BIOMASS AND BIOFUEL CHARACTERIZATION OF SOME MICROALGAL SPECIES OF ASSAM, INDIA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

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ABSTRACT

A total of twenty one microalgal strains were isolated from various water bodies of Assam and three of them were selected as potential biofuel source based on calorific value. Initially the microalgal isolates were cultured in Knops medium. Thereafter four culture media like Basal, BBM, MC-13 and BG-11 were used for the culture of microalgae for a period of 15 days. The optimized growth conditions for the microalgal isolates were determined with respect to pH and temperature. Morphological characterization of the isolates with compound and scanning electron microscopy depicts spherical shape of *Chlorella* sp and *Parachlorella kessleri* whereas, elongated concave shape of *Scenedesmus* sp. Thermo-gravimetric analysis was done at variable heating rates of 10°C/min and 30°C/min in order to study the degradation profile of the microalgal biomass. Shorter biomass degradation profile of the microalgal isolates is indicative of their credible prospects in thermo-chemical conversion. The major absorption bands as revealed by FTIR spectroscopy for the microalgal biomass were 3100-2800 cm^{-1}, 1800-1500 cm^{-1} and 1200-900 cm^{-1}, respectively which corresponded to lipid, protein and polysaccharides moieties in the microalgal biomass[1].

Heavy metals like Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+} and Cr^{2+} were supplemented in varying concentrations of 0.5, 1 and 2 mM in the culture medium of the microalga *Scenedesmus* sp. and the resultant biomass was subjected to FTIR spectroscopy to assess the lipid content. No, marked difference was observed in the spectra of the treated biomasses except in the case of Pb^{2+}.

One bloom forming microalgae *P. kessleri* was cultured in collected water samples from three eutrophic water bodies earmarked at the bank of the river Luit (Brahmaputra), Dhemaji, Assam. The growth kinetics of the species was monitored in open laboratory conditions which revealed the feasibility of mass culture of *P. kessleri* directly in the aforesaid water bodies.

Properties like calorific value, elemental content, proximate content and biochemical content of the microalgal biomass were determined in order to investigate their potential as bioenergy feedstocks. All biomasses were found to be potential for

bio and thermo-chemical conversions. Biodiesel was derived from the microalgal species and a yeast strain *Saccharomyces cerevisiae* by *in situ* transesterification. GC-FID/GC-MS analysis was carried out to determine their FAME content. All species showed variable FAME composition. *Chlorella* sp. and *P. kessleri* biodiesel mostly comprised of oleic acid (C18:1) with the percent composition 65.84% and 77.81%, respectively. Eicasopentanoic acid (C20:5), one of commonest poly unsaturated fatty acids (PUFA) was present as a constituent in *P. kessleri* and *S. cerevisiae* biodiesel with 1.85% and 2.06%, respectively. *Scenedesmus* sp. biodiesel possessed a high proportion of PUFA (57.53%).

The fuel properties like density, viscosity, calorific value and cetane number of the microbial biodiesel were determined and compared with ASTM specifications. *S. cerevisiae* biodiesel had a viscosity of 5.797 mm^2/s which was slightly higher than the ASTM specification. *S. cerevisiae* biodiesel also possessed a high cetane number (71.58).

Bio-oil from *P. kessleri* de-oiled cake was subjected to FTIR analysis for assessing the functional groups present in the aqueous and organic phases. Over 50 peaks were observed in the TIC of the bio-oil. ^1H NMR spectroscopic analysis of *P. kessleri* bio-oil was done to examine the complete intact pyrolytic bio-oil rather than a selected fraction. The integrated regions in the ^1H NMR spectra were from 0.5-1.5, 1.5-3, 3-4.5, 4.5-6, 6-8.5 and 8.5-10 ppm, respectively. The spectra showed higher proportion of resonating protons in the integrated region from 1.5-3 ppm. Higher aliphatic content in the bio-oil is indicative of high energy content.

The 18S rRNA gene was amplified using ITS primers and sequenced. All the microalgal sequences were deposited in NCBI Genbank with specific accession numbers[2]. BLAST[3] search was performed for the microalgal isolates and their identification was done on the basis of BLAST results. Homologous search of 18S-rDNA sequence of the microalgal strains was also done to find their similarity with other species of the same genus. From the BLAST results a maximum identity score of 91% was observed in the case of *Scenedesmus* sp. MPBK-2 and 99% in the case of *Scenedesmus obliquus* isolate IB-05. The nucleotide sequence of *P. kessleri* FR865655 was found to be similar with that of *P. kessleri* MMPBKK-1 with a maximum identity

score of 99% whereas, *Chlorella* sp. MP-1 showed a maximum identity score of 99% with that of *Chlorella* sp. AF514413. *P. kessleri* MMPBKK-1 and *Scenedesmus* sp. MPBK-2 belonged to the same cluster as that of *Parachlorella* and *Scenedesmus* species with a bootstrap value of 100. *Chlorella* sp. MP-1 belonged to the same cluster of the genus *Chlorella* with a bootstrap value of 87.

Transesterification of kitchen chimney dump lard (KCDL) was done as a reference to the biodiesel production from the microalgal isolates. A two stage transesterification scheme was used for the production of biodiesel. The biodiesel yield was optimized by response surface methodology (RSM). The fuel properties of the KCDL derived biodiesel like viscosity, density, calorific value, cloud point, pour point and cetane number were determined. The FTIR spectra of the KCDL feedstock and its biodiesel were found to be similar since both had almost the same chemical groups. The main absorption for the KCDL derived biodiesel was in the order of 2928, 2856 and 1748 cm^{-1}. The saturated and unsaturated FAME in the biodiesel sample were 41.86 and 58.14% respectively. The KCDL derived biodiesel possessed high density and viscosity with an appreciable calorific value.

The *P. kessleri* bio-oil was found to be ineffective against *C. albicans* and *S. cerevisiae*, whereas moderately effective against *E. coli* and *S. aureus* (12 mm ZOI).

A positive correlation was observed between % DPPH scavenging and total phenolic content in the deoiled cake extracts of *Chlorella* sp. with 0.739 (R^2); *P. kessleri* 0.511 (R^2) and *Scenedesmus sp.* 0.558 (R^2), respectively. On the contrary, negative correlation was observed between FRAPs and TPC of the deoiled cake extracts of *Chlorella* sp. (R^2= -6.41), *Scenedesmus* sp. (R^2= - 9.55) and *P. kessleri* (R^2= -9.19). The aqueous extract of all the tested samples showed the highest % DPPH scavenging potential whereas methanolic extract showed the lowest. Among all the tested extracts, methanolic extract of *Scenedesmus* sp. deoiled cakes possessed the highest FRAP activity whereas the aqueous extract of *Chlorella* sp. deoiled cake the lowest.

The molecular docking studies revealed that the compounds inhibit the bacterial and fungal enzymes (PDB ID: 1AC4, 1AV8, 1T2P, 1ZAP, 2QDF, 4JQC)

exhibiting strong molecular interaction and hydrogen bonding at the active site of the corresponding enzyme.

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DEDICATION

Dedicated to late Dr. Durgeshwar Phukan (Father)

&

Mrs. Mrinalini Buragohain Phukan

(Mother)

with love and respect.

Mayur Mausoom Phukan

DECLARATION

I do hereby declare that the thesis entitled "***Biomass and biofuel characterization of some microalgal species of Assam, India***", being submitted to the Department of Molecular Biology and Biotechnology, Tezpur University, is a record of original research work carried out by me. All sources of assistance have been assigned due acknowledgment. I also declare that neither this work as a whole nor a part of it has been submitted to any other University or Institute for any other degree, diploma or award.

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This is certify that the thesis entitled "**BIOMASS AND BIOFUEL CHARACTERIZATION OF SOME MICROALGAL SPECIES OF ASSAM, INDIA**" submitted to the school of Science, Tezpur University in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research carried out by Mr. Mayur Mausoom Phukan under my supervision and guidance.

All help received by him from various sources have been duly acknowledged.

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This is to certify that the thesis entitled **"BIOMASS AND BIOFUEL CHARACTERIZATION OF SOME MICROALGAL SPECIES OF ASSAM, INDIA"** submitted to the Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science in partial fulfillment for the award of the Degree of Doctor of Philosophy in Science, has been examined by us on 04/05/15 and found to be satisfactory.

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List of abbreviations

BBM = Bold basal medium

MC-13 = Modified Chu-13 medium.

BG-11 = Blue green-11 medium.

PMCS = Prospective mass culture sites.

FTIR = Fourier transformed infra-red spectroscopy.

KBr = Potassium bromide.

GCV = Gross calorific value.

NCV = Net calorific value.

GC-FID = Gas chromatography- flame ionization detector.

TMG = 1, 1, 3, 3- Tetramethylguanidine

FAME = Fatty acid methyl ester.

MUFA = Mono unsaturated fatty acid.

PUFA = Poly unsaturated fatty acid.

GC-MS = Gas chromatography mass spectroscopy.

PCR = Polymerase chain reaction.

BLAST = Basic local alignment search tool.

TG-DTG = Thermogravimetric - Differential thermogravimetric analysis.

DNA = Deoxyribonucleic acid.

rRNA = Ribosomal ribonucleic acid.

rpm = Rotations per minute.

ppm = Particles per million.

SEM = Scanning electron microscopy.

ASTM = American society for testing and materials.

TIC = Total ion chromatogram.

NMR = Nuclear magnetic resonance spectroscopy.

EtBr = Ethidium bromide

NCBI = National centre for Biotechnology information.

KCDL = Kitchen chimney dump lard.

RSM = Response surface methodology.

BPCDC = Bio-oil from *P. kessleri* deoiled cake

ZOI = Zone of inhibition.

DPPH = 2, 2-diphenyl-1-picrylhydrazyl.

TPC = Total phenolic content.

FRAP = Ferric reducing antioxidant potential.

PLP = Piecewise linear potential.

MVD = Molegro virtual docker

Chapter 1

Introduction

Chapter 1: Introduction

The term algae have no formal taxonomic standing and is routinely used to indicate a polyphyletic (including organisms that do not share a common origin, but follow multiple and independent evolutionary lines), non-cohesive, and artificial assemblage of oxygen-evolving, photosynthetic organisms (with several exceptions of colorless members undoubtedly related to pigmented forms [1]. Algae are recognized as one of the oldest life forms [2]. They are 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 [3]. Time and again various workers have attempted to classify algae. As of now there is no one definable classification system for algae. Algae can be both micro and macro ranging in size from single unicells to multicellular forms. The term microalgae encompass all unicellular oxygen evolving photosynthetic microorganisms (up to 200μm in size) inhabiting almost every aquatic environment on Earth like fresh water springs, salt lakes and oceans. Microalgae are generally free living, but they may establish symbiotic association with a variety of other organisms [4]. In applied phycology, the term microalgae refer only to the microscopic eukaryotic algae, and the oxygenic photosynthetic cyanobacteria [4]. The three most abundant classes of microalgae on basis of abundance are diatoms (Bacillariophyceae) green microalgae (Chlorophyceae) and the golden algae (Chrysophyceae) [5]. On the other hand macroalgae (generally called seaweeds) are multicellular algae which can grow both in fresh and salty water. Macroalgae are fast growing species and can reach sizes of up to 60m in length [6]. Macroalgae are broadly classified into three distinct groups on the basis of their pigmentation: Phaeophyceae (brown seaweed), Rhodophyceae (red seaweed) and Chlorophyceae (green seaweed). While considering the general term algae the number of species has been estimated to be between one to ten million, most of which are microalgae [1]. Table 1.1 presents a general scheme for classification of algae.

Algae can either be autotrophic or heterotrophic; the former require only inorganic carbon sources like CO_2, salts and a light energy source for growth, whereas

the later are non photosynthetic and therefore require an external source of organic compounds as well as nutrients as an energy source [7]. Algae are one of the most efficient captors of sunlight. Some photosynthetic algae are mixotrophic i.e. they have the ability to carry out both photosynthesis and acquire exogenous organic nutrients [3].

Table 1.1 A general scheme for classification of algae [1]

Kingdom	Division	Class
Prokaryota eubacteria	Cyanophyta	Cyanophyceae
	Prochlorophyta	Prochlorophyceae
	Glaucophyta	Glaucophyceae
	Rhodophyta	Bangiophyceae
		Florideophyceae
	Heterokontophyta	Chrysophyceae
		Xanthophyceae
		Eustigmatophyceae
		Bacillariophyceae
		Raphidophyceae
		Dictyochophyceae
		Phaeophyceae
	Dasycladophyceae	Haptophyceae
	Cryptophyta	Cryptophyceae
Eukaryota	Dinophyta	Dinophyceae
	Euglenophyta	Euglenophyceae
	Chlorarachniophyta	Chlorarachniophyceae
	Chlorophyta	Prasinophyceae
		Chlorophyceae
		Ulvophyceae
		Cladophorophyceae
		Bryopsidophyceae
		Zygnematophyceae
		Trentepohliophyceae
		Klebsormidiophyceæ
		Charophyceae
		Dasycladophyceae

1.1 Applications of microalgae

Some of the thrust areas of microalgal applications include animal and human nutrition, cosmetics, high value molecules such as fatty acids and pigments as well as natural dyes [8]. Microalgae and cyanobacteria have a long history of use as food [9]. Edible blue-green algae including *Nostoc, Arthrospira (Spirulina)* and *Aphanizomenon* species have been used for food for thousands of years [10]. In the 14th century the Aztecs of Mexico harvested *Arthrospira* from Lake Texcoco and used to make a sort of dry cake called *tecuitlatl*, and very likely the use of this cyanobacterium as food in Chad dates back to the same period, or even earlier, to the Kanem Empire (9th century A.D) [1]. Some species of microalgae are suitable for preparation of animal feed supplements. Microalgal species such as *Scenedesmus, Chlorella* and *Spirulina* has numerous beneficial aspects including improved immune response, better weight control, improved fertility, healthier skin and a lustrous coat [11]. Microalgal species that are cultivated commercially for hatcheries in the aquaculture field include *Chaetoceros, Crypthecodinium, Isochrysis, Nannochloris, Nitzschia, Schizochytrium, Tetraselmis,* and *Skeletonema* [12].

Microalgae are a major natural source of valuable macromolecules like carotenoids, poly unsaturated fatty acids, phycocolloid etc. The production of asthaxanthin (carotenoid pigment and an excellent antioxidant) from *Hematococcus* and β-carotene (precursor of vitamin A) from *Dunaliella* are now already well established commercial processes [13]. Many algal species are rich in omega-3-fatty acids (brain nutraceuticals) and as such are used as diet supplements and components of livestock feed [14] long with the source of iron, potassium, iodine, magnesium and calcium [15] that makes microalgae as ideal sources of nutrients for functional food preparations, food additive or in nutraceuticals [16]. The development of active biomolecules from microalgae is another speedily emerging area of interest [17].

Microalgae can be used to trap carbon dioxide from atmosphere, emissions from power plants and industrial processes and from soluble carbonate (Na_2CO_3 and $NaHCO_3$ [18]. The capability of algae to release oxygen from water and utilize light energy with high efficiency spiked early interest in algal life support systems [19]. They may potentially be used in algal life support systems for absorbing carbon dioxide and replacing it with oxygen in confined atmospheres such as those in space stations [20]. Microalgae are of special importance with regard to bioremediation of organic and inorganic compounds. Microalgae can hyper accumulate heavy metals from waste water [21] and some are capable of degrading polyaromatic hydrocarbons and other organics [22, 13]. Microalgae play a very important role in waste water treatment; they enhance the removal of nutrients, organic contaminants, heavy metals and pathogens from domestic waste water besides furnishing an interesting raw material for production of high value chemicals or bio-gas [23]. Many species of blue green algae are recognized as important biofertilizers. Nitrogen fixing blue green algae can reduce the chemical nitrogen fertilizer by 15% and can also add up to 20-30% of biologically fixed nitrogen per season per hectre [24]. Biomass conversion processes like pyrolysis results in the production of bio-char that has prospective agricultural application as biofertilizers. Some algal species can be used as an organic fertilizer either in raw or semi decomposed form in land [25]. In recent years attempts have been made to develop composite materials using microalgae (*Chlorella vulgaris*) as filler in various polymers such as polypropylene, PVC, polystyrene and polyethylene [26].

Microalgae have also attracted tremendous attention as a feedstock for biofuel production. They are promising biomass species that can serve as feedstock for the upcoming biofuel industry. Production of lipids from oleaginous microalgae offers tremendous yield advantage relative to oilseed crops with the option for cultivation on non-arable land with non-potable water sources [27].

1.2 Choice of microalgae for biofuel production

Even ages and centuries before the systematic study of Microbiology, mankind has exploited microbial processes for his own benefit. But it was only in the preceding century when he was enlightened upon with the idea "use of micro-organisms to work for him on a larger scale". One such micro-organism that has drawn the attention of mankind and has been the topic of considerable scientific interest over the past few decades is microalgae. Microalgae, the ancestors of plants are an assemblage of chlorophyll bearing autotrophic thallophytes of microscopic dimensions inhabiting diverse environments. They are sunlight driven biochemical factories which convert carbon dioxide into potential biofuels, foods, feeds and high value bio-actives [9, 28-33]. They are a major natural source for a spectrum of valuable compounds, including a diversity of pigments, for which these photosynthetic micro-organisms represent an almost exclusive biological resource [34]. Microalgae (inclusive of cyanobacteria) are photosynthetic micro-organisms that are responsible for at least 50% of the photosynthetic biomass production on Earth [13]. In wake of advancements in bioenergy research microalgae have gained tremendous scientific attention as a bio-energy feedstock. Microalgae have been studied extensively on account of their various potential advantages for biofuel production when compared to terrestrial energy crops. The superior attributes of microalgae in this direction are

1) Biomass doubling times in microalgae during exponential growth are commonly as short as 3.5 h [33]
2) The lipid content in microalgae can be adjusted through altering growth media composition [35]
3) Atmospheric carbon dioxide is the source of carbon for growth of microalgae [36].
4) Salty or waste water can be used for the culture of microalgae [36].

5) Owing to their simple cellular structure algae have higher rates of biomass and oil production than conventional crops [37]
6) Oil content in microalgae can exceed 80% by weight of dry biomass [33].
7) Microalgae can be harvested batch wise nearly all year around thereby providing a reliable and continuous supply of oil [36].
8) Depending upon the species microalgae are known to produce many kinds of lipids, hydrocarbons and other complex oils [38].
9) Biofuel production from algae can be coupled with flue gas carbon dioxide mitigation, waste water treatment and production of high value chemicals [39]
10) Algae can produce 30-100 times more energy per hectare as compared to terrestrial crops [39]

Table 1.2 Oil content in some microalgal species

Microalga	Oil content (% dry wt.)
Schizochytrium sp	50-77
Botrycoccus braunii	25-75
Nannochloropsis sp	31-68
Neochloris oleoabundans	35-54
Nitzschia sp	45-47
Cylindrotheca sp.	16-37
Nannochloris sp	20-35
Isochrysis sp	25-33
Chlorella sp	28-32
Phaeodactylum tricornutum	20-30
Dunaliella primolecta	23
Tetselmis suecica	15-23
Crypthecodinium cohnii	20
Monollanthus salina	>20

1.3 The US Department of Energy's Aquatic Species Program (ASP)

With the mission of developing renewable transportation fuels from algae the US Department of Energy's Office of Fuels Development launched the Aquatic Species Program (ASP) in 1978. This research program investigated the production of biodiesel from oleaginous algae grown in ponds, utilizing waste carbon dioxide from coal fired power plants [40]. Over an eighteen years period from 1978-96 considerable progress was made with regard to engineering of algae production systems and manipulation of algal metabolism. The ASP was discontinued in 1996 due to budget cuttings, but the salient findings and technical highlights of this research program are available in the form of a status report (A Look Back at the U.S. Department of Energy's Aquatic Species Program—Biodiesel from Algae, July 1998). The two decade long research findings of the ASP has served as a first-rate blue print for futuristic microalgal biofuel research and empowered researchers to have an overall grasp of the research matters from a higher perspective, apart from facilitating them to take advantage of the hitherto potential of algal biomass as feedstocks for bio-energy generation.

The achievements of the ASP [41] were

- Establishment of a collection of 300 species (mostly Chlorophyceae and Bacillariophyceae), having high content of oils in Hawaii; some species were capable of growth under extreme conditions of temperature, pH, and salinity.
- A greater understanding of the physiology and biochemistry of intracellular oil accumulation, more particularly, the complex relationships among nutrient starvation, oil content, cell growth rate, and overall oil productivity.

- Marked advances in the molecular biology and genetics of algae, including the first isolation from a photosynthetic organism of the gene encoding acetyl-CoA carboxylase, the first step towards fatty acid biosynthesis.

- The development of large-surface-area ($1000m^2$) pond systems capable of utilizing 90% of the injected CO_2.

1.4 Renewable biofuels from microalgae

The concept of using microalgae as a source of fuel is not new [42] but presently is being taken more seriously because of the escalating petroleum prices and, more importantly, the emerging concern about global warming associated with burning of fossil fuels [43]. The production of methane from algal biomass was studied at Berkeley, USA during 1950s, and an initial systems analysis for open ponds published [44]. The energy shocks of the 1970's sparked renewed interest in microalgal biofuels, predominantly hydrogen and methane in combination with waste water treatment [45]. There are considerable scientific endeavors underway worldwide to investigate the feasibility of renewable biofuel production from microalgae. The most prominent renewable biofuels in this regard are methane produced by anaerobic digestion of algal biomass [32] biodiesel from microalgal oil [33, 40, 46-48] photobiologically produced bio-hydrogen [49-52] and bio-oil produced by pyrolysis [53, 54]. The production of biofuels from microalgae is technically feasible and is of worldwide interest. Renewable biofuels from microalgae undeniably seems to be promising in context of the existent energy crisis, but however a crucial obstacle to their successful commercial implementation is the comparatively cheaper rates of conventional petro fuels.

1.5 Biodiesel from microalgae

In August 1893, Rudolf Diesel tested peanut oil as a fuel for his engine for the first time [55]. Rudolf Diesel envisaged that "although the practical applicability of

vegetable oils for fuel engines was unimportant during his days, the day was not far when they would be as important as petroleum". This foresightedness of Diesel became a reality after the Arab embargo of the 1970's. The energy crisis of the 70's triggered extensive research endeavors to investigate vegetable oils as a possible substitute for petrodiesel. Conventionally biodiesel is mostly produced from plant oils such as corn, canola, soybean, rape seed, palm oil, *Jatropha*, *Pongamia*, coconut, ground nut, sunflower, mustard etc. But none of these aforementioned feedstocks can even credibly satisfy a fraction of the existing demand for biodiesel. Moreover, the conundrum of the Food Vs Fuel debate has geared up the search for newer, sustainable, cost effective and environmentally benign feedstocks for biodiesel production. A possible exception that may roll up the dice in favor of sustainability in near future is biodiesel production from microalgae.

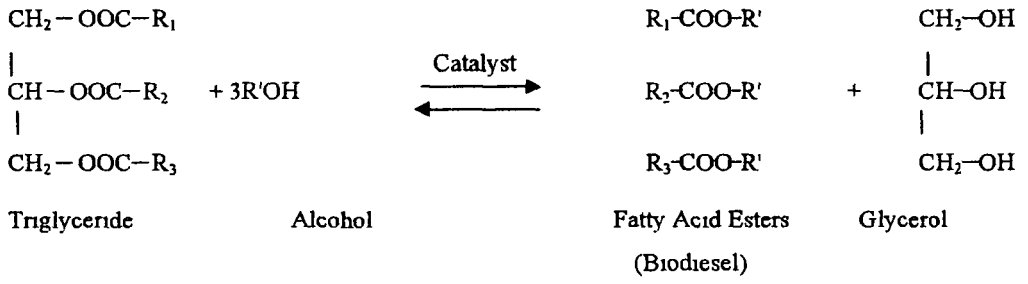
Over the past few decades microalgae have been the center of research attention as an emerging feedstock for biodiesel production. Microalgal biodiesel research is now one of the top notch research topics especially in the context of escalating petro fuel prices and climatic changes. Microalgae have an exceptionally fast growth rate in comparison to terrestrial energy crops and moreover a significant percentage of their weight is comprised of oil. This microalgal oil following extraction can directly be converted into biodiesel which is renewable and an environmental friendly biofuel. Theoretically microalgae offer strong candidature as a promising feedstock for biodiesel production. The per-unit area yield of oil from algae is estimated to be between 20,000 and 80,000L/acre/year; which are 7 to 31 times greater than the next best crop, palm oil [56]. Microalgae have been proposed to be the only source of renewable biodiesel that is capable of meeting the global demand for transportation fuels [33]. Table 1.3 presents a list of startup companies attempting to commercialize microalgal biofuels.

Table 1.3 Startup companies attempting to commercialize algal biofuels [57]

Company	Website	Location
Algenol Biofuels	www.algenolbiofuels.com	Bonita Springs, FL, USA
Aquaflow	www.aquaflowgroup.com	Nelson, New Zealand
Aurora Algae, Inc	www.aurorainc.com	Hayward, CA, USA
Bioalgene	www.bioalgene.com	Hayward, CA, USA
Bionavitas, Inc	www.bionavitas.com	Hayward, CA, USA
Bodega Algae, LLC	www.bodegaalgae.com	Boston, MA, USA
LiveFuels, Inc	www.livefuels.com	San Carlos, CA, USA
PetroAlgae Inc	www.petroalgae.com	Melbourne, FL, USA
Phyco Biosciences	www.phyco.net	Chandler, AZ, USA
Sapphire Energy, Inc	www.sapphireenergy.com	San Diego, CA, USA
Seambiotic Ltd	www.seambiotic.com	Tel Aviv, Israel
Solazyme, Inc	www.solazyme.com	South San Francisco, CA, USA
Solix Biofuels, Inc	www.solixbiofuels.com	Fort Collins, CO, USA
Synthetic Genomics Inc	www.syntheticgenomics.com	La Jolla, CA, USA

1.6 Microalgal biodiesel synthesis via transesterification

Transesterification (also called alcoholysis) is the chemical conversion of oil into its corresponding fatty ester (biodiesel) The transesterification reaction involves the displacement of alcohol from an ester by another alcohol in a process very similar to hydrolysis, except that an alcohol is employed in place of water as depicted below [58].



where, R_1, R_2, R_3 are the fatty acid chains. The main product of the transesterification process is biodiesel and glycerol is another important byproduct produced which can be burnt for heat or can be used as a feedstock in cosmetic industries [59].

Conventionally, biodiesel is produced from microalgae by the transesterification process. The process of making biodiesel occurs as follows [56]:

1) Triglycerides, alcohol (mostly methanol) and catalyst (acidic or alkaline) are placed in a controlled reaction chamber to undergo transesterification.
2) The initial product is placed in a separator to remove the byproduct, glycerine.
3) The excess methanol is removed from the methyl esters via evaporation
4) The final biodiesel is rinsed with water, neutralized and finally dried

Methanol is the most commonly used alcohol for the transesterification process due to its low cost, polar nature and short chain length, but however other alcohols like ethanol, butanol, propanol and amyl alcohol may also be used. The transesterification reaction can be catalyzed by alkalis (NaOH, KOH, carbonates and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide), acids (sulphuric, phosphoric, hydrochloric and organic sulfonic acids) and even biocatalyst (lipases). Alkali catalyzed transesterification reaction is about 4000 times faster than the acid catalyzed reaction and as such most often used commercially [58]. The chemically catalyzed transesterification reaction despite having appreciable conversion levels within short reaction time has numerous drawbacks viz., it is energy intensive, recovery of glycerol is challenging, the alkaline or acidic catalyst has to be removed from the product, alkaline waste water requires treatment and moreover free fatty acids and water interfere with the reaction [60]. As such, biocatalysts are now becoming an increasingly attractive catalyst option for transesterification because they can overcome many of these constraints. Lipases (triacylglycerol ester hydrolases, EC

3.1.1.3) are biocatalysts which are efficiently capable of catalyzing the transesterification reaction. In lipase catalyzed transesterification reaction the glycerol byproduct can be recovered without any complex process, and furthermore free fatty acids contained in fats and waste oils can completely be converted into methyl esters [58]. Low temperature is employed in lipase catalyzed transesterification reaction for preventing loss of lipase activity which in the other way round is important in reducing the economics of biodiesel production. Lipases offer significant prospects for the biodiesel industry but as of now the production cost of lipase catalyst is the major impediment for its extensive commercial deployment.

1.7 Bio-oil from microalgae

Production of biofuels from microalgae has been an area of intensive scientific focus over the past few years. But however, currently a significant fraction of the research attention in microalgal biofuel research is focused on biodiesel production from microalgae. There are tons of scientific literatures available in the scientific repository which specifically pertains to the production of biodiesel from microalgae. Biodiesel production from microalgae involves the extraction of lipids, following which the microalgal deoiled cake or remnants are left. How to best use these microalgal remnants is one of the greatest challenges for algal biorefineries [61]. One viable option in this regard which would be instrumental in reducing the economics of feedstock utility is pyrolysis of microalgal remnants (residue or deoiled cake) to obtain renewable bio-oil and other value added products. A few studies done in this direction Pan *et al.* [54], Wang *et al.* [62] warrants the feasibility of bio-oil production from microalgal remnants.

In the wake of recent advancements in bioenergy research pyrolytic bio-oils have already gathered the attention of the scientific community in that they offer potential candidature not only as a chemical feedstock but also as an increasingly attractive fuel option, but however it is interesting to note that there is paucity of

scientific information about pyrolysis of direct microalgal biomass or its remnants in comparison to the pyrolysis of lignocellulosic biomass. A few studies done in this regard Du *et al.* [63], Miao *et al.* [64] and Miao and Wu [53] suggest that pyrolysis of microalgae can produce bio-oil which in some respect is superior to bio-oil obtainable via pyrolysis of lignocellulosic biomass [63].

A major drawback of microalgal biomass as a pyrolysis feedstock is the high nitrogen content in the bio-oil product. Becker [65], reported that most of this nitrogen is present as protein in fast growing autotrophic microalgae. Other nitrogenous constituents of microalgae include nucleic acids, chlorophyll, glucosamides and cell wall materials although at comparatively low levels (less than 0.6 wt%) when compared to protein (10 wt%) [65, 66].

1.8 Bioethanol from microalgae

Over the years microalgae have been extensively investigated as a feedstock for biofuel production, and bioethanol production in this regard is no exception (although to a lesser extent in contrast to microalgal biodiesel). There has been an incredible surge in research to investigate the utilization of microalgae as an advanced energy feedstock for bioethanol production [67]. The interest in microalgae as a feedstock for bioethanol production is because of the fact that they do not require arable land for cultivation and as such do not contribute to the Food Vs Fuel debate as is the case with current bioethanol crops such as sugarcane, soybean and corn. Microalgae are reported to store considerable amounts of carbohydrates in the form of starch/ cellulose, glycogen, pentoses and hexoses which can be converted into fermentable sugars for bioethanol production via fermentation [68]. Bioethanol can be produced from microalgae by using amylolytic enzymes which facilitate starch hydrolysis and subsequent formation of fermentable sugars. Following fermentation of these sugars they can be distilled using distillation technology to obtain anhydrous bioethanol.

Although there is paucity of scientific literature with regard to bioethanol production from microalgae the process offers certain distinct advantages. Algal fermentation processes involves less energy intake and the process is much simple in contrast to biodiesel production system, moreover carbon dioxide produced by fermentation process can be recycled as carbon sources for microalgae cultivation, thereby reducing greenhouse gas emissions as well [69].

In today's scenario much of the scientific attention in microalgal biofuel research is concentrated in the production of biodiesel from microalgae. A large scale production scheme for biodiesel production from oleaginous microalgae would generate enormous quantum of microalgal remnants. Conversion of these microalgal remnants (mainly comprising of carbohydrates and proteins) into bioethanol may be an attractive option in this regard. However production of bioethanol from microalgae is at its infantile stage and warrants further scientific investigation.

1.9 Microalgal Biotechnology: an expanding horizon

Microalgae, the ancestors of present day plants are untapped biological resources that have a vast array of biotechnological applications. Microalgae have been exploited by mankind since millennia. Men first learnt to use microalgae as food, in fact the first use of microalgae dates back to 2000 years when the Chinese used to feed on *Nostoc* during famines [32]. But today with developments and diversifications in the field of microalgal biotechnology there are numerous commercial applications of microalgae. The commercial use of microalgae as sources of specific chemicals began with *Dunaliela salina* for the production of β-carotene in the 1970s followed by the use of *Haematococcus pluvialis* Flotow as a source of astaxanthin and *Crypthecodinium cohnii* Seligo for docosahexaenoic acid (DHA) and long-chain polyunsaturated fatty acid (PUFA) [12]. Today microalgal biotechnology has the potential to produce a spectrum of valuable products, such as animal and aquaculture feeds, health foods, bioplastics, fertilizers, cosmetics, high value bioactives

(asthaxanthin, poly unsaturated fatty acids, β-carotene, antivirals, antifungals, antibiotics etc) and even biofuels. As of now there is a host of companies selling products developed from microalgae. In Myanmar tablets, chips, creams and liquid extracts of *Spirulina* are sold [70]. *Spirulina* also contributes significantly to the economy of Chad as the local trading value of 'dihe' (dry cake) is worth more than US$ 100,000 [71]. In inner Mongolia, Tianjin (China), India, Mexico, Cuba, Taiwan, Iran and Japan, industries culture the microalga *Dunaliella salina* to obtain β-carotene [72], whereas the largest producers of *Haematococcus pluvialis* for asthaxanthin production on a commercial scale in the world are located in Kailuai-Kona (USA) and Chennai (India) [73]. The world sale of one of the microalgae *Chlorella* used in human food, animal feed and as food additive was higher than US$ 38 billion per annum, while the annual estimated market for docosahexanoic acid produced by microalgae (*Crypthecodinium* or *Schizochytrium*) is about US$ 10 billion [32]. The annual worldwide aquaculture market for asthaxanthin is worth US$ 200 million with an average price of US$ 2500/kg [74]. The microalgal biomass market is reported to produce about 5000t of dry matter/year while generates a turnover of approximately US$ 1.25×10^9/year [32].

In the present scenario much of the research attention in microalgal biotechnology is diverted towards biofuels. Microalgal biotechnology holds great promise for the biofuel industry. Biofuels from microalgae are poised to be sustainable alternatives to conventional petro fuels, but however they need to overcome numerous obstacles in order to compete in the fuel market and for an extensive deployment. According to a report of the SBI bulletin, a combination of various factors (public and private R&D, industry investment from the public sector and other industries, fossil fuel prices and general regulatory support for biofuels) indicate a total global market for algae biofuel technologies with double digit growth potential over the short term future for a projected annual average growth of 43.1%

that will lead the market volume from US$ 271 million in 2010 to US$ 1,627 million (US$ 1.6 billion) in 2015 [75].

Research endeavors in microalgal biotechnology has geared up in the past few decades and the industry is poised to extend into new areas. However, it is noteworthy to mention here that microalgae are still not a well studies group from biotechnological point of view [32] among thousands of species that are believed to exist only a few thousand species are kept in collections, a few hundred are investigated for their chemical content and just a handful are cultivated in industrial quantities [76]. Numerous developments in microalgal biotechnology are already in progress as diverse as biofuels, animal feeds, cosmetics, bioplastics, industrial biomolecules, pharmaceuticals, health foods etc. to name only a few. The industry may well reach the center of scientific and political attention with key technical challenges being well addressed, technological progress, superior optimized production systems, good regulating frameworks coupled with the goodwill of policymakers, thereby highlighting the increasing economic significance of these wondrous microbial creatures, the forefathers of modern day plants "microalgae".

Objectives

On the basis of the above background information the following objectives were undertaken for the present research work:

1) Isolation and the culture of lipid rich microalgae
2) Characterization of microalgal biomass
3) Investigation of the feasibility of microalgal biodiesel as fuel and its subsequent characterization
4) Molecular characterization of microalgae

Such a study may be of help in identifying certain potent microalgal isolates and subsequently investigating their potential as a biofuel feedstock.

Chapter 2

Review of Literature

Over the years algal biofuel research has been a topic of intense scientific focus and is likely to play a significant role in the ensuing future. With both developed and emerging economies gearing up research in this challenging field, optimists have started envisioning possible future triumphs of algal biofuels substituting petroleum. Most of the market based applications of microalgae is still under development and the biotechnological applications of microalgae is poised to expand into new areas. Microalgae represent an almost inexhaustible repertoire of biomass whose true biotechnological potential has not been fully realized and henceforth requires promising research in similar lines.

2.1 Characterization of microalgal biomass

In general biomass characterization involves proximate and ultimate analysis. Proximate analysis involves the determination of moisture, volatile matter, ash and fixed carbon content whereas, ultimate analysis includes determination of carbon, nitrogen, hydrogen, oxygen and sulfur in biomass. In available literature, ultimate and proximate analyses have been among the most recurrently conducted tests for investigating algal biomass [77-79]. Determination of elemental composition of whole biomass, including various minerals in ash, is also helpful in understanding microalgae as a feedstock for biofuel production [80]. The prospective value of any biomass as a biofuel feedstock is largely governed by physico-chemical properties of the molecules from which it is constituted. Various properties such as calorific value, elemental composition, bulk density, ash content, moisture content, volatile matter content etc affects the performance of a biomass fuel and as such proper determination of these properties is a must for choice of conversion technologies.

Calorific value is one of the most important properties of biomass. Possession of high calorific value has already been proposed as must for use of microalgae as a fuel [81]. Ilman *et al.* [81] performed calorimetric characterization of *Chlorella* biomass. They reported that calorific values of *Chlorella* strains increased when grown in low nitrogen medium. The calorific values of five different strains of *Chlorella*

namely *C. vulgaris* Beijerinck (CCAP 211/11B), *C. emersonii* Shihira and Kraus (CCAP 211/11N), *C. protothecoides* Kruger (CCAP 211/8D), *C. sorokiniana* (UTEX 1230) and *C. minutissima* (UTEX 2341) grown in Watanabe and low nitrogen medium were determined The best growth was obtained with *C. vulgaris* with a growth rate of 0.99/day and the highest calorific value (29KJ/g) was obtained with *C. emersonii*. The cellular components were assayed at the end of the growth period. Their findings suggest that calorific value is linked to the lipid content rather than any other component.

Scragg *et al.* [82] cultured *Chlorella vulgaris* and *C. emersonii* in a 230L pumped tubular photobioreactor in Watanabe's medium and a low nitrogen medium. The low nitrogen medium induced higher lipid accumulation in both the tested algae, which increased their calorific value. The highest calorific value was obtained with *C. vulgaris* (28KJ/g) grown in low nitrogen medium. The biomass productivity was 24 mg dry wt/L/day in the low nitrogen medium whereas 40mg dry wt/L/day in Watanabe's medium which represents reduced energy recovery.

Marcilla *et al.* [83] characterized *Nannochloropsis* sp by TGA/FTIR analysis. On-line combination of Thermogravimetric Analysis (TGA) and Fourier Transform Infrared Spectrometry (FTIR) was applied to study the evolution of volatile products with time evolved in thermal degradation of *Nannochloropsis* sp. The microalgal cells were treated in order to separate the lipid fraction, by breaking the cells and extracting the fraction soluble in hexane. Both fractions i.e., the extract and the solid residue, were also analyzed by TGA/FTIR. Their findings revealed that decomposition of microalgal biomass occurs in three different steps.

