can be configured in a vertical, horizontal or inclined plane. The major difference between the configurations is that the vertical design allows greater mass transfer and a decrease in energy usage, while the horizontal reactor is more scalable, but requires a large area of land (Ugwu *et al,* 2008).

A number of studies have been reported for the application of all types of tubular photobioreactors in culturing microalgae: vertical (Babcock et al, 2000), horizontal (Richmond *et al,* 1993) and helical (Hall *et al,* 2003). However, flat-plate photobioreactor is broadly in use due to narrow light path, which helps maintaining higher cell densities compared to other photobioreactors (Rodolfi *et al,* 2009). Additionally, these types of reactors are favorable due to (1) low power energy consumption and high mass transfer capacity, (2) reduction in oxygen build up and (3) high photosynthetic efficiency (Gitelson *et al,* 1996). Advantages and limitations of open ponds and photobioreactors are listed in Table 1.2. An appropriate reactor design is required to obtain the maximal cell mass. Various designs of flat-plate photobioreactors to cultivate microalgae have been constructed: glass types (Qiang *et al,* 1998), thick transparent PVC materials (Ortega and Roux, 1986), V-shaped (Iqbal *et al,* 1993) and inclined (Hu *et al,* 1996). The glass and PVC types are more transparent for maximum light penetration while other designs are cheap and easy to construct.

Photosynthesis process generates oxygen if not removed from the reactor can harm biomass production. In an open raceway system, this is not a problem as the oxygen is simply returned to the atmosphere. However, in a photo bioreactor, the oxygen build up can inhibit the growth of algae and hence the culture must periodically be returned to a degassing zone, where the algal broth is bubbled with air to remove the excess oxygen. Also, the algae use up carbon dioxide, which can cause carbon starvation and an increase in pH. Therefore, carbon dioxide must be fed into the system in order to successfully cultivate the microalgae on a large scale. Photobioreactors require cooling during daylight hours, and the temperature must be regulated in night hours as well. This may be done through heat exchangers located either

in the tubes themselves or in the degassing column. The obvious advantages of photobioreactors are that they can overcome the problems of contamination and evaporation encountered in open ponds (Grima *et al,* 1999). The biomass productivity of photobioreactors can average 13 times more than that of a traditional raceway pond. Harvest of biomass from photobioreactors is less expensive since the typical algal biomass is about 30 times as concentrated as the biomass in raceways (Chisti, 2007). However, photobioreactors also have some disadvantages. For example, the reactors are difficult to scale up. Moreover, light limitation cannot be entirely overcome because light penetration is inversely proportional to the cell concentration. Attachment of cells to the tubes' walls may also prevent light penetration. Although enclosed systems can enhance biomass concentration, the growth of microalgae is still sub-optimal due to variations in temperature and light intensity.

Table: 1.2 Comparison of raceway ponds and different photobioreactors for cultivation of microalgae

Production system	Advantages	Limitations
Raceway pond	Relatively cheap Easy to clean Utilizes non-agricultural land Low energy inputs easy maintenance	Poor biomass productivity Large area of land required Limited to a few strains of algae Poor mixing, light and CO_2 utilization Cultures are easily contaminated
Tubular photobioreactor	Large illumination surface area Suitable for outdoor cultures Relatively cheap Good biomass productivities	Some degree of wall growth Fouling Requires large land space Gradients of pH, dissolved oxygen and CO_2 along the tubes
Flat plate photobioreactor	High biomass productivities Easy to sterilize Low oxygen build-up Readily tempered Good light path Large illumination surface area Suitable for outdoor cultures	Difficult scale-up Difficult temperature control Small degree of hydrodynamic stress Some degree of wall growth
Column photobioreactor	Compact High mass transfer Low energy consumption Good mixing with low shear stress Easy to sterilize Reduced photo inhibition and photo-oxidation	Small illumination area Expensive compared to open ponds Shear stress Sophisticated construction

1.4. Algal biomass recovery

Although oil-accumulating microalgae are a promising feedstock for biodiesel production, large-scale production of biofuels from microalgae is not yet economically viable. This is mainly due to the high-energy inputs required for harvesting of the algal cells (Godos *et al,* 2011) Microalgal biomass production system includes growing microalgae in an environment that favors accumulation of target product and recovery of the microalgal biomass for downstream processing. However, due to the small size (5~50 μm) (Grima *et al,* 2003), the negative surface charge (about −7.5~−40 mV) on the algae that results in dispersed stable algal suspensions especially during the growth phase (Moraine *et al,* 1979; Edzwald 1993; Packer, 2009), low biomass concentrations (0.5~5 g/L) and densities similar to that of water (Reynolds 1984), their harvesting is extremely difficult. Harvesting microalgal biomass from growth medium is a significant challenge in many of the industries dealing with microalgal biomass production. In some commercial production systems, the culture broths have biomass densities below 0.5 kg/m^3, which mean that huge volumes need to be handed before algal oil extraction (Chen *et al,* 2014). Developing a cost effective harvesting method is one of the most challenging areas in the algal biofuel research (Greenwell *et al,* 2009) and a key factor that limit the commercial use of microalgae. It has been reported that 20–30% of the total production cost is involved in the biomass harvesting (Mata *et al,* 2010; Molina *et al,* 2003). Other researchers have reported that the cost of the recovery process in their study contributed about 50% to the final cost of oil production (Chisti.2007; Greenwell *et al,* 2009). Many of the studies on the microalgal biofuel production have been focused on the yield and composition of biomass rather than harvesting process. Therefore, it is necessary to develop effective and economic technologies for harvesting. There are currently several harvesting methods tried, including mechanical, electrical, biological and chemical based methods (Christenson and Sims, 2011). In mechanical methods, microalgal cells are harvested by mechanical external forces, such as centrifugation (Shelef and Sukenik, 1984), filtration (Vonshak and Richmond,1988), sedimentation (Shen *et al,* 2009), dissolved air flotation (Greenwell *et al*, 2009; Zhang *et al,* 2012) and usage of attached algal biofilms and ultrafiltration membranes (Johnson and Wen 2010; Zhang *et al*, 2010). Electrical based methods are based on electrophoresis of the algae cell (Sridhar et al, 1988; Vandamme *et al*, 2011). Because of the negative charge of microalgal cells, they can be concentrated by being moved in an electric field (Kumar *et al,* 1981; Zhang *et al,* 2012). Biological based methods are flocculation caused by extracellular polymeric substance such as polysaccharides and

proteins, originating from microalgae and microorganisms (Nie *et al,* 2011). Chemical based methods mainly refer to chemical flocculation induced by inorganic and organic flocculants. Electrolytes and synthetic polymers are typically utilized (Zheng *et al,* 2012). Among these, commonly used methods for harvesting algal biomass are flocculation, centrifugation and filtration.

1.4.1. Flocculation

Flocculation is the result of the particle collision and charge interaction between charges of the flocculants and cell surface in a liquid medium. Flocculation has been suggested as a superior method to separate algae as it can handle large quantities of micro-algal suspension and a wide range of microalgae (Uduman *et al,* 2010). Increasing the size of particles by the aggregation of algal cells through flocculation can increase the rate of settling or flotation (Mata *et al,* 2010; Williams and Laurens, 2010). Zeta potential is the apparent surface charge of the cells, which may affect the efficiency of flocculation (Henderson *et al*, 2008). Presence of negative charge on the surface of microalgae prevents them from self aggregation within the suspension. The surface charge on the algae can be neutralized by the addition of chemicals known as flocculants. It has been reported that flocculation is a most reliable and cost effective method, although unfortunately it is still quite expensive process (Benemann *et al,* 1980). Flocculation can be induced by chemicals, both inorganic and organic or by microorganisms. An ideal flocculent for microalgal flocculation should be inexpensive, nontoxic and effective in low concentrations (Grima *et al,* 2003). Multivalent inorganic metal salts like ferric chloride, ferric sulphate, and aluminum chloride (alum) and aluminum sulphate are commonly used in wastewater treatment to remove algae, and alum has been found effective in flocculating both *Chlorella* and *Scenedesmus* (Grima *et al,* 2003). Aluminum salts have been found more effective in the flocculation of *Chlorella* than Ferric salts (Papazi *et al,* 2010). 90% efficiency of flocculation was reported in the case of *Schizochytrium limacinum*, *Chlamydomonas reinhardtii,* and *Scenedesmus sp* when aluminum sulphate is used as flocculating agent (Gerde *et al,* 2014). Flocculation mediated by Ferric chloride could attain 66-98% efficiency in the case of fresh water microalgae at a concentration of 150-250 mg/l (Godos *et al,* 2011). Many reports have suggested that inorganic flocculants can also have negative effect on the viability of algal cells and coloration, and they may modify the growth medium preventing its recycling and reuse (Grima *et al,* 2003; Schenk *et al,* 2008; Papazi *et al,* 2010). Although Alum and other

inorganic flocculants are relatively cheap compared to organic flocculants, the higher dosage rates required can result in a higher cost per unit of microalgal cells flocculated than more expensive organic flocculants (Mohn, 1988).

Auto-flocculation is the flocculation that can occur naturally in certain micro algae and microalgae may flocculate in response to some environmental stress, changes in nitrogen and pH and dissolved oxygen (Schenk *et al,* 2008; Uduman *et al,* 2010). Auto-flocculation does not occur in all micro-algal species and the process can be slow and unreliable (Schenk *et al,* 2008). It has been reported that this phenomenon was associated with elevated pH due to photosynthetic CO_2 consumption corresponding with precipitation of magnesium, calcium, phosphate, and carbonate salts with algal cells (Sukenik and Shelef, 1984). In the case of calcium phosphate used in the medium excess calcium ions (positive charged) tends to react with the algal cells (negative charged) and binds them together enhancing the auto-flocculation process (Sukenik and Shelef, 1984).

In comparison to inorganic flocculants the organic flocculants are reported to give an advantage in terms of lesser sensitivity to pH, non toxic nature, wide range of applications and requirement of lower dosages for flocculation process. Chitosan is a natural flocculent commonly used in wastewater treatment for suspended solid separation. It's low cost and nontoxic nature make it one of the preferred flocculants in microalgae- based biotechnologies (Lersutthiwong *et al*, 2009). 91.3% of flocculation efficiency was achieved for flocculation of *Tetraselmis sp.*(Kwon *et al,* 2014). However for large scale harvesting of algal biomass, usage of chitosan will be costlier and the requirement of higher dosages compared to chemicals would appear to make it uneconomic for harvesting of micro-algae for biofuel production (Mohn 1988; Vandamme *et al,* 2010). Cationic starch was used for flocculation of the microalgae *Scenedesmus* sp., *Chlamydomonas reinhardtii, and Schizochytrium. limacinum* (Gerde *et al,* 2014). The other type of flocculants used are polyelectrolytes, which are cationic polymers having the property of physically linking cells together. The extent of aggregation by the polyelectrolytes will depend on the specific properties of the polymer. Increasing the molecular weight and charge on the polymers has been shown to increase their binding capabilities. The type of polymer chosen will also depend on the properties of the algal culture, such as charge in broth, pH and biomass concentration. The cationic polyelectrolyte gave better flocculation results for *Chlorella* (Tenney *et al,* 1969), whereas no flocculation was found with the anionic polyelectrolyte.

Bio-flocculants have emerged as a new research trend in flocculation technology. Generally, bioflocculation is a dynamic process resulting from synthesis of extracellular

polymer substances (EPS) by living cells (Salehizadeh *et al,* 2000). Upto now, bacteria, fungi and actinomycetes have been identified as bio-flocculent-producing microorganisms, as these microorganisms are capable of producing EPS such as polysaccharides, functional proteins and glycoprotein, which act as bioflocculants (Gao *et al,* 2006). A recent report has underlined that bioflocculant from *Pseudomonas stutzeri* and *Bacillus cereus* was effective in flocculating the marine microalga *P. carterae* (CCMP647) (Lee *et al*, 2009). Fungal assisted flocculation of microalgae was also reported (Muradov *et al,* 2015). Bio-flocculation by using flocculating microalga to concentrate the non-flocculating microalga was also reported (Salim et al, 2011). The advantages of this method are that no addition of chemical flocculants is required and similar cultivation conditions can be used for the flocculating microalgae for harvesting of the target microalgae. This method is as easy and effective as chemical flocculation which is applied at industrial scale. It is also sustainable and cost-effective as no costs are involved for pre-treatment of the biomass for oil extraction and for pre-treatment of the medium before it can be re-used.

1.4.2. Centrifugation

Centrifugation is preferred method for harvesting of high value metabolites from microalgae (Benemann *et al*, 1980; Heasman *et al*, 2000 and Grima *et al,* 2003). Centrifugation involves the application of centripetal acceleration to separate the algal growth medium into regions of greater and less densities. Once the biomass is separated, the algae can be removed from the culture by simply draining the excess medium. Sim *et al* (1988) compared different techniques in harvesting microalgae and found that centrifugation is most efficient method for biomass recovery as compared to other techniques such as dissolved air flotation and drum filtration. It was reported that 88–100% cell viability and around 95–100% harvesting efficiency can be achieved by centrifugation at 13,000g (Heasman *et al,* 2000). Nevertheless, laboratory centrifugation was reported more suitable when the concentration of the suspended sediment was above 30 mg/l (Horowltz, 1982). But this method has some limitations- high gravitational and shear forces during the centrifugation process can damage cell structure thus limiting the speed of centrifugation. The process is rapid and energy intensive; biomass recovery depends on the settling characteristics of the cells, slurry residence time in the centrifuge, and settling depth (Grima *et al,* 2003). The major disadvantages of the process include high energy costs and potentially higher maintenance requirements due to the freely

moving parts (Bosma *et al,* 2003) and thus it may not be cost effective when considering large volumes of culture.

1.4.3. Filtration

Filtration is one of the harvesting options for biomass recovery that has proved to be the most viable compared to other concentration methods. This process however, can be obstructed by low throughput and rapid clogging (Mohn 1988; Oswald 1988). There is a wide variety of filter designs and membrane filters that can be simply classified by their pore or membrane size; macro filtration >10 μm, micro-filtration 0.1–10 μm, ultra filtration 0.02–0.2 μm and reverse osmosis ~0.001 μm are used for filtration. For this process, pressure to force fluid through a membrane is required therefore the operational energy is applied, and generally energy increases with reducing membrane pore size. As the size range of micro-algae is typically between 2 and 30 μm (Grima *et al,* 2003; Brennan and Owende 2010) this would suggest that micro-filtration has the most appropriate pore size for the majority of common species such as *Chlorella* and *Cyclotella* which are 5–6 μm in diameter (Edzwald, 1993), while macro filtration is the most appropriate for flocculated cells and larger cells like those of *Coelastrum* and *Spirulina*. There are many different types of filtrations used such as dead end filtration, microfiltration, ultra filtration, pressure filtration, vacuum filtration and tangential flow filtration (TFF). Commonly, filtration involves running the broth with algae through filters on which the algae will accumulate and allow the medium to pass through the filter.

Ultra filtration is a possible alternative for recovery of very fragile cells, but has not been generally used for microalgae (Grima *et al,* 2003; Mata *et al,* 2010), though the operation and maintenance costs are very high (Purchas 1981; Mata *et al,* 2010). It has been suggested that ultra- filtration of micro-algae will extend in a similar way as desalination of seawater by reverse osmosis. The energy input for an optimised micro-algal ultra filtration plant could be 3 kWh m^{-3} which is equivalent to the lowest current energy usage for reverse osmosis desalination (Gouveia, 2011). Major problem with this process is the rapid clogging of ultra filtration membranes by extracellular organic matter (Rossi *et al,* 2004). A wide range of macro-filtration units have been used for water treatment. Vibrating screens were able to separate *Coelastrum* and *Spirulina,* although not considered to be the optimum method for *Spirulina* (Mohn, 1988). The energy cost to produce 6 % dry weight of micro-algae has been estimated at 0.4 kWh m^{-3} (Van den Hende *et al,* 2011). A modified filter press with plastic

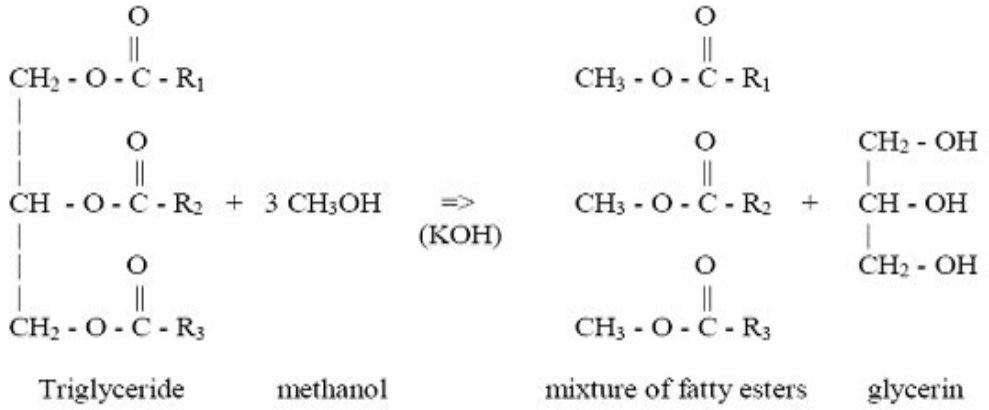
diaphragms that inflate to remove the micro-algae from the filter membrane has been found to be effective in the filtration of *Scenedesmus sp*, but capital cost are approximately one third higher than conventional filter presses and pre-coating of membrane with starch was required to prevent clogging (Mohn,1988). Rotary vacuum filters are used for the filtration of *Coelastrum*, a micro-alga that forms small colonies, which can be filtered to obtain a algal cake containing 18 % dry weight solids without a filter pre-coat, but filtration rates fall rapidly and high energy inputs are required. Vacuum belt filters can filter larger or colonial micro-algae, but investment and energy costs are very high (Mohn 1988). Larger species of micro-algae such as *Spirulina* and *Micractinium* can also be filtered on a rotary vacuum filter with a 12 μm pore diameter yielding 1–3 % dry weight micro-algal slurry, but smaller species of micro-algae such as *Chlorella* cannot filter effectively even if the pore size was reduced to 5 μm (Goh, 1984). Reports on filtration of microalgae suggest that filtration methods are suitable for micro-algae with larger cells, but inadequate to recover micro-algal species with diameters of less than 10 μm (Grima *et al,* 2003; Uduman *et al,* 2010). Always low cost harvesting technologies for microalgae are recommended for large scale operations.

1.5 Applications of Microalgae

1.5.1 Biodiesel

Biodiesel is fatty acid methyl esters derived from oil crops and biomass which can be used directly in conventional diesel engines (Clark and Deswarte, 2008). Oils rich in triglycerides are preferred substrate for biodiesel conversion (Chisti, 2007). It is a mixture of monoalkyl esters of long chain fatty acids (FAME) derived from a renewable lipid feedstock such as algal oil (Demirbas, 2009). Algal oils can be converted to biodiesel by a chemical reaction called trans-esterification, in which triglycerides and alcohol react with catalyst to produce mono alkyl esters (Chisti 2007; Hu *et al,* 2008; Mata *et al,* 2010). Trans-esterification is a multi step process with three reversible reactions in which triglycerides are first converted to diglycerides, and then diglycerides are converted to monoglycerides. Finally monoglycerides are converted to esters of fatty acids (diesel) and glycerol (by-product). Reactions of transesterifications are described in (Fig 1.3). Radicals R1, R2, R3 represent long chain fatty acids.

Figure: 3 Trans-esterification of Triglycerides to form Biodiesel



Adapted from Mata *et al,* (2010)

For the trans-esterification reaction, oil and a short chain alcohol (usually methanol) are used as reagents in the presence of a catalyst (usually NaOH). Although the alcohol: oil theoretical molar ratio is 3:1, the molar ratio of 6:1 is generally used to complete the reaction accurately. The relationship between the feedstock mass input and biodiesel mass output is about 1:1 which means that theoretically, 1 kg of oil results in about 1 kg of biodiesel (Mata *et al,* 2010). For trans-esterification, homogeneous or heterogeneous acid or basic catalyst can be used to enhance the reaction rate, even though for some processes using supercritical fluids (methanol or ethanol) it may not be necessary to use a catalyst (Warabi *et al,* 2004; Silva and Vladimir 2014). Industrial processes dealing with trans-esterification of oils uses homogeneous alkali catalysts (e.g. NaOH or KOH) in a stirred reactor operating in batch mode. The primary advantages of biodiesel are that it is one of the most renewable fuels and also non-toxic, biodegradable and can be used in existing diesel engines without any modification, and it suitable for blending at any ratio with petroleum diesel (Van Gerpen 2005). When compared to 1st generation biodiesel, algal biodiesel is more suitable for use in the aviation industry where low freezing points and high energy densities are key criteria (NREL, 2006). Another major advantage of algal biodiesel is in reduced CO_2 emissions of up to 78% compared to emissions from petroleum diesel (Sheehan, 1998). For algal biodiesel to be an accepted substitution for fossil fuels, its properties must match or exceed the International Biodiesel Standard for Vehicles (EN14214). Algal oils contain a high degree of polyunsaturated fatty acids when compared to vegetable oils, which makes it susceptible to oxidation in storage and therefore limits its utilization.

1.5.2 Bioethanol

Bio ethanol can used as an alternative fuel because its use reduces the emission levels of sulfur, carbon monoxide, lead, particulates etc. With the increasing demand on fossil fuel, USA, Europe and other states are using or considering the substitution of petrol with ethanol (Willke and Vorlop, 2004). Biomass feed stock used for the generation of bioethanol production is corn and sugarcane which have same problem associated as biomass for biodiesel. The primary problem of these feed stocks is they have food application and large area of land required. Thus, both of these compete with food chain as well as land use, which pose constraint to expand production of these biofuels (Sun and Cheng, 2002). Traditionally, the ethanol is produced by fermentation of sugars, and biomass sources varying from agriculture and energy crops to organic wastes (Xuan *et al,* 2009). Alcoholic fermentation is one of the destinations of pyruvate at the end of the glycolytic pathway, and consisting of anaerobic conversion to ethanol and CO_2. The reactions occur in two steps. In the first step, pyruvate is decarboxylated by the enzyme pyruvate decarboxylase, releasing CO_2 and forming acetaldehyde, which is then reduced to ethanol by the enzyme alcohol dehydrogenase (Lehninger *et al,* 2004). The fermentation can be represented by the equation.

$$C_6H_{12}O_6 + 2ADP + 2Pi = 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O$$

Micro algae are gaining attraction as alternative feed stock for bioethanol production. Micro algae are rich in carbohydrates and proteins and fermentable carbon compound in their composition which is varied depending on the growth conditions directly available for fermentation or after pretreatment. The prospective feedstock and feasibility of the various processes for ethanol production have been reported by various authors. However bioethanol production by fermentation of algal biomass has not been reported extensively. The principle of ethanol production by microalgae consists of cultivation of microorganisms, harvesting of cells, preparation of biomass for fermentation and extraction process of ethanol. The preparation of the biomass can be carried out through mechanical equipment or enzymes that break down the cell walls, making the carbohydrates more available, as well as breaking down large molecules of carbohydrates. When cells are broken down, the yeast *Saccharonmyces cerevisiae* is added to the biomass and fermentation begins. Finally distillation is used to purify the ethanol (Amin, 2009). The comparatively recent work on fermentation for bioethanol production was reported by Harun *et al,* (2010). They described

the use of the microalga *Chlorococum sp*. as a substrate for ethanol production using yeast with 38% productivity of bioethanol which revealed the suitability of microalgae as a promising substrate for ethanol production. Besides ethanol, fermentation produces other product such as CO_2 and H_2O. The theoretical maximum is 0.51 kg of ethanol and 0.49 kg CO_2 per kg of glucose. Carbohydrate rich microalga *Chlorella vulgaris* was used for bioethnol production with 87.6% theoretical yield at a concentration of 11.7g/l (Ho *et al,* 2013). Bioethanol production yield of 16.32% has been reported using carbohydrate-enriched biomass of the cyanobacterium *Arthrospira platensis* (Markou et al, 2013). Microalgal ethanol can be purified and used as fuel and the CO_2 can be recycled by using it as a nutrient for the cultivation of microalgae and residual biomass in the process of anaerobic digestion (Harun *et al,* 2010b).

Although limited reports on algal biomass fermentation are available, advantages of using microalgal biomass for bioethanol lies in the process that involves less intake of energy and process is simple when compared with biodiesel process. Furthermore CO_2 produced as a byproduct from the fermentation process can be recycled back as carbon source for biomass production thus reducing the green house gas as well. However, the technology for the commercial production of bioethanol from microalgae is yet under development stage and is being further investigated.

1.5.3. Biomethane

Biomethane is a product of anaerobic digestion of organic matter, consisting primarily of 55.0–65.0% methane (CH_4), 30.0–45.0% carbon dioxide (CO_2), traces of hydrogen sulfide (H_2S) and water vapor (Kapdi *et al,* 2005; Cooney *et al,* 2007), and may contain small amounts of H_2 and CO (Bailey and Ollis, 1976). The energy content of biogas produced through anaerobic digestion is 16,200–30,600 kJ m^{-3} depending on the nature of the source of biomass processed (Chisti, 2008). Several bacteria act together during the anaerobic process, which initially involves hydrolytic bacteria (mainly cellulolytic and proteolytic) for the degradation of raw material, later the acidogenic bacteria, hydrogenated and sulfate-reducers are involved. Organic acids, gases, salts and oxidized organic matter (among others) are formed from these microorganisms. In a final stage, the methanogenic bacteria form methane, CO_2 and reduced organic compounds (Omer and Fadalla, 2003; Cooney *et al,* 2007).

Biomethane from anaerobic digestion can be used as fuel gas and can be burned to generate electricity (Holm-Nielsen *et al,* 2009). Residual biomass left over after anaerobic

digestion can also be processed to make fertilizers. In addition to being renewable and sustainable, this would encourage sustainable agricultural practices in providing greater efficiencies and reducing algal production costs. Absence of lignin and lower cellulose content in the algal biomass exhibit good process stability and high conversion efficiency for anaerobic digestion (Vergara-Fernandez *et al,* 2008). The anaerobic digestion process is desirable when one uses compounds with moisture content between 80.0% and 90.0%, and the use of microalgal biomass is highly appropriate for this process (Brennan and Owende, 2010). In southern Brazil, the biomass from *Spirulina* LEB 18 grown in an 18.0 m^3 raceway-type photobioreactor under environmental conditions was used as a substrate for biogas production in a semi-continuous anaerobic bioreactor. The feed rate of microalgal biomass was 0.1 tons day^{-1} and the initial concentration of Spirulina LEB 18 was 7.2 g l^{-1}. The biogas content obtained in the methane was 77.7% (Costa *et al,* 2008). The conversion of microalgal biomass into biogas, recovers energy through the extraction of lipids that can be used for biodiesel production (Brennan and Owende, 2010). Many of the micro algae are enriched with high protein content which can result in increased production of ammonia that inhibits the anaerobic microorganisms. This can be overcome by pre-adapting the microorganism for this process. Presence of high proportion of proteins in the algal biomass results in lowering the C/N ratio, which affects the efficiency of anaerobic digestion. This problem can be solved by co-digestion with products containing a high C/N ratio (Brennan and Owende, 2010). Biogas production can be increased by addition of residues from paper recycling to microalgal biomass (Yen and Brune, 2007). The integrated technology that combines algal cultivation and wastewater treatment system for methane production can be most suitable approach to reduce production cost and to make it more viable. It may be concluded that waste water ponds could avoid eutrophication process, improve waste nutrient treatment and low cost algal biomass for biogas production.

1.5.4. Bio-oils

Currently, bio-oil obtained from algae is considered a promising alternative to fossil fuels due to its high energy content and low life-cycle emissions of greenhouse gases (Li *et al,* 2011; Azadi *et al,* 2014). Thermochemical liquefaction and pyrolysis are two methods used for the production of bio-oils from wet algal biomass. Thermochemical liquefaction is a process that can be employed to convert wet algal biomass into liquid fuel (Patil *et al,* 2008).

This process happens at high-temperature (300–350 °C) and high pressure (5–20 MPa) aided by a catalyst in the presence of hydrogen to yield bio-oil (Goyal *et al,* 2008). Reactors for thermo-chemical liquefaction and fuel-feed systems are complex and therefore the process is expensive (McKendry, 2002), but advantage of the process is their ability to convert wet biomass into energy (Clark and Deswarte, 2008). The process utilizes the high water activity in sub-critical conditions to decompose biomass materials down to shorter and smaller molecules with a higher energy density (Patil *et al,* 2008). This process is fast and eco-friendly (Peterson *et al,* 2008) and is energetically efficient because it does not include a drying step (Bridgwater *et al,* 1999; Jena and Das 2011).

Thermochemical liquefaction of *B. braunii* at 300 °C resulted in a maximum yield of 64% dry wt. basis of oil with HHV of 45.9 MJ kg^{-1} and also declared a positive energy balance for the process (output/input ratio of 6.67:1) (Dote *et al,* 1994). In a similar study conducted in *Dunaliella tertiolecta*, it showed an oil yield of 42% dry wt. obtained from giving a HHV of 34.9 MJ kg^{-1} and positive energy balance of 2.94:1 (Minowa *et al,*1995). Hydrothermal liquefaction of macro alga *Ulva fasciata* shows bio-oil yield of 40% (Singh *et al,* 2015.)

Pyrolysis is the conversion of biomass to bio-oil, syngas and charcoal at medium to high temperatures (350–700 °C) in the absence of air (Goyal *et al,* 2008). For biomass-to-liquid fuel conversion, it is considered to have the potential for large scale production of biofuels that could replace petroleum based liquid fuel (Demirbas, 2006). Few studies are conducted for the pyrolysis of microalgal biomass for possibilities of bio-oil productions (Miao *et al,* 2004; Grierson *et al,* 2009). Studies conducted with the microalgal species *C. protothecoides* and *M. aeruginosa*, yielded 17.5 and 23.7% oils respectively by the pyrolysis process whereas the yield of bio-oil was 57.9% from heterotrophic *Chlorella protothecoides* (Miao *et al,* 2004). The property of bio-oils from microalgae was more suitable for fuel use compared to those from lignocellulosic materials. Research on the influence of temperature and catalyst was conducted for the pyrolysis of *Nannochloropsis* sp. The result showed maximum bio-oil yield of 31.1 %.

1.5.5. Environmental application

Production of biofuels and other value added products from microalgae can be more environmentally sustainable, cost- effective and profitable, if combined with processes such as wastewater and flue gas treatments.

1.5.5.1 Bio- mitigation of CO_2

There are two main CO_2 mitigation strategies normally used -the chemical reaction-based approaches and the biological mitigation (Wang *et al,* 2008), Chemical reaction-based CO_2 mitigation approaches are energy-consuming, use costly processes, and have disposal problems because both the captured carbon dioxide and the wasted absorbents need to be disposed. On other hand, the biological CO_2 mitigation has attracted much attention in the recent past since it leads to the production of biomass energy in the process of CO_2 fixation through photosynthesis (Pulz and Gross, 2004). Flue gases from power plants are responsible for more than 7% of the total world CO_2 emissions in the atmosphere (Kadam, 1997) and industrial exhaust gases contains up to 15% CO_2 (Maeda *et al,* 1995; Kadam 2001). Microalgal cultivation by supplementing of CO_2-rich flue gases or other exhaust gases is potentially more efficient route for CO_2 bio-fixation process. Therefore, the use of flue gas from an industrial process unit as source of CO_2 for the microalgal growth has the potential to mitigate CO_2 in a much greener way than the existing strategies. Studies conducted using the green microalga *Monoruphidium minutum* shows that this alga can efficiently utilize flue gas containing high CO_2 as well as sulfur and nitrogen oxides, as a feedstock for the production of biomass (Zeiler *et al,* 1995).

Microalga tolerant to high CO_2 levels are beneficial to be used for its biofixation from the flue gases. *Chlorococcum littorale* a marine alga, showed tolerance to high CO_2 concentration of up to 40%-50% (Iwasaki *et al,* 1998; Ota *et al*, 2009). *Chlorella s*trains from hot springs and tolerant to high temperatures up to 42 °C, was demonstrated for for CO_2 fixation from industrial flue gases containing up to 40% CO_2 (Sakai *et al,* 1995). In terms of carbon savings, the use of microalgae for biodiesel can lower considerably the CO_2 emissions because the CO_2 released during the combustion should equal the CO_2 fixed during plant or algal photosynthesis and growth. Algal biomass production in CO_2 rich environment reduces the level of CO_2 in the environment, which can further decrease the level of green house gases in the atmosphere, thus reducing the global warming.

1.5.5.2. Waste water treatment

Microalgal biomass production and wastewater treatment (e.g. of amino acids, enzyme, or food industries wastewaters) seems to be quite promising for microalgal growth combined with biological cleaning. This provides nutrients for microalgae in the form of organic

compounds nitrogen, phosphorous and carbon. Additionally, microalgae can mitigate the effects of sewage effluent and industrial sources of nitrogenous waste such as those originating from water treatment or dairy waste water. Moreover, by removing nitrogen and carbon from water, microalgae can help reduce the eutrophication in the aquatic environment. For mass cultivation of microalgae for biofuel application, waste water linked algal cultivation offers low cost biomass and phycoremediation of the waste water. Aslan and Kapdan (2006) reported *C. vulgaris* for nitrogen and phosphorus removal from wastewater with an average removal efficiency of 72% for nitrogen and 28% for phosphorus. Dairy effluent used as medium for the growth of *Chlorococcum* sp.R-AP13 showed COD and BOD removal of waste water in the range of 93 and 82% (Sabeela and Sukumaran, 2014). Other widely used microalgae cultures for nutrient removal are *Chlorella* (Gonzales *et al,* 1997; Lee and Lee 2001; Azeez 2010) *Scenedesmus* (Martınez *et al,* 2000; Zhang *et al*, 2014; Alvarez-Diaz *et al,* 2015), and *Spirulina* species (Olguin *et al,* 2003). Nutrient removal capacities of *Nannochloris*, *Botryococcus brauinii* (Jimenez-Perez, 2004), and the cyanobacterium *Phormidium bohneri* (Dumas *et al*, 1998; An *et al,* 2003) have also been investigated. These studies reveal that dual use of microalgal cultivation for wastewater treatment and production of value added compounds or biofuel is an attractive option, in terms of reducing the energy cost, and the nutrient and freshwater resource costs for biofuel production.

1.5.6 Microalgae as source of polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are essential fatty acids for human development and physiology. PUFAs have been used in clinical purposes, such as for treatment of heart and inflammatory diseases; asthma, arthritis, migraine, headache and psoriasis (Singh *et al*, 2005). PUFAs has important role in reducing the risk of many diseases. Omega-3 fatty acids are widely obtained from fish oil, but in recent years, problems associated with unpleasant taste and poor oxidative stability of fish oil, makes it less favorable (Luiten *et al,* 2003). Also, inadequate of fish oil supplies contribute to limit its use and it is not suitable for vegetarian diet. Microalgae are found to be primary source of PUFAs and the higher order organisms get these fatty acids through food chain. Since humans do not have the enzymes for the synthesis of PUFAs, it should be supplied through the diet. Docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) are important omega-3 fatty acids involved in many metabolic activities in animals. A number of literature have been reported the mass culture of

microalgae to produce EPA. Cultivation of marine microalga, *Pavlova viridis* in 60L outdoor photobioreactor was reported for the production of EPA (Hu *et al,* 2008). The outdoor photobioreactor system gave a lower total fatty acid content but higher EPA compared to the indoor system. In another study outdoor photobioreactor cultivation of *Nannochloropsis sp*. was employed also for EPA production (Cheng-Wu *et al,* 2001). Heterotrophic microalgae are well established as an alternative source of DHA and the essential fatty acid is added to infant milk formula and other foods (Van *et al,* 2009). Heterotrophic microalgae producing DHA and EPA include *Chlorella, Nitzschia, Cyclotella, Tetraselmis, Schizochytrium, Phaeodactylum tricornutum* and *Crypthecodinium* species. Heterotrophic algae produce omega-3 LC-PUFA and its production mainly depends on the species and cultivation conditions. Heterotrophic DHA, predominantly obtained from *Schizochytrium* species, is already commercially available as dietary supplement, but is also used in health foods, infant food, animal feed and aquaculture (Ryckebosch *et al,* 2012).

1.5.7. Livestock feed

Another important application of microalgae is livestock feed**.** Many researchers have examined biochemical composition of microalgae for their suitability as a substitute for the primary livestock feed. It has been reported that microalgae also play a key role in high-grade animal nutrition food, from aquaculture to farm animals. Microalgal nutritional and toxicological evaluations have demonstrated suitability of algal biomass as a valuable feed supplement (Dhargalkar, 2009). It was also reported that some edible seaweeds can be used as healthy food due to lower calories, high concentration of minerals, vitamins and proteins and a low fat content. *Spirulina* is still used as food supplement due to its excellent nutritional features and digestibility (Kumar *et al*, 2005). In addition to the high protein content of 60–70 % dry cell weight, it is a rich source of vitamins, particularly vitamin B12, β-carotene and minerals (Kumar *et al*, 2005; Thajuddin and Subramanian, 2010). *Chlorella* is found as potential food, because it contains the complete nutrients required for human nourishment. Conventional protein feed of 5-10% can be replaced by micro algal sources in poultry feed. *Porphyridium sp.* as feed supplement on metabolism of chicken showed that cholesterol of egg yolk was reduced about 10% and the colour of egg yolk became darker, indicating higher content of carotenoids (Spolaore *et al*, 2006; Ginzberg *et al*, 2000).