Matsunaga *et al.* [84] reported the characterization of marine microalga *Scenedesmus* sp. strain JPCC GA0024 towards biofuel production. Hexadecane ($C_{16}H_{34}$) and 1-docosene ($C_{22}H_{44}$) were the main hydrocarbons present in the lipid fraction which were detectable by gas chromatography/mass spectrometry analysis. According

to calorimetric analysis the energy content of strain JPCC GA0024 was 6160 Kcal/kg (25.8MJ/kg) which was equivalent to the coal energy.

Phukan *et al.* [85] reported the physico-chemical characterization of indigenously isolated *Chlorella* sp. biomass by bomb calorimetery, TGDTA, FTIR and CHN analysis. The elemental content of carbon, hydrogen, oxygen and nitrogen in the algal biomass was 47.54%, 7.1%, 38.63% and 6 73% respectively. *Chlorella sp.* MP-1 biomass showed low ash (5.93%), whereas high energy (18.59 MJ/kg), carbohydrate (19.46%), and lipid (28.82%) content. They also reported the characterization of the algal de-oiled cake by FTIR spectroscopy and thermogravimetric study at 10 and 30°C/min. Their findings suggest that the algal biomass can be used as a feedstock for bio and thermo-chemical conversion whereas the de-oiled cake for only thermo-chemical conversion.

Bi and He [80] characterized different species of microalgae for biofuel production. Standard ASTM methods were implemented to examine the microalgal properties, including proximate and ultimate analyses. Among the microalgae studied, green microalgae had more volatile matter than brown microalgae, while the latter contain much higher ash content (as high as 43.4% wt. ± 0.20% wt. dry basis). The lowest ash content was found in green microalgae (14.3% wt. ± 0.10% wt. dry basis). Ultimate analysis showed that brown microalgae had less carbon content (approx. 25% wt. dry basis) as compared to green microalgae (49% wt. to 58% wt. dry basis). All samples of microalgae were high in sulfur content (0 4% wt. to 1.0% wt dry basis). Mineral contents of all microalgal samples were similar to those commonly present in other biomass. Brown microalgae contained significantly higher amounts of carbohydrates (72.9% wt. to 75.5% wt. dry basis) than green microalgae. On the other hand, green microalgae had more crude fat (17.1% wt. to 27.8% wt. dry basis) than brown microalgae. The fatty acid profiles revealed that the primary fatty acids in microalgal lipids were similar to those of vegetable oils such as soybean oil. However,

there were also many odd-numbered fatty acids, such as C15:0, C17:0 and C19:0 which are not typically present in other seed oils.

2.2 Microalgal biodiesel by direct (*in situ*) transesterification

Conventionally, biodiesel synthesis from microalgae involves drying of microalgal biomass and extraction of lipids using proper organic solvents, followed by their conversion to FAMEs using suitable catalyst(s). But however such a method is time consuming, expensive and difficult to be implemented in algae's crushing step because of the rigid cell walls [86]. A biodiesel production system which can circumvent the use of organic solvents for lipid extraction would contribute considerably towards cost and net energy savings, besides ease of operation. In context of the aforesaid *in situ* transesterification which reduces the number of unit operations by direct contact of the microalgal biomass with the catalyst-alcohol mixture for biodiesel production is gaining serious attention for algae based biodiesel production. The *in situ* transesterification process is focused and directed at the oleaginous microalgal biomass rather than the pre-extracted lipid.

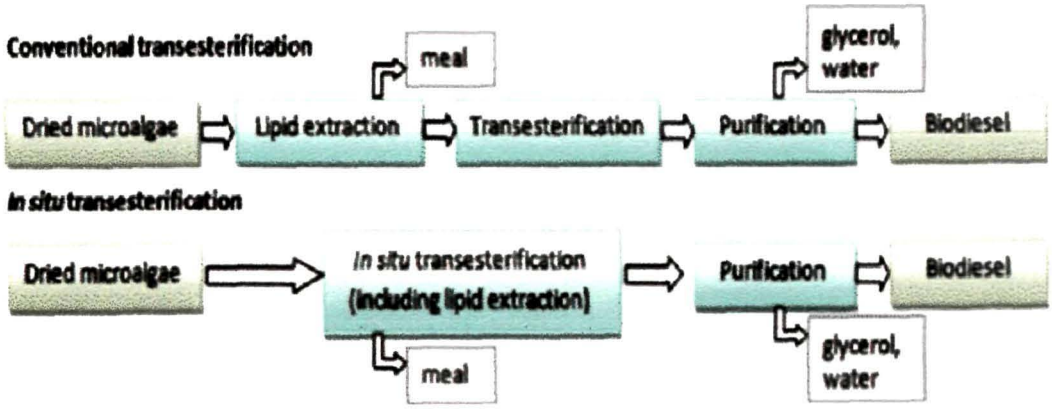


Fig. 2.1 Comparison between *in situ* transesterification and conventional transesterification. Green squares indicate initial and final products, blue squares are main processes required, and grey squares indicate by-products obtained [87]

Johnson and Wen [86] studied production of biodiesel from microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. They found that when freeze-dried biomass was used as feedstock, the two-stage method resulted in 57% of crude biodiesel yield (based on algal biomass) with a FAME content of 66.37%. The one-stage method (with chloroform, hexane, or petroleum ether used in transesterification) led to a high yield of crude biodiesel, whereas only chloroform-based transesterification led to a high FAME content. When wet biomass was used as feedstock, the one-stage method resulted in a much-lower biodiesel yield. Direct transesterification of oleaginous biomass resulted in a higher biodiesel yield and FAME content in comparison to extraction-transesterification method. The biodiesel produced from direct transesterification also meet most of the ASTM specifications.

Ehimen *et al.* [88] studied the influence of potentially important economic reaction factors on the progress of conversion of microalgae oil to biodiesel using the acid-catalysed *in situ* transesterification process. These variables include the influence of reacting alcohol volumes, temperature, reaction time, biomass moisture content and stirring. Their results indicate that increasing the reacting alcohol volume and the temperature lead to improved FAME conversions. With the exception of *in situ* transesterification carried out at ambient temperature (23°C) the equilibrium FAME conversions appeared to approach asymptotic limits for reaction times greater than 8 hrs for all temperatures investigated.

Griffiths *et al.* [89] tested and compared the effectiveness of direct transesterification (DT) to alternative methods of quantifying total fatty acid content in different species of microalgae namely *Chlorella vulgaris*, *Scenedesmus* sp. and *Nannochloropsis* sp. The Folch method was the most effective of the extraction methods tested, but comparison with DT illustrated that all extraction methods were incomplete. Higher levels of fatty acid in the cells were obtained with DT in comparison with the extraction-transesterification methods. A combination of acidic

and basic transesterification catalysts was more effective than each individually when the sample contained water. DT proved a convenient and more accurate method than the extraction techniques for quantifying total fatty acid content in microalgae.

Carvalho Junior *et al.* [90] performed in situ methanolysis of microalgae *Nannochloropsis oculata*. Biodiesel was obtained using methanol as the methylation reactant for transesterification reaction and hydrochloric acid as the catalyst precursor, at 80°C for 2h of reaction. A process energetic efficiency value of 1.17 was obtained for microalgae derived biodiesel, which is higher than from soybean and sunflower, reportedly 1.06 and 1.12. They advocated that microalgae *in situ* methanolysis process showed greater fuel available energy than energy consumption, and henceforth is energetically sustainable.

Li *et al.* [91] studied *in situ* biodiesel production from fast growing *Chlorella pyrenoidosa* in rice straw hydrolysate. They used rice straw hydrolysate as a lignocellulose-based carbon source for *Chlorella pyrenoidosa* cultivation. They reported over 99% methyl ester content and 95% biodiesel content (under optimized conditions) for 1g algal powder, 6mL *n*-hexane, and 4mL methanol with 0.5M sulfuric acid at the temperature of 90°C in 2h reaction time. Their method using (n-hexane) for *in situ* biodiesel production showed better performance than the traditional two step method.

Wahlen *et al.* [92] reported biodiesel production from microalgae, cyanobacteria, and wild mixed-cultures by simultaneous extraction and conversion of total lipids. The optimized reaction conditions for dry biomass were 2ml methanol, 1.8% (v/v) H_2SO_4 and 80°C for 20min. From the optimized conditions they demonstrated that quantitative conversion of triglycerides from different microalgal and cyanobacterial species could be achieved, including those from mixed microbial biomass collected from municipal wastewater lagoon.

Xu and Mi [93] investigated the production of biodiesel from *Spirulina* by *in situ* transesterification technology. The overall biodiesel yield (OBY) of *Spirulina* was determined for benchmarking purposes, using the Bligh and Dyer protocol for oil extraction, and transesterification with potassium hydroxide. The performance in *in situ* transesterification of the selected co-solvents toluene, dichloromethane and diethyl ether, as well as the solvent combinations petroleum ether/toluene, toluene/methanol and dichloromethane/methanol, was evaluated by OBY. Among all the co-solvents tested, the toluene/methanol system, 2:1 by volume ratio, demonstrated the highest efficiency, achieving a biodiesel yield of 76% of OBY for the first in situ transesterification cycle and 10% for the second in situ transesterification cycle. However, the authors suggested that the co-solvents used in their study may not be suitable for other algal species and the best solvent system may differ for different algal species.

Laurens *et al.* [94] developed a robust whole-biomass *in situ* transesterification procedure for quantification of algal lipids (as fatty acid methyl esters, FAMEs) which could be carried out on small scales (using 4–7mg of biomass), was applicable to a range of different species, consisted of a single-step reaction, was robust over a range of different temperature and time combinations, and also tolerant to at least 50% water in the biomass. They reported comparison of the yield of FAMEs by using different catalysts and catalyst combinations. The acid catalyst HCl provided a consistently high level of conversion of fatty acids with a precision of 1.9% relative standard deviation. Their approach (whole biomass transesterification) reflects the true potential fuel yield of algal biomass whereas gravimetric lipid quantification can under or over estimate the lipid content.

Velasquez- Orta *et al.* [87] performed alkaline *in situ* transesterification of *Chlorella vulgaris* biomass and found that in situ transesterification (alkaline) of algal biomass can achieve high conversion in less time than an acid catalyst, using a lower

ratio of catalyst. Three process variables (catalyst ratio, solvent ratio and reaction time) were studied, based on their process significance. Maximum FAME recovery of 77.6 ± 2.3 wt.% was obtained at a reaction time of 75min, using a catalyst: lipid (NaOH) molar ratio of 0.15:1 and a methanol: lipid molar ratio of 600:1.

2.3 Properties of microalgal biodiesel

Biodiesel is defined by ASTM International as a fuel composed of monoalkyl esters of long-chain fatty acids derived from renewable vegetable oils or animal fats meeting the requirements of ASTM D6751 [95]. Biodiesel is characterized by their viscosity, density, cloud and pour points, cetane number, carbon residue, sulfur content, copper corrosion and higher heating value (HHV) [96]. In order to access the potential of microalgal biodiesel as an alternative for conventional petrodiesel the fuel properties of biodiesel must be determined. Table 2 presents some of the important properties of microalgal biodiesel and petrodiesel with respective ASTM standards.

Miao and Wu [48] produced and characterized biodiesel from heterotrophically cultured *Chlorella protothecoides*. The best process combination for biodiesel production were 100% catalyst quantity (based on oil weight), 56:1 molar ratio of methanol to oil at 30°C, which reduced product specific gravity from an initial value of 0.912 to a final value of 0.8637 in 4h of reaction time. The saponification and acid value for *Chlorella protothecoides* oil were 189.3mg KOH/g and 8.97mg KOH/g of the oil. The properties of the investigated microalgal biodiesel were viscosity 5.2 mm^2/s, density 0.864 Kg/L, flash point 115°C, solidifying point -12°C, cold filter plugging point -11°C and heating value 41MJ/kg.

Xu *et al.* [97] reported the production of high quality biodiesel from heterotrophically cultured *Chlorella protothecoides*. They used corn powder hydrolysate as carbon source instead of glucose for achieving a high cell concentration. The oil (55.2%) could be efficiently extracted using n-hexane as the

solvent and converted to biodiesel by acid catalyzed transesterification. The microalgal biodiesel was characterized by a high heating value of 41MJ/kg, a density of 0.864kg /L, and a viscosity of 5.2×10^{-4} Pa s (at 40°C).

Bucy *et al.* [98] investigated the effects of various levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on algal methyl ester fuel properties. Density, viscosity, bulk modulus, cetane number, oxidative stability, cloud point and cold filter plugging point were measured for algal methyl esters produced from various microalgae feedstocks as well as model algal methyl ester compounds formulated to match the fatty acid composition of *Nannochloropsis* sp., *Nannochloropsis oculata* and *Isochrysis galbana* subjected to varying levels of removal of EPA and DHA. Their findings suggest that removal of 50-80% long chain poly unsaturated fatty acids (LC-PUFA) from *Nannochloropsis*-based methyl esters was sufficient for meeting existing specifications for oxidative stability. However, to produce fuels with acceptable cetane number higher levels of (LC-PUFA) removal from *Nannochloropsis*-based methyl esters was necessary. The removal of EPA and DHA was shown to have a detrimental effect on cold flow properties since the algal methyl esters are high in fully saturated fatty acid content.

Chen *et al.* [99] determined the fuel properties of biodiesel from the microalga *Chlorella protothecoides* and its blends with petroleum. Microalgae oil methyl esters (MAMEs) showed satisfactory fuel properties, and their predominant components were 65.2 wt.% methyl oleate and 18.5 wt.% methyl linoleate. The MAMEs had a cold filter plugging point of -13°C, an iodine value of 112.2g I_2/100g, kinematic viscosity of 4.43mm^2/s at 40°C, and an oxidation stability of approximately 4.5h. Furthermore, both the cold filter plugging point and oxidation stability of the MAMEs-diesel blends decreased with a higher blending ratio of the MAMEs.

Mostafa and El-Gendy [100] evaluated fuel properties of biodiesel from *Spirulina platensis* and its blends with Egyptian petrodiesel. They found that with

increase of biodiesel concentration in the blends, the viscosity, density, total acid number, initial boiling point, calorific value, flash point, cetane number and diesel index increases, whereas the pour point, cloud point, carbon residue and sulfur, ash and water contents decreases. The properties of the microalgal biodiesel blends were within the recommended petro-diesel standard specifications which are suggestive of better engine performance.

2.4 Microalgal biomass and fermentation

The concept of microalgal biomass as a fermentation feedstock for bio-alcohol production is not new. In fact early in the 1980's it was realized that microalgae such as the genus *Dunaliella* had potential as a rich, renewable biomass for use in the fermentative production of butanol and ethanol [101]. Additionally, the increased worldwide pressure on the resource sector to produce fuel from renewable and environmentally sustainable sources has led to ongoing research in methods to produce ethanol from microalgae since the late 1990's [102- 104]. Although the potential of microalgal biomass as a feedstock for bioethanol fermentation has been widely considered but still as of date only a modest level of research has been reported in this area [105].

Hirayama *et al.* [106] reported on ethanol production from carbon dioxide by fermentative microalgae. They isolated an excellent strain *Chlamydomonas* sp. YA-SH-1 which had a growth rate of 30gm dry biomass/m^2/day, a starch content of 30% (dry wt. basis) and a conversion rate from intracellular starch to ethanol of 50% both in dark and anaerobic condition. Microalgal cultures were carried out in flat culture bottles. The optimum temperature range for ethanol production was 30-35°C. The ethanol concentration increased during the initial 44 h following which it remained constant. They proposed a new ethanol production system and suggested that if microalgal productivity, starch content and ethanol conversion rates are improved the system may be an effective means for CO_2 fixation and energy production.

Shirai *et al.* [107] reported the cultivation of microalgae in solution from the desalting process of soy sauce waste treatment and utilization of the algal biomass for ethanol fermentation. The algal cells were disrupted and saccharified with glucoamylase. The saccharified solution was fermented using *Saccharomyces cerevisiae* IAM 4140 following which 11mg of ethanol was produced from 1gm (dry cell wt.) of *Dunaliella* cells.

Hirano *et al.* [103] studied CO_2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. A marine microalgal strain *Chlorella vulgaris* (IAM C-534) was found to possess a high starch content of 37%. A 65% ethanol conversion rate was obtained as compared to the theoretical rate from starch. They also investigated intracellular starch fermentation under dark and anaerobic conditions. Maximum ethanol concentration of 1 (w/w) % was obtained with *Chlamydomonas reinhardtii* (UTEX2247) and sak-1 isolated from sea water.

Harun *et al.* [108] investigated the suitability of microalgae (*Chlorococum sp.)* as a substrate for bioethanol production via yeast (*Saccharomyces bayanus*) under different fermentation conditions. They found a maximum ethanol concentration of 3.83g/L obtained from 10g/L of lipid-extracted microalgal debris.

Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production was reported by Choi *et al.* [109]. Microalgal biomass was converted into fermentable feedstock by two commercial hydrolytic enzymes (ά-amylase and amyloglucosidase). They reported an ethanol production of 235mg from 1gm of microalgal biomass by a separate hydrolysis and fermentation (SHF) method. The main advantages of their process were low cost of chemicals, short residence time and simple equipment system, all of which is sought after with regard to large scale application.

Harun and Danquah [110] studied the influence of acid pretreatment on microalgal biomass for bioethanol production. Different parameters such as acid concentration, temperature, microalgae loading and pre-treatment time were investigated. Furthermore a central composite design technique was employed to optimize the acid pre-treatment conditions. The highest bioethanol concentration of 7.20g/L and this was achieved when the pre-treatment step was performed with 15g/L of microalgae at 140°C using 1% (v/v) of sulphuric acid for 30min. In terms of ethanol yield ~52wt.% (g ethanol/g microalgae) maximum was obtained using 10g/L of microalgae and 3% (v/v) of sulphuric acid under 160°C for 15min.

2.5 Thermochemical conversion of microalgae

Pyrolysis is the thermal decomposition of materials in the absence of oxygen or when significantly less oxygen is available than required for complete combustion. Among various existent biomass conversion processes pyrolysis is considered to be an effective technology by which biomass can be converted into valuable bio-oils, char and gaseous products [111]. Though numerous lignocellulosic biomass feedstocks have been extensively investigated with regard to pyrolysis, recent years have seen intense scientific focus on pyrolysis of microalgal biomass. Microalgae in possession of faster growth rate in comparison to lignocellulosic feedstocks offer attractive candidature as a prospective pyrolysis feedstock.

Peng *et al.* [112] studied the pyrolytic characteristics of autotrophic microalgae (*Chlorella protothecoides* and *Spirulina platensis*) by thermogravimetric analysis. The degradation profile of the microalgal biomass was characterized by three stages (dehydration, devolatilization and solid decomposition). The activation energy value for *Chlorella* and *Spirulina* pyrolysis were 4.22-5.25×10^4 and 7.62-9.70×10^4 J/mol respectively. Their findings warrant the usefulness of the data for pyrolytic processing systems using planktonic microalgae.

Peng *et al.* [113] pyrolyzed heterotrophic microalgal cells (*Chlorella protothecoides*) in a thermogravimetric analyzer to investigate the pyrolytic characteristics and determining the kinetic parameters. The main pyrolysis reactions took place between 160 and 520°C with a volatile yield of approximately 80%. They reported a devolatilization stage comprising of two temperature zones (I and II) with a transition at 300-320°C and suggested that the obtained data may be useful for design, operation and modeling of the pyrolysis systems for microalgae.

Miao *et al.* [64] reported fast pyrolysis of microalgae to produce renewable biofuels. They obtained bio-oil yields of 18 and 24% from fast pyrolysis of *Chlorella protothecoides* and *Microcystis aeruginosa* in a fluid bed reactor. The bio-oil from microalgae was characterized by a higher heating value and lower oxygen content than bio-oil from lignocellulosic biomass.

Miao and Wu [53] also reported that manipulating the metabolic pathway in microalgae through heterotrophic growth can be a viable approach for increasing the yield of bio-oil production by fast pyrolysis. They reported a bio-oil yield of 57.9% from heterotrophic *Chlorella protothecoides* cells which was 3.4 times higher than that from autotrophic cells by fast pyrolysis.

Grierson *et al.* [114] studied the thermal characterization of microalgae (*Tetraselmischui, Chlorella* like, *Chlorella vulgaris, Chaetocerous muelleri, Dunaliella tertiolecta* and *Synechococcus*) under slow pyrolysis conditions. The samples were analyzed with a Computer Aided Thermal Analysis (CATA) technique at a standard heating rate of 10°C/min, and the pyrolysis oil product yields were estimated at 500°C. For each of the microalgal species the energy required to achieve thermal conversion was found to be approximately 1MJ/kg. The species were found to produce up to 43% by volume of bio-oils and the char fraction remained one third of the total sample weight.

Shuping *et al.* [115] investigated the pyrolytic characteristics and kinetics of the marine microalga *Dunaliella tertiolecta* using thermogravimetric analysis. The kinetic analysis of the main pyrolysis process was performed using a composite procedure involving the iso-conversional method and the master-plots method. The iso-conversional method indicated that the pyrolysis reaction should conform to a single reaction model with activation energy of 145.713KJ/mol using Kissinger's method and 146.421KJ/mol using Flynn–Wall–Ozawa's method, respectively. Their findings are helpful in designing a pyrolytic processing system using microalga *Dunaliella tertiolecta* as a feedstock.

Wang *et al.* [62] investigated fast pyrolysis of microalgae *Chlorella vulgaris* remnants in a fluidized bed reactor at 500°C for bio-oil and bio-char production. Bio-oil from *C. vulgaris* remnants was found to be a complex mixture of aromatics and straight chain hydrocarbons, amides, amines, carboxylic acids, phenols and other compounds with molecular weights ranging from 70-1200Da. The biochar produced was high in inorganic content (potassium, phosphorus and nitrogen) and henceforth was suggested to provide nutrients for crop production.

2.6 Molecular taxonomy of microalgae

One of the fundamental aspects of applied phycology research is proper identification and enumeration of the algal species of interest. Unlike other groups of organisms, initial attempts to identify and classify microalgae were based on morphological observations. But however microalgae possess very few morphological characteristics that are useful for species characterization and this leads to the possibility of numerous cryptic species [116]. Additionally, microalgae are of very small size (often <5μm) and consequently electron microscopy is required to identify microalgae to the species level.

Many researchers strongly advocate the use of molecular techniques for reliable enumeration of microalgae. In the realm of modern biology molecular approach based taxonomy has gathered more scientific attention as an accurate science in comparison to the conventional morphological approach. Molecular techniques permit an assessment of the validity of the morphological species concept for common microalgae and eventually these techniques allow for niche partitioning and distribution of these organisms [116]. Molecular identification provides a useful tool to distinguish between inter- and intra-specific morphologically similar species and mixed populations [117]. For microbes the gene most commonly employed for diversity studies is the small-subunit ribosomal RNA gene, 16S rRNA in prokaryotes and 18S rRNA in eukaryotes [118]. The conserved and variable regions of 16S–18S rDNA sequences are used as targets for primer-directed DNA amplification by polymerase chain reaction (PCR) for the identification of micro-organisms [119, 120].

Olmos *et al.* [121] characterized five species of the microalgal genus *Dunaliella* on basis of 18S ribosomal RNA genes. PCR-amplified 18S rDNAs of the respective *Dunaliella* sp. were examined for their restriction fragment length polymorphism (RFLP). Their findings demonstrated that with the utilization of conserved and specific primers and with the development of RFLP analysis of 18S rDNAs, it was possible to identify *D. salina* (M84320), *D. parva and D. bardawill* as three different species containing one, two and two introns, respectively.

Rasoul-amin *et al.* [122] performed PCR amplification of the 18S rRNA gene in microalgae. The universal eukaryotic primers 5'-GTCAGAGGTGAAATTCTTGGATTTA-3' as forward primer and 5'-AGGGCAGGGACGTAATCAACG-3' as reverse primer were used to amplify an ~700-bp region of the 18S rRNA gene. The amplified product was sequenced and deposited in NCBI. The results of BLAST analysis with other sequenced microalgae in

NCBI showed 99-100% similarity with the 18S small subunit rRNA of other microalgae.

Cha *et al.* [123] identified *Chlorella* isolates on basis of 18S rDNA analysis. The 18S rDNA of the isolates, UMT-M1 and KS-MB2 were amplified with forward (5'ACGGAGGATTAGGGTTCGATTCCG-3') and reverse (5-'GCTTCCATTGGCTAGTCGCCAATA-3') primers. Phylogenetic analysis revealed that UMT-M1 was closely clustered with *C. vulgaris* (strains KMMCC/C-88 and KMMCC/C-108) and KS-MB2 was closely clustered with *C. sorokiniana* (strains UTEX2805, Prag A14, SAG 211-8k and BE1). Phylogenetic and BLAST analysis also revealed the 18S rDNA sequence of KS-MB2 to be identical (100% homology) and closely related to other microalgae species such as *Actinastrum hantzschii* strain CCAP 200/3 and *Micractinium pusillum* strain CCAP 248/15.

Liu *et al.* [124] used universal green algal primers 18SF (forward, 5'-CCTGGTTGATCCTGCCAG-3') and 18SR (reverse, 5'-TTGATCCTTCTGCAGGTTCA-3'), for PCR amplification of the 18S rDNA in the microalgae *Coelastrum* sp. The amplified 18S rDNA gene sequence had >99% identity with that of previously sequenced *Coelastrum* sp. strains in NCBI database. On the basis of 18S rDNA gene phylogenetic analysis, HA-1 was found to be close with *Coelastrum proboscideum* var. gracile strain SAG 217-3 (GQ375099.1) and named *Coelastrum* sp. HA-1.

In addition to 18S ribosomal rRNA gene ribosomal spacer sequences, including inter transcribed spacer regions (ITS) have been reported to be used for proper identification and discrimination of genetic variation in Chlorophyceae. In one such study Gonzalez *et al.*, [125] carried out phylogenetic analyses of 15 strains representing 8 taxa of *Dunaliella* (*D. salina, D. bardawil, D. pseudosalina, D. tertiolecta, D. parva, D. viridis, D. peircei* and *D. lateralis*) belonging to both subgenera and all sections of the genus using the sequences of the nuclear rDNA

spacers (ITS-1 and ITS-2). Their findings revealed that *D. parva* UTEX 1983 was misidentified and should be renamed as *D. viridis*. Similarly, they suggested that the strains *D. parva* CCAP 19 / 9, CCMP 362 and UTEX 2192 of *D. peircei* should be renamed as *D. tertiolecta*.

2.7 Work done in India

The government of India has undertaken some initiatives for promoting algal biofuel research in India. The government of India in 2008-09 launched a "National Algae Biofuel Network" with the participation of 12 national institutes to work on algal biofuel, focusing on aspects, such as collection and characterization of algal strains from different ecological niches and deposition of the same in three repositories, development of different production systems, improved algal strains for more oil/lipid content, and lastly, design development and fabrication of low-cost and pilot-scale bioreactors for the cultivation of algae for biofuels and technology [140]. Presently, algal biofuel research in India is mostly confined to only some of the Indian institutes (government funded research laboratories and a few other Indian universities) such as Central Salt and Marine Chemicals Research Institute (CSMCRI, Bhavnagar), Department of Marine Living Resources, (Andhra University, Vishakapatnam), International Center for Genetic Engineering and Biotechnology (ICGEB –New Delhi), National Institute of Oceanography (NIO, Goa), National Chemical Laboratory (NCL, Pune), Indian Institute of Technology, Khargpur (IIT, Kharagpur), Indian Institute of Chemical Technology (IICT, Hyderabad), Calcutta University (CU, Kolkata), Energy and Wetlands Research Group, Centre for Ecological Sciences/Centre for Sustainable Technologies (Indian Institute of Science, Bangalore), Bharathidarsan University (Tiruchirappalli), Institute of Chemical Technology (ICT –Mumbai), CAS in Botany (University of Madras), National Institute of Interdisciplinary Science and Technology (NIIST, Thiruvananthapuram),

Institute of Ocean Technology (NIOT, Chennai) and Defense Research Laboratory (DRL, Tezpur) [139].

India is passing through a phase of impending fuel crisis and the country's economy is quiet susceptible to international price fluctuations and supply of petroleum. As a solution to the nation's unprecedented energy crisis, fuels from microalgae undoubtedly look promising but requires committed efforts from both government and corporate sectors. The Indian policy makers need to roll up the dice in favor of algal biofuel research looking into the commercial prospects of the algae based industry. India should also take advantage of its prevalent tropical climate and vast coastline (a marked bonus from the Indian perspective for facilitating mass scale cultivation of algae). It's the need of the hour that India should join the global race for research and development on algae based biofuel whereby enabling the country to find a solution to its impending energy crisis [140]. On a precedence basis the possible challenges in this regard should be addressed by initiating required R & D efforts.

Dayananda *et al.* [126] isolated a hydrocarbon producing *Botrycoccus braunii* (green alga) from Bear Shola Falls at Kodaikanal (latitude 10.31 N and longitude 77.32 E), Tamil Nadu, India. They reported hydrocarbon content in the range of 13-18% dry cell weight. Genetic similarity of the indigenously isolated strain (*B. braunii* CFTRI- Bb1) was compared with an authentic strain (*B. braunii* N-836) by Inter simple sequence repeats (ISSR) finger printing.

Dayananda *et al.* [127] reported on the autotrophic cultivation of *Botrycoccus braunii* in various culture media for the production of hydrocarbons and exopolysaccharides. They found that the tested organism could acclimatize to different culture conditions and to a wide range of culture media with production of more than one metabolite.

Ranga Rao *et al.* [128] studied the effect of salinity on the growth of the green alga *Botrycoccus braunii* and its constituents and found that the species was adaptable to lower levels (17mM to 85mM) of salinity with an increased production of biomass, fat, hydrocarbon, carbohydrate and carotenoids.

Chinnasamy *et al.* [129] studied the biomass production potential of waste water microalga *Chlorella vulgaris* ARC 1 under elevated levels of CO_2 (0.036 to 20%) and temperature (30, 40 and 50°C). They reported 30°C as the optimum temperature for biomass production under 6% elevated CO_2 level. Although increases in temperature above 30°C resulted in concomitant decrease in growth responses their adverse effects were significantly subdued at elevated CO_2 levels. Their results indicate that *Chlorella vulgaris* grew better at elevated CO_2 level at 30°C, albeit with lesser efficiencies at higher temperatures.

Mandal and Mallick [130] investigated the microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Marked increase in the lipid content (43% dcw) was observed when the microalga was cultured in nitrogen deficient conditions. Similarly, culture of the microalga under phosphate deficiency and thiosulphate supplementation also resulted in an appreciable increase in lipid content (30% dcw). They suggested that *Scenedesmus obliquus* offers strong candidature as a suitable feedstock for biodiesel production on account of the presence of palmitate and oleate as the major biomass constituent.

Sivasubramanian *et al.* [131] reported the use of pulsed magnetic field for improving the quality of *Desmococcus olivaceous* biomass. The microalga was grown in photo bioreactors (10L) with facility to pump the culture through magnetic field. Solenoid coil system was set to a highly homogenous sinusoidal magnetic field of intensity 15mG for a period of 3h duration per day for 15 days.The submersible pump providing the circulation was kept in operation for a period of 12h/day. The motor of the submersible pump contributed to a secondary source of magnetic field exposure

(near field) having an intensity of approximately 600mG. Samples were drawn at regular intervals to assess the productivity and biomass quality in terms of division rate, dry weight, pigments and other biochemical parameters.

Kumar *et al.* [132] reported physico-chemical characterization of oil from one Cyanophyceae (*Tolypothryx*) and five chlorophycean (*Pithophora, Spirogyra, Hydrodictyon, Rhizoclonium and Cladophora*) isolates. The physico-chemical parameters of oil such as pH, viscosity, density were analyzed by standard methods of analysis (AOAC, 1995) and algal oil properties were compared with biofuel standards in ISO15607and EN14214. The properties of the algal oil were within the ranges set by American society for testing and materials (ASTM) D6751, ISO 15607and EN14214- Europe. Gas chromatographic analysis revealed higher percentage of methyl palmitate, methyl stearate, methyloleate and methyl linoleate.

Venkata Mohan *et al.* [133] studied the possibility of using mixed microalgae existing in ecological water bodies for harnessing biodiesel. Microalgal cultures from different water bodies were cultivated in domestic waste water in open ponds and after harvest were processed through acid catalyzed transesterification. The functional characteristics of water bodies influenced both species diversity and lipid accumulation. Algal oil comprised of 33 different types of saturated and unsaturated fatty acids possessing both food and fuel characteristics. The study suggested that ecological water bodies can serve as a fine platform to harness energy through carbon sequestration besides providing a sustainable route to biodiesel production

Sudhakar and Premalatha [134] theoretically accessed algal biomass potential for carbon mitigation and biofuel production. They identified six best regions in each continent for algal biomass cultivation considering both sunlight and local climatic conditions into account. The mean hourly meteorological data, sunlight, ambient temperature and rainfall information for the identified potential site was combined to estimate annual biomass production, lipid production and carbon mitigation potential.

Maximum possible algal biomass yield and oil productivity were estimated for six global sites at three different scenarios of photosynthetic efficiency 11.42, 6 and 3% respectively. The upper optimistic biomass, oil yield and carbon fixation potential was calculated to be 533 T/ha/yr, 1, 25, 333 L/ha/yr and 95 Tons CO /ha/yr. Their study provides a baseline data for theoretical maximum, minimum and best estimates of open pond microalgae production systems.

Talukdar *et al.* [135] studied the effects of salinity on growth on total lipid content of the microalga *Ankistrodesmus falcatus* (corda) ralfs (a prospective biofuel producing strain). Influence of salinity (gradation from 40mM to 320mM of NaCl) in BG-11 medium on growth (μ), total lipid (TL) content and calorific value (CV) were studied in triplicate batch mode culture at light intensity of 35μmol/m^2/s, temperature 25 ± 2°C and 16:8h light and dark diurnal cycles. A close correlation (R^2 = 0.955) between lipid content and calorific value was observed with a maximum energy value of 27.9 ± 0.15 KJ/g.

Elumalai and Sakthivel [136] reported the spectroscopic determination of FAMEs of fresh water microalgae isolated from cement industries of Tamil Nadu, India. The lipid fractions were extracted from microalgal biomass by different solvent extraction procedures and the fractions were analyzed by means of FTIR spectroscopy and GC-MS. Among, the sixteen investigated microalgal groups, eight produced SFA (Saturated Fatty Acids) in high percentage and seven groups had high yields of PUFA (Polyunsaturated Fatty Acid) and only one group of microalgal contain MUFA (Mono Unsaturated Fatty Acid).

Talukdar *et al.* [137] characterized an indigenously isolated strain of microalga *Botrycoccus braunii* Kutzing from Assam, India towards biofuel production. A total lipid content of 57.14% and hexane extractable crude hydrocarbon of 52.6% were recorded maximum at 56 and 28 days respectively, which varied upon culture durations. The energy value (54.69KJ/g) of the strain's sundried biomass was found

higher than that of petroleum diesel fuel and nearly twice than other microalgae strains compared. The strain GUBIOTJTBB1 was found superior in terms of total lipid and hydrocarbon content compared to the previously reported Indian strains of *Botrycoccus braunii*.

Ramachandra *et al.* [138] explored the prospects of microalgal lipids from urban waste water in India for biofuel production. They collected *Euglena* sp., *Spirogyra* sp. and *Phormidium* sp. from selected locations of sewage fed urban lakes and urban treatment plants in Bangalore and Mysore. The total lipid content of *Euglena* sp. was higher (24.6%) compared to *Spirogyra* sp. (18.4%) followed by *Phormidium* sp. (8.8%) and their annual lipid yield potential was 6.52, 1.94 and 2.856 t/ha/yr respectively. The investigated species showed higher content of fatty acids (palmitate, stearate followed by oleic and linoleic acids) with the desirable biofuel properties.

Over the years algal biofuel research has geared up, but still much is to be achieved in order to attain full scale commercial deployment. Much work needs to be accomplished in specific domains such as genetic and metabolic engineering, productivity, harvesting, bioreactor designing, enzymatic hydrolysis, in expensive mass culture approaches, efficient biomass conversion technologies, value addition etc. for a better comprehensive understanding. A complete biorefinery based approach where biofuel production is coupled with co-product generation (when resolutely exploited) is likely to improvise the economic viability of algal biofuels. Nevertheless, many recent developments are already in the pipeline, and fuels from algae certainly appear to be promising. Already on the global forefront there are more than 100 firms dedicated to cultivation and harvesting of algal biomass [140]. There are also a few startup companies attempting to commercialize algal biofuels [57]. Although concept to commercialization of algal biofuels is a matter of time, in the ensuing future algae is likely to play a significant role in the global transportation fuel mix.

Chapter 3

Materials and Methods

3.1 Plastic/glassware used

All the sterilized polystyrene tubes were purchased from Tarson, India. The Erlenmeyer flasks and beakers were purchased from Borosil, Mumbai, India.

3.2 Chemicals used

All chemicals and reagents used in the present study were of analytical grade and procured from Merck India Ltd., SRL, Qualigen, Himedia, Sigma aldrich, and Bangalore Genei. Chromatography grade reagents were used for gas chromatography mass spectroscopy (GC-MS) analysis and nuclear magnetic resonance (NMR) analysis.

3.3 Equipment used

Equipment used in the present investigation are listed below

1. Laminar Hood – Reico
2. Autoclave – Dainanlabtech, Co. Ltd
3. Digital weighing balance – Metler Toledo
4. Water bath - Rectangular water bath, JSGW, India
5. Lyophilizer – Lyodel
6. Hot air oven –Remi
7. Vortexer - Vortex shaker, JSGW, India
8. pH meter - Cyberscan 500
9. Heating mentle - Rivotech, India
10. Incubator - EN500, Labtech
11. Incubator shaker - Scigenics, Orbitech, Biotech
12. Centrifuge - Remi instruments
13. Cooling centrifuge – Remiinstruments
14. Deep freezer - New Bunswick Scientific, C34085
15. Sonicator - Ultrasonic homogenizer, OMNI International
16. UV spectrophotometer – Beckman DU530 and CECIL 7400

17. Fourier transformed infrared spectrophotometer (FTIR) – Perkin Elmer, Spectrum 100
18. GC-MS- Perkin Elmer Clarus 600 gas chromatograph (GC) coupled with a Perkin Elmer Clarus 600C mass spectrometer (MS)
19. TRACE™ 1300 (Thermo Scientific) gas chromatograph equipped with FID detector and TRACE™ TR-FAME GC column.
20. NMR - JEOL JNN-ECS 400
21. Thermo gravimetric analyzer (TGA) - Pyris diamond TG/DT analyzer (PERKIN ELMER)
22. CHN analyzer - PERKIN ELMER, 2400 Series-II analyzer
23. Bomb calorimeter - Changsha Kaiyuan Instruments Co., 5E-169 1AC/ML.
24. Compound microscope – Leica ATC 2000 with attached AVC 561 Digital Color CCD Camera
25. Scanning electron microscope (SEM) - JEOL JSM Model 6390 LV Asia PTE Ltd. Singapore model
26. Polymerase chain reaction (PCR) thermal cycler - Applied Biosystem
27. Electrophoresis system for DNA - Genaxy, horizontal electrophoresis chamber, Genaxy Scientific Pvt. Ltd.
28. DNA sequencer - ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

3.4 Microalgal culture media

Different culture media used in the present investigation are presented in Table 3.1

Table 3 1 Composition of microalgal culture media (mg/L)

Composition	Modified Chu- 13	BBM	BG-11	Basal
KNO_3	200	-	-	100
$NaNO_3$	-	250	1500	-
K_2HPO_4	40	74	40	-
KH_2PO_4	-	17 5	-	-
$CaCl_2$ $2H_2O$	80	24	36	-
$MgSO_4$ $7H_2O$	100	73	75	40
Na_2CO_3	-	-	20	-
NaCl	-	25	-	-
$FeSO_4$	-	5	-	-
EDTA	-	45		-
Citric acid	100	-	6	-
Ammonium Ferric Citrate	-		6	
Ferric citrate	10	-	-	-
$Ca(NO_3)_2$ $4H_2O$	-	-	-	150
β-Na_2gly serophosphate	-	-	-	50
EDTA-Na_2	-	-	1	2 71
Vitamin B_{12}	-	-	-	0 0001
Biotin	-	-	-	0 0001
Thiamine-HCL	-	-	-	0 01
H_3BO_3	-	-	2 86	-
$MnCl_2$ $4H_2O$	-	-	1 81	0 108
Composition	Modified Chu- 13	BBM	BG-11	Basal
$ZnSO_4$ $7H_2O$	-	-	0 22	0 066
Na_2MoO_4 $2H_2O$	-	-	0 39	0 0075
$CuSO_4$ $5H_2O$	-	-	0 08	-
$Co(NO_3)_2$ $6H_2O$	-	-	0 05	-
$FeCl_3$ $6H_2O$	-	-	-	5 888
$CoCl_2$ $6H_2O$	-	-	-	0 012
Trisaminomethane	-	-	-	500

3.5 Sample collection

The procedure of collection was designed in Prof B. K. Konwar's laboratory prior to collection. The samplings were carried out with the assistance of researchers from Prof. Konwar's research group. The rationale behind selecting the sampling sites were

1) All the sampling sites (fresh water bodies) were identifiable by Google Earth

2) Sites having easy access and located nearby the national highway were selected.

Fig. 3.1 shows the location of sampling sites as depicted in the map of Assam. The sample sites with their respective geographic coordinates are presented in Table 3.2.

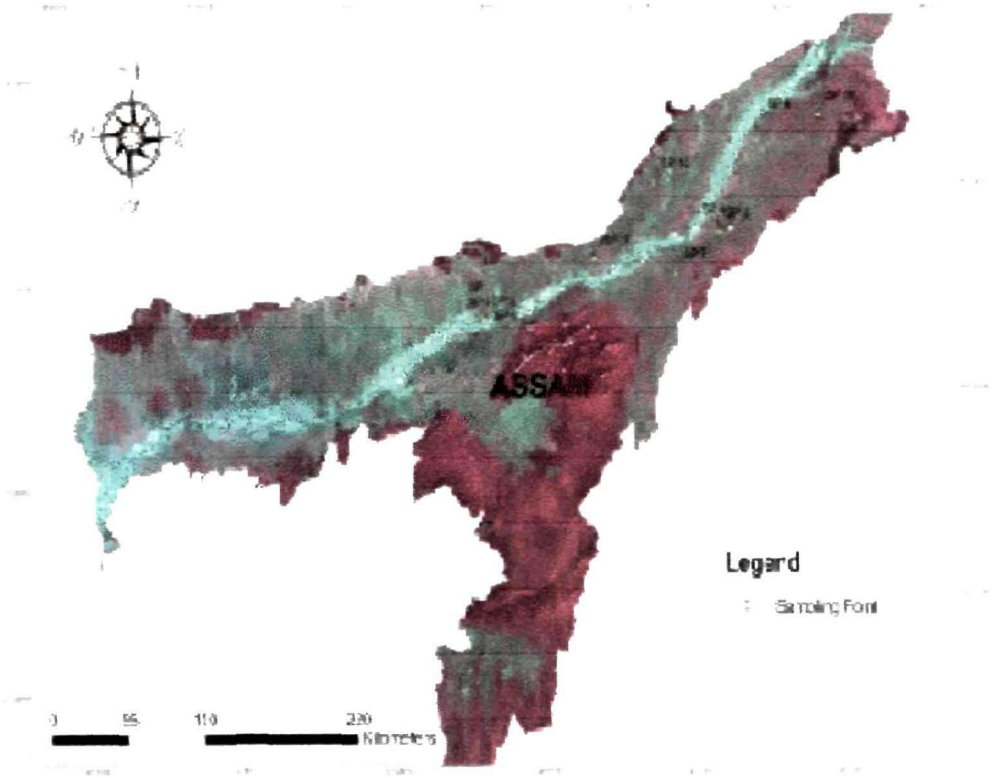


Fig. 3.1. The map of Assam, India with indications of sampling sites in it.

Table 3.2 Sampling sites with respective geographical coordinates

Sampling Sites	Location	Latitude	Longitude
SS 1	Tezpur University Lake	26°41'55"N	92°49'50"E
SS 2	Tezpur University (School of Engineering) water body	26°42'10"N	92°49'47"E
SS 3	Eutrophic Water Body, Poruwa, Tezpur	26°40'9"N	92°47'51"E
SS 4	Padum Pukhuri, Tezpur	26°37'18"N	92°47'31"E
SS 5	Hazara Pukhuri, Tezpur	26°37'45"N	92°46'53"E
SS 6	Polo Field Lake, Dibrugarh	27°29'25"N	94°55'17"E
SS 7	Tinikuniya Pukhuri, Jorhat	26°45'5"N	94°12'34"E
SS 8	Gaurisagar Pukhuri, Sibsagar	26°56'38"N	94°32'11"E
SS 9	Jaysagar Pukhuri, Sibsagar	26°57'7"N	94°37'24"E
SS 10	Tinikuniya Pukhuri, Tinsukia	27°29'8"N	95°21'6"E
SS 11	Gohpur PS Pond, Gohpur	26°52'58"N	93°36'54"E
SS 12	Rodhola Pukhuri, Lakhimpur	27°14'8"N	94° 6'33"E

Replicative water samples (50ml) were collected from the respective water bodies in amber glass bottles. Following the collection and containerization of the samples, the external surface of the glass bottles were cleaned with absorbent materials in order to ensure removal of spilled samples. The samples were collected only from the epilimnetic layer of the water bodies. Each of the glass bottles were labeled with a unique identification code number and associated information. The field samples were brought to the laboratory for processing within 24h of collection.

3.6 Compound microscopy

Following collection, each sample of microalgal isolate were microscopically observed for generic identification and record. The microscopic observations were carried out using a Leica ATC 2000 microscope and the images were captured with an AVC 561 Digital Color CCD camera attached to the microscope. The images were viewed microscopically and photographed without staining.

3.7 Isolation and purification of microalgae

The microalgal isolates were subjected to purification by serial dilution followed by quadrant streaking. The plates were incubated at 28 ± 2°C to obtain individual discrete colonies. The individual colonies were isolated by a sterile inoculation loop and inoculated into liquid medium (BG-11). The purity of the microalgal cultures were established by repeated streaking and routine microscopic observation.

3.8 Screening of microalgae for biofuel production

Microalgal isolates were screened on the basis of their energy content i.e. calorific value. The isolates were harvested by centrifugation and their producible quantum in terms of cell dry weight was determined gravimetrically. From the fresh water bodies a total of twenty one (21) strains of microalgae were isolated. Three (3) on the basis of calorific value were selected for potential biofuel applications.

3.8.1 Biomass estimation

The microalgal cultures were harvested by centrifugation at 10,000 rpm for 15 min. Cells were washed twice with distilled water after centrifugation. The pellet was dried at 80°C for 24h. The dry weight of the microalgal biomass was determined gravimetrically and growth was expressed in terms of dry weight.

3.8.2 Determination of Gross Calorific Value (GCV)

Calorific value is one of the foremost considerations in bioenergy studies. Microalgae shall have a high calorific value in order to be a fuel [82]. Screening of microalgae was done on basis of the energy content (MJ/kg).

The Gross calorific value (GCV) was determined using an automatic adiabatic bomb calorimeter. The sample was oxidized by combustion in an adiabatic bomb containing 3.3Mpsi oxygen under pressure. The assays were done in triplicates and the mean values were recorded.

3.9 Optimization of cell growth conditions

3.9.1 Preference of media for microalgal growth

The suitability of growth media for the microalgal isolates was assessed by culturing the isolates in four different media viz., modified Chu-13 [141], Bold Basal Medium [142], BG-11 [143] and Basal media [144].

3.9.2 Optimization of cell growth conditions

Optimization of growth conditions of the microalgal isolates was carried out with regard to temperature and pH. The microalgal isolates were cultured in suitable growth media at variable temperatures 20, 25, 30 and 35°C and at pH 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9. The cultures were carried out in 500ml Erlenmeyer flasks with shaking at 100rpm; light intensity 1200lux; 16:8 light and dark cycle; and inoculation at 10% v/v.

3.9.3 Monitoring of growth

The growth of the microalgal species were monitored by counting the microalgal cells in a Nuebaeur haemocytometer.

3.9.4 Determination of growth rate of microalgal cultures

The specific growth rate was determined by use of the following equation [145]

$$\mu = \ln N_t - lnN_o/T_t - T_0 \quad (1)$$

where, N_t= Number of cells at the end of log phase

N_o= Number of cells at the beginning of log phase

T_t= Final day of log phase

T_o= Starting day of log phase

If 'T' is expressed in days, then the growth rate (μ) can be converted to doublings per day (k) by dividing (μ) by the natural log of 2.0, according to the equation:

$$K = \mu/0.6931 \quad (2)$$

Doubling time T_2 for the microalgal cultures were calculated by the use of the following equation

$$T_2 = 0.6931/\mu \quad (3)$$

3.10 Role of heavy metal in lipid content

A preliminary investigation was carried out to study the impact of heavy metal (Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+},Co^{2+} and Cr^{2+}) induced stress on the lipid content of microalgal cells. The heavy metals were added in varying concentrations of 0.5, 1 and 2mM to the culture medium. The cultures were done in 500ml Erlenmeyer flasks with shaking at 100rpm; light intensity 1200lux; 16:8 light and dark cycle; and inoculation at 10% v/v. Once cultures reached the steady phase cells were harvested by centrifugation at 8000 rpm for 15min. The resultant microalgal pellet was lyophilized and subjected to FTIR analysis. An increase in lipid content was detected with the increase in the intensity of absorbance in the range of 3100-2800cm^{-1} against the control sample (FTIR spectra of untreated biomass).

3.11 Collection of representative water samples from prospective mass culture sites (PMCS)

An attempt was made to identify and map the PMCS with special reference to eutrophic water bodies formed as a result of chronic floods in the floodplains of the

Brahmaputra Valley, Assam, India. Ground verification for the sites was done by field visit and subsequent collection of representative water samples. The species under investigation (*P. kessleri*) was cultured in the representative water samples from PMCS and the subsequent microalgal growth kinetics were monitored in open laboratory conditions as against the control. Three such eutrophic water bodies in Dhemaji district of Assam namely S1 ($94^0$24'05"E $27^0$24'23"N), S2 ($94^0$27'25"E $27^0$25'22"N) and S3 ($94^0$37'31"E $27^0$29'09"N) were identified and mapped using satellite images and Google Earth. The study investigates the feasibility of mass culture of microalgae in the permanently inundated areas which are otherwise considered as wastelands.

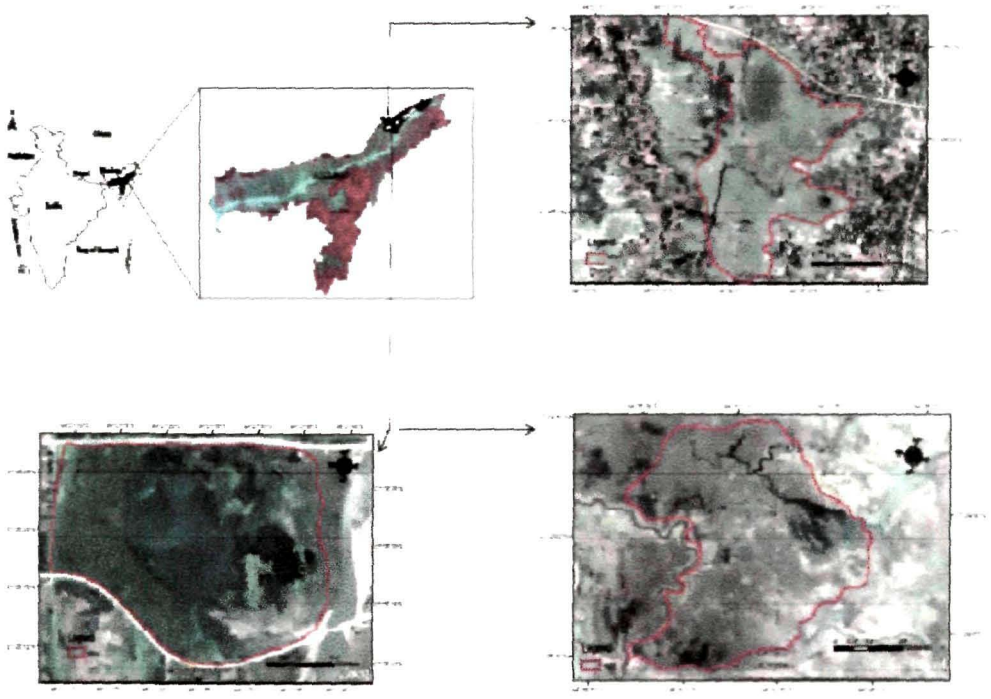


Fig. 3.2. Satellite images of prospective mass culture sites in Dhemaji district, Assam

Representative water samples from the aforesaid water bodies were collected in 5L conical flasks (Borosil, Mumbai, India) and passed through 40-mesh sieve to

remove grass and fine solids. The collected water samples were used as the natural medium for the culture of microalgae.

3.12 Determination of dissolved oxygen, nitrate and phosphate

The dissolved oxygen was measured using a multi parameter probe (HANNA HI-9828 multi parameter water quality probe). Nitrate was determined by the ultraviolet spectrophotometric screening method and phosphate concentration was determined by the stannous chloride method [161].

3.13 Determination of cell contents

3.13.1 Protein estimation

Microalgal culture 10mL was centrifuged at 6000rpm for 15min. The cell pellet was resuspended in 5mL of 1M NaOH and boiled for 5min. The protein content was determined by the Lowry method [146]. A calibration curve was prepared using BSA dissolved in distilled water.

3.13.2 Carbohydrate estimation

Total carbohydrate was determined by the anthrone method [147]. A calibration curve was prepared using D-glucose dissolved in distilled water.

3.13.3 Determination of total lipids

Total lipid was determined by the method described by Bligh and Dyer [148]

3.14 Scanning electron microscopy

Microalgal cells were harvested by centrifugation at 7000rpm for 10min, washed with PBS and fixed with 0.5% glutaraldehyde in 0.1M cacodylate buffer. Dehydration was carried out in an acetone series with 30 min changes (30, 50, 70, 90 and 100%). The scanning electron micrographs were acquired using a (JEOL JSM-6390 LV PTE Ltd. Singapore model) scanning electron microscope. For analysis, the dehydrated microalgal biomass was sprinkled on the carbon tape and then coated with 30 nm platinum coat using JOEL auto fine coater (model no. JFC-1600). The SEM was taken at an acceleration voltage of 15KV (maximum) and under 1 Pascal pressure.

3.15 Carbon, hydrogen and nitrogen analysis

Carbon, nitrogen and hydrogen content of the samples were determined in a Perkin Elmer, 2400 Series-II analyzer. The percentage of oxygen was calculated by difference.

3.16 Determination of Net Calorific Value (NCV)

The NCV was calculated from the following equation [149]

$$NCV = GCV \times \left(1 - \frac{w}{100}\right) - 2.444 \times \left(\frac{w}{100}\right) - 2.444 \times \left(\frac{H}{100}\right) \times 8.936 \times \left(1 - \frac{w}{100}\right); \left[\frac{MJ}{Kg}, w.b.\right]$$

(4)

where, 2.444=Enthalpy difference between gaseous and liquid water at 25 ^{0}C.

$$8.936 = \frac{M_{H_2O}}{M_{H_2}}; \text{i.e. the molecular mass relation between } H_2O \text{ and } H_2$$

where,

NCV= Net calorific value

GCV= Gross calorific value

h= Concentration of hydrogen in wt.%

w= Moisture content of the fuel in wt.%

3.17 FTIR analysis

For FTIR analysis the microalgal cells were harvested by centrifugation at 10,000rpm for 10min. The supernatant was discarded and the algal pellets were washed twice with phosphate buffer (pH 7.1mM Na_2HPO_4/NaH_2PO_4 buffer). After second wash in phosphate buffer, samples for FTIR analysis were stored at -20°C until lyophilisation. The dried algal biomass of 1mg was taken in an agate mortar and mixed thoroughly with 2.5mg of dry potassium bromide (KBr) using a pestle. The IR spectra were recorded at room temperature (26°C±2°C) in the mid infrared range (4000-400 cm^{-1}).

3.18 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was done in order to study the degradation profile of the microalgal biomass. For the analysis, microalgae from the late exponential phase were harvested by centrifugation at 10,000rpm for 10min. The

supernatant was discarded and the pellet was washed twice with distilled water and then finally dried at 80°C for 24h. The dried pellet was pulverized in a mortar to fine particles and then finally stored in a dessicator. Thermogravimetric analysis (TGA) was done to study the degradation profile of the microalgal biomass. The algal biomass was subjected to TGA in nitrogen atmosphere at heating rates of 10 and 30°C/min. Approximately, 10mg sample was heated at the preselected heating rates from ambient temperature to 900°C. A high purity nitrogen gas (99.99%) was fed at a constant flow rate of 100ml/min. The continuous on-line records of weight loss and temperature were obtained to plot the TGA curve and the derivative thermogravimetric analysis curves.

3.19 *Proximate analysis of microalgal biomass*

3.19.1 Determination of moisture content

Moisture content of the microalgal biomass was determined according to the ASTM D4442-07 method. The collected samples were weighed to determine their "green weight". After initial weighing the samples were taken in an aluminum container and placed in an oven which was preheated at 105 ± 3°C. The samples were kept in the oven until a constant weighed was reached. The weight of the samples at this point was considered as the direct measurement of the dry weight of cells which was subtracted from the green weight to obtain the moisture content. The following equation gives the moisture content:

$$Moisture\ content(\%) = \frac{Greenweight - Ovendryweight}{Greenweight} \times 100 \quad (5)$$

For each sample, the experiment was done in triplicate and only the mean value is reported

3.19.2 Determination of volatile matter

Volatile matter of the microalgal samples was determined by the method described in ASTM Test No. D-271-48. A platinum crucible of 10ml capacity was taken and its surface was cleaned by rubbing with fine steel wool and heated in a

furnace at 950^{0}C for 2min. The sample was allowed to cool in a desiccator for 15min and the weight of the platinum crucible was taken. $^1/_2$ to $^3/_8$ inch on top of the crucible was filled up with the ground oven dried sample and gross weight was taken, and then heated in the furnace at 950^{0}C for 2min. After the escape of the volatile matter, the crucible was removed from the furnace and cooled in air for 2 to 5min. The sample was then kept in a desiccator for 15min. The percentage of weight loss of the sample was reported as volatile matter as follows:

$$\%Volatile\ matte = \frac{Weight\ loss\ of dry\ sample}{Net\ weight\ of dry\ sample} \times 100 \quad (6)$$

For each sample, the assay was done in triplicate and only the mean value has been reported.

3.19.3 Determination of ash content

For determination of ash content, TAPPI standard method, T211 om-85, was followed. At first, an empty 25ml. silica crucible was heated in a muffle furnace at 575 ± 25^{0}C for 15min and allowed to cool in desiccators for 45 min and then weighed accurately. Ground and oven dried sample of the respective microalgal biomass was weighed and transferred into the crucible and kept in a muffle furnace at 575 ± 25^{0}C to ignite for a period of 3h to burn the carbon (completion of which was indicated by the absence of black particles). The crucible was then removed from the furnace and kept in a desiccator and weighed accurately. The percentage of ash content was calculated as follows

$$\%Ash = (Weight of ash) / (Weight of sample) \times 100 \quad (7)$$

For each sample, the assay was done in triplicate and only the mean value recorded.

3.19.4 Determination of fixed carbon

Fixed carbon content of the respective microalgal biomass was done by a simple calculation as given in ASTM Test No. D-271-48 which is as follows

$$\%\, F.C.(on\ dry\ basis) = 100 - [volatile\ matter\ (\%) + ash\ (\%)] \quad (8)$$

3.20 *In situ* transesterification

In situ transesterification was carried out by the method described by Wahlen *et al.* [92] with modifications. Tetramethyl guanidine (TMG) was used as the catalyst in place of concentrated H_2SO_4. In short, transesterification reactions were carried out with 100mg of lyophilized microalgal biomass. The sample biomass was reacted with 2ml of CH_3OH containing 25% (v/v) TMG for 30 min at 60°C in 50ml conical flasks. Upon completion, the reaction was quenched with the addition of $CHCl_3$ (1ml) whereby forming a single-phase solution with methanol. Phase separation was achieved by washing the methanol–chloroform solution with 5ml distilled water, followed by centrifugation at 2000rpm for 2min. TMG and CH_3OH partitioned with water in the upper phase, while TAG, FAME, and other lipids partitioned with $CHCl_3$ in the lower, organic phase. The remnant biomass formed a layer at the boundary between two phases. The chloroform phase was removed by a syringe to a 10ml centrifuge tube. The remaining biomass was washed twice with 2ml of chloroform to recover residual FAMEs and lipids. The total volume of $CHCl_3$ was brought to 5ml and mixed by inversion following which aliquots of 100µl used for GC-MS analysis.

The *in situ* approach was also carried out for a yeast strain *Saccharomyces cerevisiae* 0A03 (Genbank entry KF650433). The strain was obtained from lab-1 of the department of Molecular Biology and Biotechnology, Tezpur University, Assam, India.

3.21 Determination of fatty acid profile

3.21.1 Gas chromatography (GC) analysis

The fatty acid profile was determined by GC of the corresponding methyl ester on a TRACE™ 1300 (Thermo Scientific) gas chromatograph equipped with FID detector and TRACE™ TR-FAME GC column. The column temperature was held at 50°C for 2min, raised to 200 °C @10°C/min then to 300 °C@5°C/min and held for

10min at this temperature. The carrier gas used was helium and the flow rate was maintained at 1ml/min. The individual components were identified by comparison of retention time with the standard FAME mix and the wt% of the individual fatty acids was calculated based on the internal standard

3.21.2 GC-MS analysis

The fatty acid profile was analyzed by a Perkin Elmer Clarus 600 gas chromatograph (GC) coupled with a Perkin Elmer Clarus 600C mass spectrometer (MS) equipped with Elite 5MS capillary column (30m × 0.25mm × 0.25μm). The oven temperature was programmed from 85°C (5min) to 265°C at a rate of 5°C/min and finally held at 265°C for 10min. Helium was used as the carrier gas with a flow rate of 1ml/min. The MS was operated in the electron ionization mode at 70eV. The interface temperature was 265°C and the mass range was 15-650 m/z. The identification of fatty acids was performed, comparing the obtained mass spectra with NIST library.

The fatty acid profile of *Scenedesmus* sp. was determined by GC-MS analysis.

3.22 Theoretical determination of fuel properties of FAME

The fuel properties of FAMEs were determined by the theoretical method of Ramirez- verduzco *et al.* [150]

3.22.1 Determination of viscosity

The kinematic viscosity of the individual FAME was expressed as function of M_i and N is

$$\ln(\eta_i) = -12.503 + 2.496.\ln(M_i) - 0.178.N \quad (9)$$

where, M_i represents the molecular weight of the ith FAME, and N is the number of double bonds in a given FAME η_i is the kinematic viscosity at 40°C of the ith FAME in mm^2/s.

3.22.2 Determination of density

The density of the individual FAME was determined from the following equation

$$\rho_i = 0.8463 + {}^{4.9}/_{M_i} + 0.0118.N(10)$$

where, M_i represents the molecular weight of the ith FAME, and N is the number of double bonds in a given FAME, ρ_i is the density at 20°C of the ith FAME in g/cm^3.

3.22.3 Determination of Cetane number

The cetane number of the individual FAME was calculated from the following equation

$$\emptyset_i = -7.8 + 0.302.M_i - 20.N \quad (11)$$

where, M_i represents the molecular weight of the ith FAME, and N is the number of double bonds in a given FAME.

3.22.4 Determination of Higher Heating Value (HHV)

The HHV of the individual FAME was calculated from the following equation

$$\delta_i = 46.19 - \frac{1794}{M_i} - 0.21.N \quad (12)$$

where, M_i represents the molecular weight of the ith FAME, and N is the number of double bonds in a given FAME

The physical properties of biodiesel was estimated from the individual physical properties of FAMEs using the following general expression

$$f_b = \sum_{i=1}^{n} z_i.f_i \quad (13)$$

where, f is a function that represents any physical property (the subscripts b and i refer to the biodiesel and the pure ith FAME, respectively), z_i is the mass or mole fraction of the ith FAME. The function f_b must be replaced by the variables $\emptyset_b$, $\ln(\eta_b)$, ρ_b and δ_b in order to specify the cetane number, natural logarithm of kinematic viscosity, density and higher heating value of biodiesel, where as the function f_i must be interchanged by

the variables in order to specify $\emptyset_i$, $\ln(\eta_i)$, ρ_i and δ_i the properties of the individual ith FAME

3.23 Pyrolysis of microalgal deoiled cake

The bio-oil was produced in a vertical tubular lab scale fixed-bed glass reactor (ambient to 500°C at a heating rate of 40°C in nitrogen atmosphere) and the product yield were calculated on the basis of the following equations [151].

$$Bio-oil yield(wt\%) = \frac{M_{bio-oil}}{M_{microalgae}} \times 100 \quad (14)$$

$$Char\ yield\ (wt\%) = \frac{M_{char}}{M_{microalgae}} \times 100 \quad (15)$$

$$Gaseous\ yield\ (wt\%) = M_{microalgae} - (M_{bio-oil} + M_{char}) \quad (16)$$

where, $M_{microalgae}$ is the mass of the dried microalgal de-oiled cake (powder), $M_{bio-oil}$ is the mass of bio-oil, and M_{char} is the mass of char.

3.24 GC-MS analysis of bio-oil

The bio-oil profile was analyzed by a Perkin Elmer Clarus 600 gas chromatograph (GC) coupled with a Perkin Elmer Clarus 600C mass spectrometer (MS) equipped with DB5MS capillary column (30m × 0.25mm × 0.25μm). The oven temperature was programmed from 70°C (2min) to 300°C at the rate of 10°C/min. High purity helium (99.9995%) was used as the carrier gas at a flow rate of 1ml/min. The MS was operated in electron ionization mode at 70eV. The interface temperature was 240°C and the mass range was 40-1000m/z. The compounds were identified by comparing the obtained mass spectra with NIST library.

3.25 NMR spectroscopy

The ^{1}H NMR spectrum of the microalgal bio-oil was recorded using JEOL JNN-ECS 400 spectrophotometer. A sample of 20μL bio-oil was dissolved in 1ml

deuterated chloroform ($CDCl_3$) and analyzed with ^1H NMR (400 MHz) at 25°C. Chemical shifts were reported in ppm relative to the signal of tetramethylsilane.

3.26 Molecular characterization of microalgae

3.26.1 DNA extraction

Microalgal DNA was extracted using a QIAGEN plant DNA extraction kit (DNeasy Plant Mini Kit). The following are the steps followed for extracting the DNA:

- The microalgal biomass (≤ 100 mg fresh weight) was disrupted using a mortar and pestle and the content was transferred to a 2mL collection tube.
- Buffer AP1 400µl and RNaseA 4µl was added to the collection tube. The resulting solution was vortexed and incubated at 65°C for 10 min. The tube was inverted 2-3 times during incubation.
- Buffer P3 130µl was added to the mixture. The solution was mixed thoroughly and incubated for 5min on ice.
- The lysate was centrifuged at 14,000rpm for 5min.
- The lysate was pipetted into a QIAshredder spin column placed in a 2mL collection tube following which centrifugation was done at 14,000rpm for 2min.
- The flow-through was transferred to a new tube without disrupting the pellet. Buffer AW1 1.5 volumes was added to the mixture and mixed by pipetting.
- 650µl of the mixture was transferred into a DNeasy Mini spin column placed in a 2mL collection tube. The mixture was centrifuged at 8000rpm for 1min. The flow-through was discarded. (This step was repeated with the remaining samples).
- The spin column was placed in a 2mL collection tube. Buffer AW2 500µl was added to the mixture and centrifuged at 8000rpm for 5min. The flow-through was discarded.

- Another 500µl of Buffer AW2 was added to the mixture and centrifuged for 2min at 14,000rpm. The spin column was removed from the collection tube with utmost care in order to avoid contact between the column and flow-through.
- The spin column was transferred to a new 2mL microcentrifuge tube.
- Following this 100µl Buffer AE was added to the mixture for elution and incubated for 5min at room temperature (25°C). Finally, centrifugation was done for 1min at 8000rpm.

3.26.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as follows

- Agarose gel 0.8% was prepared with 1X TAE buffer and poured into the gel caster sealed with adhesive tape and fitted with comb.
- The comb and adhesive tape were removed when agarose gel got solidified.
- Following this the gel was placed in the electrophoresis chamber filled with 1X TAE buffer.
- DNA sample preparation: The DNA samples were mixed with 2.0µl of loading dye (6X) for 5.0-10.0µl of sample.
- The sample was run at 75volt for 30 min.
- The gel was removed from the electrophoresis chamber and examined on a UV transilluminator.

3.26.3 PCR amplification of 18 S-rRNA gene

Primers used for amplification and sequencing are presented in Table 3.3

Table 3.3 Primers for amplification and sequencing

SL. No	Primers	Sequence
1	ITS1	5′ TCCGTAGGTGAACCTGCGG 3′
2	ITS5	5′ GGAAGTAAAAGTCGTAACAAGG 3′
3	ITS4	5′ TCCTCCGCTTATTGATATGC 3′

3.26.4 PCR reaction mixture

Each PCR reaction was set in 15µl reaction volume consisting of 1.5µl of 10x Taq buffer (1.5µl of 15mM $MgCl_2$ was added separately to the reaction mixture), 1.5µl of 2mM dNTP mix, 0.2µl of Taq polymerase (5U/µl), 1µl of 10µM primer (Sigma Aldrich, India) and finally the volume was adjusted to 15µl with sterile de-ionized water. To the reaction mixture, 1µl microalgal genomic DNA was added.

3.26.5 PCR conditions

The PCR reaction was performed with 20ng of genomic DNA as the template in a 30µl reaction mixture by using a EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2min, 35 cycles of 95°C for 1min, 55°C, and 72°C for 1min each were performed, finishing with a 10min step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA).

3.26.6 DNA sequencing

Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

3.26.7 Nucleotide sequence analysis

A BLAST [151] search was performed for the microalgal species and identification was done on the basis of BLAST result. Further, the sequences of 18S r-RNA genes were deposited in the NCBI GenBank [152]. Additionally, the sequence alignment was carried out with the related species using CLC Main Workbench and the respective phylogenetic trees were generated using the neighbour joining method implemented in CLC Main Workbench [153].

3.27 Antioxidant analysis of microalgal deoiled cake

3.27.1 Total phenolic content (TPC)

The TPC of microalgal deoiled cake was determined using the standard Folin-Ciocalteu method [154] with slight modifications. The sample extract 100µl was reacted with 5ml of 50% Folin-Ciocalteu's reagent. 4ml of 20% sodium carbonate was added after 15min. The blank sample was prepared by replacing 100µl sample extract with 100µl deionised water. The reaction mixture was incubated in the dark at room temperature for 30min. Absorbance was measured against the blank sample at 765 nm using a Thermo Scientific UV-10 (UV-Vis) spectrophotometer. Gallic acid was used as the reference standard. A calibration curve of gallic acid was plotted by using the absorbance values against the various concentrations (100-1000µg/ml) of gallic acid. All the experiments were done in triplicate and the results obtained were averaged and expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of the algae material.

3.27.2 DPPH free radical scavenging assay

The antioxidant activity of the extracts were determined using DPPH (1, 1-diphenyl 2-picryl hydrazyl) free radical scavenging assay with modifications [155]. DPPH 0.004% solution in methanol was freshly prepared prior to analysis. The sample extract 1ml was added to 3ml DPPH solution, vortexed and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a DPPH blank using a Thermo Scientific UV-10 (UV-Vis) spectrophotometer. BHT (Butylatedhydroxytoluene) was used as the reference standard. Ability to scavenge DPPH radical was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [(Abs_{blank} - Abs_{sample})]/\ (Abs_{blank}) \times 100 \quad (17)$$

where, Abs_{blank} is the absorbance of DPPH solution in methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract/standard.

3.27.3 Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay was done for determining the total antioxidant activity of the sample extracts with modifications [156]. Solutions of 300mmol/L, pH 3.6 sodium acetate buffer, TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) 10mmol hydrochloric acid 40mmol and $FeCl_3.6H_2O$ 20mmol/L were prepared. A fresh working solution was prepared by mixing the three solutions in ratio of 10:1:1 respectively. The temperature of the solution was raised to 37°C before use. Each sample extract of 100µl was allowed to react with 3ml of FRAP solution for 30min in the dark at room temperature. Absorbance of the reaction mixture was measured at 593nm against a reagent blank using a Thermo Scientific UV-10 (UV-Vis) spectrophotometer. The standard curve was prepared using $FeSO_4$ (100-2000µg/ml) solution and the results were expressed as µmol Fe(II)/g dry weight of the algae material. BHT was used as the reference standard.

3.28 Antimicrobial assay of microalgal bio-oil

3.28.1 Preparation of cultures

E. coli MTCC723 and *Staphylococcus aureus* MTCC96 were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Fresh bacterial cultures (*S. aureus* and *E. coli*) were prepared by adding a loopful of stock culture to sterilized nutrient broth. The media was incubated at 37°C for 24h and used for assaying antibacterial activity. Fresh cultures of *C. albicans* and *S. cerevisiae* were prepared by adding a loopful of stock culture to Potato Dextrose Broth (PDB) and YPD medium respectively. The former culture was incubated at 37°C (48h) and the later at 28°C (48h), respectively for evaluating antiyeast and antifungal activity.

3.28.2 Agar well diffusion method

The antimicrobial assay was done by using the agar well diffusion method. The assay was carried out to find out if the thermally converted products (TCP) had any antimicrobial activity. Microbial cultures were adjusted to 0.5 McFarland standards

before the tests. The media used for antibacterial assay was Muller Hinton Agar, whereas PDB and YPD media were used to access the antifungal and antiyeast activity. The standard solutions of the samples were 500µl/ml DMSO (Merck). Sterile liquid culture media (25mL) were poured into petriplates and after solidification were inoculated by spread plate method with an inoculum corresponding to 0.5 McFarland standards. Three (3) wells of 5mm diameter were punched into the agar with the help of a sterilized well puncturer (5mm diameter) and 50µl of the sample was added to the sample well. Chloramphenicol (30µg/mL) and 1% DMSO was taken as the positive and negative control, respectively. The plates were then sealed with paraffin and kept for incubation at 37°C for 24h (for *S. aureus*, *E. coli* and *S. cerevisiae*) and 28°C for 48h (for *C. albicans*). The antimicrobial activity was evaluated by measuring the ZOI diameter with the zone scale (Antibiotic Zone Scale, HIMEDIA). The tests were carried out in triplicate and only the mean values were recorded.

3.28.3 Minimum inhibitory concentration

The MIC was determined following the protocol of Wang *et al.* [157] with slight modification. The MIC activity was determined using a 96-well microtitre plate. The stock solution for the sample bio-oil was 100µl/ml DMSO). A volume of 10ml culture of *E. coli*, *S. aureus* were prepared in LB broth, whereas the same amount of culture of *C. albicans* and *S. cerevisiae* were prepared in PDB and YPD broth, respectively. Saturated cultures of all four strains were then diluted to form an approximately 1×10^6 colony forming units (CFU)/ml. In the 1st well 200µl of stock solution was added and serially diluted by adding the respective culture medium. Following this 100µl of culture was added to the respective wells. Kanamycin at the concentration of 50mg/ml was used as the positive control for bacteria, whereas Indofil M-45 (commercial fungicide) at the concentration 50mg/ml as the positive control for *C. albicans* and *S. cerevisiae*. DMSO (1%) was used as negative control for all the test samples. The plates were covered and incubated overnight at 37°C (for *S. aureus* and *E. coli*), and 28°C for 48h for *C. albicans* and *S. cerevisiae*. At the end of the

incubation period, 40µL of MTT solution at 0.2 mg/mL was added into each well and then further incubated at 37°C for 45min. The culture absorbance was recorded spectrophotometrically at 570nm.

3.29 Production of biodiesel from kitchen chimney dump lard (KCDL)

3.29.1 Collection of KCDL feedstock for biodiesel production

KCDL was collected from the kitchen chimney of Nilachal Men's Hostel, Tezpur University, Assam-784028, India (26.7008°N and 92.8303°E). KCDL is primarily a kitchen waste which can be collected from the horizontal collecting ducts present in a kitchen chimney. It is formed as a result of frying food items where the vapors of the cooking oil after condensation gets collected in semi solid form in the collecting ducts of a kitchen chimney. KCDL is light brown in color and has a murky odor. A clean spatula was used to collect the feedstock from the kitchen chimney. After collection the feedstock was kept in a beaker and labeled properly with necessary information.

3.29.2 Determination of acid value of KCDL

The acid value of the sample was determined by titrating it against KOH using phenolphthalein as an indicator. The calculations was done using the following formula: [162]

Acid value (mg KOH/gm) = Titre value × Normality of KOH × 56.1/Weight of the sample (gm)

where, the factor 56.1 is taken from equivalent weight of KOH since 1ml 1N KOH contains 56.1mg wt. of KOH.

3.29.3 Transesterification

A two stage transesterification process was used for the production of biodiesel from KCDL. In the first stage, concentrated H_2SO_4 (97% purity) was used as an acid catalyst to convert high FFAs to esters and in the second stage NaOH was used as an

alkali catalyst to convert triglycerides into biodiesel. The standard biodiesel reaction mixture comprised of KCDL, methanol and appropriate concentration of catalysts (acid and basic).

3.29.4 Heat treatment of feedstock

Prior to acidic esterification the KCDL feedstock was heated at 120°C for 30 min. This step is required to melt all the solid fat present in the feedstock.