1.6. Objectives of the current study

Biodiesel from algal oil is considered as a prime candidate for short to medium term replacement or substitution of petro diesel. Algal biomass can be of nutritional value, besides being a source of oil that can be converted into biodiesel. The work focused on isolation of micro algae from virgin forests of Silent Valley National Park, in the Western Ghats, which is a biodiversity hotspot harbouring several endemic species. It was speculated that micro algae from such habitats may be sources of algal biomass rich in novel lipids and other value added products for both biofuel and nutraceutical applications. Microalgae are comparatively easier to cultivate and are amenable for continuous cultivation, but the major challenges in algal cultivation is the cost for production of biomass and harvesting of algal biomass from the production medium. Algal culture systems can be open or closed. In open pond culture systems, exposure to too much sunlight may result in cell bleaching and death and the temperatures must be maintained at the optimal levels for growth of the cultivated species. Cell densities achieved in phototrophic cultivation are generally low, making it essential to have large areas for cultivation to obtain sufficient biomass. Associated with such cultivation systems are the requirement of huge volumes of water. Though the yields of oil from microalgae are reported to be 15-300 times more than the traditional crops on area basis, one of the most important concerns is the need to provide substantial amounts of water and nutrients to the algal populations undergoing mass cultivation. Major challenges in algal cultivation include 1) Strains capable of high growth rate and oil production 2) Ability to cultivate the microalgae in brackish or sea water avoiding competition with drinking water 3) Algae with growth features suitable for mass cultivation and easy mechanical harvesting 4) Ability to cultivate the algae heterotrophically on industry wastes and by-products etc. Fresh water is a valuable resource for the future and hence algal biomass production in non potable waters like seawater or waste water is highly desirable. Main aim of this work was to explore the potential of novel microalgae isolated from Silent valley national park as source for algal oil for biodiesel and nutraceutical applications, and the possibilities of culturing potent microalga (e) in non-potable water for biomass and lipid production.

The specific objectives included

1. Isolation of Western *Ghats* microalgae and evaluation of microalgae for growth, biomass and oil production in fresh water and sea water and identification of a potent microalga for production of oil.
2. Evaluation of phototrophic, heterotrophic and mixotrophic cultivation of the potent algal isolate for biomass and oil production
3. Development of strategies and their optimization for cultivation of the potent alga in industrial effluents/wastes like dairy effluent, biodiesel industry waste glycerol and biomass biorefinery effluents.
4. Extraction of algal oil from the potent algal isolate cultivated under different modes of cultivation and their characterization to establish suitability for various applications including biodiesel and nutraceuticals
5. Evaluation of flocculation strategies for harvesting of the algal cells from culture medium.

Chapter 2: Materials and Methods

2.1. Materials

All solvents and analytical reagents were either HPLC grade or AR grade from commercial sources -either Merck India or Sigma-Aldrich, India. Media components were either reagent grade or industrial grade from commercial sources. Biodiesel Industry Waste Glycerol (BDWG) was a kind gift from Prof KB Ramachandran, Indian Institute of Technology, Chennai, India.

2.2. Microalgae and culture conditions

Unicellular microalgae were isolated in our laboratory from water samples of the virgin forests of Silent Valley National Park in Kerala, India. Water samples were collected from different locations in the Silent Valley National Park. Samples were serially diluted and plated on a minimal medium containing 2.0% agar. The medium designated as CTM contained in mg/l: KH_2PO_4.30, $MgSO_4$ $7H_2O$-50, $(NH_4)_2SO_4$ -100 and 500µl of trace element stock solution which contained in mg/ml: $ZnSO_4.7H_2O$ – 3.45, $CoCl_2.6H_2O$ -2.0, $MnSO_4.H_2O$ -1.6, and $FeSO_4$ $7H_20$ – 5.0. Medium pH was adjusted to 6.8 using either 1N NaOH or 1N H_2SO_4. Incubations were carried out at room temperature (30 ± 2 °C) for 7 days with alternating 12h light and dark cycles. Isolated colonies from the plates were plated again on CTM agar and purified. The colonies of pure culture were maintained on CTM agar slants at 4 °C. Screening of the microalgal isolates for biomass and lipid production was done in the MA medium (Table 2.1). All the isolates were further maintained in the MA medium in both slant and plates stored at 4ºC. Subculturing was done every 3 months.

Table 2.1 Composition of MA medium

Components	mg/l
$Ca(NO_3)_2.4H_2O$	50
KNO_3	100
$NaNO_3$	50
Na_2SO_4	50
$MgCl_2.6H_2O$	50
Na-β-glycerophosphate.$5H_2O$	100
Na_2-EDTA.$2H_2O$	5
$MnCl_2$	5
$FeCl_2.6H_2O$	5
$Na_2MoO_4.2H_2O$	0.8
H_3Bo_3	20
pH 6.8	

2.3. Phototrophic cultivation

Medium for photoautotrophic culture was prepared as above. Sterilized media (200ml in 500ml Erlenmeyer flasks) were inoculated with 10% v/v of an inoculum containing 3×10^6 cells/ml unless otherwise specified and were incubated in an environmental chamber equipped with fluorescent lamps (Illumination-3000 Lux) at a temperature of 30 ± 2 °C for 25 days with a diurnal cycle of 13/11h light/darkness. CO_2 was bubbled through the medium at low pressure with an aeration rate of 0.8 vvm.

2.4. Heterotrophic cultivation

All heterotrophic cultivations of *Chlorococcum sp.*R-AP13 were performed in 500 ml Erlenmeyer flasks containing 200 ml medium supplemented with glucose or biodiesel industry waste glycerol (BDWG). Medium in each flask was inoculated with 10% v/v of an inoculum containing 3×10^6 cells /ml unless otherwise specified. Counting of the cells was done with hemocytometer under a phase contrast microscope for preparation of inoculum. The cultures were incubated at a temperature of 30 ± 2˚C for 12 -14 days without light exposure.

2.5. Mixotrophic cultivation

All mixotrophic cultivations were performed in 500 ml Erlenmeyer flasks containing 200 ml medium supplemented with glucose or biodiesel industry waste glycerol (BDWG), acid pretreatment liquor (APL) or dairy effluent (DE) as carbon source. Medium in each flask was inoculated with 10% v/v of an inoculum containing 3×10^6 cells /ml unless otherwise specified, and were incubated in an environmental chamber equipped with fluorescent lamps (Illumination -3000 lux) at a temperature of 30 ± 2 °C for 25 days with a diurnal cycle of 13/11h light/darkness.

2.6. Cell morphology and growth

Morphological changes in algal cells were observed under a phase contrast microscope (Leica DMLS2000, Germany) and scanning electron microscopic analyses was done according to method described by Dayananda et al (2010). Briefly, the exponentially growing cells were fixed with 2% glutaraldehyde solution in 0.2M Phosphate buffer (pH 6.8) for 2h followed by sequential dehydration in varying alcohol concentrations (30-100 %). Completely dried cells were sputter coated with gold and was examined in a Scanning Electron Microscope (Zeiss Evo 17 SE, or JEOL JSM5600LV, Germany). Algal growth was monitored under all cultivation modes at two days interval by taking the cell counts for each mode of cultivation and growth was expressed as cell densities (cell numbers/ml). For measurement of biomass quantities, known volume of evenly dispersed algal culture was centrifuged (8,000 rpm for 10 min), washed and was lyophilized to remove water. Biomass dry weight was measured using a moisture analysis balance (AND, USA) and was expressed as mg/l or g/l.

2.7. Analytical Methods

2.7.1. Intracellular lipid accumulation

Nile red staining was conducted to detect intracellular lipid droplets in micro algal cells as specified by Chen *et al* (2009). Briefly, the cells were harvested by centrifugation (8000 rpm for 10 min), washed with 0.2M phosphate buffer (pH 6. 8) and 40µl Nile red solution in acetone (containing 25% DMSO) was added. The cell suspension was vortexed for 2 min and was incubated at room temperature (30 ± 2 °C) for 10 min. Fluorescence intensity of neutral

lipid was measured in a multimode reader (Tecan Infinite M200 Pro, Switzerland) at a wave length of 575nm. Cell images were visualized by fluorescence microscopy with excitation and emission filters of 530 and 595 nm respectively. Fluorescence images of the stained cells were captured using a camera attached to fluorescence microscope

2.7.2. Lipid production by Alga

For total lipid extraction, the algal cells grown phototrophically, mixotrophically or heterotrophically were harvested by centrifugation for 10 min at 8000 rpm. Biomass was washed with distilled water and was lyophilized to remove water. Dry cell weight of the biomass was determined using a moisture balance. Total lipids were extracted from the dried biomass as per the method described by Bligh and Dyer (1959). Briefly, a 2:1 mixture of chloroform and methanol was added to the biomass taken in a stoppered flask and was sonicated for 10 min. The mixture was then filtered through Whatman® no 1 filter paper. The chloroform layer containing lipid fraction was separated using a separating funnel and to this fraction 2g of anhydrous Na_2SO_4 was added for removing moisture. The chloroform fraction was then taken in a pre-weighed round bottom flask and was then concentrated using a Rotavapor (Buchi, Switzerland). The weight of lipids was determined after evaporation of chloroform and was expressed as percentage of the dry cell weight of algal biomass (% DCW).

2.7.3. Identification of the lipid components

HPTLC analyses were carried out to identify the lipid components of the extracted lipids in an HPTLC system (CAMAG, Switzerland). HPTLC aluminium sheets coated with silica gel 60F254 (EMerck, India) was spotted with 5µl each of the extracted lipid samples using a Linomat 5 automatic sample spotter. The solvent system used for separation of the sample was hexane: diethylether: acetic acid in the ratio 80:20:1. Plates were developed in a CAMAG glass twin chamber. Bands were detected by CAMAG “Win CATS 1.3.0” planar chromatography manager software. CAMAG TLC Scanner3 was used to detect the bands. Bands of lipids was visualised in either iodine vapours or UV light

2.7.4. Fatty acids analysis

Fatty acid profile of the *Chlorococcum* sp. RAP-13 lipids was determined as fatty acid methyl esters (FAME). Trans-esterification of the lipids was done using acid catalyst. Briefly, reactions were performed with 2 % H_2SO_4 in dried methanol at 100˚C for 6 hours. FAMEs were extracted with hexane and dried over anhydrous Na_2SO_4. The samples were analyzed in a gas chromatograph (6850N, Agilent Technologies) equipped with FID detector and using an Agilent DB-225 capillary column. The oven temperature was programmed as 160 ˚C (2 min) - to - 230˚C at a ramping rate of 5°C/min and final hold at 230 °C for 20 min. The carrier gas (N_2) flow rate was 1 ml/min and the sample injection volume used was 1µl. The injector and detector temperature were maintained at 230 ˚C and 270˚C respectively. The peak area percentages were recorded with a standard HP-Chemstation data system. The FAMEs were identified by comparing their fragmentation pattern with internal standards (Sigma Aldirch, India).

2.8. Genomic DNA isolation from micro algae

Algal cells were inoculated into 200ml MA medium in 500ml Erlenmeyer flasks and were incubated phototrophically for 20 days at room temperature (30 ± °C). DNA was extracted from the biomass according to Doyle and Doyle (1984). Briefly 0.8 g wet weight of the biomass was frozen in liquid nitrogen and was ground to a fine powder. It was suspended in 10 ml of the lysis buffer (250 Mm NaCl, 25 mM EDTA, 0.5% w/v CTAB and 200 Mm Tris-HCl, pH 8.5). Suspension was incubated at 60 °C for 30min with occasional gentle mixing. After centrifugation at 13,000 rpm for 15min (4°C), the supernatant was transferred to a new tube and polysaccharides and proteins were precipitated by adding an equal volume of Phenol: Chloroform: Iso-amyl Alcohol (25:24:1). Clear aqueous fraction was transferred with wide bore pipette into a nuclease free centrifuge tube. DNA was precipitated by adding 3/2 volume of chilled iso-propanol to aqueous fraction. The solution was gently mixed by inversion, placed at -20 °C for 20 min and DNA was recovered by centrifugation at 12000 rpm for 15 min (4°C). The DNA precipitate was washed twice with 70% ethanol and was allowed to air dry after which it was re-suspended in 10 mM Tris-EDTA buffer (pH 8.0). DNA was stored at -80°C.

2.9. Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA was conducted in a Biorad Horizontal Gel apparatus. 1% agarose gel was used for electrophoresis of PCR amplicons and 0.8% gel was used for Genomic DNA. Ethidium bromide (0.5µg/ml) was included in the gel for visualization of DNA under UV light. 0.5µg of 1 kb DNA ladder (Fermentas, USA) was run as a molecular weight marker for determination of approximate size of DNA fragments.

Chapter 3: Screening of microalgae from Silent Valley and identification of a potent strain for biomass and lipids production

3.1 Introduction.

Micro algal cultivation has recently gained immense popularity due to its potential as a source of valuable products like proteins, carbohydrates and lipids. Micro algal lipids rich in triglycerides are considered as a future feedstock for renewable fuels with a significant amount of research being directed to its studies. Several photosynthetic microalgae have been identified as efficient biological systems for producing a wide variety of high-value chemicals and pharmaceuticals, such as phycobiliproteins, astaxanthin and poly unsaturated fatty acids (PUFAs) (Huerlimann *et al*, 2010; Martin *et al*, 2014). Consequently, several processes have been developed to obtain these and many other compounds on a commercial scale. Most of these processes are based on phototrophic cultivation of the algae using CO_2 as the carbon source (Kumar *et al*, 2010). Algae are generally defined as photosynthetic eukaryotes even though prokaryotic cyanobacteria, also known as blue-green algae, are sometimes included under this broad category (Lee, 1989). Micro algae are unicellular organisms and are fast growing with the ability to complete their entire growth cycle in few days, if adequate amount of sunlight, water, CO_2 and nutrients are available in the growth medium (Brennan and Owende, 2010). Reports indicate that the aquatic microalgae have high oil contents (up to 60% of their biomass dry weight) and hence the potential to become more productive sources of biofuels, in comparison to terrestrial crops like soybeans (Chisti, 2007; Mata *et al*, 2010). Algal biomass is a non food resource that may be cultivated on non productive land using saline or waste waters.

One of the most important concerns in algal biomass production is the need to provide substantial amounts of water and nutrients to the algal population undergoing mass cultivation (Murphy and Allen, 2011). It is estimated that ~6000 litres of water is consumed per litre of algal oil produced in the conventional systems of cultivation (Ozkan *et al*, 2012). Water provides the physical environment for growth of the algae, delivers the nutrients, removes waste products and acts as a thermal regulator (Murphy and Allen, 2011). Normally the fresh water micro algae are cultivated in fresh water based media or in waste waters. Large scale algal cultivation in fresh water may not a viable option for future due to the competing demand of this resource for potable applications, and hence algal biomass generation in non potable water like sea water or waste water is highly desirable. There have

been several studies on micro algal cultivation using seawater (Reitan *et al*, 1994; Takagi *et al,* 2006; Chen *et al*. 2013) but the studies on cultivation of fresh water microalgae in seawater are rare (Iijima *et al*, 2015)

Increasing demand for biofuels have exposed a great need for discovery of more productive non food sources of biomass and oil that may be converted to biodiesel or other transportation fuels and novel species of algae that gives high productivities of biomass and oil are highly desired (Lee *et al*, 2014). Microalgae are a very diverse and heterogeneous group of organisms which can even belong to different phyla, and consequently with diverse growth requirements. Therefore, location is a key determining factor for selection of microalgae for biodiesel or biomass (De Morais and Costa, 2007). Of the different algal strains, the ideal strain will likely be different for different location, especially when outdoor cultivation methods are employed. The environmental conditions of a specific area can greatly influence microalgal populations and their growth dynamic and the most logical approach is to screen for strains with high biomass and lipid productivities from selected environmental regimes (Lee *et al*, 2015).

Tropical regions with abundant availability of solar radiation throughout the year like in the south west of India are suitable for mass cultivation of algae, but strains adapted for this region have not been studied extensively. Silent Valley National Park, which forms part of the protected Nilgiri Biosphere in Western Ghats, is a very less explored geographical strip with respect to floral, faunal and microbial biodiversity, and hence the chances for finding novel fresh water microalgae adapted to the tropical climate may be considered high. The objective of the present study was therefore to screen the fresh water micro algae isolated from tropical rain forests of this region, for production of biomass and lipids in fresh water and natural sea water based medium under phototrophic condition.

3.2 . Materials and Methods

3.2.1. Isolation of microalgae and cultivation for screening of biomass and lipid production

Fresh water microalgae used in the study were isolated from water samples collected at different locations in the Silent Valley National Park, Kerala, India and the sample IDs and location of collections were recorded (Table 3.1, Fig 3.1). The water samples were serially diluted and plated on a basal medium containing 2.0 % agar which is described in section 2.2.

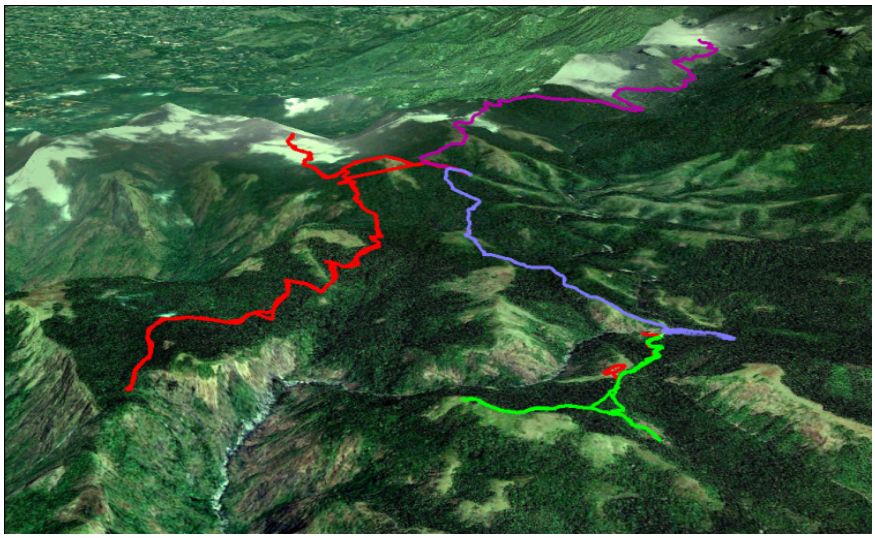
Incubations were carried out at room temperature (30 ± 2 °C) for 14 days with alternating 12h light and dark cycles. Isolated colonies were plated again on the same medium and were maintained at 4 °C.

For screening of the algal isolates for biomass and lipid production in fresh water medium, each alga was grown in MA medium (Section 2.2). For evaluation of growth in sea water, the isolates were transferred to medium containing sea water in concentrations ranging from 30% to 100% and were cultivated as mentioned above Sea water medium contained (in mg/l) KH_2PO_4-200, K_2HPO_4 -400, $MgSO_4$ -200, Urea-200, KNO_3-200, F1/2 trace metal mix -1.0 ml, vitamin mix -0.5ml (Guillard and Ryther, 1962) in 50 % aged natural sea water as a base for phototrophic cultivation.

Table 3.1: Sample ID and locations of where samples were collected

Sample ID	Location
R1-60	
R1-67-3	
R1-67-1	Neelikkal
S1-78	
R1-70	
R1-71	Valakkad
R1-91	
R1-44	Puchi Para
R AP-13	Arikan para

Figure 3.1. GPS Mapping of the Trek paths for sample collection in Silent Valley



3.2.2. Phototrophic culture conditions

Phototrophic cultivation of 10 isolates from the Silent Valley water samples were done in 500 ml Erlenmeyer flasks containing 200 ml of fresh water/sea water based medium under static conditions. Cultivation was carried out with exposure to natural light, and the illumination and temperature were not controlled; so as to simulate the real life conditions. There was an approximately 12h day and 12h night light cycle for cultivation of algae and the water temperatures varied between 28 -33 °C. Sterilized media were inoculated at 5 % level with an inoculum containing 3×10^6 cells/ml.

3.2.3. Identification of potent strain

Molecular identification of the potent isolate was done by sequencing of its 18S rDNA. Algal cells were frozen and homogenized in liquid nitrogen and genomic DNA was extracted using the CTAB method described in Section 2.8 (Doyle and Doyle, 1984). DNA precipitate was washed twice with 70% ethanol, allowed to air dry and was re-suspended in 10mM Tris EDTA buffer (pH 8.0). A portion of 18S rDNA was amplified from the genomic DNA by polymerase chain reaction (PCR) using universal primers PROTO5R and EK28F (White *et al*. 1990). PCR reactions contained 0.5 units of *Taq DNA polymerase*, 1x *Taq* buffer, 200 µM of each of dNTPs, 2 µM of $MgSO_4$ (All from Fermentas, USA), 0.2 µg of genomic DNA, and 0.5 µM of forward and reverse primers. Reaction conditions for PCR amplification were an initial temperature of 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1.0 min and a final extension step at 72°C for 10 min. An Eppendrof gradient PCR system was used for amplification. PCR products were separated by electrophoresis in a 1.0% Agarose gel containing Ethidium Bromide and the product was visualized in long range UV trans-illumination for documentation. PCR product was purified from the Agarose gel using a commercial DNA purification kit (Nucleospin, Macherey-Nagel, Germany) according to manufacturer's protocol. Sequencing of the amplicon was done using forward and reverses primers in an ABI 3730 XL cycle sequencer. Database similarity searches were performed using NCBI BLAST (Altschul *et al*. 1990).

3.2.4. Algal Cell Morphology by Microscopy

Morphology of the algal cell grown in sea water medium was checked under a phase contrast microscope. Scanning electron microscopy (SEM) analysis of the potent strain was carried out as described previously in Section 2.6 to find out the difference between algal cells grown on fresh water and sea water based media (Dayananda *et al*, 2010). Completely dried cells were sputter coated with gold and was examined by the Scanning Electron Microscope. Biomass production was determined as dry cell weight (DCW). For this, the cells were harvested by centrifugation at 8000 rpm for 10 min, washed with distilled water to remove the salt and were lyophilised to remove moisture. Lyophilized cells were weighed in an analytical balance and the dry cell weight was expressed as mg/l

3.2.5. Analytical Methods

3.2.5.1. Intracellular lipid content by Nile Red Fluorescence

Neutral Lipid accumulation inside the algal cells was monitored by Nile red staining (Chen *et al*, 2009) using the protocol described in Section 2.7.1. Lipid accumulation inside the cells was measured as fluorescence of the Nile Red stained algal cell suspension using a Spectrofluorometer at 575 nm. Fluorescence of stained lipid bodies in algal cells was also visualized under a fluorescence microscope with excitation and emission wavelengths of 530 and 575 nm respectively, and was captured using an attached digital camera.

3.2.5.2 Total lipid content of algal cells and the lipid yield

Total lipids were extracted from dried biomass by the solvent extraction method as described in Section 2.7.2. The lipid extract was taken in a pre-weighed round bottom flask and the lipid weight was determined after evaporation of chloroform in a rotary evaporator. Lipid yield was expressed as percentage of the dry cell weight of algal biomass (% DCW). Lipid yield was expressed as mg of lipid produced per litre (mg/l) of culture fluid calculated from the biomass yield and lipid content of biomass.

3.2.5.3. Fatty acids characterization

Fatty acid profile of the algal oil was determined as fatty acid methyl esters (FAME). Trans-esterification reactions were performed with 2.0 % HCl in dried Methanol at 100 °C for 1h. FAME were extracted with hexane and dried over anhydrous Na_2SO_4. The fatty acids profiling was done in gas chromatography as described in Section 2.7.4 The FAMEs were identified by comparing their fragmentation pattern with internal standards (Sigma Aldrich, India).

3.3 Results and discussion

3.3.1 Algal growth in fresh water and sea water based medium

A total of 10 different algal isolates were obtained from the Silent Valley water samples. For selection of the best algal isolate for oil production, the biomass and lipid production potential of the isolates in MA medium was evaluated. Maximum biomass (299 mg/l) was produced by isolate R-AP13. While maximum lipids production was by R 1-27 (79.6% DCW). However, the yield of lipids was highest for R-AP13 (100mg/l), followed by R1-67-3 and R-167-1 respectively (Table 3.2). Lipid profile analyzed by TLC showed Triglycerides and free fatty acids.

Table 3.2: Biomass and lipid production by micro algal isolates in MA Medium.

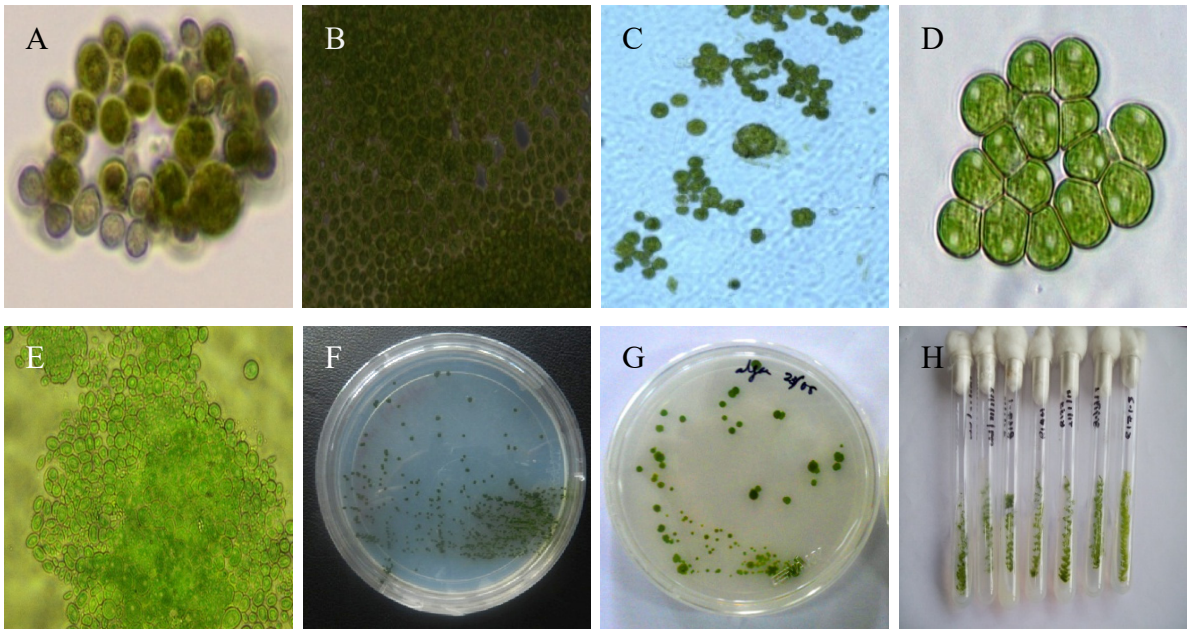
Algal Isolate	DCW of biomass (mg/l)	Lipids yield (mg/l)	Cellular Lipid content (% DCW)
R-AP13	299.0 ± 12.16	100 ± 07.42	33.5 ± 01.11
R1-67-1	191.5 ± 22.62	79.5 ± 02.12	41.3 ± 05.93
R1-67-3	233.2 ± 08.83	86.2 ± 10.96	32.0 ± 10.36
S1-78	150.5 ± 07.77	47.2 ± 06.01	31.3 ± 02.41
R1-71-3	137.2 ± 02.47	56.5 ± 06.36	41.2 ± 05.38
R1-44	111.0 ± 05.72	58.2 ± 01.76	52.5 ± 04.31
R127	80.72 ± 06.71	64.2 ± 02.47	79.6 ± 03.60
R1-70	133.0 ± 01.41	67.0 ± 02.82	50.3 ± 01.59
R160-2	82.25 ± 23.68	55.0 ± 12.72	55.2 ± 12.42
R1-191	70.71 ± 06.01	43.5 ± 13.08	61.3 ± 01.76

Large scale cultivation of microalgae in fresh water may not a practical option due to the increasing demand of fresh water for consumption and the cost of processing of water. Evaluation of growth in media containing 30-100% sea water indicated that 50% seawater concentration supported maximal algal growth, and hence this concentration was selected for conducting the screening studies in seawater based medium. The microalgal isolates were screened in 50% seawater medium for biomass and lipid production. Biomass from the phototrophically grown algal isolates was harvested on the 20^{th} day of incubation by centrifugation. Among the 10 isolates, R-AP13, R1-67-1 and RI-67-3 produced the maximum biomass amounts of 161 mg/l, 165 mg/l and 168 mg/l respectively (Table 3.3). However, the total lipid yield and % DCW of lipid was high for R-AP13 which is 67 mg/L with 42 % cellular lipid accumulation followed by R167-1 (60 mg/l and 36% of accumulation). Biomass and lipid production by other cultures were low. Microscopic examination of the cells revealed that all are unicellular green micro algae, some existing in colonial forms (Fig 3.2 A, B, C, D, E). All isolates produced colonies on fresh water and sea water based agar plates (Fig 3.2F and G) and was maintained in both media (Fig3.2H). Total lipid yield, maximum biomass production, ability to produce biomass in natural climatic conditions, resistance to infection by other micro organisms and self flocculation are important criteria for choosing an alga for mass cultivation for biofuel application (Wijffels and Barbosa, 2010; Lee *et al*, 2014). Mass cultivation of algae requires huge volumes of water and fresh water may not be a practical option in future. Therefore, cultures that can adapt to marine water are highly advantageous for mass cultivation for biomass and lipids production; especially for biodiesel application. Sea water is considered as an attractive medium for cultivation of microalgae since it contains most of the mineral nutrients. Besides, sea water offers the advantage of lesser bacterial and fungal contamination which is the main cause of decreased biomass productivity in open pond cultivation of fresh water microalgae.

Table 3.3: Biomass and lipids content of algal isolates in 50% sea water medium.

Algal isolates	Biomass concentration (mg/l)	Lipid yield (mg/l)	Cellular lipid content (% DCW)
R-AP13	161.0 ± 0.63	67.5 ± 1.0	42
R1-67-1	165.0 ± 2.76	60.0 ± 1.6	36
R1-67-3	168.0 ± 1.22	55.0 ± 0.2	33
S1-78	148.0 ± 1.41	49.5 ± 1.0	33
R1-71-3	98.0 ± 1.20	37.5 ± 1.0	38
R1-44	118.0 ± 3.96	40.0 ± 2.6	33
R1-27	146.0 ± 3.05	55.0 ± 3.0	37
R1-70	87.5 ± 0.98	18.5 ± 0.7	21
R160-2	100.0 ± 6.70	35.0 ± 1.2	35
R1-191	89.0 ± 0.84	23.5 ± 0.7	26

Figure 3.2: Cell morphology of representative algal isolates by light microscopy

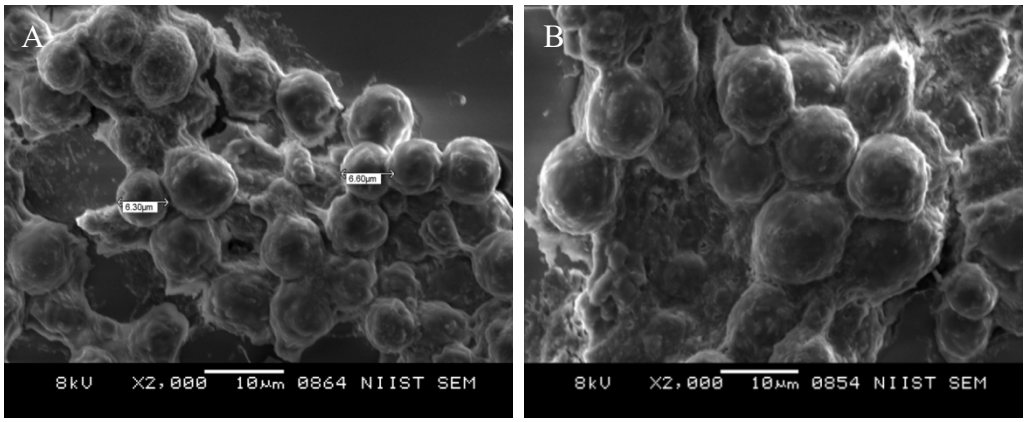


(A) R1-67-1, (B) R1-67-3, (C) R-AP13, (D) R-170, (E) R-191, (F) Algal colonies on sea water plate, (G) Colonies on fresh water medium plate and (H) Isolates maintained on slants.

3.3.2 Scanning Electron microscopy analysis

Morphological features of algal cells grown in fresh water and sea water based media, was compared by scanning electron microscopic (SEM) analysis of the best performing algal isolate R-AP13. SEM analysis showed that the size of the algal cells grown in seawater was increased in comparison to that grown in fresh water (Fig 3.3 A and B). Salinity of sea water could influence the cell enlargement or it could be an adaptation of the fresh water alga to survive in sea water. Kaewkannetra *et al* (2012) has reported that change in salinity conditions affects the morphology of the fresh water alga *Scenedesmus obliquus,* and cell enlargement was reported as an adaptation to salt stress environment in *Skeletonema* (Balzano *et al,* 2010).

Figure 3.3: Scanning electron microscopy of algal cells



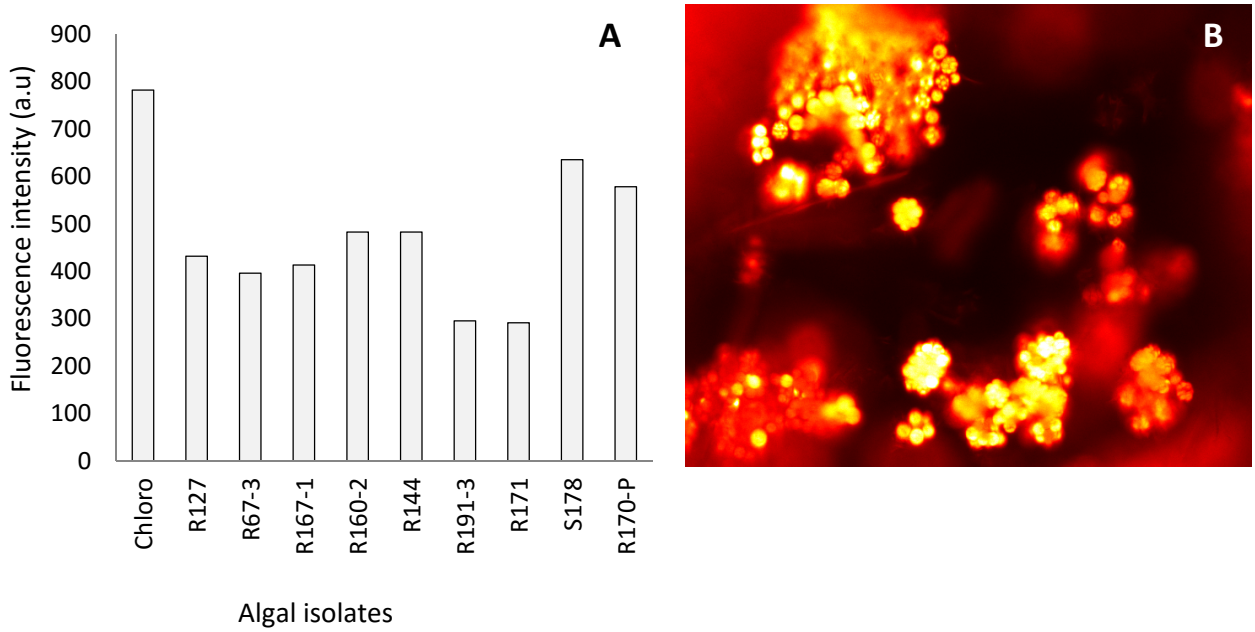
(A) Fresh water medium (B) Sea water medium.

3.3.3 Nile red assay

Nile red is a lipid-soluble dye and is staining with Nile red is a widely employed strategy to determine lipid production by microalgae (Chen *et al*, 2009). Nile red assay was used to assess the intracellular neutral lipid accumulation in the algal cells, since the fluorescence intensity is proportional to lipid content. Assay was carried out on 18th day of incubation when the cells were in stationary phase. Strain R-AP13 showed strong fluorescence of lipid (782 au) followed by isolate SI-78 (635 au) and RI-70P (578 au) (Fig 3.4.A), suggesting that these isolates accumulated significant amounts of neutral lipids inside their cells. Fluorescence microscopic images confirmed this and strong yellow florescence of the

intracellular lipid droplets were visible inside the cells (Fig 3.4.B). Neutral lipids production is important in the context of biodiesel (Chen *et al*, 2009; Hu *et al*, 2008).