3.29.5 Two stage transesterification and statistical optimization

The sample from the previous step was taken and allowed to cool to a temperature of 50°C and the required amount of methanol (60% by vol, 99% purity) was added. Following this, H_2SO_4 (3% v/v, 97% purity) was added to the mixture and the reaction was commenced at 50°C with 600rpm for 60min. Once completed the reaction mixture was placed in a separating funnel and left to stay overnight. The upper layer being a mixture of unreacted triglycerides, unreacted methanol and esters was separated from the lower layer. This layer was washed thrice with warm water (~60°C) to remove the unreacted catalyst and methanol. Finally, the product (esterified) was heated at 115°C for 20min to ensure moisture removal.

The second phase of the two stage transesterification process (alkali catalyzation) was optimized using response surface methodology (RSM). RSM explores the relationship between the explanatory variables with the response variables. Its main idea is to obtain an optimal response using a sequence of designed experiments [158, 159]. In the present investigation, Box–Behnken design [160] experiment for RSM was employed for four independent variables viz. methanol (χ_1), NaOH (χ_2), temperature (χ_3) and time (χ_4) for the optimal biodiesel yield during the second stage transesterification step. The range of four variables studied is shown in Table 3.4 and the factors of highest confidence levels were represented in three levels, coded −1 for low, 0 for middle and +1 for high.

Table 3.4 Variable ranges for CH_3OH, NaOH, temperature and time

Variables	Variable Code	-1	0	+1
CH_3OH	χ_1	40	50	60
NaOH	χ_2	0.5	1.5	2 5
Temperature	χ_3	52	62	72
Time	χ_4	80	90	100

As per the optimization response, 1.86% NaOH (by wt) was dissolved in 40% (by vol) methanol (99% purity) to prepare the alkali catalyst-methanol mixture. This mixture was prepared freshly to maintain catalytic activity and prevent moisture absorbance. Following this the alkali catalyst-methanol mixture was added to the esterified product and alkali transesterification was commenced at 52°C with 600rpm for 100min. The reaction mixture was then placed in a separating funnel and left to stay overnight. Two distinct layers were formed, the upper layer (biodiesel) and the lower layer (glycerol). The two layers were gravity separated with glycerol simply drained off from the bottom of the separating funnel. The separated biodiesel layer was subjected to the same washing and heat treatment as of the acidic esterification stage. Biodiesel yield (wt %) relative to the weight of KCDL was estimated.

3.29.6 Determination of fatty acid profile

The fatty acid profile was determined by GC-MS analysis and the same is described in section 3.21.2

3.29.7 Analysis of KCDL based biodiesel

The fuel properties of KCDL based biodiesel such as density, viscosity, cloud point, pour point, flash point and calorific value were determined using standard methods and the same has been presented in Table 3.5.

Table 3.5 Standard test methods for determination of fuel properties of KCDL derived biodiesel

Properties	Standard test method used
Density (40°C, g/cm^3)	ASTM D 5002
Viscosity (mm^2/s, cSt at 40 °C)	ASTM D 445
Cloud point(°C)	ASTM D 2500
Pour point (°C)	ASTM D 97
Gross calorific value (MJ/kg)	Adiabatic Bomb Calorimeter
Net calorific value (MJ/kg)	Equation for calculation of NCV (section 3.16)
Flash point (°C)	ASTM D 93
Ash content (Wt %)	ASTM D 874

3.30 Molecular docking

Molecular docking simulation studies were carried out using Molegro Virtual Docker 5.5 (MVD) with regard to compounds identifiable by GC-MS analysis for bio-oil derived from *P. kessleri* against certain proteins of *S. aureus* [Sortase A (PDB ID: 1T2P)] and *E. Coli* [Ribonucleotide reductase R2 (PDB ID: 1AV8), enoyl reductase (PDB ID: 4JQC) and YaeT (PDB ID: 2QDF)] which exhibit anti microbial activity. Molecular docking simulation studies were also carried out also against cytochrome c peroxidase of *S. cerevisiae* (PDB ID: 1AC4), and aspartic proteinase of *C. albicans* (PDB ID: 1ZAP). MVD is based on a differential evolution algorithm and the solution of the algorithm considers the sum of the intermolecular interaction energy between the ligand and the protein, and the intramolecular interaction energy of the ligand. The docking energy scoring function is based on the modified piecewise linear potential (PLP) with new hydrogen bonding and electrostatic terms.

Chapter 4

Results

A total of twenty one microalgal strains were isolated from various water bodies of Assam. The microalgae were subjected to purification by serial dilution and streaking. The individual colonies were isolated and inoculated into Knops medium [163]. Stock cultures of the microalgae were maintained routinely on both liquid and agar slants of Knops medium by regular sub culturing at 15 days interval. The cultures were harvested by centrifugation at 7000 rpm for 15 min. The cell pellets were washed twice with distilled water. The pellets were dried at 80°C for 24 h in a hot air oven. The dried microalgal pellets were subjected to GCV analysis in a bomb calorimeter. Three microalgal strains were selected as potential biofuel source on the basis of calorific value.

4.1 Microalgal cultures

4.1.1 Growth

Initially the microalgal isolates were cultured in Knops medium. Following literature survey, four culture media like Basal, BBM, MC-13 and BG-11 were used for culturing the microalgae. The microalgal isolates were cultured in the stated media for a period of 15 days.

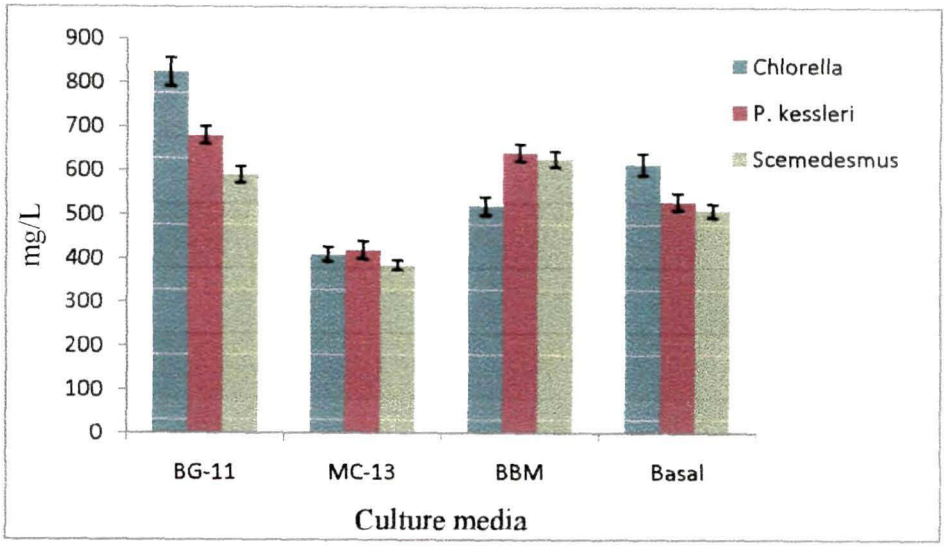


Fig. 4.1 Microalgal biomass yield in various culture media by gravimetric analysis .

The cultures were harvested by centrifugation at 7000 rpm for 15 min. The cell pellets were washed twice with distilled water. The pellets were dried at 80°C for 24 h in a hot air oven. The dry weight of the algal biomass was determined gravimetrically and growth was expressed in terms of dry weight. Data obtained for gravimetric analysis is presented in Fig. 4.1. Out of four, the medium BG-11 was found to be the best for *Chlorella* sp. and *P. kessleri* with a total biomass yield of 824 and 680 mg/L, respectively. For *Scenedesmus* sp. BBM was the best medium with a biomass yield of 625 mg/L.

The growth of microalgae in their respective suitable media was monitored by measuring the cell concentration (cells/mL) by haemocytometer against time duration (h). The results thus obtained are presented in Fig. 4.2 (a), (b) and (c).

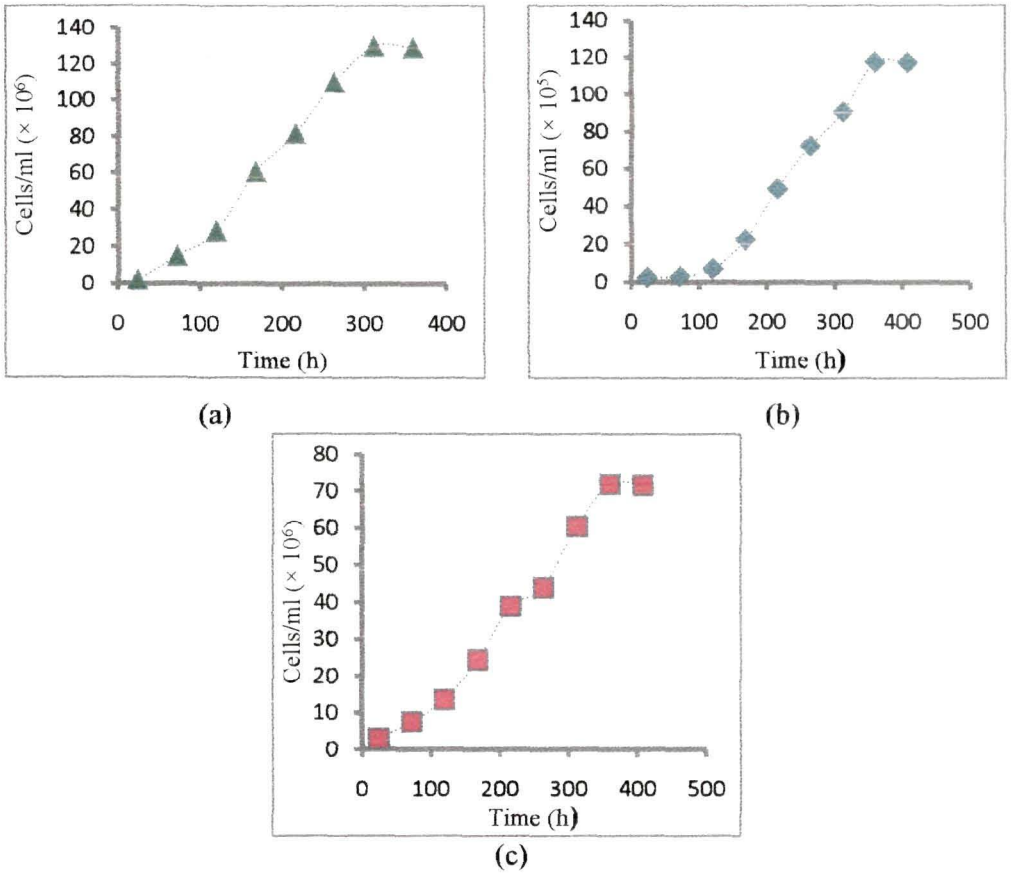


Fig. 4.2 Growth curve of (a) *Chlorella* sp. (b) *Scenedesmus* sp. and (c) *P. kessleri*. in suitable culture media.

After the initial lag phase 120-130 h, the microalgal species exhibited exponential growth. *Chlorella* sp. maintained growth up to 336 h, whereas *Scenedesmus* sp. and *P. kessleri* up to 408 h and thereafter there was a gradual decline in growth of the microalgal species.

4.1.2 Optimization of growth conditions and determination of growth kinetics of microalgae

Each microalgal isolate was cultured in suitable medium with variable pH range of 6.5, 7, 7.5, 8 and 8.5 and incubated at 25, 27, 30, 33 and 35°C to determine the optimum growth conditions. The cultures were carried out in 500 ml Erlenmeyer flasks and incubated in an orbital shaker keeping the photoperiod (16:8), light intensity (1200 lux) and rpm (100) constant. The optimized culture conditions for the microalgal isolates are presented in Table 4.1 (a). The specific growth rate, divisions per day and doubling time were determined and the same are presented in Table 4.1(b).

Table 4.1 (a) Optimized growth conditions for culture of microalgae

Microalgae	Growth media	pH	Temperature
Chlorella sp.MP-1	BG-11	7.5	28°C±2
Scenedesmus sp. MPBK-2	BBM	8.2	28°C±2
P. kessleri MMPBKK-1	BG-11	8.0	30°C±2

Table 4.1 (b) Growth rate in microalgae

Sl. No	Microalgae	Specific growth rate (μ)	Divisions/day (k)	Doubling time (T_2)
1	*Chlorella*	0.21	0.31	3.21
2	*Scenedesmus*	0.27	0.40	2.48
3	*P. kessleri*	0.20	0.29	3.37

From the above two tables, the temperature and pH preference by the microalgal isolates were found to be within the range of 7.5-8.2 and 28-30°C±2. The isolate *Scenedesmus* sp. showed the fastest growth rate with the shortest doubling time of 2.48 day whereas, *P. kessleri* the slowest with 3.37 day.

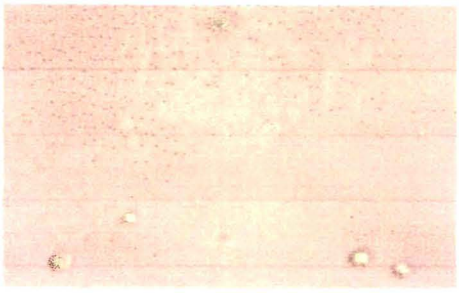
4.2 Morphological characterization of microalgal isolates

4.2.1 Compound microscopy

The compound microscopic images of the microalgal isolates are presented in Fig 4.3 (a), (b) and (c). *Scenedesmus* sp. cells were found in clusters, whereas *Chlorella* sp and *P. kessleri* cells were in solitary. All the isolates were dense green and bounded by cell wall.

(a)

(b)

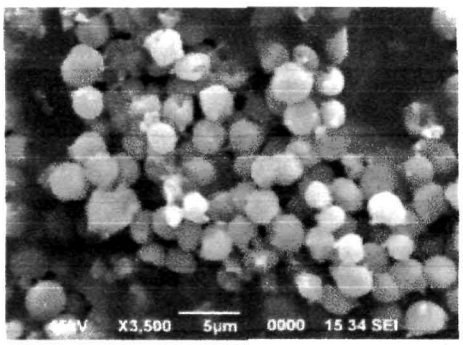


(c)

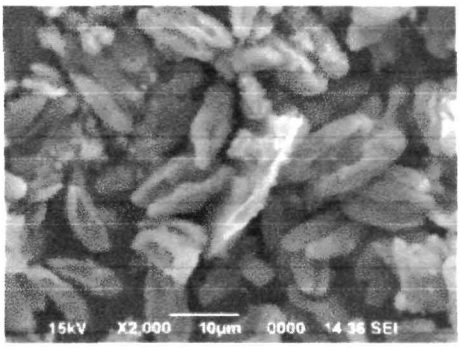
Fig. 4.3 Compound microscopic images of (a) *Chlorella* sp. (b) *Scenedesmus* sp. and (c) *P. kessleri* at 400 X

4.2.2 Scanning electron microscopy

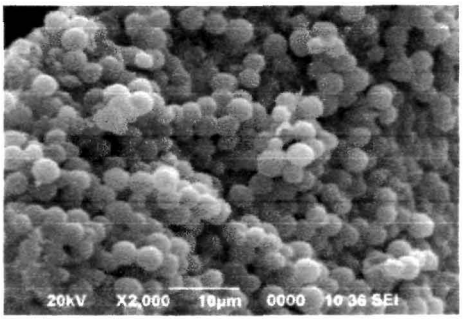
Scanning electron microscopy was done in order to study the surface morphology of the microalgal isolates. The SEM micrographs for the respective microalgal isolates are shown in Fig. 4.4 (a), (b) and (c). SEM magnification was altered from 2000-3500X. The morphological characterization of the microalgal isolates as revealed by SEM are presented in Table 4.2.



(a)



(b)



(c)

Fig. 4.4 Scanning electron micrographs of (a) *Chlorella* sp. at 3500X (b) *Scenedesmus* sp. at 2000X and (c) *P. kessleri* at 2000X

The photographs depict spherical shape of *Chlorella* sp and *P. kessleri* whereas, elongated concave shape of *Scenedesmus* sp. The morphological characterization of the microalgal isolates as revealed by SEM are depicted in Table 4.2.

Table 4.2 Morphological characterization of *Chlorella, Scenedesmus* and *P. kessleri* by scanning electron microscopy

Microalgae	Size		Shape	Organization
	Length (μm)	Diameter (μm)		
Chlorella sp.		1.5 - 4.5	Spherical	Solitary
Scenedesmus sp.	6 - 20	2.5 - 3	Fusiform, slightly acute, boat shaped cavities	Colonial, 4 cells in a single series
P. kessleri		2 - 3.5	Spherical	Solitary

4.3 Thermal analysis of microalgae

Thermogravimetric analysis was done at variable heating rates of 10°C and 30°C in order to study the degradation profile of the microalgal biomass and the data thus obtained is shown in Fig.4.5. From the figure it is clear that *Scenedesmus* sp. had the shortest biomass degradation profile with the major degradation ending up at 300°C

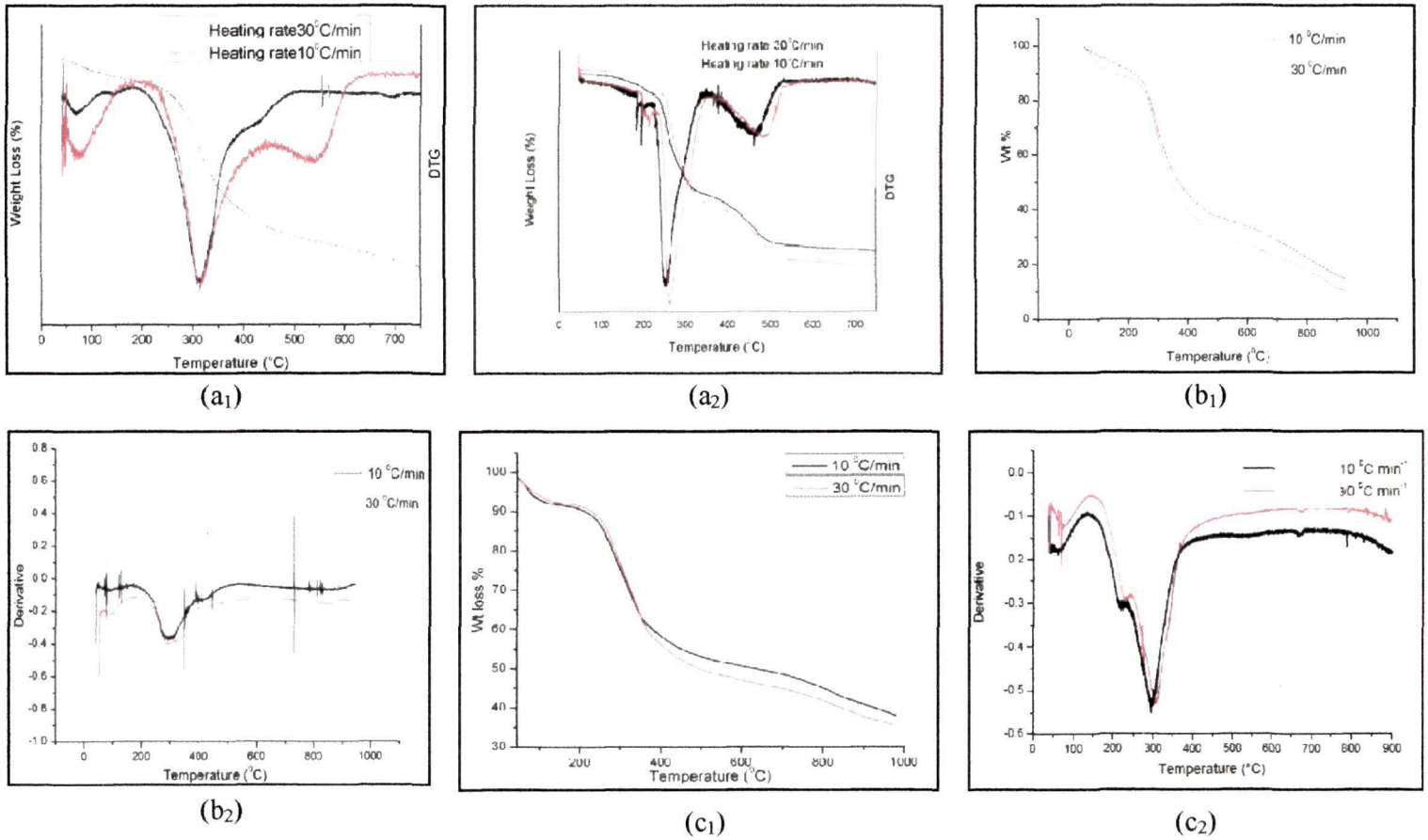
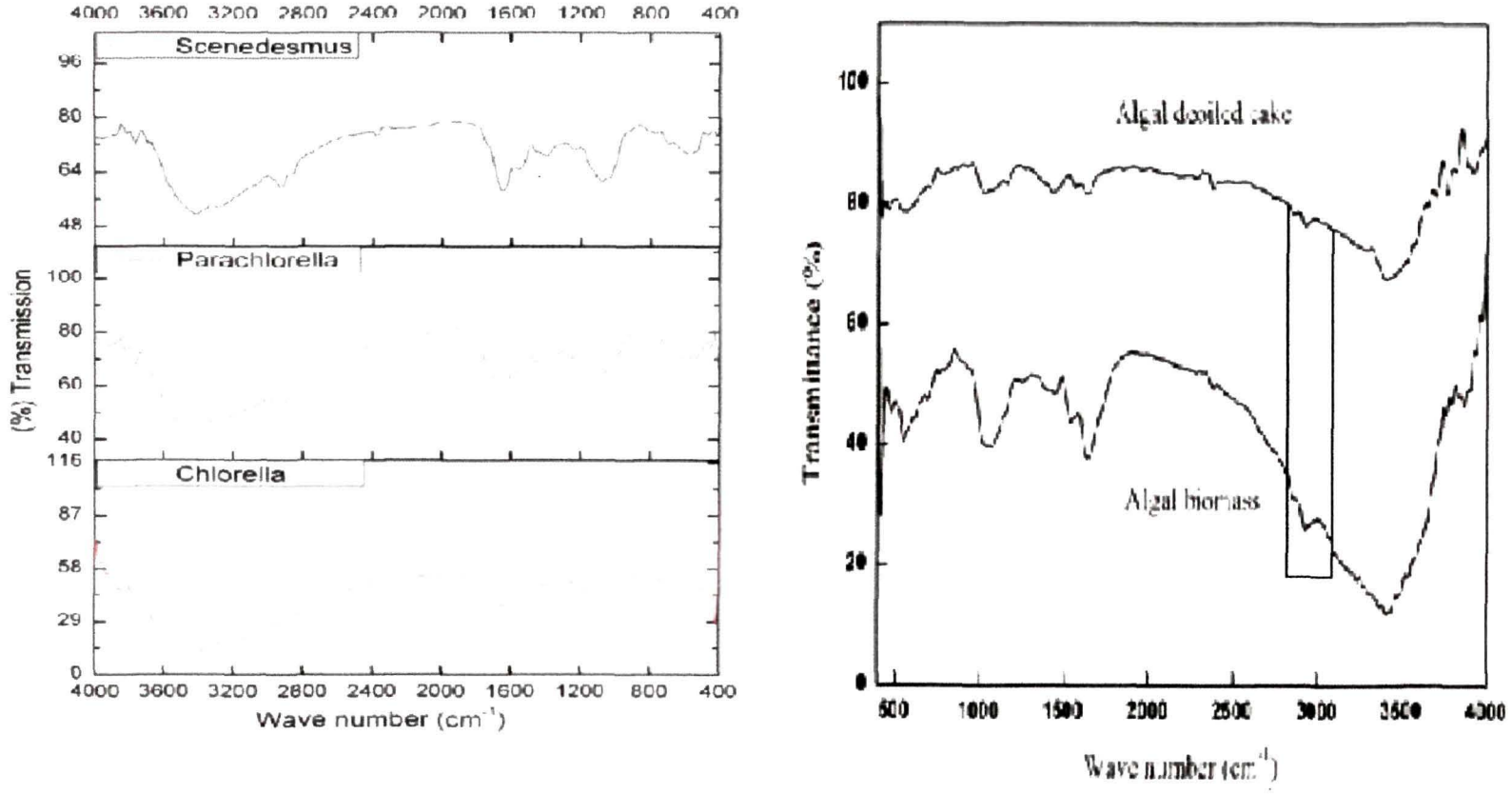


Fig. 4.5 Biomass degradation profile (a_1) = TG-DTG profile of *Chlorella* sp. biomass, (a_2) = TG-DTG profile of *Chlorella* sp. deoiled cake, ($b=b_1+b_2$), (b_1) = TG profile of *Scenedesmus* sp. biomass, (b_2) = DTG profile of *Scenedesmus* sp. biomass and ($c= c_1+c_2$), c_1= TG profile of *P. kessleri* biomass , (c_2) = DTG profile of *P. kessleri* biomass

4.4 Assessment of functional groups in microalgal cellular mass

The functional groups in the microalgal cellular mass were determined by FTIR spectroscopy. The cells were subjected to lyophilization and then properly ground by mixing with KBr. The FTIR spectra of the microalgal biomasses are shown in Fig 4.6 (a). Fig 4.6 (b) shows the FTIR spectra of *Chlorella* sp. biomass and the deoiled cake.

The major absorption bands for the microalgal biomass were 3100-2800 cm^{-1}, 1800-1500 cm^{-1} and 1200-900 cm^{-1}, respectively. In Fig. 4.6 (b) the zone of the spectra within the line shows the absorbance for lipid.



(a) (b)

Fig. 4.6 FTIR spectra of (a) microalgal cellular mass (b) *Chlorella* sp. biomass and deoiled cake

4.5 Properties of microalgal biomass for their potential in bio-oil extraction

Properties of microalgal biomass were studied to determine their potential as bioenergy feedstock The important properties of biomass like calorific value, elemental content (carbon, nitrogen, hydrogen, oxygen), proximate content (moisture, volatile matter, fixed carbon, ash) and biochemical content (carbohydrate, protein, lipid) were determined and data thus obtained are presented in Table 4 3

Table 4 3 Biomass properties of *Chlorella* sp , *P kessleri* and *Scenedesmus* sp biomass

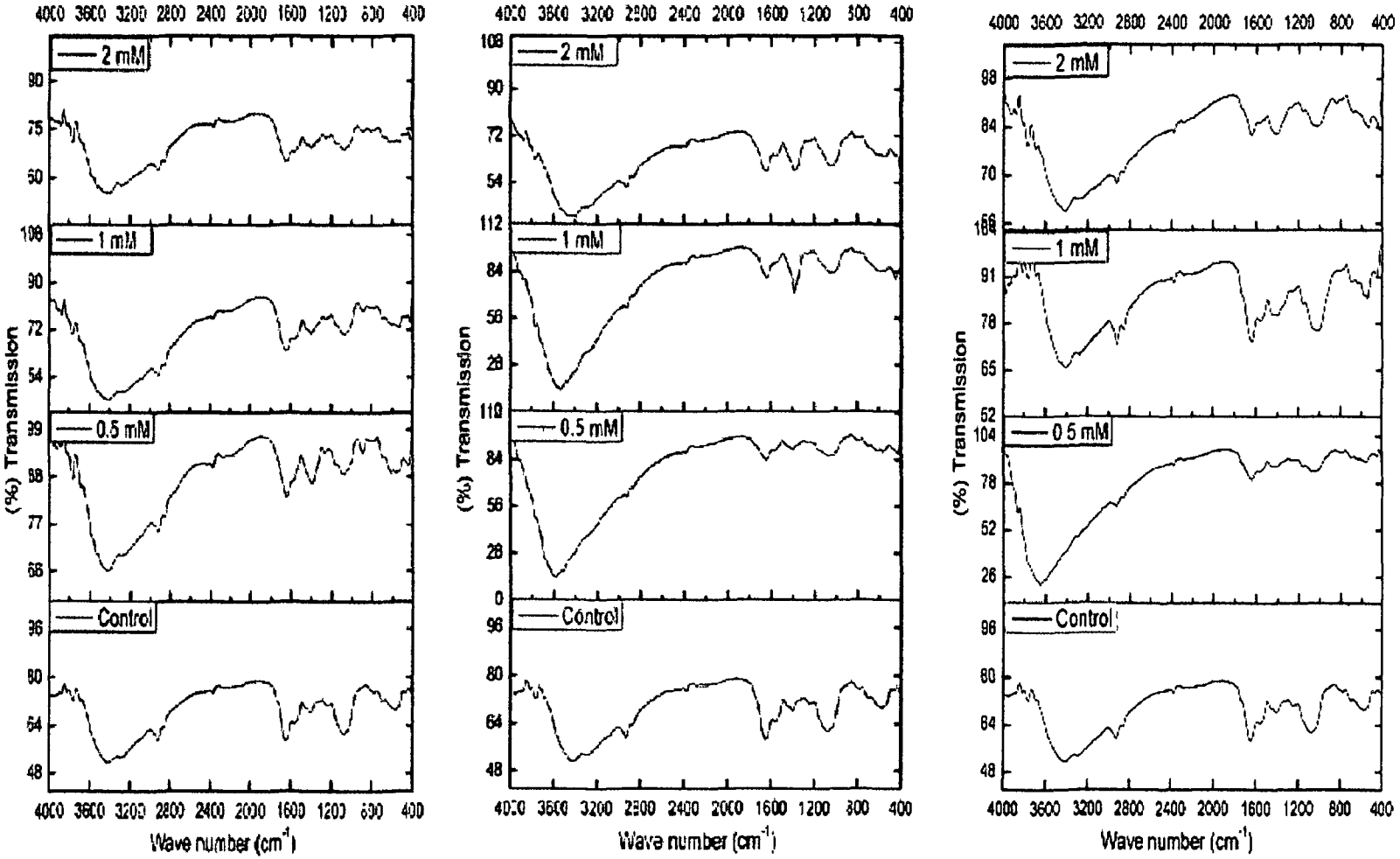
Properties		*Chlorella* sp	*P kessleri*	*Scenedesmus* sp
Gross calorific value (MJ/kg)		18 59	17 89	15 42
Net calorific value (MJ/kg)		15 88	15 11	13 00
Empirical formulae (on ash free basis)		$C_{8\,25}H_{14\,79}NO_{5\,02}$	$C_{9\,1}H_{17\,37}NO_{6\,16}$	$C_{8\,25}H_{14\,79}NO_{5\,02}$
H/C ratio (on ash free basis)		1 79	1 9	1 86
O/C ratio (on ash free basis)		0 6	0 67	0 93
Elemental analysis (wt %)	Carbon Hydrogen Nitrogen Oxygen (by difference)	47 54 7 1 6 73 38 63	45 68 7 26 5 85 41 21	39 37 6 12 5 25 49 26
Proximate analysis (wt %)	Moisture content Volatile matter Fixed carbon Ash content	6 8 ± 1 11 72 19 ± 1 73 15 08 ± 1 21 5 93 ± 0 81	6 33 ± 0 45 70 63 ± 0 75 17 44 ± 0 46 5 5 ± 0 36	6 5 ± 0 29 72 15 ± 1 35 16 13 ± 1 64 5 21 ± 0 16
Biochemical analysis	Total carbohydrate Protein content Lipid content	19 46 ± 0 25 43 22 ± 0 33 28 82 ± 0 72	30 46 ± 0 29 41 31 ± 0 65 16 2 ± 0 63	34 21 ± 0 27 40 56 ± 0 42 15 3 ± 0 36

From the above table, calorific value was found to be the best in the case of the isolate *Chlorella* sp.

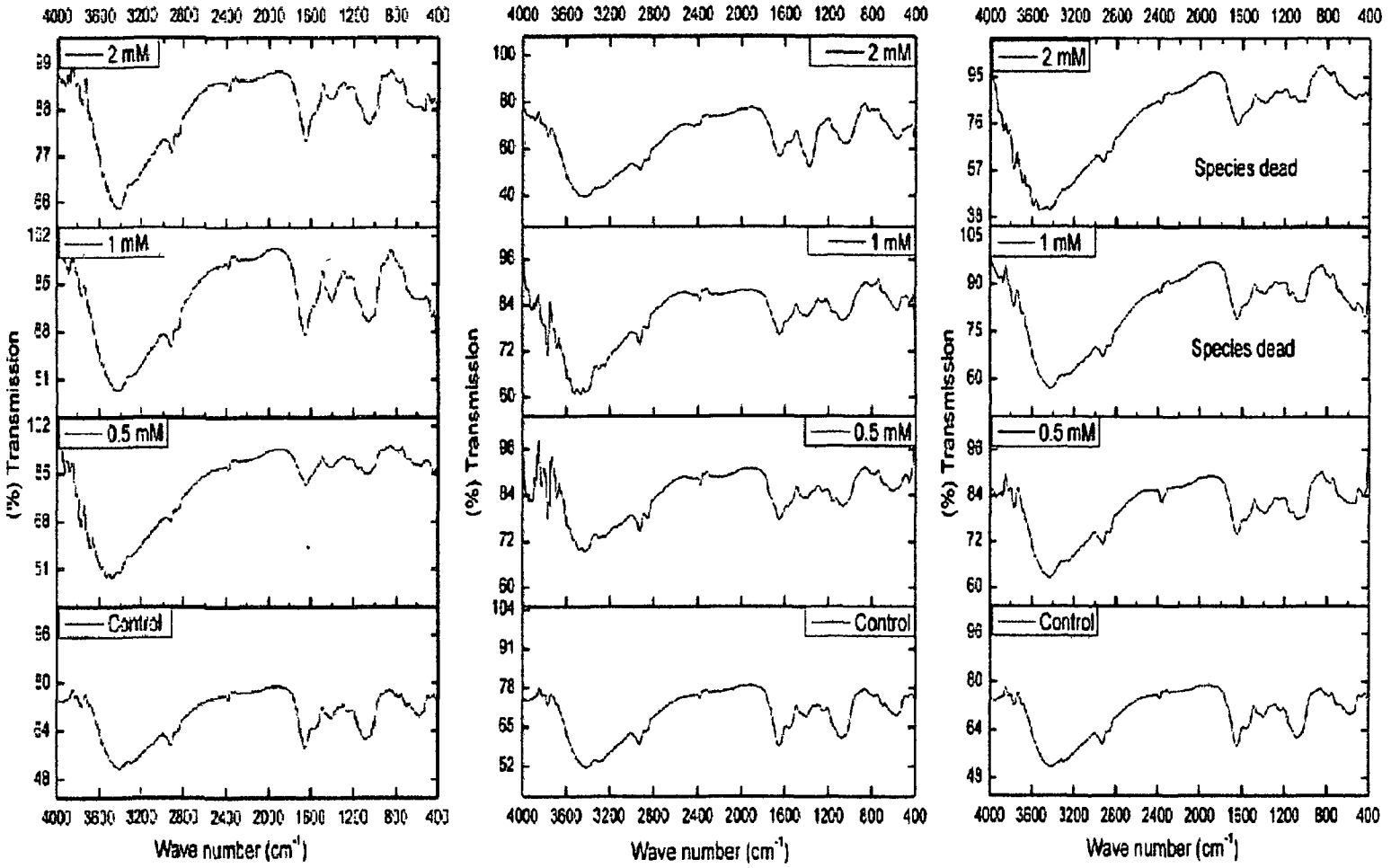
4.6 Role of heavy metal on lipid content of microalgae

The microalga *Scenedesmus* sp. was isolated from a heavy metal contaminated site in Tezpur University and accordingly it was expected to be tolerant to heavy metals with their role on the content of lipid. An attempt was made to study the influence of heavy metals like Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Cr^{2+} and Co^{2+} on lipid content of the microalga. The heavy metals were supplemented in varying concentrations of 0.5, 1 and 2 mM in the culture medium of the microalgae (BG-11) and their resultant biomass was subjected to FTIR spectroscopy to assess the lipid composition Fig. 4.7 (a-f).

No, marked degree of differences were observed in the spectra for the control and the treated biomass except in the case of Pb^{2+}.



(a) (b) (c)



(d) (e) (f)

Fig. 4 7 FTIR spectra of *Scenedesmus* sp in (a) Chromium (b) Cobalt (c) Lead (d) Nickel (e) Cadmium and (f) Mercury supplemented media

4.7 Assessment of open mass culture of microalgae

A bloom forming microalga *P. kessleri* was isolated from a drain adjacent to the department of Molecular Biology and Biotechnology, Tezpur University, Naapam, India. The species was expected to have eutrophic growth in natural water bodies. Three such eutrophic water bodies were identified at the bank of the river Brahmaputra, Dhemaji, Assam and mapped using satellite images and Google Earth. Ground verification of the sites was done by field visit and subsequent collection of representative water samples. Accordingly, the species was cultured in three representative water samples collected from the prospective mass culture sites of Dhemaji district. The microalgal growth kinetics was monitored in open laboratory conditions (open batch cultures). The microalgal growth data thus obtained are presented in Fig. 4.8 and Table 4.4.

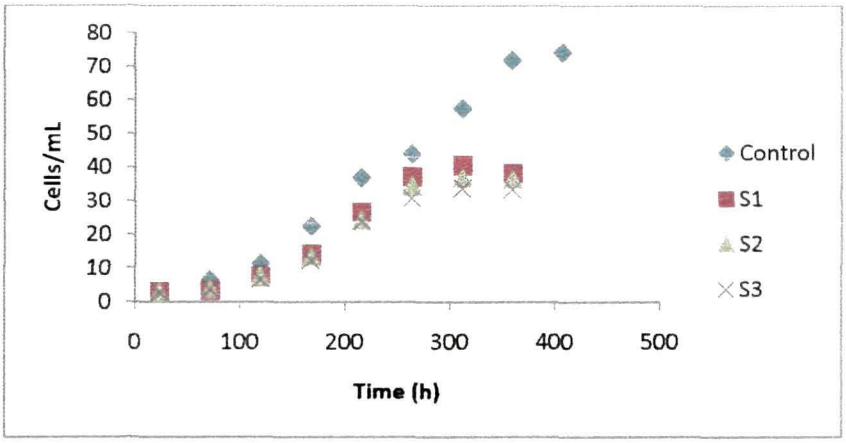


Fig. 4.8 Growth curve of *P. kessleri* in representative water samples as against control

Table 4.4 Growth kinetics of *P. kessleri* in representative water samples

Culture	Specific growth rate (μ)	Divisions/day (k)	Doubling time (T_2)
Control	0.188	0.271	3.681
S1	0.248	0.358	2.791
S2	0.219	0.317	3.150
S3	0.220	0.318	3.143

The growth of the *P. kessleri* cultured in representative water samples from Dhemaji district of Assam, reached steady state at an earlier time duration as compared to the control. Site S1 (Bordoloni, Dhemaji, Assam) was found to be the most favorable one for the mass culture of the species on the basis of microalgal growth kinetics.

Table 4.5. Characteristics of permanently inundated water bodies.

Study site	Geographical coordinates	Water flow pattern	Type of Waste	Water temperature	pH	Total nitrate mg/L	Total phosphate mg/L	Dissolved oxygen mg/L
S1	$94^0$24'05"E $27^0$24'23"N	Stagnant	Agricultural and household	19^0C	7.9	0.4	1.447	10.09
S2	$94^0$27'25"E $27^0$25'22"N	Stagnant	Agricultural runoff	18^0C	7.6	0.294	1.262	10.56
S3	$94^0$37'31"E $27^0$29'09"N	Stagnant	Agricultural runoff	19^0C	7.6	0.375	1.412	10.23

From Table 4.5 it is clear that site S1 had the highest nitrate and phosphate content.