Figure 3.4: Intracellular lipid accumulations in the algal isolates



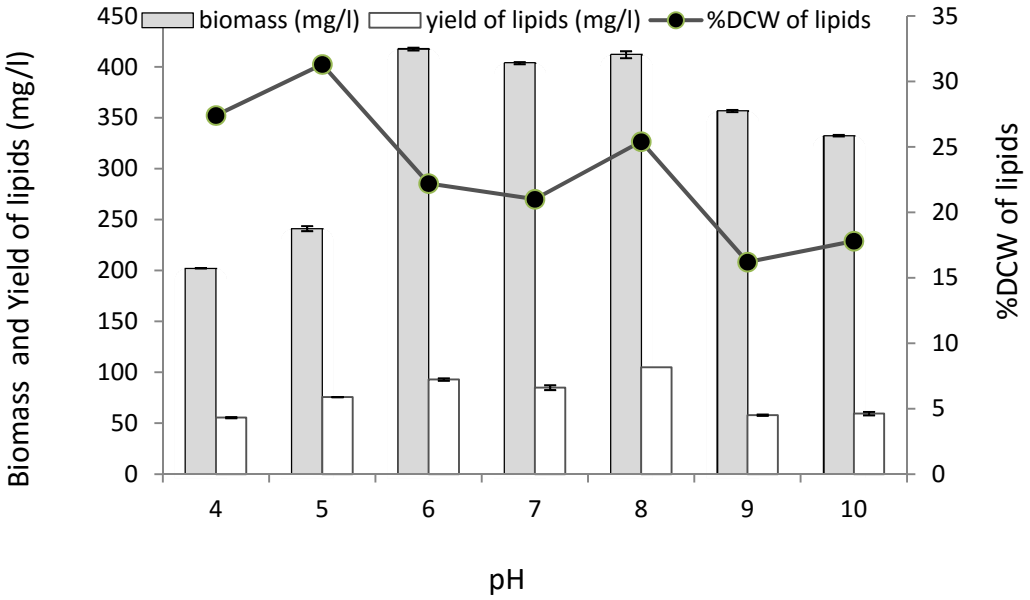
(A) Intracellular lipid accumulation of isolates measured as Nile red fluorescence (B) Fluorescence image showing lipid accumulation inside representative algal isolate R-AP13

3.3.4. pH tolerance

Effect of medium pH on the biomass and lipid production by potent isolate R-AP13 was evaluated since it is an important factor which affects the microbial surface properties and flocculation, and can have significant influence on the metabolism (Ren *et al*, 2013). Initial pH of the sea water medium (7.0) was varied from 4.0 to 10.0 (Fig 3.5). Interestingly, the strain R-AP13 had a strong pH tolerance and could grow in a wide range of pH (4.0-10.0). Furthermore, the total biomass production of algal cells was similar from pH 6.0 to pH 8.0, while the total lipid yield was high in cells grown at pH 8.0. Here, the biomass production was doubled in comparison to the screening studies indicating that the growth of fresh water microalgal cells gradually adapted to salinity is not affected by the salt stress. In fact, salinity stress in this case had resulted in enhanced biomass production. At the two extremes of pH (4.0 and 10.0), the algal cells showed poor growth and lipid productivity. Lipid content

measured as % DCW was high in cells grown at pH 5.0 and pH 4.0, probably indicating that this could be an adaptation to acidic conditions. The results indicated that the algal growth and lipid accumulation were significantly affected by the pH of the medium. The strain R-AP13 could tolerate a wide change in pH of the medium which is advantageous in real life conditions, since the change in environmental conditions also affects the pH of cultivation medium. Therefore it may be speculated that this strain can survive in a range of environmental conditions without serious impacts on biomass and lipid production. Similar phenomenon has been observed in other algae like *Chlorococcum sp, Scenedesmus sp, C. acidophila, and Chlorella sorokiniana* in autotrophic growth (Wong *et al*, 2013; Liu *et al,* 2013; Elias and Spijkerman, 2005). pH tolerance of the algae might be caused by self-regulation of metabolism or special adaptations of the microalgae to survive changes in environmental conditions. Tolerance of fresh water algae (*Clamydomonas sp* and *Picochlorum atomus)* to saline conditions was reported by many researchers (Ho *et al*, 2014: Alvensleben *et al*, 2013)

Figure 3.5: Biomass and lipid production by algal isolate RAP-13 under different pH



3.3.5. Molecular identification of potent isolate

Molecular identification of the potent isolate (R-AP13) was done by sequencing of its 18S rDNA. Genomic DNA was isolated from fresh algal biomass according to Doyle and Doyle (1984). PCR amplification of the 18S rDNA sequence was performed using universal

eukaryotic primers for 18S rDNA (White *et al*. 1990) which yielded a 317 bp nucleotide fragment.

```
>R-AP13 18SrRNA Amplicon
AAAGAAATTAAATAAATAAATAGTTTAAAGCATAAAATTCGTAAAAAAAAAAAAAAACCAACCTTATTAAT
AGAGGAAATTGTCATCCAGTCAGAAGTATTAGGCAACAATAACTCTGTTGTGATCTCATAAGATGTTGTG
GGCACCACGCGTACTACAATAGAGCATTCAACTAGCCCATCTTTGGCCAAGAAATCCGGTTAATTTGTGA
AACTGCATCGTGATGGGGATAGAGTATTGCAATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAG
TCATCAGCTGCTATTCTGGAATATATTTTTCGGCTTT
```

Database similarity searches of nucleotide sequences were performed using NCBI BLAST (Altschul *et al*. 1990). The partial 18S rRNA sequence of the isolate R-AP13 matched those of *Chlorococcum* sp with highest identities (Fig 3.6). The sequence was deposited in Genbank with accession number KC560011 and the isolate was designated as *Chlorococcum* sp.R-AP13

Figure 3.6: BLAST results showing R-AP13 18s rDNA sequence homology with *Chlorococcum* 18s rDNA

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chlorococcum minutum strain SAG 213-7 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	KM020099.1
Chlorococcum sp. GRK7-WB5 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	KF144187.1
Chlorococcum sp. GRK7-WB4 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	KF144186.1
Chlamydomonas sp. NIES-2211 gene for 18S ribosomal RNA, partial sequence	215	215	69%	3e-52	82%	AB701516.1
Chlamydomonadaceae sp. KMMCC 249 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	JQ315635.1
Chlamydomonadaceae sp. KMMCC 370 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	JQ315634.1
Chlamydomonadaceae sp. A10 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	KC492081.1
Chlamydomonas debaryana genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene,	215	215	69%	3e-52	82%	FR865523.1
Chlamydomonadaceae sp. KMMCC FC-97 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	GQ122379.1
Chlorococcum sp. RK261 gene for 18S rRNA, partial sequence	215	215	69%	3e-52	82%	AB490286.1
Chlorococcum sp. KNU-F-2002-C1 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	DQ303098.1
Chlamydomonad sp. Tow9/21T-1w 18S ribosomal RNA gene, partial sequence	215	215	69%	3e-52	82%	AY220568.1

3.3.6 Fatty Acid Characterization.

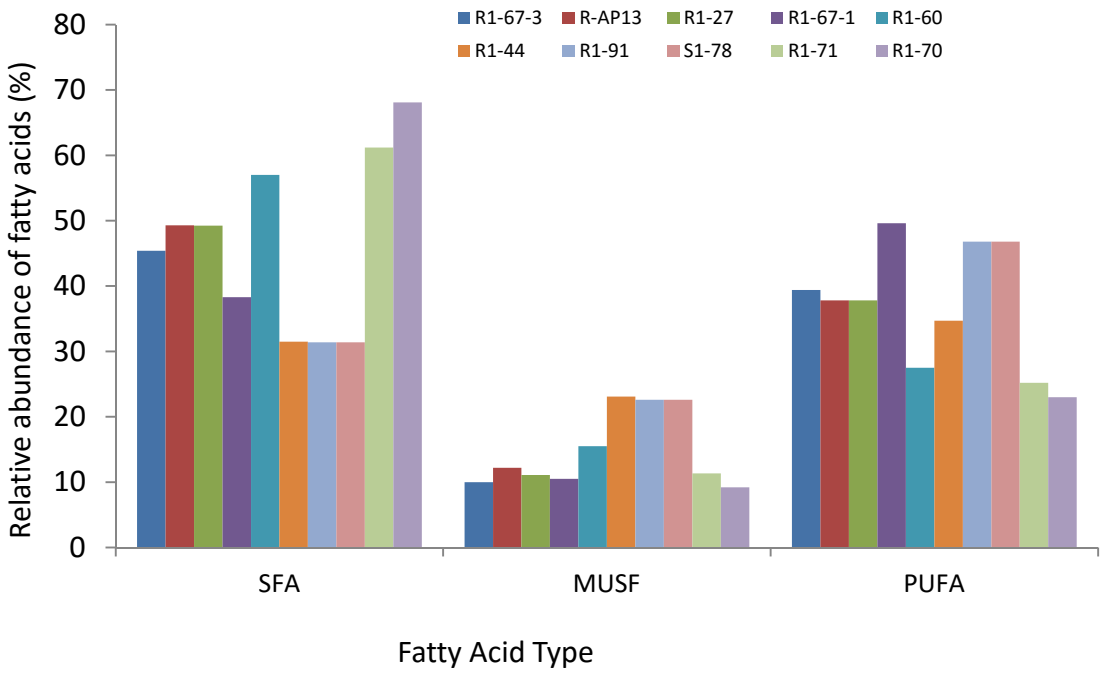
Gas chromatographic analysis of the fatty acid methyl esters (FAME) of algal oil from phototrophic cultures of the isolates revealed that the major fatty acid in all the cases is palmitic acid (C16:0), followed by oleic acid (C18:1), linoleic (C18:2) and linolenic (C18:3) acids (Table 3.4). Isolate R-AP13 and RI-27 had similar fatty acid profiles and contained the long chain C24 fatty acid. Poly unsaturated fatty acids (PUFAs) like C18:2 and C18:3 were produced in the range of 10-38% and 9-16% respectively. Linolenic and linoleic acids are essential fatty acids for humans and animals, and therefore oil produced by this algal isolate may have application in food and feed supplementation.

Table 3.4. Fatty acid profile of Phototrophically cultivated *Chlorococcum* sp. R-AP13

FA	R1-67-3	R-AP13	R1-27	R1-67-1	R1-60-2	R1-44	R1-91	S1-78	R1-71-3	R1-70-P
C12	2.0±0	2.4±.07	2.4 ±0.1	2.5±0.14	4.6±0.3	2.7±0	-	-	8±0	9.2±1.3
C14	0.9±0.1	1.6±0	1.6±0	1±0.1	4.5±0.4	2±0	3.2±0.1	-	5.7±0.1	-
C15	-	3±0.3	2.9 ±0.1	-	-	2.3±0.1	-	-	0.3±0	-
C16	28.8±0.3	30.6±0	30.6±0.1	24.3±0.2	28.6±0.4	13.5±0.5	15.4	46.5±0.8	29.6±0.1	30.5±1.6
C60-1	0.8±0	1.1±0	1.1±0	0.9±0.42	7.9±0.3	1.7±0.1		-	7.35±0.2	-
C16-2	8.6±0.2	8.3± 0.1	8.3±0.3	19.7±0.9	-	6.4±0.1	1.0±0	-	-	-
C17	1.8±1	1.7±0.1	1.7±0.3	1.8±0.42	-	2±0	-	2.4±0	2. 8±0.2	-
C18	8.3±0.1	8.7 ±0.2	8.7±0.3	7.2 ±0.3	18.3±0.1	10.6±0.1	12.8±0	-	14.2±0.4	28.4±0.6
C18-1	9.2±0	9.5±0.1	9.5±0.3	9.6±0	7.6±0.3	21.2±0.1	22.6±0	24.9 ±1	4.0±0.14	9.2±0.2
C18-2	12±0.12	11.7±0	11.7±0.1	14±1.9	11.2±0.1	17.8±0.1	37.9±0.6	10±0.1	13.1±0	7.6±2.6
C18-3	18.8±0.3	17.8±0.1	17.8±0.1	15.9±1.2	16.3±0.1	10.5±0.1	7.9±0.6	14.5±0.3	12.1±0.1	15.4±0
C20	3.4±0	0.5±0	0.5±0.1	1.5±0	1	0.7±0.1		-	0.6±0	-
C22	0.19±0	-	-	-	-	-	-	1.1±0.1	-	-
C22-1	-	1.6±0	0.5±0.1	-	-	-	-	-	1.4±0	-
C23	-	-	-	-	-	-	-	-	-	-
C24	-	0.8±0	0.85±0.1	-	-	-	-	-	-	-

Saturated fatty acids (SFA) production by these algal isolates was in the range of 31-68%, while the monounsaturated fatty acids (MUFA) and poly unsaturated fatty acids (PUFA) were in the ranges 10-25% and 23-50% respectively (Fig.3.7). Lipids quantity and composition are the key properties that determine the biodiesel oxidative stability and performance properties. In order to produce a biodiesel of high quality, the following fatty acid profiles are desirable: (1) Lowest possible saturated fatty acid levels (eg. C16:0 and C18:0) for improved winter operability, (2) highest possible monounsaturated fatty acid levels (such as C18:1) for good stability and winter operability and (3) lowest possible polyunsaturated fatty acids levels (such as C18:3) to increase oxidation stability (Cuellar-Bermudeza *et al*, 2014).

Figure 3.7: Variation in the relative abundance of fatty acid types in algal isolates



Fatty acids profile of oils used for biodiesel application influences the quality of the fuel and the preferred ones include C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2 (Schenk *et al*, 2008). When all the requirements for biodiesel quality are considered, it becomes clear that not a single fatty acid methyl ester could satisfy every requirement for biodiesel production. However, a balance of different fatty acids containing higher amounts of mono-unsaturated fatty acids such as Oleate (18:1Δ9), and fewer saturated and polyunsaturated fatty acids would yield a better biodiesel (Durrett *et al*, 2008). Oxidative stability is another important

quality of biodiesel influenced by the FAME component. Allylic and particularly bis-allylic double bond positions can provide greater oxidative instability (Gopinath *et al,* 2009). Linolenate (C18:3) contains two bis-allylic groups and a 12 % limit for this FAME has been set in the European B100 biodiesel standard (EN 14214). The standard also limits the amount of FAMEs with four or more double bonds to 1%. Based on the fatty acids profile of oils produced by the 10 algal isolates, it is apparent that most of them may be useful for biodiesel production.

Microalgal lipids may contain large amount of poly unsaturated fatty acids, which includes the α- Linolenic acid (ALA 18:3 ω-3), γ-Linolenic acid (GLA, 18:3 ω-6), eicosapentaenoic acid (EPA, 20:5 ω-3), arachidonic acid (ARA, 20:6 ω-6) docosapentaenoic acid (DPA, 22:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3). Long chain poly unsaturated fatty acids like EPA and DHA are synthesised in the humans from 18:2 and 18:3 fatty acids (Huang *et al*, 2010; Muhlroth *et al*, 2013). Fatty acids profile of algal isolates indicated that oils from the isolates – R1-91, R1-61-3, R-AP13, R1-27, R1-67-1, R1-60-2 and R1-44 may be used as source of healthy fat for food and feed applications.

Results showed that algal isolate is able to produce biomass and lipids while growing phototrophically under natural climatic conditions in both fresh water and natural sea water based media. Potential of fresh water algal cells to produce biomass and oils in sea water as a base medium is highly advantageous due to economic and environmental benefits. Mass production of algal biomass consumes large amount of fresh water, particularly in phototrophic mode, which otherwise is the best strategy for cultivation due to the minimal cost of medium and potential environmental benefits due to carbon dioxide recycling. Sea water is a complex medium consisting of several known elements and a large number of organic compounds whose composition can vary significantly, especially when collected from the surface (Harrison and Berges 2005). However, it is also an abundant natural source of most of the major and minor elements required for algal mass cultivation. It is known that several fresh water algal species can grow in high concentrations of sea water, if certain mineral nutrients are absent or present in low concentrations (Wetherell, 1961). Mass cultivation of fresh water algae in sea water reduces contamination by other micro organisms. Use of sea water as the basal medium helps in reducing the requirement of mineral supplements and more importantly helps to save fresh water- an important resource.

3.4 Conclusions

Isolates of fresh water microalgae could grow and produce lipids in both fresh water and sea water. Among 10 algal isolates screened in the present study, the isolate R-AP13 produced maximum biomass and lipids in both media. The alga was highly adaptive to change in pH and salinity and holds great potential as a source of oil for both biodiesel as well as neutraceutical applications. The potent isolate R-AP13 was identified as *Chlorococcum sp*. by molecular methods. This isolate was used for further studies. Use of sea water as a basal medium instead of fresh water was demonstrated to be possible albeit with lesser biomass and lipid yields. Nevertheless, the use of sea water helps in reducing the requirement of mineral supplements and contamination with other micro organisms and more importantly helps to saves fresh water**.**

Chapter 4: Evaluation of phototrophic and heterotrophic growth and lipid production of *Chlorococcum* sp. R-AP13 in fresh water medium

4.1. Introduction

Oil from microalgae is expected to be an important future feedstock for biodiesel and is often considered as the only one with potential to meet the global demand for transportation fuels (Schenk *et al,* 2008). Microalgae have the potential to grow very rapidly and produce more oil per unit area of cultivation than any other feedstock (Dermirbas, 2009). They are known to accumulate more than 60% of their dry cell biomass as oil, and often the yield of oil from algae is more than 200 times the corresponding yields from terrestrial plants (Sheehan *et al,* 2008; Chisti, 2007). Since they have a submerged growth condition, they have better access to nutrients and are more efficient in converting solar energy to biomass (Chisti, 2007). Microalgae with high lipid or polyunsaturated fatty acid content are desirable in production of biodiesel and nutraceutical/pharmaceutical purposes respectively (Mendes *et al,* 2007; Gong and Jiang, 2011).

Many algae are rich in oil which can be converted to biodiesel and the use of algae as oil source has the advantage of their easy adaptability to growth conditions, lack of requirement for arable land and the ability to cultivate using minimalistic media with only sunlight and CO_2 as primary requisites (Campbell, 2008). However, such photo autotrophic cultivation of algae is not free from limitations and often the biomass yields in such cultures are very low. Typical dry cell densities achieved in the large scale cultivations are between 0.3-0.5g/l and are seldom greater than 5g/l (Wang *et al,* 2008). Autotrophic cultivation is often limited by light deficiency caused by the lack of light penetration especially at high cell densities, photo inhibition due to excessive light, contamination by organisms that feed on algae or compete with them for nutrients, large areas needed in the case of open cultivation systems and harvesting challenges among many other factors (Huang *et al,* 2010).

Heterotrophic cultivation of microalgae on the other hand offers several advantages, especially the increase in biomass and lipid accumulation. These are cultivation systems where the algae are grown on some cheap organic substrate without light (Wen and Chen, 2003). Heterotrophic cultivation also possess the advantage of high cell densities, easy scalability, possibility for better control being a closed system, ability to control contamination and lower cost of harvesting due to high cell densities etc (Huang *et al,* 2010). Carbon source is the most important factor for heterotrophic cultivation of microalgae and

several algae which can grow either phototrophically or heterotrophically grows better and achieves a higher biomass when grown using acetate, glucose or other organic carbon sources in the medium (Wu *et al,* 1994). Waste glycerol from biodiesel industry is a potential feedstock for heterotrophic cultivation of microalgae and production of oil (Sabeela and Sukumaran, 2015), but all microalgae cannot grow and produce oil on waste glycerol. Biodiesel waste glycerol contains varying amounts of methanol, concentration of which can be greater than 20% which is inhibitory to several microalgae (Chen and Walker, 2011).

In the present investigation, potential of phototrophic and heterotrophic cultivation on growth and lipid production of the microalga *Chlorococcum* sp. R-AP13 in fresh water medium was studied. Supplementation of glucose, pure glycerol or biodiesel industry waste glycerol (BWG) was used as carbon source for heterotrophic condition. The suitability of the extracted oil for biodiesel applications was assessed by measuring type and relative proportions of fatty acids and oils by both HPTLC and GC analyses.

4.2. Materials and Methods

4.2.1. Algal cultivation

Chlorococcum sp. R-AP13 was cultivated either phototrophically or heterotrophically in 500 ml Erlenmeyer flaks containing 200ml of sterilized MA medium. The flasks were inoculated at 5 % v/v level using an exponentially growing algal culture. For phototrophic growth, the flasks were bubbled with 0.5 vvm of CO_2 while for heterotrophic cultivation; the medium was supplemented with 5.0 % (w/v) of glucose, pure glycerol or biodiesel waste glycerol (BDWG). Phototrophic cultivation was carried out with exposure to light and the illumination and temperature was not controlled so as to simulate the real life conditions. There was an approximately 12h day and 12h night, light cycle for cultivation of algae and the temperatures varied between 28 -33°C. The entire flask for heterotrophic mode was kept under static condition without light exposure. All experiments were performed in triplicates.

4.2.2. Cell Morphology and Growth

Morphological changes in algal cells were observed under a phase contrast microscope and scanning electron microscopy (SEM) analyses was performed as described under section 2.6. Completely dried cells were sputter coated with gold and was examined in a Scanning Electron Microscope (Zeiss Evo 17 SE, Germany). Algal growth was monitored under both cultivation modes at two days interval by taking the cell counts and biomass production was expressed as cell concentration (no. of cells/ml).

4.2.3. Total lipid extraction

For total lipid extraction, the algal cells grown either phototrophically or heterotrophically for 2 weeks in MA medium were harvested by centrifugation at 8000 rpm for 10 min. Biomass was washed with distilled water and was lyophilized to remove water. Dry cell weight of the biomass was determined using a moisture analysis balance. Total lipids were extracted from the dried biomass as per the method described in section 2.7.2. The chloroform containing the lipid fraction was then taken in a pre-weighed round bottom flask and was then concentrated using a Rotavapor. The weight of lipids was determined after evaporation of chloroform and was expressed as percentage of the dry cell weight of algal biomass (% DCW).

4.2.4. Thin layer chromatography analysis

HPTLC analyses were carried out to identify the lipid components of the extracted oil in an HPTLC system (CAMAG, Switzerland). HPTLC aluminium sheets coated with silica gel 60F 254 (Merck, India) was spotted with 5µl each of the extracted algal oil samples using a Linomat 5 automatic sample spotter. The solvent system used for separation of the sample was hexane: diethylether: acetic acid in the ratio 80:20:1. Plates were developed in a CAMAG glass twin chamber. Bands were detected by CAMAG “Win CATS 1.3.0” planar chromatography manager software. CAMAG TLC Scanner3 was used to detect the bands.

4.2.5. Fatty Acids Analyses

Fatty acid profile of the *Chlorococcum* sp.R-AP13 oil produced either phototrophically or heterotrophically was determined as fatty acid methyl esters (FAME). Trans-esterification of lipids was done using acid catalyst as outlined in section 2.7.4. The FAMEs were identified by comparing their fragmentation pattern with internal standards (Sigma Aldirch, India)

4.3. Results and discussion

4.3.1. Growth and oil production by *Chlorococcum* sp *R-AP13*

Chlorococcum sp. *R-AP13* could grow and produce biomass under both phototrophic and heterotrophic conditions. The maximum cell number attained was 9.1×10^6 cells/ml for the heterotrophic cultures with BDWG in 16 days, whereas in phototrophic mode with CO_2 supplementation, similar cell numbers (9.8×10^6 cells/ml) were attained only on the 24^{th} day of cultivation. In phototrophic cultivation without any carbon supplementation, maximum cell number attained was only 5.5×10^6 cells/ml on the 24^{th} day (Fig.4.1). Under phototrophic cultivation, the algal biomass and lipid production was higher when cultivated with CO_2 supplementation. The biomass yield was ~23 % higher in the case of cells grown with CO_2 compared to the ones cultivated without CO_2 supplementation. Similarly the lipid accumulation was higher (28% DCW) when cultivated with CO_2 compared to without it (21 % DCW). CO_2 is essential for photosynthesis and its supplementation during phototrophic cultivation of algae is known to improve the biomass yield and lipid production (Mata *et al,* 2010; Ota *et al,* 2011; Vidyashankar *et al,* 2013). *Chlorocccccum* sp *R-AP13* isolated from the virgin tropical evergreen forests was highly adaptable to the culture conditions to which it was exposed. The culture had a basal lipid accumulation of 21 % under natural phototrophic cultivation which was enhanced to 28 % upon supplementing CO_2 in the culture. There was also a significant enhancement (23%) in biomass yield. Industrial exhaust gases rich in CO_2 have effectively been used as CO_2 supplement for algal cultivation (Negoro *et al,* 1991; Chiu *et al,* 2011) and such systems provide the dual advantage of CO_2 removal and oil production. *Chlorococcum* sp *R-AP13* have shown significant improvement in both biomass and oil production on CO_2 supplementation during phototrophic cultivation, and shows the potential for further development of such culture systems.

Figure: 4.1 Growth of *Chlorococcum sp*.R-AP13 under phototrophic and heterotrophic cultivation in fresh water based medium

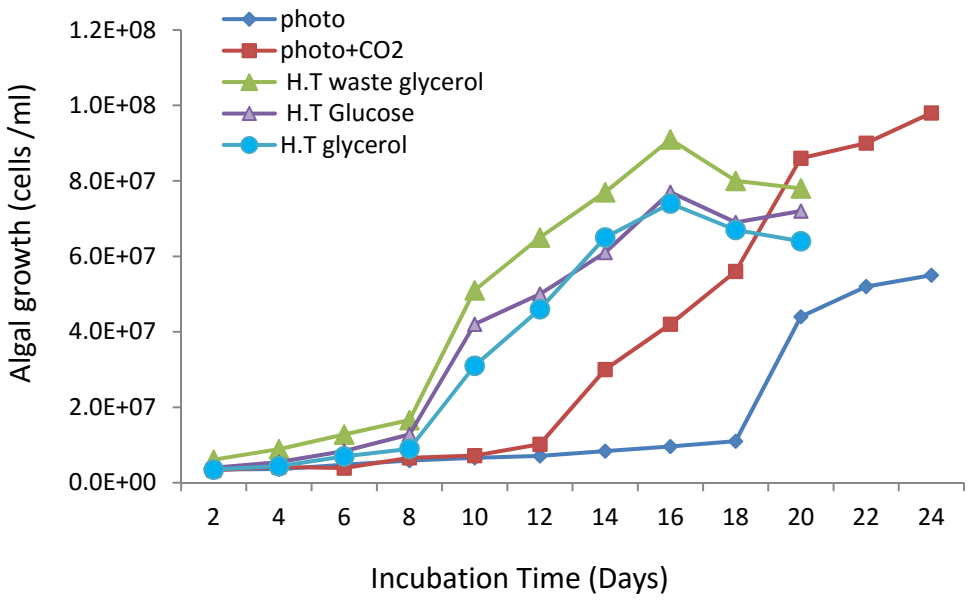


Photo: phototrophic cultivation, Photo + CO_2: phototrophic cultivation with CO_2 supplementation. H.T Glucose: Heterotrophic with glucose supplementation, H.T waste Glycerol: Heterotrophic with waste glycerol supplementation. H.T Glycerol Heterotrophic with glycerol

Lipid production was significantly higher under heterotrophic cultivation in all the three different carbon sources (glucose, pure glycerol and biodiesel industry waste glycerol (BDWG)) compared to the phototrophically grown cells (Fig.4.2). The highest lipid accumulation (~47% DCW) was obtained when pure glycerol was used as carbon source with a biomass yield of 356 mg/l. However, the culture produced the highest biomass yield of 575 mg/l with ~42 % DCW of lipid accumulation in media supplemented with BDWG as carbon source indicating that the overall lipid yield was high when crude glycerol is used as carbon source. The total lipid yield from the heterotrophic cultivations using glucose, glycerol and BDWG was 164, 167 and 242 mg/l respectively. It may be noted that the lipid accumulation of cells cultivated in pure or waste glycerol was very similar and the latter carbon source supported a higher biomass yield indicating its suitability for heterotrophic cultivation of the alga. Biomass yield was lowest in cells cultivated using pure glycerol as carbon source. In heterotrophic culture system, there was a bleaching of chlorophyll pigment (Fig 4.3). Morphology of algal cells was varied in different culture conditions. Microscopic and scanning electron micrograph showed difference in the morphology of algal cells under phototrophic and heterotrophic conditions (Fig 4.3).

Figure: 4.2 Biomass and lipid production during phototrophic and heterotrophic cultivation of *Chlorococcum* sp R-AP13 in fresh water medium.

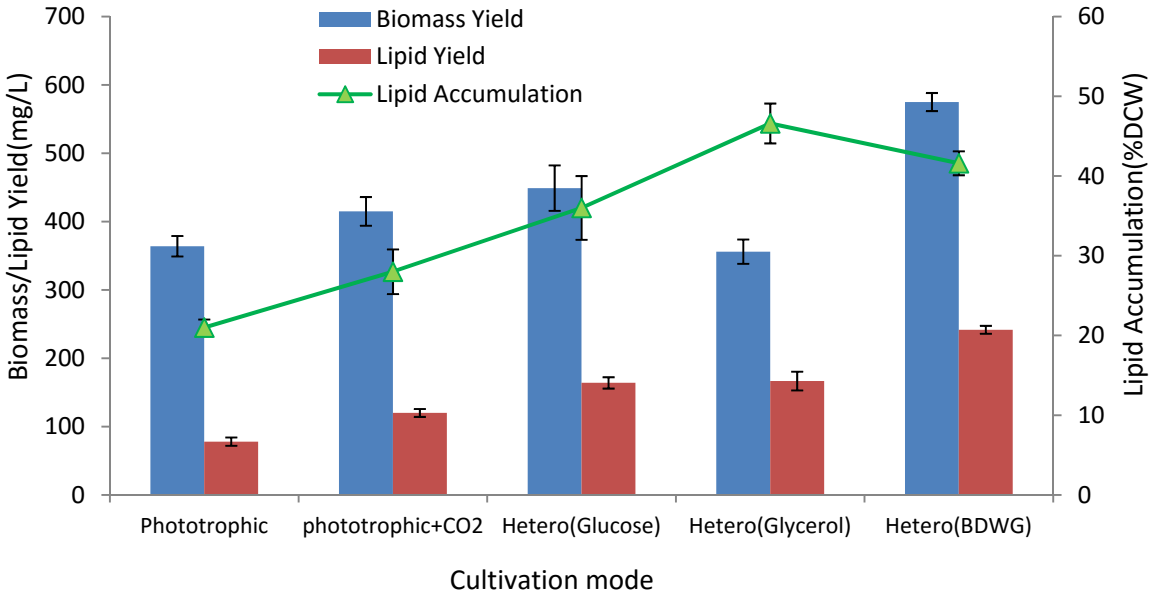
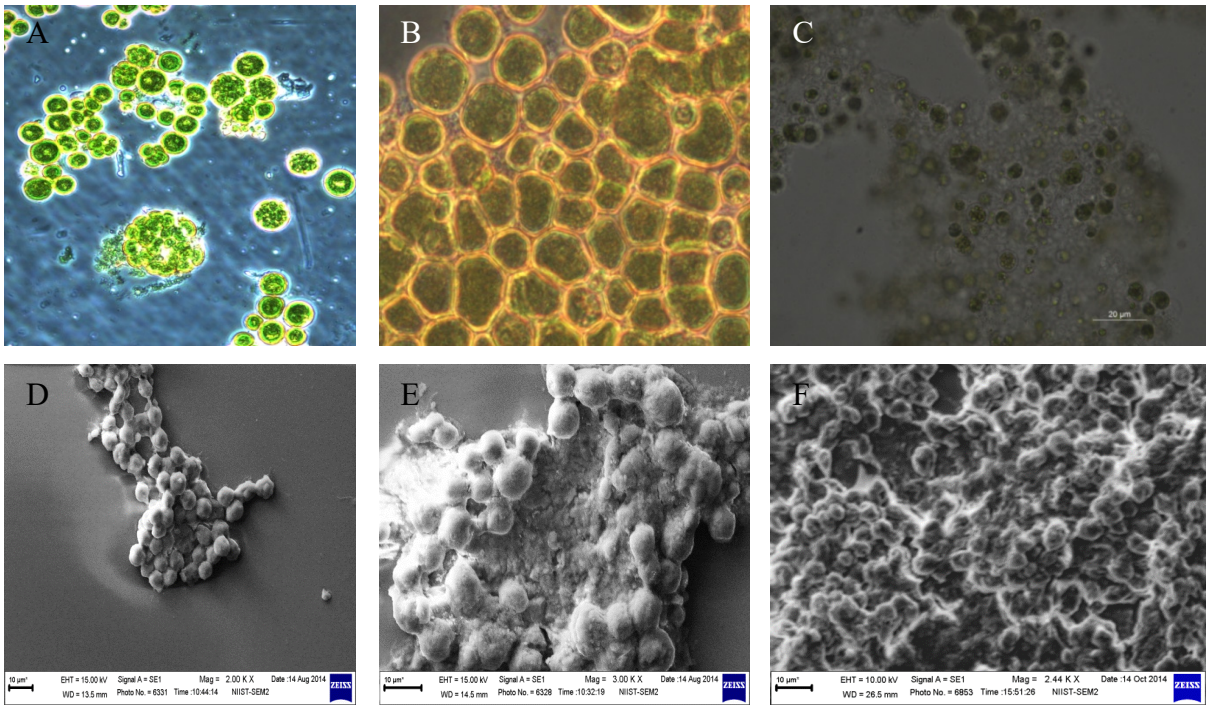


Figure 4.3: Microscopic observation of the morphology of *Chlorococcum* sp. R-AP13 under different modes of cultivation



A) Phototrophic cultivation B) Heterotrophic cultivation on glucose, C) Heterotrophic cultivation on glucose Lipid accumulation inside phototrophically grown cells D, E, and F are the corresponding SEM analysis of alga under both conditions

The data on heterotrophic growth and lipid production indicated that biodiesel industry waste glycerol is a promising carbon source for generation of biomass using the *Chlorococcum sp* R-AP13. Growth of the alga under heterotrophic conditions resulted in the disappearance of chlorophyll, but it also led to high accumulation of lipid in the cells. Waste glycerol from biodiesel production has been used for lipid production by micro algae (Chen and Walker, 2011; Liang *et al,* 2010). Crude glycerol is the major waste product of the biodiesel industry and as the biodiesel industry is growing, the problem of its disposal is also growing. Biodiesel industry waste glycerol contains high levels of methanol which may prevent its efficient treatment and disposal. This also affects the ability for cultivating algae in crude glycerol and it was reported that the maximal concentration of untreated glycerol that could be tolerated by the alga *Schizochytrium limacinum* SR21 was 25 g/L (Liang *et al,* 2010). *Chlorococcum* sp *R-AP13* could grow well in 50g/L of waste glycerol and biomass and lipid yield was superior to that obtained both in phototrophic cultivation and in heterotrophic cultivation with similar concentration of either glucose or pure glycerol. This indicates that the crude glycerol may be used directly without any pre-treatment for cultivation of the alga which would simplify the process and reduce the operating costs for biomass production.

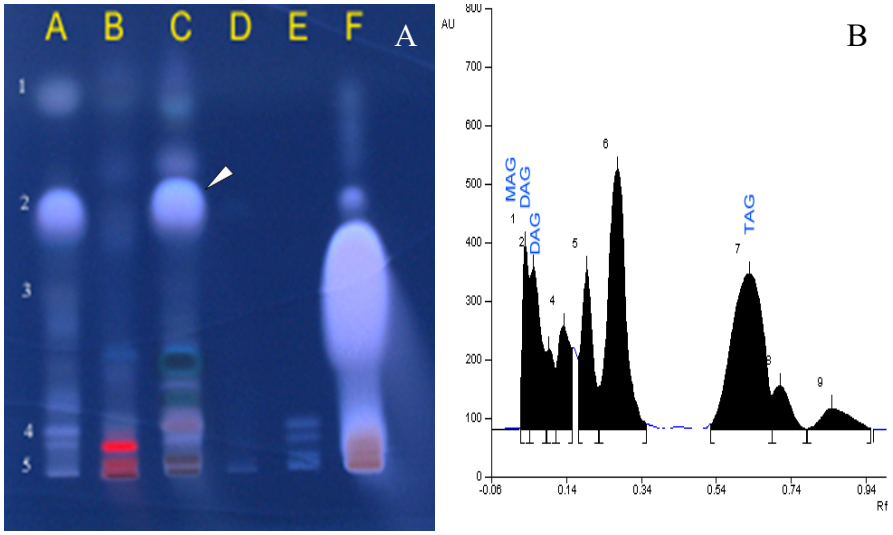
Chlorococcum sp. *R-AP13* had different lipid contents and fatty acid compositions depending on the mode of cultivation (phototrophic vs heterotrophic). Lipid content (47%) in heterotrophic cells of *Chlorococcum sp*. was doubled in comparison to that in the autotrophic cells (28%). Heterotrophic cultivation of microalgae has been reported to provide not only high algal biomass production but also high cellular lipid content (Bumbak *et al,* 2011; Morales-Sa´nchez *et al,* 2015). This may be due to the carbon abundance in heterotrophic cultures which leads to increased proportion of storage lipids, mainly in the form of triglycerides in the stationary phase of their growth. The production of high amount of lipid in crude biodiesel industry waste glycerol (BDWG) indicated that there is great potential to develop the alga as a feedstock for producing oil used in biodiesel production. It is proposed that for the microalgae to become competitive as a liquid fuel source, the organism should be capable of accumulating 50-60% of its dry cell weight as lipids (Xu *et al,* 2005). Even without any optimizations, *Chlorococcum* sp R-AP13 produced high biomass and accumulated 42-47 % lipid in the cells which demonstrated its potential for further development as a feedstock organism for biodiesel production.