The species was harvested by centrifugation (8000 rpm for 15 min) following attainment of steady phase. The microalgal biomass was oven dried and subjected to FTIR analysis for assessing changes in the functional composition. The FTIR spectra of *P. kessleri* is shown in Fig. 4.9

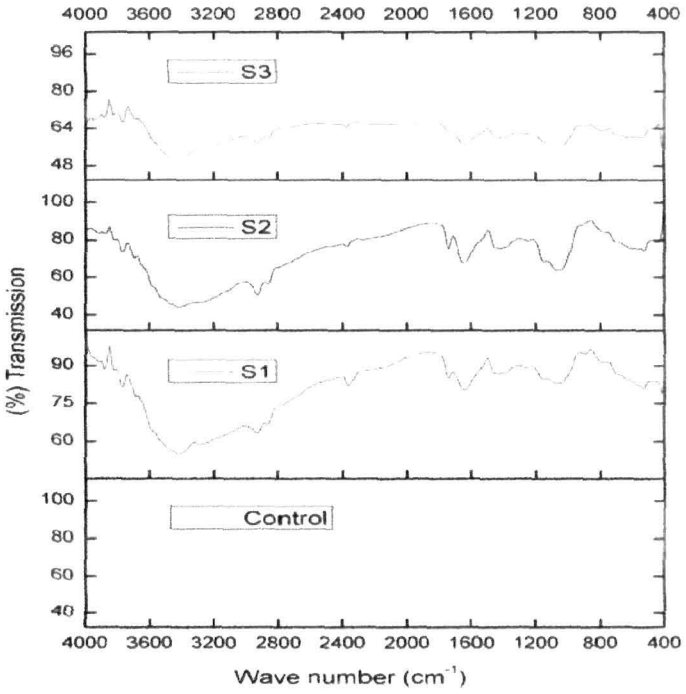


Fig. 4.9 FTIR spectra of *P. kessleri* biomass cultured in the representative water samples from S1, S2 and S3 PMCS against the control

As evident from the above FTIR results, there was no marked difference among the spectra of the biomass samples. This clearly indicated that the species maintains chemical homogeneity (in terms of functional groups) over a wide range of culture environments.

4.8 Production of biodiesel from microalgae and yeast and analysis of their FAME content

Biodiesel was derived from microalgae and yeast through *in situ* transesterification. GC-FID analysis was carried out for *Chlorella* sp., *P. kessleri*, and *S. cerevisiae*, whereas GC-MS analysis was done for *Scenedesmus* sp. for analyzing the FAME content. Data thus obtained are presented in Table 4.6. The standard GC of the FAME mix is presented in Fig. 4.10. Fig. 4.11 shows the GC-MS profile of *Scenedesmus* sp. biodiesel. A comparative FAME composition of the microalgal isolates and *S. cerevisiae* biodiesel has been presented in Table 4.7

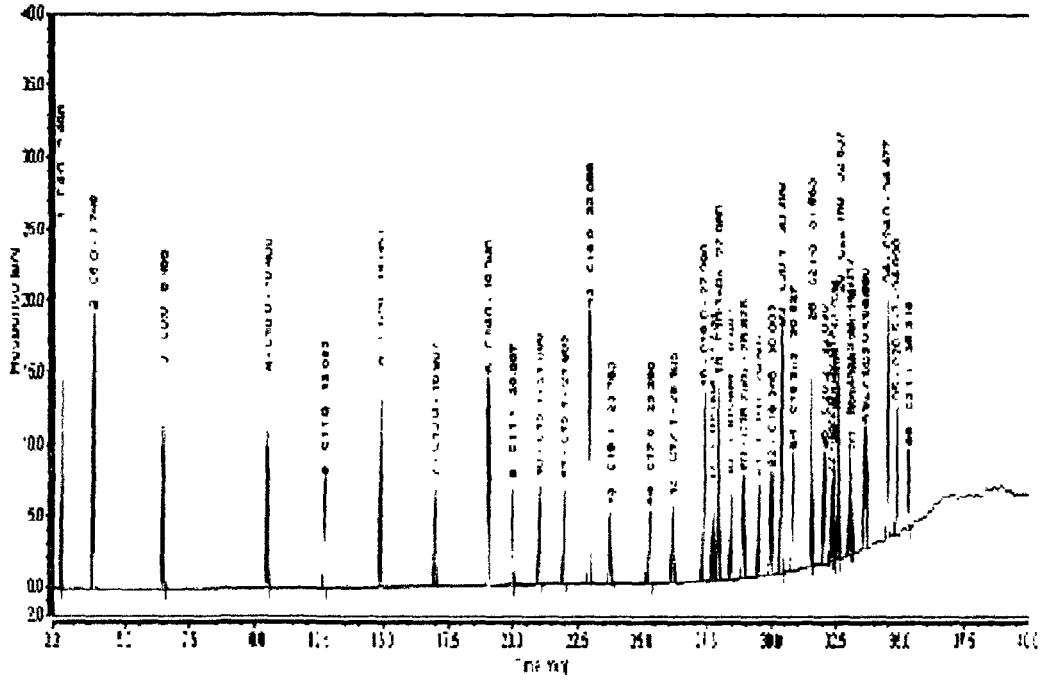


Fig 4.10 Gas chromatogram of FAME-mix (standard)

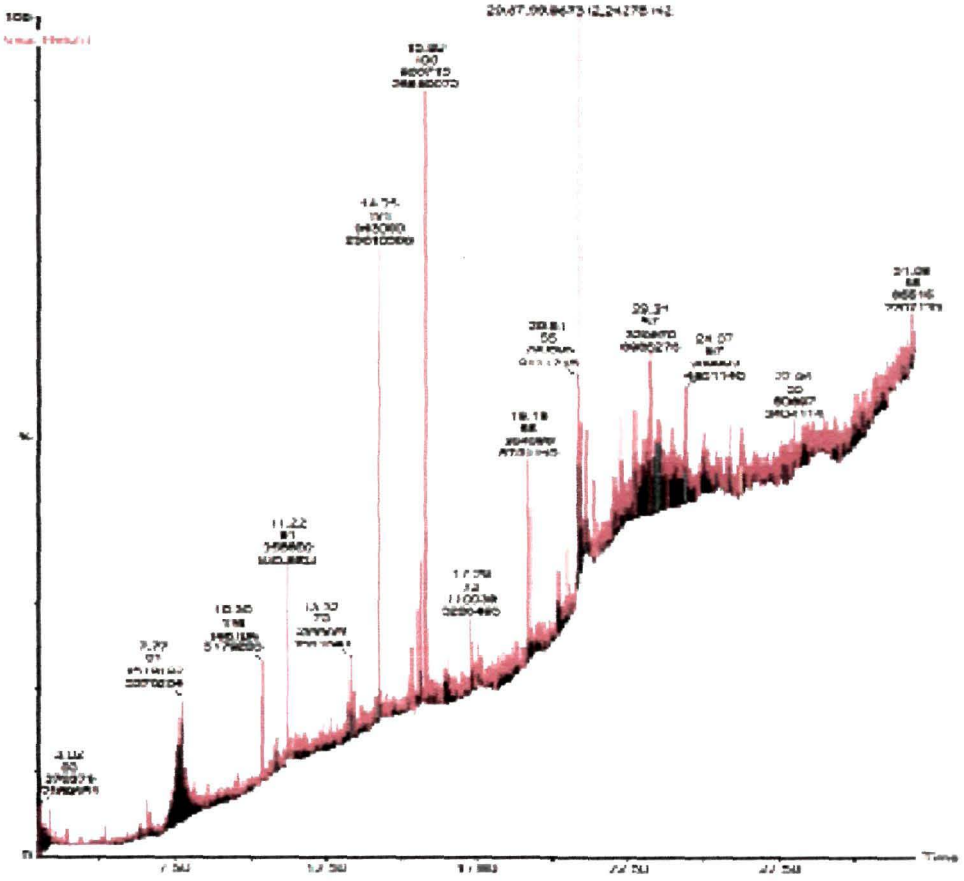


Fig. 4.11. GC-MS profile of *Scenedesmus* sp. biodiesel

Table 4 6 FAME composition of microalgal species and *S cerevisiae* (yeast)

Sl No	Retention time	FAME	Wt (%)
(a) *Chlorella* sp biodiesel			
1	21 243	C14 1	0 75548
2	25 703	C16 0	16 0665
3	28 278	C18 3	0 62956
4	28 538	C18 2	5 74163
5	28 633	C18 1 isomer	2 59381
6	28 887	C18 1	63 2586
7	29 173	C18 0	7 70587
8	31 218	C20 6	1 86351
9	37 942	C24 1	1 41022
(b) *P kessleri* biodiesel			
1	21 235	C14 1	0 88331
2	25 637	C16 0	3 20781
3	28 325	C18 3	2 69642
4	28 533	C18 2	13 5286
5	28 608	C18 1 isomer	9 01906
6	28 88	C18 1	68 8052
7	31 232	C20 5	1 8596
(c) *Scenedesmus* sp biodiesel			
1	19 19	C14 0	10 29
2	20 5	C16 0	3 58
3	20 81	C16 1	8 74
4	20 87	C16 2	29 71
5	21 07	C16 3	5 29
6	22 66	C16 4	8 69
7	23 21	C18 0	9 84
8	24 37	C18 1	9 32
9	25 82	C18 2	3 76
10	26 2	C18 3	8 02
11	27 95	C18 4	2 06

(d) *S. cerevisiae* biodiesel			
1	17 443	C12 0	13 3501
2	25 155	C16 1	28 9673
3	25 642	C16 0	5 03778
4	28 513	C18 2	2 01511
5	28 685	C18 1 isomer	18 136
6	29 248	C18 0	1 76322
7	31 22	C20 5	2 01511
8	37 183	C23 0	28 7154

Microalgal biodiesel is comprised of both saturated and unsaturated fatty acids. All the tested microalgal biodiesel samples contained C16:0, C18:1 and C18:2 FAMEs whereas, a significant percentage of C20:5 and C23·0 FAMEs (30.73 %) were observed in the case of yeast biodiesel.

Table 4 7 Comparison of FAME percentage in microbial biodiesel

FAME's	Species			
	Chlorella sp	*Scenedesmus* sp	*P kessleri*	*S cerevisiae*
C12				• (13 35)
C14		• (10 29)		
C14 1	• (0 75)		• (0 88)	
C16 0	• (16 06)	• (3 58)	• (3 2)	• (5 03)
C16 1		• (8 74)		• (28 96)
C16 2		• (29 71)		
C16 3		• (5 29)		
C16 4		• (8 69)		
C18 0	• (7 70)	• (9 84)		• (1 76)
C18 1 (isomer)	• (2 59)		• (9 01)	• (18 13)
C18 1	• (63 25)	• (9 32)	• (68 80)	
C18 2	• (5 74)	• (3 76)	• (13 52)	• (2 01)
C18 3	• (0 62)	• (8 02)	• (2 69)	
C18 4		• (2 06)		
C20 5			• (1 85)	• (2 01)
C20 6	• (1 86)			
C23 0				• (28 71)
C24 1	• (1 41)			

From the above table it can be observed that the FAME composition was variable in all the species and also yeast (*S cerevisiae*). *Chlorella* sp and *P kessleri* biodiesel mostly comprise of oleic acid (C18 1) The percent composition of oleic acid in *Chlorella*.sp was 65 84% and 77.81% in *P kessleri*, respectively Eicasopentanoic acid (C20 5), which is one of commonest PUFA (Poly unsaturated fatty acids) was

present as a constituent in *P kessleri* and *S. cerevisiae* biodiesel with two percentages 1 85 and 2 06, respectively. On the other hand *Scenedesmus* sp. biodiesel had a high proportion of PUFA (57 53%)

4.9 Fuel properties of biodiesel

The fuel properties of biodiesel like density, viscosity, calorific value and cetane number were determined in the biodiesels derived from microalgae and yeast The data thus obtained are presented in Table 4 8

Table 4.8 Comparison of fuel properties of microalgal (*Chlorella* sp., *P. kessleri* and *Scenedesmus* sp) and yeast (*S cerevisiae*) biodiesel

Fuel properties	*Chlorella* sp biodiesel	*P kessleri* biodiesel	*Scenedesmus* sp biodiesel	*S cerevisiae* biodiesel	ASTM standard
Density (kgL^{-1})	0 874	0 876	0 884	0 870	0 86-0 9
Viscosity (mm^2/s)	4 591	4 445	3 437	5 797	3 5-5
Calorific value (MJ/kg)	39 884	39 861	39 248	40 142	-
Cetane number	62 476	58 463	40 960	71 58	-

The fuel properties of microalgal biodiesel were within the ASTM ranges *S. cerevisiae* biodiesel had a viscosity of 5 797 mm^2/s which was slightly higher than the ASTM specifications. It also possessed a high cetane number (71.58)

4.10 Thermochemical conversion of deoiled cake of microalgae (pyrolytic approach)

The deoiled cake derived from the microalgal species *P kessleri* was subjected to thermochemical conversion by using the pyrolytic approach. The resultant bio-oil was subjected to FTIR analysis for assessing aqueous and organic phases The FTIR spectrum of both the phases of the bio-oil is presented in Fig 4.12. Following FTIR analysis, the bio-oil was subjected to GC-MS analysis and the TIC profile is presented in Fig 4 13

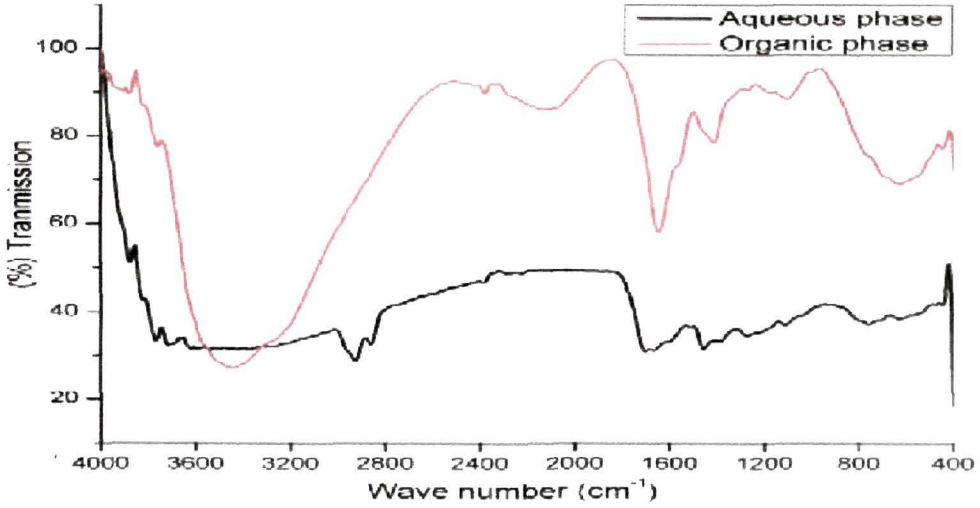


Fig. 4.12 FTIR spectra of *P. Kessleri* bio-oil (aqueous and organic phase)

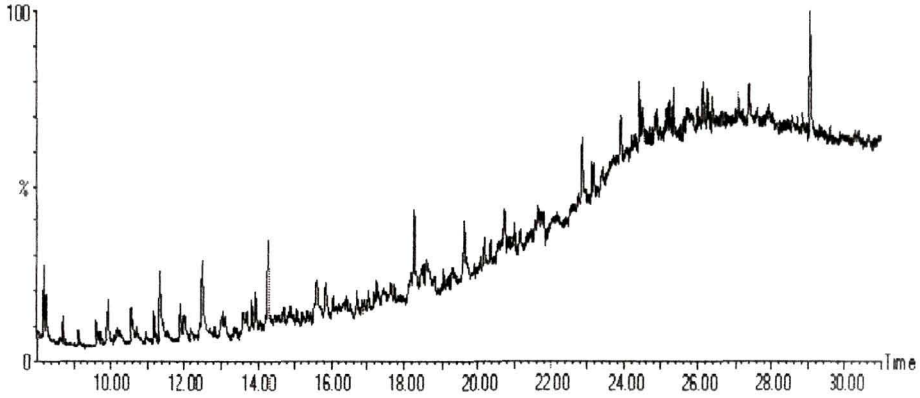
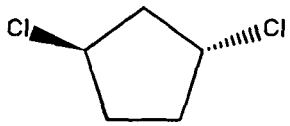
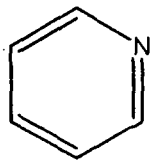
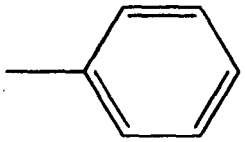
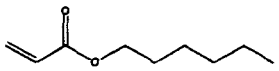
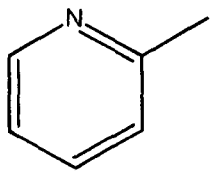
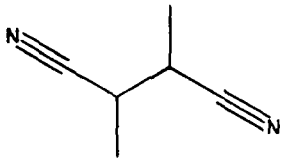
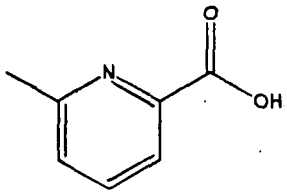
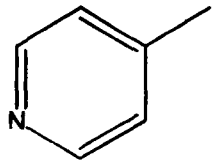
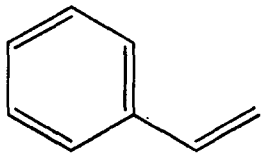
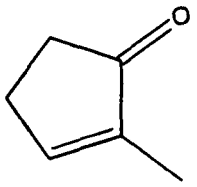
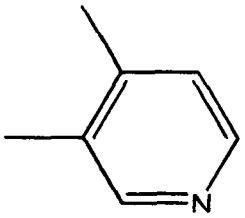
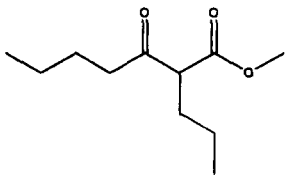
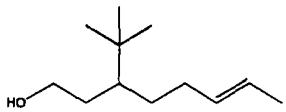
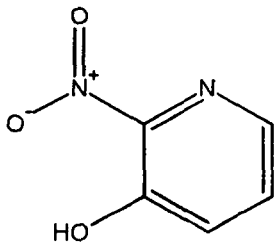
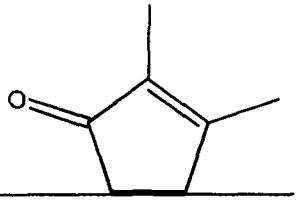


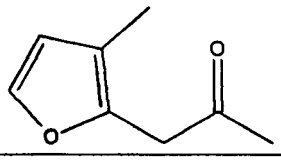
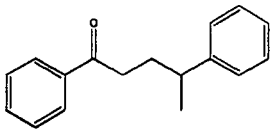
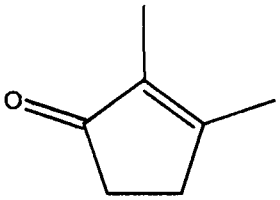
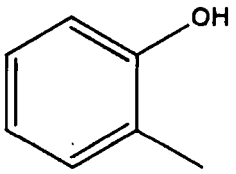
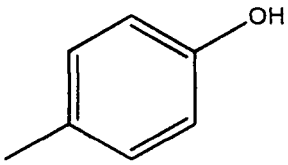
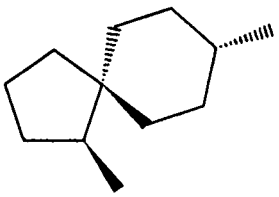
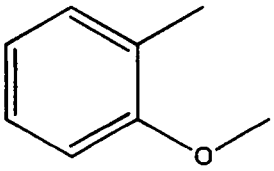
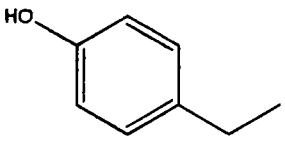
Fig .4.13 TIC of *P. kessleri* bio-oil

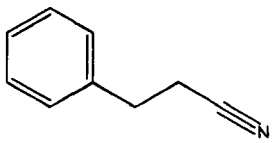
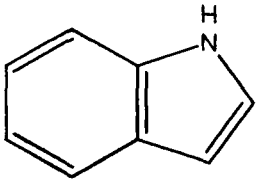
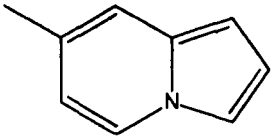
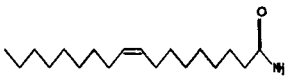
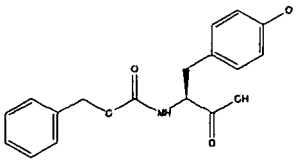
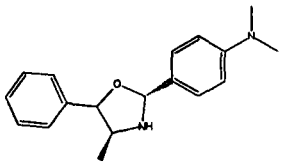
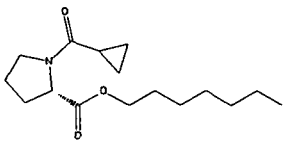
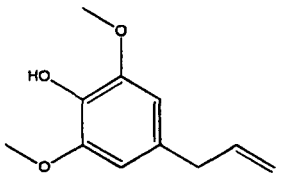
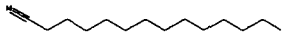
The main absorption for the bio-oil was 3600-3200, 950-650 and 1640 cm^{-1} in the FTIR spectra. The aqueous phase showed a broad peak in the range of 3600-3200 cm^{-1}. More than 40 peaks were observed in the TIC of bio-oil of the microalgal species *P. kessleri*. The products identified in the bio-oil derived from *P. kessleri* were identified by GC-MS analysis and the same are presented in Table 4.9.

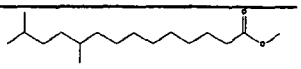
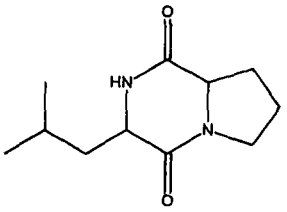
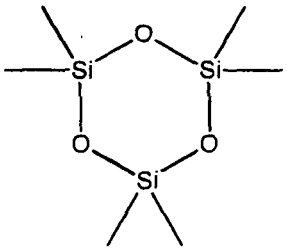
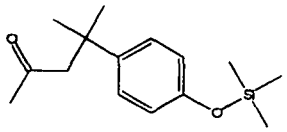
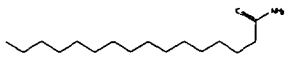
Table 4.9 Pyrolytic products identifiable by GC-MS for *P. kessleri* bio-oil

Retention time	Relative Abundance Area (%)	Compound name	Compound class	Molecular formulae	Molecular weight	Structure
8.21	3.990	CYCLOPENTANE, 1,3-DICHLORO-, TRANS-	Cyclic Halogenated hydrocarbon	$C_5H_8Cl_2$	139.02	
8.28	Trace	PYRIDINE	Heterocyclic Aromatic Hydrocarbon	C_5H_5N	79.10	
8.73	Trace	TOLUENE	Aromatic Hydrocarbon	C_7H_8	92.14	
9.13	Trace	N-HEXYL ACRYLATE	Unsaturated Ester	$C_9H_{16}O_2$	156.22	
9.65	Trace	PYRIDINE, 2-METHYL-	Heterocyclic Aromatic hydrocarbon	C_6H_7N	93.13	
9.97	Trace	BUTANEDINITRILE, 2,3-DIMETHYL-	Cyan Compound	$C_6H_8N_2$	108.14	
10.58	Trace	2-PYRIDINECARBOXYLIC ACID, 6-METHYL-	Heterocyclic Aromatic Carboxylic Acid	$C_7H_7NO_2$	137.14	

10.63	Trace	PYRIDINE, 4-METHYL-	Heterocyclic Aromatic Hydrocarbon	C_6H_7N	93.13	
11.19	Trace	STYRENE	Aromatic Hydrocarbon	C_8H_8	104.15	
11.35	3.651	2-CYCLOPENTEN-1-ONE, 2-METHYL-	Cyclic Ketone	C_6H_8O	96.13	
11.94	Trace	PYRIDINE, 3,4-DIMETHYL-	Heterocyclic Aromatic Hydrocarbon	C_7H_9N	107.15	
12.04		HEPTANOIC ACID, 3-OXO-2-PROPYL-, METHYL ESTER	Aliphatic Ester	$C_{11}H_{20}O_3$	200.27	
12.08		3-T-BUTYL-OCT-6-EN-1-OL	Unsaturated Branched Alcohol	$C_{12}H_{24}O$	184.32	
12.53	4.039	3-PYRIDINOL, 2-NITRO-	Heterocyclic Aromatic Nitro Compound	$C_5H_4N_2O_3$	140.10	
13.08		2-CYCLOPENTEN-1-ONE, 2,3-DIMETHYL-	Cyclic Unsaturated Ketone	$C_7H_{10}O$	110.15	

13.62		3-METHYL-2-(2-OXOPROPYL) FURAN	Heterocyclic	$C_8H_{10}O_2$	138.16	
13.72		1,4-DIPHENYL-1-PENTANONE	compound	$C_{17}H_{18}O$	238.32	
13.85		2-CYCLOPENTEN-1-ONE, 2,3-DIMETHYL-	Cyclic Unsaturated Ketone	$C_7H_{10}O$	110.15	
13.96		PHENOL, 2-METHYL-	Phenolic Compound	C_7H_8O	108.14	
14.29	3.976	PHENOL, 4-METHYL-	Phenolic Compound	C_7H_8O	108.14	
14.93		TRANS,CIS-1,8-DIMETHYLSPIRO[4.5] DECANE	Spiro Compound	$C_{12}H_{22}$	166.30	
15.64	3.367	BENZENE, 1-METHOXY-2-METHYL-	Aromatic Compound	$C_8H_{10}O$	122.16	
15.87		PHENOL, 4-ETHYL-	Phenolic Compound	$C_8H_{10}O$	122.16	

17 27		BENZENEPROPANENITRILE	Mixed Cyan Compound	C_9H_9N	131 17	
18 27	2 712	INDOLE	Heterocyclic Aromatic Hydrocarbon	C_8H_7N	117 15	
19 64	3 734	INDOLIZINE, 7-METHYL-	Heterocyclic Aromatic Hydrocarbon	C_9H_9N	131 17	
20 75	2 691	9-OCTADECENAMIDE, (Z)-	Unsaturated Aliphatic Amide	$C_{18}H_{35}NO$	281 48	
21 65	2 106	N-BENZYLOXYCARBONYL-L-TYROSINE	Amino Acid derived Compound	$C_{17}H_{17}NO_5$	315 32	
22 87	3 654	1,3-OXAZOLIDINE 4-METHYL-TRANS-5-PHENYL-2-[4-(DIMETHYLAMINO) PHENYL]-	Heterocyclic Compound	$C_{18}H_{22}N_2O$	282 38	
23 13	4 478	L-PROLINE, N-(CYCLOPROPYLCARBONYL)-, HEPTYL ESTER	Amino Acid derived Compound	$C_{16}H_{27}NO_3$	281 39	
23 19	6 744	PHENOL, 2,6-DIMETHOXY-4-(2-PROPENYL)-	Phenolic Compound	$C_{11}H_{14}O_3$	194 23	
24 40	3 557	Z,Z-6 28-HEPTATRIACTONTADIEN-2-ONE	Unsaturated Ketone	$C_{37}H_{70}O$	530 95	
25 24	4 648	TETRADECANENITRILE	Cyan Compound	$C_{14}H_{27}N$	209 37	

25.35	8.514	TETRADECANOIC ACID, 10,13-DIMETHYL-, METHYL ESTER	Aliphatic Ester	$C_{17}H_{34}O_2$	270.45	
26.15	7.007	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(2-METHYLPROPYL)-	Heterocyclic Compound	$C_{11}H_{18}N_2O_2$	210.27	
27.13	11.060	CYCLOTRISILOXANE, HEXAMETHYL-	Siloxane Compound	$C_6H_{18}O_3Si_3$	222.46	
27.42	9.733	TRIMETHYL[4-(2-METHYL-4-OXO-2-PENTYL)PHENOXY] SILANE	Siloxane aromatic compound	$C_{15}H_{24}O_2Si$	264.44	
29.06	8.812	HEXADECANAMIDE	Saturated Aliphatic Amide	$C_{16}H_{33}NO$	255.44	

The main compounds identifiable in the bio-oil of *P. kessleri* by GC-MS analysis were phenolics and aromatic compounds.

^{1}H NMR spectroscopic analysis of *P kessleri* bio-oil was done to examine the complete intact pyrolytic bio-oil rather than a selected fraction and the resultant NMR spectrum is presented in Fig. 4.14.

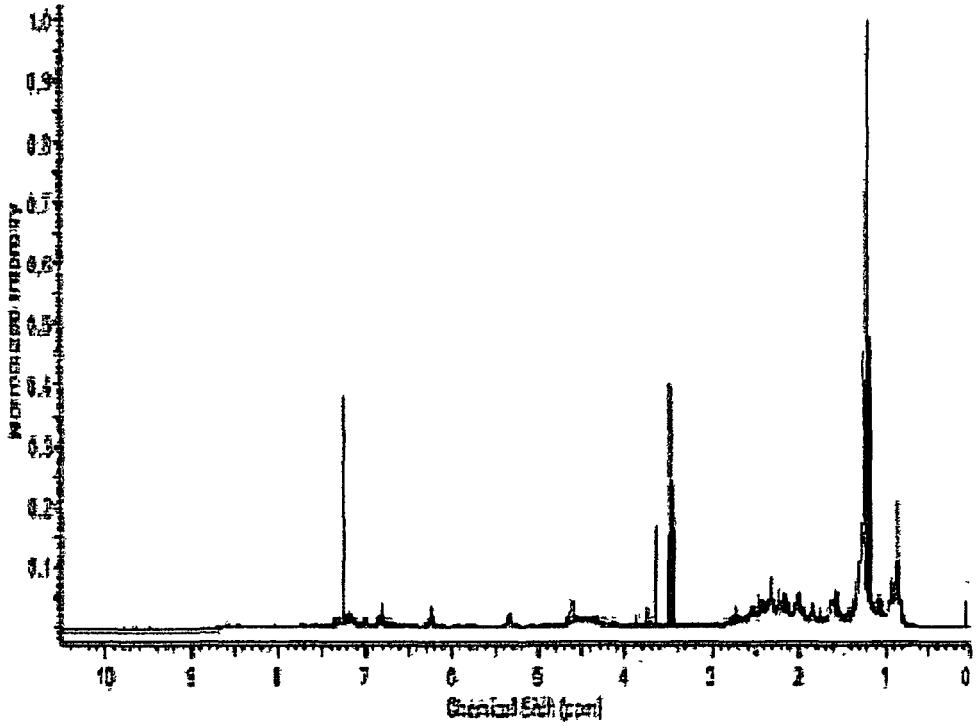
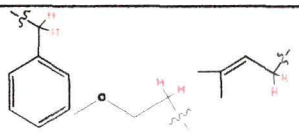
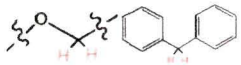
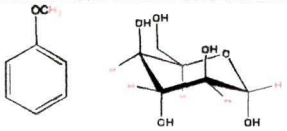
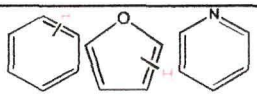
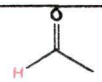


Fig. 4.14 ^{1}H NMR spectra of *P. kessleri* bio-oil

Table 4.10 Percentage of hydrogen based on ^{1}H-NMR analysis according to chemical shift range

Chemical shift (ppm)	Proton assignment	P. *kessleri* bio-oil (%)
0.5-1.5	 Alkanes	52
1.5-3	 á-heteroatoms or unsaturation	26
3-4.5	 Alcohols, methelene diabenzene	10
4.5-6	 Methoxys, carbohydrates	7
6-8.5	 (Hetero)-aromatics	3
8.5-10	 Aldehydes	2

A higher aliphatic content (presence of short, long and or branched chain hydrocarbons) was evident in *P. kessleri* bio-oil.

4.11 Molecular characterization of microalgae

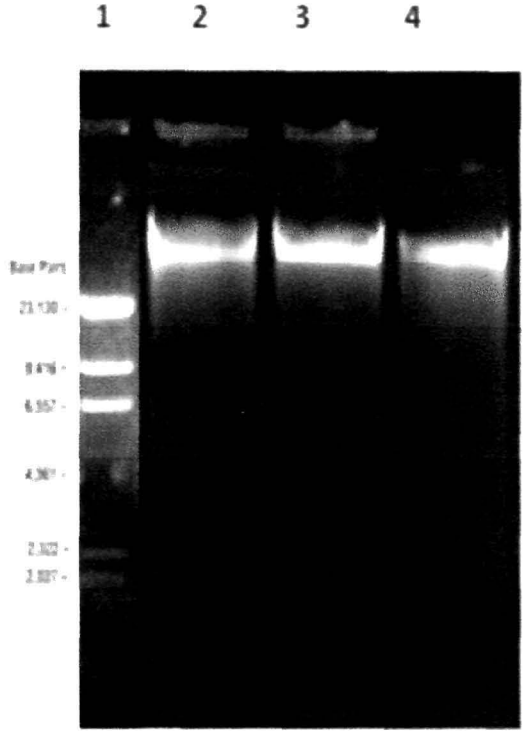


Fig. 4.15. Gel depicting genomic DNA of microalgal isolates. Lane 1= λ DNA/HindIII digest (Marker), Lane 2= *Chlorella* sp., Lane 3= *P. kessleri* and Lane 4= *Scenedesmus* sp.

The genomic DNA isolated from the microalgal isolates was used for amplification of 18S rRNA gene by PCR. The 18S rRNA gene was amplified using ITS primers and sequenced. Fig 4.16 shows the partial DNA sequences of (a) *Scenedesmus* sp. MPBK-2, (b) *P. kessleri* MMPBKK-1 and (c) *Chlorella* sp. MP-1

CTCTGAGTGACTGCGGAGGATCATTGTATGTGTTAAAAGCGTGAAAGCTCTGTTGTACCACGTCTGC
TGTGCTGGCAGCACACTCGGGCTTGCATTGATTAAGCCCGCGGCAAATGCCGGTATTGATGCCCGTT
AGCCTCGCGATTTGGCATGCCGGTGAACCCGTGCTAACCCTCTTCGGAACCAAACTTAGAAGTTTTG
TTTTCTATCAATTGGCAATCCTAACCAAAGACAACTCTCAACAACGGTTCTTATGCCTCTCGCAACGA
AGACGAACTCTGAGAATTGCGATACGTAGTGTGAATCGCAGAATTCCGTCAATCACCGAATATTTGA
ACGCCTATTGCGCTCGAGCCCTCGGGCAAGAGCATATCAGCCTCACCGTCGGTTTACACCCTCACCC

CTCTTTCCTTTTGGATCGCATGTTATCTTCTCAGCTGGCCCTAGGGGTGGATCTGGCTTTCCCGATCCT
TTCTGGGTTGGGTTGGCTAAAGTGTAGAGGCTTAATCAAGGACCCGATATGGGCTTCAACTGGATAG
GTAGCAACGGCTATAGCCGACTACACGAAGTTGTGGCTTGTGGACTTTGATAGGAGCCAAGCAGGA
AACGTGCTTGCACGTTTTAAACTTTCGACCTGAGCTCAGGCAAGGCTACCCGCTGAACTTAAGCTAT
AAAAAGGGGGGAGGAAAAAAAAACACCAAA

(a) *Scenedesmus* sp. MPBK-2

The sequence was deposited in the NCBI Genbank repository as *Scenedesmus* sp. MPBK-2 partial 18s rRNA gene and assigned a Genbank accession No. KF279644.

GGGGAAAATTAGTGTGAATTGCAAATTCCGTGAACCATCGAATCTTTGAACGCAACTTGCGCCCGAGG
CTCCGGCCGAGGGCTTGCCTGCCTCAGCGTCGGTTTACACCCCCTCGCCCCCACCCCCTTGTGGGTAGT
GGGAGCGGACCTGGCACCCTCGGGCTGCTGTCGGAAACCCTCCGACTGCCGGCCCGGGTCTGCTGAA
GTTCAGAGGCTTGAGCATGGACCCCGTTTGCAGGGCACCGGCTTGGTAGGTAGGCGTCAGCCTGCACC
CCGCCTGCCGGTACCCGAGGGGACTTTGCTGGGAGCCCGACCGGGAGCCGATTCGTCGACCTCCCACC
CTTCAAACCTTCGACCTGAGCTCAGGCAAGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
AACCAG

(b) *P. kessleri* MMPBKK-1

The sequence was deposited in the NCBI Genbank repository as *P. kessleri* strain MMPBKK-1 partial 18s rRNA Gene and assigned a Genbank accession No. KF163441.

AACAGTAATCATATGCTTGTCTAAAATTAAGCCATGTGTCTAAGTATAAACTGCTTTAATACTGTGAA
ACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTAACTACTCGGATAACCGTAGT
AATTCTAGAGCTAATACGTGCGTAAATCCCGACTTCCGGAAGGGACGTATCTATTAGATAAAAGGCCG
ACCGGGCTATGCCCGACTCGCGGTGAATCATGATAGCTTCACGAATCGCATGGCCTTAGTGCCGGCGA
TGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGG
GTGACGGAGGATTAGGGTCCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG
GCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATACTGGGC
TTTTCAGGTCTGGTAATTGGAATGAGTACAATCTAAAACCCTTAACGAGGACCAATTGGAGGGCCAST

CTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCT
CGTAGTTGGATTTCGGGTGGGTTCCGTCGTTCGGCGTTCGGTGTGCACTGGCGGCGCCTATCTTGCTGT
CGGGGACGGGCTCCTGGGCTTCACTGTCCGGGCTCGGAGTCGCCGAGGTTACTTTGAGTAAATTAGAG
TGTTCAAAGCAGGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACCCTGGCCTATCTT
GTTGGTCTGTAGGACCGGAGTAATGATTAAGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCAGAG
GTGAAATTCTTGGATTTATGAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATC
AAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCCTAGTCTCAACCATAAACGATGCCGA
CTAGGGATTGGTGGATGTTTATTAGATGACTTCACCAGCACCTTATGAGAAATCAAAGTTTTTGGGTT
CCGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAA
CCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGACATAGTGAGGATTGACAGATT
GAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGG
TTGATTCCGGTAACGAACGAGACCTCAACCTGCTAAATAGTCACGATCGATTTTTGCGGTTGGCCGAC
TTCTTAGAGGGACTCTTGGCGACTAGCCAATGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTA
GATGTTCTGGGCCGCACGCGCGTTACACTGATGCATGTAACGAGCCTATGCTTGACCGAGAGGTCCGG
GTAATCTGCGAAACTGCATCGTGATGGGGATAGATTATTGCAATTATTAATCTTCAACGAGGAATGCC
TAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCT
AGCGATTGGATGTGCTGGTGAAGCGTCCGGACTGGTGAGGGGCGACGGTTCGCCGCTGCTTGTCGCCG
AGAAGTTCGTTAAACCCTCCCATCGAGGAAGGAGAGTCGTAACAAGGGTTCCAGTAGGTGATTATCG
AT

(c) *Chlorella* sp. MP-1

The sequence was deposited in the NCBI Genbank repository as *Chlorella* sp. Strain MP-1 partial 18s rRNA gene and assigned a Genbank accession No. KJ499988.