4.3.2. Characterization of lipids produced by *Chlorococcum* sp *R-AP13*

4.3.2.1. HPTLC analysis of lipids

Analysis of the lipids extracted from *Chlorococcum* by HPTLC indicated that the major lipid fraction present in the oil is Triacylglycerols (TAG) (Fig.4.4 A and B). This is advantageous if the oil is to be considered for biodiesel applications since TAGs are the raw materials for trans-esterification reactions to produce biodiesel (Chisti, 2007; Durret *et al,* 2008). Thus the higher proportion of TAGs in *Chlorococcum* sp R-AP13 is advantageous, if it were to be used as a feedstock organism for biodiesel production.

Figure 4.4: HPTLC profile of the lipids produced by *Chlorococcum* sp *R-AP13* grown phototrophically



A: Scanned image showing HPTLC profile of *Chlorococcum* sp R-AP13 oil, B: HPTLC profile of *Chlorococcum* sp. lipids showing relative proportion of the different oil types (as the signal intensities of corresponding bands in HPTLC plate)

4.3.2.2. Fatty acids profiling of lipids

Apart from differences in lipid production, the different culture conditions also had an effect on the type and quantities of fatty acids produced by the alga. Oil produced by the phototrophically grown cells contained linolenic acid (18:3), palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (18:2) and oleic acid (18:1) as the principal components. The most abundant unsaturated fatty acid was linolenic acid (C18:3) which constituted 27.1% of the total fatty acids (Table 4.1). Oil from heterotrophically grown cells contained a greater

percentage of C16 fatty acids (18-36%) in comparison with phototrophically grown cells, and the actual amount varied also depending on the type of carbon source. The percentage of linolenic acid (C18:3) was almost same in the oils from both phototrophic and glycerol grown cells. Lipids produced by *Chlorococcum* sp grown on BDWG contained longer chain fatty acids of up to C24. The fatty acids were predominantly C16:0, C18:1, C18:2 and 18:0 while C24:0 was produced in lesser quantities (Table 4.1). Linolenic acid (a polyunsaturated fatty acid), was completely absent in the oils produced in BDWG medium.

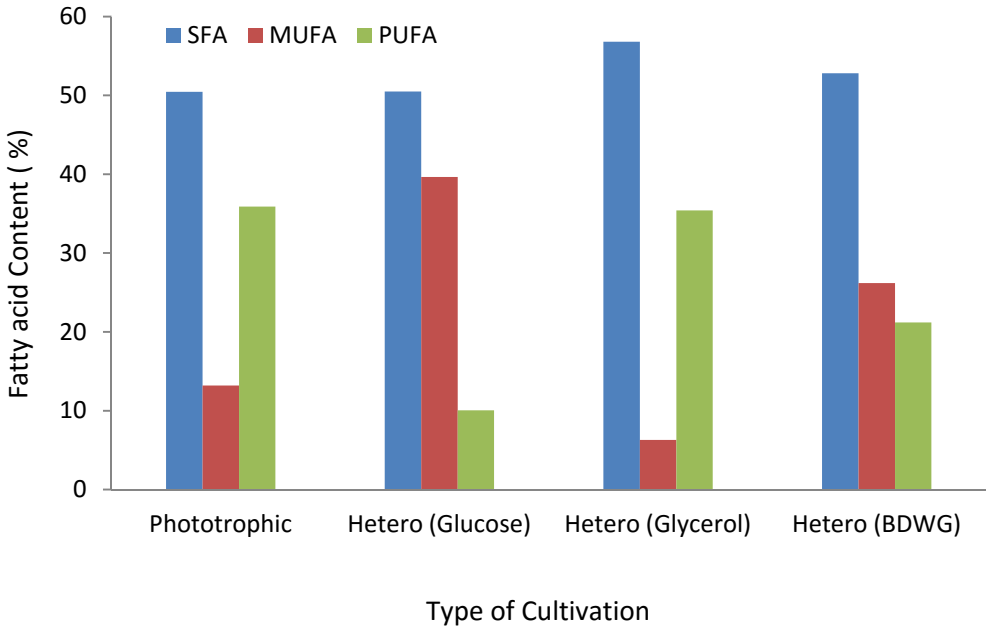
Table 4.1: Fatty acid profile of the lipids produced by *Chlorococcum* sp *R-AP13* cultivated phototrophically or heterotrophically in different carbon sources

Fatty Acid Type	Percentage of Total Lipids (%)			
	Phototrophic	Heterotrophic (Glucose)	Heterotrophic (Pure Glycerol)	Heterotrophic (BDWG)*
C12	2.50 ± 0.14	-	2.45 ± 0.21	-
C14	3.05 ± 0.07	0.60 ± 0.00	2.55 ± 0.07	0.75 ± 0.07
C15	1.75 ± 0.07	0.14 ± 0.00	0.95 ± 0.07	-
C16	18.25 ± 0.21	36.75 ± 0.3	18.75 ± 0.77	28.40 ± 0.7
C16-1	5.75 ± 0.49	0.65 ± 0.07	6.30 ± 0.28	0.55 ± 0.07
C16-2	-	-	-	-
C17	2.10 ± 0.00	0.17 ± 0.03	-	0.45 ± 0.07
C18	13.90 ± 0.35	9.70 ± 0.10	15.00 ± 0.14	14.00 ± 0.00
C18-1	5.85 ± 0.07	38.50 ± 0.28	-	24.7 ± 0.00
C18-2	8.80 ± 0.28	10.05 ± 0.07	7.55 ± 0.35	20.65 ± 0.07
C18-3	27.10 ± 0.14	-	27.85 ± 0.07	-
C20	8.90 ± 0.0	1.25 ± 0.07	10.00 ± 0.14	1.85 ± 0.07
C20-1	-	0.50 ± 0.00	-	0.39 ± 0.00
C20-2	-	-	-	0.55 ± 0.07
C22	-	0.95 ± 0.07	7.1 ± 0.14	2.35 ± 0.07
C22-1	1.60 ± 0.00	-	-	-
C23	-	-	-	0.55 ± 0.07
C24	-	0.95 ± 0.07	-	4.45 ± 0.35

* BDWG: Biodiesel industry waste glycerol

Saturated fatty acids production by *Chlorococcum* sp.RAP-13 was in the range of 50-57 % in the different media, whereas the levels of mono unsaturated fatty acids (MUFA) and poly unsaturated fatty acids (PUFA) production varied more, depending on the carbon source used for heterotrophic cultivation (Fig 4.5). Total poly unsaturated fatty acid (PUFA) levels were similar in phototrophic and pure glycerol grown cultures, while the total PUFA content was significantly lower in BDWG and glucose grown cultures.

Figure 4.5: Variation in the relative abundance of fatty acid types in the oil produced by *Chlorococcum* sp *R-AP13* cultivated under different conditions



Moreover, fatty acid profiling of the oils produced under different cultivation systems indicated that the oil produced using waste glycerol was rich in C16:0, C: 18, C18:1 and C18:2, and the poly unsaturated fatty acids like C18:3 were completely absent (Table 4.1). Saturated fatty acids production was in the range of 50-56% regardless of the culture conditions. MUFA production was higher in heterotrophic systems supplemented with glucose. Recommended fatty acids for good biodiesel properties include C14:0, C16:0, C16:1, C18:0, C18:1and C18:2 (Schenk *et al,* 2008; Ahmad *et al,* 2011). European Biodiesel Standards EN 14214 for vehicle use, also specifies that the content of methyl linolenate (C18:3) and the polyunsaturations (≥4 double bonds) content are to be limited to a maximum of 12 and 1%, respectively (Knothe 2008). The lipid profile of oil from *Chlorococcum* sp R-AP13 cultivated in BDWG therefore seems to be ideal as a feedstock for biodiesel production.

The high percentage of oleic acid (18:1) linoleic acid (18:2) and linolenic acid (18:3) which are essential fatty acids makes *Chlorococcum sp*. R-AP13, a good source of healthy food fats for both food and feed applications. The human body can synthesize the long-chain PUFAs (EPA and DHA) from linolenic acid (Doughman *et al,* 2007; Stefan and Boussiba, 2014) and therefore the supplementation of algal oil rich in this fatty acid may help to serve the requirement of EPA and DHA. Because of its unique fatty acid profile, the oleaginous *Chlorococcum sp* R-AP13 may be considered a suitable nutritional supplement for humans and a feed additive for animals, besides being a starting material for biodiesel production. Ability to grow both phototrophically and heterotrophically, latter on waste glycerol indicates the importance of this novel isolate as a potent source of algal oil; the composition of which may be modulated by adjustments in growth mode and conditions.

4.4. Conclusions

Chlorococcum sp R-AP13, a new algal isolate was capable of efficient growth and accumulation of significant amount of lipids during the heterotrophic growth in biodiesel industry waste glycerol. The cells displayed different growth characteristics, lipid content and fatty acid profiles depending on the carbon substrates used for heterotrophic cultivation. The heterotrophically grown algal cells demonstrated much higher yield of total lipids and biomass than the photoautotrophic cells, and the alga could grow very efficiently in 50g/L waste glycerol with 42% DCW lipid accumulation. The lipid profile of the oil produced from waste glycerol grown cultures indicated even more interesting features like high triglycerides content, a fatty acid profile with medium chain length fatty acids enriched and a high content of the essential fatty acid-linolenic acid. These features project the alga as a potent source of oil which can be used as feedstock for biodiesel and also as a food or feed supplement.

Chapter 5: Phototrophic and heterotrophic cultivation of *Chlorococcum sp* R-AP13 in sea water based medium for biomass and lipid production

5.1. Introduction

Microalgae can be cultivated phototrophically or heterotrophically depending on the availability of light and the ability of the cultured species to utilize organic carbon. Photoautotrophic cultivation is considered the most desirable method of culturing microalgae, since this requires only sunlight, CO_2 and water. Furthermore, the photosynthetic efficiency of microalgae is substantially higher than the higher order plants (Chisti, 2007). Heterotrophic growth of microalgae eliminates the requirement for light and so offers the possibility of increasing algal cell density and productivity on a large-scale with the cell densities reaching as high as 100 gL^{-1}(Bumbak *et al,* 2011). However, the cost of carbon source becomes a limiting factor here, and costs as high as 80 % of the total medium cost has been attributed to the carbon source (Li *et al,* 2007; Chen & Walker, 2011). The cost of algal biodiesel depends on several factors- the most important of which include substrate cost (heterotrophic culture), cost of harvesting the biomass, lipid yield from the cells and the quality of lipid with respect to biodiesel specifications (Yang *et al,* 2006).

While commercial scale test-facilities are operational for algal biodiesel production, significant improvements are still needed to make algal biofuels economically viable (Georgiana and Mayfield, 2012). Though the yields of oil from microalgae are reported to be 15-300 times more than the traditional crops on area basis (Schenk *et al,* 2008), algae grown under nutrient replete conditions and subjected to dry oil extraction processes have an unfavorable net energy balance (Lardon *et al,* 2009). One of the most important concerns is the need to provide substantial amounts of water and nutrients to the algal populations undergoing mass cultivation (Murphy and Allen, 2011). Algal cultivation requires significant amount of water and it has been stated that ~ 6000 litres of water is consumed per litre of algal oil produced in conventional systems of cultivation (Ozkan *et al,* 2012). Water provides the physical environment for growth of the algae and also delivers the nutrients, removes waste products and acts as a thermal regulator (Murphy and Allen, 2011). Fresh water is a valuable resource for the future, and hence algal biomass production in non-potable waters like seawater or waste water is highly desirable. There have been several studies on micro algal cultivation using seawater (Raitan *et al,* 1994; Takagi *et al,* 2006; Chen *et al,* 2013).

However, most of these studies have reported culturing of marine microalgae and reports on heterotrophic cultivation of microalgae in sea water are rare.

Previous chapter discussed about the heterotrophic growth and lipid production by a potent fresh water micro algal isolate – *Chlorococcum* sp RAP13, in a synthetic medium in fresh water. The alga was screened in sea water medium which showed that this isolate can produce biomass and lipids in sea water. The culture was therefore tested for growth and lipid production in 50% sea water under phototrophic and heterotrophic modes. Biomass and lipid production under heterotrophic growth with glucose or waste glycerol supplementation was compared with phototrophic cultivation in sea water based medium. The oil produced was characterized for lipid composition to determine suitability for biodiesel applications.

5.2. Materials and Methods

5.2.1. Growth adaptation in seawater and inoculum preparation

The fresh water micro alga *Chlorococcum sp* R-AP13 was able to produce biomass and lipids in fresh water medium under heterotrophic cultivation. Potent algal cells were adapted to grow in marine water by repeated culturing in 50% natural sea water base medium and were maintained in the same medium. Stock culture was inoculated into the sea water base medium and was incubated phototrophically at 30 ± 2 ˚C and agitation of 100 rpm for 15 days with a light/dark period of 13/11h. The cells were re-suspended in fresh medium to a concentration of 3×10^6 cells /ml and the suspension was used at the required concentration as inoculum.

5.2.2. Sea water based cultivation medium

A minimal medium of the following composition (in mg/l) KH_2PO_4-200, K_2HPO_4-400, $MgSO_4$-200, Urea-200, KNO_3-200, F1/2 trace metal mix -1 ml, and Vitamin mix -0.5ml (Guillard and Ryther, 1962) in 50 % aged sea water was used as a base medium for both phototrophic and heterotrophic cultivation of the alga. pH of the sea water medium was adjusted to 7.0. All microbiological-grade carbon and nitrogen sources used in the experiments were known to be stable when subjected to sterilization at 121 °C, 15 psi for 15 min. However, some of the components were autoclaved separately to prevent any

precipitation reaction while the heat labile components such as the vitamin mix were filter sterilized and added to the pre-sterilized medium.

5.2.3. Phototrophic cultivation

Medium for photoautotrophic culture was prepared as above but with buffering (10 mM Na-Phosphate buffer in the 50% sea water base). Sterilized media (200ml in 500ml Erlenmeyer flasks) were inoculated with 5% (v/v) of the inoculum containing 3 x 10^6 cells/ml, and were incubated in an environmental chamber equipped with fluorescent lamps (Illumination - 3000 lux) at a temperature of 30 ± 2˚C and with 100 rpm agitation for 25 days with a diurnal cycle of 13/11h light/darkness. CO_2 was bubbled through the medium at low pressure with an aeration rate of 0.8 vvm.

5.2.4. Heterotrophic cultivation

All heterotrophic cultivations of *Chlorococcum sp.* R-AP13 were performed in 500 ml Erlenmeyer flasks containing 200ml sea water base medium supplemented with either glucose or biodiesel industry waste glycerol (BDWG) at 5% (v/v) level. Medium in each flask was inoculated with 5% (v/v) of the inoculum containing 3 x 10^6 cells /ml. Cell counts were performed using a haemocytometer for inoculum preparation. The cultures were incubated at a temperature of 30 ± 2˚C and with 100 rpm agitation for 12 days without exposure to light.

5.2.5. Total lipid extraction

Total lipids were extracted from dried biomass by the solvent extraction method described by (Bligh and Dyer, 1959). Lipid extraction methods were performed according to section 2.7.2. Lipid yield was expressed as percentage of the dry cell weight of algal biomass (% DCW).

5.2.6. Analytical Methods

5.2.6.1. Monitoring cell growth and utilization of carbon source

Cell growth was monitored as increase in cell numbers at two days interval, and by measuring the dry cell weight (DCW). For DCW determination, the cells were harvested by centrifugation at 8000 rpm for 10 min, washed with distilled water to remove the salt and were lyophilised to remove water. Lyophilized cells were weighed in an analytical balance and the dry cell weight was expressed as mg/l. Utilisation of glucose and glycerol were determined by monitoring their depletion from medium by HPLC, using RI detector as outlined by Sluiter (2006).

5.2.6.2. Intracellular lipid accumulation

Lipid accumulation in the algal cells was monitored by Nile red staining (Chen *et al,* 2009). As outlined under section 2.7.1. Nile red fluorescence was visualized in a fluorescence microscope with excitation and emission wavelengths of 490 and 595 nm respectively, and was captured using an attached digital camera.

5.2.6.3. Thin Layer Chromatography (TLC) analysis

TLC analysis was carried out to identify the lipid components present in the extracted oil. Samples were spotted on TLC aluminium sheets coated with silica gel $60F_{254}$ of 0.2mm thickness (E Merck, India). The solvent system used for separation of sample was hexane: diethyl ether: acetic acid in the ratio of 80:20:1. Lipids were developed in a glass chamber and the spots of lipids were visualised using iodine vapours.

5.2.5.4 Fatty acids analysis

Fatty acid profile of the algal oil was determined as fatty acid methyl esters (FAME). Trans-esterification reactions were performed with 2 % HCl in dried methanol at 100˚C for 1h. FAMEs were extracted with hexane and dried over anhydrous Na_2SO_4. The samples were analyzed in a gas chromatograph (6850N, Agilent Technologies) as discussed under section

2.7.4. The FAME was identified by comparing their fragmentation pattern with internal standards (Sigma Aldrich, India).

5.3 Results and discussion

5.3.1 Heterotrophic growth on glucose and waste glycerol

Chlorococcum sp R-AP13 was previously found to utilize both glucose and waste glycerol efficiently for growth and lipid production in fresh water medium discussed in chapter 4. In this study, the culture adapted in 50 % sea water based medium was found to grow well and produce oil in sea water-based medium heterotrophically with either glucose or BDWG supplementation. The maximum cell numbers attained was 2.7×10^6 cells/ml for the heterotrophic cultures with BDWG in 9 days, whereas in phototrophic mode with CO_2 supplementation, the same numbers were attained only on the 15th day (Fig. 5.1). Though the maximal cell numbers attained under heterotrophic or phototrophic modes were similar, cell dry weight was higher under heterotrophic cultivation, indicating that the cell sizes were larger and probably with higher lipid accumulation under heterotrophic cultivation. The latter was confirmed since the lipid yield and cellular lipid accumulation was 330 mg/l and 38.9 %, respectively, for medium with waste glycerol, which was the highest compared to the other growth modes evaluated (Table 5.1). *Chlorococcum sp* R-AP13 could adapt very well to a wide range of culture conditions and was effective for biomass as well as lipid production under heterotrophic cultivation using glucose and waste glycerol. The current study demonstrated its potential to adapt to high salinity and in fact it produced more biomass and lipids when grown in the sea water based medium. While heterotrophic cultivation of *Chlorococcum* sp in sea water based medium was better in terms of both biomass and lipid yield, it was also observed that there is a bleaching of cells after about 7.0 days of heterotrophic cultivation, which could not be reversed by exposure to light. Nevertheless, the cells continued to grow efficiently under heterotrophic mode utilizing either glucose or waste glycerol supplemented in sea water based medium. The differences in biomass yields supported by these carbon sources were very small. Since BDWG is an industrial waste product, its utilization for algal oil production can be highly advantageous both to reduce the production cost and as a waste treatment and value addition method. Crude glycerol from biodiesel industry is known to be used for lipid production by heterotrophic cultivation of microalgae (O 'Gradey and Morgan, 2011; Chen and Walker, 2011).

Figure: 5.1. Growth of *Chlorococcum sp*.RAP-13 in sea water medium under phototrophic and heterotrophic cultivation

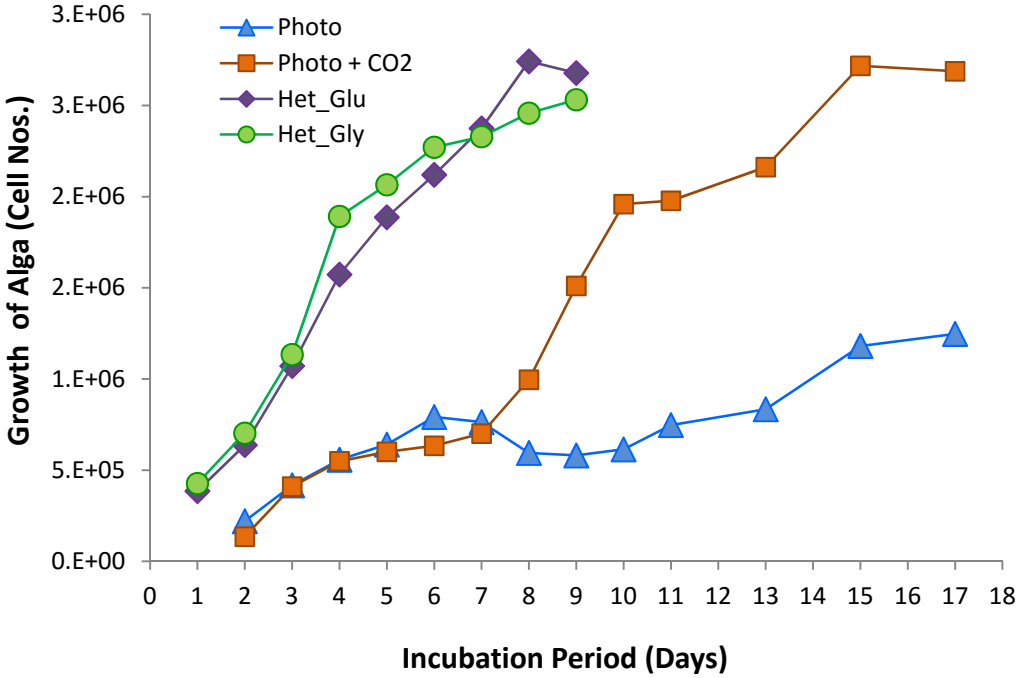


Photo: phototrophic cultivation, Photo + CO_2: phototrophic cultivation with CO_2 supplementation. Ht_Glu: Heterotrophic with glucose supplementation, Ht_Gly: Heterotrophic with glycerol supplementation

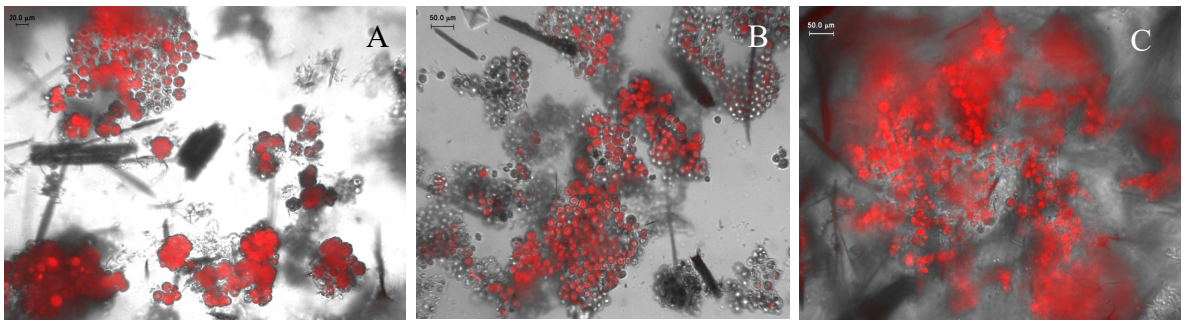
Table 5.1: Biomass and lipid production of *Chlorococcum sp RAP13* under phototrophic and heterotrophic mode on 50% sea water medium

Cultivation conditions (carbon supplemented)	Biomass conc. (mg/l)	Yield of lipids (mg/l)	Cellular Lipid Accumulation (% DCW)
Phototrophic	152.5 ± 0.7	31.0 ± 0.65	20.8 ± 2.60
Phototrophic + CO_2	301.0 ± 0.3	72.5 ± 0.40	24.0 ± 0.84
Heterotrophic + Glucose	1008 ± 7.7	304.0 ± 2.00	30.5 ± 0.35
Heterotrophic + BDWG	850.0 ± 7.0	330.0 ±1.00	38.9 ± 1.90

Data are expressed as mean ± standard deviation of triplicates.

Nile red staining of cells confirmed lipid accumulation inside the cells in the form of intra cellular lipid droplets (Fig 5.2). Fluorescence intensities were indicative of the presence of neutral lipid storing vesicles or vacuoles in the cells. Interestingly, compared to *Chlorococcum* sp. R-AP13 grown heterotrophically in fresh water base medium, the culture grown in sea water yielded higher amounts of biomass and lipids. The biomass yields for heterotrophic growth in sea water medium supplemented with glucose and BDWG were 1008 mg/l and 850 mg/l and the lipid yields were 304 mg/l and 330 mg/l respectively. The lipid yields were significantly higher than those obtained for corresponding experiments using fresh water based medium which is discussed in chapter 4.

Figure 5.2: Intracellular lipid accumulation in algal cells grown in sea water under different modes of cultivation.



Images of Nile red fluorescence and phase contrast images merged to show intracellular lipid accumulation in algal cells, A) Phototrophically grown algal cells B) Heterotrophically grown algal cells with glucose as C source C) Heterotrophically grown algal cells with glycerol as C source

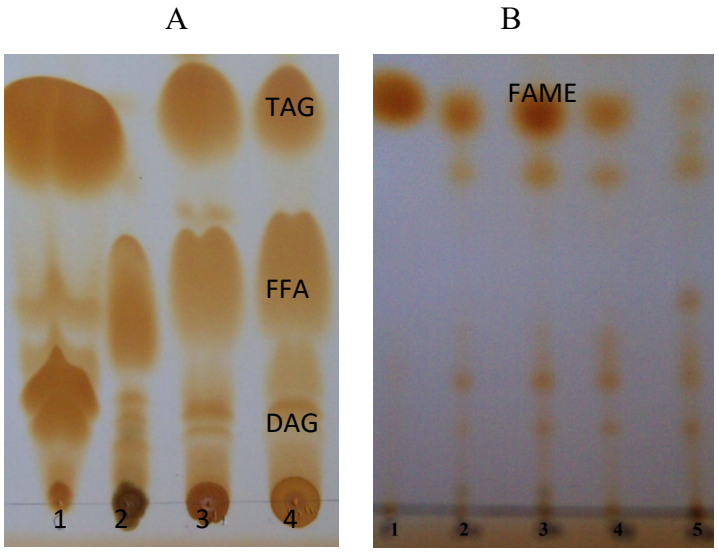
5.3.2 Phototrophic cultivation

Phototrophic cultivation of *Chlorococcum* sp. RAP13 was not as efficient as its heterotrophic cultivation, both in terms of biomass and lipid yields. However, phototrophic cultivation with CO_2 bubbling was comparatively better than the cultivation performed without CO_2 supplementation (Table 5.1). Though the initial pH of the sea water medium used for algal cultivation was 7.0, there was a shift in pH of the medium from 7.0 to 3.5 during the continuous bubbling of CO_2 in the medium. Interestingly, the alga could survive this change in pH. Cellular lipid accumulation in cultures supplemented with CO_2 (24% DCW) however, was only marginally better than where CO_2 was not supplemented (20% DCW). Biomass and lipid accumulation was very low under phototrophic cultivation.

5.3.3. Analyses of the algal oil

Lipid profiling of oils extracted from algal cells grown phototrophically or heterotrophically indicated presence of Triacylglycerols (TAG), Diacylglycerols (DAG) and Free fatty acids (FFA) as the major lipids (Fig 5.3). The TAG content was comparatively lower for oil from phototrophically grown cells. Oils rich in triglycerides are preferable starting materials for biodiesel production via trans-esterification reactions (Hu *et al,* 2008). Oil from heterotrophically cultivated *Chlorococcum sp.* R-AP13 had a higher content of TAGs and since the yield of lipids per unit volume of culture was also higher, this mode of growth would be better for oil production suited for biodiesel applications. Oil produced from the heterotrophic cultures were enriched with neutral lipids-especially TAG. Higher TAG content of the oil may be due to the abundance of carbon in heterotrophic cultivation, or it could be influenced by the salinity stress due to use of sea water in the medium

Figure 5.3: Lipid profiling of oil from *Chlorococcum* sp. grown under heterotrophic mode



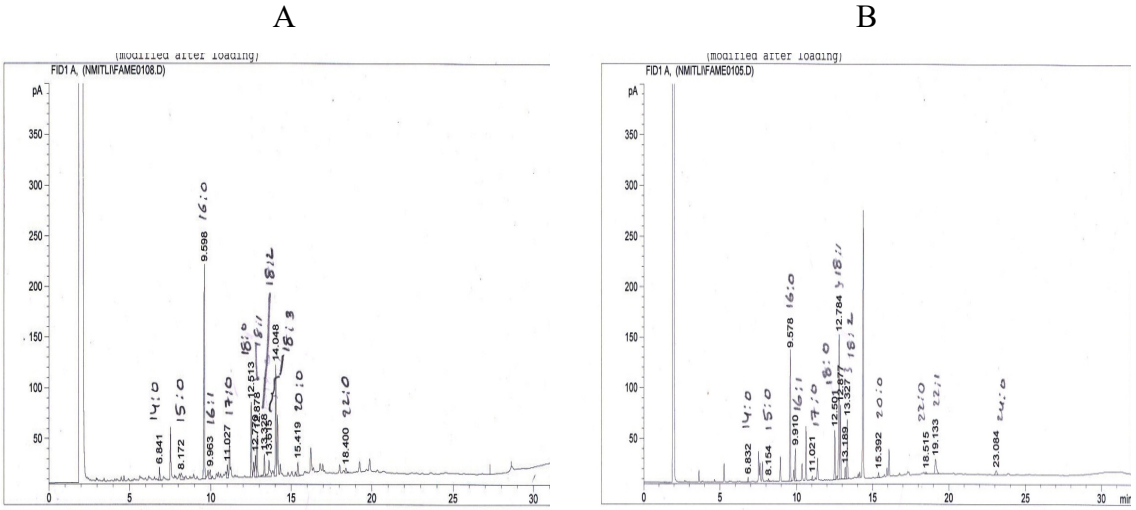
A) TLC analysis of oil produced by *Chlorococcum* grown under different modes of cultivation: Lane Information: 1) Control (Trioleate), 2) Oil from phototrophically grown cells, 3) Oil from heterotrophically grown cells with BDWG as C source, 4) Oil from heterotrophically grown cells with glucose as C source. TAG – Triacylglycerol, DAG – Diacyl glycerol, FFA- Free fatty acid; B) TLC analysis showing fatty acids methyl esters formation after trans-esterification reaction.

Gas chromatographic analysis of the fatty acid methyl esters of algal oil from heterotrophic cultures indicated that the major fatty acid is oleic acid (C18:1), followed by palmitic (C16:0), palmitoleic (C16:1), linoleic (C18:2) and linolenic (C18:3) acids (Table 5.2). Very long chain fatty acids of chain length C24 were also produced under heterotrophic conditions (Fig 5.4). Poly unsaturated fatty acids (PUFA) like C18:3 were either very less or absent in heterotrophically produced oil. There were significant differences in the type of fatty acids produced under phototrophic and heterotrophic cultivation and even between heterotrophic cultures where different carbon sources were supplemented. In the case of photoautotrophic growth, the principal fatty acid produced was palmitic acid (C16:0) followed by linolenic (C18:3) and stearic (C18:0) acids, while other fatty acids were produced in very low quantities. Saturated fatty acids production was high under phototrophic growth.

Table 5.2: Fatty acid profile of algal oil produced under various modes of cultivation

Fatty Acid Type	Fatty acid content in the oil (%)		
	Phototrophic	Hetero + Glucose	Hetero + BDWG
C14	1.90	0.285	0.64
C15	1.50	0.335	0.00
C16	36.00	2.40	16.40
C16: 1	4.60	6.90	8.95
C17	2.60	0.78	0.55
C18	12.80	11.00	8.60
C18: 1	11.70	53.95	41.05
C18: 2	4.40	9.90	8.05
C18: 3	19.20	0.79	6.25
C20	0.00	0.00	5.40
C22	2.90	0.69	3.65
C22: 1	1.40	0.00	0.00
C24	0.00	2.10	1.60

Figure 5.4. Gas chromatogram showing fatty acids profile of lipids from *Chlorococcum sp.*R-AP13 grown heterotrophically in sea water based medium



A) Phototrophic and B) Heterotrophic

Fatty acids profile of the oil from *Chlorococcum sp* varied depending on the culture conditions and substrate used for biomass production. The change in fatty acid composition of oil synthesized by algae in response to the cultivation conditions may have a protective role and may help in adaptation to the changed environmental conditions. Fatty acids preferred for biodiesel include C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2 (Schenk *et al,* 2008). When all the requirements for biodiesel quality are considered, it becomes clear that not a single fatty acid methyl ester could satisfy every requirement for biodiesel production. However, a balance of different fatty acids containing higher amounts of mono-unsaturated fatty acids such as oleate (18:1Δ9), and fewer saturated and polyunsaturated fatty acids would yield a better biodiesel (Durrett *et al,* 2008). For quality biodiesel, based on European Biodiesel Standards EN14214 for vehicle use, content of methyl linolenate (18:3) and the PUFAs containing greater than four double bonds should be limited to a maximum of 12 and 1% respectively.

Micro algal oils are rich source of Omega 3 and Omega 6 fatty acids which are conditionally essential and help in preventing many diseases including cancer (Altenburg and Siddiqui, 2009). Phototrophically produced lipids from *Chlorococcum sp.* R-AP13 had a higher percentage of PUFAs like the C18:3 linolenic acid (19%), C18:2 linoleic (4.4%) and the monounsaturated oleic acid (C18:1) which makes this micro algal isolate a source of healthy fats for both food and feed applications.

Production of algal oils for biodiesel is technically and economically challenging. Major limitations for algal oil production are in harvesting, fresh water source for cultivation and the biomass productivity. Selection of fast growing, productive strains adapted to local climatic conditions is important for the success of mass cultivation of algae, especially for low value products such as biodiesel (Griffiths and Harrison, 2009). Choosing an algal species well suited for a biorefinery mode of operation, for example- for production of valuable co-products such as fine chemicals, nutraceuticals or a nutrient-rich biomass, contributes to both economic success and environmental sustainability.

Ability to grow algae phototrophically and heterotrophically using sea water as the base for cultivation medium is highly advantageous due to the economic and environmental benefits. Algal cultivation consumes huge amounts of fresh water, especially in phototrophic mode which otherwise is probably the best strategy for algal cultivation due to the minimal cost of medium and potential environmental benefits due to carbon dioxide recycling. Sea water is a complex medium consisting of several known elements and a large number of organic compounds whose composition can vary significantly; especially when collected from the surface (Harrison and Berges, 2005). However, it is also an abundant natural source of most of the major and minor elements required for algal mass cultivation. It is known that several fresh water algal species can grow in high concentrations of sea water, if certain mineral nutrients normally absent or present in low concentrations are supplied (Wetherell 1961). While phototrophic cultivation of the alga in 50% seawater resulted in an accumulation of oil rich in PUFA, heterotrophic cultivation in sea water medium with either glucose or waste glycerol supplementation resulted in oil that is rich in medium chain length saturated fatty acids and monounsaturated fatty acids which is ideal for biodiesel production. Biomass productivity and lipid yield from heterotrophic cultures were significantly higher compared to those from phototrophic cultivation, and the highest lipid yield and cellular lipid accumulation was recorded with use of waste glycerol as carbon source. Cultivation of microalgae using crude glycerol provides added advantages of the value addition of this by-product besides generating oil for biodiesel production ensuring that the major waste/by-product from biodiesel industry can be recycled back to the fuel.

5.4. Conclusion

The fresh water microalga -*Chlorococcum sp* R-AP13 was capable growth and lipid production in sea water. While tolerance to salinity has been reported for fresh water microalgae, better performance in terms of growth and lipid production in sea water is rare in fresh water algae. *Chlorococcum* sp RAP13 had significantly higher growth and lipid accumulation in sea water based medium than in fresh water and moreover, under heterotrophic cultivation in a sea water based medium, it produced an oil rich in monounsaturated fatty acids and medium chain length saturated fatty acids, ideal for biodiesel. Mode of culture influenced the oil composition with higher percentage of PUFAs produced while being cultivated phototrophically in seawater based medium. The alga was highly adaptive to pH and salinity as observed from this study and holds great potential as a source of oil for both biodiesel as well as nutraceutical applications. Use of sea water as a the basal medium helps in reducing the requirement of mineral supplements and more importantly helps to save fresh water- an important resource. The alga could efficiently convert waste glycerol from biodiesel industry back to oil demonstrating the potential to recycle this waste. To the best of our knowledge this is the first report on cultivation of the fresh water alga *Chlorococcum* sp. under heterotrophic mode in sea water.

Chapter 6: Evaluation of mixotrophic culture for biomass and lipids production by *Chlorococcum* sp. R-AP13

6.1. Introduction

Depending on the type of alga and growth conditions, microalgae can accumulate various metabolites in their cells, such as proteins, carbohydrates, and lipids, which can serve as important raw materials for biofuel, food, feed or industrial applications. Furthermore, different culture conditions affect microalgal biomass productivity and lipid composition. The most commonly used mode of microalgal cultivation is the photoautotrophic mode. Autotrophically cultivated microalgae in the presence of light use the inorganic carbon source CO_2. Many algal species can grow in the presence of light by using an organic carbon source such as glucose, maltose, lactose, and glycerol (Cheirsilp and Torpee, 2012; Kong *et al,* 2013). Such nutritional condition is a combination of autotrophic and heterotrophic modes and is called the mixotrophic mode (Li *et al,* 2014). By cultivating under such conditions, microalgae can fix carbon dioxide as well as consume organic substrate added into the growth medium. In this case, the light energy does not play the role of limiting factor, and higher biomass concentrations can be achieved compared to autotrophic growth conditions (Zhang *et al,* 2011).

Low-cost feedstock production plays an important role in the commercialization of biofuel production from algae. Taking into account that the addition of organic carbon such as sugar alcohols, sugars, and organic acids in the growth medium of microalgae increases the cost of microalgae biomass cultivation, recently, great attention has been given to replacing them with cheaper carbon sources for algal cultivation (Skorupskaite *et al,* 2015). Therefore optimization of algal biomass production on low cost carbon source is important. Lipid accumulation in microalgae can be enhanced by changing cultivation conditions and optimizing the available nitrogen and carbon sources in the culture medium. Ammonia, urea and nitrate are often selected as the nitrogen (N) sources for the mass cultivation of microalgae (Hsieh and Wu, 2009; Lin and Lin, 2011; Kim *et al,* 2013). Nitrogen source is crucial to the cultivation of microalgae. Organic nitrogen may be found in numerous biological substances which include peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials (RNA, DNA) (Cai *et al*, 2013). The most appropriate nitrogen source and concentration must be identified for each microalgal species as this is a vital factor which affects growth and lipid regulation (Hsieh and Wu,

2009; Chia *et al*, 2013). Nitrogen is available in several different forms. The form of nitrogen that is supplied to the microalgae may affect the cellular composition of the algae, altering lipid content and fatty acid composition, as well as decreasing the growth rate and reducing culture stability (Borowitzka and Moheimani, 2013). All microalgae assimilate inorganic nitrogen in the forms of nitrate and ammonium (Cai *et al,* 2013). Selection of cheap nitrogen sources for cultivation of micro algae is an important parameter for biomass production.

This study is focused on optimization of the mixotrophic cultivation of *Chlorococcum sp.* R-AP13 for biomass and lipids production. Effect of different nitrogen sources, carbon sources, incubation time and inoculum concentration was evaluated to arrive at the optimal concentration/levels of these parameters to enhance biomass and lipid production. Elucidation of lipid profiles and FAME analysis of oil produced at varying concentration of carbon sources under mixotrophic cultivation was carried out for comparison of type of lipids produced

6.2 Materials and Methods

6.2.1 Medium and culture condition

The culture medium used for the study was MA medium with the composition as specified under section 2.2. The medium was supplemented with various concentrations of glucose and biodiesel industry waste glycerol (BDWG) ranging from 2-10% (w/v or v/v respectively for glucose and BDWG) as carbon source. Screening of nitrogen sources was done by replacing the nitrogen sources in the medium with Urea, KNO_3, $CaNO_2$, NH_4NO_3 or $(NH_4)_2SO_4$, at equivalent total concentration. Further evaluation of urea as a nitrogen source was performed at a concentration of 0.2-1g/l. Effect of incubation time for biomass and lipids production was tested for up to 10 days. Effect of inoculum concentration for biomass was tested from 10 to 50% (v/v) levels.

6.2.2 Mixotrophic cultivation

All mixotrophic cultivation studies were performed in 500 ml Erlenmeyer flasks containing 200ml medium supplemented with either glucose or BDWG as carbon sources. Sterilized medium in each flask was inoculated with 10% v/v of an inoculum containing 3×10^6 cells/ml

and were incubated in an environmental chamber equipped with fluorescent lamps (Illumination -3000 lux) at a temperature of 30 ± 2˚C for 10 days with a diurnal cycle of 13/11h light/darkness.

6.2.3. Biomass recovery and lipid extraction

For total lipid extraction, the algal cell grown under mixotrophic condition was harvested by centrifugation at 8000 rpm for 10min. The biomass was washed with distilled water and was lyophilized to remove water. Dry cell weight of the biomass was determined using a moisture analyzing balance. Total lipids were extracted from the dried biomass as detailed under section 2.7.2. The weight of lipids was determined after evaporation of solvent and was expressed as a percentage of the algal dry cell weight (% DCW).

6.2.4. Thin Layer Chromatography (TLC) analysis

TLC analysis was carried out to identify the lipid components present in the extracted oil from mixotrophic biomass. Samples were spotted on TLC aluminum sheets coated with silica gel $60F_{254}$ of 0.2 mm thickness (E Merck, India). The solvent system used for separation of sample was hexane: diethyl ether: acetic acid in the ratio of 80:20:1. Samples were developed in a glass chamber and the spots of lipids were visualized using iodine vapors.

6.2.5. Fatty acid profiling

Fatty acid profile of the mixotrophic algal oil was determined as fatty acid methyl esters (FAME). Trans-esterification reactions were performed with 2 % H_2SO_4 in dried methanol heated at 100˚C for 1h. FAME were extracted with hexane and dried over anhydrous Na_2SO_4. The samples were analyzed by gas chromatograph (6850N, Agilent Technologies) discussed in section 2.7.4. The FAME was identified by comparing their fragmentation pattern with internal standards (Sigma Aldrich, India).

6.3. Results and Discussion

6.3.1. Evaluation of nitrogen sources enhancing biomass and lipids production

Of all the tested nitrogen sources, urea and potassium nitrate produced considerable effect on the biomass and lipids yield. Maximum biomass production was 970 mg/l and lipid yield was 210 mg/l respectively with KNO_3 supplementation, while in the case of urea, biomass and lipid yields were 915 mg/l and 200 mg/l respectively. Calcium nitrate also showed positive effect on biomass and lipid yields under mixotrophic cultivation. Ammonium sulfate and ammonium nitrate did not show any significant enhancement of biomass or lipid production (Fig 6.1). Biomass and lipid yields in the case of urea and potassium nitrate supplementation were almost equal. Since urea is a cheap nitrogen source available also as a fertilizer, it was selected for further studies. Various concentrations of urea were studied for their effects on biomass and lipids production by the alga. Results showed that optimum concentration of urea for biomass and lipids production was 0.4 g/l. The maximum biomass and lipid yield obtained with this concentration was 1280 mg/l and 315 mg/l (24.5%) respectively. Further increasing the concentration of urea did not have any influence on the lipid production (Fig 6.2). Micro algal lipid production is enhanced in media with low nitrogen concentration.

Figure 6.1: Effect of various nitrogen sources for biomass and lipid production under mixotrophic condition

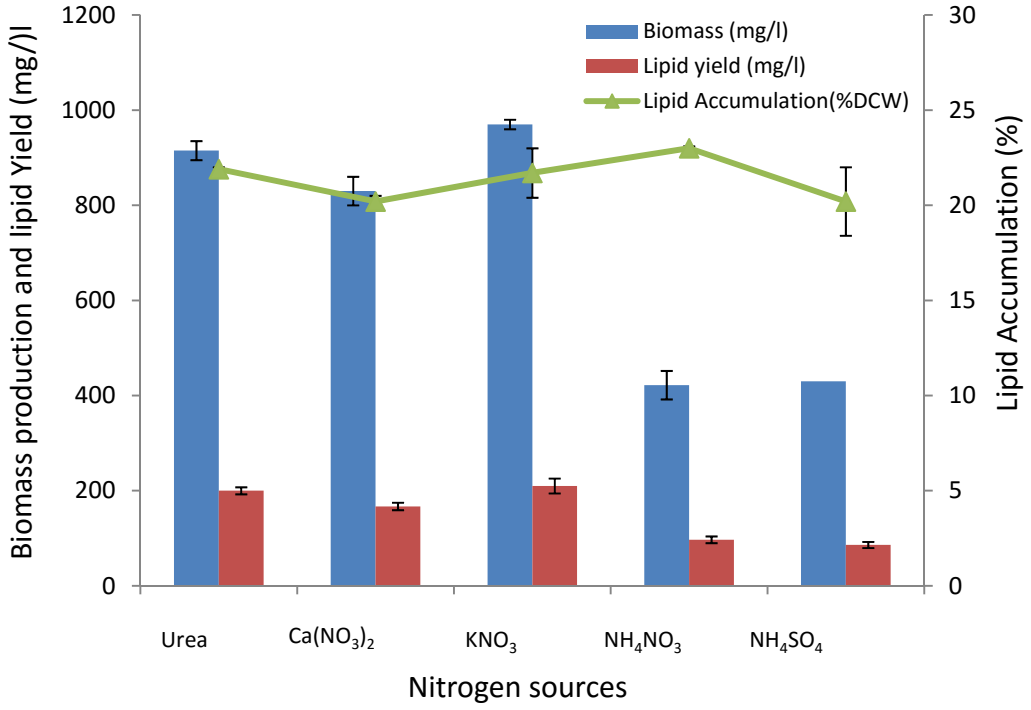
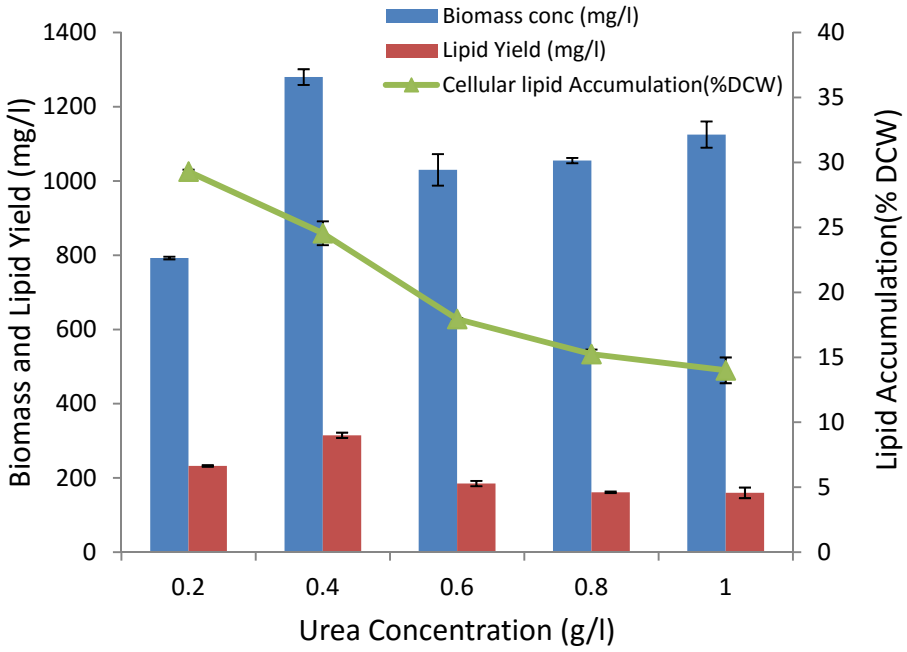


Figure 6.2: Optimization of urea concentration for biomass and lipid production by *Chlorococcum* sp R-AP13



Many studies have reported the role of various nitrogen sources in the growth of microalgae (Hsieh and Wu, 2009; Lin and Lin, 2011; Arumugam *et al,* 2012; Hulatt *et al,* 2012). Although ammonium sulfate and urea are used for mass cultivation of microalgae due to the relatively lower cost, urea and nitrate are more suited for the growth and lipid accumulation in microalgae (Lin and Lin, 2011). Urea dissociates to form CO_2 and Ammonium in solution via the urea amido hydrolase pathway (urease enzyme). This ammonium is directly absorbed into the cell and accumulates to form amino acids which are beneficial in the formation of chlorophylls which are essential in the photosynthetic process (Wijanarko, 2011; Kim *et al,* 2013). It was also found that biomass yields and lipid production increased with CO_2 addition. The additional CO_2 dissociated in solution may have a dual effect by enhancing micro algal growth as well as providing excess carbon flux towards lipid production. This could be the reason for urea proving to be the most effective nitrogen source resulting in the highest biomass concentration (Arumugam *et al,* 2013; Lizzul *et al,* 2014).

6.3.2. Effect of carbon sources on biomass and lipid production by *Chlorococcum* sp.

Effect of different types and concentrations of carbon sources on biomass and lipid production by *Chlorococcum* sp.R-AP13 was evaluated under mixotrophic condition. High concentrations of biomass and lipids were obtained when 6% (w/v) of glucose was used as carbon source. Maximum biomass and lipids production were 1720 mg/l and 710 mg/l (41%) respectively (Fig 6.3). Increase in the concentration of glucose has an inhibitory effect on biomass and lipids production by *Chlorococcum* sp.R-AP13. Higher concentrations of glucose were reported to be inhibitory for biomass production earlier by Azma *et al,* (2011). Various concentrations of Biodiesel industry waste glycerol (BDWG) as carbon source was tested for maximizing biomass production by *Chlorococcum* under mixotrophic conditions. The biomass production was maximal when medium was supplemented with 6% BDWG. Optimum biomass and lipids production was recorded as 845 mg/l and 272 mg/l (32%) respectively (Fig 6.4). Further increasing the concentration does not lead to any improvement in biomass and lipids production (Fig 6.4). Commonly used organic substrates for micro algal biomass production are glucose and acetate (Ren *et al,* 2013; Rai *et al,* 2013; Kumar *et al,* 2014). But the report on the cultivation of algal biomass production in BDWG is limited. BDWG is a cheap carbon source for algal cultivation, which also leads to the value addition of this waste/byproduct stream coming from biodiesel manufacturing. For every 10 tons of biodiesel produced, around 1 ton of impure glycerol with high sodium concentration is produced as the by-product. Microalgal species with better performance in waste glycerol are good candidates for fuel production. Results of the present study showed that *Chlorococcum* sp R-AP13 can utilize both glycerol and glucose as the substrate for growth and oil production. Production of biomass from glucose is relatively costly and therefore, a cheap substrate like waste glycerol is attractive and its use as feedstock for microalgal cultivation opens up future opportunities to develop algal biodiesel from industrial waste streams. Glycerol, a by-product from the biodiesel industry, can be utilized to produce oil which eventually goes back to the biodiesel production completing the full cycle with minimal wastes being generated in the process.

Figure 6.3: Optimization of glucose concentration for concentration for biomass and lipid production by *Chlorococcum* sp R-AP13

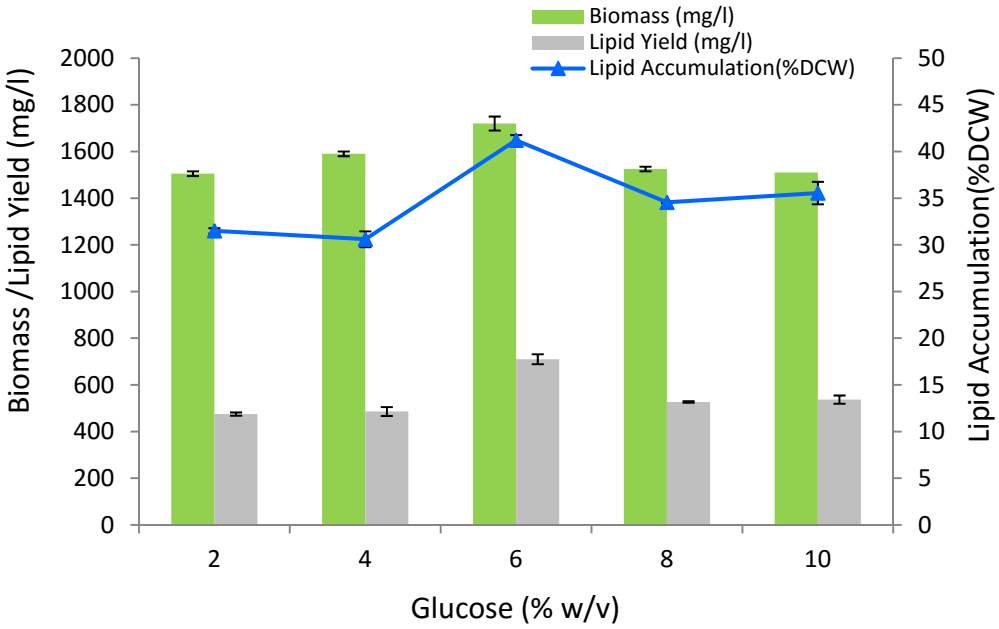
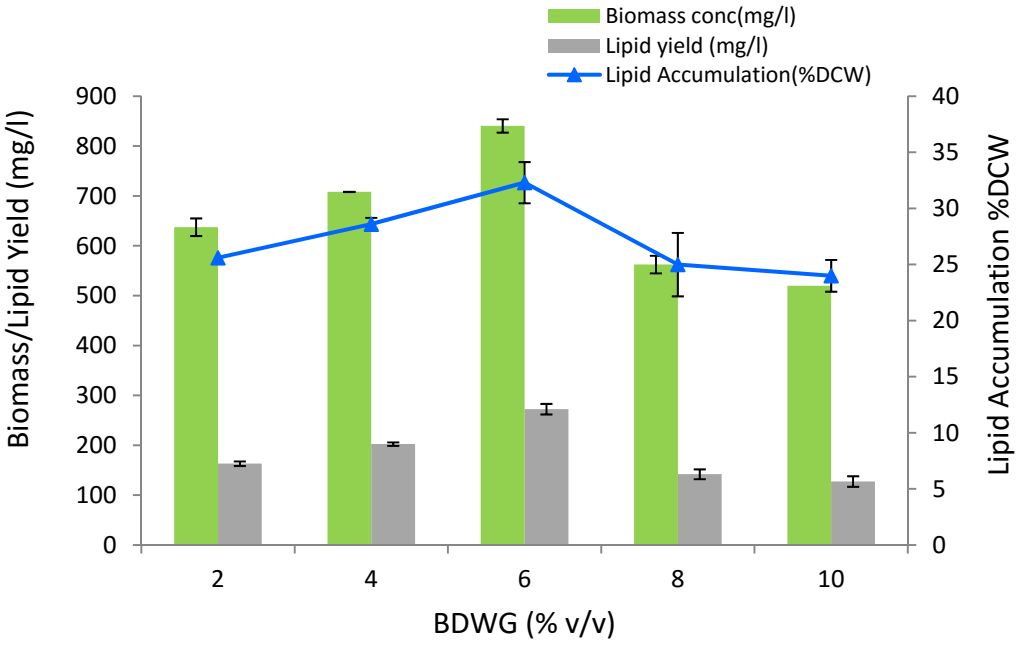


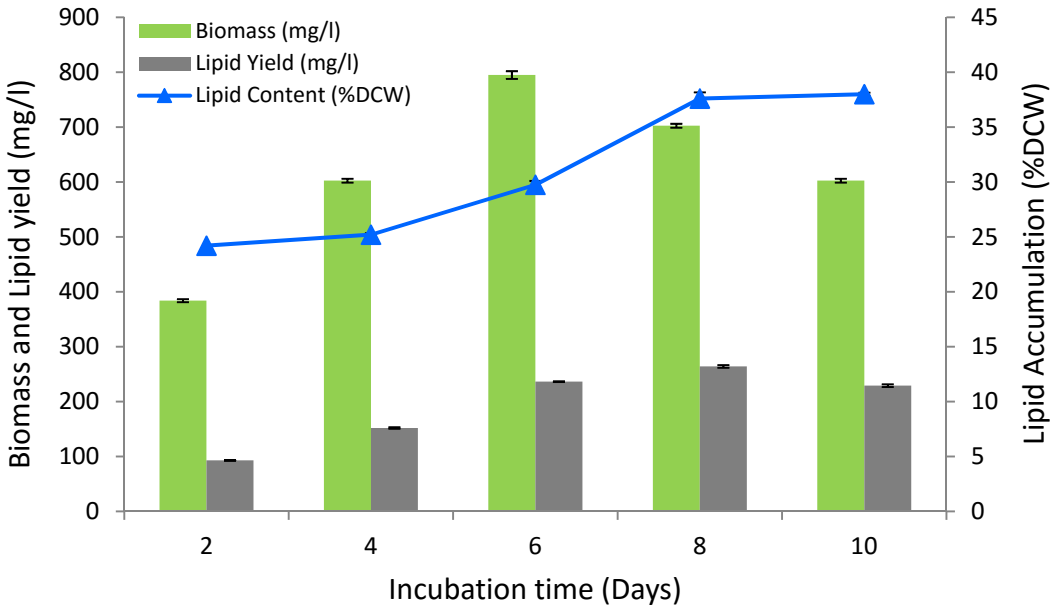
Figure 6.4: Optimization of BDWG concentration for concentration for biomass and lipid production by *Chlorococcum* sp R-AP13



6.3.3. Effect of incubation time on biomass and lipid production by *Chlorococcum* sp.

Effect of incubation time on biomass and lipids production by *Chlorococcum* sp. was evaluated under mixotrophic condition. Medium was supplemented with 6.0% BDWG as carbon source and inoculated with 10% v/v of an algal culture containing 3×10^6 cells/ml and were incubated for 10 days. Biomass and lipid production was recorded from second day onwards at 2 Day intervals. Maximum biomass was produced on the 6th day of incubation which was 795 mg/l whereas the maximum lipid yield was obtained on the 8th day (264 mg/l) (Fig 6.6). As the incubation time increased, biomass production decreased and the lipid production remained almost unaltered from 06th to 10th day of incubation, indicating that there is an enhanced intracellular lipid accumulation with increased incubation. The studies demonstrated that for obtaining algal oil for biofuel applications from *Chlorococcum* sp R-AP13, mixotrophic condition is the best, since high biomass and lipid production from the alga was obtained in a shorter period of time of about 10 days, while under phototrophic mode, the incubation time is usually much longer (~20-25 days)

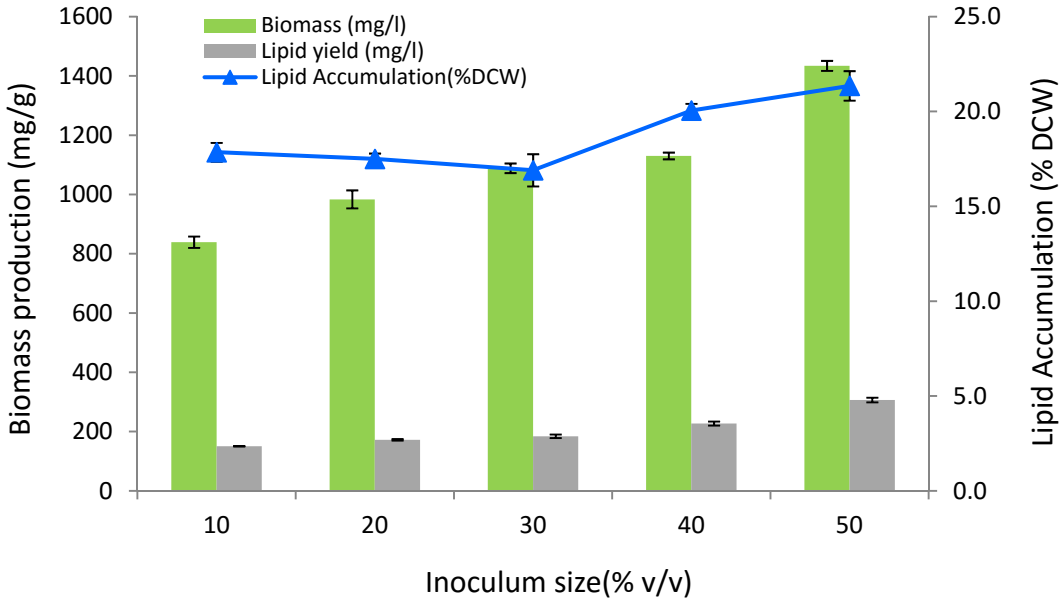
Figure 6.6: Effect of incubation time on biomass and lipid production by *Chlorococcum* sp under mixotrophic cultivation



6.3.4. Effect of inoculum size for biomass and lipids

Inoculum size is one of the important parameters for large scale culturing of microalgae and has great effect on the growth, lipid accumulation and metabolism of microalgae. One of the possible ways to improve the biomass production is the use of appropriate inoculum size, which is shown to be a critical factor influencing microalgal growth, especially for outdoor cultivation systems (Rodolfi *et al,* 2009). Therefore, the effect of inoculum size on the biomass and lipid production by *Chlorococcum* sp. R-AP13 was evaluated under mixotrophic cultivation with 6 % BDWG as carbon source. Inoculum size ranging from 10-50% (v/v) of an inoculum containing 3×10^6 cells/ml was used for the current study. Incubations were carried out as specified under section 6.2.2 for 10 days. Results indicated that biomass production and lipid yield improved as the inoculum size increased. Maximum biomass production was achieved when the medium was inoculated with 50% inoculum. Biomass production increased from 839 mg/l to 1439 mg/l and lipid yield from 150mg/l to 306 mg/l as the inoculum size increased from 10-50% (Fig 6.5). For large scale microalgal cultivations outdoor, 50% inoculum is typically used and the size of the inoculum is recommended to be large enough for the desired species of microalga to establish itself against the competing species (Demirbas and Demirbas, 2010). Moreover, large inoculum size also reduces the risk of contamination by other micro organisms in the open systems.

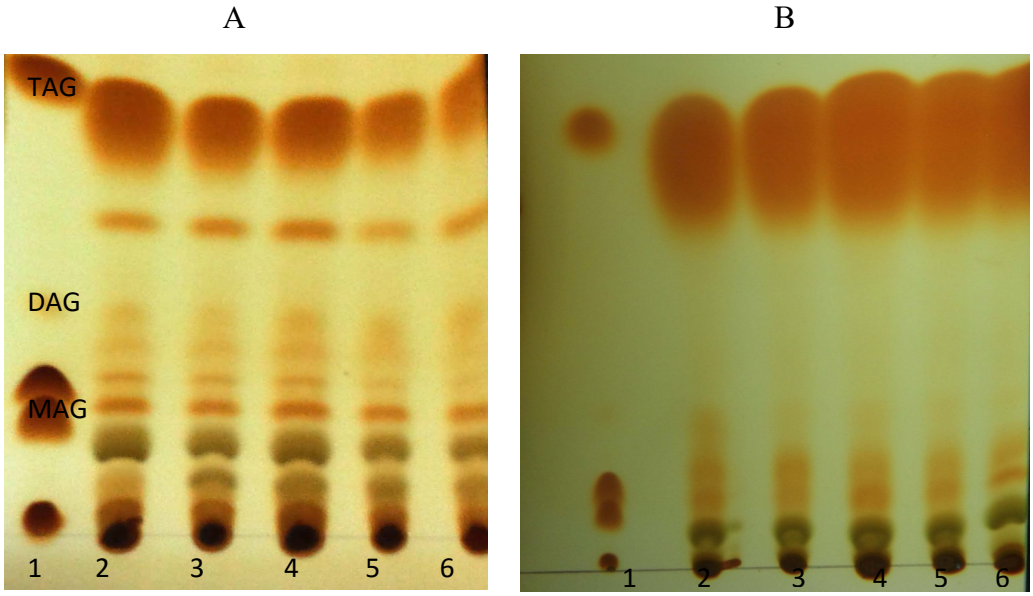
Figure 6.5: Effect of inoculum size on biomass and lipid production by *Chlorococcum* sp under mixotrophic cultivation



6.3.5. Lipids profiling by TLC and GC analysis

Type of lipids produced under mixotrophic cultivation was analyzed by TLC. Results showed that the algal cells under mixotrophic condition produced oil enriched in triglycerides. The Triacylglycerol (TAG) spot was more prominent in the oil from mixotrophically grown cells. This is advantageous, if the oil is to be considered for biodiesel applications since TAGs are the raw materials for trans-esterification reactions to produce biodiesel (Chisti, 2007). In order to profile the fatty acids in the oil from cells grown mixotrophically with glucose or waste glycerol as carbon source, trans-esterification was carried out and resultant FAMEs were analyzed by gas chromatography.

Figure 6.7: Lipid profiling of mixotrophically grown algal cells



TLC of oil produced by algal cells grown mixotrophically with A) different concentrations of waste glycerol B) different concentrations of glucose as carbon source. Lane information: 1 Lipid standards, 2: 2%, 3: 4%, 4: 6%, 5: 8%, 6: 10% of carbon source

Fatty acids profile from mixotrophically grown algal cells showed that the profiles of lipids varied depending upon the carbon source supplemented in the medium. The major fatty acids produced with glucose as C source were palmitic acid, oleic acid, linoleic acid and linolenic acid. Saturated fatty acid (71.5%) production was elevated in glucose medium. In the oil produced with waste glycerol as C source, the predominant fatty acids were palmitic acid, oleic acid, eicosapentaenoic acid (EPA), decosahexaenoic acid (DHA), and stearic acid.

Other long chain fatty acids were produced in low percentage (Table 6.1). Saturated and unsaturated fatty acids were produced in almost equal proportion by *Chlorococcum* sp. cultivated in waste glycerol as carbon source. Results showed that C16 and C18 were the major fatty acids produced under mixotrophic condition. In terms of the quality of biodiesel, oils containing C16 or C18 fatty acids are desirable and the results from this study indicated compliance with oil composition required for high quality biodiesel (Huang *et al,* 2010).

Table 6.1 Fatty acids profile of *Chlorococcum* sp R-AP13 grown on mixotrophic conditions

Fatty Acid Type	FAME Content (% of Total FAME)	
	Mixotrophic with Glucose	Mixotrophic with BDWG
C12	0.8	0.8
C14	0.4	-
C16	65.5	41.5
C16:1	-	1.1
C17	-	-
C18	4.8	3.6
C18:1	20.8	20.0
C18:2	6.2	2.5
C18:3	1.6	3.1
C20:2	-	1.1
C20:3	-	4.1
C20:5	-	3.9
C21:0	-	4.0
C22:0	-	1.6
C22:2		3.7
C22:6	-	4.2
C24:0	-	-
SFA	71.5	51.5
USFA	28.5	48.5

Oil produced using the waste glycerol supplemented medium shows DHA, EPA and precursor fatty acids of EPA and DHA. EPA and DHA are long chain omega 3-fatty acids (LCPUFA), which is conditionally essential for humans. Omega-3 LCPUFA reduce the risk of heart disease, and has a positive impact on the incidence of stroke, rheumatoid arthritis, kidney function, in the growth and development in infants/children, and are able to lower high contents of blood fats. In addition to these physical health benefits, omega-3 LC-PUFA may have benefits for neuropsychiatric disorders including depression and dementia

(Simopoulos, 2008; Ruxton *et al,* 2004). Therefore, the supplementation of algal oil rich in this fatty acid may help to serve the requirement of EPA and DHA. Because of its unique fatty acid profile, the oleaginous *Chlorococcum sp* R-AP13 may be considered a suitable nutritional supplement for humans and a feed additive for animals, besides being a starting material for biodiesel production.

6.4. Conclusion

This study demonstrated that urea as nitrogen source and glucose and waste glycerol as carbon sources are efficient for mixotrophic cultivation of *Chlorococcum* sp. R-AP13 using fresh water based MA medium. Urea at 0.4 g/l was found to be the optimal for biomass and lipids under mixotrophic condition. Biomass production was maximal when the medium was supplemented with 6% glucose compared to waste glycerol. However, production of algal biomass in cheap substrate like waste glycerol and relatively cheaper nitrogen sources like urea can certainly improve the techno-economics of large scale biodiesel production. Optimization of incubation time and inoculum size resulted in high biomass and lipid yields (1439 and 306 mg/l respectively) with BDWG as carbon source for mixotrophic cultivation. Interestingly it was also demonstrated that the fatty acid profile of the alga could be modulated by altering the carbon source used in mixotrophic cultivation and the culture produced high value essential fatty acids while growing in BDWG as carbon source. The results demonstrated the use of this culture for the production of oil suited for biodiesel as well as nutraceutical applications.

Chapter 7: Cultivation of *Chlorococcum* sp R-AP13 in dairy effluent (DE) for biomass and oil production

7.1. Introduction.

The major challenges in mass production of algal biomass are the costs of fertilizers, harvesting and the availability of a suitable source of water besides the limitations imposed by lighting especially when the algae are grown phototrophically (Danquah *et al,* 2009). Recent advances in methods of systems biology, genetic engineering, and bio-refinery approach present opportunities to expand algal lipid production from a craft to a major industrial process in near future (Wijffels and Barbosa, 2010).

Microalgal cultivation is either phototrophic, mixotrophic or heterotrophic depending on the algal strain's adaptation with their environment. Microalgae have immense potential for adapting with the fluctuating environmental conditions. While phototrophic cultivation of microalgae can be limited in terms of achieving high biomass yield and hence oil production, heterotrophic growth of microalgae eliminates the requirement for light and hence offers the possibility of increasing algal cell density and productivities. Therefore micro algae adapted to heterotrophic growth on cheap carbon substrates are of great significance in production of algal oil or other value added products.

An economic process for algal oil based fuel also depends on source of water, fertilizers and organic carbon source used for cultivation (Yang *et al,* 2006). As fresh water is projected to be a valuable commodity in the future, spending of this important resource for algal cultivation may be considered as a luxury, and the use of industrial effluents or sea water could be a viable alternative. Many effluents such as those from food processing industries are rich in nitrogen and phosphorus besides having high carbon content. Increase in population and industrialization in developing countries like India, generate large amount of effluent that must be treated before being discharged into natural water bodies. Dairy industry is ubiquitous all over the world, but their manufacturing processes vary tremendously. This sector generates huge volume of wastewater and its pollution is primarily organic (Briao and Tavares, 2007). Dairy industry generates about 0.2–10 litres of effluent per litre of milk processed (Vourch *et al,* 2008). In general, the liquid waste stream from dairy industry has high organic content with high levels of protein, nitrogen, phosphorous, dissolved sugars and nutrients. Organic waste present in the dairy effluent is a serious environmental threat due to the high COD and BOD and problems associated with rapid putrefaction. There are a large number of studies on treatment of industrial, municipal and agricultural waste waters by

microalgal culture systems (Zhang *et al,* 2012; Ji *et al,* 2013; Samori *et al,* 2013; Zhu *et al,* 2013). Micro algae can grow on effluent and produce valuable biomass while they remove organic content and minerals for building the biomass. Cultivation of micro algae on dairy effluent offers several advantages such as 1) biomass production utilizing the organic carbon, nitrogen and minerals without need for any additional nutrients 2) reduction of COD and BOD of the effluent 3) oxygenation of the treated effluent and 4) possibility to extract high value products like, lipids, proteins and carbohydrates for fuel, pharmaceutical/nutraceutical and chemical industries. Integrated strategies to enhance the cost effectiveness and environmental sustainability of algal cultivation involves combining the benefits of biofuel production, CO_2 mitigation, wastewater treatment.

The present study was undertaken to evaluate the use of untreated dairy effluent as a source of nutrition for the intensive microalgal growth. The fresh water microalga *Chlorococcum sp.* R-AP13 was used for this investigation to evaluate biomass and lipid accumulation on dairy effluent. The culture was studied for growth and lipids production under mixotrophic and heterotrophic conditions, the latter with supplementation of biodiesel industry waste glycerol (BWG). The suitability of the extracted oil for biofuel production was assessed by measuring type and relative proportions of fatty acids by GC analysis. Removal of organic pollution load was monitored as reduction in of BOD and COD of effluent.

7.2. Materials and Methods

7.2.1. Sample collection

Dairy effluent for this study was obtained from milk processing factory of Kerala co-operative milk marketing federation (MILMA) at Ambalathura, Trivandrum India. Fresh Dairy effluent before it is transferred to the effluent treatment plant was collected in sterilized containers and was brought to the laboratory. The dairy effluent (DE) was stored at 4˚C until used.

7.2.2 Organism and growth condition

*Chlorococcum sp.*R-AP13 was used for the study. Stock culture was inoculated into dairy effluent and was incubated for 15 days with an approximately 13h light and 11h dark cycle at 30 ±2˚C and with 100 rpm agitation. Microalgal culture so adapted to DE was used for the

mixotrophic and heterotrophic cultivation experiments. Mixotrophic cultivation experiments were performed in 500ml Erlenmeyer flasks, each containing 200ml of untreated sterilized DE. Flask was inoculated with 10% (v/v) of an inoculum containing 3×10^6 cells /ml produced as above and was incubated under static conditions in an environmental chamber with temperature and diurnal light cycle as above. For heterotrophic cultivation, the dairy effluent was supplemented with 2, 4 or 6% (v/v) of BDWG. Sterilized medium (200ml) taken in 500ml flasks were inoculated at 10 % v/v level with the same inoculum as above and the cultivation was carried out as for heterotrophic growth studies but without light . All experiments were carried out in triplicates.

7.2.3. Cell growth, morphology and lipid accumulation

Growth of algal cells in DE under both mixotrophic and heterotrophic cultivation was monitored as change in optical density at 680nm compared to an un-inoculated DE sample. Morphological changes of *Chlorococcum sp.*R-AP13 grown on dairy effluent was observed under phase contrast microscope and scanning electron microscopic analyses was done according to method described by Dayananda et al (2010). Protocol for sample processing was as briefed in section 2.6. Completely dried cells were sputter coated with gold and was examined in a Scanning Electron Microscope (Zeiss Evo 17 SE, Germany). Lipid accumulation inside the cells grown in DE was monitored by Nile Red assay (section 2.7.1). Fluorescence intensity of neutral lipid was measured in a multimode reader (Tecan Infinite M200 pro, Switzerland) at a wave length of 575nm.