A BLAST search was performed for the microalgal isolates and their identification was done out from the BLAST result. The results for BLAST analysis are presented in Table 4.11 (a), (b) and (c). Homologous search of 18S-rDNA sequence of the microalgal strains was also done to find their similarity with other species of the same genus. The sequence alignment was carried out with the related species using CLC Main Workbench and the respective phylogenetic trees were generated using Neighbor Joining Method implemented in CLC Main Workbench. The sequence alignments are presented in Fig. 4.17 (a), (b) and (c) and the phylogenetic tree generated are presented in Fig. 4.18 (a), (b) and (c).

Table 4.11 (a) BLAST results of nearest 10 organisms with *Scenedesmus* sp. MPBK-2

SN	Description	Accession	Alignment score	Query coverage	E-value	Max Identity
1	*Scenedesmus obliquus* isolate IB-05	JQ782745	736	76%	0.0	91%
2	*Coelastrum proboscideum*	JQ898144.	708	96%	0.0	86%
3	*Scenedesmus bajacalifornicus*	JX456466	702	76%	0.0	90%
4	*Scenedesmus acutus* f. *costulatus* strain KMMCC	JQ315789	651	70%	0.0	90%
5	*Scenedesmus bajacalifornicus* isolate IB-10	JQ782744	647	89%	0.0	85%
6	*Scenedesmus bajacalifornicus* strain ZA1-2	HQ246448	573	78%	7e-160	86%
7	*Acutodesmus obliquus* isolate Iso2	JX041598	568	73%	3e-158	87%
8	*Scenedesmus obliquus*	FR865738	566	78%	1e-157	85%
9	*Scenedesmus sp.*	JX046428	562	78%	1e-156	85%
10	*Scenedesmus acutus*	AJ249509	562	75%	1e-156	86%

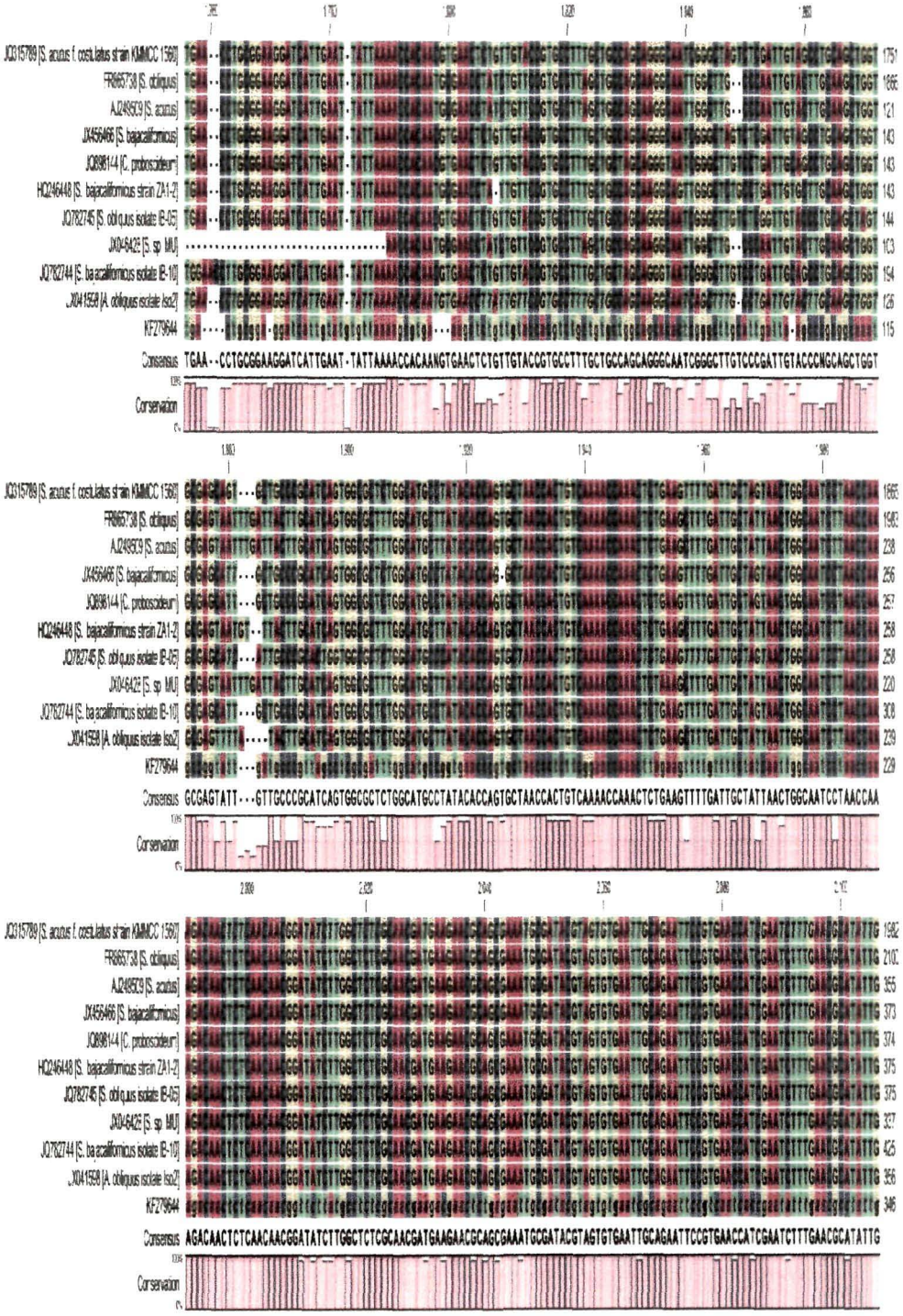


Fig4.17 (a) Sequence alignment of the nearest 10 organism with the microalgal species *Scenedesmus* sp. *Strain* MPBK-2 [NCBI Accession: KF279644] showing the consensus sequences

Fig. 4.18 (a) Tree based on neighbor joining pointing KF279644 with the same cluster of other species of *Scenedesmus* showing a bootstrap value of 100.

Table 4.11 (b) BLAST results of nearest 10 organisms with *P. kessleri* MMPBKK-1

SN	Description	Accession	Alignment score	Query coverage	E-value	Max Identity
1	*Parachlorella kessleri*	FR865655	723	96%	0.00	99%
2	*Chlorella vulgaris*	AY948419	723	96%	0.00	99%
3	*Parachlorella kessleri* strain HY-6	JQ797561	708	96%	0.00	99%
4	*Parachlorella kessleri* strain BAFC CA10	HM744739	652	87%	0.00	99%
5	*Parachlorella kessleri*	FM205885	652	87%	0.00	99%
6	*Parachlorella kessleri*	FM205846	652	87%	0.00	99%
7	*Parachlorella hussii* strain ACOI 473	HM126550.	462	91%	9e-127	88%
8	*Parachlorella hussii* strain ACOI 1508	HM126548	459	86%	1e-125	89%
9	*Parachlorella hussii* strain ACOI 939	HM126551.1	457	76%	4e-125	935
10	*Parachlorella beijerinckii*	FM205845	453	86%	6e-124	93%

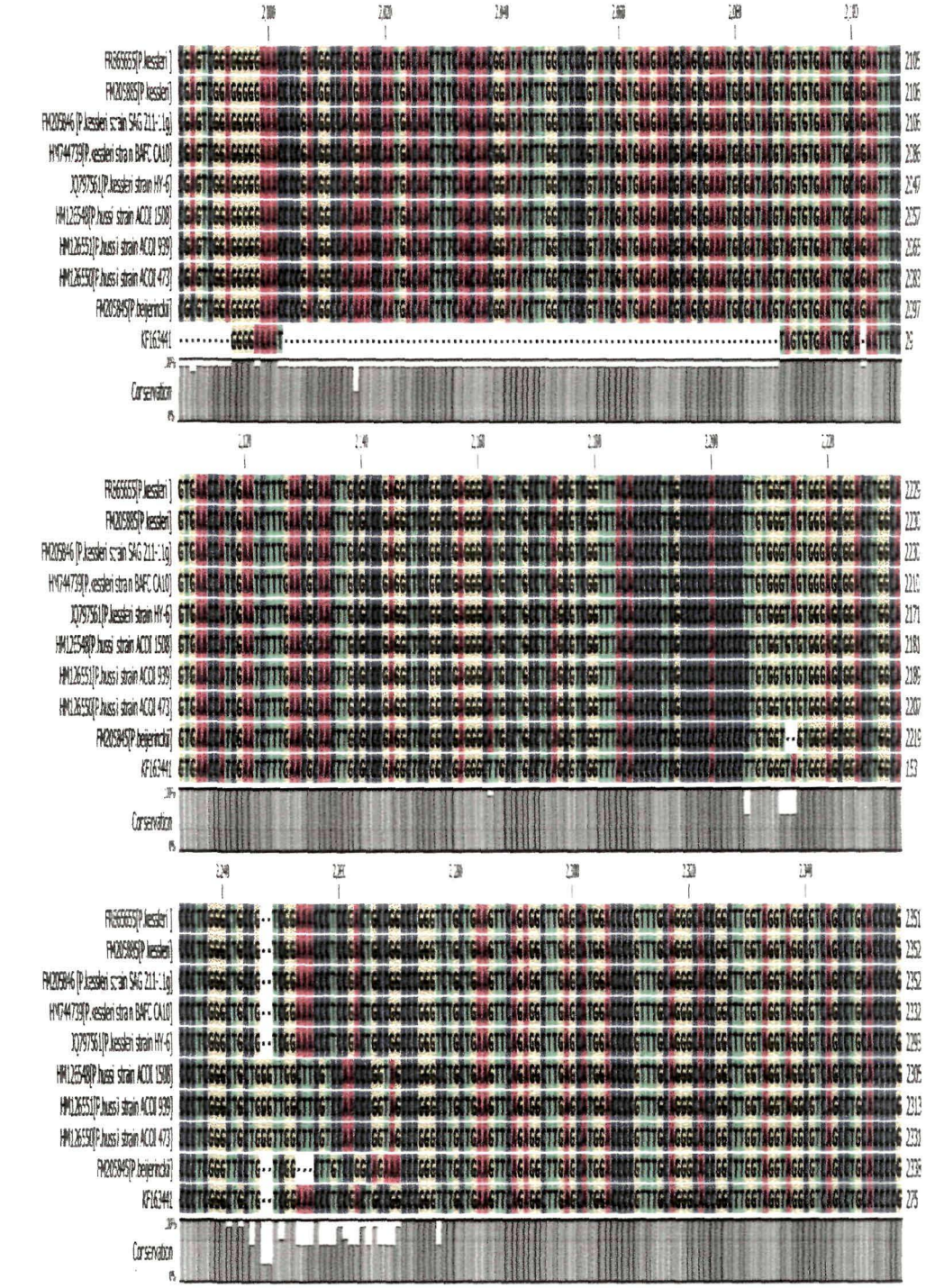


Fig. 4.17 (b) Sequence alignment of the nearest 10 organism with the microalgal species *P. kessleri* strain MMPBKK-1 [NCBI Accession: KF163441] showing the consensus sequences

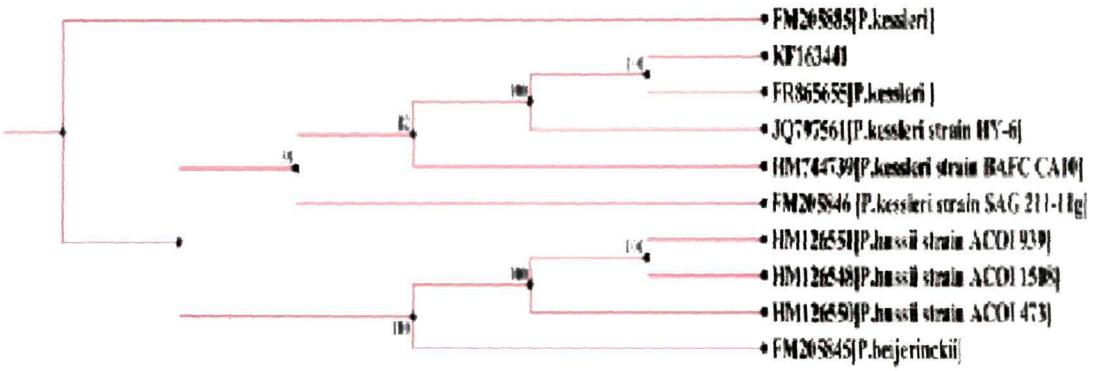


Fig 4.18 (b) Tree based on neighbor joining pointing KF163441 with the same cluster of other species of *Parachlorella* showing a bootstrap value of 100

Table 4.11 (c) BLAST results of nearest 7 organisms with *Chlorella* sp. MP-1

SN	Description	Accession	Alignment Score	Query coverage	E-value	Max Identity
1	*Chlorella* sp. 18S ribosomal RNA gene, partial sequence	AF514413	3055	98%	0.0	99%
2	*Raphidonema nivale* 18S ribosomal RNA gene, partial sequence	AF448477	2952	97%	0.0	97%
3	*Chlorella ellipsoidea* 18S rRNA gene, strain SAG 211-1a	X63520	2939	97%	0.0	97%
4	*Chlorella' ellipsoidea* gene for small subunit rRNA, partial sequence	D13324	2933	97%	0.0	97%
5	*Pabia signensis* partial 18S rRNA gene, strain SAG 7.90	AJ416108	2929	97%	0.0	97%
6	*Pseudochlorella subsphaerica* gene for 18S rRNA, partial sequence	AB006050	2928	97%	0.0	97%
7	*'Chlorella' saccharophila* genomic DNA containing 18S rRNA gene	FR865676	2885	97%	0.0	97%

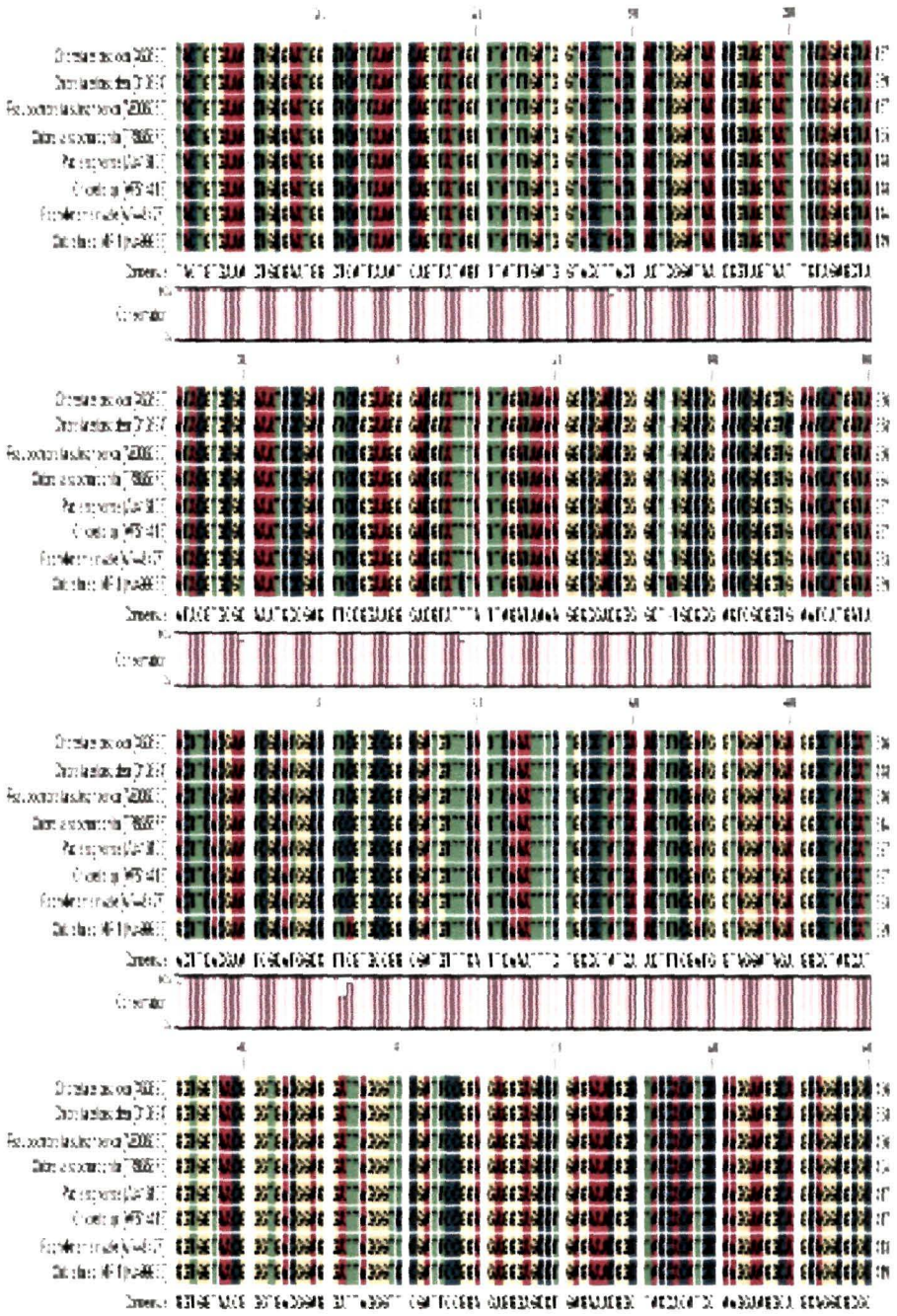


Fig 4.17 (c) Sequence alignment of the nearest 8 organism with the microalgal species *Chlorella* sp. Strain MP-1 [NCBI Accession: KJ499988] showing the consensus sequences

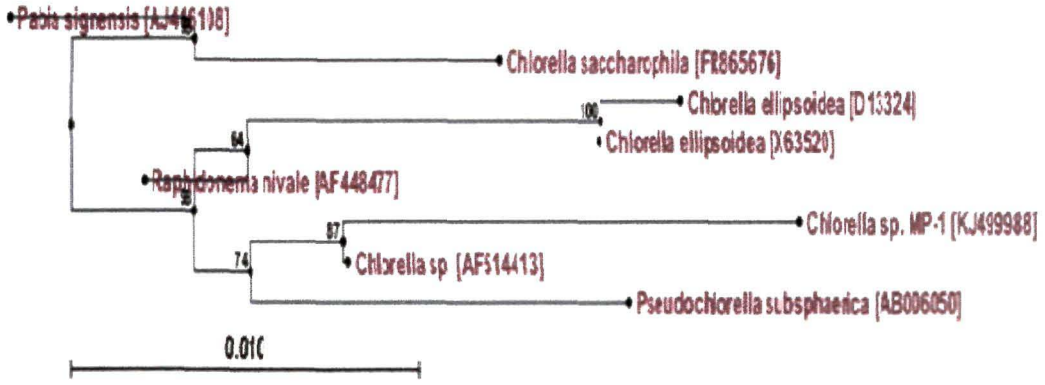


Fig. 4.18 (c) Tree based on neighbor joining pointing KJ499988 with the same cluster of other species of *Chlorella* showing a bootstrap value of 87

From the BLAST results [Table 4.11, (a), (b) and (c)] a maximum identity score of 91% was observed in the case of *Scenedesmus* sp. MPBK-2 and 99% in the case of *Scenedesmus obliquus* isolate IB-05. The nucleotide sequence of *P. kessleri* FR865655 was found to be similar with that of *P. kessleri* MMPBKK-1 with a maximum identity score of 99% whereas, *Chlorella* sp. MP-1 showed maximum identity score of 99% with that of *Chlorella* sp. AF514413.

P. kessleri MMPBKK-1 and *Scenedesmus* sp. MPBK-2 belonged to the same cluster as that of *Parachlorella* and *Scenedesmus* species with a bootstrap value of 100 whereas, *Chlorella* sp. MP-1 belonged to the same cluster of the genus *Chlorella* with a bootstrap value of 87.

4.12 Production and characterization of biodiesel from KCDL

In the kitchen oils from biomasses are used for cooking purpose. Droplets of such cooking oils are usually deposited in the kitchen chimney as lard dump (kitchen chimney dump lard, KCDL). An effort has been made to recollect the oil from KCDL following the schematic design presented below as a reference to the biodiesel extraction from the microalgal isolates through transesterification of the lipid components. The schematic design of biodiesel from KCDL is presented in Fig. 4.19 along with feedstock and subsequent characterization.

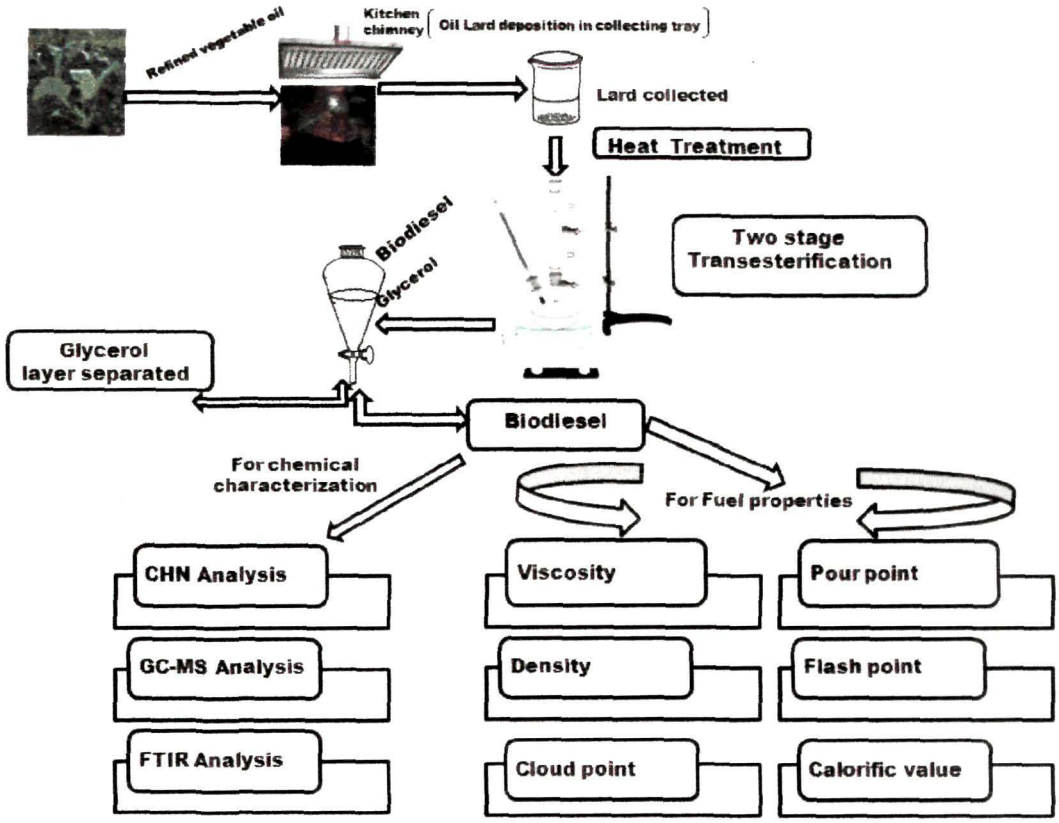


Fig. 4.19 Schematic design of biodiesel production from KCDL feedstock and subsequent characterization [164]

The feedstock was collected from the kitchen chimney of Nilachal Men's Hostel, Tezpur University, Assam-784028, India. A two stage transesterification scheme was used for the production of biodiesel. The base-catalysed transesterification was optimized statistically by using the response surface methodology (RSM). The fuel properties of the biodiesel like viscosity, density, calorific value, cloud point, pour point and cetane number were determined to assess the quality of the biodiesel.

4.12.1 Statistical optimization

The current investigation included optimization of biodiesel yield by RSM method. The level of variables chosen for the Box– Behnken optimization experiment for biodiesel yield is presented in Table 3.4 (Materials and Methods section). Statistical optimization with Box-Behnken design [160] also shows the level of four

independent variables [Methanol (χ_1), NaOH (χ_2), Temperature (χ_3) and Time (χ_4)] which were further investigated as listed in Table 4.12.

Table 4.12 Box–Behnken experimental design, representing the response of biodiesel yield from KCDL as influenced by methanol, NaOH, temperature and time

Run Order	Methanol	NaOH	Temperature	Time	Yield (%)	Predicted Yield (%)
1	0	-1	0	1	80	81.179
2	0	1	0	-1	75	75.647
3	0	0	0	0	83	84.202
4	0	0	-1	-1	84	83.904
5	1	1	0	0	81	81.772
6	0	0	-1	1	85	86.571
7	1	0	0	-1	80	78.821
8	-1	1	0	0	75	73.396
9	0	1	0	1	79	79.314
10	-1	0	-1	0	84	85.648
11	1	-1	0	0	84	81.733
12	0	0	0	0	82	84.202
13	0	-1	1	0	95	94.083
14	-1	0	1	0	87	87.424
15	0	1	-1	0	84	83.355
16	0	-1	-1	0	95	89.316
17	-1	0	0	-1	89	89.349
18	1	0	-1	0	78	81.206
19	0	1	1	0	80	79.313
20	0	0	0	0	84	84.202
21	1	0	1	0	82	83.982
22	1	0	0	1	85	82.487
23	0	0	1	1	79	79.433
24	0	-1	0	-1	93	94.513
25	-1	-1	0	0	94	94.166
26	-1	0	0	1	77	76.016
27	0	0	1	-1	93	91.766

The design matrix of variables in the Box-behnken design could highlight the predicted yield of biodiesel from KCDL. The contour plots are known to explore the relationship between the variables like methanol, NaOH, temp, and time for biodiesel yield.

The biodiesel yield was described with contour plots against NaOH, methanol, temperature and time as shown in Fig.4.19

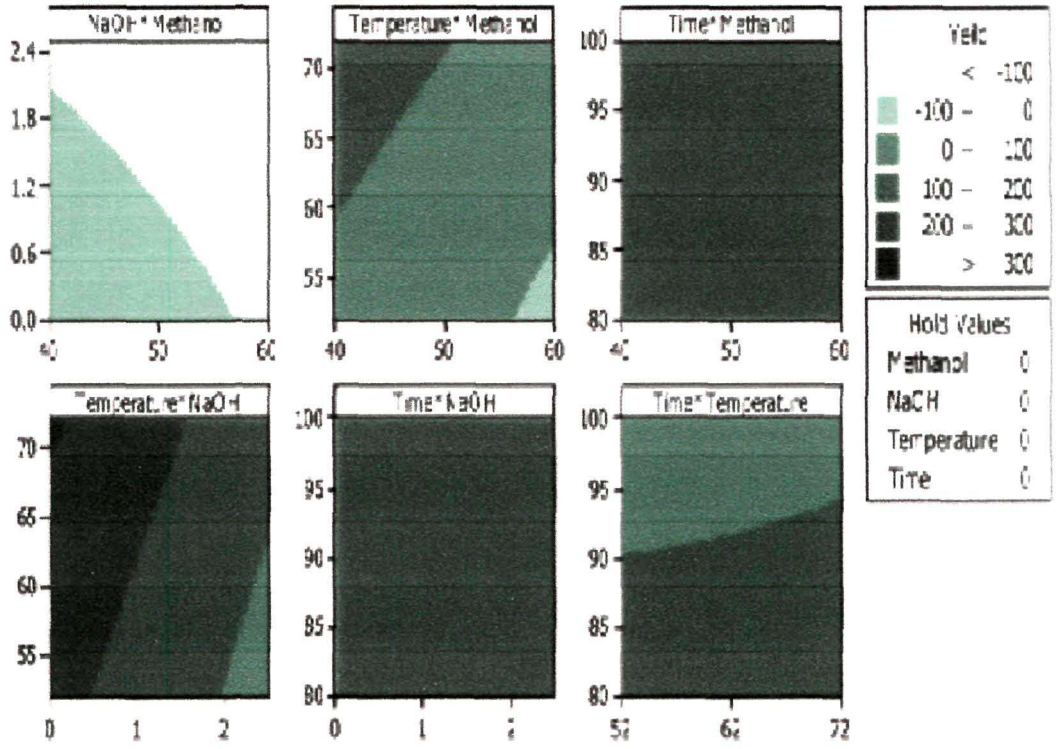


Fig 4.20 Contour plots of KCDL biodiesel yield. The darker green contour region represents higher biodiesel yield (>300) while the lighter green color represents lower biodiesel yield (<−100).

4.12.2 Characterization of KCDL derived biodiesel

The biodiesel derived from the KCDL was characterized by FTIR spectroscopy and GC-MS analysis. The FTIR spectra and GC-MS profile of KCDL biodiesel is presented in Fig. 4.21 and 4.22. The FAMEs identifiable by GC-MS analysis is shown in Table 4.13.

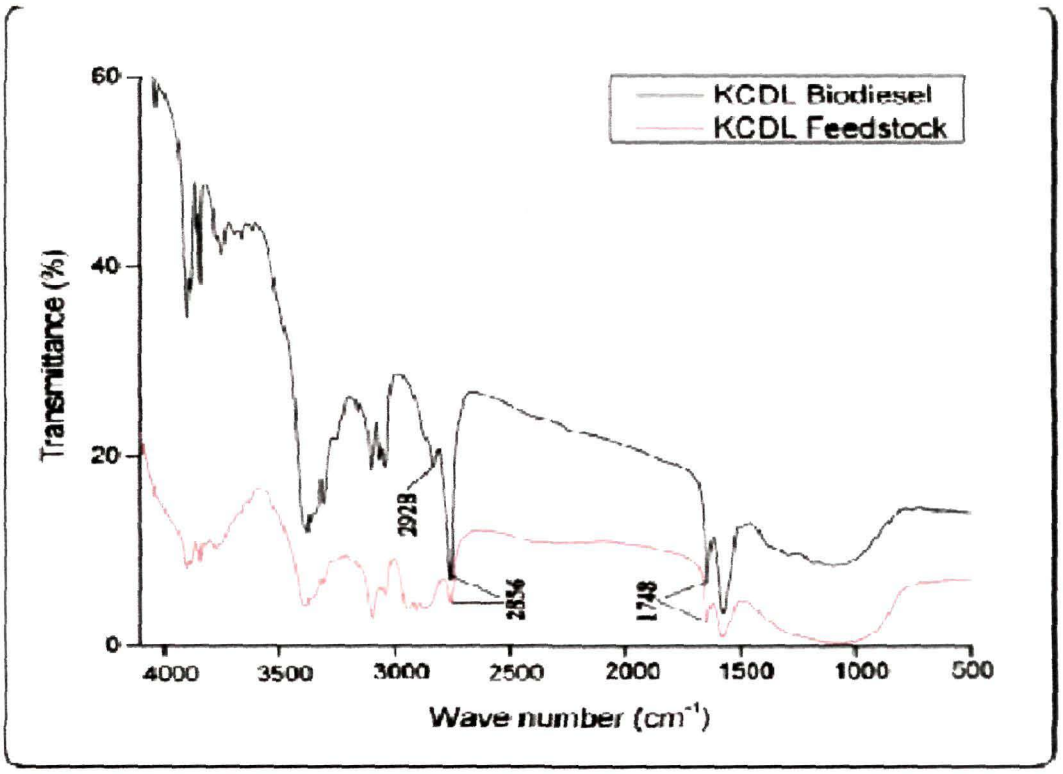


Fig. 4.21 FTIR spectra of KCDL feedstock and biodiesel

The FTIR spectra of the feedstock and biodiesel were found to be similar since both had almost the same chemical groups. The main absorption for the KCDL derived biodiesel was in the order of 2928, 2856 and 1748 cm^{-1}.

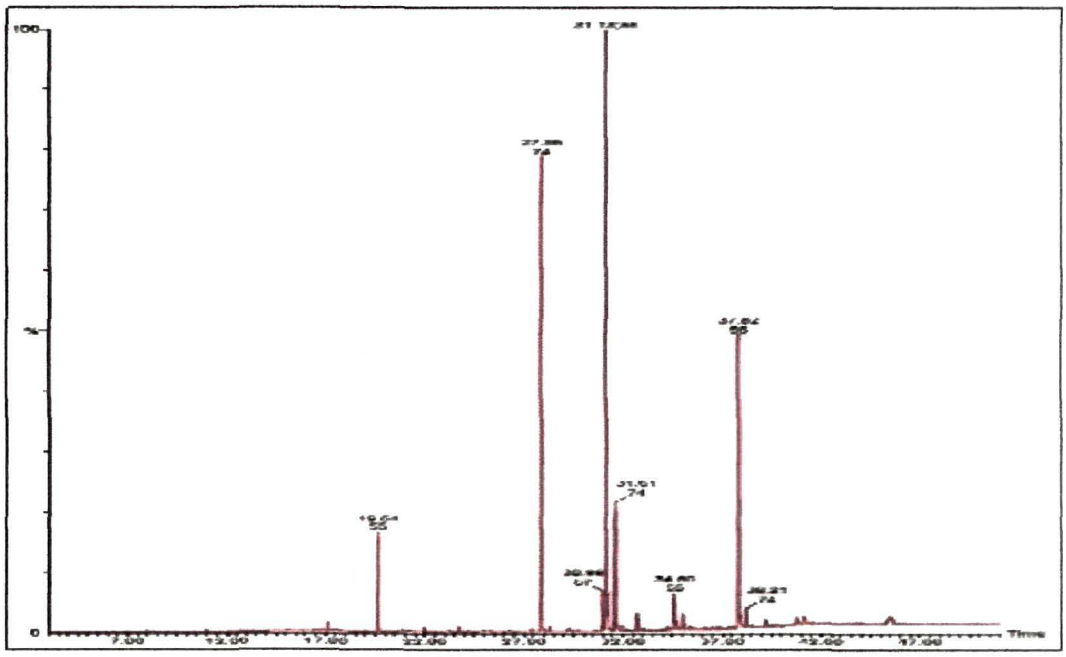


Fig. 4.22 GC-MS profile of KCDL biodiesel

Table: 4.13 FAME analysis of KCDL biodiesel

SN	Fatty acid methyl ester (FAME)	Molecular formula	Wt (%)
1	Nonanedioic acid, dimethyl ester	$C_{11}H_{20}O_4$	5.68
2	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	$C_{17}H_{34}O_2$	34.95
3	9-octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	39.92
4	9-hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	18.82
5	Heptacosanoic acid, 25-methyl, methyl ester	$C_{29}H_{58}O_2$	1.21

The identification of FAME's by GCMS analysis was done by comparing mass spectra with that in NIST library. The saturated and unsaturated FAME in the biodiesel sample were 41.86 and 58.14%, respectively.

The fuel properties of KCDL biodiesel were determined by Standard Test Methods (STM) and the results obtained are presented in Table 4.14

Table 4.14 Properties of KCDL biodiesel

Properties	KCDL Biodiesel	Standard test method used
Density (40°C, g/cm^3)	0.9	ASTM D 5002
Viscosity (mm^2/s, cSt at 40 °C)	8.7	ASTM D 445
Cloud point(°C)	8	ASTM D 2500
Pour point (°C)	11	ASTM D 97
Gross calorific value (MJ/kg)	36.5	Adiabatic Bomb Calorimeter
Net calorific value (MJ/kg)	34.40	Equation for calculation of NCV[36]
Flash point (°C)	123	ASTM D 93
Ash content (Wt %)	0.05	ASTM D 874

From the above table it is clear that KCDL derived biodiesel possessed high density and viscosity with an appreciable calorific value.

4.13 Antimicrobial assay of bio-oil obtained from the microalgae *P. kessleri*

The antimicrobial activity of the bio-oil obtained from the microalgal species *P. kessleri* was assessed against *E. coli* (gram –ve bacteria), *S. aureus* (gram +ve bacteria), *C. albicans* (fungus) and *S. cerevisiae* (yeast) and the results for antimicrobial assay are shown in Fig. 4.23.

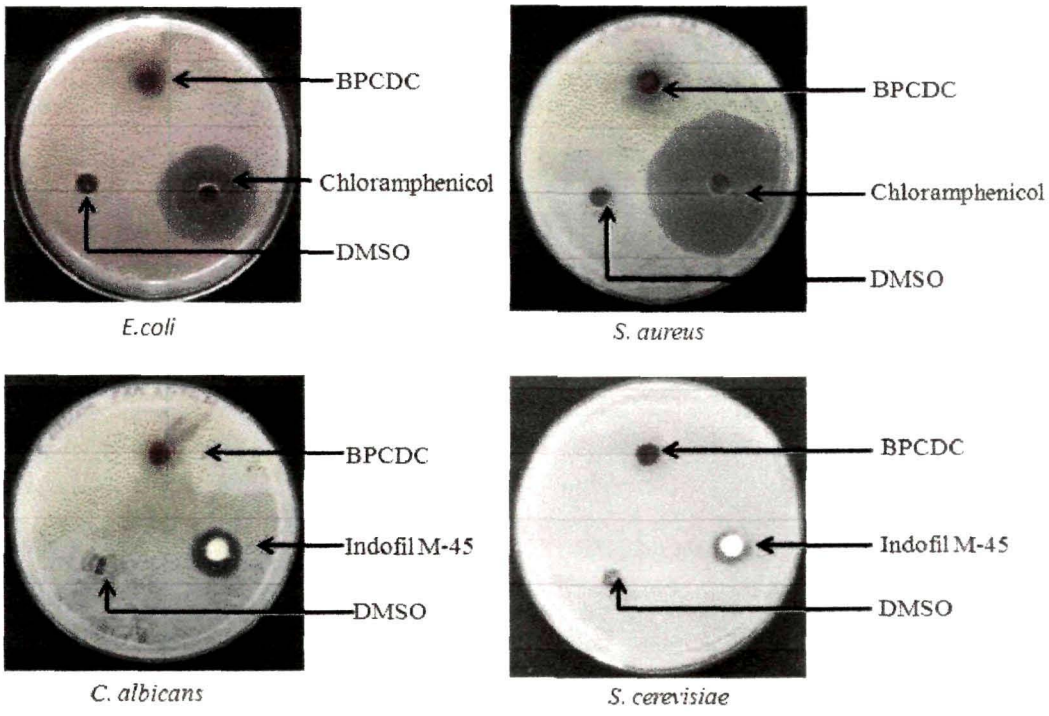


Fig. 4.23 Inhibition zones of BPCDC against *E. coli* and *S. aureus*. No, ZOI was observed against *S. cerevisiae* and *C. albicans*.

As evident from the above figure no ZOI was observed against *C. albicans* and *S. cerevisiae*, whereas only a moderate ZOI (12 mm) was observed against *E. coli* and *S. aureus*.

4.14 Antioxidant activity of microalgal deoiled cake

The microalgal deoiled cake (biomass left after lipid extraction) was accessed for possession of possible antioxidant activity. The antioxidant activity of the extracts was determined by DPPH free radical scavenging assay and the total antioxidant activity of the extracts was determined by FRAP. Total phenolic content of the microalgal deoiled cakes was also determined to establish their correlation with % DPPH scavenging potential and FRAP activity. The results obtained are shown in Fig 4.24 (a), (b) and (c). Similarly the correlation of phenolic content with FRAP activity are presented in Fig. 4.25 (a), (b) and (c).