7.2.4. Physicochemical analysis of dairy effluent

The physicochemical parameters of DE used for algal cultivation were analyzed every 3 days starting from the third day of inoculation for 12 days to monitor the reduction in organic pollution load. The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) were analyzed according to the standard method described in APHA (1998). Changes in pH during algal growth were monitored by using a pH meter. The reduction in organic pollution load was expressed as percentage of the values for control (un-inoculated fresh DE).

7.2.5. Biomass and lipid extraction

Micro algal biomass from both mixotrophic and heterotrophic cultures was harvested by centrifugation at 8000 rpm for 10 min on the 12^{th} and 15^{th} day of incubation. Biomass was washed with distilled water to remove the salt and was lyophilized to remove water. Weights of the lyophilized cells were taken and were expressed as mg/l. Total lipids were extracted from the dried biomass by solvent extraction method as described section 2.7.2. Lipid yield was expressed as percentage of the dry cell weight of algal biomass (% DCW).

7.2.6. Thin Layer Chromatography (TLC) Analysis

TLC analysis was carried out to identify the lipid components in the extracted oil. TLC aluminum sheets coated with 0.2mm thickness silica gel $60F_{254}$ (E Merck, India) were spotted with 5ul of each lipid sample. Samples were separated using a solvent system with hexane, diethyl ether and acetic acid in the ratio of 80:20:1. Lipids were developed in a glass chamber and the spots were visualized using iodine vapors.

7.2.7. Fatty acids Analysis

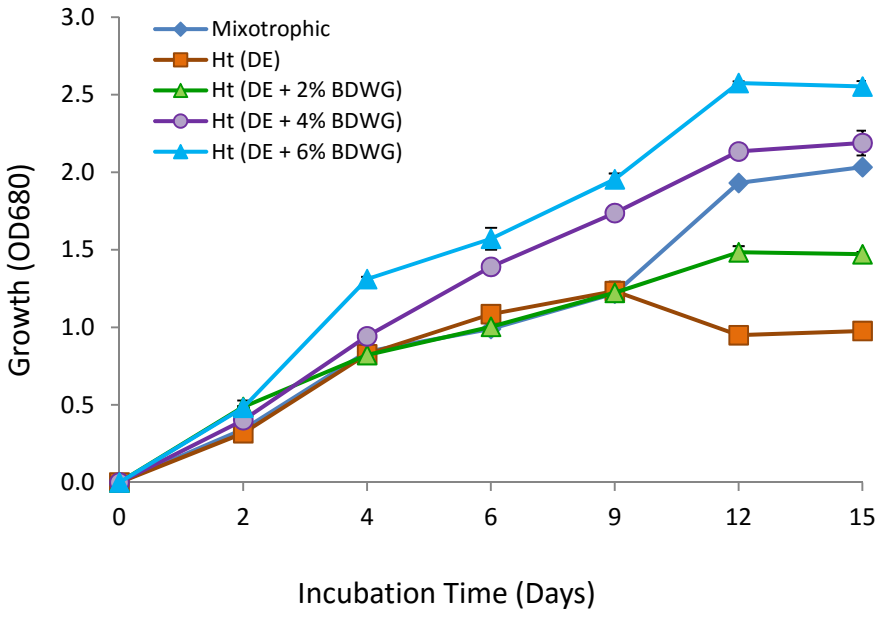
Fatty acid profile of the algal oil was determined as fatty acid methyl esters (FAME). Trans-esterification reactions were performed with 2 % HCL in dried methanol at 100˚C for 1 h. FAME were extracted with hexane and dried over anhydrous Na_2SO_4. The samples were analyzed in a gas chromatograph according to the method mentioned in section 2.7.4. (The FAMEs were identified by comparing their fragmentation pattern with internal standards (Sigma Aldrich, India).

7.3. Results and discussion

7.3.1 Algal growth in dairy effluent

The diary effluent had an off white colour with offensive odour during collection, and was highly alkaline with a pH of 10.6. The effluent was used for algal cultivation without any conditioning or pH adjustments. *Chlorococcum sp*-R-AP13 could adapt to the effluent and produce biomass under both mixotrophic and heterotrophic cultivation in this medium. Growth was higher under mixotrophic mode with a 7 fold increase in biomass in 12 days while the increase in biomass for same duration was only 3 fold in the case of heterotrophically grown cells (Fig 7.1). Supplementation of BDWG in dairy effluent as additional carbon source improved the algal growth under heterotrophic cultivation. Maximum growth for heterotrophic cultivation was obtained with 6% supplementation of BDWG in the DE (6.25 fold increase in 12 days). Dairy effluent contained lesser amounts of sugars (0.02 g/L) compared to the normal levels used for heterotrophic cultivation of microalgae, even though the effluent was used without any dilution. Additional carbon source in the form of glycerol would have provided a relatively easy to assimilate energy source and hence the biomass would have enhanced on supplementation of it.

Figure 7.1: Growth of *Chlorococcum* sp R-AP13 in dairy effluent



DE- Dairy effluent, Ht-Heterotrophic, BDWG – Biodiesel industry waste glycerol

Microscopic observation of the heterotrophically grown algal cells showed differences in colour and morphology. The cells were brownish in comparison with the mixotrophically grown cells which were green (Fig 7.2). Changes in pigment production in heterotrophic cultures of green algae are reported by many. It has been reported that under heterotrophic conditions, reduction in chlorophyll and carotenoids and a decrease in chlorophyll a/b ratio is part of the dark adaptation (Young, 1993). Phase contrast images and scanning electron micrograph showed that algal cells are aggregated when growing in DE (Fig 2B &C). Aggregate formation is an important quality of micro algae that helps in the self sedimentation of microalgae, which may lead to a lower cost of harvesting the cells. These results showed that cultivation of the alga in heterotrophic mode improved the cell density in comparison to the mixotrophic condition and there were visible differences in the cell properties. Similar results were obtained for dairy waste water treatment by the micro algae *Botryococcus* (Shen *et al*, 2008) and *Chlamydomonas polypyrenoideum* (Kothari *et al,* 2013).

Figure 7. 2: Morphological changes of *Chlorococcum* sp RAP13 cultivated under mixotrophic and heterotrophic modes in dairy effluent.

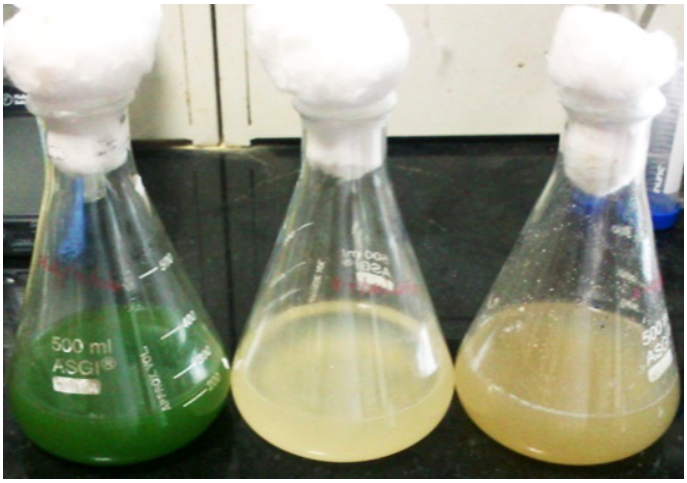
Phase contrast images of A) mixotrophically grown algal cells B) Heterotrophically grown algal cells and C) SEM image of algal cells growing in dairy effluent (Mixotrophic)

7.3.2 Biomass and lipid production

Biomass and lipid production by *Chlorococcum sp* R-AP13 was evaluated for both mixotrophic and heterotrophic cultivation. The alga grew better and accumulated more lipid under heterotrophic cultivation in DE with glycerol supplementation compared to mixotrophic cultivation. Under mixotrophic growth, the algal cells were lemon green in color

which gradually turned to dark green by the 2nd week of cultivation (Fig 7.3). Heterotrophic cells on the other hand were brownish. While the biomass yield was 0.8 g/L for mixotrophic cultivation, it increased to 1.478 g/L and 1.94 g/L in heterotrophic cultivation with 4 % or 6 % BDWG supplementation respectively (Fig 7.4). Heterotrophic growth on raw DE resulted in the lowest biomass yield 0.58 g/L which was lower than even the mixotrophic growth. It may be speculated that the available carbon in raw DE may not be sufficient to support enhanced growth under heterotrophic cultivation of the alga and supplementation of additional carbon source improves the biomass yield significantly. For the economic production of algal biomass, heterotrophic growth on a cheap carbon substrate is often suggested as an alternative to conventional low density phototrophic race way cultivation (Doucha & Livansky, 2012). The cost of carbon source represents 50% of the cost of medium in algal cultivation (Cheng *et al,* 2009) and effluents like those from dairy industry can provide a potent feedstock for cultivation of algae. Similarly, BDWG is again a waste product from biodiesel industry, which may be used in algal cultivation without incurring high cost for the carbon supplementation

Figure 7.3: Cultivation of *Chlorococcum* sp R-AP13 in Dairy Effluent

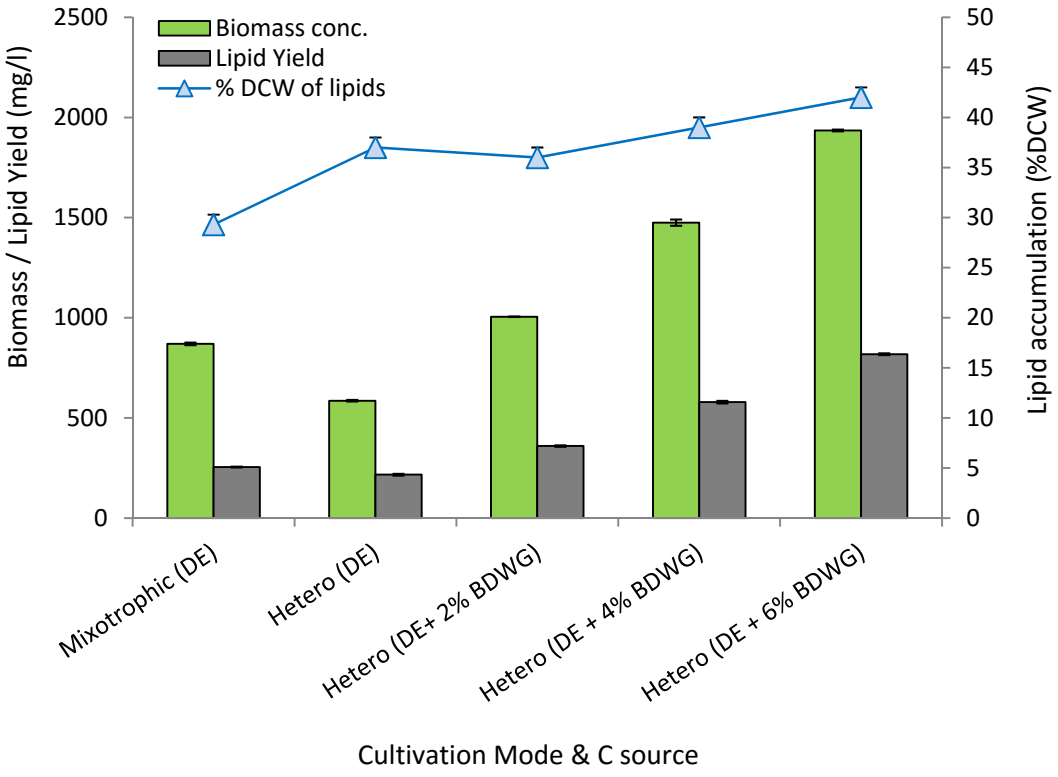


Flasks showing algal cultures in DE under (from left to right) mixotrophic, heterotrohic in DE and heterotrophic in DE supplemented with 6% BDWG

Lipid accumulation in the cells was enhanced with the increase in supplementation of BDWG in DE medium. While the cells accumulated 31 % of their dry cell weight as lipid in mixotrophic cultivation, the lipid content improved to 36, 39 and 42 % for cells cultivated heterotrophically with 2, 4 or 6 % BDWG respectively (Fig 7.4). The lipid yields from heterotrophic cultivations were 0.36, 0.58 and 0.8g/l for the 2, 4 and 6% glycerol

supplementation respectively. Previous work on *Chlorococcum sp*. grown in municipal effluent had reported 30% lipid accumulation in the cell (Mahapatra and Ramachandra, 2013). Similarly, studies on green algae grown in dairy and municipal wastewaters had yielded a maximum lipid content of 29% (Woertz *et al,* 2009). Recently, the micro alga *Chlorococcum polypyrenoideum* was shown to accumulate 42 % of its dry cell weight as lipid when grown on dairy effluent (Kothari *et al,* 2013). Oleaginous green algae typically have an average total lipid content of 25 % DCW in normal phototrophic mode, which can be raised to 55 % DCW when they are grown under stress conditions or heterotrophically (Xu *et al,* 2006; Hu *et al*, 2009).

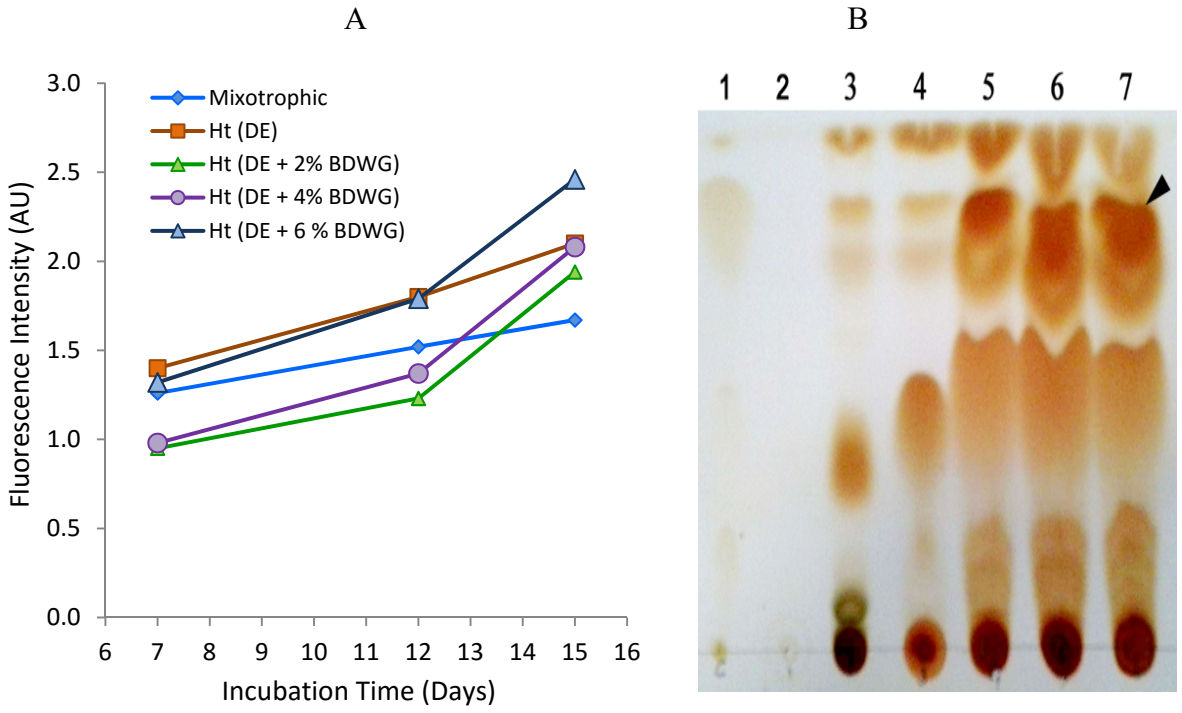
Figure 7.4: Biomass and lipid production by *Chlorococcum* sp-R-AP13 in DE



The intracellular neutral lipid accumulation of algal cells was measured by the 'Nile Red' fluorescence. Staining was performed on cells starting from the 7th day of incubation. The fluorescence intensity of cells increased in proportion to the day of harvesting, indicating that the age of cells may be a critical factor in lipid accumulation with older cells accumulating more lipids (Fig 7.5). In the case of heterotrophically grown cells, lipid accumulation increased both with age and the amount of carbon supplied. Maximum accumulation of lipid was observed for 6% BDWG supplemented medium on the 15th day of incubation. TLC

analysis of the extracted lipid indicated that neutral lipids form the major fraction (Fig 7.5). The Triacylglycerides (TAG) spot was more prominent in the oil from heterotrophic cells. This is advantageous if the oil is to be considered for biodiesel applications since TAGs are the raw materials for trans-esterification reactions to produce biodiesel (Chisti, 2007). The cultivation of *Chlorococcum* sp R-AP13 in dairy effluent with crude glycerol supplementation may therefore be considered to provide the added advantage of value addition of the effluent and waste glycerol.

Figure 7.5: Cellular lipid accumulation under various growth conditions and TLC profiling of the extracted algal oil.



A) Accumulation of neutral lipids by the alga under various modes of growth monitored as Nile red fluorescence. B) TLC profile of the oil produced by alga under different modes of growth. Lane information- 1: control oil (tripalmitate), 2: DE –negative control, 3: oil from mixotrophic, 4: heterotrophic in DE, 5: heterotrophic in DE with 2% BDWG, 6: heterotrophic in DE with 4% BDWG, 7: heterotrophic in DE with 6% BDWG.

7.3.3. Fatty acid profiling

GC analysis of FAMEs from the algal oil showed that saturated fatty acid content was high in the oil regardless of the growth mode. Major fatty acid from *Chlorococcum sp.*R-AP13 oil produced in DE was palmitic acid (44-53%) followed by oleic acid (21-27%) and stearic acid

(2-16%). Linoliec acid (C18:2), linolenic acid (C18:3) and other fatty acids were low in the oil (Table 7.1). Saturated fatty acids were 61-69% depending on the conditions for growth, whereas unsaturated fatty acid content was 28-33%. Stearic acid content increased from 3.6% in the oil from mixotrophic cells to 16.9 % for the oil from heterotrophically grown cells. There are numerous studies targeting to maximize growth of microalgae under cultivation, and to enhance the level of oil or other value added products. However, the reliability of the algal oil for biodiesel applications depends not only on the quantity of oil produced, but also on the type and structure of the fatty acids present in the oil. Hence the lipid content and fatty acid profile are key factors to consider when selecting algae for fuel production. FAMEs of saturated fats tend to have poor low-temperature operation, while biodiesel containing high levels of polyunsaturated fatty acids (PUFAs) have a shorter shelf life due to their tendency to undergo oxidation (Chen *et al,* 2012). Fatty acids indicated for good biodiesel properties include C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2 (Schenk *et al,* 2008). When the requirements for biodiesel quality considered, any single fatty acid methyl ester (FAME) cannot satisfy every requirement of biodiesel. A mixture of different fatty acids containing higher amounts of mono-unsaturated fatty acids such as oleate ($18:1\Delta^9$), and fewer saturated and polyunsaturated fatty acids is considered to be better for reliable biodiesel (Durrett *et al*, 2008). Based on European Biodiesel Standards EN14214 for vehicle use, content of methyl linolenate (18:3) and the poly unsaturated fatty acids containing greater than four double bonds are should be limited to a maximum of 12 and 1% respectively (Knothe , 2005).

Previous studies have indicated that, by alternating the environmental conditions that the microalgae is exposed during growth, it is possible to change the biosynthesis of fatty acids significantly (Los and Murata, 2004).The change in fatty acid composition of the algal oil on cultivation in effluent may have a protective role, helping the alga to adapt to the change in environmental conditions. *Chlorococcum sp* has the potential to change its fatty acid profile depending up on the cultivation mode and change in the medium. Therefore, tuning of the culture conditions, especially the medium can be considered as a strategy for obtaining desired fatty acid profile for the specific end application-either for biofuel or other industries like nutraceuticals. Algal oils are a rich source of omega 3 and omega 6 fatty acids which are essential fatty acids and have therapeutic and nutritional benefits in humans. Human body can synthesize long chain PUFAs (EPA, DHA) from C18 fatty acid precursors like stearic acid, oleic acid and linolenic acid (Pereira *et al*, 2004). *Chlorococcum sp*.R-AP13 grew on dairy effluent and produced more high levels of 18:1 fatty acids (20-27 %), 18:2

fatty acids (10%), and 18:3 fatty acids (4.7%) which make this microalgal oil a good source of good fats for both human and animal consumption.

Table 7.1: Fatty acid profile of the lipids produced by *Chlorococcum* sp RAP13 cultivated mixotrophically or heterotrophically in dairy effluent

Fatty Acid Type	Relative proportions (%)		
	Mixotrophic DE	Heterotrophic DE	Heterotrophic DE with 6 % BDWG
C12	6.91	9.00	5.90
C14	2.40	3.96	1.82
C16	53.96	46.25	44.30
C16:1	-	1.59	1.23
C16:2	-	-	1.56
C18	2.35	2.30	16.90
C18:1	27.00	22.50	20.90
C18:2	3.10	10.80	-
C18:3	-	-	4.70
SFA	65.61	61.51	68.92
USFA	30.00	33.30	28.39

SFA – saturated Fatty acids, USFA – unsaturated fatty acids

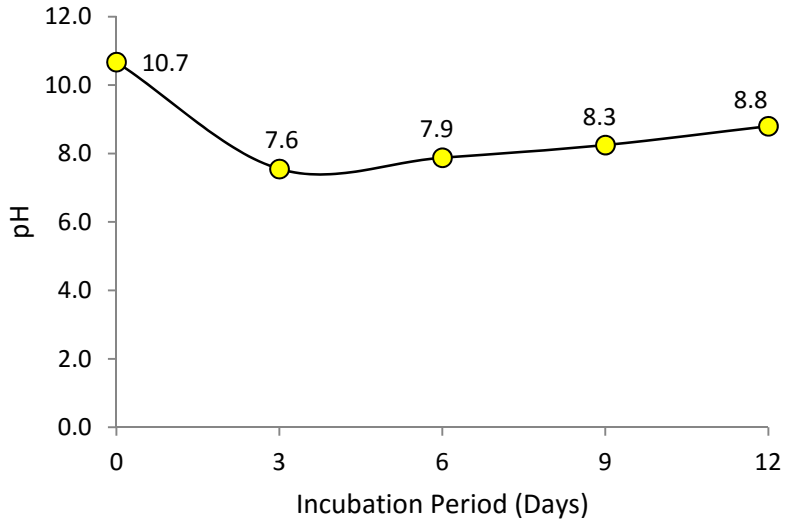
For any feasible technology for micro algal oil production, achieving high levels of oil and biomass is critical. A suitable algal strain should have high lipid productivity either by having high basal productivities or the ability to accumulate significant amounts of lipid in response to induction conditions. Other desirable features include the easiness of harvesting and oil extraction, adaptability to the cultivation conditions and resistance to other organisms which might invade their culture systems. Open phototrophic cultivation systems also requires that the culture has the machinery to efficiently harvest and convert solar radiation to oil and is highly adaptable to prevailing climatic conditions, at the same time achieving high biomass levels in culture. Considering the above, it is natural that a locally isolated microalgal strain would adapt better to the culture system and provide a better biomass and oil yield (Rodolfi *et*

al, 2009). *Chlorococcum* sp. R-AP13 cultivation in DE may be considered as effective in producing biomass and oil useful for both biodiesel or nutraceutical applications and the culture was highly adaptable to growth in the effluent without any conditioning of the medium.

7.3.4. Reduction in pollution load of dairy effluent

Changes in organic pollution load were monitored as COD, BOD and pH during the experiment to evaluate the effect on algal cultivation on effluent treatment process. pH is one of the physical parameters used to assess the quality of water. Initial pH of the DE was 10.6, which gradually reduced to the range 7.5 -8.8 after algal growth on effluent (Fig.7.6). According to environment protection and pollution control board standards for industrial effluent discharged into water bodies, pH should be in the range of 5.5 - 9.0. Several factors influence the pH of the medium such as algal growth (pH increase as a result of CO_2 uptake), and the excretion of acidic or basic metabolites from organic matter biodegradation (Gonzalez *et al*, 2008).

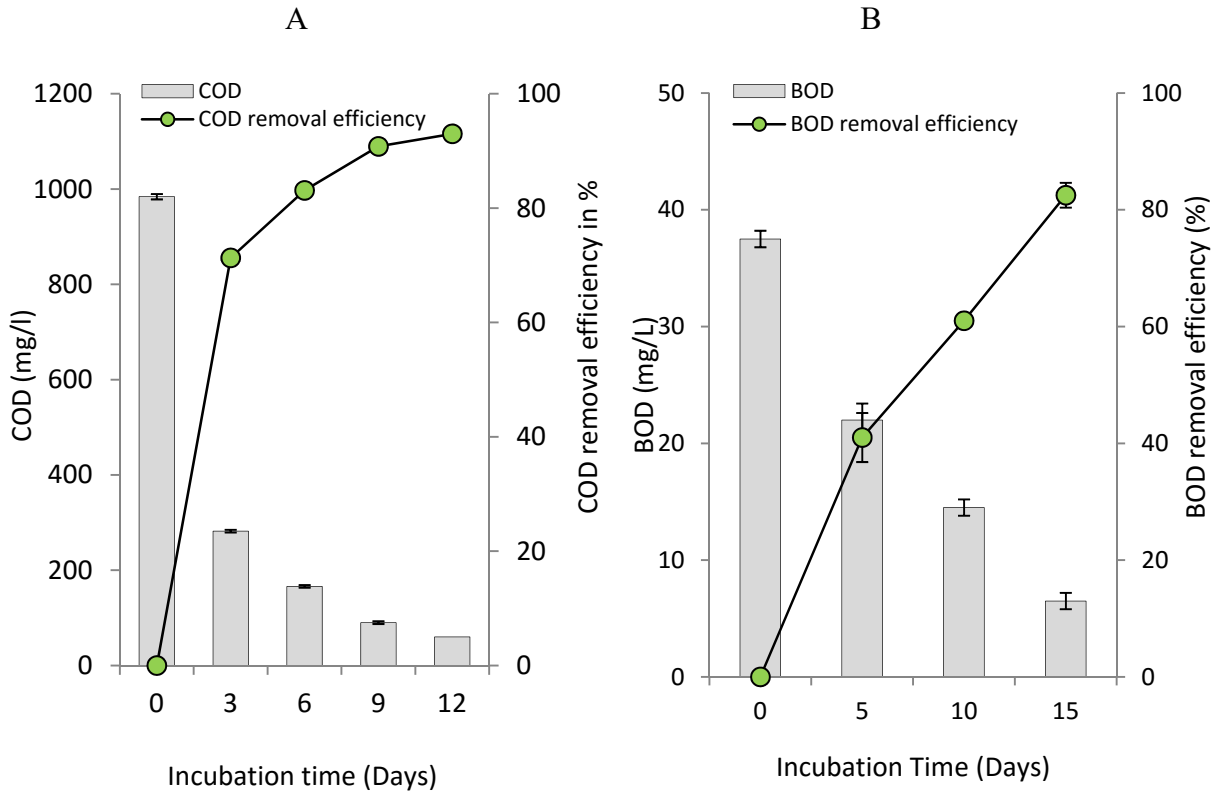
Figure 7.6: Changes in pH of the DE during mixotrophic cultivation of *Chlorococcum* sp R-AP 13



Primary parameters for monitoring effluent quality are COD and BOD. COD gives the total pollution load in the form both organic and inorganic matter, whereas BOD gives an estimate for biologically degradable matter in the effluent. Initial COD and BOD of the DE were very high and beyond the permissible limits. Permissible limit of BOD and COD for municipal and industrial effluent discharge is 30mg/l and 250 mg/l respectively, according to the state

pollution control board standards. COD of the dairy effluent decreased from 984mg/l to 60mg/l after 12 days of algal culture in it. COD reduction was rapid during the initial period with 73% of COD removed on 3rd day while 93% of reduction was observed after 12 days (Fig 7.7A). The slow reduction in COD for latter period could be attributed to presence of remaining carbon as some colloidal form or as slowly biodegradable material.

Figure 7: Removal of the organic pollution load of dairy effluent by mixotrophic cultivation of *Chlorococcum* sp RAP13.



A) COD reduction B) BOD reduction, during mixotrophic cultivation of the alga in DE.

BOD is an indicator of biologically degradable content. From the initial value of 37mg/l, the BOD was reduced gradually during the algal growth in the effluent and reached 7mg/l after 15 days of incubation (82% reduction) (Fig 7.7 B). Similar COD and BOD removal efficiencies of 88 and 89.60% respectively from industrial waste water using *Chlorella vulgaris* was reported by Azeez (2010). Apart from reducing the COD and BOD of DE significantly, *Chlorococcum* sp R-AP13 could also remove the offensive smell of the effluent rapidly. Biomass production in the alga relies on rapid utilization of the organic content in the effluent and can be considered as an attractive, efficient and eco friendly means for

treating this waste water, since in-addition to removing the pollution load, algal cultivation adds value to the process by generating commercially valuable products- the algal biomass and oil.

Existing technologies used for algal cultivation to generate oil for biofuel application are largely limited in viability due to the many challenges associated with attaining high biomass and hence oil yields. Major bottlenecks include the source of water, fertilizers used for the algal cultivation and costs associated with harvesting. However, dual use of microalgal cultivation for wastewater treatment and production of value added compounds/biofuel is an attractive option, in terms of reducing the energy cost, and the nutrient and freshwater resource costs. The high biomass productivity of *Chlorococcum sp*. R-AP13 on dairy effluent suggests that this cultivation method offer real potential as a viable means for algal biomass generation along with phycoremediation and value addition of this waste stream. The culture produced high amount of biomass (~2.0g/l) and oil (42%) while cultivated heterotrophically with waste glycerol supplementation, providing an added advantage of value addition of this waste/byproduct from biodiesel industry by converting it back to usable oil for fuel applications in a cost effective green process.

7. 4. Conclusions

Chlorococcum sp RAP13 cultivated on dairy effluent produced high amount of biomass and accumulated significant amount of lipids, especially under heterotrophic growth with supplementation of biodiesel industry waste glycerol. The cells displayed different growth characteristics, lipid content and fatty acid profiles based on differences in the growth conditions and carbon substrate. Biomass yields increased from 0.8g/L to ~ 2g/L and lipid yield enhanced from 30 to 42% when shifted from mixotrophic to heterotrophic mode in DE with waste glycerol supplementation. The lipid profile of the oil produced from algal biomass grown in DE indicated interesting features like high triglycerides content, a fatty acid profile with high proportion of medium to long chain fatty acids and a high content of the essential fatty acid linolenic acid. These features project the alga as a potent source of oil which can be used as feedstock for biodiesel and also as a food or feed supplement. Moreover, algal cultivation in the dairy effluent could reduce the effluent's pollution load significantly indicating potential for its use as an effective effluent treatment program with value addition of the waste stream.

Chapter 8: Mixotrophic cultivation of *Chlorococcum* sp R-AP13 in Acid pretreatment liquor (APL) from lignocellulose biorefinery

8.1. Introduction

Oleaginous microalgae have been in focus for wastewater treatment, carbon dioxide (CO_2) mitigation, or as a feedstock for biofuel production (Brennan and Owende, 2010; Abreu *et al,* 2012; Ramaraj *et al,* 2014; Cuellar-Bermudez *et al,* 2015). The phototrophic microalgae can be cultivated either in open ponds or closed photo bioreactors (PBR) using CO_2 and light as carbon and energy sources, respectively (Chen *et al,* 2011). However, photoautotrophic culture mode presents several disadvantages including low cell densities and long duration of cultivation. Hence, heterotrophic and mixotrophic growth regimes have been proposed as feasible alternatives for the production of micro algal biomass (Yu *et al,* 2009). Heterotrophic cultivation of algae involves the assimilation of organic carbon compound as sole carbon and energy source and major disadvantages of this system are frequent contamination with other micro organisms, limited number of microalgal species that can grow heterotrophically, energy expenses and costs involved for organic substrate, inhibition by an excess of organic carbon substrate, and inability to produce light-induced metabolites (Chen, 1996; Perez-Garcia *et al,* 2011). The mixotrophic system on the contrary, use inorganic CO_2 and organic compound simultaneously as a carbon source for biomass production (Sabeela and Sulumaran, 2014; Venkata Mohan *et al,* 2015). Hence, microalgae cultivated under mixotrophic conditions synthesize compounds characteristic of both phototrophic and heterotrophic metabolisms at high production rates. Additionally, lower energy costs have been associated with mixotrophic cultivation in comparison with photoautotrophic cultures, due to its relatively lower requirements for light intensities.

Although mixotrophic cultivation of microalgae provides higher biomass density and lipid production than cultivation under photoautotrophic conditions, the cost of the organic carbon substrate is estimated to be about 80% of the total cost of the cultivation medium (Li *et al,* 2007). As a result, less costly organic sources have to be found in order to overcome the high carbon cost (Liang *et al,* 2009). In this context, crude glycerol from biodiesel production, acetate from anaerobic digestion, and carbohydrates from agricultural and industrial wastes offer great promise as inexpensive organic substrates for the cultivation of microalgae on mixotrophic cultivation of micro algae (Bhatnagar *et al,* 2011; Heredia-Arroyo *et al,* 2011; Leitr *et al,* 2015).

Acid pretreatment liquor (APL) is one of the waste streams that are generated in the lignocellulosic biomass processing plants for bioethanol production. Processing of lignocellulosic biomass, especially the unit operation of pretreatment, directly generate huge quantities of waste water/byproduct streams which are rich in lignocellulose breakdown products including cellulose and hemicellulose derived sugars like glucose, xylose, arabinose, their derivatives like furfural, hydroxy methyl furfural (HMF), and organic acids etc. Biorefineries utilizing acid pre-treatment (as is the case with most of the current pilot scale and commercial facilities) will be generating the acid pre-treatment liquor (APL) containing a minimum of about 1.5-2.0% sugars besides furfural, HMF, organic acids and a fair amount of phenolics in huge quantities. While this effluent could be unfavorable for growth of most bacteria and yeast, microalgae are known to grow in similar harsh environments. APL is rich in C5 and C6 sugars which can be used as organic carbon source for microalgal cultivation under mixotrophic condition. Utilization of APL for micro algal cultivation is not reported so far. Use of this type of waste stream can not only provide cheap carbon for augmenting algal growth and lipid production, but can also serve as a value addition of the pentose stream from lignocelluloses. Here we describe the growth of fresh water microalga *Chlorococcum sp* R-AP13 in APL from the acid pretreatment of Rice straw and Sorghum Stover, evaluation of utilization of C5 and C6 sugars and the utilization/removal of sugar degradation products present in the APL medium. Fatty acid profiling of oils produced under mixotrophic mode was performed and the suitability of the oil for biodiesel were analyzed based on the physico-chemical parameters and FAME content.

8.2. Materials and Methods

8.2.1. APL recovery and APL based media preparation

Acid pretreatment liquor (APL) from biomass pretreatment was procured from the bioethanol pilot plant at the Centre for Biofuels, CSIR-NIIST. Rice straw biomass was pretreated with 20% (w/w) biomass loading and 2% acids loading (w/v) where as Sorghum Stover biomass was pretreated with 20% (w/w) biomass and 1.4% (w/v) acid (H_2SO_4) loading. Pretreatment was performed in a Nauta mixer heated by steam injection at 120°C for 15 min. After the pretreatment, slurry was neutralized to pH of ~5.0 with NaOH. Solid liquids separation was done using a vibra sifter and plate and frame filter in series (Final separation through 200 mesh filter cloth) and the liquid fraction was collected and used a the APL. Algal growth and

lipid production was studied in APL alone, APL with supplementation of MA medium (composition as described in section 2.2), and in APL supplemented with agricultural grade NPK mixture (18:18:18) at a concentration of 0.2g/l.

8.2.2. Microalgal strain and Inoculum preparation

Phototrophically grown *Chlorococcum* sp.R-AP13 cells were inoculated into APL based medium for adaptation of cells in the media under mixotrophic condition. Microalgae adapted in the APL were used for conducting the experiment. Experiments were performed in 500ml flasks containing 200 ml media 10% v/v of an inoculum containing and 3×10^6 cells /ml was used. Flasks were incubated mixotrophically with 40.5 μmol photons m^2/s illumination and a light/dark cycle of 10/14 h at a temperature of 30±2°C.

8.2.3. Cell growth and morphology

Cell growth was monitored by checking the cell count using hemocytometer at two days interval. Morphological changes of *Chlorococcum sp.*R-AP13 grown in APL was observed under phase contrast microscope, and scanning electron microscopic analyses was done according to method described in section 2.6. Completely dried cells were sputter coated with gold and was examined in a Scanning Electron Microscope (JEOL JSM5600LV, Germany). For dry cell weight (DCW) determination, the cells were harvested after two weeks of incubation by centrifugation (8,000 rpm for 10 min), washed with distilled water to remove the salt and were lyophilized. Lyophilized cells were weighed in an analytical balance, and the dry cell weight was expressed as milligrams per liter (mg/l). Nile red assay was conducted for the evaluation of lipid production by algal cells after the cells reached to the stationary phase of growth (8^{th} day) as described in section 2.7.1 and fluorescence intensity of the stained cells were analyzed in micro titer plate reader.

8.2.4. Analytical Methods

8.2.4.1. Assay of sugars and inhibitors

Sugars present in the APL medium before and after algal cultivation was analyzed by HPLC methods as outlined by Sluiter *et al*, (2006). Sugars were analyzed and quantified on a Rezex ® RPM monosaccharide analysis column (Phenomenex) using an HPLC (Shimadzu prominence UFLC) with RI detector. Oven temperature was maintained at 85 °C, and de-ionized water at a flow rate of 0.8 ml/min was used as mobile phase. Rezex® ROA organic acid analysis column (Phenomenex) and photodiode array (PDA) detector were used for separation and detection of inhibitors present in APL. Oven temperature was kept at 50 °C, and mobile phase used for separation was 0.05M H_2SO_4 at a flow rate of 0.6 ml/min

8.2.4.2. Total lipid extraction and analyses

Total lipids were extracted from dried biomass by the solvent extraction method as described in section 2.7.2. Lipid yield was expressed as percentage of the dry cell weight of the algal biomass (% DCW). HPTLC analyses were carried out to identify the lipid components of the extracted lipids in a CAMAG HPTLC system (Switzerland). HPTLC aluminium sheets coated with silica gel 60F254 (EMerck, India) was spotted with 5µl each of the extracted lipid samples using a Linomat 5 automatic sample spotter. The solvent system used for separation of the sample was hexane: diethylether: acetic acid in the ratio 80:20:1. Plates were developed in a CAMAG glass twin chamber. Spots of corresponding lipids were detected by UV light.

8.2.4.3. Fatty acid analysis

Fatty acid profile of the algal oil was determined as fatty acid methyl esters (FAME). Transesterification reactions were performed with 2 % H_2SO_4 in dried methanol at 100 °C for 6h for complete methylation. FAMEs were extracted with hexane and dried over anhydrous Na_2SO_4. Analysis was performed as described in section 2.7.4

8.2.5. Biodiesel specification compatibility of algal oil

In order to evaluate the suitability of oils from alga grown in APL for biodiesel application, several chemical and physical properties specified for establishing quality of biodiesel were estimated from the FAME profile directly by comparing the FAME profile against standards specified by in EN14214 and ASTM –D6751-02. Biodiesel properties of oils were analyzed theoretically with the help of biodiesel analyzer software version 1.1 (available on "http://www.brteam.ir/biodieselanalyzer")

8.3. Results and Discussion

8.3.1. Evaluation of algal growth.

APL was directly used for the growth of *Chlorococcum sp.*R-AP13 under mixotrophic condition. pH of the liquor was 4.7 and no further conditioning or adjustment of the pH was done for the experiment. Algal cells were gradually adapted to this medium cells adapted to APL for at least one generation were used as inoculum. *Chlorococcum sp.*R-AP13 could adapt to the APL and was able to produce biomass in this effluent under mixotrophic condition. Cell growth was enhanced in APL and the maximum cell density achieved was 7×10^7 cells /ml on the 10^{th} day, whereas in the control culture (phototrophic growth in MA medium), the cell density on the same day was only 4.8×10^6cell/ml (Fig 8.1). Biomass production was enhanced when the APL was supplemented with nutrients; maximum cell density was achieved on 10^{th} day in the APL from Sorghum Stover Biomass (SBAPL) in comparison to APL from Rice Straw (RSAPL). Growth of the cells was further enhanced when the APL medium was supplemented with agricultural fertilizer grade NPK mixture. Maximum growth was in the Sorghum Stover Biomass APL where a cell density of containing 10×10^7cells/ml was achieved in comparison to Rice Straw which supported a cell density of 9×10^7 cells/ml on the same day

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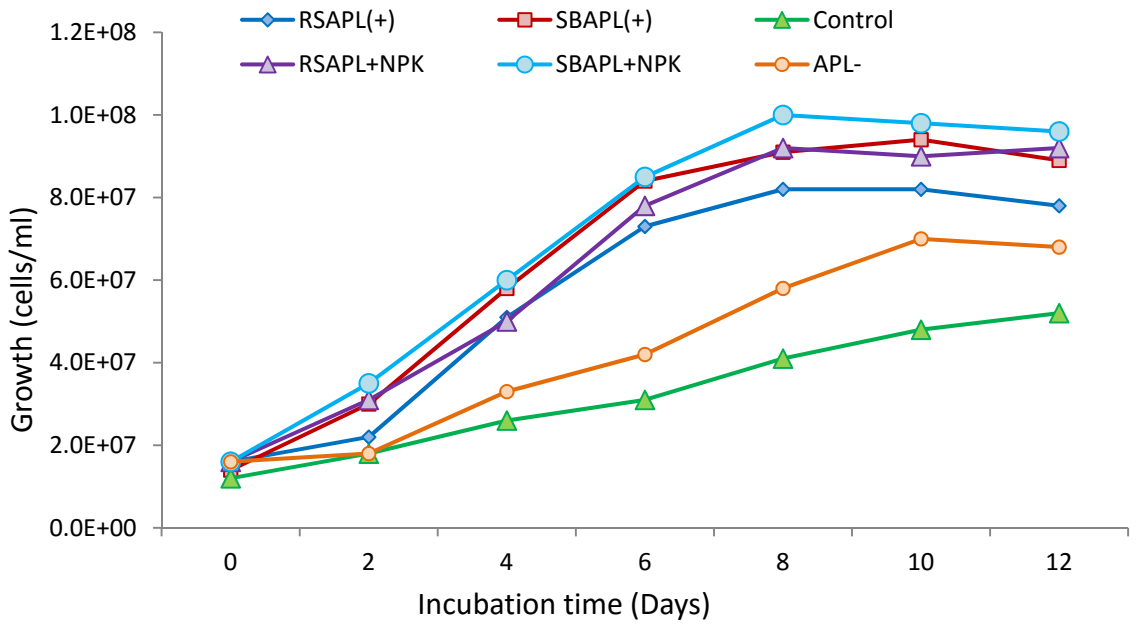
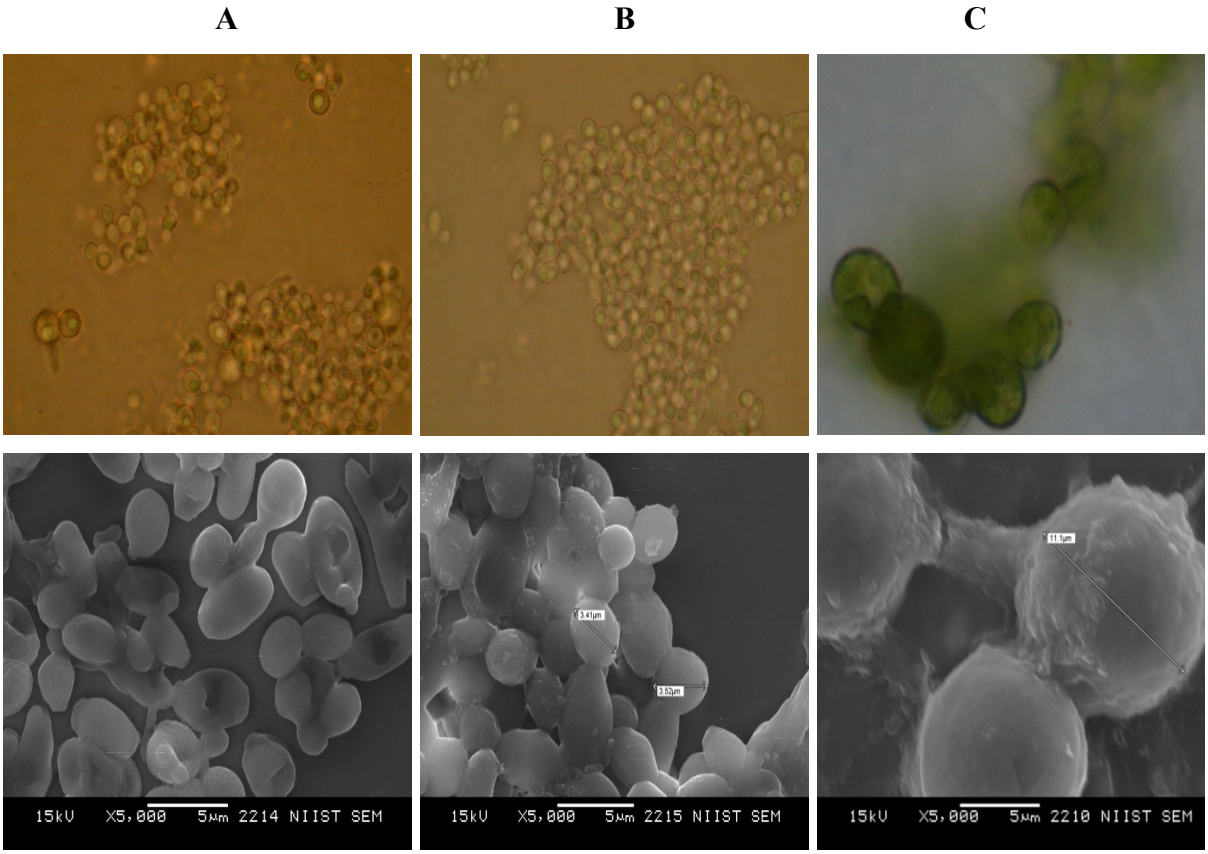
Figure 8.1: Growth of *Chlorococcum* sp RAP13 in Acid Pretreatment Liquor



APL- APL alone, RSAPL: Rice straw APL, SBAPL: Sorghum stover Biomass APL, RSAPL/SBAPL+: APL with MA medium, RSAPL/SBAPL+NPK: APL with NPK mix

Microscopic observation of the cells grown mixotrophically in APL showed that the cells were brownish green when compared with the phototrophic cells which were green (Fig 8.2). This may indirectly due to lesser light penetration in the medium due to the dark color of APL which may lead to decreased pigment production or bleaching of chlorophyll and carotenoids and shifting of metabolism at least partially towards a heterotrophic growth. Changes in pigment production by algal cells under heterotrophic condition have been reported by many. It has been reported that under heterotrophic conditions, there is reduction in chlorophyll a/b ratio and carotenoids which form part of the dark adaptation (Young, 1993), reduction in number of chloroplasts (Scheerer and Parthier, 1982) and alteration of photosynthetic membrane proteins (Sasidharan and Gnanam, 1990). Scanning electron microscopic observation of the cells grown mixotrophically in APL indicated that the size of the cells was reduced from 10µm for phototrophic cells to about 4 µm for the cells grown in APL (Fig 8.2).

Figure 8.2: Morphological changes of *Chlorococcum* sp R-AP13 cells cultivated under mixotrophic condition in APL



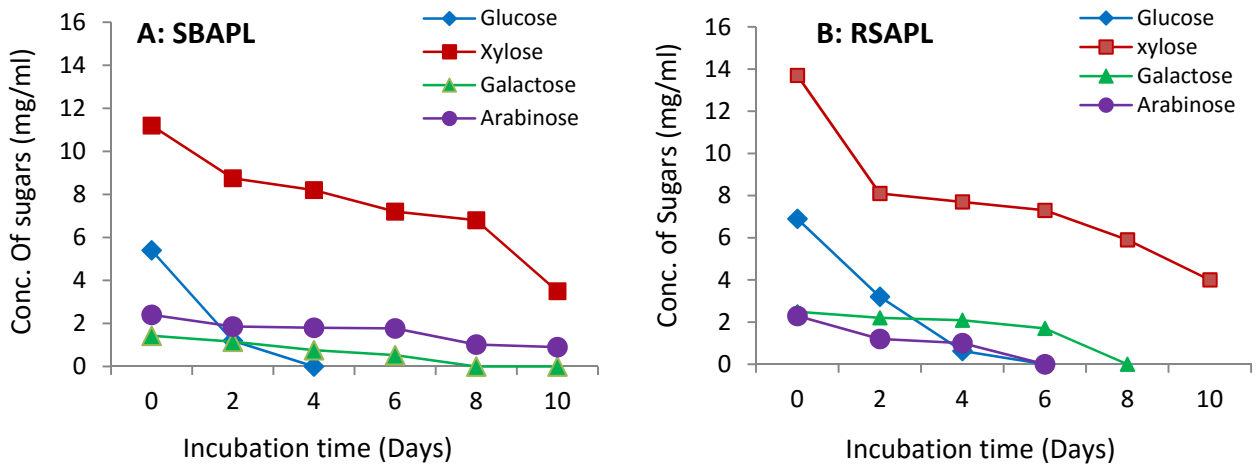
Phase contrast (Row1) and scanning electron micrograph (Row2) images of algal cells grown mixotrophically in A) RSAPL B) SBAPL and phototrophically in C) MA medium (Control)

8.3.2. Evaluation of sugar consumption and inhibitor removal

APL medium is rich in hexose sugars like glucose and galactose and pentose sugars like xylose, and arabinose. Xylose is the major carbon substrate present in the APL. Sugar consumption of algal cells in the APL was analyzed after the algal growth at two days interval. Results indicated that *Chlorococcum* sp. R-AP13 was able to completely utilize glucose and arabinose in APL by the 6th day of incubation, whereas galactose was utilized completely by the 8th day. Xylose was consumed gradually in the case of APL from sorghum biomass (Fig 8.3A). In the case of RSAPL glucose utilization was rapid and the sugar was utilized completely on the 4th day of its growth. Other sugars were consumed gradually under mixotrophic mode (Fig 8.3B). Arabinose and galactose utilization by algae are not reported so far and xylose utilization was reported only in the case of prokaryotes or fungi. However, utilization of xylose by *Scenedesmus sp* under mixotrophic condition was reported recently

by Yang *et al* (2014) and mixotrophic cultivation of microalgae using the xylose rich waste stream from paper and pulp industry was reported by Leite *et al* (2015). *Chlorococcum* spR-AP13 was able to utilize all the four major sugars present in the APL medium under mixotrophic condition

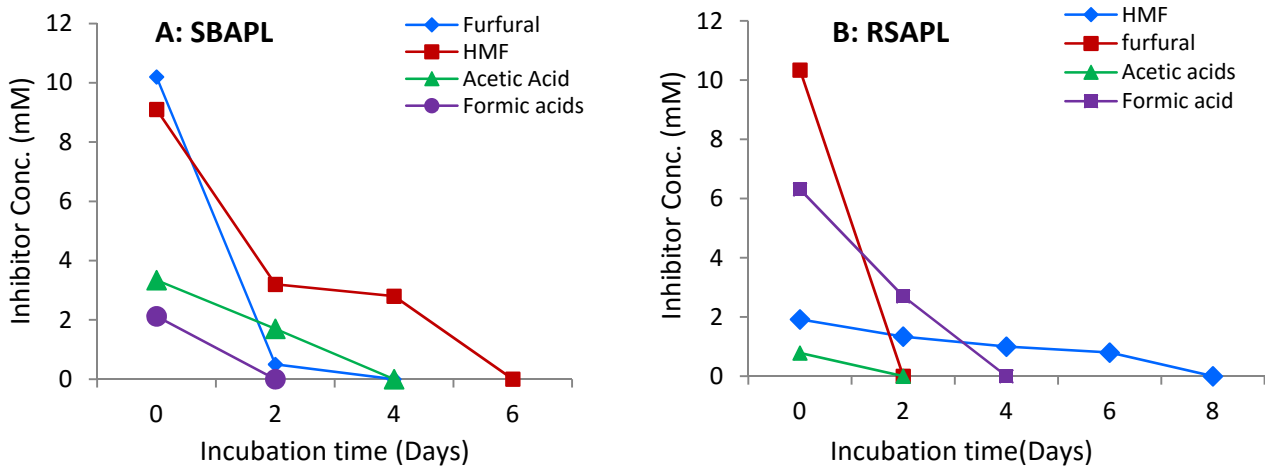
Figure 8.3: Utilization of C6 and C5 sugars present in the APL:



Furan derivatives, phenolics, and weak acids are common inhibitors of microbial growth found in APL. Furan derivatives like furfurals and hydroxy methyl furfurals (HMF) are mainly derived by degradation of hexoses and pentoses present in the cellulose and hemicellulose fractions of biomass, and lignin releases phenolic compounds on degradation (Palmqvist and Hahn-Hfagerdal, 2000). Acetyl groups present in the hemicelluloses are released as acetic acid, while furan derivatives under high temperatures break down in to formic acid (Liu and Blaschek, 2010). Inhibitors present in the APL was utilized or removed by *Chlorococcum sp.*R-AP13 while growing mixotrophically in this which is an added advantage in treatment of the biorefinery effluents. Initial concentration of acetic acid in SBAPL and RSAPL were 3.3 mM and 0.7 mM respectively while the corresponding formic acid concentrations were 2.1 mM and 6.3 mM. Formic acid was removed on 2nd day of growth while furfural and acetic acid were removed on the 4th day and HMF was removed on the 6th day of incubation from the SBAPL. However the removal of furfural and acetic acid is on the 2nd day of growth period, formic acid on the 4th day and HMF is removed gradually taking 8 days from the RSAPL (Fig 8.4). So far, information about the effect of furans on microalgal growth has been rather scarce. Inhibitory effect of HMF and furfural on cyanobacteria was reported at a concentration of 9 mM and 7 mM (Yu *et al,* 1990). Recently,

it has been reported that furfural at 0.6 g/l can cause 30% biomass reduction during mixotrophic acetate-based cultivation of *Chlamydomonas reinhardtii* (Liang *et al,* 2013). This novel microalga was able to utilize these furan derivatives for its growth and biomass production under mixotrophic mode at lower concentrations. More recently, it was reported that pyrolytic bio-oil rich in acetic acids is used as substrate for the growth of *Chlamydomonas reinhardtii* under heterotrophic mode of growth. Acetic acid concentrations above 4g/l inhibited the growth of micro algae in this case (Zhao *et al,* 2015).

Figure 8.4: Removal of Inhibitors from APL by *Chlorococcum* sp.



8.3.4. Biomass and lipid production

Biomass and lipid production by *Chlorococcum sp* R-AP13 under mixotrophic growth in APL was evaluated against phototrophic cultivation in control (MA) medium. The alga grew better and accumulated more lipid under mixotrophic cultivation in APL. Biomass and lipid production was enhanced when APL was supplemented with NPK mix. Under mixotrophic growth, the algal cells were greenish brown in color. While the biomass yield was 0.8g/l for the phototrophically grown control, it increased to 1.832 g/l in APL from sorghum biomass with NPK and 1.795 g/l in APL from rice straw biomass (Table 1). Mixotrophic growth in raw un-supplemented APL yielded the lowest biomass of 1.088 g/L which was lower than APL supplemented with growth medium component (1752 and 1630 mg/l respectively for SBAPL and RS APL respectively). It may be speculated that the available carbon in raw APL may not be sufficient to support enhanced growth, and the supplementation of macro elements for the growth may be necessary for enhanced biomass production. For the

economic production of algal biomass, growth on a cheap carbon substrate is often suggested as an alternative to conventional low density phototrophic race way cultivation (Doucha & Livansky, 2012). The cost of carbon source represents approximately 50% of the cost of medium in algal cultivation (Cheng *et al,* 2009) and effluents like APL from biomass processing industry can provide a potent feedstock for cultivation of algae. Similarly, commercial grade NPK mix is again a low cost supplement which may be used for economic algal cultivation. Therefore the pentose sugar rich effluent -APL is a potential alternative medium for the cultivation micro algae, which will also aid in the biorefinery mode operation of lignocellulosic and algal biofuel plants.

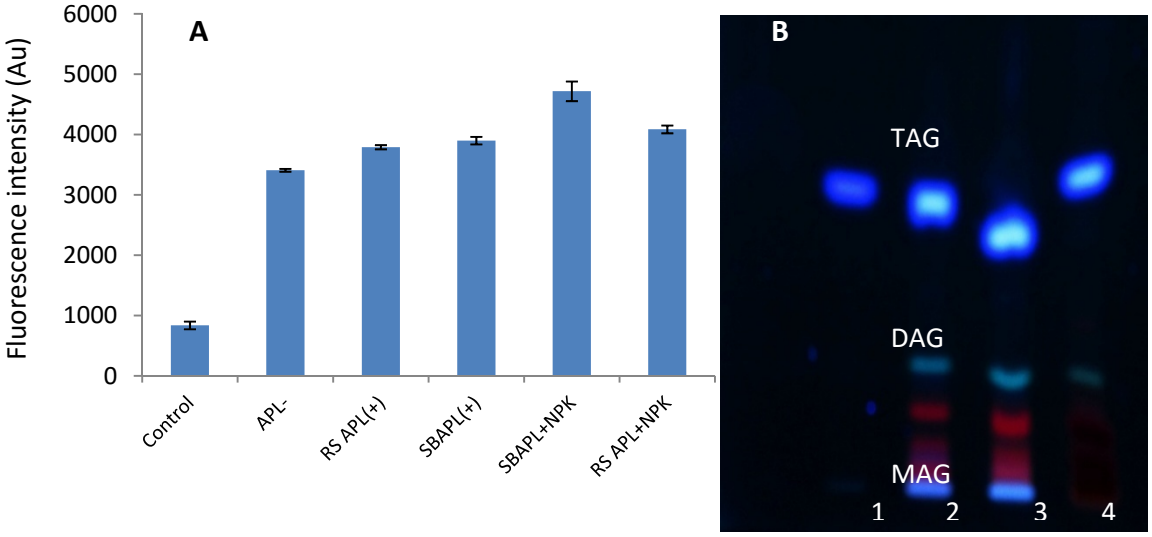
Table 8.1: Biomass and lipid production of *Chlorococcum sp RAP13* under mixotrophic condition in Acid pre-treated liquor

Cultivation conditions	Biomass conc. (mg/l)	Yield of lipids (mg/l)	Cellular Lipid Accumulation (% DCW)
APL-	1088 ± 33.9	298 ± 0.9	27.4 ± 0.7
SBAPL+	1752 ± 36.1	484.8 ± 11.2	28.0 ± 0.8
RSAPL+	1630 ± 20.0	443.1 ± 12.0	27.0 ± 0.4
SBAPL(NPK)	1832 ± 24.7	568 ± 15.2	30.8 ± 0.0
RSAPL(NPK)	1795 ± 21.2	538.2 ± 20.5	29.5 ± 2.12
Control	816 ± 34.0	190 ± 14.0	23.0 ± 0.7

Data are expressed as mean ± standard deviation of triplicates.

Lipid accumulation was enhanced in algal cells grown in SBAPL compared to those from the RSAPL medium. Algal cells accumulated 27 % of their dry cell weight as lipid in APL without any supplementation, the lipid content improved to 30% in both SBAPL and RSAPL supplemented with NPK. Supplementation of medium component resulted in an almost similar lipid accumulation of 27% and 28% respectively for RSAPL and SBAPL respectively (Table 8.1). The lipid yields from mixotrophic cultivations in SBAPL and RSAPL were 0.484 g/l and 0.443 g/l respectively for MA medium supplementation, which was enhanced to 0.568 g/l and 0.538 g/l with NPK supplementation. Phototrophic control produced 0.19 g/l of lipid when compared to APL alone as medium which showed significantly higher production 0.298 g/l.

Figure 8.5: Lipid accumulation studies



A) Nile red fluorescence B) TLC profiling of lipids

The intracellular neutral lipid accumulation of algal cells was measured by the 'Nile Red' fluorescence emission. Staining was performed on cells on the 10th day of incubation. The fluorescence intensity of cells increased in the APL medium with NPK mix in comparison to control medium. Fluorescence intensity was high in cells grown on the APL from the sorghum biomass (Fig8.5A). TLC analysis of the extracted oil indicated that neutral lipids form the major fraction (Fig 8.5B). The Triacylglyceride (TAG) spot was more prominent in the oil from mixotrophic cultures. This is advantageous if the oil is to be considered for biodiesel applications since TAGs are the raw materials for trans-esterification reactions to produce biodiesel (Christi, 2007). The cultivation of *Chlorococcum* sp R-AP13 on APL medium with NPK therefore may be considered to provide an efficient method for algal biomass and oil production with the added advantage of the value addition of pentose stream from lignocellulose.

8.3.5. Fatty acids profiling

GC analysis of FAMEs from the algal oil showed that oleic acid content was high in the oils produced through mixotrophic cultivation in APL. In phototrophically grown cells the content of palmitic acid was elevated in the algal oil. Major fatty acid from *Chlorococcum sp*-RAP-13 oil produced in APL was oleic acid (41-45%) followed by palmitic acid (30-

31%), stearic acid (15%), Linoliec acid (2.6%) and linolenic acid (2.2-4.8%), and other fatty acids were low in the oil (Table 8.2). Saturated fatty acids were 46-47%, unsaturated fatty acid content was 4.8-7.3% and monounsaturated fatty acids production was 43-47% under mixotrophic cultivation. Under phototrophic growth, saturated fatty acids production was 62% which is high compared to the mixotrophic modes in APL. There are numerous studies targeting to maximize growth of microalgae under different cultivation modes and to enhance the level of oil or other value added products. However, the reliability of the algal oil for biodiesel applications depends not only on the quantity of oil produced, but also on the type and structure of the right fatty acids for fuel application. Hence the lipid content and fatty acid profile are key factors to consider when selecting algae for fuel production.

Table 8.2. Fatty acid profile of the lipids produced by *Chlorococcum* sp R-AP13 cultivated mixotrophically in APL medium in comparison to phototrophic control

Fatty Acid Type	Relative proportions (%)		
	Mixotrophic RSAPL	Mixotrophic SBAPL	Phototrophic control
C12	0.5	0.4	1.6
C14	0.4	0.2	1.5
C16	31.4	30.1	52
C16:1	-	1.83	2.7
C17	-	1.9	-
C18	15.84	15.7	-
C18:1	41.5	44.7	20
C18:2	2.6	2.7	7
C18:3	4.8	2.2	4.70
C20:1	0.4	-	-
C20:2	0.4	-	-
C22:0	0.5	-	-
C24:0	-	-	-
SFA	47.8	46.7	62.4
MUFA	43.2	47.4	20.4
USFA	7.36	4.88	14.6

SFA: saturated Fatty acids, USFA: unsaturated fatty acids, MUFA: Monounsaturated fatty acids

8.3.5. Evaluation of biodiesel properties from FAME profile

Twelve important biodiesel fuel properties of oils from *Chlorococcum* spR-AP13 was analyzed by biodiesel analyzer software (Table 8.3). FAME composition analysis and matching of fuel property specifications are very important in choosing an alga for biodiesel production. Tested CV value for algal oil was in the range of 60-61 which comes within the limit of biodiesel fuel standards, minimum CN value for diesel application is ≥ 47 and ≥51 according to the ASTM D4751-02, EN14214 and IS15607 biodiesel standards. The cetane number (CN) is indicative of the time delay in the ignition of fuel of diesel engines. The higher the CN value shorter is the ignition time, CN increases with the length of the unbranched carbon chain of the FAME components (Knothe, 2005). Another biodiesel quality parameter is iodine value (IV) which is not included in the ASTM or Indian standards but important in the EN 14214. IV represents the DU (degree of unsaturation) by weighted sum of the masses of MUFA and PUFA which play an important role in biodiesel oxidative stability. IV of oil from this alga was 51-56 which falls within the limit of the standard. High unsaturation levels of oils may result in the polymerization of glycerides, formation of deposits and susceptibility to oxidation attack (Francisco et al, 2010).

Table 8.3: Compatibility to Biodiesel specifications of Oil from *Chlorococcum* sp. R-AP 13 cultivated mixotrophically in APL based on FAME profile

	CN	SV	IV	DU	LCSF	CFPP	CP	APE	BAPE	V	P	HHV
EN14214	≥51	-	≤120	-	-	≤5/-20	-	-	-	3.5-5	0.86	NA
ASTM-D6751-02	≥47	-	NA	-	-	NA	-	-	-	1.9-6	0.86	NA
IS-15607	≥51	-	NA	-		6/18	-	-	-	2.5-6	0.86	NA
RS-APL	60.4	202.49	56.83	58.01	11.02	18.14	11.31	56	12.1	1.36	0.85	38.87
SB-APL	61.0	203.25	53.77	57.16	10.89	17.7	5.46	54.4	7.08	1.38	0.86	39.08
Control	60.9	207.88	51.79	49.6	5.95	2.22	22.36	49.2	22.2	1.27	0.85	38.21

DU: Degree of unsaturation, CN: Cetane Number, SV: Saponification value, IV: Iodine value, LCSF: Long-chain saturated factor, CFPP: cold filter plugging point, CP: cloud point, HHV: Higher heating value, υ: kinematic viscosity, ρ: Density, APE: Allylic position equivalents, BAPE: Bisallylic position equivalent

The key low temperature flow properties for winter fuel specification are CFPP and CP. There are no specifications given in European and US specifications for low temperature properties. Each country is free to determine its own limits according to their local climatic conditions. CFPP of oils from *Chlorococcum sp* falls within the range of Indian standards (Table 8.3). Long chain saturated factor (LCSF) of lipid feedstock is a critical parameter for oxidation stability, cetane number, IV and cold filter plugging point (CFPP) of the biodiesel. CP value was closely affected by the solid phase consisting mainly of the saturated methyl esters at the equilibrium point and can be accurately predicted only by the amount of saturated methyl esters regardless of the composition of unsaturated ester components (Imahara *et al*, 2006). Here the cloud point of oils from this organism grown in APL was 5.4-22°C. The APE and BAPE are effective in predicting the oxidation stability of biodiesel. For oils produced from both feedstocks, APE shows higher values and BAPE shows lower values. There are no specifications given for the higher heating value in any of biodiesel standards. Energy content of fatty acids methyl esters is directly proportional to the chain length of fatty acids. HHV from the FAME profile shows 38-39 MJ/Kg in the phototrophic control and APL derived fatty acids. Density (p) for biodiesel standards values are set in a range of 8.6-9g/cm^3 according to EN14214. Density value of oils from mixotrophic and phototrophic cultures were found to be within this range. Furthermore, biodiesel must have an appropriate kinematic viscosity (υ) to ensure that an adequate fuel supply reaches injectors at different operating temperatures (Ramirez-Verduzco et al, 2012). Viscosity of fuel is inversely proportional to the temperature, which also affects the CPFF of fuel at low temperature. Viscosity of oils from *Chlorococcum sp* was in the range of 1.27-1.38mm^2/s which is in the limit of biodiesel standards.

8.4. Conclusions

Chlorococcum sp R-AP13 cultivated in APL- a major effluent from lignocellulosic biomass processing plant, produced high amount of biomass and accumulated significant amount of lipids, particularly under mixotrophic culture. The cells displayed different growth characteristics, lipid content and fatty acid profiles, based on differences in the growth conditions and supplementation of nutrients. Biomass yields increased from 1g/l to 1.83g/l and lipid yield enhanced from 27 to 30% when shifted from APL alone to APL supplemented with nutrients/fertilizers like NPK mixture. The lipid profile of the oil produced from algal biomass grown in APL indicated interesting features like high triglycerides content and a

fatty acid profile with high proportion of oleic acids. This alga was able to survive in the medium, in the presence of inhibitors like the furan derivatives –HMF and Furfural; acetic acid and formic acid. These features project the alga as a potent source of oil which can be used as feedstock for biodiesel and also as a food or feed supplement. Moreover, algal cultivation in the biorefinery effluent can reduce the use of fresh water and nutrients for algal cultivation, at the same time allowing reducing of the pollution load of this effluent. To the best of our knowledge, this is one of the first reports of pentose rich acid pretreatment liquor as a medium for micro algal oil.

Chapter 9: Microalgal biomass recovery by flocculation

9.1. Introduction

Generally algal biomass production systems include cultivating microalgae in an environment that stimulate the accumulation of target metabolites and the recovery of the biomass for the downstream processing (Cheng *et al,* 2011). However major bottleneck in the algal biomass based product is the recovery of biomass from the production medium, mainly due to the smaller size (5~50 μm), presence of negative surface charge, low biomass concentrations, and similarity of the density of algal cells to the growth medium (Garzon-Sanabria,2012; Reynolds 1984; Milledge and Heaven 2013). Key factor limiting the commercial use of microalgal biomass is cost effective harvesting which is considered to be most challenging area in algal based biofuels (Greenwell *et al,* 2009; Olguın 2003; Georgianna and Mayfield 2012). It has been suggested that 20-30% of the cost of algal biomass is due to the cost of harvesting (Grima *et al,* 2003; Mata *et al,* 2010; Zittelli *et al,* 2014). However harvesting technology is an important factor in the production of algal based biofuels, and an effective and economical method of microalgal harvesting has not yet been developed (Kim *et al,* 2005). These high costs can only be acceptable in cases where microalgae can yield high value products. For low-value bulk products, both the investment as well as the operational costs should be drastically decreased to make commercial production feasible (Wijffels and Barbosa, 2010). So it is necessary to develop cost effective techniques that can permit efficient harvesting.

There are several methods which have been tested for the harvesting of algal biomass, which includes centrifugation, filtration, flotation and flocculation (Chen *et al,* 2011; Uduman *et al,* 2010; Milledge *et al,* 2013). Flocculation is a chemical based separation process that needs less energy than centrifugation and filtration, and thus it is regarded as one of the most promising means of dewatering algal biomass. Flocculation technology to recover microalgal biomass has also been of great interest (Salim *et al,* 2012; Wan *et al,*, 2013; Guo *et al,* 2013; Alam *et al,* 2014). A large number of chemical products are tested as flocculants including various inorganic multivalent metal salts (Duan and Gregory, 2003) and organic polymer/polyelectrolyte (Vandamme *et al,* 2010). A variety of flocculation strategies, such as physical, chemical and biological methods have been developed for microalgal harvesting as summarized in recent reviews (Vandamme *et al,* 2013; Wan *et al,* 2015). It is well known

that flocculation of algal biomass is sensitive to pH of the culture medium. pH increase enhances flocculation efficiency of algal biomass as reported by a few (Wu *et al,* 2012; Henderson *et al,* 2010). Change in pH of the medium may also influence the charge of microalgal cells (Danquah *et al,* 2009). In this aspect, flocculation simply by increasing the pH could be an attractive alternative because it is low cost, low energy and non toxic to microalgal cells and the use of flocculants can be avoided. Another advantage of this strategy is that the growth medium can be recycled after flocculation, since no flocculants are used and medium is not contaminated by toxic chemicals. However, this method was tested only in a few number of microalgal strains (Harith *et al,* 2009; Wu *et al,* 2012). The mechanism of flocculation depends on the interaction of cell surface charge and flocculent charges. Metal salts such as aluminum sulphate, ferric chloride, ferric sulphate, etc. are generally preferred in flocculation processes, because they lead to improved harvesting efficiency. One of the disadvantages of these inorganic flocculants is that they are required in high doses and results in contamination of the biomass with aluminum or iron (Becker 1994; Wyatt *et al,* 2012). Chitosan has emerged as a favorable organic flocculating agent in its use as a cationic polysaccharide in the harvesting of microalgae. Compared with other flocculants, it presents various advantages, including formation of larger flocs hence resulting in faster sedimentation of biomass and providing a clearer residual solution. Chitosan is also non toxic and biodegradable which makes it possible to reuse the flocculated medium for algal cultivation (Chen *et al,* 2014).

In the present investigation, potential of different flocculants including aluminum sulphate, ferric chloride, chitosan, pH change of medium and auto flocculation was compared for harvesting of *Chlorococcum sp.R-AP13* biomass. Flocculation efficiency, dose, and zeta potential of algal biomass during flocculation were tested and fatty acids profiling was conducted in the optimized flocculated biomass. Recycling of flocculated medium for cultivation of alga was also investigated.

9.2. Materials and methods

9.2.1. Microalga and culture condition

Chlorococcum sp RAP-13 was used for flocculation studies. The alga was maintained in MA medium as described in section 2.2. Cells for the study were grown in 5L flasks containing 3L medium and incubated in a climate controlled chamber at 30°C with diurnal cycle of 14/10h. Flocculation studies were performed after the cells reached stationary phase of the growth. Flocculants tested were procured either from Merck, India or Sigma-Aldrich, India.

9.2.2. Flocculation experiment

The effect of the flocculent type and concentration on flocculation efficiency was determined using a jar test (Vandamme *et al,* 2010; Gerde *et al,* 2014). Briefly, the algal suspension (100 ml) was stirred at 250 rpm in a 100 ml beaker while the flocculent was added slowly. After this, the stirring was continued for 2 min, then stopped and allowed to settle for 10 min. Then an aliquot of the supernatant was taken 2.0 cm from the surface of the liquid and its absorbance was measured at 680 nm in UV visible spectrophotometer. Absorbance of the original suspension was also taken before addition of the flocculent. The absorbance values were extrapolated to cell numbers based on a standard curve constructed with algal cell suspensions having different cell densities. Flocculation efficiency of *Chlorococcum* sp was calculated based on

$$\frac{\text{(Initial cell concentration – Cell concentration in supernatant)}}{\text{Initial cell concentration.}} \times 100$$

9.2.3. Zeta potential measurement

Zeta potential of the *Chlorococcum sp*.R-AP13 was measured before and after the addition of various flocculants into the medium in Malvern Zetasizer 90 (Malvern Instruments Ltd., USA). Zeta potential was analyzed in triplicates at room temperature and the mean values were taken.