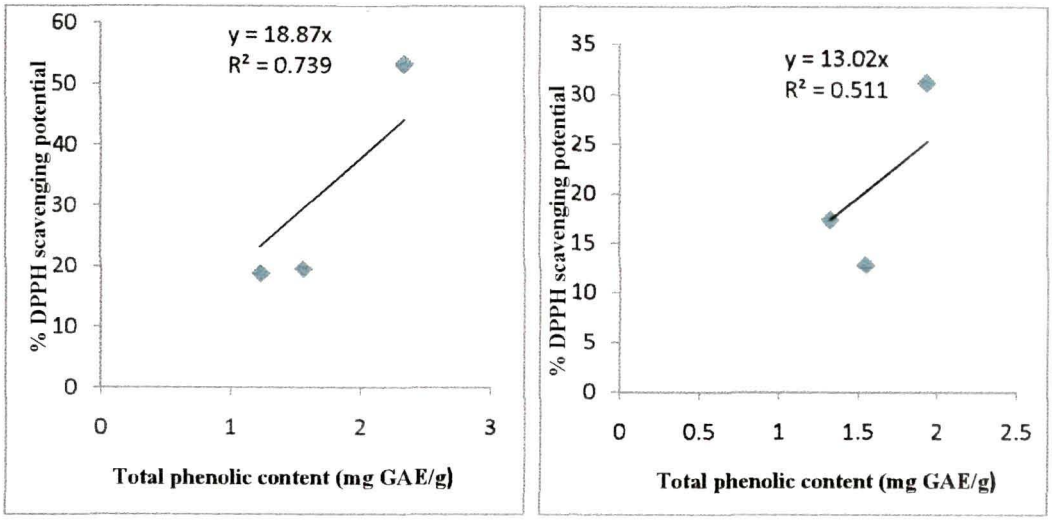
As depicted in Fig. 4.24 (a), (b) and (c) positive correlation was observed between % DPPH scavenging and total phenolic content of the deoiled cake extracts

of *Chlorella* sp. 0.739 (R^2); *P. kessleri* 0.511 (R^2) and *Scenedesmus sp.* 0.558 (R^2), respectively.

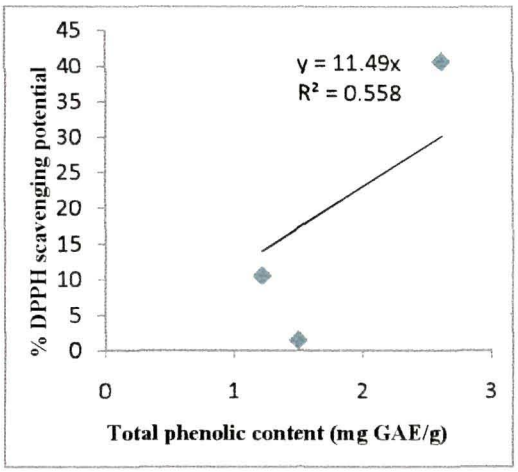
As shown in Fig. 4.25 (a), (b) and (c) negative correlation was observed between FRAPs and TPC of the deoiled cake extracts of *Chlorella* sp. (R^2= -6.41), *Scenedesmus* sp. (R^2= - 9.55) and *P. kessleri* (R^2= -9.19), respectively.

The total phenolic content (TPC) and % DPPH scavenging potential in the deoiled cake of the microalgal isolates were determined and the same are presented in Table 4.15 (a).

The TPC and FRAP activity in the deoiled cake of microalgal isolates were determined and the data obtained are presented in Table 4.15 (b).

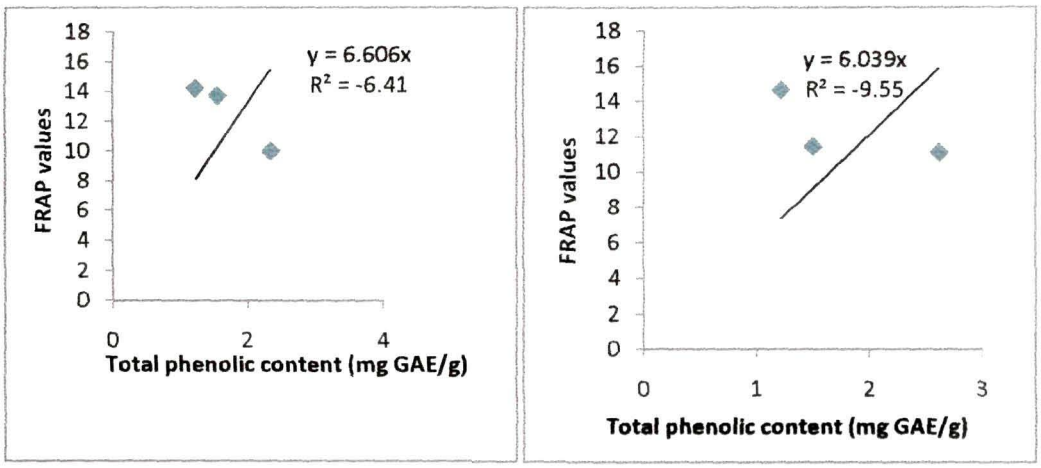


(a) (b)

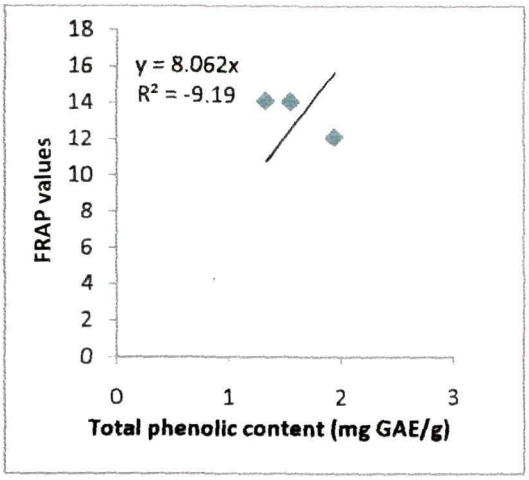


(c)

Fig. 4.24 Correlation between DPPH scavenging activity and TPC of deoiled cake extracts of (a) *Chlorella* sp. (b) *P. kessleri* and (c) *Scenedesmus* sp.



(a) (b)



(c)

Fig. 4.25 Correlation between FRAPs and TPC of deoiled cake extracts of (a) *Chlorella* sp. (b) *P. kessleri* and (c) *Scenedesmus* sp

Table 4 15 (a) TPC and % DPPH scavenging potential of the investigated microalgal de-oiled cake sample extracts

Sample code	Extract	TPC (mg GAE/g)	%DPPH scavenging potential
Chlorella sp	Aq	2 34	53 20
	EtOH	1 56	19 44
	MeOH	1 23	18 70
Scenedesmus sp	Aq	2 62	40 75
	EtOH	1 50	14 90
	MeOH	1 22	10 57
P kessleri	Aq	1 94	31 17
	EtOH	1 55	17 35
	MeOH	1 33	12 80

[Aq= Aqueous, EtOH= Ethanol and MeOH=Methanol]

From the above table it is evident that the aqueous extract of all the tested samples showed the highest % DPPH scavenging potential whereas methanolic extract showed the lowest

Table 4 15 (b) TPC and FRAP activity in the microalgal de- oiled cake sample extracts

Sample code	Extract	TPC (mg GAE/g)	FRAP values (µmol Fe (II)/g dw)
Chlorella sp	Aq	2 34	10 00
	EtOH	1 56	13 69
	MeOH	1 23	14 22
Scenedesmus sp	Aq	2 62	11 09
	EtOH	1 50	11 42
	MeOH	1 22	14 63
P kessleri	Aq	1 94	12 10
	EtOH	1 55	14 04
	MeOH	1 33	14 09

[Aq= Aqueous, EtOH= Ethanol and MeOH=Methanol]

From the above table it is clear that among all the tested extracts, methanolic extract of *Scenedesmus* sp. deoiled cake possessed the highest FRAP activity whereas the aqueous extract of *Chlorella* sp. deoiled cake showed the lowest FRAP activity.

4.15 Molecular docking of the compounds present in the bio-oil of microalgae

Molecular docking simulation study was carried out with regard to compounds identified by GC-MS analysis in the derived bio-oil from the microalgal species *P. kessleri* against certain proteins possessing anti microbial activity. Docking simulation was carried out against cytochrome c peroxidase of *S. cerevisiae* (PDB ID: 1AC4) [top scores shown in Table 4.16 (a)], ribonucleotide reductase R2 of *E. coli* (PDB ID: 1AV8) [top scores shown in Table 4.16 (b)], sortase A of *Staphylococcus aureus* (PDB ID: 1T2P) [top scores shown in Table 4.16 (c)], secreted aspartic proteinase of *C. albicans* (PDB ID: 1ZAP) [top scores shown in Table 4.16 (d)], N-terminal domain of YaeT of *E. coli* (PDB ID: 2QDF) [top scores shown in Table 4.16 (e)], enoyl reductase of *E. coli* K-12 (PDB ID: 4JQC)) [top scores shown in Table 4.16 (f)] . The snaps of top docking hits of each enzyme are shown in Fig. 4.26 (a), (b), (c), (d), (e) and (f).

Table 4.16 (a) Docking result of PDB ID: 1AC4

Ligand	MolDock Score	Rerank Score	HBond
UNK0-TYR1	-89.6731	-64.1891	-8.51982
3-pyradinol, 2-nitro-	-34.9496	-21.649	-11.074
phenol,2,6-dimethoxy-4-(2-propenyl)-	-38.9893	-28.015	-10.4618
3-pyradinol, 2-nitro-	-43.6947	-31.3687	-15.0505
3-pyradinol, 2-nitro-	-29.0745	-22.5982	-10
2-pyridinecarboxylic acid, 6-methyl-	-41.6872	-30.5634	-8.59072
3-T-Butyl-oct-6-en-1-ol	-41.2725	-14.2004	-7.5
9- octadecenamide, (Z)-	-73.5624	-52.1104	-4.94835
Phenol, 4-ethyl-	-23.1061	-17.8525	-4.47476

Table 4.16 (b) Docking result of PDB ID: 1AV8

Ligand	MolDock Score	Rerank Score	HBond
l- proline n- (cyclopropylcarbony)-heptyl ester	-76.9198	-18.0652	-5.32248
Heptanoic acid 3-oxo-2-propyl-methyl ester	-32.5376	-8.4619	0
Pyridine 34-dimethyl-	-27.0364	-3.12932	-1.81223
Pyridine	-14.9532	-0.292629	0
Cyclopentane 13- dichloro- trans-	-24.0947	-4.91998	0

Table 4.16 (c) Docking result of PDB ID: 1T2P

Ligand	MolDock Score	Rerank Score	HBond
Cyclotrisiloxane hexamethyl-	-44.2172	-18.5866	-9.99848
3-pyradinol 2-nitro-	-46.2216	-39.0308	-3.03719
2-pyridinecarboxylic acid 6-methyl-	-30.2104	-2.51673	-6.24447
LEU1-PRO2	-22.9113	-8.59668	-5.97849
Phenol4-methyl-	-46.5493	-36.1653	-3.13241
Phenol 4-ethyl-	-41.2184	-27.199	-2.77695
3-pyradinol 2-nitro-	-38.5243	-30.9809	-5.99903
Phenol 2-methyl-	-50.4013	-39.3883	-5
Phenol4-methyl-	-41.2493	-36.5151	-4.06338

Table 4.16 (d) Docking result of PDB ID: 1ZAP

Ligand	MolDock Score	Rerank Score	HBond
UNK0-TYR1	-73.7242	-21.6237	-6.16179
3-T-Butyl-oct-6-en-1-ol	-58.7082	-44.7315	-5.45941
3-pyradinol 2-nitro-	-49.4047	-45.7671	-7.41054
Phenol 4-ethyl-	-47.1378	-41.4912	-4.99721
]Phenol 2-methyl-	-39.436	-37.2419	-5.78459
Phenol 2-methyl-	-39.0708	-36.6867	-5
Phenol4-methyl-	-37.0928	-33.5056	-4.99214
Phenol 4-ethyl-	-29.6682	-14.209	-5.70592
Phenol4-methyl-	-26.0685	-5.89295	-5.80753
2-pyridinecarboxylic acid 6-methyl-	-50.5865	-45.2827	-5.06341

Table 4.16 (e) Docking result of PDB ID: 2QDF

Ligand	MolDock Score	Rerank Score	HBond
UNK0-TYR1	-51.4033	-37.8776	-4.93563
Phenol, 2-methyl-	-14.7255	-6.98441	-3.16878
trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane	-35.5216	-13.3262	-2.5
cyclotrisiloxane, hexamethyl-	-21.1014	3.66056	-1.73517
3-methyl-2-(2- oxopropyl) furan	-38.137	-30.265	-0.392104
Phenol, 4-ethyl-	-29.1874	-26.3348	-2.5
Phenol,4-methyl-	-28.4042	-25.351	-2.49898
Phenol,4-methyl-	-27.6813	-21.3815	-2.5
2-pyridinecarboxylic acid, 6-methyl-	-24.6224	-16.5662	-2.39403
Phenol,4-methyl-	-24.4752	-16.375	-3.50554

Table 4.16 (f) Docking result of PDB ID: 4JQC

Ligand	MolDock Score	Rerank Score	HBond
3-pyradinol, 2-nitro-	-40.1681	-32.8728	-3.91223
2-Cyclopenten-1-one, 2-methyl-	-45.1465	-39.0434	-2.563
Phenol,4-methyl-	-46.1277	-39.0284	-4.89517
cyclotrisiloxane, hexamethyl-	-69.4069	-49.1511	-7.49432
Phenol, 2-methyl-	-47.2904	-42.7517	-5
2-Cyclopenten-1-one, 2-methyl-	-47.5634	-40.3864	-4.70303
Pyridine	-40.0626	-37.8629	-2.27401
Pyridine	-30.7021	-29.2011	-2.09861
2-cyclopenten-1-one, 2,3-dimethyl	-59.0411	-50.9745	-0.105997
Indole	-55.9825	-48.0049	-1.50295

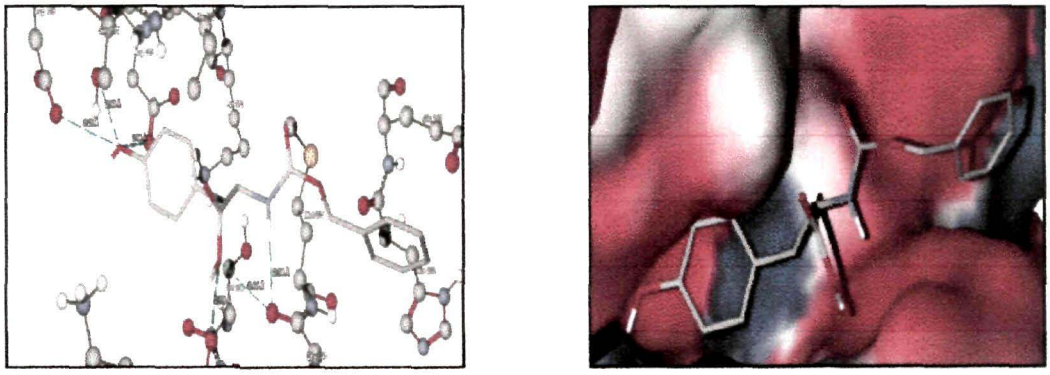


Fig. 4.26 (a) Molecular interaction and electrostatic interaction of UNK0-TYR1 with cytochrome c peroxidase of *S. cerevisiae* (PDB ID: 1AC4)

The interaction of UNK0-TYR1 with cytochrome c peroxidase of *S. cerevisiae* (PDB ID: 1AC4) had a MolDock score of -73.7242, Rerank score of -21.6237 and HBond -6.16179

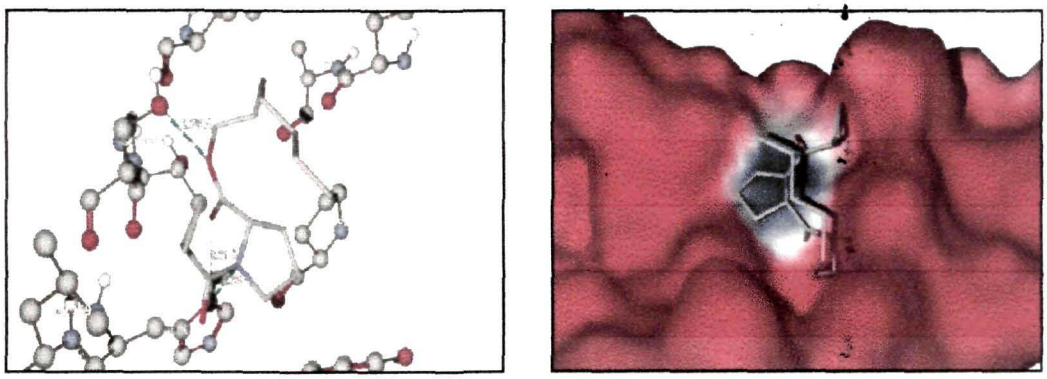


Fig. 4.26 (b) Molecular interaction and electrostatic interaction of l- proline n-(cyclopropylcarbony)- heptyl ester with ribonucleotide reductase R2 of *E. coli* (PDB ID: 1AV8)

The interaction of l- proline n- (cyclopropylcarbony)- heptyl ester with ribonucleotide reductase R2 of *E. coli* (PDB ID: 1AV8) had a MolDock score of -76.9198, Rerank score of -18.0652 and HBond -5.32248

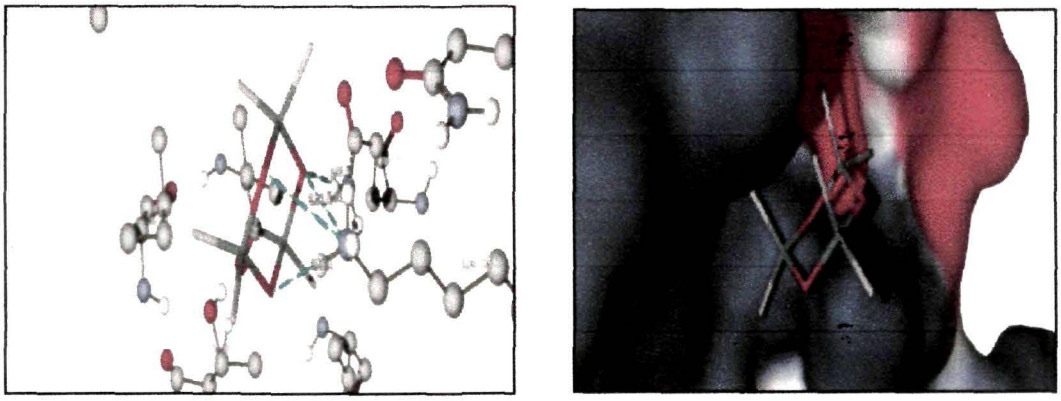


Fig. 4.26 (c) Molecular interaction and electrostatic interaction of cyclotrisiloxane hexamethyl with Sortase A of *S. aureus* (PDB ID: 1T2P)

The interaction of cyclotrisiloxane hexamethyl with Sortase A of *S. aureus* (PDB ID: 1T2P) had a MolDock score of -44.2172, Rerank score of -18.5866 and HBond -9.99848

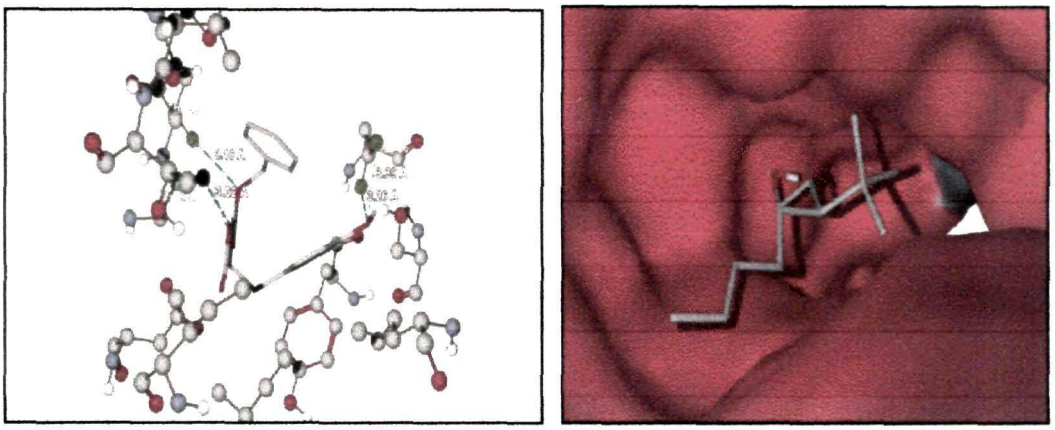


Fig. 4.26 (d) Molecular interaction and electrostatic interaction of UNK0-TYR1 with secreted aspartic proteinase of *C. albicans* (PDB ID: 1ZAP)

The interaction of UNK0-TYR1 with secreted aspartic proteinase of *C. albicans* (PDB ID: 1ZAP) had a MolDock score of -73.7242, Rerank score of -21.6237 and HBond -6.16179

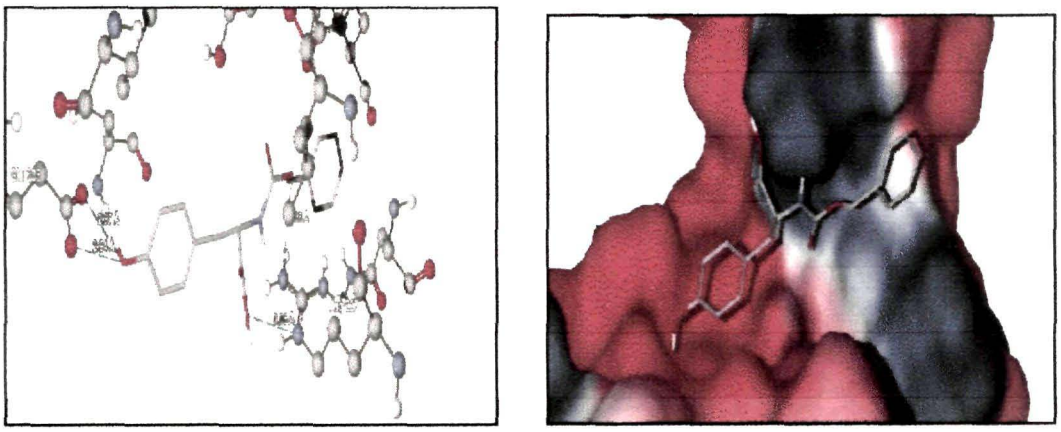


Fig. 4.26 (e) Molecular interaction and electrostatic interaction of UNK0-TYR1 with N-terminal domain of YaeT of *E. coli* (PDB ID: 2QDF)

The interaction of UNK0-TYR1 with N-terminal domain of YaeT of *E. coli* (PDB ID: 2QDF) had a MolDock score of -51.4033, Rerank score of -37.8776 and HBond -4.93563

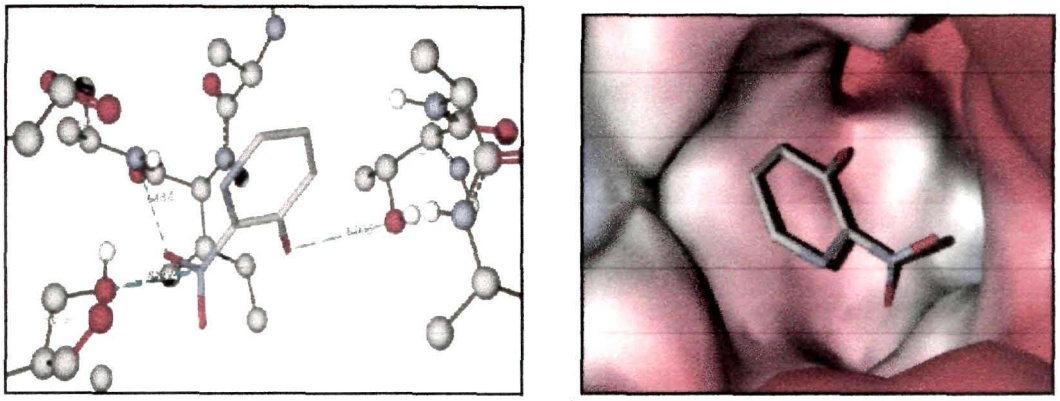


Fig. 4.26 (f) Molecular interaction and electrostatic interaction of 3-pyradinol, 2-nitro- with enoyl reductase of *E. coli* K-12 (PDB ID: 4JQC)

The interaction of 3-pyradinol, 2-nitro- with enoyl reductase of *Escherichia coli* K-12 (PDB ID: 4JQC) had a MolDock score of -40.1681, Rerank score of -32.8728 and HBond -3.91223

The molecular docking studies revealed that the compounds inhibit the bacterial and fungal enzymes (PDB ID: 1AC4, 1AV8, 1T2P, 1ZAP, 2QDF, 4JQC) exhibiting strong molecular interaction and hydrogen bonding at the active site of the corresponding enzyme as evident from the rerank Score and hydrogen bonding score. The molecular interaction distance of each of the docked compounds is depicted in Fig. 4.26 (a-f).

Chapter 5

Discussion

5.1 Microalgal growth

Microalgal preference for growth are quite variable, some require complex nutrient regime whereas others prefer simple requirements [1]. Choice of proper culture media is also of prime importance in microalgal cultures. A good microalgal culture media must have proper carbon source (CO_2 or carbonates), nitrogen source (nitrate, ammonia and urea), vitamins, metal chelators (mostly EDTA), salt (NaCl, $CaCl_2.2H_2O$) and trace elements in order to support growth. In this study BG-11 was found to be the most suitable media for the growth of *Chlorella* sp., and *P. kessleri* (Fig. 4.1). BG-11 is a well balanced culture medium with high concentration of nitrogen ($NaNO_3$). A high concentration of nitrogen source in BG-11 culture media (1500mg/L) in comparison to others might serve as the key contributor in this regard.

In the present study, microalgal cultures were carried out in 500 ml Erlenmeyer flasks. Optimization of microalgal growth parameters was carried out with regard to temperature and pH, whereas other parameters such as light intensity (1200 lux), photoperiod (16:8) and mixing rate (100 rpm) were kept constant. The optimum temperature for *Chlorella* sp. and *Scenedesmus* sp. growth were 28±2°C whereas, for *P. kessleri* it was 30±2°C [Table 4.1 (a)]. The optimum pH requirement for *P. kessleri* and *Scenedesmus* sp. (8 and 8.2) was slightly higher in comparison to *Chlorella* sp (7.5). Abu Rezq *et al.* [215] reported on the optimum production conditions for three different marine algae viz. *Nannochloropsis*, *Tetraselmis* and *Isochrysis*. The optimum production conditions were *Nannochloropsis* (19-21°C, pH 7.4-7.8), *Tetraselmis* (19-21°C, pH 7.6-7.8) and *Isochrysis* (24-26°C, pH 8). Dauta *et al.* [165] reported the optimal temperature range for growth of fresh water microalgae to be within the range of 25-32 °C. The optimal temperature ranges for our strains were within the range of 28-30 ± 2°C [Table 4.1 (a)] which is in complete agreement with the findings of Dauta *et al.* [165]. For most strains of microalgae, pH tolerance lies between 7 and 9 while the optimal has been reported to be 8.2-8.7 [166]. The optimum pH range for *Scenedesmus* sp. and *P. kessleri* was within the reported range, whereas *Chlorella* sp

showed a relatively lesser alkaline pH of 7.5 for effective growth. In addition to temperature and pH, light and photoperiod (light: dark cycle) are two other important factors which are of fundamental importance in microalgal culture. Lavens and Sorgeloos [166] reported light intensities of 100-200 µmol/s/m^2 as a minimum for microalgal biomass production. Generally, for phototrophic Chlorophyceae light energy is a growth limiting substrate. The major concern in regard to light is its intensity. With increase in cell density, light penetration into the culture (expressed as a percent of total incident light impinging on culture surface) decreases exponentially [210]. A study by Ogbonna *et al.* [209] reports an increase in microalgal biomass yield (*Chlorella* sp.) from 2g/L/day to 4g/L/day with increase in the light intensity from 163 µmol/s/m^2 to 310 µmol/s/m^2. This clearly indicates the importance of light intensity in microalgal growth. A light intensity of 1200 lux and a light: dark cycle (16:8) was maintained during the entire culture period of the microalgal species.

The growth curve of the strains [Fig. 4.2 (a), (b) and (c)] follows the characteristic sigmoidal pattern consisting of a lag phase, an exponential phase and a steady phase. The attainment of steady state in the microalgal isolates was due to increase in cell numbers and depletion of nutrients in the culture media. It is clear that *Scenedesmus* sp. [from Table 4.1 (b)] was the most aggressive and fastest grower with minimum doubling time of 2.48 day followed by *Chlorella* sp (3.21 day). *P. kessleri* was the slowest grower with doubling time of 3.37 day. The specific growth rates and divisions/day were recorded to be 0.21 and 0.31 for *Chlorella* sp, 0.27 and 0.40 for *Scenedesmus* sp and 0.20 and 0.29 for *P. kessleri*, respectively. The fast doubling time in *Scenedesmus* sp in comparison to the other species is indicative of the fact that the species had a higher adaptive ability marked by its ecological success in the imposed culture environment. However, it is noteworthy to mention that supply of CO_2 and exposure of microalgal cultures to higher light intensities can trigger faster exponential growth in microalgae.

Productivity is one of the major concerns in microalgal cultures. A high volumetric productivity is important as it can suffice for small microalgal culture systems. High volumetric productivity means a high microalgal biomass density which

can be significant with regard to downstream processing for biorefining applications. Microalgae can reach up to 300 times more oil productivity for biodiesel production in comparison to traditional crops on an area basis [33]. Microalgae also have areal productivity much superior to traditional agricultural crops [33]. High volumetric productivities and high biomass yields on sunlight are needed to decrease production system volumes and to lower production costs which can be fulfilled by cultivating microalgae in photobioreactors with high surface to volume ratio [211]. Hempel *et al.* [212] reported the influence of light intensity and culture temperature on biomass productivity of *Chlorella* sp. 800 when cultured in glass photobioreactors. At varied light intensity, the biomass productivity was 0.423 g L^{-1} day^{-1} at 200 µmol photons $m^{-2}s^{-1}$ and 0.707 g L^{-1} day^{-1} at 500 µmol photons $m^{-2}s^{-1}$. The biomass productivity was 0.538 g L^{-1} day^{-1} compared to 0.435 g L^{-1} day^{-1} at 35°C and 0.34 0.538 g L^{-1} day^{-1} at 15°C. However, productivity was not in the scope of the present study.

5.2 Morphological characterization

As observed under compound microscopy all the microalgal strains were green in color, unicellular, non-motile and non-filamentous. *Chlorella* sp and *P. kessleri* strains were spherical in shape whereas, *Scenedesmus* sp. was fusiform with the presence of boat shaped cavities (Fig. 4.3). Microscopic images revealed that each of the individual strain can remain single or even in clusters. The colonies appeared to be round, dense green, and full-sized enough to be visible to the naked eye.

Scanning electron microscopy revealed the surface morphology and size of the microalgal cells. In general species belonging to the genus *Scenedesmus* remain in clusters of 4, 8, 16 and 32 cells [Fig. 4.4 (b)]. The SEM micrographs revealed that *Scenedesmus* sp. cells are arranged in a flat plate. The cells are around 2.5 - 3 µm in diameter and 6 – 20 µm in length (Table 4.2), with a thick cell wall. Both smooth and rough cell walls were observed in the species and each colony was covered by an irregular network, but no spines were visible. Boat shaped cavities were also observed on the upper side of the cells and apparently they were of similar size. The *Chlorella* sp. cells [Fig. 4.4 (a)] were spherical in shape. The *Chlorella* sp. cells are always in solitary and apparently were of the same size (1.5 - 4.5µm in diameter). No spines, and

filaments were observed in the SEM micrographs for *Chlorella* sp. SEM micrographs of *P. kessleri* [Fig. 4.4 (c)] reveals that the cells are spherical in shape with absence of spines and flagella.

5.3 Thermal analysis

Thermo gravimetric analysis (TGA) was done in nitrogen atmosphere to study the degradation profile of microalgal biomass. The TG-DTG profile of *Chlorella* sp. [Fig. 4.5 (a_1)] biomass reveals an initial weight loss between ambient temperature and about 130°C and 160°C for 10 and 30°C/min. This could possibly be due to elimination of physically absorbed water in the biomass and due to external or superficial water bounded by surface tension. This was followed by continuous decrease in sample weight (where main degradation occurred) which ended by approximately 380–390°C for the lower heating rate and 410–435 °C for the higher heating rate. These zones (130–390°C) and (160–435°C) have been referred to as the zone of active pyrolysis. For the two heating rates significant changes in the slope of the thermogram were observed at around 400°C and 440°C, which indicated the initiation of the passive pyrolysis zone that terminated at around 525°C for the former and 650°C for the later. A very slow loss of weight occurred until 750°C which indicates that there was further reaction involving char. This implies that the main pyrolysis reactions occurred between 160–525 °C and 650 °C for the stated heating rates.

The TG-DTG profile [Fig. 4.5 (a_2)] of *Chlorella* sp. de-oiled cake reveals an initial weight loss between ambient temperature and 110°C for 10 and 30°C/min. This could possibly be due to moisture evolution. This was followed by continuous decrease in sample weight (where main degradation occurred) which ended by approximately 330–340°C for the lower heating rate and 350–365°C for the higher heating rate. No major observable difference was noticed in the thermogram of the de-oiled cake at the two heating rates. The degradation of de-oiled cake terminated earlier than algal biomass due to loss of crude cell components in the lipid extraction procedure. The shorter thermal degradation profile of the algal de-oiled cake suggests that it can be an ideal feedstock for thermo-chemical conversion.

Analysis of the thermogram [Fig 4.4 (b_1 + b_2)] showing the TG-DTG profile of *Scenedesmus* sp. reveals the following.

(a) Evolution of moisture and light volatiles: 140°C for 10°C and 142°C for 30°C /min and

(b) Zone of active pyrolysis: (130-385°C) for the lower and (130-400°C) for the higher rate.

Analysis of the *P. kessleri* thermogram [Fig 4.5 (c_1 + c_2)] reveals the following

(a) Evolution of moisture and light volatiles: 137°C for 10°C and 142°C for 30°C /min and

(b) Zone of active pyrolysis: (150-390°C) for the lower and (155-400°C) for the higher rate.

The temperature range for the active pyrolysis zones of *Chlorella* sp., *Scenedesmus* sp. and *P. kessleri* are in agreement with the findings of Shuping *et al.*, [115]. The analysis of the thermograms shows that during the main pyrolysis process, only one strong peak and henceforth only one decomposition process corresponding to the degradation of crude protein was observed. Microalgae contains very high amount of proteins, (*Chlorella* sp = 43.22%, *Scenedesmus* sp = 40.56% and *P. kessleri* = 41.31%). Therefore the major degradation corresponds to protein. A lateral shift towards higher temperature values was visually obvious in all the thermograms with increase in heating rate. A very slow weight loss was evident after termination of the active pyrolysis stage which is indicative of further reactions involving char. The short thermal degradation profile of *Chlorella* sp, *Scenedesmus* sp and *P. kessleri* renders them to be ideal feedstocks for thermo-chemical conversion.

5.4 FTIR analysis

FTIR analysis was done in order to determine the functional groups present in microalgal biomass. No major difference was observed in the IR spectra of *Chlorella* sp, *Scenedesmus* sp and *P. kessleri* [Fig. 4.6 (a)]. The region 3300–3000 cm^{-1}

is characteristic for C-H stretching vibrations of C ≡ C, C, C=C and Ar-H, whereas the region from 3000 – 2700 cm^{-1} is dominated by C-H stretching vibrations of $-CH_3$, $>CH_2$, CH and CHO functional groups, respectively [167, 168]. The olephinic C-H stretching vibration between 3600 – 3300 cm^{-1} indicates unsaturation. The absorption at 1652 cm^{-1} implies the presence of C=O of carboxylic acid and derivatives. The region between 1800 and 1500 cm^{-1} demonstrate characteristic bands for proteins, whereas 1700–1600 cm^{-1} is specific for amide-I bands [168] which is mainly due to C=O stretching vibrations of peptide bond [169]. The bands in the amide-I region provide insight into the protein secondary structure [170]. On the other hand the region from 1600–1500 cm^{-1} is specific for amide-II bands, which is due to N-H bending vibrations [171]. The region from 1200 - 900 cm^{-1} signifies a sequence of bands due to C-O, C-C, C-O-C and C-O-P stretching vibrations of polysaccharides [172] well as CH_3, CH_2 rocking modes [173]. CH_2 stretching vibrations in the range of 3100–2800 cm^{-1} imply the presence of lipid. The absorption at 2928 and 2860 cm^{-1} implies CH_2 asymmetric and symmetric stretching in lipid [167].

Bands prominent in *Chlorella* sp. biomass showed progressive degradation in their intensity in the spectra of the de-oiled cake [Fig. 4.6 (b)]. There was a general decrease in protein and carbohydrate content indicated by a decrease in the intensity of absorption bands in the 1800–800 cm^{-1} region. This region is specific for proteins and carbohydrates [168]. Declination in the intensity of absorption in the range of 3100–2800 cm^{-1} is indicative of decrease in the lipid content.

5.5 Role of heavy metal on lipid composition in microalgae

Anthropogenic activities are responsible for the release of heavy metals such as Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Cr^{2+} etc into the water bodies. Investigating the feasibility of microalgal culture directly in these contaminated water bodies which otherwise are considered as abandoned may provide interesting information on the biochemical profile of microalgae. FTIR based analyses were carried out to study the impact of heavy metals such as Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+} and Cr^{2+} induced stresses on lipid content of the microalgal cells. Absorption or adsorption of heavy metals by microalgae was not within the scope of the present investigation. It is also worth

mentioning here that the investigated microalgal species was not cultured in representative water samples from heavy metal contaminated water bodies Heavy metals at varying concentrations of 0 5, 1 and 2mM were directly added to the microalgal culture media and the FTIR spectra of the resultant biomass were recorded for preliminary interpretation regarding lipid modulation

Many algal species are well capable of trapping and concentrating heavy metals like Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} and Cr^{2+} In general, the region from 3100-2800 cm^{-1} [167] implies the presence of lipid, 1200-900 cm^{-1}[172] corresponds to polysaccharides whereas, 1800-1500 cm^{-1} corresponds to proteins [168] Variation in the intensity of absorption in the aforementioned regions is indicative of fluctuation in the biochemical (lipid, protein and carbohydrate concentration) profile However, from the point of view of lipid modulation only 3100 – 2800 cm^{-1} is important in our study

No, marked degree of differences were observed in the investigated spectra for the control and treated biomass except in the case of Pb^{2+} An increase in the intensity of absorption at 2928 cm^{-1} was observed for Pb^{2+} induced stress Fig 4 7 (c), which is indicative of increase in lipid content On the other hand Hg^{2+} was toxic for the growth of *Scenedesmus* sp at concentrations of 1mM and 2mM in culture media [Fig 4 7 (f)] However, *Scenedesmus* sp could resist mercury concentrations of 0 5mM. From the above findings it can be inferred that *Scenedesmus* sp can be cultured in Pb^{2+} contaminated water bodies for a possible hike in lipid quantum Culture of *Scenedesmus* sp in Cr^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Hg^{2+} contaminated water bodies may not provide productive results with regard to lipid modulation However, these findings must be supported by proper assessment of water quality data and subsequent investigation of the effects of environmental factors on lipid and biomass yield of the respective microalgae Field trials are also a must for proper verification of selected sites (heavy metal contaminated) for production of lipid rich microalgae Besides, heavy metals must be removed from the microalgal oils prior to biodiesel synthesis The presence of phenol, oxidized compounds, phosphatides and heavy metals in oils alter storage life and influences transesterification in the biodiesel process [174]

5.6 Culture of microalgae in PMCS

One of the facets for enhancing the economic viability and sustainability of the microalgal biofuel is undoubtedly large scale open mass culture of microalgae in natural eutrophic abandoned water bodies for reducing the cost of the microalgal culture.

The Brahmaputra (Luit) valley is dotted with numerous water bodies and is highly susceptible to flood. As a result of river processes and annual floods numerous permanently inundated areas are formed, serving no use to man, being wastelands only. Dhemaji district in the north banks of the river is chronically flood affected. Consequently the district harbors numerous permanently inundated areas most of them being eutrophic. Microalgae have ubiquitous growth in nature; consequently it was assumed that these eutrophic water bodies might serve as suitable blooming sites for the direct mass culture of the microalgae. Accordingly, a case study was designed to identify some prospective mass culture sites (PMCS) in Dhemaji district. Three eutrophic water bodies were identified and mapped using satellite images and Google Earth. Ground verification for these sites was done by field visit and subsequent collection of representative water samples to culture the microalgae as against control media to evaluate the microalgal growth kinetics and to investigate its growth feasibility in natural conditions.

A bloom forming microalgal strain later identified as *P. kessleri* was isolated from an algal bloom flourishing in the water drain adjacent to the Department of Molecular Biology and Biotechnology, Tezpur University. The microalgal species showed marked degree of ecological success in stringent aquatic environmental conditions by virtue of its ability to form algal blooms. As a scientific forethought for succeeding investigations it was arguable to expect a high degree of ecological success of this species when subjected to direct culture in permanently inundated water bodies.

Table 4.4 shows the growth kinetics of *P. kessleri* as against control (in BBM culture medium). As expected the growth pattern of *P. kessleri* shows a progressive decline in comparison to the control, when cultured in representative water

samples from PMCS The control sample showed a maximum attainable cell density of 121 6 × 10^6 cells/ml and a specific growth rate of 0 302/day The strain showed its best performance in S1 water samples with a maximum attainable cell density of 45 2 × 10^6 cells/ml and a specific growth rate of 0 255/day High concentration of nitrate (0 4 mg/L) and phosphate (1 447 mg/L) (Table 4 5) in S1 water samples may be the key contributor in this regard The cell density and specific growth rate of *P. kessleri* culture in S2 and S3 water samples were 36 3 × 10^6 and 38 5 × 10^6 cells/ml and 0 272 and 0 275/day The decline in growth pattern of *P. kessleri* in comparison to the control may be due to two following reasons

(i) Absence of nutrients in water samples (excess of NO_3^{2-} and PO_4^{3-} due to permanent inundation), (ii) Possible presence of growth inhibitors in representative water samples (either zooplanktons or certain chemicals) and (iii) The strain was cultured in open laboratory conditions There were variations in the culture environment with regard to temperature, light intensity etc

Fig 4 9 shows the FTIR spectra of the control (*P. kessleri* grown in BBM culture medium) and *P. kesslen* grown in representative water samples viz , S1, S2 and S3 The 1800-1500 cm^{-1} [168] zone demonstrates characteristic bands for proteins, the 1200-900 cm^{-1} zone signifies a sequence of bands due to C-O, C-C, C-O-P and C-O-C stretching vibrations of polysaccharides [172] whereas the CH_2 stretching vibrations from 3100-2800 cm^{-1} implies the presence of lipid [167] A slight variation was observed in the intensity of transmission (in all the four spectra) in the 1800-800 cm^{-1} region This region is specific for proteins and carbohydrates which indicate that minor changes in protein and carbohydrate content occurs when the species is subjected to variable culture environments As evident from FTIR, it is clear that no marked difference was observed in the spectra of the biomass samples This clearly indicates that the species maintains chemical homogeneity (in terms of functional groups) over a wide range of culture environments The species can be cultured both *in-vitro* and *in-vivo* without significant observable changes in its chemical profile

The species *P kessleri* showed a meager growth profile in comparison to the control (Fig 4 8), it showed acceptable degree of ecological success when cultured in

representative water samples from S1, S2 and S3. Our findings suggest that *P. kessleri* may be directly cultured in the aforementioned permanently inundated water bodies for biomass production. However, the experimental findings must be validated by field trials for a comprehensive understanding of direct mass culture of microalgae in such water bodies.

5. 7 Physico-chemical characterization of microalgal biomass

The prospective value of any biomass depends on the physico-chemical properties of the molecules from which it is made. Every biomass feedstock has distinctive properties which determine their performance and reactivity during conversion processes. The present investigation makes an attempt to study the biomass properties of all the investigated microalgal species as several characteristics affects the performance of a biomass fuel including calorific value, moisture content and physico-chemical properties and to evaluate their potential as bio-energy feedstocks for biofuel production.

Table 4.3 shows the properties of *Chlorella* sp., *P. kessleri* and *Scenedesmus* sp. biomass with their average characteristic composition. The moisture content of any biomass varies considerably depending on the type of biomass and its storage. Moisture content is of great significance in proper selection of biomass conversion technology as it influences physical, thermal and mechanical properties of biomass fuels. In general, biomass fuels with low moisture content are more suited for thermal conversion whereas those with elevated moisture content are more suited for biochemical processes such as fermentation conversion [175]. On this basis of moisture content *Chlorella* sp., *P. kessleri* and *Scenedesmus* sp. are potential candidates for direct thermo-chemical conversion.

The high volatile matter content in all the microalgal species strongly influences their combustion behavior and thermal decomposition. The high volatile matter is suggestive of their ideal potential for energy production by pyrolysis and gasification [7]. The ash content (inorganic component) in biomass affects both the

processing and handling costs of overall biomass energy conversion. The ash content in microalgae may vary depending on the species and geographical location.

Determination of elemental composition of biomass is one of the most frequently conducted tests for characterizing biomass. The elemental content of carbon, hydrogen, nitrogen and oxygen in the microalgal biomass were as follows: *Chlorella* sp. (47.54, 7.1, 6.73 and 38.63%), *Scenedesmus* sp. (39.37, 6.12, 5.25 and 49.26%) and *P. kessleri* (45.68, 7.26, 5.85 and 41.21%). The GCV and NCV of the investigated biomass were *Chlorella* sp 18.59 and 15.88 MJ/kg, *Scenedesmus* sp. 15.42 and 13 MJ/kg and *P. kessleri* 17.89 and 15.11 MJ/kg, respectively. The GCV of *Chlorella* sp. was higher than 18 MJ/kg as reported by Illman *et al.* [81] Matsunaga *et al.* [84] reported a calorific value of 6,160 kcal/mg (i.e. 25.8 MJ/kg) for marine *Scenedesmus* sp. strain JPCC GA0024 which was higher than that of the present *Scenedesmus* strain. High lipid content (73%) is the possible reason for higher energy content in *Scenedesmus* sp. strain JPCC GA0024. An increase in calorific value in microalgae is linked to increase in lipid content rather than any change in other cell components such as carbohydrates and proteins [81]. The empirical formulae for *Chlorella* sp., *P. kessleri* and *Scenedesmus* sp. biomass were $C_{8.25}H_{14}NO_{5.02}$, $C_{8.74}H_{16.32}NO_{8.21}$ and $C_{9.1}H_{17.37}NO_{6.16}$, respectively. The H/C and O/C molar ratios calculated from elemental composition for the microalgal biomass were *Chlorella* sp. 1.79 and 0.6, *Scenedesmus* sp. 1.86 and 0.93 and *P. kessleri* 1.9 and 0.67, respectively.

Determination of cell constituents like carbohydrate, protein and lipid is an integral part of biomass characterization. Generally, microalgal biomasses with high lipid content are the usual feedstocks for biodiesel production, whereas those with elevated carbohydrate levels are ideal for bio-alcohol production. In addition to potential fuel valuation, microalgae are also an increasingly attractive option for human food and animal feed. Microalgae have long been recognized as an invaluable source for human nutrition. Ever since millennia man has exploited microalgae as a source of food. Existent markets for microalgae are largely in the sectors of human health food and animal feed owing to high concentrations of long-chain unsaturated fatty acids, such as omega-3 and omega-6 [177, 178]. High concentrations of

carbohydrates and proteins make microalgae an ideal source of nutrients for preparing functional foods, food additives, and even nutraceuticals. Consequently, determination of cell constituent's carbohydrate, protein, and crude fat in microalgae is attractive from nutritional point of view [80]. The content of carbohydrate, protein and lipid for the microalgal biomass were *Chlorella* sp. (19.46, 43.22 and 28.82%), *Scenedesmus* sp. (34.21, 40.56 and 15.3%) and *P. kessleri* (30.46, 41.31 and 16.2%), respectively which is quiet attractive from both fuel and food point of view.

5.8 Fatty acid composition and biodiesel fuel properties of microalgae and yeast

Determination of fatty acid profile is crucial for biodiesel analysis since the properties of the individual esters influences the overall properties of the biodiesel fuel. The fatty acid composition of *Chlorella* sp., *P. kessleri* and *Scenedesmus* sp. biodiesel were determined by GC-FID, whereas, *Scenedesmus* sp biodiesel by GC-MS analysis. For fatty acid analysis methyl esters were prepared by in situ transesterification technique using 25% methanolic 1, 1, 3, 3-Tetramethylguanidine (TMG). TMG was chosen as the reaction catalyst as TMG catalyzed reaction is faster than conc. H_2SO_4 even under mild reaction conditions [179]. Microalgae comprises of both saturated and unsaturated fatty acids (Table 4.6and 4.7). All the tested microalgal biodiesel samples contained C16:0, C18:1 and C18:2 fatty acids, which are generally the major constituents of their fatty acid profiles [80]. Eicasopentanoic acid (C20:5), which is one of commonest PUFA was present as a constituent in *P. kessleri* and *S. cerevisiae* (yeast) oil.

Chlorella sp. and *P. kessleri* biodiesel mostly comprise of oleic acid (C18:1). The oleic acid percentage in *Chlorella* sp. was 65.84% and 77.81% in *P. kessleri*. *Chlorella* sp. biodiesel was mostly composed of unsaturated fatty acids (76.25%). The percentage of unsaturation in *P. kessleri* biodiesel was exceptionally high (96.79%). Microalgal oil generally contains high degree of unsaturation. Gouveia and Oliveira [180] reported the presence of 51.91% of unsaturated fatty acids in *Chlorella vulgaris*, which was significantly than our findings for *Chlorella* species (*Chlorella* sp, 76.25%). The presence of long chain fatty acids C24:1 (*Chlorella* sp.) and C20:5 (*P. kessleri*) are expected to have poor oxidative stability. Biodiesel from

highly unsaturated sources oxidizes more rapidly than conventional diesel, resulting in formation of insoluble sediments which interfere with engine performance, consequently proper percentage of saturated and unsaturated fatty acid is very important for investigating microalgae as a biodiesel feedstock [181]

The most dominant fatty acids in *Scenedesmus* sp biodiesel were C16 2 (29 71%), C16 4(8 69%), C18 0 (9 84%), C18 1 (9 32%), C18 3 (8 02%) and C14 0(10 29%) *Scenedesmus* sp oil had a high proportion of polyunsaturates (57 53%) Algae in general contains high amount of polyunsaturates which may present a stability problem since higher levels of polyunsaturated fatty acids tend to decrease the stability of biodiesel, conversely polyunsaturates also have much lower melting points than monounsaturates or saturates, and as a consequence algal biodiesel should have much better cold weather properties than many other bio-oils [182]

The fatty acids present in *S. cerevisiae* biodiesel were as follows C12 (13 35%), C16 1(28 96%), C16 0 (5 03), C18 2 (2 01), C18 1isomer (18 13), C18 0 (1 76), C20 5 (2 01) and C23 (28 71), respectively Yeast biodiesel is composed primarily of lignoceric acid (C23, 28 71%), palmitoleic acid (C16 1, 28 90%), and oleic acid (C18 1, 18 13%) High percentage of saturated fatty acids and monounsaturated fatty acids were observed in yeast oil This may be considered optimal from a fuel quality stand point in that fuel polymerization during combustion would be substantially less than what would occur with polyunsaturated fatty acid-derived fuel [40]

In general the properties of biodiesel are quiet similar to conventional petrodiesel The most significant properties of biodiesel are cetane number, calorific value, viscosity, oxidative stability, cold flow properties and lubricity [183] The biodiesel densities for each of the tested strains were within the ASTM range (0 86-0 9 kg/L), *S. cerevisiae* biodiesel had the lowest density of 0 870 kg/L whereas, *Scenedesmus* sp the highest density (0 884 kg/L) Determination of kinematic viscosity is crucial for the proper assessment of a biodiesel fuel A higher kinematic viscosity is likely to create engine problems like engine deposits [184] The kinematic viscosities for *Chlorella* sp and *P. kessleri* were well within the ASTM range (3 5-5

mm^2/s) Conversely, *Scenedesmus* sp biodiesel had a lower kinematic viscosity (3 437 mm^2/s) whereas yeast biodiesel had a slightly higher viscosity (5 797 mm^2/s) Unlike the cetane number, viscosity increases with the number of carbon and decreases with the degree of unsaturation [183] The presence of C23 (28 71%) in yeast FAMEs could be attributed to the rise in kinematic viscosity of yeast biodiesel The use of biosurfactants could be helpful in reducing the high viscosity of biodiesel fuels Calorific value for petrodiesel ranges between 39-41 MJ/kg [96], whereas the values for *Chlorella* sp, *Scenedesmus* sp, *P kessleri* and *S cerevisiae* biodiesel were 39 88, 39 24, 39 86 and 40 12 MJ/kg (Table 4 8) which being well within the range for petrodiesel The calorific values of the present microbial biodiesels are in close proximity of previous reports [97] Cetane number (CN) is one of the most important properties of biodiesel It is extensively used to assess the ignition quality of fuels The CN of petrodiesel fuels are within the range of 47 to 51 [185] The CN for *Scenedesmus* sp biodiesel was the lowest (40 96) whereas highest for yeast biodiesel (71 58) [Table 4 8] According to a previous study by Stanshell *et al* [186], the approximated CN for some of the microalgal species were within the range of 39-54 Mostafa and El – gendy [187] reported a CN of 70 for *Spirulina platensis* biodiesel A higher CN results in higher combustion efficiency, improve engine performance, clean up emissions and can be recommended for use in the high speed engine (speeds above 800 rpm), whereas fuels with low CN will cause difficulty in starting, higher noise and exhaust smoke [187] Increase in saturated FAMEs content and its chain length positively enhance the CN of biodiesel [187]

5.9 Microalgal deoiled cake and pyrolysis

Pyrolysis is one of the most promising thermochemical processes which produce solid and volatile products [188] The yield of char was 32% whereas bio-oil was 25% Chemically bio-oil is a complex mixture of water, guaiacols, catechols, syringols, vanillins, furancarboxyaldehydes, isoeugenol, pyrones, acetic acid, formic acid and other carboxylic acids [111] Bio-oils also contain other major group of compounds including hydroxyaldehydes, hydroxyketones, sugars, carboxylic acids and phenolics [111] Due to this chemical complexity of pyrolytic bio-oils their

characterization has been a challenging problem and in general requires the combined use of several analytical techniques, chromatography (GC, HPLC, GPC) and spectroscopy (IR, MS) [111].

In the recent years GC and IR analysis have been extensively used for assaying the chemical composition of bio-oils. But these techniques have major drawbacks in that they cannot provide insights into the overall chemical make-up of bio-oils. In the FTIR spectra [Fig 4.12] the absorption in the range of 3600 to 3200 cm^{-1} corresponds to O-H stretching and is indicative of polymeric involvement of - OH, H_2O and NH_2. The absorption at 1640 cm^{-1} corresponds to C=O stretching in aldehydes, ketones, esters, carboxylic acid etc. Absorption in the range of 950 to 650 cm^{-1} corresponds to C-H in plane bending in aromatic compounds. Spectroscopic techniques like FTIR analysis can only provide qualitative insights (functional groups present in bio-oils). Only 25-40% of bio-oil compounds are observable by GC analysis since a significant fraction of these pyrolytic bio-oils comprise of lignin and carbohydrate oligomers, which are not volatile enough to be observed by GC [189]. Fig. 4.13 shows the TIC of bio-oil whereas Table 4.9 shows the compounds identifiable by GC-MS analysis. The pyrolytic liquids are acidic in nature. Presence of acids in bio-oil is undesirable due to its corrosive nature; however, they could be used as chemical feedstock if separated from bio-oil. Phenols, amides, aliphatic ester, ketones, aromatic hydrocarbons, carboxylic acids, carbonyls, cyano and spiro compounds were determined as the main compounds forming the microalgal pyrolytic bio-oil.

The ^1H NMR spectrum of *P. kessleri* bio-oil is presented in Table 4.10 with the respective integral values of the selected regions of the spectra on percentage basis. The integrated regions were from 0.5-1.5, 1.5-3, 3-4.5, 4.5-6, 6-8.5 and 8.5-10 ppm, respectively. *P. kessleri* bio-oil has high percentage of resonating protons in the integrated region from 1.5-3 ppm [Table 4.10]. A higher aliphatic content is indicative of high energy content in the bio-oil. *P. kessleri* bio-oil had a higher proportion of aromatic ether protons. The integrated region 6-8.5 ppm corresponds to aromatic portion (aromatic carbon from benzene rings and heteroaromatics) of bio-oils. The

integrated region. 8.5-10 ppm corresponds to oxygenated compounds. (aldehydes, ketones, carboxylic acids, esters and amides). A higher proportion of resonating protons for *P. kessleri* bio-oil in this region is suggestive of an increased corrosive nature of *P. kessleri* bio-oil. The presence of oxygenated compounds may be useful for applications involving the synthetic modification of bio-oils and their potential utilization as chemical feedstock.

Chemical stability is a key issue in pyrolysis. It has been previously documented that among the limiting factors affecting pyrolysis are the unknown parameters which effect pyrolytic oil stability [213]. In pyrolysis when biomass is rapidly heated in the absence of oxygen it produces unstable free radical volatiles which quickly condense to form the oil. The products formed do not reach thermodynamic equilibrium and in turn the products react with each other, until product stability is reached [214]. Fahmi *et al.* [214] defined commercial pyrolysis oils from biomass as oil which maintains its chemical and physical properties such as viscosity and stability (which can only be achieved when the oil exhibits high inhomogenity, possessing lower molecular weight compounds).

5.10 Molecular characterization of microalgae

The BLAST result [Table 4.11 (a)] of *Scenedesmus* sp. showed 91% similarity with *Scenedesmus obliquus* isolate IB-05 [NCBI Accession No. JQ782745] with a query coverage of 76%. The BLAST result for the nearest 10 similar organisms with *Scenedesmus* sp. [Table 4.11 (a)] showed their closeness. The microalgal species was deposited in the Genbank database as *Scenedesmus* sp. MPBK-2 partial 18s rRNA gene and assigned the accession No. KF279644. Additionally, the nucleotide sequence of 10 similar organisms from the BLAST result was retrieved from the GenBank database. Their nucleotide sequence was aligned using CLC main workbench [Fig. 4.17 (a)] and the tree created based on neighbor joining method [Fig 4.18 (a)] with bootstrap values. It is evident that all 10 algal species belong to the same cluster [Fig. 4.18 (a)] belonging to *Scenedesmus* with a bootstrap value of 100 and the validation of the BLAST result revealed the algal species as *Scenedesmus* sp. MPBK-2.

The BLAST results of *P. kesslerı* [Table 4 11 (b)] shows a 99% similarity with *Parachlorella kesslerı* [NCBI Accession No FR865655] with a query coverage of 96% The result reports the presence of the nearest 10 similar organism with *P. kesslerı* [Table 4 11 (b)] The algal species *P. kesslerı* was deposited in the Genbank database as *Parachlorella kesslerı* Strain MMPBKK-1 partial 18s rRNA Gene and assigned the accession No KF163441 Additionally, the nucleotide sequence of these 10 similar organisms from the BLAST result was retrieved from the GenBank database Their nucleotide sequence was aligned using CLC main workbench [Fig 4 17 (b)] and a tree created based on neighbor joining method [Fig 4 18 (b)] with bootstrap values From Fig 4 18 (b) it is evident that the algal species belong to the same cluster of *Parachlorella* with a bootstrap value of 100 which is a validation of the BLAST result revealing the algal species as *Parachlorella kesslerı* strain MMPBKK-1

The BLAST results for *Chlorella* sp [Table 4 11 (c)] show a 99% similarity with *Chlorella* sp [NCBI Accession No AF514413] with a query coverage of 98% The BLAST result of the nearest 7 similar organisms with *Chlorella* sp is shown in Table 4 16 (c) *Chlorella* sp was deposited in the Genbank database as *Chlorella* sp strain MP-1 partial 18s rRNA gene and assigned an accession No KJ499988 Nucleotide sequence of these 10 similar organisms from the BLAST result was retrieved from the GenBank database Their nucleotide sequence was aligned using CLC main workbench [Fig 4 17 (c)] and the tree was created based on neighbor joining method [Fig 4 18 (c)] with bootstrap values From Fig 4 18 (c) it is evident that the algal species belongs to the same cluster as that of *Chlorella* with bootstrap value of 87 which is a validation of the BLAST result revealing the algal species to be *Chlorella* sp strain MP-1

5.11 Production and characterization of biodiesel from KCDL feedstock

KCDL feedstock (light brown in color and murky odor) had a density of 2 01 gm/cc, average molecular weight of 825 02 and an acid value of 28 mg KOH/gm However, there may be variations in physical properties of the feedstock which is mainly governed by the product purity and time of exposure of the feedstock to atmospheric oxygen

5.11.1 Two stage transesterification

Special procedures are required for transesterification of fat/oils containing significantly high amount of free fatty acids. Pretreatment processes using strong acid catalyst have been shown to provide good conversion yield and high quality products [190]. One of the common methods to reduce the free fatty acid (FFA) content of a feedstock when present in excess of wt% is a two step process, where acidic pretreatment of the feedstock is first done to lower the FFA followed by homogenous base catalyst based transesterification to produce biodiesel [191-194]. KCDL had an unusually high acid value of 28mg KOH/g and thus biodiesel was produced by a two stage transesterification process. The first stage esterification (acidic) was catalyzed by concentrated H_2SO_4 whereas the second stage (basic) by NaOH.

5.11.2 Elemental composition and calorific value

The elemental composition of carbon, hydrogen, nitrogen and oxygen in the KCDL biodiesel sample were 59.98, 9.51, 0.69 and 29.82%, respectively. The low nitrogen content in the biodiesel sample is important especially with regard to environmental protection. The main difference between biofuels and petroleum feedstocks is the oxygen content. The oxygen content in biofuels ranges from 10 to 45% while petroleum has essentially none, making the chemical properties of biofuels quiet different from those of petrofuels [96]. The Gross calorific value (GCV) and Net calorific value (NCV) for KCDL biodiesel was 36.5 MJ/kg and 34.40 MJ/kg respectively, which is slightly lower than that reported for other biodiesels (39 to 41 MJ/kg [96]. The GCV for KCDL biodiesel was less than that reported for methyl esters (biodiesel) derived from veg fried oil and non-veg fried oil viz., 42.59 MJ/kg and 42.38 MJ/kg, respectively [195].

5.11.3 Response surface methodology (RSM)

The RSM experiment was not carried out for the pre-treatment step (acid catalyzed esterification). Statistical optimization using Box-Behnken design [158]

shows the level of four independent variables [Methanol (χ_1), NaOH (χ_2), Temperature (χ_3) and Time (χ_4)] which were further investigated at three different levels

The contour plots [Fig 4 20] explores the relationship between the variables used i e methanol, NaOH, temperature, and time for biodiesel yield The plot displays the three dimensional relationship in the form of a two dimensional plot

The salient findings of RSM methodology [164]

(i) gradual increase in biodiesel yield with high level of NaOH and temperature (ii) increase in biodiesel yield with increase in the level of methanol and time (iii) increase in yield with increment in the level of NaOH and decrease in yield with increment in time and (iv) decrease in biodiesel yield with increase in the level of methanol and NaOH

For predicting the optimal point, a second order polynomial function was fitted to the experimental results of biodiesel yield

$$Y\ activity = 113.430 + 3.053\ \chi_1 + 55{:}130\ \chi_2 + 0{:}934\ \chi_3 + 1{:}694\ \chi_4 + 0.520\ \chi_1 \chi_2 + 0.007\ \chi_1 \chi_3 + 0.043\ \chi_1 \chi_4 + 0.220\ \chi_2 \chi_3 + 0.425\ \chi_2 \chi_4 + 0.038\ \chi_3 \chi_4$$

Where, χ_1, χ_2, χ_3 and χ_4 are the methanol, NaOH, temperature and time respectively

The regression model (eq 1) fits the experimental data well with a high R^2 (coefficient of determination) value of 90 81% The value of R^2 thus obtained suggests a high degree of correlation between the experimental and the predicted values The closer the value of R^2 to 1, the stronger is the correlation between the measured and the predicted values We found that the calculated coefficient of determination value indicates that the model could explain 90 81% of variability in the response

To determine the optimum condition and to confirm the validity and accuracy of the model, an experiment was performed under the basal and the predicted optimal conditions where biodiesel yield was monitored against the calculated data

from the model The optimized values were 40 08 for methanol (χ_1), 1 86 for NaOH (χ_2), 52 for temperature (χ_3) and 100 for time (χ_4) from the response surface optimizer The estimated biodiesel yield was 82% compared to 80% of the predicted value from the polynomial model Thus, it points out the accuracy of the model with more than 97 56 % which is an evidence for the model validation However, this variance of biodiesel yield might be because of slight variation in the experimental conditions Statistical optimization was applied to find out the optimal biodiesel yield and found to be an effective statistical technique RSM can be quiet helpful in design of experiments for biodiesel production and allied approaches

5.11.4 FAME composition analysis

The FAME's (Table 4 13) were identified by GCMS analysis following comparison of their mass spectra with those in NIST library The unsaturated and saturated FAME in KCDL biodiesel were 58 14 and 41 86% respectively High degree of saturation is likely to make the biodiesel fuel more stable The average molecular weight of KCDL methyl esters was 276 34

5.11.5 FTIR analysis

A marked degree of similarity was observed in the FTIR spectra of KCDL feedstock and the biodiesel (Figure 4 21) owing to the presence of the same chemical groups The IR spectra of the biodiesel sample shows a C=O stretching band of methyl esters at 1748 cm^{-1} and C-O bands at 1130 and 1196 cm^{-1} [196] The aliphatic hydrogen at 2928 cm^{-1} and 2856 cm^{-1} are indicative that the main components of KCDL biodiesel are long carbon chains [197] C-H deformations in the range of 1500–1300 cm^{-1} further verify the presence of long carbon chains [198]

5.11.6 Fuel properties of KCDL biodiesel

The chemical composition of biodiesel feedstocks is different and consequently variations in the fuel properties are a must The high viscosity (8 7 mm^2/s) of KCDL biodiesel (Table 4 14) in contrast to others like waste cooking oil (4 89 @40°C, mm^2/s [199], methyl esters of fried oil (5 11 @40°C, mm^2/s) and methyl esters of non-veg fried oil (5 81 @40°C, mm^2/s [199], is the major bottleneck for its

practical usability in the diesel engine. As a suggested measure, the use of biosurfactants (environmentally benign in contrast to chemical surfactants) may be used to reduce the high viscosity of KCDL biodiesel.

5.12 Antimicrobial assay of bio-oil from microalgal deoiled cake

There is paucity of scientific information pertaining to the antimicrobial activity of bio-oils. Only a few reports regarding antifungal activity of bio-oil is available till date [200, 201]. The prokaryotic system included *Staphylococcus aureus* (MTCC96) and *Escherichia coli* (MTCC723), whereas the eukaryotic system included *Candida albicans* (ATCC 183) and *Saccharomyces cerevisiae* (ATCC 4126) as the test organisms for the antimicrobial assays. The ZOI of BPCDC [Fig. 4.23] extract was compared with the standard antibiotic chloramphenicol for antibacterial and Indofil M-45 (commercial antifungal) for antifungal and antiyeast assays.

BPCDC showed varying degrees of antimicrobial activity against all the tested microbial strains. No, antiyeast and antifungal activity were shown by BPCDC [Fig. 4.23]. However, BPCDC showed moderate inhibition zone (12mm) against *E. coli* and *S. aureus*. MIC of BPCDC at varying concentrations was determined against both prokaryotic and eukaryotic systems. BPCDC was found to be effective only against prokaryotes, *E. coli* (25µg/ml) and *S. aureus* (12.5µg/ml).

Antimicrobial activity is conferred by the presence of bioactive agents. The antibacterial activity of BPCDC may be due to the presence of carboxyl and phenolic groups detected by FTIR and GC-MS. Chemically, bio-oil is a complex mixture of water, guaiacols, syringols, furancarboxyaldehydes, pyrones, isoeugenol, vanillins, catechols, acetic acid, formic acid and other carboxylic acids [111]. Bio-oils also contain other major group of compounds including hydroxyaldehydes, hydroxyketones, sugars, carboxylic acid and phenols [202]. Looking into the chemical complexity it is quiet arguable to except a diversity of bioactive agents in various bio-oils which might lead to the development of new pharmaceuticals. The antimicrobial assays suggest that bio-oil from the different plant and algal feedstocks which remain

grossly unexplored should be evaluated for antimicrobial activity against phytopathogens and multidrug resistant bacteria (MDR)

5.13 Antioxidant analysis of microalgal deoiled cake

Free radical scavenging potential of the microalgal deoiled cake was done as a part of waste valorization The antioxidant properties of the sample extracts were assayed by % DPPH scavenging and FRAP assay Among all the tested extracts (aqueous, ethanol and methanol), the aqueous extract of all the tested samples showed the highest % DPPH scavenging potential [Table 4 15 (a)] The higher antioxidant activity of the aqueous extracts can be attributed to the presence of high phenolic compounds in the extract In the previous reports the antioxidant activity shown by plant extracts is due to the presence of flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins [203] As evident from Fig 4 24 (a), (b) and (c) a positive correlation [R^2= 0 7390 *Chlorella* sp deoiled cake], R^2 = 0 5580 [*Scenedesmus* sp deoiled cake], R^2 = 0 5110 [*P. kessleri* deoiled cake] between the total phenolic contents and % DPPH scavenging potential of all the sample extracts further support the experimental findings The positive linear correlation is indicative of the importance of phenolic compounds in the antioxidant behavior as evident from the DPPH radical scavenging potential of the tested sample extracts

The FRAP activity of the aqueous and alcoholic extracts [Table 4 15 (b)] of the three test samples shows that the methanolic extract of the sample *Scenedesmus* sp deoiled cake has the highest FRAP activity Correlation study between the total phenolic content and FRAP activity of the samples was done to establish the role of phenolic compounds present in the sample extracts in reducing the ferric ions and thus acting as reducing agents However, as evident from the correlation studies [Fig 4 25 (a), (b) and (c)], the negative correlation R^2= - 6 41 (*Chlorella* sp deoiled cake), R^2 = - 9 55 (*Scenedesmus* sp deoiled cake), R^2 = - 9 19 (*P. kessleri* deoiled cake) between the total phenolic content and FRAP activity of the extracts suggests that the phenolic compounds present in the extracts are not acting as reducing agents in this case Lower or negative correlation between TPC and FRAP activity can also be explained by the fact that Folin–Ciocalteu reaction used

for the estimation of TPC is based on the redox reactions [204]. The assay not only detects polyphenolic compounds, but also other biological substances like amino acids, carbohydrates and ascorbic acid which are reactive towards Folin–Ciocalteu reagent [204-206]. According to the previous reports the antioxidant activity of the sample extracts is not always limited to phenolics [207]. Other antioxidant secondary metabolites such as volatile oils, carotenoids, and vitamins can also show the reducing activity. The reducing power as evident from the FRAP assay indicates that the antioxidant secondary metabolites can act as the electron donors and thus reduce the oxidized intermediates to a more stable form [208]. The resultant difference in activity of the tested assays (DPPH and FRAP) can be attributed to the difference in reaction mechanisms and the different reaction conditions.

The findings relating to the free radical scavenging and ferric ion reducing potential of the respective microalgal de-oiled cakes can serve as an incremental step in the identification of active antioxidant components present in the samples and their subsequent appraisal of commercial prospects.

5.14 Molecular docking

Molecular docking simulation was carried out against Cytochrome C peroxidase of *S. cerevisiae* (PDB ID: 1AC4) [top scores shown in Table 4.16 (a)], ribonucleotide reductase R2 of *E. coli* (PDB ID: 1AV8) [Top scores shown in Table 4.16 (b)], Sortase A of *S. aureus* (PDB ID: 1T2P) [Top scores shown in Table 4.16 (c)], secreted aspartic proteinase of *C. albicans* (PDB ID: 1ZAP) [top scores shown in Table 4.16 (d)], N-terminal domain of YaeT of *E. coli* (PDB ID: 2QDF) [top scores shown in Table 4.16 (e)], enoyl reductase of *E. coli* K-12 (PDB ID: 4JQC)) [top scores shown in Table 4.16 (f)] . Rerank scoring was done so as to provide an estimate of the strength of the protein ligand complex interaction (it is not calculated in chemical units and does not take entropy into account). The snaps of only the top docking hits of each enzyme are presented. The molecular docking results revealed that the compounds possess strong molecular interactions and hydrogen bonding interaction as mandated by the hydrogen bonding scores [Table 4.16 (a-f)]. Moreover, the compounds inhibit most of the bacterial proteins. The ligand-protein interaction

[Fig. 4.26 (a-f)] was also exhibited for the bacterial enzymes. Additionally, from the docking scores, the compound UNK0-TYR1 (MolDock score of -73.7242, Rerank score of -21.6237 and HBond -6.16179) exhibited the top docking hits against the bacterial proteins used in the investigation. As evident from the rerank scores and hydrogen bonding scores it is quite evident that the compounds inhibited the bacterial and fungal enzymes PDB ID: 1AC4 (cytochrome c peroxidase of *S. cerevisiae*), 1AV8 (ribonucleotide reductase R2 of *E. coli*), 1T2P (sortase A of *Staphylococcus aureus*), 1ZAP (aspartic proteinase of *C. albicans*), 2QDF (YaeT of *E. coli*) and 4JQC (enoyl reductase of *E. coli* K-12).

Chapter 6

Conclusion and future prospects

Assam, India with its unique climatic profile encourages and facilitates the ubiquitous growth of numerous species of microalgae. The atmosphere is packed with high humidity, has an alteration of summer and winter of which neither is extreme. A strong correlation seems to exist between the high humidity and temperature with the prevalence of microalgal vegetation in the region. The fresh water ecosystem of the state is the natural abode for countless species of microalgae (Chlorophyceae, Bacillariophyceae and Cyanophyceae) which have hitherto remained unexploited.

The present investigation explores the feasibility of biodiesel production from the indigenously isolated microalgal strains. Economic evaluation or feasibility studies of fresh water microalgae for biodiesel production are unlikely to make economic sense if the focus is on the final product. As such the study also takes into account the feasibility of production of bio-oil from the microalgal deoiled cakes (low value biomass refuse) which are otherwise used as aquaculture feeds. The non energy prospects (antimicrobial and antioxidant properties) from microalgae are also taken into consideration in the present study as a part of value addition to the microalgal biomass. Finally, the study also reports the practicability of biodiesel production from yeast (*S. cerevisiae*) and kitchen chimney dump lard (KCDL) as reference to biodiesel production from microalgae.

6.1 Conclusion

The following conclusions were drawn from the present investigation:

- High biomass yield, attractive biochemical profile and high energy content in the microalgal strains namely *Chlorella* spp. KJ499988, *Scenedesmus* spp. KF279644 and *Parachlorella kessleri* KF163441 offers strong candidature as bioenergy feedstocks.

- Culture of *P. kessleri* KF163441 in representative water samples from PMCS suggested the practicability of mass culture of *P. kessleri* in permanently inundated water bodies which are otherwise considered as wastelands.
- *Chlorella* spp. KJ499988 biomass could be used as feedstock for bio and thermochemical conversions, whereas the deoiled cake for thermochemical conversion.
- The fuel properties of microalgal biodiesel such as density, calorific value and cetane number were within ASTM ranges.
- Biodiesel from yeast was superior to microalgal biodiesel with regard to calorific value and cetane number.
- *P. kessleri* KF163441 deoiled cake could be directly used as a feedstock for bio-oil production.
- Production of biodiesel from KCDL is feasible. The application of RSM is quite helpful in designing conversions for biodiesel production.
- Bio-oil from *P. kessleri* KF163441 deoiled cake was found to be moderately effective against the prokaryotic system, whereas completely ineffective against eukaryotic system. The study suggests that microalgal bio-oil offer prospective applicability as a bioactive agent besides being an increasingly attractive fuel option. The study also suggests that bio-oil from the different algal feedstocks can be evaluated for antimicrobial activity against multidrug resistant bacteria (MDR) and phytopathogens.
- Free radical scavenging potential of the aqueous extracts of *Chlorella* spp. KJ499988, *Scenedesmus* spp. KF279644 and *Parachlorella kessleri* KF163441deoiled cakes was 53.20, 40.75 and 31.75% respectively.
- Molecular docking studies revealed that the compounds present in *P. kessleri* bio-oil inhibit the bacterial and fungal enzymes (PDB ID: 1AC4, 1AV8, 1T2P, 1ZAP, 2QDF, 4JQC).

6.2 Future prospects

In the context of the present investigation the following research may be undertaken to improve the prospects of microalgae:

- Genome sequencing of microalgal strains would enable comprehensive understanding of nucleotide sequences for the novel genes, proteins and important metabolites of commercial interests.
- Identification of suitable stress triggers for lipid modulation in microalgae will lead to better understanding of biofuel productivity.
- Process parameters for biodiesel production needs to be optimized.
- Co-culture of the strains and assessment of their feasibility as bioenergy feedstock needs to be initiated.
- Microalgal culturing in raceways and tubular photobioreactors needs to be initiated for comparative analysis of their capabilities with regard to biomass production.
- There is a need to assess the prospect of omega-3-fatty acid production from the microalgal strains

The proposed future work has direct biorefinery applications. The choice of proper microalgal strains for biofuel production, combined with cheap methods of culturing, following optimization of growth parameters, in conjugation with co-product generation should lead to rich dividends in biorefining endeavors.

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