9.2.4. Cell Viability

The viability of flocculated cells was tested by dye exclusion method using 1% Trypan blue, which is excluded by viable cells. One milliliter samples of each experiment were centrifuged at 6000 rpm for 5min and the supernatant was discarded. Then 100 µl of the 1.0% Trypan blue solution was added, and the cells were incubated for 3 h at room temperature. Next, the cells were washed twice using deionized water to remove excess of unbound dye. Finally, the fresh preparations of cells were examined for dye exclusion under a Phase contrast Microscope (Leica DMLS2000, Germany). Cells with intact cell wall (live cells) exclude Trypan Blue, while the dead cells take up the dye differentiating viable and non-viable cells.

9.2.5. Recycling of flocculated medium

Flocculated biomass and medium was separated by aspirating the medium. pH of the flocculated medium was adjusted to 6.8-7.0 using 1N NaOH or HCl. After that, components of MA medium were added and used for cultivation of the next batch of cells after sterilization. Fresh MA medium was used as control. The control medium and recycled media were inoculated with 10% v/v of an inoculum containing 3×10^6 cells/ml. Biomass production was monitored as cell density at two days interval.

9.2.6. Fatty acids profiling

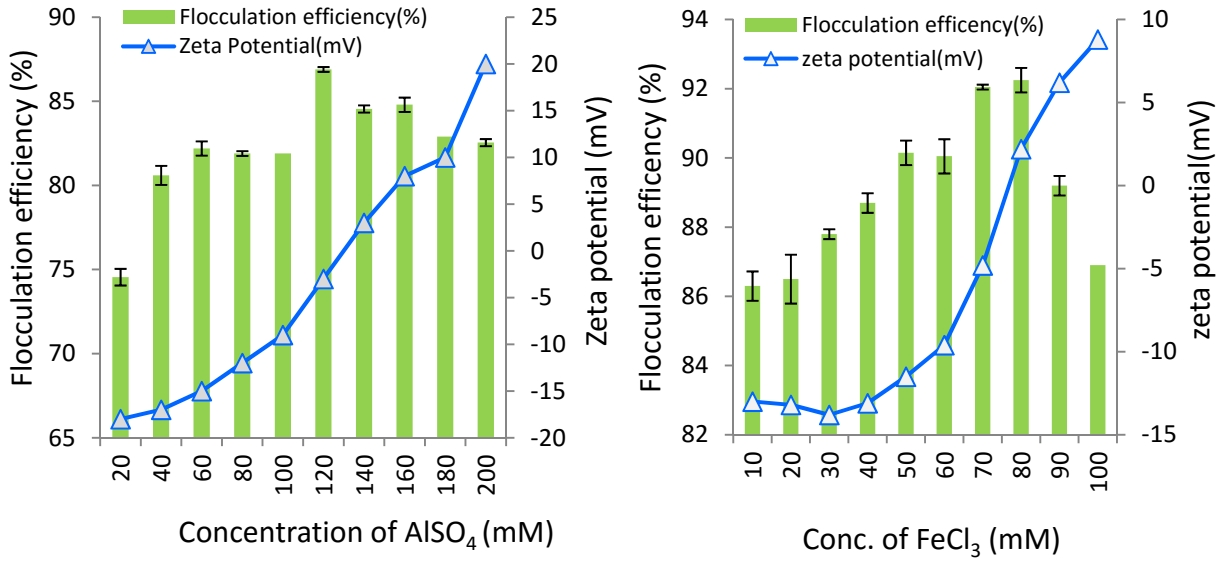
Fatty acid profiles of oil from different flocculated biomass were done by acid mediated trans-esterification for FAME generation (section 2.7.4) followed by gas chromatography. The FAME was identified by comparing their fragmentation pattern with internal standards (Sigma Aldirch, India).

9.3. Results and discussion

9.3.1. Evaluation of inorganic flocculants for harvesting microalgal cells

The inorganic flocculants-aluminum sulphate and ferric chloride were tested for flocculation of *Chlorococcum* sp R-AP13 cells. Ferric chloride was found to be more effective than aluminum sulfate. Ferric chloride supported a flocculation efficiency of 92% at concentrations of 70-80mM while aluminum sulphate had an efficiency of 87% 120 mM concentration (Fig. 9.1). Initial zeta potential of the algal cells was found to be -20 mV. The surface charge of the cell changed after the addition of flocculants. Flocculation efficiency was increased near to the neutralization point. Higher concentrations of aluminum sulfate and ferric chloride increased the positive charges in the medium which affected the flocculation efficiencies of cells (Fig 9.1). Possible explanation for this could be that the amount of flocculent that exceeded the optimum concentration could contribute to excess of positive charges, thus stabilizing the cell particles in suspension by charge repelling, as well as by steric hindrance (Vandamme *et al,* 2010).

Figure 9.1: Flocculation of *Chlorococcum* sp.RAP-13 cells using inorganic flocculants



A: Aluminum sulphate, B; Ferric chloride

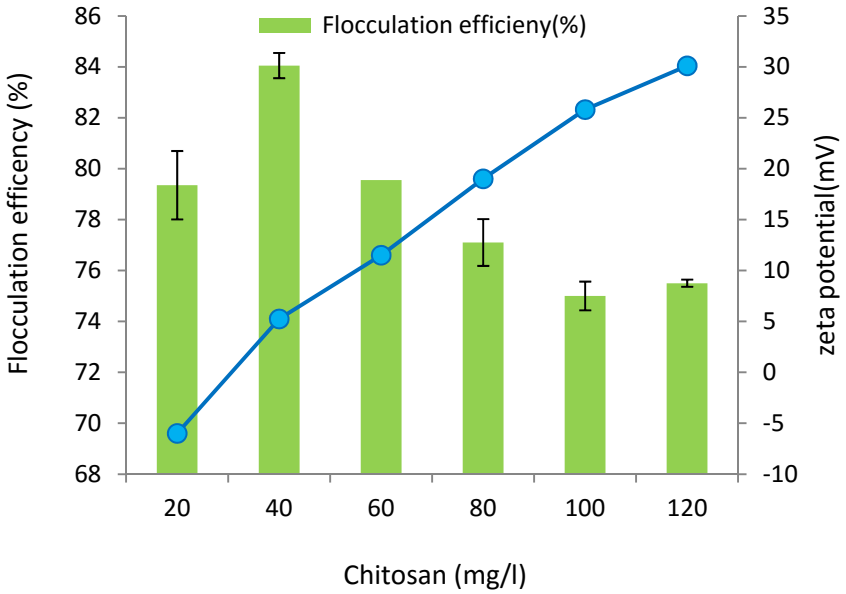
The flocculation mechanism depends on the nature of the algal cells and the charge of the flocculent. Numerous chemical coagulants or flocculants have been tested for microalgal flocculation (McGarry 1970; Papazi *et al,* 2010; Rakesh *et al,* 2014). Metal salts (aluminum sulphate, ferric chloride, etc.) are generally preferred because they lead to improved harvesting efficiency. The results of ferric chloride as flocculent showed an almost comparable efficiency with the reported literature on the flocculation of *Chlorella zofingiensis* (Wyatt *et al,* 2012). For any given algae species, effective flocculation with $FeCl_3$ might be obtained, if the conditions of negative surface charge and sufficient ferric chloride concentration are available in the medium. Since different algal species vary in their concentrations of functional groups on the cell surface, the minimum amount of $FeCl_3$ required for effective flocculation may differ (Wyatt *et al,* 2012; Rakesh *et al,* 2014). When compared with aluminum sulphate, ferric chloride is generally required in minimum concentrations to promote coagulation of algal cells. In solution, ferric chloride forms positively charged hydroxide precipitate (at pH <8) which associates with the negative algal cell surface. The ferric hydroxide precipitates form bridges between algal cells which bind them together into flocs. At low algal concentrations, the amount of $FeCl_3$ required to achieve coagulation increases linearly with algal concentration. However, at higher concentrations, the minimum amount of $FeCl_3$ required for flocculation becomes independent of algal concentration, as the dominant mechanism changes from electrostatic bridging to sweep flocculation by large coagulated algal flocs (Wyatt *et al,* 2012). Major disadvantage of inorganic flocculants such as alum and iron chloride is that it may lead to contamination of growth medium with aluminum or iron (Oh *et al,* 2001); however, they may be useful in treatment of wastewaters, wherein the spent water after mass multiplication of microalgae can be passed through columns to remove the Fe ions and then reused for growth purposes. In this present study compared to alum, ferric chloride was found to be effective flocculent for harvesting of microalgae.

9.3.2. Evaluation of Chitosan for flocculation

Chitosan is a cationic polysaccharide, which has emerged as a favorable flocculating agent in the harvesting of microalgae (Zeng *et al,* 2008; Xu *et al,* 2013; Tran *et al,* 2013). Compared with other commercial flocculants, it has various advantages, including production of larger flocs (Zeng *et al,* 2008) resulting in faster sedimentation rates and providing a clearer residual solution after harvesting, and being nontoxic and biodegradable (Knuckey *et al,* 2006). Use

of chitosan as flocculent makes it possible to reuse the residual solution to grow microalgae. Chitosan mediated flocculation of *Chlorococcum* sp R-AP13 was tested at concentrations of 20-120 mg/l. Flocculation efficiency of 84 % was obtained at a concentration of 40 mg/l and the zeta potential of algal cell was changed from -20 mV to +5mV (Fig 9.2). Further increase in the concentration of chitosan increased the positive charges on the cells which affected further flocculation. This drastic decrease in performance could have resulted when the chitosan overdose caused an overload of positive charges, which were retained on the surface of the cell causing repulsion between positively charged microalgal cells resulting in re-stabilization.

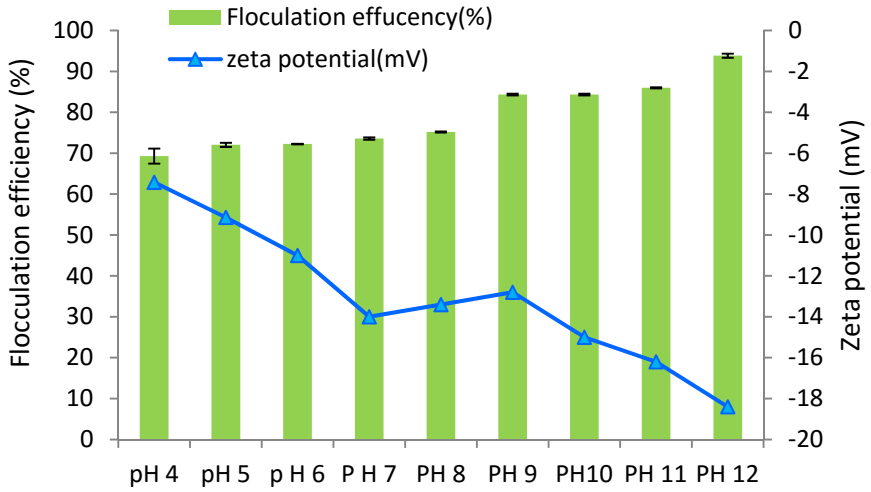
Figure 9.2: Flocculation of *Chlorococcum* sp RAP-13 cells using chitosan



9.3.3. Flocculation of algal cells by changing pH of the medium

Recently, flocculation induced by increase in pH is gaining more attention for algal flocculation (Wu *et al,* 2013; Rakesh *et al,* 2014). In this current study, increase in medium pH as a flocculation agent was evaluated for *Chlorococcum* sp R-AP13 cells. Flocculation efficiency was increased as the medium pH was increased to the alkaline range of 11 and 12. Maximum efficiency of 94% was obtained with the pH increased to 12. Zeta potential of the algal cell varied with different pH, but the surface charge of the algal cells was negative in the alkaline pH (Fig 9.3).

Figure 9.3: Flocculation of *Chlorococcum* sp R-AP13 by increase in medium pH



The zeta potentials were pH dependant and negative at different pH values. For freshwater microalgal systems, the zeta potential was shown to initially decrease with increase in pH, but increasing on further increase of pH. The decrease in zeta potential with pH increase indicated the decrease of the cell surface charges, possibly due to charge neutralization in this range. Possible mechanisms of pH mediated flocculation is the formation $Mg(OH)_2$ precipitate from Mg^{2+} in the growth medium as the pH increased. The $Mg(OH)_2$ precipitate has a large adsorptive surface area and a positive superficial charge (Parks, 1967). This precipitate attracts the negatively charged microalgal cells, thus resulting in the compression of the electrical double- layer and causing them to become destabilized and hence to flocculate. For freshwater microalgae, zeta potential increased after the initial decline, which was attributed to the dissociation of carboxylic acid groups on the surface of microalgal cells (Henderson *et al,* 2010). However, the flocculation efficiency was significantly higher, indicating that sweep flocculation was active in this pH range (Wu *et al,* 2013). $Mg(OH)_2$ precipitates tended to have a rather open structure, so that even a small mass could give a large effective volume concentration and hence it has a high probability of capturing microalgal cells (Duan and Gregory, 2003). The flocculation efficiency was therefore considerably improved, than when particles were destabilized just by charge neutralization. Present results agreed with the previous reports by Wu *et al* (2012) and Vandamme *et al,* (2012).

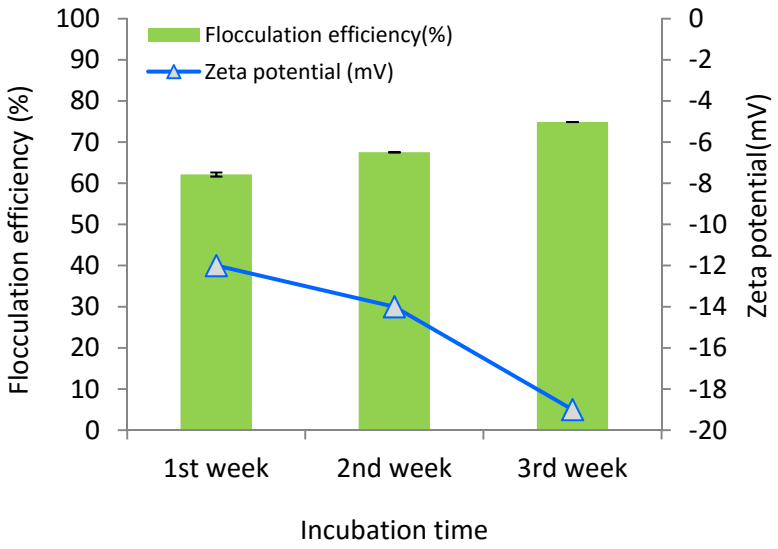
Flocculation induced by high pH is considered as a potentially useful method to pre-concentrate fresh water microalgal biomass during harvesting (Vandamme *et al,* 2012). However, as microalgae usually carry a negative surface charge, an increase in pH will cause an increase in surface charge rather than a decrease, which might be the possible cause for flocculation induced by high pH. The use of flocculation induced by high pH for harvesting microalgae may have an additional advantage that the high pH may effectively sterilize the microalgal biomass as well as the process water. This may be advantageous when microalgae are used in wastewater treatment, as the high pH may kill pathogenic microorganisms (Semerjian and Ayoub, 2003). It has been reported that an increase in pH within the range of 8.5 to 11.0 allows the recovery of microalgae such as *Phaeodactylum tricornutum* (Sirin *et al,* 2012), *Anabaena marina* (Lopez *et al,* 2009), or *Dunaliella tertiolecta* (Horiuchi *et al,* 2003) and has biomass recovery efficiencies higher than 90 %.

9.3.4. Effect of auto flocculation

Auto flocculation of *Chlorococcum* sp R-AP13 was evaluated by culturing the cells up to 3 rd week of incubation under phototrophic condition and flocculation efficiency was tested at every week of incubation time. Flocculation efficiencies increased as the incubation time increased from 62% in the initial 1st week to maximum efficiency of 75% in the 3rd week of incubation (Fig. 9.4). Zeta potential of cells became more negative with increase in incubation time.

Auto flocculation refers to the cell aggregation and adhesion of cells to each other in liquid culture, due to special cell surface properties or some other factors. Cell flocculation widely occurs in microorganisms and several self-flocculating microalgae have also been discovered, such as *Chlorella vulgaris* JSC-7 (Alam *et al,* 2014), *Scenedesmus obliquus* AS-6-1 (Guo *et al*, 2013), *Ankistrodesmus falcatus* (SAG202-9) and *Ettlia texensis* (SAG79.80) (Salim *et al,* 2012).

Figure 9.4: Auto flocculation of *Chlorococcum sp* R-AP13

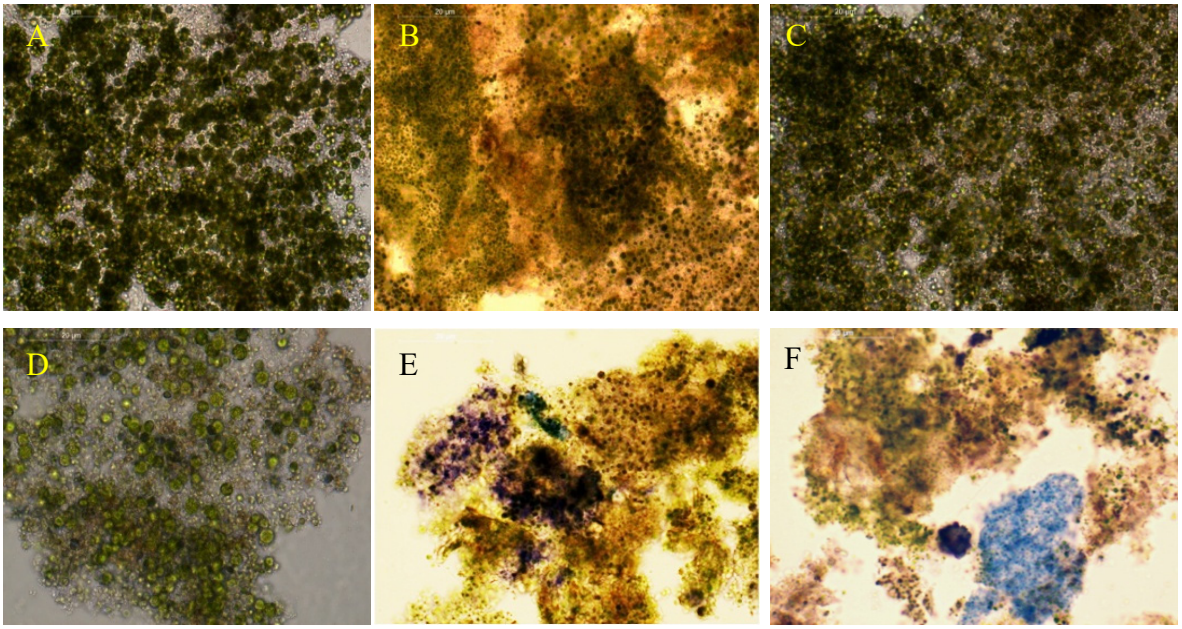


Limited reports are available in the literature regarding the auto flocculation of cells and actual mechanism of auto flocculation is still obscure. Alam *et al,* (2014) and Guo *et al,* (2013) had studied the biochemical basis of auto flocculation of two micro algae *C. vulgaris* JSC-7 and *S. obliquus AS-6-1*. They found that the polysaccharides biosynthesized by these two strains were responsible for self-flocculation. Another recent report proposed that glycoprotein is involved in cell flocculation of green microalga *E. texensis* SAG79.80 (Salim *et al,* 2014). Therefore, microalgal self-flocculation may occur when the flocculating agents (e.g., polysaccharides and glycoprotein) produced by microalgal cells themselves patch adjacent cells, or it may be due to formation of bridges between the cells via charge neutralization with changes in medium pH, promoting self-flocculation. More research is needed in this area to understand the exact mechanism of self flocculation of microalgal cells. Microalgal cell self-flocculation, differing from the flocculation induced by pH adjustment, can occur naturally via interaction of adjacent cells without acid, alkaline, or metal ion addition. Moreover, harvesting microalgae using self-flocculation, which requires no extra expenditure in cultivation of microalga or purification of bio-flocculent, is a promising method for low-cost harvesting.

9.3.5. Viability of flocculated biomass

Viability assay of flocculated biomass was carried out by Trypan blue staining of the cells. Auto flocculated cells, cells flocculated by chitosan and through change in medium pH were found to be viable. Cells flocculated through aluminum sulphate and ferric chloride showed dye uptake indicating the presence of dead cells and the percentage of dead cells were proportionate to the concentration of the flocculent (Fig 9.5). Inorganic flocculants, including alum and iron chloride, may also lead to contamination of the growth medium with aluminum or iron (Oh *et al,* 2001). Flocculation by alum or ferric chloride therefore cannot be considered as a preferred method for algal biomass recovery in this case, since it was found to be toxic to the cells besides contaminating the residual medium. Flocculation mediated by chitosan was very effective for harvesting the biomass, with the added advantages of non toxicity and complete clarification of medium after flocculation. However, the cost of chitosan is high making it not a feasible option for large scale usage. Flocculation mediated by auto flocculation or induced by pH increase was found to be effective strategies for harvesting the microalgal biomass since these are low cost processes and no extra flocculants are required for harvesting of the biomass.

Figure 9.5: Viability staining of flocculated cells with trypan blue assay:

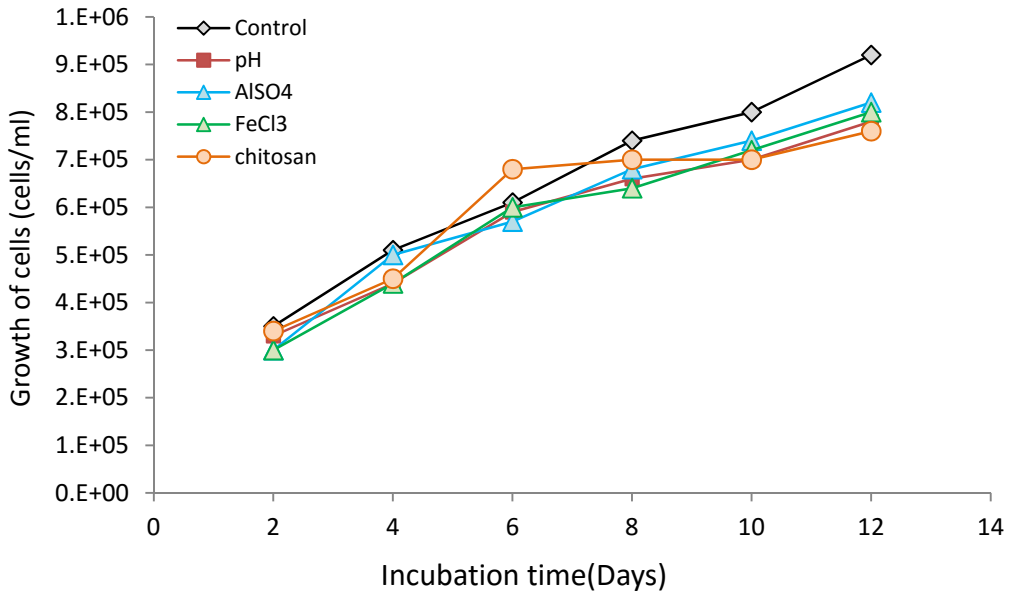


A) Chitosan, B) pH 12, C) $FeCl_3$, D) Auto flocculated cells, E) $AlSO_4$ F)100 mM $FeCl_3$

9.3.6. Recycling of flocculated medium for algal cultivation

Medium recovered from flocculation could preferably be recycled for next round of cultivation. In the flocculation studies performed, the medium was recovered after flocculation and then were supplemented with nutrients (Components of MA medium). The medium pH was adjusted to 6.8-7.0, and was used for algal cultivation so as to evaluate the possibility for medium recycling. *Chlorococcum* sp R-AP13 cells were cultivated in the recycled medium and results are shown in Figure 9.6. It was observed that the cell densities of *Chlorococcum* sp R-AP13 cultivated in the recycled growth medium were close to that cultivated in fresh MA medium, indicating that the residual medium after flocculation and separation of cells could be successfully recycled for cultivation of the alga.

Figure 9.6: Growth of *Chlorococcum sp* R-AP13 in recycled medium



9.3.7. Fatty acids profiling of flocculated biomass

Fatty acids profiling of flocculated biomass was carried out to check any changes in the lipids profile of biomass after the addition of flocculants in the medium. Results showed that fatty acids profile of auto flocculated biomass, pH induced and chitosan mediated flocculated biomass were not affected while the biomass from aluminum sulphate and ferric chloride flocculated cultures showed differences in the fatty acids profile. Some of the fatty acids were

not detected which could be due to the toxic effect of the flocculants. Therefore flocculation induced by auto flocculation or pH are proposed as the effective methods for final application in cell harvesting of *Chlorococcum* sp. R-AP13

Table 9.1: Fatty acid profile of flocculated algal biomass

Fatty Acid Type	Auto flocculation	Chitosan	pH12	$AlSO_4$	$Fecl_3$
C12	3.7	2.8	4.0	1.6	3
C14	2.3	3.2	3.0	-	-
C15	1.7	1.8	3.2	-	1.8
C16	39.7	42	41.2	38	44
C16:1	3.6	2.8	3.69	-	-
C17	2.1	3.7	3.1	-	2.1
C18: 0	8.1	6.2	3.8	18	20
C18: 1	22.0	20	16.5	26	24
C18: 2	3.2	7.8	7.1	12	8
C18:3	7.5	3.1	8.2	8	12
C22	0.0	0.0	0.0	0.0	0.0
C22: 1	5.9	5.83	5.0	-	-
C24	-	-	-	-	-

9.4. Conclusion

Development of economically feasible flocculation technology for microalgal harvesting significantly contributes to cost reduction and energy saving in bulk microalgal biomass production. Chemical flocculation has high efficiency, but may cause contamination in the biomass and the environment. Among the inorganic flocculants, compared to aluminum sulphate, ferric chloride was the most promising, with a flocculation efficiency of 92% at 80 mM concentration. Chitosan showed only 84% efficiency, but is environmentally safe. In these experiments, by raising medium pH did emerge as a feasible strategy with a flocculation efficiency of 94%. Moreover, pH change and auto flocculation does not

contaminate the residual medium allowing its re-use. Therefore, pH-induced flocculation and auto flocculation method could be possible options for cost effective and efficient harvesting of algal cells. The flocculated medium could be reused, thereby minimizing the demand for water and reducing the cost of biodiesel production from algae. Finally, the method was only conducted at laboratory scales and tested to one microalgal strain. Therefore, further research would have to be looked for application development at larger scales and for other algal groups. The self flocculating novel *Chlorococcum* sp R-AP13 can be used for various applications such as biofuels and nutraceuticals.

Chapter 10: Summary and Conclusion

Biodiesel from algal oil is considered as a prime candidate for short to medium term replacement/substitution of petro-diesel. Algal biomass can be of nutritional value, besides being a source of oil that can be converted into biodiesel. The potential advantages of microalgae as oil source and other value added products include, their high growth rate, ability to switch from photo autotrophic to heterotrophic conditions, ability to synthesize and accumulate large amounts of oils and lipids, capability to survive in saline, brackish and sea water, sequestering of CO_2 from flue gas emitted from fossil fuel fired power plants and other sources thereby reducing the green house gases. Photoautotrophic cultivation is considered the most desirable method of culturing microalgae, since this requires only sunlight, CO_2 and water. Heterotrophic growth of microalgae eliminates the requirement for light and so offers the possibility of increasing algal cell density and productivity on a large-scale with cell densities as high as 100 gL-1 achieved. However, the cost of carbon source becomes a limiting factor here, and costs as high as 80 % of the total medium cost has been attributed to the carbon source. Though the yields of oil from microalgae are reported to be 15-300 times more than the traditional crops on area basis, one of the most important concerns is the need to provide substantial amounts of water and nutrients to the algal populations undergoing mass cultivation. Fresh water is a valuable resource for the future and hence algal biomass production in non potable waters like seawater or waste water is highly desirable.

The current study therefore was undertaken to isolate an alga suitably adapted to the climatic regime of tropics and capable of heterotrophic and mixotrophic growth so as to develop processes for biomass and oil production using the alga utilizing cheap carbon sources and non-potable waters. Ten microalgal isolates from Silent Valley National Park in Kerala, India were purified and screened for biomass and oil production in both fresh water and sea water, under phototrophic mode of cultivation. Maximum biomass obtained was 299 ± 12.16 and lipid production was 100 ± 7.42 mg/l respectively. One of the isolates designated as R-AP13 was the best performer in 50% sea water medium producing 161 ± 0.63 mg/l and 67.5 ± 0.98 mg/l biomass and lipid respectively. Based on the biomass and lipid yield, isolate R-AP13 was selected for further studies and was identified as *Chlorococcum sp*. based on 18S rDNA analysis.

Chlorococcum sp. R-AP13 grown phototrophically and heterotrophically in fresh water based medium possessed differences in lipid content and fatty acid composition of the

lipid. Lipid content in heterotrophically grown cells of *Chlorococcum sp*. (47%) was 1.6 fold higher than those grown photoautotrophically (28%). Saturated fatty acids were high in lipids from fresh water cultivated alga (50-56%) and PUFA and MUFA contents were 10-36 % and 6-39% respectively. Experiments conducted for growth in 50% sea water based medium indicated that maximal biomass production was under heterotrophic mode with glucose as carbon source (1.0 g/l), followed by waste glycerol (0.850 g/l). Lipid yield was high in heterotrophically grown cells with waste glycerol as carbon source (330 mg/l). Lipids from heterotrophic cultures contained triglycerides as major lipids. The production of high amount of lipid in waste glycerol containing medium indicated that there is a good potential to develop the alga as a biofuel source through its heterotrophic cultivation. Cultivation on waste glycerol provides added advantage of the value-addition of this by-product from biodiesel industry. Results also indicated that the fatty acid profile of the alga could be altered by the mode of cultivation and carbon source, and this offers an opportunity for modulating the fatty acid content of algal oil. Also since the alga could grow well in 50% seawater, it would be advantageous for mass cultivation due to the lesser requirement of fresh water.

Optimization of mixotrophic cultivation was performed by evaluating different carbon and nitrogen sources, their concentrations, inoculum size and incubation time towards enhanced yield of biomass and lipids. Maximum biomass was achieved when 50% inoculum was used, which resulted 1.4 g/l biomass and 0.3 g/l lipids, respectively. Urea, potassium nitrate, calcium nitrate, ammonium nitrate and ammonium sulfate were used as nitrogen sources for algal cultivation. Among these, urea and potassium nitrate supported significant biomass and lipid production under mixotrophic conditions, yielding 970 and 915 mg/l biomass (210 and 200 mg/l lipids), respectively. Various concentrations of urea (0.2 – 1.0 g/l) were evaluated for biomass and lipids production by the alga. Maximum biomass and lipid was obtained with 0.4 g/l of urea as N source. Biomass production was 1.28 g/l and lipid yield was 315 mg/l. Effect of various concentrations of glucose (2-10%) and waste glycerol as carbon sources were evaluated for biomass and lipid production by the alga. Maximum biomass was obtained from 6.0% of glucose (1.72 g/l biomass and 710 mg/l lipids) and glycerol as carbon source (0.8 g/l and 273mg/l), respectively. Saturated fatty acid production was elevated under mixotrophic condition.

Mixotrophic and heterotrophic growth and lipid production by *Chlorococcum* sp RAP-13 was evaluated in dairy effluent (DE) as medium. Mixotrophically grown cells produced more biomass and lipids (0.87 and 0.25 g/l respectively). Biomass and lipid production was enhanced under heterotrophic condition, when the percentage of waste

glycerol supplemented as additional carbon source increased. Maximum biomass and lipid production (1.94 and 0.82 g/l, respectively) was obtained in DE with 6 % waste glycerol supplementation. Saturated fatty acids production was enhanced in the waste water medium. Algal growth in DE could also reduce significantly the organic pollution load. BOD and COD removal were 82 and 93 %, respectively. Dual use of microalgal cultivation for wastewater treatment and production of value added compounds/biofuel could be an attractive option, in terms of reducing the energy cost, and the nutrient and freshwater resource costs.

Acid pretreatment liquor (APL) from the lignocellulosic ethanol pilot plant was used as medium for biomass and lipid production using *Chlorococcum* sp R-AP13 under mixotrophic condition. The alga utilized C5 (xylose, arabinose) and C6 (glucose, galactose) sugars present in APL as carbon sources, and could survive in the presence of inhibitors like furans and sugar breakdown products. *Chlorococcum sp.* R-AP13 utilized almost completely the sugars and the inhibitors -furfural and HMF in the APL during its growth and produced 1.8 g/l of biomass. The lipid yield was 0.568 g/l when supplemented with agricultural grade NPK (18:18:18) fertilizer mixture. FAME profile of fatty acids indicated that the oil was rich in oleic acid (18.1) with~ 45 % of the oil being this fatty acid, followed by palmitic acid (16.1) and stearic acid (18.0) which formed respectively ~30 and 15 % and of the oil. Biodiesel (FAME) was synthesized from the algal oil using acid catalysis and was evaluated for its physicochemical properties and compatibility for biodiesel specifications using BiodieselAnalyzer® software which indicated its compliance to EN 14214 and ASTM D6751. Ability of the alga to grow in biomass acid pretreatment liquor is particularly interesting for value addition of this byproduct which is otherwise difficult to process. Cultivation of microalgae that can grow and produce oil in this medium would be immensely beneficial to the biorefineries since it will address both waste water treatment and value addition of the resource.

One of the major challenges in micro algal cultivation is the harvesting of biomass. 20- 30% of production cost is involved in biomass harvesting in the microalgal industry. Therefore studies were performed on flocculation of *Chlorococcum* sp.R-AP13. Chitosan, aluminum sulfate, variation in medium pH and ferric chloride were evaluated as flocculation agents. Results showed that higher concentration of flocculent led to decreased negative charges on the cell surface, which reduced flocculation efficiency. Chitosan and aluminum sulfate showed maximum efficiencies of 84 and 87% respectively while $FeCl_3$ and alkaline pH showed maximum efficiencies of 92% and 94% respectively. High concentration of $FeCl_3$

and $AlSO_4$ was toxic to the cells. Flocculation induced by pH increase was effective for harvesting microalgae. Reusability of residual medium after flocculation was demonstrated and there were no significant differences in cell growth between fresh and recycled media.

From these studies the novel microalga *Chlorococcum* sp.R-AP13 was demonstrated as a very potent organism to be used for biomass and oil production. Ability to grow both phototrophically and heterotrophically in sea water, industrial waste waters and most importantly on biodiesel industry waste glycerol and the acid pretreatment liquor from lignocellulosic biorefinery under mixotrophic conditions indicated the importance of this novel isolate as a potent source of algal oil, the composition of which may be modulated by adjustments in growth mode and conditions. The biomass yield under mixotrophic growth in APL was close to 2g/l and lipid yield was 570mg/l which are significant quantities considering the nature of the effluent. Till date there are no reports on cultivation of microalgae in acid pretreatment liquor.

The culture could also synthesize the essential fatty acids like DHA and EPA which are high value compounds with nutraceutical importance while being cultivated mixotrophically in waste glycerol. This is also important as it opens up the possibility of producing high value metabolites using a waste stream thereby adding value to it.

Considering the biomass production, lipid content and fatty acid composition of *Chlorococcum sp* R-AP13, in the industrial waste streams like dairy waste water, biorefinery effluent and biodiesel industry waste glycerol, the alga can be considered as efficient in growth and lipid production using no-potable waters and holds potential for future exploitation as an economic means of oil production for biodiesel and nutraceutical industries.

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APPENDIX

LIST OF ABBREVIATIONS

#	Number
%	Percentage
°C	Degree Celsius
µ	Micron/Micrometer
µg	Microgram
µl	Micro liter
APHA	American Public Health Association
APL	Acids pretreated liquor
ASTM	American Society for Testing and Materials
BDWG	Biodiesel derived waste glycerol
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical oxygen demand
bp	Base Pair
COD	Chemical oxygen demand
CSIR	Council for Scientific and Industrial Research
DCW	Dry cell weight
DE	Dairy effluent
DHA	Docosa hexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy Ribonucleotide triphosphate
DWW	Dairy waste water
EDTA	Ethylene Diamin Tetra Acetic acids
EPA	Eicosa pentaenoic acid
FAME	Fatty acids methyl esters
FID	Flame ionization detector
Fig	Figure
GC	Gas chromatography

HMF	Hydroxy methyl furfural
HPTLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
L	Liter
mg/L	Milligram per liter
mM	Millimolar
mV	Microvolt
NIIST	National institute for Inter Disciplinary Science and Technology
nm	nanometer
NREL	National Renewable Energy Laboratory
PCR	Polymerase chain reaction
PUFA	Poly unsaturated fatty acid
rpm	Rotation per minutes
RS	Rice straw
SB	Sorghum biomass
SEM	Scanning electron microscopy
SFA	Saturated fatty acids
sp	Species
TAG	Triacylglycerides
USFA	Unsaturated fatty acids
UV	Ultra violet
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight