

APPLICATION OF LIPID EXTRACTED ALGAE IN FEED AND ENERGY PRODUCTION

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Declaration by student

Application of lipid extracted algae in feed and energy production

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I confirm that this thesis is composed of my original work and contains no material previously submitted to the Durban University of Technology or any other institution for academic qualifications. The content of my thesis consists of work I have carried out since the commencement of my Ph.D. studies.

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I hereby approve the final submission of the following thesis.



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SUMMARY

Microalgae are well considered to be promising feedstocks for biodiesel production. Microalgae can be grown under different types of cultivation conditions and their biomass has tremendous potential to be used as biofuel feedstock and for other applications such as feed, food, cosmetics, pharmaceutical etc. Despite the many benefits and the significant development in the field of microalgal biodiesel production, there are several challenges including high cultivation cost and developing efficient downstream processing methods. The biomass production cost is high, which significantly hinders the use of microalgae as a feedstock. Most of the available literature is focused on upstream, single strain and single product strategy, where mainly algal lipids are used for biofuel production. Hence, for improving the sustainability of the algal biofuel production processes and related process economics, a multiple applications approach using integrated biorefinery and exploiting microalgae for environmental benefits is required.

To explore the microalgal biorefinery concept it is vital to understand the various cultivation conditions and applications of biomass in different sectors. There are various strategies, which have potential to make algal biofuel technologies more economically feasible and environmentally sustainable. Use of alternative culture media, improving the biomass production and the efficiency of downstream processing (drying, cell disruption, lipid extraction etc.) algal biofuel technology economical. Utilizing lipid-extracted algae (LEA) for energy and aqua feed application will maximize overall economic return and will leave minimal residues as by-product.

The major focus of this thesis was to utilize LEA as substrate for biomethane production and protein source in aquaculture feed. However, effect of preceding steps such as microalgae cultivation, biomass drying and cell disruption on major metabolites extraction was also studied. Microalgae were cultivated in different medium (domestic wastewater and BG11) and their

biomass yields and biochemical composition (lipid, protein and carbohydrate) were compared. Different drying and cell disruption techniques were employed for lipid extraction and their effect on lipid, protein and carbohydrate yields were evaluated. The yield of major metabolites on whole cell and LEA were also compared. Suitable solvent systems were selected for optimum lipid extraction from wet and dry biomass with minimal toxic effect on LEA metabolites so that LEA can be further used for biomethane and aquaculture feed production.

The choice of microalgae at large scale depends upon the number of factors such as their adaptability to large-scale cultivation, biomass production, major metabolites content, robustness towards the open system cultivation and contamination. In this study, *S. obliquus* and *C. sorokiniana* were cultivated in wastewater and BG11 medium at laboratory scale. Both strains are indigenous to KwaZulu-Natal. *C. sorokiniana* showed lower biomass and major metabolites (lipid, protein and carbohydrate) production at large scale compared to *S. obliquus*. Considering better adaptability to open cultivation, high biomass and metabolites yields, *S. obliquus* strain was selected for the LEA application study.

Microalgae species, *C. sorokiniana* and *S. obliquus* were cultivated on BG11 and using different ratios of raw domestic wastewater and post-chlorinated wastewater as nutrient media. The cultivation of *S. obliquus* and *C. sorokiniana* showed biomass yield of 1.2-3.5 and 0.78-1.8 g L⁻¹ in BG11 medium, respectively. While biomass yield observed in wastewater was 0.59-1.59 g L⁻¹ for *S. obliquus* and 0.67-1.45 g L⁻¹ for *C. sorokiniana*. The higher biomass yield in BG11 medium attributed to the higher nutrient contents in this medium compared to wastewater. The lipid contents for *S. obliquus* and *C. sorokiniana* were 20 and 16.5% dry cell weight (DCW), respectively when grown using BG11 medium. While increases in lipid contents of 26.25 and 29.4% DCW were found for *S. obliquus* and *C. sorokiniana*, respectively when cultivated using

wastewater. Similarly, carbohydrate contents for *S. obliquus* and *C. sorokiniana* were 18 and 17% DCW, respectively for BG11 medium. Increased in carbohydrate contents of 25% for *S. obliquus*, 28.4% DCW for *C. sorokiniana* were observed for wastewater. Microalgae tend to accumulate more lipids and/or carbohydrates under nutrient stress condition. The nitrogen and phosphorus contents in wastewater are lower than BG11 medium, which were responsible for stressed condition for microalgae. With limited nutrients in wastewater compared to BG11 medium, growth of microalgae is also lower which resulted in lower protein content. Protein content for *S. obliquus* and *C. sorokiniana* in BG11 medium were 37.83-48.8 and 25-35.3% DCW, respectively. The protein contents for *S. obliquus* and *C. sorokiniana* in wastewater medium were 16.4-27.29 and 15.8-27.3% DCW, respectively. The biochemical composition depends upon the nutrient composition of the medium and cultivation conditions.

The two selected microalgae have shown potential for nutrient removal while cultivated in wastewater. The removal efficiency by *S. obliquus* was found to be 76.13% for COD, 98.54% for nitrogen and 97.99% for phosphate. Microalgae *C. sorokiniana* cultivation in wastewater removed 69.38% COD, 86.93% nitrogen and 68.24% phosphates. Increased lipid accumulation in the cells was also recorded in stressed conditions due to low nutrient availability from wastewater.

After harvesting of microalgae from culture media, the water content in thick algal slurry (>85% DCW) lowers the products recovery. To overcome this challenge drying and cell disruption are required to enhance the efficiency of lipid extraction. Where drying and cell disruption increase the viability of biomass for lipid extraction process.

Three biomass-drying techniques viz. sun, oven and freeze-drying and four-cell disruption techniques viz. microwave, sonication, osmotic shock and autoclave disruption were studied for their effect on recovery of major metabolites from *S. obliquus*. Microalgae metabolites recovery

from whole cell and LEA were analysed and compared. The results showed that after lipid extraction, LEA still contained comparable protein to whole algae biomass however, the carbohydrate concentration was reduced. Oven drying exhibited the highest recovery of all the major metabolites followed by freeze-drying; sun drying however, showed lower yields. Despite lower metabolites recovery sun-drying technique is preferable at large scale due to its easy application and cost-effective nature. The main drawback of sun drying technique is weather dependence and required longer period to dry.

The microwave and autoclave microalgal cell disruption improved the lipid yield but loss of other compounds was observed. In osmotic shock treatment, due to poor cell disruption efficiency low lipid were obtained and comparably lower protein loss was noticed during lipid extraction.

Lipid extraction is crucial step for microalgae biodiesel production. Solvent-assisted lipid extraction is widely used technique for lipid recovery from dry or wet algae biomass. In a biorefinery approach, it is vital to choose appropriate solvents for the optimum lipid extraction whilst having minimal effect on the remaining metabolites (protein and carbohydrates) in LEA. LEA could be used for energy generation or aquaculture feed applications.

Six commonly used organic solvents/ solvent systems were used for lipid extraction from wet and dry biomass. The results showed that the lipid extraction efficiency depends strongly on types of biomass as well as solvent systems selected. Lipid extraction from wet algal biomass could reduce the processing steps and save energy incurred in drying. However, the water present in wet algal slurry acts as a barrier, which results in lower lipid yield compared to the dry biomass. The results revealed that among all six-selected solvents, chloroform: ethanol (1:1 v/v) was most effective if wet biomass used specifically for lipid purpose only. To explore the biorefinery concept, isopropanol/hexane composition is the most suitable solvent system because it is less toxic and

resulted in high protein (20.07% DCW) and carbohydrate (22.87%) yields in LEA. For dry algal biomass, chloroform: methanol (2:1 v/v) is an appropriate solvent system if biomass used especially for lipid (19.25%) extraction. If LEA to be used for energy and/or aquaculture feed application, DCM: methanol was found to be a suitable solvent system, which gave 32.79% protein and 26.92% carbohydrate yield. Comparatively hexane has lower lipid recovery but shown higher protein and carbohydrate yield in LEA. Due to less toxic, easy to scale up and inexpensive, hexane is preferable as a solvent for lipid extraction if LEA is to be further utilized at large scale for energy or feed application.

Anaerobic digestion (AD) of organic residues is well-researched technology for biomethane production. Whole microalgae and LEA has promising potential for biomethane production. The anaerobic sludge used as inoculum for microalgal biomass digestion. Biomethane production from whole algae and products extracted algae highly depends on sludge to algae biomass ratio for higher methane production. The extraction of metabolites also changes the biochemical composition of residual biomass, which can affect the biomethane production. It is vital to understand the effect of various product-extracted algae and as well as pre-treated algae on the biochemical methane potential.

In order to compare biomethane potential, four types of biomass were selected namely sun dried powder algae (SDPA), mild heat-treated algae (MHTA), LEA (using hexane as lipid extracting solvent) and protein-extracted algae (PEA). The average methane (CH_4) production rate was ~ 2.5 times higher for protein and lipid extracted algae than for whole algae SDPA and MHTA whilst the cumulative CH_4 production was higher for pre-treated algae. Highest cumulative CH_4 production ($318.7\text{ mL CH}_4\text{ g}^{-1}\text{ VS}$) was found for MHTA followed by SDPA ($307.4\text{ mL CH}_4\text{ g}^{-1}\text{ VS}$). The CH_4/CO_2 ratios of 1.5 and 0.7 were observed for MHTA and LEA, respectively. Outcome

of this objective revealed that pre-treatment process disrupts the microalgae cell walls, exposing intracellular material and increasing the surface area. The product-extracted algae changes the elemental composition, which decreases the cumulative gas yield CH_4/CO_2 ratio. Presence of high nitrogen in the form of protein produces ammonia (NH_3) which inhibits the methane production. Therefore, it is imperative to use PEA biomass to improve the methane production yield than the whole cell biomass.

Due to escalating price and unstable supply of fish meal (FM), alternative protein sources are used in aqua feed, however these sources do not meet to the requirement. The use of less expensive protein source in aquaculture feed as alternative to FM is required. Microalgae are primary producers in the food chain as well as a natural food for fish. Microalgal biomass is comprised of proteins, lipids, carbohydrates, pigments and many other bioactive compounds. The microalgal proteins have an appropriate balance of all essential amino acids, while lipids are rich in polyunsaturated fatty acids (omega-3 fatty acids, EPA, DHA). Whole algae contain all required ingredients while LEA also contain protein, carbohydrates, vitamins, bioactive compounds even though most of the lipid soluble nutrients have been removed. Thus, microalgae have promising potential to be used in aquaculture feed. Aquaculture production continues to increase globally, to meet the aquaculture feed demand algae supplemented aquaculture feed will play an important role in providing good quality fish.

In this study, approximately 200 kg of microalgal biomass was harvested for the feed application. Due to lower toxicity, ease of availability and ease of recovery from mixture, hexane was used as a lipid extracting solvent at pilot scale to generate LEA. The 44 weeks (from juvenile to finisher stage) feeding trials were conducted to evaluate the effect of whole and LEA supplementation of *S. obliquus* strain on growth performance, disease tolerance, feed utilization, physiological

activity, and fillet biochemical composition of Nile tilapia (*Oreochromis niloticus*). In the first trial, fish were fed with an algae free diet (control) and four experimental diets (2.5, 5, 7.5 and 10 wt%) as protein source of dried *S. obliquus*. The study showed that microalgae could be used as a protein supplement in the Tilapia feed for enhancement of morphological characteristics and nutritional value. The 7.5% and 10% supplementation of whole algal biomass in tilapia feed showed significant improvement in weight and length of the fish compared to the control. The daily body weight gain was 0.25 g higher in experimental groups than the control. The hepatosomatic index percentage was also higher in fish feed when 7.5% whole algae was used in fish feed as a protein source. The results also showed that 7.5% and 10% have better specific growth rate (1.57 and 1.5%), daily body weight gain (1.1 and 0.86 g), overall body weight gain (427.16 and 331.48 g), protein assimilation (43.96 and 40.46%) higher than the control diet fed fish. The survival rate of fish were 100% at every inclusion level. In second trial (44 weeks), two supplementations (7.5 and 10 wt%) of LEA as protein source were used in Nile tilapia diets. Results showed 7.5% and 10% LEA supplemented feed shown better growth performance than control. The protein content were 42.2%, 41.3% and 36.1% in tilapia fed with 7.5%, 10% LEA and control feed, respectively. The body weight gain, tilapia fed with 7.5% LEA shown 357 g while 10% LEA and control have 331.78 g, and 330.08 gm, respectively. The application of whole and LEA of *S. obliquus* in tilapia feed, shown appropriate supplementation level for tilapia feed at demonstration scale.

This thesis presents advances in knowledge in the field of microalgae biorefinery research for pilot scale operations. This research work has covered various aspects such as effect of drying, cell disruption and lipid extraction on whole and LEA metabolites yield. The extraction of lipid from wet and dry microalgal biomass using various solvent systems provides a new insight for the

selection of appropriate solvent systems, which can be used for the large-scale lipid extraction. The study on LEA for biomethane production enhances the understanding about the effects of different pre-treatments and product extractions on biomethane production. The results revealed that the supplementation of whole cell and LEA using *S. obliquus* for tilapia feed is safe therefore, can be used as an alternative protein source. The findings of this study have both academic and industrial value.

Dedication

*This thesis is dedicated to my beloved
family specially my mother, father, Wife
and son Ashaz Ahmad*

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My experience as a Ph.D. student at Durban University of Technology has been more than pleasurable. Whilst this thesis has a single author, the work involved a great many people, without whom I would not have made it this far. In this section, I have stated as many names of those who have contributed to this work as possible. The many people who have given me emotional support throughout not only the Ph.D. years, but which came before it, I give my deepest thanks.

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LIST OF PUBLICATIONS

This thesis is based on the following manuscripts that have been published. They are referred to in the text by their Roman numerals.

- I. SANJAY KUMAR GUPTA, **FAIZ AHMAD ANSARI**, AMRITANSHU SHRIWASTAV, NARENDRA KUMAR SAHOO, ISMAIL RAWAT and FAIZAL BUX (2016). Dual role of *Chlorella sorokiniana* and *Scenedesmus obliquus* for comprehensive wastewater treatment and biomass production for biofuels. Journal of Cleaner Production. 15, 255-264. DOI:10.1016/j.jclepro.2015.12.040
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- III. **FAIZ AHMAD ANSARI**, SANJAY KUMAR GUPTA, AMRITANSHU SHRIWASTAV, ABHISHEK GULDHE, ISMAIL RAWAT and FAIZAL BUX (2017). Evaluation of various solvent systems for lipid extraction from wet microalgal biomass and its effects on primary metabolites of lipid-extracted biomass. 1-9. DOI:10.1007/s11356-017-9040-3
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- V. **FAIZ AHMAD ANSARI**, ABHISHEK GULDHE, SANJAY KUMAR GUPTA, ISMAIL RAWAT and FAIZAL BUX. Improving the feasibility of aquaculture feed by using microalgae (Ready to submit).

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CONFERENCE PRESENTATIONS

- I. **FAIZ AHMAD ANSARI**, POONAM SINGH, ABHISHEK GULDHE and FAIZAL BUX. Microalgal cultivation using aquaculture wastewater: A biorefinery approach. *Oral Presentation at 2nd International Conference on Alternative Fuels and Energy: Future and Challenges – ICAFE 2017*, Daegu, South Korea. 23-25 October 2017.
- II. **FAIZ AHMAD ANSARI**, ISMAIL RAWAT, ABHISHEK GULDHE and FAIZAL BUX. Enhanced Tilapia production using algal biomass. *Oral presentation at 2nd International Peri-Urban Conference*. Cape Town, South Africa. 26-30 November 2017.

LIST OF ABBREVIATIONS

AD	Anaerobic digestion
ADC	Apparent digestibility coefficient
ALA	α -Linolenic acid
AWW	Aquaculture wastewater
BG11	Blue green algae 11
BOD	Biological oxygen demand
COD	Chemical oxygen demand
CSM	Canola seed meal
DCW	Dry cell weight
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAO	Food and agricultural organization
FM	Fishmeal
FO	Fish oil
LEA	Lipid extracted algae
MHTA	Mild heat-treated algae
PCW	Post-chlorinated wastewater
PEA	Protein extracted algae
SAR	Sludge to algae ratio
SDPA	Sun dried algae
WW	Wastewater

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1.0 INTRODUCTION

1.1 General introduction

Energy security, increasing greenhouse gas emissions and increasing oil prices are among the main reasons for finding alternate and renewable energy resources. Globally carbon emissions are likely to increase from the current 404 ppm of atmospheric CO₂, which will create severe challenges (Wang *et al.*, 2016). The main reasons for the likely increase are rapid increase in the world's population, rapid industrialization, increasing demand of transportation etc. (Faried *et al.*, 2017). Biofuels produced from renewable feedstocks, have the potential to reduce greenhouse gas emissions. There is ample demand for renewable feedstocks for biofuels as alternatives to the fossil fuels (Chisti, 2007; Mata *et al.*, 2010). Biodiesel (mono-alkyl esters of long fatty acids chain) has potential as transportation fuel. Biodiesel feedstocks can be categorized into three generations (i) first generation biodiesel derived from edible oils (ii) second generation biodiesel generated mainly from non-edible oils e.g. *jatropha* oil, waste frying oil etc. (iii) third generation biodiesel produced from microalgae (Faried *et al.*; 2017; Shah *et al.*, 2018a; Singh *et al.*, 2014). Microalgal biodiesel has emerged as an environmental friendly alternative to the existing fossil fuels.

Despite several advantages of microalgae as potential renewable feedstocks for biodiesel production, biodiesel from microalgae still does not represent a significant share in worldwide liquid fuel supply (Park & Lee, 2016). Due to many challenges to commercial production of microalgae biodiesel which span from large-scale cultivation to biodiesel production (Fazal *et al.*, 2018). There are many challenges in downstream processes like drying, cell disruption, lipid extraction and conversion of lipids to biodiesel which makes biodiesel uneconomical (Rashid *et al.*, 2014; Sharma *et al.*, 2016; Tan *et al.*, 2018). Drying and cell disruption steps are crucial for

biodiesel production (Guldhe *et al.*, 2014). The drying and lipid extraction collectively accounts for 50-90% of the overall energy consumption (Wang *et al.*, 2013). Thus, selection of simple and efficient cell drying and disruption techniques for optimal lipid recovery are the key steps towards sustainable biodiesel production (Prabakaran & Ravindran, 2011). The drying process removes the water from microalgae biomass. Presence of water in algae cells result in the formation of water films, which reduce mass transfer and form emulsions, which further hamper the efficiency of lipid extraction from wet biomass (Dong *et al.*, 2016; Taher *et al.*, 2014).

The major practical limitations for the algal biofuel industry are high cost of cultivation and application of technology for a single purpose especially for biodiesel (Karemore & Sen, 2016). Wide ranges of microalgae biodiesel prices (1.2- 20 USD per L) have been reported which indicate that algal biodiesel is not cost competitive to fossil fuels (Park & Lee, 2016). Employing current production process for biodiesel, estimated cost of a barrel to be US\$ 300-2600, which is much higher than a barrel of petrol (Mathimami & Mallick, 2018).

Much of the recent research work has been focused on upstream processes of isolation and screening of oleaginous microalgae, cultivation and nutrient optimization for high lipid accumulation for biodiesel production (Guldhe *et al.*, 2016; Narayanan *et al.*, 2018; Sahoo *et al.*, 2017; Singh *et al.*, 2016). Very few studies have reported on systematic downstream processing on microalgal biomass and efficient use of microalgae biomass after lipid extraction for energy and feed application. The utilization of LEA has potential to produce additional revenue, which will potentially make biodiesel production more cost effective.

The production of 1 kg of microalgal biodiesel produces approximately 2.4 kg of LEA and the price of LEA is reported to be between 100- 225 USD per ton (Bryant *et al.*, 2012). It has been

also estimated that 3.6 to 4.5 million metric tons of LEA could be produced from approximately 4 billion liters of algal biofuels produced (Mirsiaghi, 2016). The LEA could be the feedstock for different products including feed and energy production. The appropriate application of LEA can provide significant benefits towards improving microalgae biodiesel cost and reducing environmental impacts. Shifting the focus from a single product strategy (biodiesel production) to multiproduct strategies have promising potential to improve algal biofuel technology.

For this approach to biodiesel production, sufficient lipid yield after different downstream processing and minimal deteriorating effects on LEA are important in order to establish the feasibility of using LEA as feed or energy substrates. Different downstream processing methods significantly affect the lipid recovery from whole algae and LEA metabolites. Cultivation of microalgae using cheap nutrient sources and effect of various down processing like drying, cell disruption, solvent selection for lipid extraction and subsequent application of LEA needs more attention from researchers.

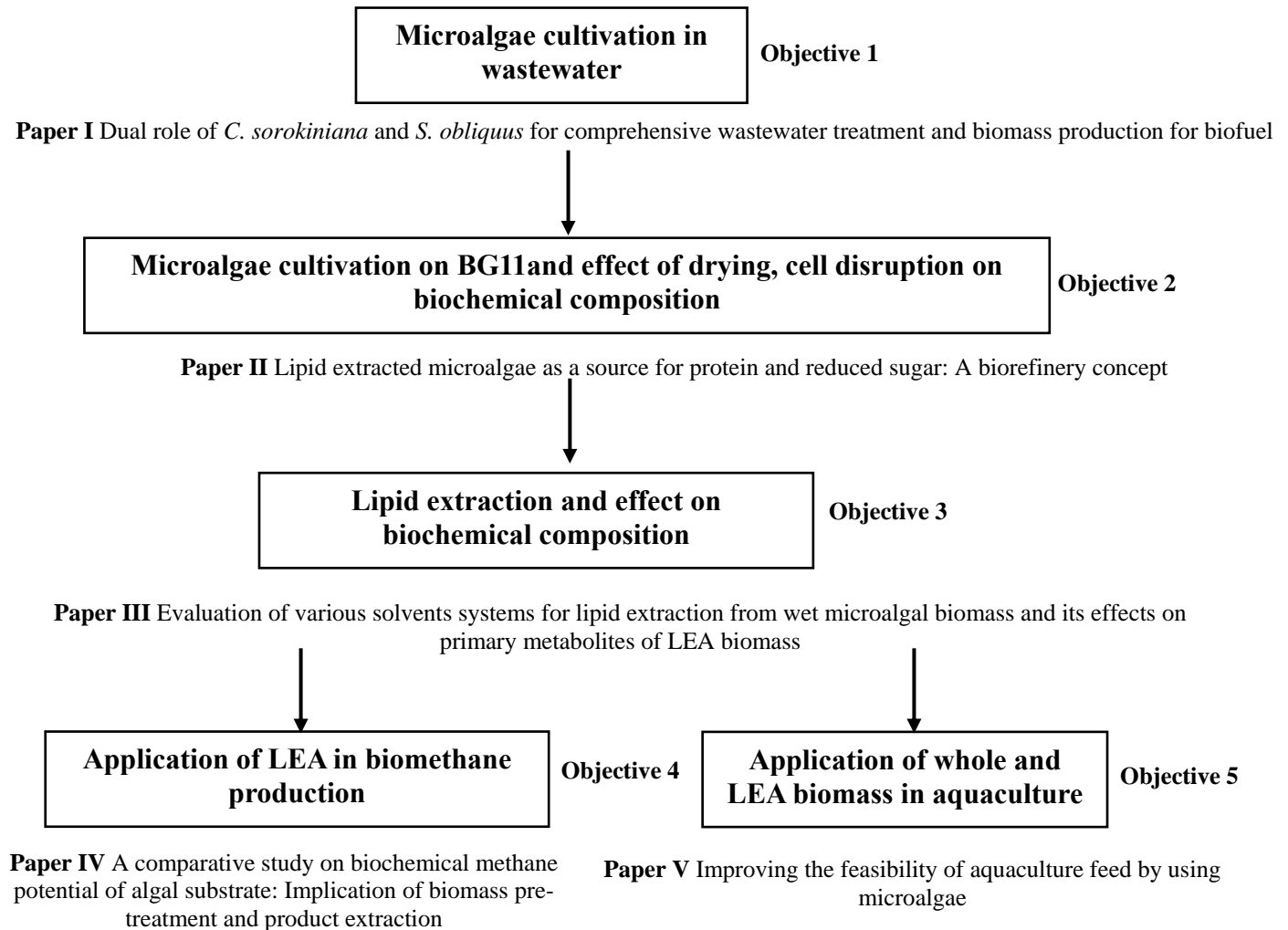
The aim of this study was to evaluate the effects of different drying, cell disruption and lipid extraction techniques on major microalgal metabolites and utilize LEA for feed and energy production. The hypothesis employed was LEA obtained after lipid extraction is still rich in proteins and carbohydrates, which can be further used as protein source in aquaculture feed and energy sources by microorganisms to produces energy. The model products were whole and LEA supplemented tilapia feed and biomethane. The other product such as fuel and value-added chemicals could be potentially be produced by selecting different microorganisms and/or processes.

Aim: To assess the effect of different downstream processing on algal biochemical composition and application of lipid extracted biomass in feed and energy production

1.2 Objectives

- Comparison of lipid, carbohydrate and protein of whole cell microalgae grown on two different growth medium (BG11 and wastewater) (**Paper I, published in Journal of Cleaner production**).
- To determine the effect of different drying and cell disruption techniques on primary metabolites of microalgal biomass (**Paper II, published in Bioresource Technology**).
- Evaluation of the effects of different lipid extraction procedures on primary metabolites composition of microalgae and lipid extracted algae (**Paper III, published in Environmental Science and pollution Research**).
- Evaluation of co-digestion of LEA with waste sludge for the production of biogas (**Paper IV, published in Bioresource Technology**).
- Assessment of nutritional value of whole cell algae and lipid extracted algal biomass as functional ingredients for animal feed (**Paper V, ready to submit, Paper VI**, Application of *S. obliquus* as a protein source in rearing Nile tilapia at demonstration scale is under preparation).

1.3 Thesis framework



2.0 LITERATURE REVIEW

2.1 Microalgae

The demand of energy in developing countries have increased tremendously due to industrialization, fast growing population and rapid modernization. Approximately 80% of the total energy consumption comes from non-renewable sources (petroleum, natural gas and coal) (EI Arroussi *et al.*, 2017; Schlagermann *et al.*, 2012). The annual consumption of fossil-based fuels is expected to rise by approximately 90% in 2030 (Steen *et al.*, 2010). Fossil-based fuels also increase greenhouse gases. The European Union has planned targets that emissions of greenhouse gases must be reduced by 20% in 2020 (Schlagermann *et al.*, 2012).

The increase in petroleum prices and concern over energy dependency and security, greenhouse gas emissions and climate change have garnered major concern to scientific world. Global pressure on non-renewable resource depletion and climate change have triggered and driven obvious advances in the development of renewable and sustainable energy. Biofuel has immense potential as green renewable alternative to fill the global energy gap.

Biodiesel is the mixture of fatty acid methyl esters, obtained after transesterification of oil (Zhu *et al.*, 2017). The main disadvantages of these feedstock sources are low oil yields and high-water demand, land, fertilizer, carbon mitigation potential and limited ability to achieve commercial targets for biofuels production (Mata *et al.*, 2010; Vassilev and Vassileva, 2016). Therefore, it is crucial to find alternative sources for biodiesel for sustainable development. Microalgae are recognized as one of the most suitable candidate for biodiesel production, which have capability to store, more lipid than other oil crops like jatropha and rapeseed (EI Shimi & Moustafa, 2018). Microalgal biomass has three major metabolites viz., lipid (20-70%), protein (6-71%) and carbohydrate (5-64%) (Becker, 2007; Jankowska *et al.*, 2017). The yield and productivity of

metabolites depends on algae species and growth conditions applied. From energy point of view, the most valuable microalgal metabolite is lipid. In optimum conditions, microalgae can accumulate lipid up to 50-70% (DCW) (Jankowska *et al.*, 2017). Microalgal biomass production can be divided in two parts, i.e. upstream and downstream processing (figure 1). Globally around 9000 tons per year microalgae biomass are produced with production cost of \$ 20-\$ 200 per kg (Wang *et al.*, 2016). Microalgae biomass has been explored as renewable feedstocks for biodiesel production (EI Arroussi *et al.*, 2017; Lan *et al.*, 2018; Song *et al.*, 2016; Yu *et al.*, 2015). Due to high production and processing cost microalgal biodiesel is not currently an economical alternative to existing technologies (Rawat *et al.*, 2013; Zhu, 2015). The cultivation of microalgae using wastewater as nutrient source could form a sustainable biorefinery with dual benefits of, nutrients remediation and biomass generation for biofuels production. At commercial level microalgal biodiesel production process generates huge amounts of LEA as waste product. The LEA is rich in proteins, carbohydrates, vitamins etc. These could be used as feedstock for the production of biomethane or as a supplement to aquaculture feed. To improve the economics of microalgae-based biorefinery it is vital to utilize LEA for different purposes (feed and energy).

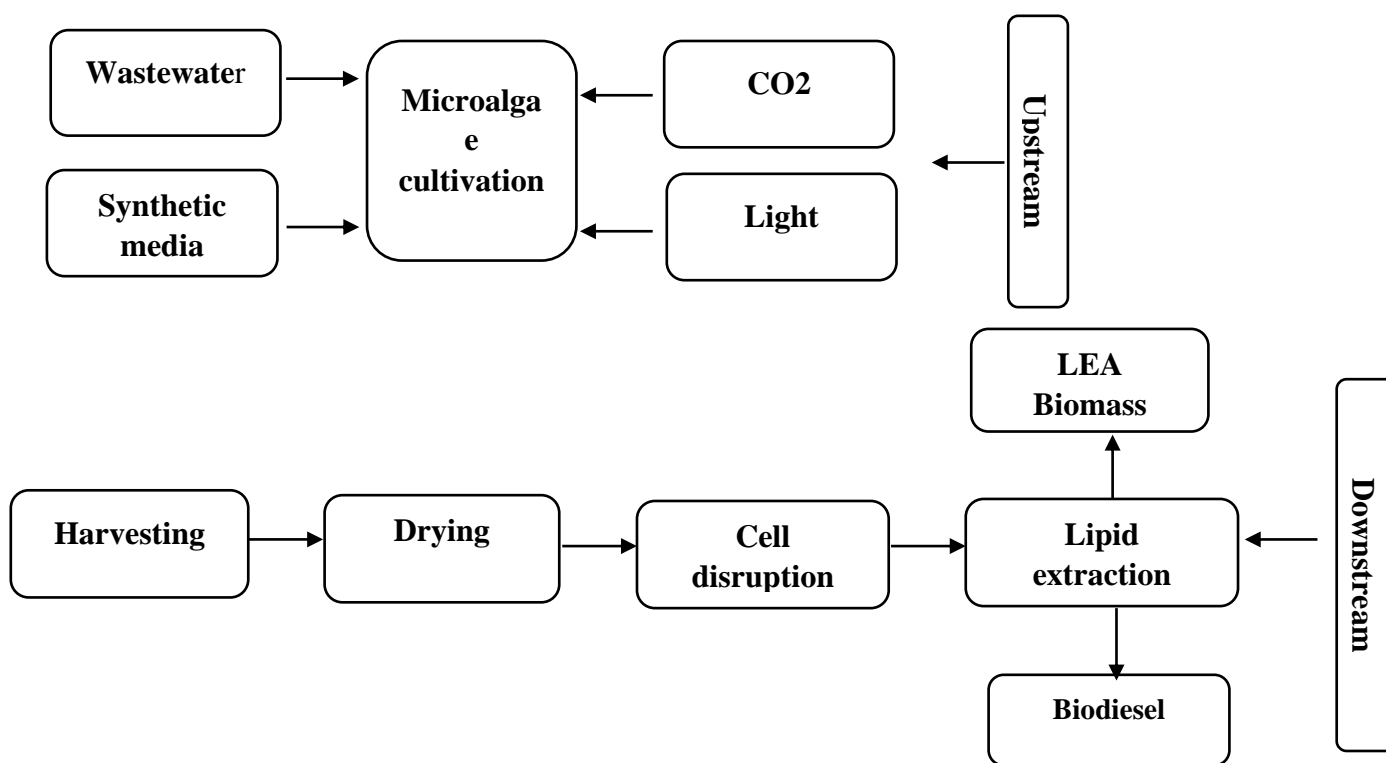


Figure1 Schematic diagram of microalgae biodiesel production

2.1.1 Microalgal cultivation in wastewater

The role of microalgae in wastewater treatment has gained attention due to their dual role of nutrient uptake from wastewater for growth and due to the value of microalgal biomass produced (Novoveská *et al.*, 2016). Microalgal cultivation at large-scale is still challenging for higher biomass productivity containing maximum possible lipid, protein and carbohydrate yields. The main challenges to large-scale cultivation are high cultivation cost, harvesting, drying, extraction and most importantly application of microalgae biomass for single purpose (Karemore & Sen, 2016; Rawat *et al.*, 2013; Shurtz *et al.*, 2017). Various types of microalgae have been cultivated in different types of wastewater (municipal wastewater, dairy and food

wastewater) for nutrients removal and biomass production for biodiesel production (Caporgno *et al.*, 2015; Farooq *et al.*, 2013; Li *et al.*, 2011; Pittman *et al.*, 2011).

Commercial scale production of microalgae biomass for biofuel, feed, food and other value-added products requires economical cultivation techniques to make process feasible and sustainable (Chisti 2007; Griffiths & Harrison 2009). Mainly, there are two suitable methods for the production of substantial microalgae biomass such as (i) raceway ponds and (ii) photobioreactors (Carvalho *et al.*, 2006). Both cultivation methods have their own advantages and disadvantages with reference to efficacy, time and ease of operation (Table 1). The selection of an appropriate technique for cultivation and production of microalgal biomass with maximum lipids, proteins and carbohydrates contents depends on microalgal strain, appropriate nutrient medium, light etc. (Ramanna *et al.*, 2018; Singh *et al.*, 2015; Srinuanpan *et al.*, 2018).

Table 1 Comparison between raceway and photobioreactor cultivation systems

	Raceway	Photobioreactor
Advantages	<ul style="list-style-type: none"> • Low construction cost • Low operating cost • Well researched • Easy to clean • Pond can be constructed in desert and non-arable land 	<ul style="list-style-type: none"> • High productivity • Concentrated biomass • Easily controlled • Small area required • Better control of gas transfer and protection from outside contamination
Disadvantages	<ul style="list-style-type: none"> • Low productivity • High possibility of contamination • Large land area required • Poor mixing • Water loss due to evaporation • Challenging to maintained monoculture 	<ul style="list-style-type: none"> • high build-up of dissolved oxygen • High operating cost • Harsh to clean • High capital cost for design and operating • Bio-fouling • Overheating • Benthic algae growth

(Brennan and Owende, 2010; Chisti, 2007; Harun *et al.*, 2010; Mata *et al.*, 2010; Narala *et al.*, 2016; Rawat *et al.*, 2013)

2.1.2 Microalgae biomass drying

Prior to lipid extraction, biomass drying is required, which increases the viability of biomass for the lipid extraction process (Show *et al.*, 2015). The major drying techniques are rotary drying, spray drying, sun drying, cross flow drying, vacuum drying, shelf drying, oven drying, freeze-drying (Show *et al.*, 2015). The biomass drying techniques should be designed to eradicate possible deterioration of the delicate algae quality arising from the dehydration process (Show *et al.*, 2015). Oven and sun drying techniques are economically better drying techniques than freeze-drying. The oven drying technique is fast but energy intensive process while sun drying takes comparably longer time also highly depends on the prevailing weather conditions (Guldhe *et al.*, 2014). The sun drying technique is economical and ease to scale up

but time consuming and weather dependent (Show *et al.*, 2015). The drying techniques influence the recovery of biochemical compositions from microalgal biomass (Chen *et al.*, 2015a, Hussain *et al.*, 2015). Several studies have been done in the recent past to investigate the effect of drying on lipid for biodiesel production (Chen *et al.*, 2015a; Guldhe *et al.*, 2014; Hussain *et al.*, 2015; Sahoo *et al.*, 2017; Show *et al.*, 2015). Freeze-drying technique maintained the structure and biochemical compositions of microalgal biomass that improved the lipid recovery (Guldhe *et al.*, 2014). The effect of different drying technique on protein and carbohydrate recovery is yet to be explored. The selection of drying technique also has an influence on target product (lipid, protein, carbohydrate, pigment etc.) and scale of microalgae production. Sun drying does not require energy intensive sophisticated instrumentation, which makes it preferable drying technique at large-scale.

2.1.3 Microalgae cell disruption and lipid extraction

Cell disruption and selection of suitable solvent/solvent systems are essential downstream processes to improve the lipid extraction efficiency (Prabakaran & Ravindran, 2011). Therefore, the suitable cell disruption and lipid extraction processes are the key to increasing the lipid extraction efficiency (Lee *et al.*, 2010). Lipid extraction from microalgae is carried out by using either mechanical or chemical methods or by using a combination of these methods. In mechanical methods, oil expeller or press, ultrasound, microwave etc. are used to extract oil from microalgal biomass (Mubarak *et al.*, 2015). Microwave, ultrasound, autoclaving and osmotic shock (10% NaCl) are cell disruption techniques commonly employed to improve the lipid extraction yields (Prabakaran & Ravindran, 2011). In chemical methods, solvent extraction, supercritical and ionic liquid extraction have done to extract the lipid from

microalgae (Dai *et al.*, 2014; Taher *et al.*, 2014; Vanthoor-Koopmans *et al.*, 2013). The combination of mechanical cell disruption techniques and solvents extraction are widely used for optimum lipid yield (Guldhe *et al.*, 2014). The common solvents used in lipid extraction are chloroform, methanol, isopropanol, ethanol, petroleum ether, dichloromethane etc. (Mata *et al.*, 2010; Ramluckan *et al.*, 2014).

Lee *et al.* (2010) studied the effect of different cell disruption techniques (autoclaving, bead-beating, microwave, sonication and addition of 10% NaCl) using chloroform: methanol (1:1 v/v) as organic solvents for lipid extraction from, *Botryococcus* sp., *C. vulgaris* and *Senedesmus* sp. The results showed that microwave cell disruption techniques were most appropriate and efficient method for lipid extraction. Similarly, Prabakaran and Ravindran (2011), conducted a comparative cell disruption (autoclaving, bead-beating, microwave, sonication and 10% NaCl) study for lipid extraction from *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp. using chloroform: methanol as lipid extracting solvent system. Results showed that sonication method was most applicable and efficient as cell disruption technique and the highest lipid was recovered from *Chlorella* sp. The major drawback of using some organic solvents (e.g. chloroform) are their toxicity to the algal cells as well as to the environment (Yun *et al.*, 2014). For example, chloroform is highly toxic and could pose serious threat during its handling and to the environment. Therefore, it is vital to select less toxic lipid extracting solvents, which are cheap, easy to scale up and have minimal affinity towards non-lipid metabolites remain left in LEA.

2.2 Microalgae major metabolites

Microalgae can be used as a feedstock to obtain different types of products. Based on the application, algal products can be categorised in to energy (biodiesel, biomethane, bioethanol, biobutanol etc.) and non-energy (feed and food application) products. Algal biomass contains substantial amounts of three major metabolites i.e. lipids, proteins and carbohydrates, therefore, it is an ideal feedstock for producing energy as well as non-energy products. The percentage of these major metabolites in the biomass depends on the microalgal strain and cultivation conditions (Table 2).

Table 2 Different metabolites and cultivation condition

Microalgae	Cultivation	Medium	Protein %	Carbohydrate %	Lipid %	Reference
<i>A. falcatus</i>	Flask	BG11	45.02	15.98	26.37	Guldhe <i>et al.</i> , 2016
<i>D. salina</i>	Flask	f/2	35	28	28	Chen <i>et al.</i> , 2013
<i>D. tertiolecta</i>	PBR	-	27.2	40.5	22	Kim <i>et al.</i> , 2015
<i>Scenedesmus</i> sp.	Flask	BG11	20.88	42.68	23.62	Pancha <i>et al.</i> , 2015
<i>Chroococuss</i> sp. 1	Flask	Wastewater	0.39 mgmg ⁻¹	0.25 mgmg ⁻¹	0.19 mgmg ⁻¹	Prajapati <i>et al.</i> , 2013
<i>Chroococuss</i> sp. 2	Flask	Wastewater	0.41 mgmg ⁻¹	0.20 mgmg ⁻¹	0.21 mgmg ⁻¹	Prajapati <i>et al.</i> , 2013
<i>C. minutissima</i>	Flask	BG11	43.78	14.59	16.32	Prajapati <i>et al.</i> , 2014
<i>C. pyrenoidosa</i>	Flask	BG11	40.92	25.3	13.65	Prajapati <i>et al.</i> , 2014
<i>C. vulgaris</i>	Flask	BG11	36.48	15.39	20.35	Prajapati <i>et al.</i> , 2014
<i>C. vulgaris</i>	PBR	F/2	54.9	-	15.5	Kebelmann <i>et al.</i> , 2013
<i>Scenedesmus</i> sp.	-	-	42.5	12.3	16.9	Ramos-Suárez <i>et al.</i> , 2014
<i>C. vulgaris</i>	Flask	f/2	13.8	29.8	-	Tibbetts <i>et al.</i> , 2015b
<i>C. vulgaris</i> UTEX	raceway	-	35.13	16.82	9.81	Zhao <i>et al.</i> , 2014
<i>N. granulate</i>	PBR	f/2	350.4 gkg ⁻¹	149.1 gkg ⁻¹	285.5 gkg ⁻¹	Tibbetts <i>et al.</i> , 2015a
<i>N. salina</i>	Flask	f/2	55	30	11	Chen <i>et al.</i> , 2013
<i>Ulva rigida</i>	-	Marine	6.64	22	12	Satpati & Pal, 2011
<i>P. elipsoidea</i>	-	-	32.96	18.82	36.13	Gao <i>et al.</i> , 2012
<i>Nannochloropsis</i> sp.	raceway	-	34.03	7.64	10.65	Zhao <i>et al.</i> , 2014
<i>M. reisseri</i>	Flask	f/2	14.6	30	-	Tibbetts <i>et al.</i> , 2015b

2.2.1 Protein

Proteins are one of the major metabolites of microalgae. Some of the algae contained up to 60% of proteins (Trivedi *et al.*, 2015). The yield of proteins in microalgae, depends highly upon the algae strains and cultivation conditions. Microalgae grown in nitrogen rich nutrient medium can accumulate high protein contents. Some of the microalga such as *Chlorella vulgaris*, *S. obliquus* contain 50-58% of protein (Becker, 2007; Bleakley & Hayes 2017). *Spirulina* is also good source of protein containing around 60% of protein. The other algae species with high protein content include *Anabeana*, *Dunaliella* and *Euglena*. Whole microalgae and LEA have great potential to be an alternative protein sources in feed and food, since they contain many essential amino acids (Becker, 2007; Ju *et al.*, 2012; Sørensen *et al.*, 2017). Amino acids (essential and non-essential) are basic constituents of proteins; 20 amino acids are common for proteins synthesis. Animals and fish cannot synthesize ten essential amino acids (methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine). Whole cell algae and LEA both are rich in essential amino acids. Table 3 shows the concentrations of essential amino acids in whole cell and LEA of different microalgae. Tibbetts *et al.* (2015a) found that, there are no significant differences in essential amino acids profile in whole and LEA of *N. granulate* (Table 3). In another study, they found that essential amino acids concentration (mg g DW^{-1}) significantly improved in LEA of *Scenedesmus* sp. (Tibbetts *et al.*, 2015c). This shows that the amino acids concentration in LEA depends on lipid extraction protocols and microalgal species. Brown *et al.* (1997) studied 40 species of microalgae and reported that all those species contain similar amino acids. Due to well balance of amino acid profile and good digestibility, microalgae have potential to be used as a protein source in aquaculture feed. Therefore, microalgae have great potential to replace fishmeal as protein source (Radhakrishnan *et al.*, 2014; Walker & Berlinsky, 2011).

Table 3 Composition of essential amino acids in whole and lipid extracted microalgae

Microalgae	Biomass	Essential amino acid											References	
		Unit	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Arg		
<i>N. granulata</i>	whole	mg/g		7.5	17.4	32.4	24.1	8.7	19.1	16.5	0.4	21.5	25.4	Tibbetts <i>et al.</i> , 2015a
<i>N. granulata</i>	LEA	mg/g		7.6	18	33.1	21.2	9	19.3	18.1	0.4	22.7	26.3	Tibbetts <i>et al.</i> , 2015a
<i>Nannochloropsis</i> sp.	whole	mg/g		26.26	47.22	94.04	68.31	23.6	55.26	48.56	-	60.24	60.82	Kent <i>et al.</i> , 2015
<i>Tetraselmis</i>	whole	g/100 protein	g	2.01	4.06	9.45	6.52	2.78	5.62	5.17	1.61	5.72	5.01	Schwenzfeier <i>et al.</i> , 2011
<i>S. pacifica</i>	whole	g.16 g ⁻¹ N		2.03	5.79	8.74	4.72	3.52	4.94	5.41	0.83	6.3	8.05	Misurcova <i>et al.</i> , 2014
<i>S. platensis</i>	whole	g/100 protein	g	1.69	6.34	9.8	4.49	2.4	5.16	4.85	1.42	6.91	6.72	Parimi <i>et al.</i> , 2015
<i>Scenedesmus</i> sp.	whole	mg/g		26.06	44.1	91.89	66.61	24.4	55.72	56.27	-	61.76	64.13	Kent <i>et al.</i> , 2015
<i>Dunaliella</i> sp.	whole	mg/g		25.03	45.08	93.22	62	25.3	59.59	50.53	-	59.83	65.92	Kent <i>et al.</i> , 2015
<i>Scenedesmus</i> sp	whole	mg g DW ⁻¹		4.7	13.7	27	18	7	19.4	17.2	7	18.4	19.2	Tibbets <i>et al.</i> , 2015c
<i>Scenedesmus</i> sp	LEA	mg g DW ⁻¹		7.2	18.3	36.3	20.7	8.7	22.3	23.7	6.5	26	25.4	Tibbets <i>et al.</i> , 2015c

2.2.2 Lipid

Microalgae can accumulate up to 70% of lipid (DCW), and under specific conditions, some microalgal species accumulate up to 90% of lipid (Mata *et al.*, 2010; Stephenson *et al.*, 2011). Microalgae cultivated in nitrogen stressed conditions are rich in lipids but have low biomass productivities (Singh *et al.*, 2016). Microalgal lipids are composed of saturated and unsaturated fatty acids. Stress conditions, environmental factors, cultivation conditions and growth phase affect the fatty acids compositions in the microalgae. Moreover, microalgal lipids contain both essential and nonessential fatty acids, and are generally rich in PUFA viz. DHA, EPA, ALA (Table 4). Rapidly grown algae can accumulate up to 14-30% of lipid, which are suitable for its use in aquaculture feed (Table 2). For preparation of aquaculture feed, it is most important to focus on the percentage of ALA, DHA and EPA as these are essential nutrients for suitable growth and development. Marine microalgae have significantly higher DHA content than fresh water algae. *Cryptocodinium cohnii* contains approximately 30-50% DHA of their constitutes fatty acids.

Gladyshev *et al.* (2016) reported that *Cryptomonas sp.* has 16.27 of ALA, 13.95% of EPA and 3.53% of DHA (Table 4). The yields and composition of PUFA in microalgae depends on the microalgae species and cultivation conditions.

Table 4 Composition of PUFA in various microalgae species

Microalgae	ALA (%)	EPA (%)	DHA (%)	Reference
<i>C. muelleri</i>	0.9	20.3	0.6	Chen <i>et al.</i> , 2015b
<i>C. vulgaris</i>	34.02	-	-	Gladyshev <i>et al.</i> , 2016
<i>Chlorella sp.</i>	0.36	8.9	3.24	Sahu <i>et al.</i> , 2013
<i>C. protothecoides</i>	7.12	0.03	-	Solana <i>et al.</i> , 2014
<i>C. calcitrans</i>	-	17.8	1.3	Delaporte <i>et al.</i> , 2003
<i>C. affinis</i>	3.1	13.2	18.6	Suh <i>et al.</i> , 2015
<i>C. didymus</i>	3.7	8.8	24.1	Suh <i>et al.</i> , 2015
<i>Cryptomonas sp</i>	16.27	13.95	3.53	Gladyshev <i>et al.</i> , 2016
<i>Chatoceros sp</i>	0.2	19.9	2.9	Suh <i>et al.</i> , 2015
<i>N. salina</i>	0.3	1.5	-	Solana <i>et al.</i> , 2014
<i>N. gaditana</i>	0.8	12.2	-	Matos <i>et al.</i> , 2015
<i>N.gaditana</i>	0.3 mgg ⁻¹	175 mgg ⁻¹	-	Ryckebosch <i>et al.</i> , 2014
<i>N. oculata</i>	0.7 mgg ⁻¹	193 mgg ⁻¹	-	Ryckebosch <i>et al.</i> , 2014
<i>N.gaditana.</i>	2.1	16.9	-	Carrero <i>et al.</i> , 2015
<i>I. galbana</i>	2.7	Trace	9.5	Chen <i>et al.</i> , 2015b
<i>Tetraselmis sp</i>	16.2	10	<0.01	Tsai <i>et al.</i> , 2016
<i>T. suecica</i>	10.5	5.4	0.1	Delaporte <i>et al.</i> , 2003
<i>T. chui</i>	13.6	4.2	Trace	Chen <i>et al.</i> , 2015b
<i>T. suecica</i>	68 mgg ⁻¹	16.3 mgg ⁻¹	0.8 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>T. suecica</i>	1.9 mgg ⁻¹	81 mgg ⁻¹	0.9 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>T. weissflogii</i>	1.4	10.1	9.6	Suh <i>et al.</i> , 2015
<i>Isochrysis sp.</i>	5.7	0.4	7.8	Delaporte <i>et al.</i> , 2003
<i>P. lutheri</i>	10 mgg ⁻¹	92 mgg ⁻¹	40.9 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>P. tricornutum</i>	0.8 mgg ⁻¹	111 mgg ⁻¹	8.3 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>P. cruentum</i>	1.42 mgg ⁻¹	35.6 mgg ⁻¹	-	Ryckebosch <i>et al.</i> , 2014
<i>P. tricornutum</i>	0.22	30.26	0.98	Qiao <i>et al.</i> , 2016
<i>S. menzelii</i>	0.62	11.42	3.6	Jiang <i>et al.</i> , 2016
<i>Hindakia sp</i>	20.08	-	-	Daroch <i>et al.</i> , 2013
<i>Isochrysis T-iso</i>	29 mgg ⁻¹	2.8 mgg ⁻¹	46 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>R. salina</i>	92mg/g	18 mgg ⁻¹	11.1 mgg ⁻¹	Ryckebosch <i>et al.</i> , 2014
<i>A. sanguinea</i>	0.6	20.1	23.8	Suh <i>et al.</i> , 2015

2.2.3 Carbohydrate

Carbohydrates are another important metabolite of microalgae (Table 2). During photosynthesis, microalgae utilize ATP/NADPH to fix and convert atmospheric CO₂, water and light to produce biomass, which are rich in carbohydrates (Chen *et al.*, 2013; Lehninger *et al.*, 2005). The main constituents of microalgal carbohydrates are starch, cellulose/hemicellulose, sugars and other polysaccharides. The carbohydrate contents in algae depends on the mode of cultivation. The carbohydrate yield in microalgae can be improved by the use of various strategies such as changing irradiance, nitrogen stress, varying temperature, pH shift and addition of CO₂ (Trivedi *et al.*; 2015). Microalgae such as *Chlorella*, *Chlamydomonas*, *Dunaliella*, *Scenedesmus*, and *Tetraselmis* accumulate high amounts of carbohydrates (>40%) (Ho *et al.*, 2013). The common sugars found in polysaccharides of many algal species are glucose, mannose, ribose/xylose, rhamnose and fucose with glucose being the major constituent and accounting for 28–86% of the total carbohydrates, while mannose was a substantial component in all cases (Roy & Pal, 2015). Microalgal starch/glucose conventionally is used for bioethanol or biohydrogen production (John *et al.*, 2011).

2.3 Whole microalgae and lipid extracted microalgae

Cultivation of microalgae can be done using different types of media. The yield of all major metabolites in whole algae depends on nutrient availability in culture the medium. Microalgae grown in wastewater containing low nitrogen or any other nitrogen stress condition shows higher lipid and carbohydrate yield but lower protein contents and biomass productivity (Singh *et al.*, 2016). Microalgae accumulate higher protein content in high nitrogen contained medium such as BG11 (NaNO₃=1.5 gL⁻¹). Becker, (2007) reported 14-22 % lipid, 51-58% protein and 12-17% carbohydrate in *C. vulgaris* whole cell biomass and 50-56% protein, 12-14% lipids, and 10-17%

carbohydrates in whole algae biomass of *S. obliquus*. Prajapati *et al.* (2014) reported that *Chlorella pyrenoidosa* can accumulate up to 40.92% protein, 13.6% lipid and 25.3% carbohydrate. The whole algae biomass can be used for energy generation (biodiesel, biomethane, bioethanol etc.) or feed application in aquaculture. Whole algae can be used as a protein, lipids, pigments etc. source with other conventional feed ingredients in aquaculture feed. It provides amino acids, vitamins and many other nutrients that are very important for fish cultivation.

The high cost of biodiesel production can be mitigated by the effective use of LEA (Maurya *et al.*, 2016). After the lipid extraction, LEA accounts for 70% of biomass of whole algae (DCW, basis), and the LEA mostly contains proteins and carbohydrates. Bohutsky *et al.* (2015) reported that LEA from *A. protothecoides* contained 14% protein and 75% carbohydrate. Tibbets *et al.* (2015b) also reported 17.3% protein and 33.6% carbohydrates in LEA of *C. vulgaris*. Similarly, 39.6% protein, 12% carbohydrate and 2.83% lipid was recorded in *C. vulgaris* UTEX (Zhao *et al.*, 2014) (Table 5). The different upstream and downstream processes such as cultivation, harvesting and lipid extraction significantly affect the yields of protein and carbohydrate in LEA (Rashid *et al.*, 2013). Due to availability of high proportion of carbohydrates and proteins, LEA has major applications for either energy or aquaculture feed. Several studies have demonstrated excellent use of LEA as a substrate for the production of biohydrogen, bioethanol, biobutanol, syngas etc. (Beneroso *et al.*, 2013; Lee *et al.*, 2015; Yang *et al.*, 2010; Zhao *et al.*, 2014). However, the LEA required pre-treatment prior to its utilization, for energy feed production. The biomass with higher C/N ratio is excellent substrate for energy production, while, the biomass with lower C/N ratio can be used for the feed application. The LEA having higher protein contents can be effectively used in aquaculture feed as an alternative to fishmeal or soybean meal. Ju *et al.* (2012) used LEA to replace fishmeal partially in the diet of Pacific white shrimp and observed improved pigmentation

in the shrimps instead of negative effects. Patterson & Gatlin (2013) found that LEA of *Navicula* sp. and *N. salina* has potential to replace up to 10% of crude protein from FM and soybean protein without showing significant reductions in juvenile red drum performance. The proteins, carbohydrates and lipids contents in whole and LEA of some major algae are summarized in table 5.

Table 5 Major metabolites in whole and lipid extracted algae

Microalgae	Whole algae			LEA Biomass			Reference
	Proteins %	Carbohydrates %	Lipids %	Proteins %	Carbohydrates %	Lipids %	
<i>A. protothecoides</i>	6	33	57	14	75	NA	Bohutskyi <i>et al.</i> , 2015
<i>C vulgaris UTEX</i>	35.13	16.82	9.81	39.96	12	2.83	Zhao <i>et al.</i> , 2014
<i>C. vulgaris</i>	13..8	29.8	NA	17.3	33.6	NA	Tibbetts <i>et al.</i> , 2015b
<i>Chlorella</i> sp	NA	NA	NA	55.28	24.77	<1	Kassim <i>et al.</i> , 2014
<i>Scenedesmus</i> sp.	56	25	13	72	21	<1	Vardon <i>et al.</i> , 2012
<i>Nannochloropsis</i> sp	34.03	7.64	10.65	32.7	9.56	3.03	Zhao <i>et al.</i> , 2014
<i>N. basillaris</i>	13.8	27.2	NA	22.2	43.9	NA	Tibbetts <i>et al.</i> , 2015b
<i>M. reisseri</i>	14.6	30	NA	17.7	35.7	NA	Tibbetts <i>et al.</i> , 2015b
<i>Tetracystis</i> sp.	13.2	27.7	NA	21.9	43.2	NA	Tibbetts <i>et al.</i> , 2015b
<i>Navicula</i> sp.	19.4	NA	18.8	13.3	NA	4.9	Patterson & Gatlin, 2013
<i>D. tertiolecta</i>	NA	NA	NA	13.42	82.08	NA	Goo <i>et al.</i> , 2013
<i>D. tertiolecta</i>	27.2	40.5	22	35	51.9	NA	Kim <i>et al.</i> , 2015
<i>T. suecica</i>	NA	NA	NA	63.04	19.81	<1	Kassim <i>et al.</i> , 2014
<i>B. braunii</i>	NA	NA	NA	46	23.9	2.71	Neumann <i>et al.</i> , 2015

2.4 Lipid-extracted algae as a feedstock for biomethane production

The anaerobic digestion process is a simple and economical approach to an improved energy recovery from LEA. Anaerobic digestion generates biogas that contains mainly methane (65%) and carbon dioxide (35%) and other gases (N₂, NO, H₂S etc.) normally are found in less than 1% (Passos *et al.*, 2014). In anaerobic digestion, there are four steps hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2). In hydrolysis, large or complex molecules break down into simple molecules such as proteins into amino acids, polysaccharides in to sugars, lipids

in to fatty acids. In acidogenesis, microorganisms further break down the remaining complex molecules into ammonia, CO₂ and H₂S. The volatile fatty acids are produced by acidogenic (or fermentative) bacteria along with ammonia, CO₂ and H₂S and other by-products (Appels *et al.*, 2008). In acetogenesis, acetoacetate, CO₂ and H₂ formed. In methanogenesis, methanogenic bacteria utilized intermediate product of other steps and transform it into methane, CO₂ and H₂O. Among all four steps, the hydrolysis process is vital and the rate limiting step.

There are several factors (pre-treatment of biomass, C/N ration, inoculum, pH, temperature etc.), which can improve the biomethane production through anaerobic digestion. Low C/N ratio feedstock are not suitable for the biomethane production, to improve the C/N ratio carbon sources can be added. After lipid extraction the LEA is still high in carbon content (Table 6), which indicates that LEA has potential to be used as a substrate for biomethane production. Zhao *et al.* (2014) studied the whole cell algae and LEA of *C. vulgaris*, they found that LEA has 44.03, 5.03 and 8.15% carbon, hydrogen and nitrogen, respectively. Similarly, Kim *et al.* (2015) found 44.78% of carbon, 6.78% of hydrogen and 8.4% of nitrogen in LEA of *D. tertiolecta*.

Table 6 Elemental composition of whole algae biomass and LEA

Microalgae	Whole algae biomass					LEA biomass					Reference
	C %	H %	N %	O %	S %	C%	H %	N %	O %	S %	
<i>C. vulgaris UTEX</i>	52.81	6.13	7.77	29.33	0.72	44.9	5.03	8.15	27.82	0.79	Zhao <i>et al.</i> , 2014
<i>Chlorella sp.</i>	-	-	-	-	-	39.34	6.6	7.91	45.5	0.65	Kassim <i>et al.</i> , 2014
<i>Senedesmus sp.</i>	52.1	7.4	8.8	31.1	0.48	49.9	7.1	9.9	32.1	0.96	Vardon <i>et al.</i> , 2012
<i>Nannochloropsis sp.</i>	52.844	6	7	23.41	0.73	43.47	5.13	6.84	34	0.8	Zhao <i>et al.</i> , 2014
<i>N. Salina</i>	56.2	8.76	3.78	25.63	0.55	47.8	6.9	5.65	31.8	0.77	Zhao <i>et al.</i> , 2014
<i>Nanofrustulum sp.</i>	27.45	4.23	2.9	29.31	0.96	20.19	2.93	2.9	33.96	0.76	Zhao <i>et al.</i> , 2014
<i>N. salina</i>	20.2	-	0.98	-	-	50	-	5.72	-	-	Quinn <i>et al.</i> , 2014
<i>D. tertiolecta</i>	38.23	6.19	11.12	44.46	-	44.78	6.78	8.4	40.04	-	Kim <i>et al.</i> , 2015
<i>D. tertiolecta</i>	-	-	-	-	-	38.63	6.41	2.54	-	-	Goo <i>et al.</i> , 2013
<i>D. tertiolecta</i>	48.1	6.33	7.33	29.04	0.59	39.3	6.53	5.03	39.89	0.8	Francavilla <i>et al.</i> , 2015
<i>A. protothecoides</i>	59	9.6	1.5	26	0.15	59	7.1	2.3	39	0.42	Bohutskyi <i>et al.</i> , 2015
<i>P. tricornutum</i>	44.12	5.14	6.43	28.3	1.29	38.62	5.7	6.8	28.7	1.28	Zhao <i>et al.</i> , 2014
<i>T. suecica</i>	-	-	-	-	-	24.09	3.64	4.12	67.54	0.61	Kassim <i>et al.</i> , 2014

Various studies have compared biomethane production from whole algae and LEA. Alzate *et al.* (2014) analyzed methane production from whole and LEA of *Nannochloropsis* sp. Higher methane production was observed in LEA than the whole algae. The lipid extraction process is itself a pre-treatment for biomass and therefore, additional pre-treatments do not significantly improve the biodegradability of LEA. Zhao *et al.* (2014) studied the anaerobic digestion of five different types of LEA which produced 300 to 380 mL CH₄ g⁻¹ VS. Kinnunen *et al.* (2014) achieved 482 and 194 mL methane production from 1 g VS of wet and dry LEA of *Nannochloropsis* sp., respectively in batch tests. They suggest that LEA produced through wet lipid extraction from microalgae could serve as a good substrate for methane production. In this study, 48% higher methane yield (220 mL CH₄ g⁻¹ VS) was recorded in thermophilic reactors compared to the mesophilic reactor (149 mL CH₄ g⁻¹ VS). Lipid extracting solvents, chloroform: methanol have high extracting efficiency however traces of these solvents remains in LEA, which can negatively affect the biomethane production (Yun *et al.*, 2014). Yun *et al.* (2016) reported that very low concentrations (1.67 µM) of chloroform inhibited methanogenic and acidogenic bacteria as well as sulfate reducing bacteria,

which are important for biomethane production. Selection of appropriate solvent/ solvent systems to extract the microalgae lipid is vital if LEA is to be used for biomethane production. Higher lipid extraction efficiency, eco-friendly, lesser toxicity and lower price solvent systems are preferable. It is important to wash or evaporate the residual solvents from the LEA before its use in biomethane production.

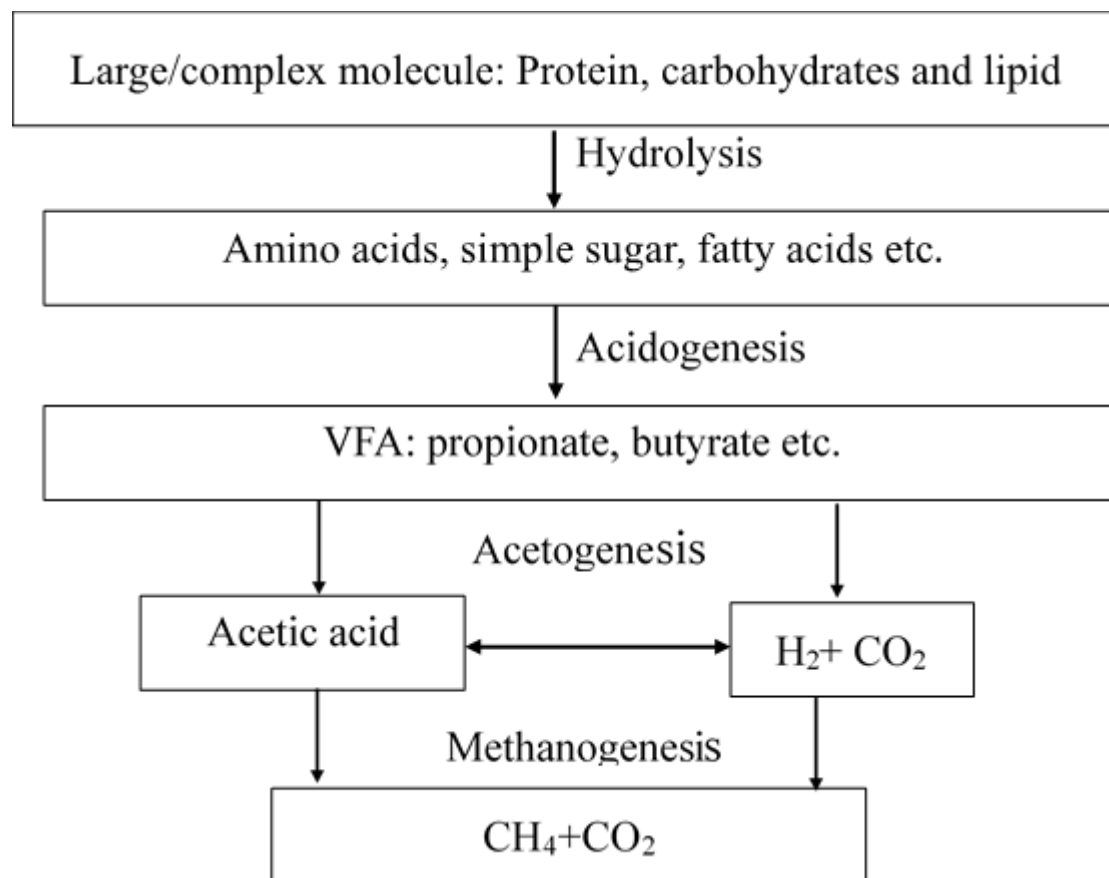


Figure 2 Steps involved in the anaerobic digestion process

2.5 Factor affecting biomethane production

2.5.1 Pre-treatment

The biomethane yield depends significantly on the substrate biochemical compositions and content of organic substances (Jingura & Kamusoko, 2017). Other than substrate quality, pre-treatment steps consequently improve the biodegradability of the substrate and thus improve the biomethane production. The different pre-treatment techniques such as thermal, chemical (alkaline and acidic hydrolysis) and enzymatic are common practice (Jankowska *et al.*, 2017; Ward *et al.*, 2014). The combination of chemical and thermal pre-treatments are also applied widely (Córdova *et al.*, 2018). The selection and combination of suitable enzyme mixtures improve the cell degradation efficiency and methane production. Pre-treatment of biomass increases the surface area and makes intra-cellular organic molecules readily available to the microorganisms for their biomethane production (Ramos-Suárez *et al.*, 2014). Alzate *et al.* (2014) observed that thermal pre-treatment of whole biomass of *Nannochloropsis* sp. and LEA improved the biogas production by 40% and 15%, respectively. In study by Golueke and Oswald (1959), heating at 100 °C for 8 h found to increase the gas productivity by the 33% as compared to untreated biomass. The main disadvantage of thermal pre-treatment is its energy intensive and time-consuming nature. In chemical pre-treatment, alkali and acid reagents are commonly used to solubilise the hemicellulose and lignin components of the microalgal biomass. Alkali treatment (NaOH, KOH etc.) are comparably more effective for hemicellulose solubilisation (Rodriguez *et al.*, 2015). The pre-treatment also depends on algal species. Bohutskyi *et al.* (2014) evaluated the combined effects of alkali (NaOH) and high temperature for the *Chlorella* sp. *Nannochloropsis*, *T. weissflogii* *Tetraselmis*, sp. and *Pavlova cf* sp. The results showed that biogas and methane production increased up to 30-

40% for *Chlorella* sp. and *Nannochloropsis* sp., whereas no effects of combined pre-treatment was observed for other species.

Enzymatic degradation is commonly accomplished using cellulase, glucohydrolase and xylanase which enhance around 126% solubilization and 15% improvement in methane yield as compared with cellulase or glucohydrolase alone (Passos *et al.*, 2016). Muñoz *et al.* (2014) used *Raoultella ornithinolytica* MA5 for pre-treatment of *N. gaditana*, which increased 158.68% of methane yield. Application of enzymes in cell degradation requires low energy, but very selective towards microalgal species and expensive to scale up.

2.5.2 C/N ratio

The composition of carbon and nitrogen available in organic material are represented by the C/N ratio. In microalgae the C/N ratio are in the range of 4.16-7.82. When the ratio is lower than 20, the biomass is not preferable for microorganism involved in anaerobic digestion (Ward *et al.*, 2014). The low C/N ratio indicate that substrate is protein rich (Jingura & Kamusoko, 2017). The suitable C/N ratio for anaerobic digestion is approximately 20-30. Methanogens rapidly consume the nitrogen from the organic material with high C/N ratio, which results decreased gas production. The C/N ratio lower than 15 produce ammonia nitrogen in the process and increase pH above 8.5, which is toxic to the methanogenic bacteria (Ehimen *et al.*, 2013).

The algal biomass contained high protein contents (and nitrogen) which produced ammonia toxicity in anaerobic digestion. The protein-extracted algae (PEA) has great potential to be used as substrate for biomethane production (Parimi *et al.*, 2015). The PEA has low nitrogen contents, which improved the C/N ratio, may also help in reducing the formation inhibitory factor such as ammonia. Thus, application of PEA for biomethane can improve the economic feasibility of algal

biotechnology. The optimum C/N ratio can be achieved by co-digestion materials of high and low C/N ratio, such as energy crops or silage mixed with sewage or animal manure.

2.6 Feedstock for aquaculture and challenges

Globally more than one million people depend on fish as source of protein in their diet. Fish contribute around 30% of animal protein intake (Elsaidy *et al.*, 2015). The worldwide demand for freshwater fish increased by 30% from the year 2003 to 2007 and approximately 37 million tons of fish will be required globally by 2020 (Hamid *et al.*, 2016). Due to high demand and limited supply of FM and fish oil (FO) from wild captures fisheries/ global fish stocks become a constraint to the development of aquaculture industry (Perez-Velazquez *et al.*, 2018; Radhakrishnan *et al.*, 2016).

Formulating the right feed and feeding correctly are the two main factors required for fast growth rates and higher yields of fish. Aquaculture feed mostly based on conventional feedstock such as FO and FM however, sole utilization of FM lead to increase in the price (Mataka and Kang'ombe, 2007). Other conventional aquaculture feed ingredients such as wheat bran, maize bran, wheat and rice bran, cotton and sunflower seed cakes and sunflower oil have been used in different types of fish feed to see their effect on growth, survival and yield (Limbu *et al.*, 2016).

Soybean meal is one of the most widely used sources of plant-based protein (47-50%) in fish feed preparation (Huang *et al.*, 2017; Sharawy *et al.*, 2016). Use of soybean meal in fish has advantages such as ease of availability, environmentally friendly and low cost. The soybean and other plant-based proteins cannot be used as sole protein sources due to factors such as palatability and digestibility, which are the main disadvantages. Soybean meal has low nutrient availability, contains unbalanced amino acid profile and lacks EPA and DHA (Table 6). The content of primary amino acids such as methionine and lysine are very low in soybean meal.

The solid-state fermentation (SSF) is biological process for bioremediation and detoxification used to eliminate/reduce saponin from whole soybean by *Rhizopus oligosporus* (Fenwick & Oakenfull, 1983). The SSF detoxificate soybean and enzymes produced by microorganisms improve nutrients bioavailability (Khan & Ghosh, 2013). Sharawy *et al.* (2016) used four-inclusion levels of solid-state fermented soybean meal with *Saccharomyces cerevisiae* to replace FM in prawns (*Fenneropenaeus indicus*). Results revealed that 50% FM could be replaced using solid-state fermented soybean meal with *S. cerevisiae*. High levels of FM replacement by soybean meal significantly decreased morphometric characteristics (Sharawy *et al.*, 2016). Sillva-Carrillo *et al.* (2012) used four different (0, 20, 40 and 60%) proportions of soybean meal to replace FM in juvenile spotted rose snapper feed. After 12 weeks of study, they found that 20% FM replacement by soybean meal did not affect weight gain, feed intake, PER compared to fish fed the control diet. They noticed that fish growth performance was reduced at higher levels (60%) of FM replacement with soybean meal as protein source. There was no significant difference observed in survival rate of fish fed different diets.

The use of poultry by-products and feather meal in fish feed has been practiced for decades. These are a good source of protein, containing 69% crude protein. Hydrolysed feather meal enhances feed digestibility of fish. Folwer (1990), reported that Chinook salmon diets containing 15% of feather meal did not affect growth and feeding efficiency. Various amino acids, lysine and methionine are present in poultry by-products (Rossi & Davis, 2012). Poultry meal is devoid of pigments and lacks essential PUFAs. The addition of earthworm powder in aquaculture feed could potentially enhance the lysine content in aquaculture feed diet (Hamid *et al.*, 2016). Limbu *et al.* (2016) used rice bran alone and mixed ingredient to feed Nile tilapia. Their results showed that growth performance, survival rate and condition factor of Nile tilapia were not affected either

feeding with mixed ingredient or rice bran alone. Application of conventional feed in aquaculture feed have its own advantages and disadvantages (Table 7). It is important to select the right diet for appropriate fish species for higher growth, survival and to improve nutritional quality.

Table 7 Aquaculture feed ingredients and their advantages and disadvantages

Ingredients	Advantage	Disadvantage
Soybean meal (SBM)	Cheap protein and oil source	Lack methionine, lysine and PUFA
Feather meal/poultry meal	Cheap protein source, easily available	Lack pigment, imbalanced essential amino acids
Wheat	High starch content	Low proteins
Barley	Digestible proteins	Low protein content, high fibre concentration
Corn gluten meal	High digestible proteins	Lack in lysine
Peas/lupins	High digestible proteins	Lysine and methionine are limited, presence of antinutrients quinolizidine alkaloids
Cottonseed meal (CSM)	Cheap protein and oil source	Gossypol might have toxic effect
Canola meal	Not widely used in aquaculture feed similar to the protein content of SBM	High cost
Canola protein concentrate	Protein content similar to fishmeal	Amino acid supplements needed to overcome limiting amino acids levels
FM	Easily digestible, rich in essential amino acids and PUFA	High cost, lack of pigments
Microalgae	Easily digestible, rich in essential amino acids, PUFA, and also contains pigments, vitamins and minerals	High cultivation and processing cost

(Hemaiswarya *et al.*, 2011; Hien *et al.*, 2015; Plaza *et al.*, 2008; Sharawy *et al.*, 2016; Sirakov *et al.*, 2015; Spolaore *et al.*, 2006; Walker & Berlinsky, 2011)

2.6.1 Microalgae in aquaculture feed

Increasing demand for protein and the high cost of FM in the recent years has resulted in the requirement of alternative protein sources (animal and plant sources) to complete the nutritional requirements in aquaculture. FM is widely used ingredient in fish feed, due to balance nutrient contents, digestibility, palatability etc. however, it is expensive which adds to the production cost. Microalgae have promising potential for use as alternative to FM in aquaculture feed as they have balanced nutritional quality and positive effect on the growth rate, disease resistivity, and improvement in protein decomposition and essential fatty acid in fillet (Ju *et al.*, 2012, Kousoulaki *et al.*, 2016; Radhakrishnan *et al.*, 2016). There are several other factors, which make microalgae

more suitable candidate for aquaculture feed than terrestrial plants, such as higher productivity than terrestrial plants, with simple nutritional requirements. The other benefits of using microalgae in aquaculture feed, significantly depends on growth stage and type of fish (Table 8).

Table 8 Application of microalgae in aquaculture feed and its effect on fish

Microalgae	Application mode of microalgae in feed	Ingredient replaced	Fish/ shrimps/ prawns/ molluscs	Comments	Reference
<i>Chaetoceros muelleri</i> and <i>Tisochrysis lutea</i>	Live with spray dried <i>spirulina</i>	-	<i>Panopea generosa</i>	Combination of <i>C. muelleri</i> and <i>T. lutea</i> give the best growth rate	Arney <i>et al.</i> , 2015
<i>A. maxima</i> or <i>D. salina</i>	Whole	-	<i>Haliotis laevis</i>	Feed supplemented with <i>A. maxima</i> or <i>D. salina</i> increased the size and body weight	Dang <i>et al.</i> , 2011
<i>Spirulina sp</i>	Whole	-	<i>Cyprinus carpio</i>	Spirulina supplemented feed increased the body weight, sensitivity, protein and lipid content	Abdulrahman <i>et al.</i> , 2014
<i>Arthrospira platensis</i>	Whole	Fishmeal	<i>Macrobrachium rosenbergii</i>	Replacement of 50% fishmeal by <i>A. Platensis</i> significantly increased the weight gain, specific growth rate, feed efficiency and, also enhanced the proteins, amino acid. Carbohydrate and oil content. No significant effect had been found on digestive enzymes activities	Radhakrishnan <i>et al.</i> , 2016
<i>Navicula sp.</i> , <i>Chlorella sp.</i> , <i>Nannochloropsis salina</i>	LEA	Protein	<i>Sciaenops ocellatus</i>	10% of FM and soy proteins could be replace by LEA proteins without causing any negative affect	Patterson & Gatlin, 2013
<i>Nannochloropsis sp.</i>	Whole	-	<i>Macrobrachium rosenbergii</i>	Suitable inclusion levels substantially improved performance of larval culture of <i>M. rosenbergii</i>	Lober & Zeng, 2009
<i>Nannochloropsis granulate</i>	Whole		<i>Litopenaeus vannamei</i>	Protein apparent digestibility for all <i>N. granulate</i> meals was moderate, potentially can use in <i>Litopenaeus vannamei</i> diet	Tibbetts <i>et al.</i> , 2017
<i>Isochrysis sp.</i>	Whole	-	<i>Tridacna noae</i>	The ingestion and digestion of microalgae by <i>T. noae</i> larvae influenced by types of microalgae and larval age	Southgate <i>et al.</i> , 2017
<i>Chaetoceros muelleri.</i>	Whole	-	<i>Ruditapes decussatus</i> larvae	Feed supplemented with <i>C. muelleri</i> significantly increased the growth and survival rate	Aranda-Burgos <i>et al.</i> , 2014
<i>Spirulina platensis</i> , <i>C. vulgaris</i> , <i>Azolla pinnata</i>	Whole	-	<i>Macrobrachium rosenbergii</i> postlarvae	Microalgae included diet significantly vit C and E in hepatopancreas and muscle tissue. These microalgae can be used as an alternative protein ingredient in <i>Macrobrachium</i> culture	Radhakrishnan <i>et al.</i> , 2014

<i>Chlorella vulgaris</i>	Whole	Synthetic pigment	<i>Oncorhynchus mykiss</i>	Inclusion of <i>C. vulgaris</i> as pigment ingredient significantly increase the color of <i>O.mykiss</i> .It has promising potential to replace synthetic pigment in <i>O. mykiss</i> feed diet.	Gouveia <i>et al.</i> , 1996
<i>Spirulina platensis</i>	Whole	Fishmeal	<i>Litopenaeus vannamei</i>	Fishmeal could be replaced (75%) by <i>A. platensis</i> without any negative effects and the lower (25%) inclusion level enhanced the immunological parameters.	Macias-Sancho <i>et al.</i> , 2014
<i>Scenedesmus almeriensis</i>	Whole	Fishmeal	<i>Sparus aurata</i>	Inclusion of 20% <i>S. almeriensis</i> biomass in <i>S. aurata</i> feed significantly improved the absorptive capacity of intestinal mucosa in the interior and posterior intestinal region	Vizcaíno <i>et al.</i> , 2014

2.6.2 Whole algae in aquaculture feed

Microalgae are being used in aquaculture feed as a supplement for either partial or full replacement of some of the important ingredients in conventional feeds (Hanel *et al.*, 2007; Ma & Qin, 2014; Radhakrishnan *et al.*, 2014). It is vital to choose the correct algal species at the optimum inclusion level. The inclusion of different microalgae species provides better nutrition and enhanced fish growth compared to feed that contains single algae species (Hemaiswarya *et al.*, 2011; Spolaore *et al.*, 2006). Whole microalgae inclusion in fish feed can provide proteins with balanced amino acid profiles, oils rich in essential unsaturated fatty acids, pigments, antioxidants, vitamins and minerals. Various studies demonstrated that inclusion of small proportion of algae, increased the morphometric characteristics such as weight and length of the fish, protein retention, omega-3 fatty acid contents and provided well-balanced essential amino acids profile in final products (Abdulrahman *et al.*, 2014; Radhakrishnan *et al.*, 2014). Potential replacement of FM, FO and pigments with microalgae could decrease the price of fish feed. Whole algal biomass of *Spirulina* has been used in feed for giant freshwater shrimp, *Penaeus japonicas* and improved growth, survival and feed utilization was observed (Nakagawa & Gomez – Diaz 1995). Kousoulaki *et al.* (2016) reported that inclusion of 5% whole heterotrophic microalgae (*Schizochtrium sp.*) in

extruded meal of salmon successfully replaced FO without any adverse effect on morphological characteristics. Vizcaíno *et al.* (2014) incorporated five different levels (0%, 12%, 20%, 25% and 30%) of *S. almeriensis* in the diets of sea bream (*Sparus aurata*). After a 45 days trial, they observed that *S. almeriensis* incorporated feed showed no negative effect on fish growth or nutrient utilization efficiency. Fish feed with 12% *S. almeriensis* incorporated diets showed higher trypsin levels than the control.

In a study by Walker & Berlinsky. (2011) three experimental diets were used to replace 0, 15 and 30% of FM proteins in diets of juvenile Atlantic cod (*Gadus morhua*). The results showed that 30% inclusion caused palatability problems so feed intake and growth were reduced. They observed that at 15% of algal biomass supplementation, the feed intake was improved. In another study by García-Ortega *et al.* (2016) FM, squid meal and FO were replaced by a combination of algal meal (*Schizochytrium limacinum*), soy protein concentrate and soybean meal. The study showed that soybean meal, soy protein concentrates and algae can replace at least 40% of marine protein sources, in which algal lipid can be used as the core source in *E. lanceolatus* diets, which significantly improved the fish performance.

Sprague *et al.* (2015) used *Schizochytrium* sp. (rich in DHA) for replacement of FO in Atlantic salmon (*Salmo salar*). Similarly, Aranda-Burgos *et al.* (2014) used four mono and multi-algal inclusions to see the effect of microalgal diets on growth, survival and fatty acid composition during larval development in grooved carpet shell (*Ruditapes decussatus*). The result showed that the feeding regime improved larval growth, increase mortality and fatty acid composition. A low level of microalgae meal inclusion in fish feed increases fish growth rate and showed positive morphometric effects. Li *et al.* (2009) used a low level (1.0-1.5%) inclusion of microalgae (*Schizochytrium* sp.) in catfish (*Ictalurus punctatus*) feed and noticed significant improvement in

feed efficiency ratio, weight gain and PUFA contents. They also observed gradual decrease in fish growth, when algal meal supplementation was higher in feed, suggesting low inclusion levels were preferable. Dallaire *et al.* (2007) incorporated three inclusion levels (12.5, 25 and 50%) of microalgae in rainbow trout fry (*Oncorhynchus mykiss*) feed to evaluate its growth rate and nutritional value. They found that higher inclusion levels of microalgae feeds negatively affected rainbow trout growth rate. Results suggested that a maximum 12.5% microalgae could be incorporated to avoid negative effects on rainbow trout fry. Inclusion of algae in high proportions increases the fibre contents in the feed, which negatively effects the digestibility and metabolic processes (Hussein *et al.*, 2013; Ju *et al.*, 2012). Most of the previous work was done at lab-scale for shortened periods. For appropriate application of whole microalgae in aquaculture feed from juvenile to finisher, it is important to know the microalgae inclusion level, biochemical composition and growth performance of the tilapia. Microalgae for fish feed has potential to become profitable in the near future (Shah *et al.*, 2018b).

2.6.3 Lipid-extracted algae in aquaculture feed

The LEA biomass obtained after lipid extraction is rich in proteins, carbohydrates and other important components (minerals, water-soluble vitamins and bioactive compounds) (Ju *et al.*, 2012). The proteins content of LEA has potential to be used in aquaculture feed as it can replace FM in aquaculture feed (Sørensen *et al.*, 2017). Ju *et al.* (2012) used LEA biomass of *Haematococcus pluvialis* as a protein source and prepared four test diets to partially replace FM at 12.5, 25, 37.5 or 50% in diets of Pacific shrimp. After 8 week feeding trails, they observed that feed with 12.5% replacement of FM showed significantly higher weight, percentage weight gain and specific growth rate in shrimp than the control diet. Patterson & Gatlin (2013) used three different LEA biomass of *Naviculla* sp., *Chlorella* sp. and *N. salina* as an alternative protein

sources for red drum diets. In their first experiment, LEA of *Naviculla* sp. was used to replace 5 and 10% of crude protein. The results showed that inclusion of 10% LEA, significantly reduced the protein and energy retention value. While weight gain, feed efficiency, survival etc. were not significantly affected to reference diet. In their second experiment, *Chlorella* sp. was used to replace 5, 10, 20, and 25% of crude protein in reference diets. The results showed that replacement of 20 and 25% crude proteins by *Chlorella* sp. significantly reduced growth and protein efficiency ratio but there were no changes in condition indices or whole-body proximate composition. In their 3rd experiment, small inclusion levels of *N. salina* was done to replace 5, 7.5, 10 and 15% of the crude protein in reference diets. Results showed that LEA of *N. salina* could not be used at more than 10% to replace crude proteins in juvenile red drum diets due to its negative effects. Gong *et al.* (2016) used LEA of *Nannochloropsis* sp. and *Desmodesmus* sp. to feed Atlantic salmon postsmolts and observed the apparent digestibility coefficient (ADC) of dry matter. The ADC estimates nutrients availability in feedstuffs, which are used to optimize nutritional value and cost of formulated diets (Fagbenro, 1999). Two sets of experiment were done, one using cold-pelleted and other employing extruded pellets to examine the ADC, protein, ash and energy. Results showed that LEA of *Nannochloropsis* incorporated cold-pelleted feed resulted higher ADC and protein, compared to the *Desmodesmus* sp. The LEA of *Nannochloropsis* sp. inclusion in extruded feed also showed higher ADC and energy than *Desmodesmus* sp. The LEA of *Nannochloropsis* sp. was more digestible than *Desmodesmus* sp. and the extrusion process improved the digestibility of certain nutrients. Bryant *et al.* (2012) employed hedonic pricing methods to calculate the value of *N. oculata* LEA-based feed for aquaculture and found that it has a lower value than soybean meal and menhaden FM. Such studies clearly revealed that the LEA has greater potential than

terrestrial plant-based feed ingredients as a replacement protein in aquaculture feed. However, the LEA biomass inclusion in fish feed highly depends on algal species and types of fish.

Most of previous research was based on lab scale and optimization of LEA inclusions level for shortened period in fish diets. Therefore, it is also very important to know the inclusion level of LEA in the diet before preparation of feed formulations and fed to fish from juvenile to finisher.

The application of LEA in aquaculture will also make algal biodiesel production process more economical and sustainable by opening a new gateway to collect revenue from residual biomass.

3.0 PUBLISHED ARTICLES

3.1 Paper I

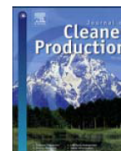
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Dual role of *Chlorella sorokiniana* and *Scenedesmus obliquus* for comprehensive wastewater treatment and biomass production for bio-fuels



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ABSTRACT

Microalgal treatment of raw sewage presents many complexities, mainly resulting from the inability of the algal species to sustain increased physiological stresses due to variable nutrient levels and high concentrations of organics. *Chlorella sorokiniana* and *Scenedesmus obliquus* have been identified to tolerate higher amounts of organic loading and physiological stresses. Nutrient removal, pathogen removal, and lipid accumulation with secondary or tertiary effluents have been demonstrated independently for these organisms. However, their potentials for accomplishing these objectives simultaneously with raw sewage have not been investigated. This study presents comprehensive investigations of applicability of *C. sorokiniana* and *S. obliquus* to wastewater treatment without the requirement for any additional treatment. *S. obliquus* showed greater potential for removing organic carbon ($76.13 \pm 1.59\%$ COD removal), nutrients ($98.54 \pm 3.30\%$ N-removal, $97.99 \pm 3.59\%$ P-removal) and comparable pathogens removal ($99.93 \pm 0.12\%$ total coliforms removal, 100% faecal coliform removal) in comparison to *C. sorokiniana* ($69.38 \pm 1.81\%$ COD removal, $86.93 \pm 3.49\%$ N-removal, $68.24 \pm 11.69\%$ P-removal, $99.78 \pm 0.12\%$ total coliforms removal, 100% faecal coliform removal) with 15 days of cultivation with filtered raw sewage, but also encountered increased levels of stress (F_v/F_m of 0.48 ± 0.03) which accounted for increased lipid accumulation in the cells ($23.26 \pm 3.95\%$ w/w) but might also affect their biomass productivity and treatment potential in longer applications. In comparison, *C. sorokiniana* demonstrated better adaptability to physiological stresses (F_v/F_m of 0.53 ± 0.01) and may be suitable for achieving comprehensive treatment and sufficient lipid accumulation ($22.74 \pm 3.11\%$ w/w) without compromising these potentials during prolonged applications. These results highlight the importance of selecting algal species with better stress resistance to extend their applicability for comprehensive wastewater treatment and lipid production.

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1. Introduction

Wastewater generation has always been associated with the development of human societies. Effective and efficient treatment of wastewater is critical to achieve sustainable growth of algae and to reduce the demand for fresh water, which already is a scarce resource in many parts of the world. Over the years, much effort has been devoted to the development of efficient wastewater treatment processes. These can be broadly categorized into primary treatment, secondary treatment, and tertiary treatment based on the

specific objectives of the processes (Arceivala and Asolekar, 2007). These processes cumulatively are very energy and cost intensive (McCarty et al., 2011), and hence have limited overall applicability in economically weaker societies. An ideal process should be able to achieve comprehensive wastewater treatment with minimum energy and cost input.

The role of microalgae in wastewater treatment has gained prominence due to their potential for nutrient uptake from wastewater for growth, and more importantly due to the value of algal biomass generated and other by-products (Christenson and Sims, 2011; Pittman et al., 2011; Rawat et al., 2011). Many algal species have demonstrated great potential for the dual applications of wastewater treatment and production of biomass for bio-fuel or other valuable products (Rawat et al., 2011). In many independent

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studies, microalgae have also demonstrated potential for removal of organic carbon and pathogens in addition to nutrients. For example, *Chlorella* sp. have been reported to remove 83% COD from domestic wastewater (Wang et al., 2010). In addition, faecal coliforms and other pathogens are sufficiently removed from wastewater with prolonged cultivation of microalgae (Ansa et al., 2012). Increase in pH, dissolved oxygen production, and excretion of algal metabolites having antibacterial properties are cited as the main reasons for pathogen removal in algal cultivations (Ansa et al., 2011, 2012). These applications have however been restricted largely to secondary or tertiary treated effluents (Ji et al., 2013a; Srirangan et al., 2011). Applications of algal cultivation to raw sewage mainly from animal livestock have been reported. These applications often encounter a number of difficulties, viz. unbalanced C:N:P ratios, chromaticity, need for pre-treatment etc (Ji et al., 2013b). Still, the applicability to raw sewage depends largely on the ability of selected algal species to tolerate the high and variable organic loading of these wastewaters.

Tolerance to the high organic loading associated with domestic wastewaters has been reported for *Chlorella* sp. and *Scenedesmus* sp. (Kümmerer, 2008; Palmer, 1969). In addition, *Chlorella* sp. have been reported to have good nutrient removal potential with sufficient lipid production for bio-fuels from a wide variety of wastewaters (Choi and Lee, 2015; Filippino et al., 2015; Ramanna et al., 2014; Ryu et al., 2014; Xu et al., 2015). Similarly, *Scenedesmus* sp. have also demonstrated good nutrient removal and lipid accumulation potentials with wastewater (Xin et al., 2010). Nutrient removal, pathogen removal, and lipid accumulation with secondary or tertiary effluents have been demonstrated in independent studies for both these organisms. However, their potentials for achieving all these objectives simultaneously, namely comprehensive wastewater treatment with sufficient lipid production using raw sewage without any additional treatment process(es), have not been investigated or are rare. The present study was designed to investigate the applicability to raw sewage and compare their respective potentials in detail for comprehensive wastewater treatment that includes nutrient removal, pathogen removal, and organic carbon removal; and lipid yield in the biomass. To the best of our knowledge, no other study has covered such detailed information i.e. removal of organic load, nutrients (N&P), coliforms as well as physiological health of algae, biomass production and its lipid yield as well. The use of raw sewage without any treatment and use of post chlorinated wastewater has not yet been reported. The success of batch experiments once translated to pilot scales would reduce the use of fresh water by several fold. In addition, it would improve the economics of the micro-algal biomass production (for bio fuel) which is a major techno-economic barrier to commercial algal bio fuel production currently.

2. Material and methods

2.1. Algae culture

Chlorella sorokiniana (genbank accession number: AB731602.1) and *Scenedesmus obliquus* (genbank accession number: FR751179.1) were isolated from the Durban region of KwaZulu Natal, South Africa and purified by subsequent sub-culturing using the streak plate method (Ramanna et al., 2014). Seed culture was prepared by inoculating BG11 medium with *C. sorokiniana* and *S. obliquus* in 1 L flasks. Cultures were maintained at room temperature ($22 \pm 2^\circ\text{C}$) with 16:8 h light:dark cycle under illumination from Gro-Lux lamps ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$). To maintain turbulent conditions, the culture was continuously shaken at 80 rpm on orbital shaker (OrbiShake Shaker, Labotec, South Africa). The pH adjustment of the medium to 7 was done using either 1 M H_2SO_4 or 1 M NaOH.

2.2. Experimental details

The growth, comprehensive wastewater treatment, and lipid accumulation potentials of *C. sorokiniana* and *S. obliquus* at different wastewater concentrations were observed for 15 days. A flow chart of the experimental design is presented in Fig. 1. The raw sewage obtained from eThekweni municipality of South Africa was used in this study and processed in following manner before actual growth experiments. The raw sewage was first filtered through 0.25 mm stainless steel filter then aerated for 8 h with commercial pump (flow rate $\geq 10 \text{ L/min}$) which resulted in some foaming due to the presence of inherent fatty components. This build-up foam was skimmed regularly, and after 8 h of aeration, foaming decreased substantially. Aeration was carried out to ensure maximum removal of oil and grease contents. This suggested that the sewage to be used in experiments was relatively free from external fatty components. Moreover, this also ensures and lipid quantification during the batch experiment would predominantly be from algal biomass. All the experiments were conducted in 2 L flasks under continuous aeration in growth chambers (at an illumination of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ with 16:8 h light:dark cycle at $22 \pm 2^\circ\text{C}$). The light intensity was measured by a light meter (MT 940, Major Tech, South Africa). The filtered raw sewage was diluted with post chlorinated effluent (residual chlorine $< 0.01 \text{ mg L}^{-1}$) from a wastewater treatment plant to achieve different nutrient concentrations (25, 50, 75, and 100% wastewater). All the experimental sets were inoculated with 5% of stock culture of *C. sorokiniana* (biomass

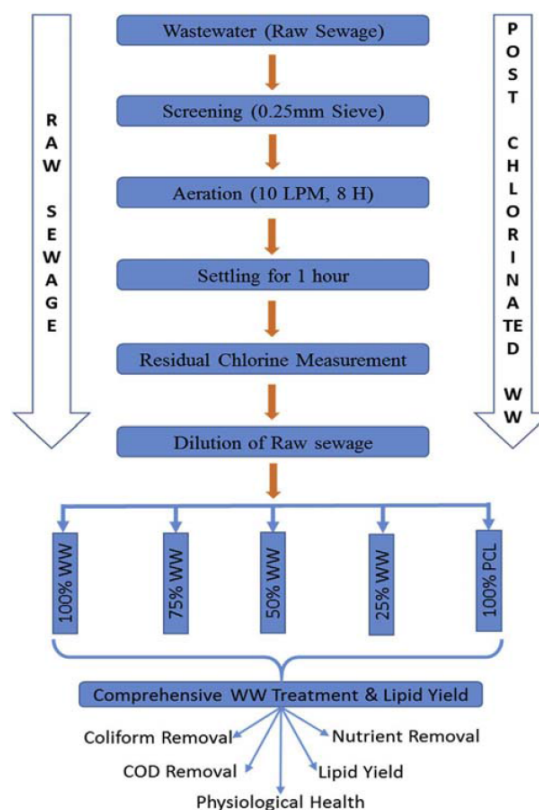


Fig. 1. Flow chart of the experimental design.

concentration was 92.46 mg L^{-1}) and *S. obliquus* (biomass concentration 96.21 mg L^{-1}). Table 1 presents the characteristics of the filtered raw sewage and the post chlorinated effluents used during experiments. Every 3rd day, 50 mL culture was withdrawn from each flask for analysis. 10 mL sample was used to determine specific growth rate (by measuring optical density at 680 nm) and physiological health by pulse amplitude modulated (PAM) fluorometry. Remaining 40 mL sample was centrifuged (20 min at 1509 g) and the supernatant was used further for physico-chemical and microbiological analysis and thick algal slurry was used for gravimetric analysis. Algal dry weight biomass of all the sets was determined (each after three days) gravimetrically according to standard method 2540-D (APHA, 1998). Consistent air supply (2 L min^{-1}) in the culture was maintained throughout the experiment by continuous aeration using portable air pumps, air valves and ceramic diffusers.

2.3. Measurement of growth and physiological health of algae

The growth of the cultures was continuously monitored by taking the optical density of the culture at 680 nm using UV visible spectrophotometer (Spectroquant Pharo 300, Merck, Germany) and gravimetric analysis of the biomass. The gravimetric analysis data is not presented. A Dual-PAM (pulse amplitude modulation) 100 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used for non-invasive fluorescence measurements. The sample was dark adapted before measurements to open all photo system-II (PS-II) reaction centres in the chlorophyll. The quantum efficiency of PS-II charge separation (F_v/F_m) was calculated as per following equation (Ramanna et al., 2014):

$$F_v/F_m = (F_m - F_o)/F_m \quad (1)$$

where F_v is the variable fluorescence resulting due to maximum fluorescence F_m and minimum fluorescence F_o in a dark adapted sample.

2.4. Physico-chemical analysis

The pH was measured with a pH meter (Orion Dual Star, Thermo Scientific). The nitrate, nitrite and orthophosphate concentrations in the samples were measured by Gallery™ Automated Photometric Analyzer (Thermo Scientific, USA). The chemical oxygen demand (COD) was determined by closed reflux titrimetric method. All measurements were done in triplicate.

2.5. Microbiological analysis

Colilert kit was used for the coliform and *Escherichia coli* analysis. The colilert is based on defined substrate technology (IDEXX Laboratories, Inc., USA). The β -galactosidase present in the colilert

medium gets metabolised in o-nitrophenyl, with a colour change from colourless to yellow; whereas, *E. coli* use β -glucuronidase to metabolize 4-methyl-umbelliferyl into fluorescent 4-methyl-umbelliferone. A 10 mL supernatant obtained from centrifugation was diluted with sterilized water. The growth media pouches provided with colilert were dissolved in the diluted supernatant and transferred into IDEXX Quanti-trays and sealed. The IDEXX Quanti-trays were incubated at 37°C and counted after 24 h. The *E. coli* counting was done in UV chamber.

2.6. Lipid extraction and quantification

At the end of the experiment, 1 L of algal culture was centrifuged (20 min at 1509 g) and lipid extraction from the biomass was carried out by microwave assisted solvent extraction. The effects of various concentrations of wastewater on the lipid yield were assessed by comparing it with control biomass grown in BG11 medium. The centrifuged biomass was kept overnight at -84°C in a bio-freezer (Glaciar NU9668E, Nuaire, Japan), followed by freeze drying using a lyophilizer (Mini Lyotrap, LTE scientific Ltd. United Kingdom). 500 mg biomass from each set was taken and mixed with 20 mL solvent mixtures (2:1 ratio of chloroform and methanol) and subjected to microwave digestion (Milestone S.R.L., Italy) at 100°C for 10 min at 1000 W following the method of Guldhe et al. (2014b). The solvent layer was decanted by filtration and residual biomass was again subjected to such repeated solvent extraction for maximum lipid recovery. All the extracted solvent aliquots were mixed and washed with distilled water followed by centrifugation. The supernatant was collected and dried to constant weight at 70°C . The percentage lipid yield per gram of dried biomass was determined gravimetrically.

3. Results and discussion

3.1. Growth potential of algae with raw sewage

The applicability of microalgae for comprehensive treatment of raw sewage is of great interest as it circumvents the need for additional treatment processes. Such applications have largely been investigated with secondary or tertiary treated effluents for major algal species (Ji et al., 2013a; Srirangan et al., 2011). Since, *C. sorokiniana* and *S. obliquus* both have predominantly been found to be suitable for nutrient removal with nutrient rich wastewaters, it is imperative to investigate their potential for such comprehensive treatment of raw domestic sewage and to compare their potentials (Pittman et al., 2011). A primary objective of this work was to investigate the growth potentials of *C. sorokiniana* and *S. obliquus* with raw sewage without any detrimental effect on culture physiology. Fig. 2 presents the growth results of *C. sorokiniana* (Fig. 2a) and *S. obliquus* (Fig. 2b) at different concentrations of raw sewage. The suitability of sewage for algal growth could be readily observed, since essential nutrients are already present in it. At all sewage dilutions, reasonable growth for both species was observed, except when nutrient levels were drastically lowered after dilution (WW-25). This further establishes the algal productivity on raw sewage qualitatively. A more detailed quantitative estimate of the effects of various sewage dilutions on the biomass production of both species could also be investigated from these figures. As observed, *C. sorokiniana* achieved maximum growth with WW-75 (75% raw sewage) and maintained a higher biomass level for the duration of the experiment (Fig. 2a). The nutrient content of such wastewater (WW-75) was sufficient to sustain the growth while the level of dilution in raw sewage provided more favourable growth conditions in comparison to raw sewage with no dilution (WW-100). This is also supported by the lag phase of two days as observed for

Table 1
Characteristics of raw sewage and post chlorinated effluent.

Parameter	Raw sewage	Post chlorinated effluent
pH	6.93 ± 0.28	7.65 ± 0.21
Alkalinity, mg L^{-1}	240.00 ± 2.35	10.67 ± 1.15
COD, mg L^{-1}	320.07 ± 3.78	48.12 ± 1.15
N-NH_3 , mg L^{-1}	52.23 ± 1.21	1.45 ± 0.86
N-NO_2 , mg L^{-1}	0.00 ± 0.00	0.36 ± 0.58
N-NO_3 , mg L^{-1}	0.40 ± 0.13	3.75 ± 0.33
P-PO_4^{3-} , mg L^{-1}	8.47 ± 0.23	0.48 ± 0.15
Total coliform, cfu 100 mL^{-1}	$1.27 \times 10^7 \pm 10^4$	0.00 ± 0.00
Fecal coliform, cfu 100 mL^{-1}	$2.00 \times 10^6 \pm 10^2$	0.00 ± 0.00

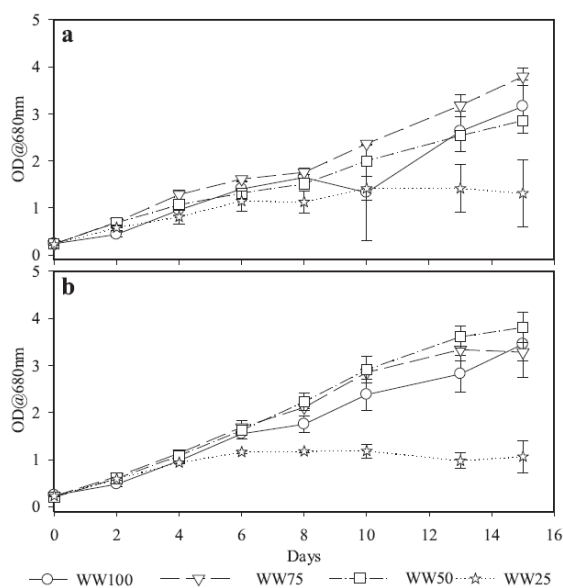


Fig. 2. Effect of different wastewater levels on the growth of (a) *C. sorokiniana*, (b) *S. obliquus*.

raw sewage, which is due to the necessary acclimatization of culture to such raw wastewater. In comparison, other dilution levels of wastewater (eg. WW-75, WW-50, and WW-25) provided more favourable growth conditions as no such lag was observed (Fig. 2a). Also, the raw sewage (WW-100) provided similar cultivation conditions for growth of *C. sorokiniana* as 50 percent diluted sewage (WW-50). The reduced nutrient content in WW-50 had the similar effects as the need for acclimatization with raw sewage, and the growth was comparable in both wastewaters. In contrast, growth of *C. sorokiniana* declined after six days with WW-25, due to exhaustion of nutrients. These results indicate the potential of raw sewage to be used for the cultivation of *C. sorokiniana* with 75% raw sewage as the optimal condition for growth. Cultivation of *C. sorokiniana* with raw effluent from palm oil mill was also found to be suitable (Nwuche et al., 2014).

In case of *S. obliquus*, comparable growth was observed for six days of cultivation with raw sewage (WW-100), and its two dilutions (WW-75 and WW-50). After six days, raw sewage performed sub-optimally in comparison to other two and this may be due to higher sustained physiological stress (refer Section 3.2) with such raw sewage (Fig. 2b). The 50% sewage was found to be optimal cultivation media for *S. obliquus* for the overall experimental duration, while WW-75 provided marginally sub-optimal conditions. In contrast, WW-25 was found to be limited in nutrients and did not support growth.

3.2. Effects of using raw sewage on the physiological conditions of algae

Since, the application of raw sewage for algal cultivation has been reported to present many complexities, it is important to investigate the stresses on culture physiology to determine their sustainable applications (Ji et al., 2013a). The effects of using raw sewage as a substrate on physiological conditions of *C. sorokiniana* and *S. obliquus* were observed by calculating the quantum efficiency (F_v/F_m) of reaction centres in PS-II of chlorophyll by non-

invasive fluorescence measurements. This parameter has now gained acceptability for indicating the stress conditions of the culture and is widely been used in identifying stresses due to various environmental conditions such as nutrient starvation (White et al., 2011). $F_v/F_m < 0.5$ generally indicates that culture is under going physiological stress. Fig. 3 presents the evolution of F_v/F_m with cultivation of both species in raw sewage and its dilutions. For *C. sorokiniana* WW-25 resulted in low F_v/F_m values which indicate that the culture is stressed due to nutrient starvation with this cultivation medium (Fig. 3a). These effects were also observed on reduced growth of *C. sorokiniana* with WW-25 (see Fig. 2a). In comparison, WW-75 resulted in continuous F_v/F_m values above 0.5, which indicates a healthy culture. The optimal growth using this wastewater (WW-75) is also an effect of such healthier culture. For raw sewage (WW-100), *C. sorokiniana* remained in a stressed condition (low F_v/F_m) during the initial phase of cultivation. Stress levels can be explained by the need for acclimatization to raw sewage. A lag phase of growth was also observed due to such need for acclimatization (see Fig. 2a). For WW-50, the culture remained healthy as observed by F_v/F_m values, but started to experience the stresses after eight days as the nutrients started to become limiting.

In comparison, the stresses when using sewage were more prominent with *S. obliquus* (Fig. 3b). Growth with WW-25 resulted in a highly stressed culture due to nutrient limitations which is evident from continually declining F_v/F_m values. Similar stresses were observed with raw sewage (WW-100) during the initial phase of experiments. This might be due to the need for acclimatization of *S. obliquus* to raw wastewater and evidence of this is also observed in lower growth with the raw sewage (see Fig. 2b). Cultivation using WW-75 maintained the culture in a relatively healthy state for 10 days of operation after which F_v/F_m started to decline. Decline was also observed with WW-50 after four days of cultivation.

These results when analyzed with the corresponding growth data of *C. sorokiniana* and *S. obliquus* provide some important insights about the suitability of cultivating these species on raw sewage without any additional treatment (see Figs. 2 and 3). As observed, *C. sorokiniana* was able to acclimatize better to raw sewage and maintain relatively lower stress levels than *S. obliquus*

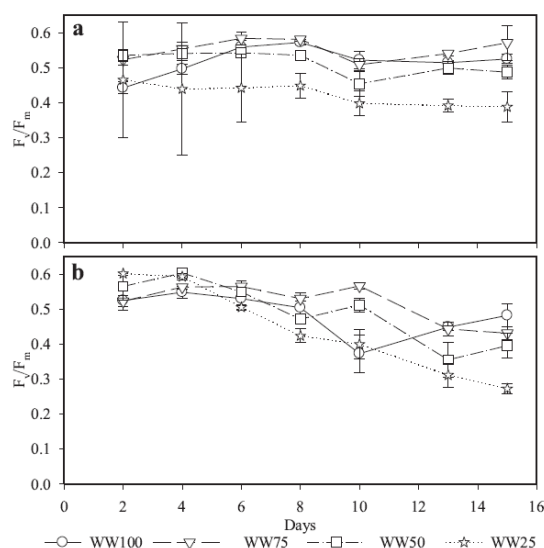


Fig. 3. Effect of different wastewater levels on the quantum efficiencies of (a) *C. sorokiniana*, (b) *S. obliquus*.

for all cultivation conditions. This better suitability is also evident by the fact that optimal growth of *C. sorokiniana* was observed with WW-75, while *S. obliquus* showed sub-optimal growth in WW-100 and WW-75. Raw sewage had to be diluted by 50% to achieve its optimal growth, which indicated the relatively unsuitable conditions using raw sewage for *S. obliquus* cultivation. Both species suffered severe nutrient stresses with WW-25 which affected their growth. Hence WW-25 is not a sustainable cultivation medium for either species. For sustainable long term cultivation, raw sewage could be diluted to 75% for *C. sorokiniana* and 50% for *S. obliquus* without the requirement for any additional treatment. These results also indicate the suitability of such sewage for the biomass production for lipid. Since *C. sorokiniana* have a well-established potential of maintaining high lipid contents (Zhang et al., 2013; Zheng et al., 2013), the application of WW-75 for their cultivation may result in a viable and economically more sustainable strategy. The need for dilution of raw sewage (WW-50) to achieve optimal growth conditions for *S. obliquus* makes such cultivation relatively more costly for this species due to additional requirements for pumping the dilution water and other involved costs, though technically feasible.

3.3. Removal of organic carbon from raw sewage

The organic carbon loading in raw sewage was $320.07 \pm 3.78 \text{ mg L}^{-1} \text{ COD}$ which was lowered with subsequent dilutions by post chlorinated effluent (see Table 1). Wastewaters were treated by cultivation of *C. sorokiniana* and *S. obliquus* to investigate the organic carbon removal performance of the two species and to compare their optimal performance. Since, *C. sorokiniana* and *S. obliquus* both can undergo mixotrophic growth while consuming organic carbon (Kim et al., 2013; Mandal and Mallick, 2009), these species also removed organic carbon from sewage as presented in Fig. 3. *C. sorokiniana* removed sufficient COD with all wastewaters (see Fig. 4a), despite the final effluent COD being the highest in raw sewage (WW-100). This is due to the initial higher level of COD in WW-100 and the need for acclimatization of *C. sorokiniana* with WW-100 and the associated stresses. In contrast, the final COD levels in effluents with WW-75, WW-50, and WW-25 are all lower than with raw sewage (Table 2). *S. obliquus* also achieved COD removal with all wastewaters (Fig. 4b). Final effluent COD with WW-100 and WW-75 achieved with *S. obliquus* were comparable while better quality effluents were achieved with WW-50 and WW-25. In this study, we used raw sewage without any pretreatment except prefiltration and aeration, therefore a relatively high bacterial contamination was expected. In the initial phases, most of the COD removal could be due to the synergistic effect of bacterial and algal consortium as culture was aerated continuously. Once bacteria have degraded most of the organic matter and mineralized the media, algae could have picked up exponential growth. Therefore at initial stage, even though both the algal species were stressed, 70–90% of the total COD removal was achieved. The best effluent quality in terms of COD removal was achieved for WW-25 with both species, which is due to the reduced level of organic carbon in the influent. Other researchers have also observed comparable COD removal with algae on different wastewaters (Wang et al., 2010). It has been observed that at low nutrient concentrations, both the algae and bacteria compete which suppresses the growth of both species (Rhee, 1972). But in a nutrient rich medium, algae and bacteria co-exist and support the growth of each other (Ma et al., 2014). Zhang et al. (2012) reported that in an algae bacterial consortia, primary degradation of complex organic compounds to its metabolites (N and P species) is done by bacterial communities and such metabolites are further utilized by algal communities. Gonzalez and Bashan (2000) also reported higher

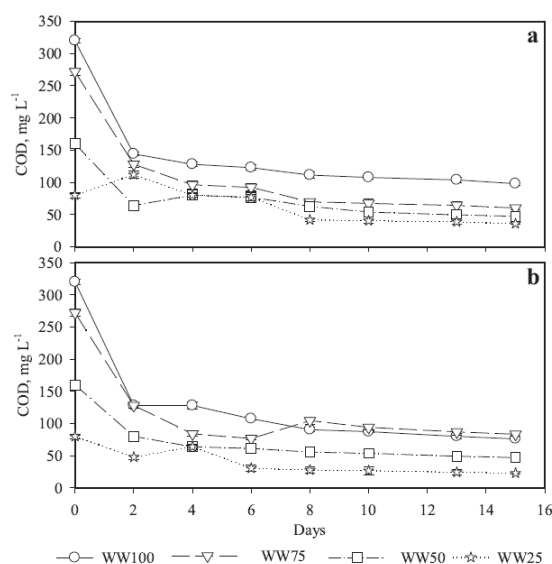


Fig. 4. Effect of different wastewater levels on the COD removal by (a) *C. sorokiniana*, (b) *S. obliquus*.

autotrophic growth of *Chlorella vulgaris* supported by some of the bacteria. Our findings are in accordance with the findings of Ma et al. (2014) who also reported that symbiotic relationships between wastewater born bacteria and algae play a significant role in the nutrient removal. Our findings are also in accordance with his studies that at initial stages, bacteria promotes the nutrient removal.

The performances of both species for COD removal are also compared to gain some insight into their potentials (Fig. 5). Lower COD in effluents after treatment with *S. obliquus* in all cases except WW-75 (Fig. 5a). Similarly, it was able to tolerate the variable COD input and the stresses associated with such variations to maintain a fairly uniform COD removal in all experiments (Fig. 5b). In comparison, COD removal potential varied with the influent COD and the growth conditions for the *C. sorokiniana*, which achieved best COD removal with WW-75 and that declined with WW-50 and WW-25. Similarly it also achieved lower COD removal for raw sewage. These results indicate the superior suitability of *S. obliquus* for COD removal with variable loading despite stressing culture conditions, possibly due to their higher mixotrophic growth potential.

3.4. Removal of nutrients from raw sewage

3.4.1. Removal of nitrogen

Nitrogen present in the raw sewage was predominantly in a reduced form as ammonia, while the post chlorinated effluents had nitrate as the dominating nitrogen form (Table 1). These compositions resulted in the changing levels of different nitrogen species with different wastewaters as per corresponding dilution. Ammonia concentrations in the influent wastewaters (WW-100, WW-75, WW-50, and WW-25) subsequently reduced with dilution (Fig. 6a), while those of nitrite and nitrate increased (Fig. 6b and c respectively). Both *C. sorokiniana* and *S. obliquus* were able to sufficiently remove ammonia from wastewaters due to uptake, with *S. obliquus* showing marginally better potential for such removal (Fig. 6a). In contrast, the treatment with both algal species resulted in an increase of nitrite levels in the final effluent after treatment

Table 2
Comparison of performance between *C. sorokiniana* and *S. obliquus*.

Parameter	<i>C. sorokiniana</i>				<i>S. obliquus</i>			
	WW-100	WW-75	WW-50	WW-25	WW-100	WW-75	WW-50	WW-25
Final Biomass as OD@680 nm	3.16 ± 0.57	3.79 ± 0.18	2.86 ± 0.07	1.31 ± 0.72	3.45 ± 0.36	3.28 ± 0.53	3.81 ± 0.31	1.06 ± 0.34
Culture condition, as F_v/F_m	0.53 ± 0.01	0.57 ± 0.05	0.49 ± 0.02	0.39 ± 0.04	0.48 ± 0.03	0.43 ± 0.02	0.40 ± 0.04	0.27 ± 0.01
% COD removal	69.38 ± 1.81	77.89 ± 2.81	70.49 ± 6.13	55.15 ± 5.63	76.13 ± 1.59	69.31 ± 2.72	070.56 ± 6.35	71.10 ± 6.28
% N-removal	86.93 ± 3.49	89.01 ± 4.28	91.85 ± 5.56	88.92 ± 9.08	98.54 ± 3.30	98.02 ± 4.23	97.29 ± 5.58	95.44 ± 8.77
% P-removal	68.24 ± 11.69	86.25 ± 5.09	96.20 ± 6.72	76.97 ± 12.60	97.99 ± 3.95	92.12 ± 5.28	89.72 ± 6.93	84.65 ± 10.49
% Total coliform removal	99.78 ± 0.12	99.88 ± 0.12	99.88 ± 0.12	99.93 ± 0.12	99.93 ± 0.12	99.98 ± 0.12	99.97 ± 0.12	99.97 ± 0.12
% Fecal coliform removal	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Final pH	11.31 ± 0.14	11.22 ± 0.24	11.21 ± 0.12	11.12 ± 0.28	10.63 ± 0.21	10.58 ± 0.32	10.02 ± 0.41	8.65 ± 0.23
% Lipid yield	22.74 ± 3.11	25.87 ± 2.62	26.11 ± 1.86	27.68 ± 3.12	23.26 ± 3.95	25.34 ± 1.55	26.59 ± 3.12	28.36 ± 2.02

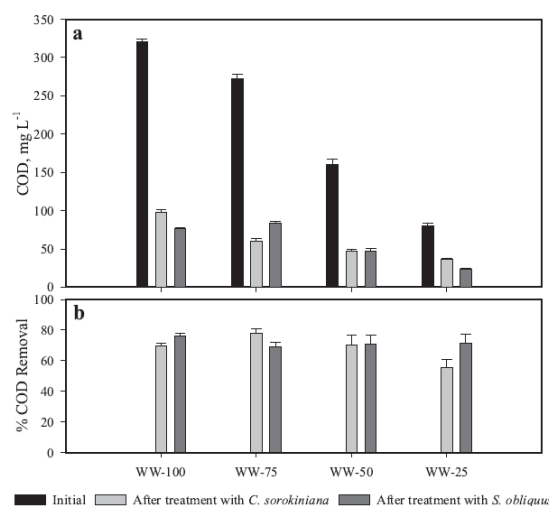


Fig. 5. Comparison of COD removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) absolute COD removal; (b) percent COD removal.

(Fig. 6b). Such increases were significantly higher with *C. sorokiniana* and may be a consequence of more favourable conditions for oxidation of ammonia to nitrite with this species. This speculation is supported by the fact that nitrate levels also increased for WW-100 and WW-75 with *C. sorokiniana* (Fig. 6c) which could also be attributed to nitrification of ammonia. Lorenzen et al. (1998) also observed similar nitrification in the presence of microalgae and attributed it to photosynthetic oxygen. Nitrate was taken up by both species as suggested by their subsequent removals from WW-50 and WW-25. Overall, *S. obliquus* demonstrated higher nitrogen removal potential in comparison to *C. sorokiniana* for all wastewaters (Fig. 6d). In addition, both species were able to maintain their nitrogen removal potential above 85 percent removal with all variations in nutrient levels and growth conditions, with *S. obliquus* showing better removal potentials (Fig. 6e). Similarly high nitrogen removal potential has also been observed by other researchers for both *Chlorella* sp. (Wang et al., 2010) and *Scenedesmus* sp. (Zhang et al., 2008).

The distribution of various nitrogen species was also analyzed after treatment with both species and compared with initial composition in the corresponding wastewater to investigate the postulate of *C. sorokiniana* providing relatively better conditions for ammonia nitrification to nitrite and nitrate (see Fig. 7). Such conditions represent better availability of photosynthetic oxygen, and indicate the corresponding photosynthetic efficiencies of the

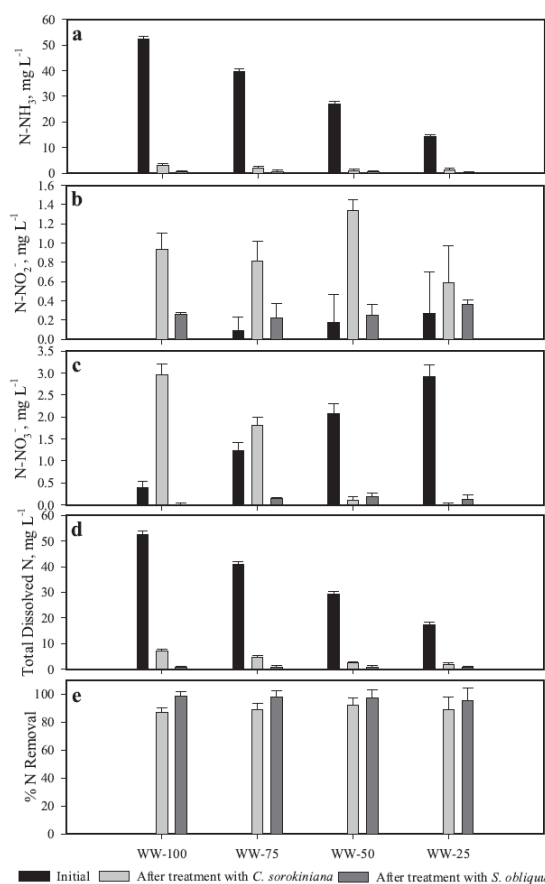


Fig. 6. Comparison of N-removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) N-NH_3 removal; (b) N-NO_2^- removal; (c) N-NO_3^- removal; (d) total dissolved N-removal; (e) percent N-removal.

concerned algal species (Lorenzen et al., 1998). The initial composition of wastewaters had ammonia as the dominant nitrogen species whose contribution declined from 99.24% for WW-100 to 81.65% for WW-25; and the nitrate levels increased with the dilution of raw sewage from 0.76% for WW-100 to 16.81% for WW-25. For a particular wastewater, the distribution of various nitrogen species in the effluent after treatment with both *C. sorokiniana* and *S. obliquus* are also quantified. In general,

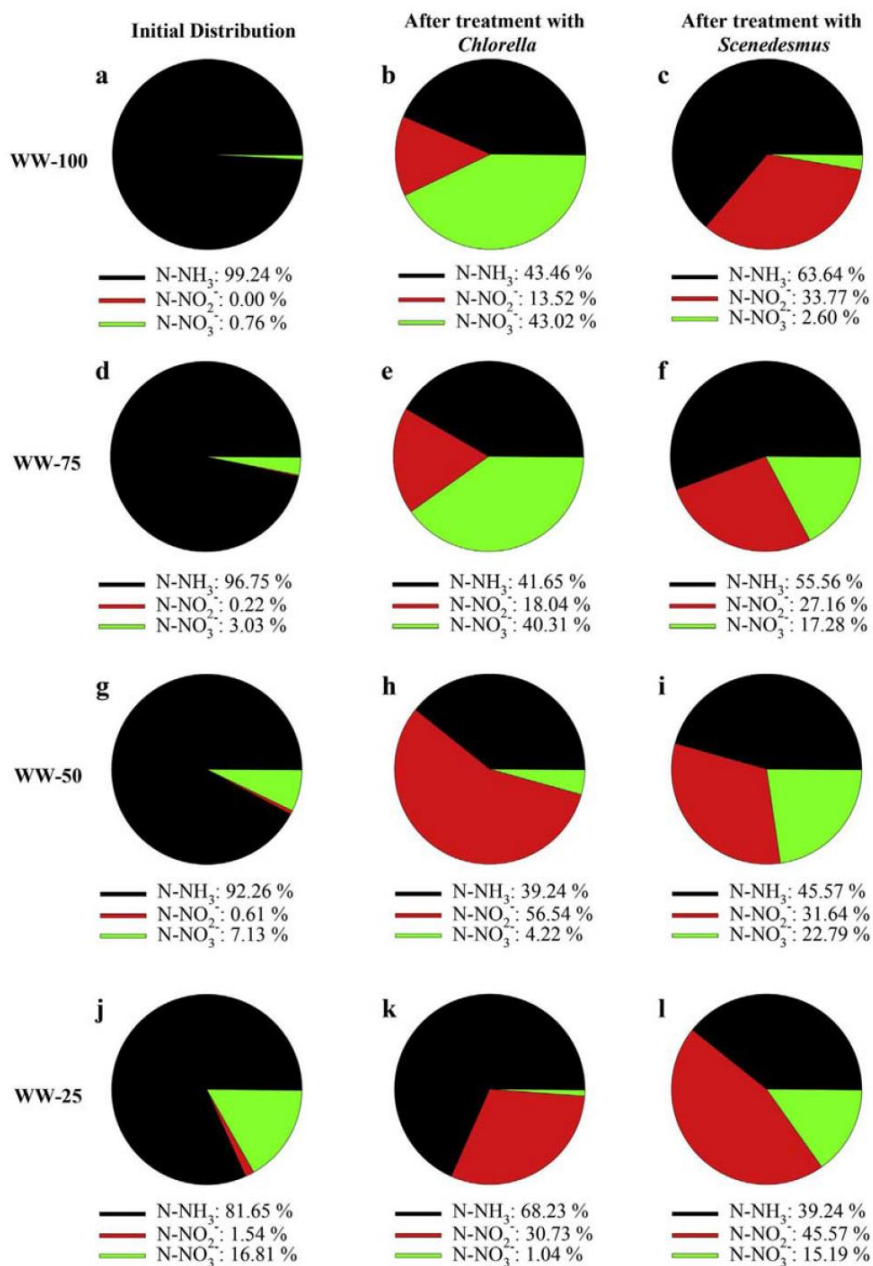


Fig. 7. Effects of treatment with *C. sorokiniana* and *S. obliquus* on the distributions of various N-species in the wastewater.

treatment with *C. sorokiniana* resulted in a more nitrified effluent with lesser ammonia and higher nitrate contributions in comparison to *S. obliquus*. The anomaly in this is observed for WW-25, where *S. obliquus* resulted in an effluent with lesser ammonia and higher nitrate and nitrite. Since, cultivation with WW-25 resulted in a stressed culture of both species after 10 days of treatment, and the absolute levels of these nitrogen species in the treated

effluents are very low, and the anomaly could be a consequence of either stressed culture behaviour or experimental errors in measuring such low nitrogen levels and their distributions. These observations qualitatively suggest a higher photosynthetic efficiency and better oxidative conditions during treatment with *C. sorokiniana*, whilst better nitrogen removal is achieved with *S. obliquus*.

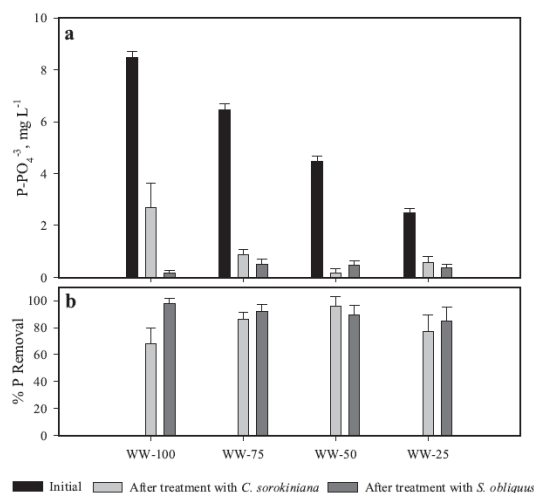


Fig. 8. Comparison of P-removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) absolute P-removal; (b) percent P-removal.

3.4.2. Removal of phosphorus

Phosphorus was present in raw sewage as orthophosphate. Similar to nitrogen, phosphorus uptake has also been widely reported for these algae species (Wang et al., 2010; Zhang et al., 2008). Phosphate removal is compared for all wastewaters to gain insights into the potential of *C. sorokiniana* and *S. obliquus* (Fig. 8). Treatment with *C. sorokiniana* resulted in lowest phosphorus removal efficiencies from raw sewage ($68.24 \pm 11.69\%$ P-removal). The stressed growth of *C. sorokiniana* and the need for acclimatization to raw sewage may have contributed to the lower removal

efficiency. Phosphorus removal potentials increased with more favourable growth conditions and reduced stress levels of the culture with increasing dilutions and highest removal efficiency ($96.20 \pm 6.72\%$) was achieved for WW-50. Removal efficiencies again declined for WW-25 which could be due to stressed growth of *C. sorokiniana* with low nutrient content of this wastewater as evident in reduced growth and increased stresses. In contrast, *S. obliquus* achieved similar P-removal efficiencies with all wastewaters. In addition, *S. obliquus* demonstrated better phosphorus removal efficiencies when compared to *C. sorokiniana*.

3.5. Removal of coliforms from raw sewage

The application of both algal species during treatment resulted in the removal of coliforms from the wastewater (Fig. 9). Pathogen removal has been observed with prolonged algal cultivation such as in maturation ponds (Oswald, 1990). Treatment with *C. sorokiniana* resulted in removal efficiencies of total coliforms from $99.78 \pm 0.12\%$ for WW-100 to $99.93 \pm 0.12\%$ for WW-25 (Table 2). In comparison, application of *S. obliquus* achieved total coliforms removals from $99.93 \pm 0.12\%$ for WW-100 to $99.97 \pm 0.12\%$ for WW-25 (Fig. 9a). Complete removal of faecal coliforms was achieved for all wastewaters with both species (Fig. 9b). These results indicate the applicability of both species to achieve a high level of pathogen removal during wastewater treatment without any additional process such as chlorination, with *S. obliquus* showing marginally better potential for pathogen removal. Cultivation of both species in wastewater resulted in the increase in pH to values above 10 (Fig. 10). The removal of coliforms can be postulated to be due to such high levels of pH which are reported to have antibacterial effects on pathogens. In addition, various metabolites excreted from these algae are also reported to have bactericidal effects on pathogens (Kümmerer, 2008). To compare the dominating reason for such coliforms removal, the effects of increase in pH without any algae on coliforms were also studied. Such increased pH in

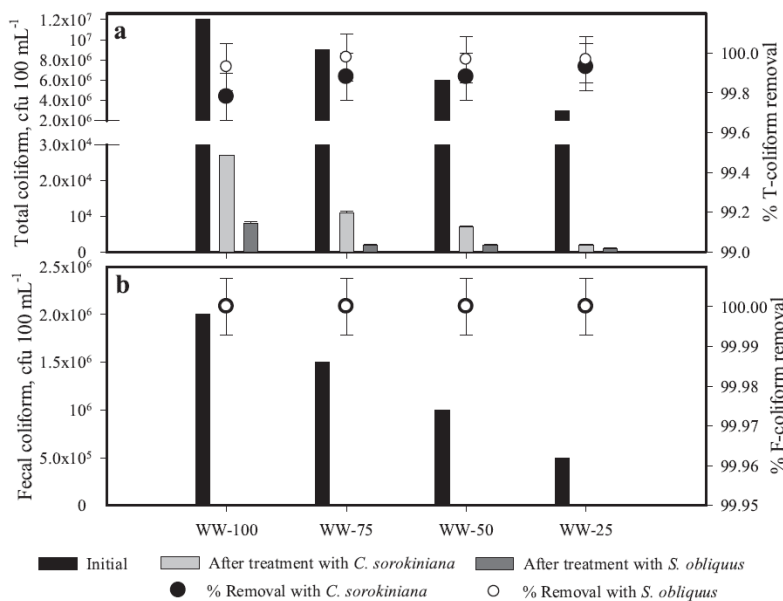


Fig. 9. Comparison of pathogen removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) total coliforms removal; (b) fecal coliforms removal.

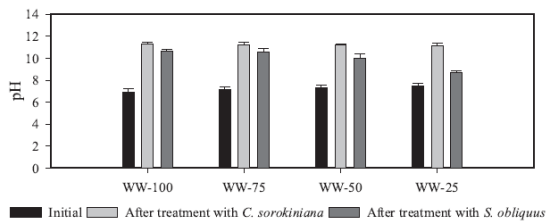


Fig. 10. Effects of treatment with *C. sorokiniana* and *S. obliquus* on the pH.

wastewaters did not result in substantial reduction of coliforms (data not shown). This suggests for the bactericidal effects of algal metabolites as the dominating factor for pathogen removal in comparison to rise in pH in the present study.

3.6. Lipid production

Algal biomass after experimental durations was used for lipid extraction. The final yields of lipids for both *C. sorokiniana* and *S. obliquus* with all wastewaters are presented in Fig. 11. In addition, the lipid production with BG11 media is also compared as a control for nutrient rich cultivation conditions. Both *C. sorokiniana* and *S. obliquus* accumulated lipids ($14.17 \pm 1.72\%$ and $15.82 \pm 2.23\%$ respectively) in BG11 media. The cultivation of these species with different wastewaters resulted in a significant (ANOVA, 95% confidence level) increase in lipid accumulation with maximum yield being achieved with WW-25 for both *C. sorokiniana* ($27.68 \pm 3.12\%$) and *S. obliquus* ($28.36 \pm 2.02\%$). Since, the nutrient levels in BG11 are higher than in actual wastewaters, the cultivation of algae with real wastewater provided relatively nutrient deprived conditions and resulted in nutrient stress on algal cell. Such stresses are extensively reported to increase the lipid accumulation in the microalgae cell (Singh et al., 2014; Xin et al., 2010; Zhang et al., 2013). Other researchers have also observed higher lipid accumulation by algae with real wastewater in comparison to BG11 medium (Ramanna et al., 2014). The successive dilution of wastewater resulted in nutrient stressed conditions and hence the highest lipid accumulation was observed with WW-25 with maximum such stress, although the effect of dilution was not significant. *C. sorokiniana* and *S. obliquus* both have been reported to accumulate such high levels of lipid under nutrient stress (Guldhe et al., 2014a; Ramanna et al., 2014). The lipid accumulation potential was comparatively higher for *S. obliquus* and may be a consequence of

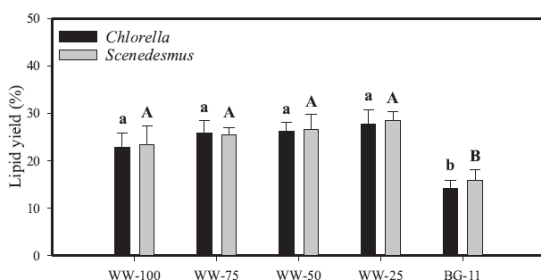


Fig. 11. Effects of different wastewater levels on the lipid yields of *C. sorokiniana* and *S. obliquus*. Letters over individual bars represent results of 1-way ANOVA at 95% confidence level. Small letters indicate ANOVA results for *C. sorokiniana*, while capital letters indicate those for *S. obliquus*. Different letters represent significant difference.

increased stress levels in the cell at similar cultivation conditions than *C. sorokiniana*.

4. Conclusion

This study investigated the role of *C. sorokiniana* and *S. obliquus* in achieving a comprehensive treatment of filtered raw domestic sewage and lipid accumulation in biomass. *S. obliquus* demonstrated better overall nutrients and pathogen removal potentials, as well as lipid accumulation in comparison to *C. sorokiniana*. However, prolonged growth on raw sewage resulted in stressed culture and which might affect the overall productivity in long term applications and hence the treatment potentials. *C. sorokiniana* demonstrated better stress resistance under similar cultivation conditions, and hence might be a suitable candidate for achieving such objectives in the long term without appreciable effects on the culture physiology. Furthermore, the additional requirement of water for dilution to provide acceptable growth conditions with *S. obliquus* might be a cost intensive alternative in comparison to *C. sorokiniana*.

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3.2 Paper II

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Lipid extracted algae as a source for protein and reduced sugar: A step closer to the biorefinery



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HIGHLIGHTS

- Lipid extracted algae (LEA) was used as a source for protein and reduced sugars.
- Comparable yields of these products were obtained from total algae and LEA.
- Microwave assisted extraction from oven dried samples provided highest lipid yield.
- Effective cell disruption for lipid extraction increased loss of other products.
- Maximizing the yields of all products requires proper process selection.

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ABSTRACT

The objective of this study was to investigate the feasibility of using lipid extracted algae (LEA) as a source for protein and reduced sugar, and the effects of various procedural treatments on their yields. LEA provided comparable yields of protein and reduced sugars to those from total algae. Oven drying provided highest yields of all products followed by freeze drying, while sun drying significantly lowered their yields. Effective cell disruption by microwave and autoclave increased the lipid yields from algae, but resulted in increased loss of other compounds with lipid extracting solvents lowering their yields during sequential extraction. Relatively inefficient cell disruption by ultrasonication and osmotic shock lowered the amount of cell protein lost to the lipid extracting solvents. These results highlight the complexity of concurrent extraction of all value added products from algae, and the need for proper selection of the processes to achieve the objectives of integrated biorefinery.

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1. Introduction

Microalgae have drawn much attention in past few decades due to their nutrient removal potential from wastewater (Di Termini et al., 2011; Sawayama et al., 1998). In addition, microalgae are considered to be important resources for biofuels and a viable alternative to limited fossil fuels due to their lipid accumulation potential (Morweiser et al., 2010; Tsukahara and Sawayama, 2005). Recent efforts have focussed on achieving the dual objectives of wastewater treatment and biofuels production from microalgae (Park et al., 2011; Wu et al., 2012). However, algal biofuels are yet to achieve economical sustainability (Lundquist et al., 2010).

In addition to lipids, microalgae also produce other compounds of great economic value (Olguín, 2012). Algal proteins are an acceptable alternative of conventional food supplements due to

their nutritional value and amino acid profiles (Becker, 2007). Similarly, algal polysaccharides can be hydrolyzed to reduced sugars which have great application in the production of bioethanol (Fu et al., 2010; Sun and Cheng, 2002). The use of residual algal biomass after lipid extraction for other applications can reduce the cost of algal cultivation and biofuel production (Rashid et al., 2013). The concurrent extraction of other valuable products in addition to lipids from algal biomass may result in optimal value extraction and economically beneficial algal technology. This is an important objective of integrated algal biorefinery approach for algal biofuels technology (Subhadra, 2010).

The application of residual algal biomass after lipid extraction has been investigated by several researchers for different objectives. These objectives can broadly be divided in two categories: first for energy production by utilizing the remaining carbon and hydrogen, and secondly for extracting products for their nutritional and economical values. Zhu et al. (2013) studied the hydrothermal liquefaction potential of lipid extracted algae (LEA) for their

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conversion to liquid fuels. Similarly, Yang et al. (2011) developed a two-stage process to produce hydrogen and methane gases from LEA. The potential of using LEA as a protein source in animal feeds was also established (Ju et al., 2012; Lodge-Ivey et al., 2014). Geun Goo et al. (2013) investigated the application of LEA for bioethanol production. The extraction of valuable compounds from LEA was found to lower the cost of biofuel production in comparison to the utilization of carbon and hydrogen in residual mass for energy production (Gao et al., 2012).

A major consideration in the concurrent extraction of all valuable products from algae is the effect of various treatments on the individual yields of such products. The algal biomass undergoes many processes during lipid extraction which invariably affects these coproducts. Theoretically, lipid extraction should not result in any loss of other cellular compounds resulting in their fractions in algal mass to increase in the LEA in comparison to whole cell algae, and thus increasing their yield (% w/w) in LEA. However, under realistic conditions, some loss of these compounds is unavoidable as cell disruption would unbind them from cell mass to some degree and the unbound fraction could be lost to the applied solvent during lipid extraction. For example, Lam et al. (2014) found reduction in the carbohydrate content by 7.2% in the algal biomass after lipid extraction in comparison to whole cell algae. The additional processes used for extracting individual products also have their process limitations, for example hydrolysis of polysaccharides to reduced sugars requires sufficient reaction time for achieving good yields (Fu et al., 2010). It is therefore important to investigate the effects of various treatments during lipid extraction and additional processes for individual product extraction on the final yields of these products. Comparison of yields from whole cell algae in order to establish the feasibility of using LEA as a source of these compounds is also beneficial. Studies investigating the effects of various treatments on product yields from LEA are not available in the literature to the best of our knowledge. This study, investigates the effects of various procedural treatments, i.e. drying and cell disruption, during extraction of lipids, and subsequently protein and reduced sugars from LEA on the product yields and comparison to yields from whole cell algae to identify the optimal treatments and establish the feasibility of LEA as a source for these products. Cell disruption by four methods: microwave, ultrasonication, autoclaving, and osmotic shock with 10% NaCl were investigated. In addition, main drying processes applied in literature include sun drying, oven drying, freeze drying, drum drying, spray drying, fluidized bed drying etc. However, not all of these are economically sustainable (Brennan and Owende, 2010; Guldhe et al., 2014). Hence in this study, focus has been on investigating the effects of sun drying, oven drying, and freeze drying processes, which are more economic in nature.

2. Methods

2.1. Algae culture

Scenedesmus obliquus (Genbank accession number: FR751179.1) used in this study was isolated from Durban region, KwaZulu Natal, South Africa (Misra et al., 2014). A raceway pond of 300,000 L was operated with BG11 medium for algal cultivation under natural sun light ($400\text{--}1200\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) and temperature ($18\text{--}27\ ^\circ\text{C}$). The grown biomass was harvested by gravitational settling and then centrifuged to obtain thick algal slurry.

2.2. Drying of the harvested algae

The thickened biomass slurry was dried by three different methods: (a) sun drying, (b) oven drying, and (c) freeze drying.

Sun dried biomass was obtained by placing the thickened slurry on a drying bed lined with $1500\ \mu\text{m}$ white plastic for three days under natural sunlight and ambient conditions ($400\text{--}1200\ \mu\text{mol m}^{-2}\text{ s}^{-1}$, $18\text{--}27\ ^\circ\text{C}$). Similarly algal slurry was placed in a hot air oven at $60\ ^\circ\text{C}$ for 24 h for oven drying. A freeze dryer (Mini Lyotrap, LTE Scientific Ltd., United Kingdom) was used to lyophilize the samples after overnight freezing at $-84\ ^\circ\text{C}$ in a bio-freezer (Glaciar NU9668E, Nuaire, Japan). Dried biomass was pulverized with mortar and pestle and stored in a desiccator.

2.3. Lipid extraction assisted by cell disruption

Dried biomass of *S. obliquus* was further subjected to various cell disruption procedures to increase the efficiency of solvent based lipid extraction. Four cell disruption methods were investigated: (a) microwave, (b) ultrasonication, (c) autoclave, and (d) osmotic shock with 10% NaCl. Total lipids were extracted by the method of Folch et al. (1957) using 2:1 (v/v) mixture of chloroform and methanol.

For microwave assisted lipid extraction, 1 g of dried biomass was mixed with 20 mL of solvent mixture (chloroform and methanol in 2:1 ratio) and heated at $100\ ^\circ\text{C}$ for 10 min at 1000 W in a microwave digester (Milestone S.R.L., Italy; 1200 W of output power). Solvent containing lipids was separated by centrifugation and then vacuum filtered. After such separation from cell debris, solvent was evaporated in oven at $60\ ^\circ\text{C}$. The remaining total lipids were quantified gravimetrically. Similarly during ultrasonication, 20 mL of solvent mixture was added to the 2 g of dried biomass in a 50 mL centrifuge tube, after which the entire mixture was sonicated for 2 min at 15 kHz (Misonix XL-2000-010; 100 W of output power, 22.5 kHz of output frequency). The mixture was centrifuged and supernatant transferred to a separate tube. A further 20 mL of the solvent mixture was added to the centrifuged cell debris, sonicated and supernatant separated by centrifugation and mixed with previously recovered solvent. The pooled solvent was vacuum filtered and evaporated in oven at $60\ ^\circ\text{C}$ to recover the extracted lipids. Autoclaving was also investigated as a cell disruption procedure in which 500 mg of dried biomass was added with 50 mL of ultrapure water and autoclaved at $121\ ^\circ\text{C}$ and 15 lbs for 5 min (Prabakaran and Ravindran, 2011). The autoclaved sample was mixed with extracting solvent mixture in 1:1 ratio and transferred to a separatory funnel after mixing for 5 min, where two layers formed. The lipid containing layer was carefully removed and lipids were recovered after drying in oven at $60\ ^\circ\text{C}$. Osmotic shock with 10% NaCl also results in cell disruption and accessibility to intracellular content due to changing osmotic pressures on both sides of cell walls. 500 mg of dried biomass was mixed with 50 mL of 10% NaCl solution and vortexed (VM-300, Gemmy, Taiwan) for 1 min, after which the biomass was left suspended in NaCl solution for two days. After two days, this suspension was added to lipid extracting solvent mixture in 1:1 ratio and transferred to a separatory funnel after 5 min of mixing. The distinct layer containing lipids was carefully removed and evaporated at $60\ ^\circ\text{C}$ in oven to recover lipids. After each extraction, the lipid mass was quantified gravimetrically and lipid yields (% w/w) of the processes were calculated.

The algal biomass remaining after lipid extraction was vacuum filtered and air dried at room temperature to obtain lipid extracted algae (LEA) from which proteins and reduced sugars were extracted. This recovered biomass was quantified and utilized in calculating the subsequent yields from LEA.

2.4. Protein extraction from LEA

Dried whole cell algae or LEA biomass was subjected to protein extraction as per Lowry method (López et al., 2010; Lowry et al.,

1951). All reagents were prepared as per the procedure outlined by López et al. (2010). The actual procedure was subjected to minor modifications to optimize the protein extraction in the present study. 200 mg of different dried LEA or whole cell algae samples were added to 25 mL of lysis buffer solution. The mixture was ground using a mortar and pestle for 5 min, and subsequently vortexed for 2 min. The mixture was then centrifuged at 3000 rpm for 10 min, and supernatant decanted into a separate tube. 25 mL lysis buffer was added to the pellet and again ground, vortexed, and centrifuged. Both supernatants were pooled and mixed. 0.5 mL of this supernatant was mixed with 0.5 mL of SDS solution in a test tube and vortex mixed. Subsequently 5 mL of reagent-C was added in the mixture and vortex mixed. The solution was left to rest for 10 min, before 0.5 mL of Folin reagent was added in the test tube and mixed. Absorbance of this mixture was measured at 750 nm using a spectrophotometer (Spectroquant Pharo 300, Merck) after 30 min of sample hold. Bovine serum albumin (BSA) was dissolved in lysis buffer and this solution was used to develop calibration curve for protein quantification, and the protein yields (% w/w) were calculated from the standard curve (López et al., 2010).

2.5. Extraction of reduced sugars from LEA

Polysaccharides present in the whole cell algae as well as LEA were hydrolyzed to reduced sugars using H_2SO_4 and autoclaving (Sun and Cheng, 2002). 500 mg of dried algae or LEA were added to 50 mL of H_2SO_4 (2% v/v) in a 100 mL conical flask. Hydrolysis was achieved by autoclaving this mixture at 121 °C for 30 min. Flasks were removed and cooled to room temperature under running tap water. The hydrolyzed mixture was neutralized with 0.1 M NaOH/ H_2SO_4 . Total reducing sugars were quantified using the DNS method (Miller, 1959). Minor modifications were incorporated to optimize the yield in the present study. 1 mL of hydrolyzed sample mixture was added with 1 mL of DNS reagent in a test tube. These tubes were capped and heated to 100 °C by keeping in boiling water for 10 min. Samples were cooled to room temperature and 8 mL of ultrapure water was added to each test tube. Absorbance of the sample was measured at 540 nm using a spectrophotometer (Spectroquant Pharo 300, Merck). Calibration was done using glucose solutions of different concentrations (Fu et al., 2010).

2.6. Chemicals and reagents

All chemicals and solvents were purchased from Sigma Aldrich, USA and were of analytical/HPLC grade. All solutions were prepared with ultrapure water (Aqua MAX Ultra 370, Younglin, Korea).

2.7. Statistical analysis

All analyses were performed in triplicate. Two-way ANOVA was carried out to investigate the effects and interactions of various treatments on product yields. Tukey's honestly significant differences (HSD) test was used for post hoc analysis of the results.

3. Results and discussion

3.1. Effects of drying and cell disruption procedures on the lipid yield

The extracted lipids from *S. obliquus* have been demonstrated to be suitable for biodiesel conversion based on their fatty acid profile and saponification value (Mandal and Mallick, 2009). *S. obliquus* strain used in this work has also been previously demonstrated to contain suitable composition and characteristics for biofuel conversion (Guldhe et al., 2014). However, the present study found the

lipid yields from *S. obliquus* to significantly depend on various drying as well as cell disruption methods applied during extraction. The resulting lipid yields after drying and cell disruption are presented in Fig. 1. Lipid yields significantly ($P < 0.05$) varied with different treatments and ranged from $8.78 \pm 2.15\%$ for sun dried samples assisted with cell disruption by osmotic shock with 10% NaCl solution, to $21.43 \pm 1.52\%$ for freeze dried samples with microwave assisted cell disruption. Mata et al. (2010) have also reported similar lipid yields for *Scenedesmus* sp. Microwave assisted extraction resulted in significantly ($P < 0.05$) higher lipid yields for all dried samples in comparison to other selected methods, and osmotic shock with 10% NaCl solution resulted in lowest lipid yields. In addition, sun dried samples had the significantly lower ($P < 0.05$) lipid yields than all the selected drying techniques.

The interactions between and within these two different treatments of drying and cell disruption were analyzed with two-way ANOVA at 95% confidence level, and results are presented in Table 1. Variations in drying methods significantly affected the lipid yields ($P = 0.005$). Similarly variability in cell disruption processes also had significant effects on the lipid yield ($P < 0.001$). No significant interaction between these two factors of drying and cell disruption was observed ($P = 0.477$). Post-hoc analysis with Tukey's test was carried out to perform pairwise comparisons within each group to investigate their impacts on lipid yields. Different drying processes were compared to all cell disruption procedures. Least square means were calculated to be 11.92 for sun dried samples, 14.19 for oven dried samples, and 15.07 for freeze dried samples with a standard error of 0.63. These comparisons

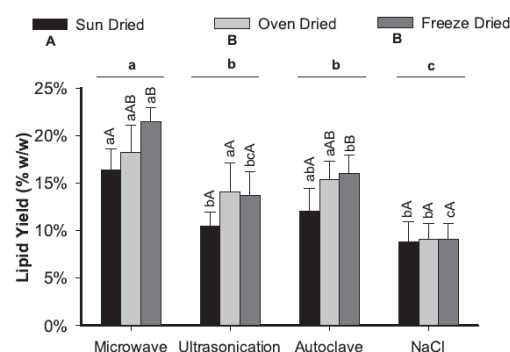


Fig. 1. Effects of different drying and cell disruption procedures on the lipid yields of *S. obliquus*. Small normal face letters signify level of significance between different cell disruption treatments for an individual drying process. Small bold face letters signify significance level between different cell disruption treatments for overall drying effect. Capital normal face letters signify level of significance between different drying methods for an individual cell disruption process. Capital bold face letters signify significance level between different drying processes for overall cell disruption effect. Processes followed by same letters have no significant difference (2-way ANOVA, Tukey's test, $P < 0.05$).

Table 1

Results of two-way ANOVA for different drying and cell disruption procedures on lipid yield from *S. obliquus*.

Source of variation	DF	SS	MS	F	P
Drying process	2	63.426	31.713	6.722	0.005
Cell disruption process	3	434.251	144.75	30.684	<0.001
Drying × cell disruption	6	26.984	4.497	0.953	0.477
Residual	24	113.22	4.717		
Total	35	637.881	18.225		

DF: degree of freedom; SS: sum of square; MS: mean of square; F: likelihood ratio; P: probability

are summarized in Fig. 1. Sun drying resulted in significantly lower lipid yields when compared to oven drying ($P = 0.044$) and freeze drying ($P = 0.005$), while no significant difference in yields was observed for oven dried and freeze dried samples ($P = 0.588$). Similar comparisons for different cell disruption procedures were also performed for all drying processes. These processes had least square means of 18.67 for microwave, 12.77 for ultrasonication, 14.48 for autoclaving, and 9.00 for osmotic shock with 10% NaCl, with an associated standard error of 0.72. Samples undergoing cell disruption with NaCl showed significantly lower lipid yields than the other three processes. Microwave assisted cell disruption resulted in significantly higher lipid yields than the other methods. No significant difference was observed between ultrasonicated and autoclaved samples for such yield ($P = 0.361$). Guldhe et al. (2014) also found better lipid yields with microwave assisted extraction in comparison to ultrasonication for *Scenedesmus* sp.

Fig. 1 also depicts the pair wise comparisons of effects of variability within both treatment groups for each individual interaction on lipid yields. For sun dried samples, microwave cell disruption resulted in significantly higher lipid yields in comparison to ultrasonication and NaCl assisted extraction. However, no significant difference in yields was observed in sun dried samples with microwave and autoclave assisted extractions ($P = 0.099$). Lipid yields of ultrasonication, autoclaving, and NaCl treatment were statistically similar. In comparison, for oven dried samples, microwave assisted extraction resulted in significantly higher yields ($P < 0.001$) from the NaCl assisted process only, while no significant differences were observed in comparison to ultrasonication ($P = 0.123$) and autoclaving ($P = 0.392$). Yields for oven dried samples with NaCl were also significantly lower than ultrasonicated ($P = 0.044$) and autoclaved ($P = 0.009$) samples, while autoclaved and ultrasonicated oven dried samples resulted in statistically similar lipid yields ($P = 0.896$). For freeze dried samples, microwave cell disruption provided significantly higher lipid yields than other processes, while NaCl assisted process had significantly lower yields than other processes with the exception of ultrasonication. Autoclaving and ultrasonication did not show any significant difference in the yields ($P = 0.582$). A pairwise comparison between different drying methods showed a significant difference in lipid yields of microwave assisted extraction of sun dried and freeze dried samples only ($P = 0.023$).

These results highlight the inefficiency of sun drying process in comparison to oven or freeze drying. In addition, the longer drying period with sun drying exerts huge land requirement for complete drying in comparison to other processes, and which might be a constraint during full scale application. Widjaja et al. (2009) also reported oven drying at 60 °C and freeze drying to provide best lipid extraction from *Chlorella vulgaris*. Careful selection of drying methods is important because oven and freeze drying are both energy intensive processes (Guldhe et al., 2014) and may have higher operating costs during long term full scale application. However, higher lipid yields were achieved by these two methods in this study, and which might also be an important factor to selecting the most appropriate drying method.

Another factor which governed the final lipid yield from algae was the cell disruption procedure assisting the solvent extraction of lipids. Disruption processes result in greater contact between solvent and the cellular lipid than with solvent only and have been found to be more efficient. Microwave, ultrasonication, autoclaving, and osmotic shock are the most common cell disruption methods used for algae (Prabakaran and Ravindran, 2011). Microwave assisted extraction resulted in significantly higher lipid yields among all dried samples in the present study. Higher lipid yields with microwave in comparison to other cell disruption processes are also reported for *Botryococcus* sp., *C. vulgaris*, and *Scenedesmus* sp. (Lee et al., 2010). In addition, they also concluded that

microwave assisted lipid extraction of oven dried samples enabled maximum lipid recovery for *Scenedesmus* sp. which is similar to the findings of the present study. Guldhe et al. (2014) also observed microwave assisted lipid extraction to provide higher yields than ultrasonication. The efficient heating of the whole sample with microwave resulted in better cell disruption, solvent movement, and hence lipid extraction efficiencies (Iqbal and Theegala, 2013). In comparison, osmotic shock with 10% NaCl resulted in lower lipid yields and which may be a consequence of insufficient cell disruption achieved using this method. The longer time required with osmotic shock (two days) is also a limitation of this process.

3.2. Extraction of protein from lipid extracted algae (LEA)

The algal biomass after extraction of lipids was further utilized to recover protein and reduced sugars. Protein contents (% w/w) of lipid extracted algal biomass should theoretically increase in comparison to whole cell algae with their increased fraction to account for the removed mass of extracted lipids. However, the cell disruption and solvent application during lipid extraction also results in loss of some protein from the biomass reducing the total amount available for extraction. Hence it is important to investigate and compare the protein yields from such lipid extracted algae (LEA) to establish their applicability as a protein source. Fig. 2 presents the protein yields from such lipid extracted algae with different treatments. Similar yields from whole cell algae without lipid extraction were also determined, and are presented in Fig. 2. Sun dried samples of *S. obliquus* without any lipid extraction resulted in the protein yield of $41.13 \pm 1.64\%$, while significantly higher ($P < 0.05$) yields of $51.42 \pm 1.01\%$ for oven dried and $51.19 \pm 0.81\%$ for freeze dried intact samples were achieved. Becker (2007) reported protein contents for *S. obliquus* to be around 50–56% which are similar to those obtained in the present study. LEA demonstrated sufficient protein recovery yields when compared to whole cell algae. Protein yields in LEA depended on the drying and cell disruption procedures for lipid extraction. The highest yield of $58.03 \pm 1.29\%$ was observed for oven dried samples assisted with ultrasonication while sun dried samples with autoclaving resulted in the lowest protein yield of $37.51 \pm 1.57\%$.

The effects of different treatments on protein yields were analyzed, and such yields were significantly affected by both different drying ($P < 0.001$) and cell disruption ($P < 0.001$) procedures

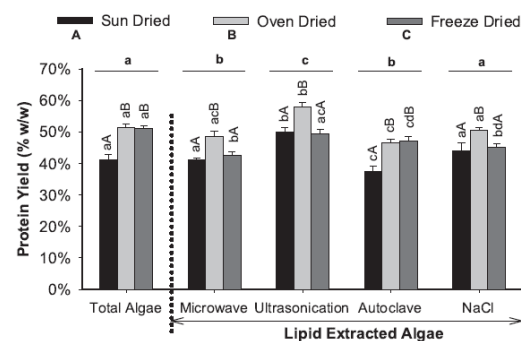


Fig. 2. Effects of different drying and cell disruption procedures on the protein yields of lipid extracted *S. obliquus*. Small normal face letters signify level of significance between different cell disruption treatments for an individual drying process. Small bold face letters signify significance level between different cell disruption treatments for overall drying effect. Capital normal face letters signify level of significance between different drying methods for an individual cell disruption process. Capital bold face letters signify significance level between different drying processes for overall cell disruption effect. Processes followed by same letters have no significant difference (2-way ANOVA, Tukey's test, $P < 0.05$).

(Table 2). In addition, a significant interaction ($P < 0.001$) was also observed between these two treatments. Further pair wise comparison was also carried out with post hoc Tukey's test (Fig. 2). For different drying processes, least square means were calculated as 42.79 for sun drying, 51.02 for oven drying, and 47.09 for freeze drying with a standard error of 0.36. Oven drying resulted in significantly higher protein yields ($P < 0.001$) than other drying methods among all cell disruption processes. Similarly, protein yields in sun dried samples were significantly ($P < 0.001$) lower than other methods. These comparisons were also performed for different cell disruption procedures to compare their effects on the protein yields. Least square mean of 47.91 was calculated for intact samples of whole cell algae without any cell disruption. Means were calculated for LEA samples as 44.11 for microwave, 52.46 for ultrasonication, 43.75 for autoclaving, and 46.53 for NaCl assisted cell disruption. Standard error of such means was calculated as 0.46. Ultrasonicated LEA samples had a significantly higher ($P < 0.001$) protein yield than whole cell algae. Such protein yields were significantly reduced for microwave ($P < 0.001$) and autoclave ($P < 0.001$) assisted LEA samples when compared to the yields from intact samples of whole cell algae. Osmotic shock with 10% NaCl resulted in LEA which had similar protein yields to whole cell algae ($P = 0.245$). From all the cell disruption procedures, ultrasonication favored highest protein yields after lipid extraction, while such yields significantly reduced for both microwave and autoclave assisted cell disruption.

Effects of variability on protein yields for each individual interaction within both treatment groups are also compared in Fig. 2. For sun dried samples, ultrasonicated LEA had significantly higher ($P < 0.001$) protein yield while autoclaved LEA showed significantly lower ($P = 0.026$) protein yield than whole cell algae. No significant differences were observed between yields of microwave and NaCl assisted LEA and yield of whole cell algae. Ultrasonication assisted LEA also showed significantly higher ($P < 0.001$) protein yields than whole cell algae for oven dried samples. Similarly autoclaving significantly lowered ($P = 0.002$) protein yield than whole cell algae. For freeze dried samples, whole cell algae showed significantly higher protein yields than LEAs assisted with autoclave ($P = 0.01$), microwave ($P < 0.001$), or NaCl ($P < 0.001$); while ultrasonicated LEA had comparable ($P = 0.533$) yields to whole cell algae. The effects of different drying methods with each cell disruption procedure is also compared in Fig. 2. Sun dried LEA as well as whole cell algae samples had significantly lower ($P < 0.001$) protein yield than oven dried samples for all cell disruption methods. Similar yields of sun dried samples were lower ($P < 0.001$) than freeze dried samples for whole cell algae and autoclaved LEA only. Oven drying also produced higher yields than freeze drying for microwave, ultrasonication, and NaCl assisted LEA.

These results further highlight the limitation of sun drying, since sun dried samples had significantly lower protein yields for both LEA and whole cell algae. In comparison, oven drying resulted in significantly higher protein yields. Since oven drying is an energy intensive process, higher yields of lipids and proteins obtained should be helpful in making such oven drying more cost effective in nature. The protein yields of different LEA were also

compared with whole cell algae, and ultrasonication was found to significantly increase yields, while microwave and autoclave reduced the protein yields. When these results are compared with those for lipid extraction, some important insights are gained into the dynamics of these processes. During lipid extraction, microwave and autoclave assisted processes had higher yields than ultrasonication and osmotic shock (Fig. 1). However, the increased cell disruption associated with microwave and autoclave during lipid extraction resulted in increased loss of protein from the disrupted cell to the solvents as higher amounts of protein became unbound and were washed with lipid extracting solvents. Though these processes resulted in the higher lipid yields, the loss of protein with applied solvent for lipid extraction reduced the protein yields from such LEA. This is further supported by the fact that ultrasonication and osmotic shock which resulted in relatively lower cell disruption and hence lower lipid yields provided higher protein yields from their LEA since the loss to the solvents was reduced. Safi et al. (2014) investigated the effects of different cell disruption methods on the extraction of protein from whole cell algae without any lipid extraction and found the protein yields to increase from manual grinding to high pressure homogenization with intermediate yields from ultrasonication and chemical treatment. These yields also follow the trends observed with lipid extraction in the present study and are a consequence of better cell disruption and availability of cellular unbound protein to the extracting solvent. Despite the loss in LEA for different cell disruption procedures, sufficient protein yields were still obtained thus establishing the feasibility of extraction of these coproducts from algae. In addition, the results also demonstrate the complexity of the objective to optimize the extraction of different products using an integrated algal biorefinery approach. The proper selection and optimization of processes involved is important for simultaneously optimizing the yields of these products.

3.3. Extraction of reduced sugar from lipid extracted algae (LEA)

Similar to protein, LEA are also rich in polysaccharides. Such polysaccharides can be hydrolyzed to reduced sugars which can be extracted for their application in bioethanol production (Sun and Cheng, 2002). Miranda et al. (2012) reported sufficient carbohydrate content from *S. obliquus*. In addition, they also characterized its composition after reduction, and observed glucose as the major fraction (70% w/w of all sugars), followed by other monosaccharides such as mannose, galactose, xylose and arabinose. The contents of carbohydrate and composition of reduced products are suitable for bioethanol production from *S. obliquus*. The feasibility of using LEA as a potential source for such sugars was also investigated in the present work to achieve improved utilization of algal biomass in accordance with biorefinery approach. The yields of such reduced sugars (% w/w) in the whole cell algae depended on the drying process and the efficacy of hydrolysis reaction needed for such conversion of total sugars to reduced sugars and ranged from $12.79 \pm 3.44\%$ for freeze dried to $14.63 \pm 3.52\%$ for sun dried samples (Fig. 3). LEA also provided statistically comparable yields of such reduced sugar for different treatments of drying and cell disruption, ranging from $12.37 \pm 3.03\%$ for freeze dried samples assisted with ultrasonication to $19.51 \pm 3.81\%$ for oven dried samples with autoclaving. The yields of reduced sugar obtained in the present study for both whole cell algae and LEA were comparable to those reported in literature (Fu et al., 2010). The effects of variability between different treatments were also analyzed by two-way ANOVA (Table 3). No significant effects on the yield of reduced sugar were observed due to variability in either drying ($P = 0.1$) or cell disruption ($P = 0.273$) methods for lipid extraction. Similarly, no significant interaction ($P = 0.981$) was observed between these two treatments on the yield of

Table 2
Results of two-way ANOVA for different drying and cell disruption procedures on protein yield from lipid extracted *S. obliquus*.

Source of variation	DF	SS	MS	F	P
Drying process	2	513.299	256.65	132.608	<0.001
Cell disruption process	4	447.578	111.894	57.815	<0.001
Drying \times cell disruption	8	174.192	21.774	11.25	<0.001
Residual	30	58.062	1.935		
Total	44	1193.131	27.117		

DF: degree of freedom; SS: sum of square; MS: mean of square; F: likelihood ratio; P: probability.

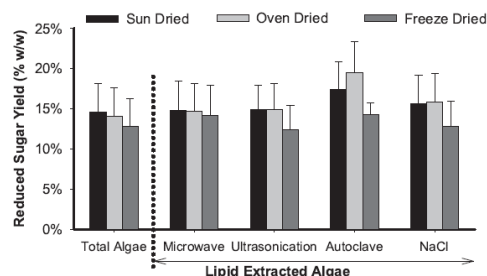


Fig. 3. Effects of different drying and cell disruption procedures on the reduced sugar yields of lipid extracted *S. obliquus*.

Table 3

Results of two-way ANOVA for different drying and cell disruption procedures on reduced sugar yield from lipid extracted *S. obliquus*.

Source of variation	DF	SS	MS	F	P
Drying process	2	55.763	27.882	2.484	0.1
Cell disruption process	4	60.822	15.206	1.355	0.273
Drying × cell disruption	8	21.001	2.625	0.234	0.981
Residual	30	336.674	11.222		
Total	44	474.26	10.779		

DF: degree of freedom; SS: sum of square; MS: mean of square; F: likelihood ratio; P: probability.

reduced sugar. Insignificant difference between all treatments for whole cell algae as well as LEA may be a consequence of increased errors introduced into the extraction due to the hydrolysis process for converting total sugar to reduced sugar in the cell as observed by Fu et al. (2010) also in their hydrolysis yields. No significant difference was observed between the yields of reduced sugar between whole cell algae and LEA undergoing different treatments. These comparable yields of reduced sugar for whole cell algae and LEA qualitatively establish the feasibility of LEA as their source.

4. Conclusion

This study demonstrated the feasibility of using LEA as source of protein and reduced sugars. Microwave assisted extraction of oven dried samples resulted in maximum lipid yield, while minimum yields were obtained from sun dried samples with osmotic shock. In comparison, oven dried LEA with ultrasonication provided significantly higher protein yields, while such yields from microwave and autoclaved samples reduced in comparison to whole cell algae. In addition, yields of reduced sugar from LEAs undergoing different treatments were comparable to those obtained from whole cell algae. These results also contribute towards integrated biorefinery approach for maximum value extraction from algae.

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3.3 Paper III

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RESEARCH ARTICLE

Evaluation of various solvent systems for lipid extraction from wet microalgal biomass and its effects on primary metabolites of lipid-extracted biomass

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Abstract Microalgae have tremendous potential to grow rapidly, synthesize, and accumulate lipids, proteins, and carbohydrates. The effects of solvent extraction of lipids on other metabolites such as proteins and carbohydrates in lipid-extracted algal (LEA) biomass are crucial aspects of algal biorefinery approach. An effective and economically feasible algae-based oil industry will depend on the selection of suitable solvent/s for lipid extraction, which has minimal effect on metabolites in lipid-extracted algae. In current study, six solvent systems were employed to extract lipids from dry and wet biomass of *Scenedesmus obliquus*. To explore the biorefinery concept, dichloromethane/methanol (2:1 v/v) was a suitable solvent for dry biomass; it gave 18.75% lipids (dry cell weight) in whole algal biomass, 32.79% proteins, and 24.73% carbohydrates in LEA biomass. In the case of wet

biomass, in order to exploit all three metabolites, isopropanol/hexane (2:1 v/v) is an appropriate solvent system which gave 7.8% lipids (dry cell weight) in whole algal biomass, 20.97% proteins, and 22.87% carbohydrates in LEA biomass.

Keywords Primary metabolites · Solvent extraction · Wet biomass · Microalgae · *Scenedesmus obliquus*

Introduction

The gradual depletion of fossil fuel causes price hikes, with increasing demand for crude oil (Chisti 2007). For the past few decades, significant attention has been paid to exploring renew-

Highlights: • Lipid yield in wet biomass was less than the dry biomass.
• Protein and carbohydrate yields were comparable in both dry and wet biomass.
• Solvents show varying efficiencies of extraction in wet and dry biomass.
• DCM/methanol (2:1 v/v) was suitable solvent for lipid extraction from dry biomass.
• Isopropanol/hexane (2:1 v/v) is appropriate solvent systems for wet biomass.

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able energy sources to as replacements to the ever increasing demand for fossil fuel and afford environmental protection (Choi et al. 2010; De Schampheleire and Verstraete 2009). Microalgae are unicellular photosynthetic organisms and have promising potential to replace fossil fuels due to high growth rates, and accumulation of lipids content up to 50–70% has been reported for selected species (Pragya et al. 2013). The added advantages are that there is no food vs fuel concern as algae can grow in wastewater and does not require arable land. Moreover, as per the estimates, production of 1 t of algal biomass would consume 1.83 t of CO₂; thus, it is environmentally sustainable as well (Chisti 2007). Therefore, due to such inherent abilities, microalgae could be a better alternate to first- and second-generation biofuel feedstocks.

Despite all these advantages, economic feasibility of microalgae biofuels is questionable due to large capital costs involved in its upstream and downstream processes. Life cycle assessment of microalgal biofuels shows negative net energy balance which means the energy required for biodiesel production is greater than energy generated (Taelman et al. 2013). There are currently some process limitations which result in inefficient use of microalgal biomass. Other than lipids, microalgae also contain other valuable compounds such as proteins, carbohydrates, pigments, vitamins, and minerals (Becker 2007; Ho et al. 2013). The proper utilization of lipid-extracted biomass (LEA) has potential to make biodiesel production cost-effective and feasible.

For biodiesel production, efficiencies of different lipid extraction methods and solvent systems have been reported in various studies (Araujo et al. 2013; Kim et al. 2013; Ramluckan et al. 2014). The microalgal cell disruption with a microbead beater followed by extraction with chloroform/methanol (2:1 v/v) has been reported as most efficient and effective in comparison to other lipid extraction processes (Balasubramanian et al. 2013). Nevertheless, Lee et al. (2010) reported that cell disruption by microwave digestion, sonication, and bead beating is more effective and comparatively, higher lipid yield was achieved than autoclaving and osmotic shock of 10% NaCl solution. Guldhe et al. (2014) also reported that microwave-assisted cell disruption is more efficient than sonication.

LEA is the residual biomass of microalgae which remains after lipid extraction. LEA contains several important components such as proteins, carbohydrates, minerals, water-soluble vitamins, pigments, and other bioactive compounds (Ju et al. 2012; Kassim et al. 2014). These residues are of great economic importance and can be used as a feed additive for various animal and aquaculture feeds, due to its high food and pharmaceutical values (Borowitzka 1997; Certik and Shimizu 1999). Ju et al. (2012) reported that the LEA can be used as a feed additive and a replacement of 50% fish meal proteins by LEA does not affect physiology and nutritional value of shrimp. The use of various algal feed additives for animal nutrition has already been approved by the European Union

without further authorization, marketing, and labeling of feed (The Regulation (EC) No. 1831/2003). The efficient use of LEA will certainly reduce the overall production cost of algal biodiesel (Christaki et al. 2011; Rashid et al. 2013; Lardon et al. 2009). Previous studies have also demonstrated that LEA biomass can also be a suitable substrate for fermentative biomethane and biohydrogen production, as an added advantage is the absence of the lignin content (Chisti 2007; Zamalloa et al. 2012). It is estimated that the energy requirement for the production of 1 kg of biodiesel from dried algal biomass is ~4000 times greater than that of wet biomass (Lardon et al. 2009). The lipid extraction from wet algal slurry for biodiesel production can save the energy utilized in dewatering of wet biomass. Lee et al. (2010) reported that 80% of lipids can be extracted from wet algal biomass by using methanol and chloroform employing microwave-assisted cell disruption technique. Several studies have been done to minimize the energy consumption and utilization of wet biomass for biodiesel production purposes (Cheng et al. 2014; Sathish and Sims 2012; Taher et al. 2014; Wahidin et al. 2014). However, the effect of various lipid extraction systems on primary metabolites such as proteins and carbohydrates of wet algal biomass is yet to be reported. Effect of different cell disruption techniques, biomass processing, and different solvent systems on qualitative and quantitative lipid yields as well as fatty acid profile has been studied and reported widely. However, literature on effects of such solvent systems on the LEA metabolites is scant.

The objective of this study was to investigate and compare the effect of different organic solvent systems on algal biomass (dry and wet slurry biomass) towards lipid yield as well as its effects on the LEA metabolites to explore algal biorefinery concept. The concurrent results may help to choose the optimal lipid-extracting solvent with minimal effect on other metabolites (proteins and carbohydrates) in LEA biomass.

Material and methods

Algae culture

Scenedesmus obliquus culture was cultivated in BG11 medium in a 3000-L pond under natural sunlight at a light intensity of 400–600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a temperature of 22–27 °C. The algal biomass was harvested in late log phase by gravitational settling and then centrifuged to obtain a thick algal slurry. The slurry was divided into two parts, one was used as is and the other was sun dried, powdered, and stored at 4 °C in sealed containers until further analysis.

Lipid extraction

A total of six solvents and solvent mixtures were used for total lipid extraction of dried and wet algal biomass. The six

solvent/solvent mixture systems were as follows: (1) chloroform/ethanol (1:1 v/v), (2) hexane, (3) hexane/isopropanol (1:2 v/v), (4) isopropanol, (5) chloroform/methanol, (2:1 v/v) and (6) dichloromethane (DCM)/methanol (2:1 v/v). Dried and wet algal biomass was processed in the following manners:

- (a) **Dry biomass:** Total lipids from the dry biomass of *S. obliquus* were extracted using mixture of chloroform/methanol (2:1 v/v) and chloroform/ethanol (1:1 v/v) following Guldhe et al. (2014) and hexane, hexane/isopropanol (1:2 v/v), isopropanol (Dai et al. 2014), and DCM/methanol (2:1 v/v) with microwave-assisted cell disruption (Balasubramanian et al. 2013). In brief, 1 g of biomass was mixed with 20 mL solvent and digested in a microwave digester (Milestone S.R.L., Italy; 1200 W of output power) at 1000 W and 100 °C for 10 min. Solvent extracts were first vacuum filtered and further subjected to oven drying at 60 °C to remove residual solvents. Total lipid was quantified gravimetrically in the dried residue.
- (b) **Wet biomass:** Stored wet biomass (moisture content $80 \pm 4\%$) was first thawed at room temperature (Wahidin et al. 2014). Wet algal biomass equivalent to 1 g dried biomass was mixed with 20 mL of abovementioned six solvent and solvent mixtures subjected to microwave digestion and lipid extraction.

Protein extraction

Protein extraction from algal and residual biomass was carried out following López et al. (2010). In brief, 25 mL lysis buffer solution per 100 mg of dried biomass was added and ground for 5 min with mortar and pestle, and then mixed by vortex for 5 min. The mixture was centrifuged (3000 rpm) for 10 min, and the supernatant was collected. An aliquot of 0.5 mL mixed with 0.5 mL SDS solution was vortex mixed. This mixture was further vortex mixed with 5 mL of reagent-C (20 mL of 4.0 g L^{-1} of sodium hydroxide and 20.0 g L^{-1} of sodium carbonate, 0.2 mL of 0.001 g L^{-1} of copper sulfate pentahydrate, and 0.2 mL of 0.002 g L^{-1} copper sulfate tartrate tetrahydrate). After 10 min, 0.5 mL of Folin reagent was added and allowed to react for the next 30 min. The absorbance of the mixture was recorded at 750 nm (Spectroquant Pharo 300, Merck) (Lowry et al. 1951). Bovine serum albumin was used as a standard, and this calibration curve was used for proteins quantification (López et al. 2010).

Carbohydrate extraction

Total carbohydrates were quantified with phenol-sulfuric acid method (DuBois et al. 1956). Five hundred milligrams of

oven-dried biomass was added in 50 mL of H_2SO_4 (2% v/v), and autoclaved for 30 min at 121 °C. The mixture was neutralized with 0.1 M NaOH/ H_2SO_4 , centrifuged ($3000\times g$) for 10 min. An aliquot of 0.1 mL of supernatant was diluted to 1 mL, and then mixed with 1 mL of phenol (5% w/v) and 5 mL of 96% H_2SO_4 was added. The mixture was kept in water bath at 25–30 °C. After 10 min, absorbance was measured at 490 nm using spectrophotometer (Spectroquant Pharo 300, Merck). Glucose was used as a standard for calibration curve preparation (Prajapati et al. 2014).

Chemicals and reagents

Ultrapure water (Aqua MAX Ultra 370, Youngling Korea) was used to prepare all solutions. All the analytical grade chemicals, reagents, and HPLC grade solvent were procured from Sigma-Aldrich, USA.

Statistical analysis

Two-way ANOVA with post-hoc analysis by Tukey's test at 95% significance level were conducted to investigate the effects of various treatments.

Result and discussion

Effect of different solvents on lipid yields in dry and wet algal biomass

Earlier studies have demonstrated that the fatty acid composition of *S. obliquus* is suitable for biodiesel production (Guldhe et al. 2014). The lipid yield and its quality depends on the algal strain, solvent system, and cell disruption technique used. The effects of solvent and cell disruption method on lipid yield of dry biomass of *S. obliquus* have been reported earlier (Balasubramanian et al. 2011; Guldhe et al. 2014; Ansari et al. 2015). In this study, effect of various solvents and solvent mixtures on the lipid yield of both dry and wet biomass of *S. obliquus* was studied. We observed that the lipid yield depends significantly on solvent/solvent mixtures if microwave-assisted cell disruption process is used (Fig. 1); moreover, lipid yield varied significantly ($p < 0.05$) in dried and wet algal biomass. In dry biomass, it ranged from 2.85 to 19.25% employing selected six solvent systems, in which chloroform/methanol gave maximum yield while hexane gave minimum lipid yield as an extracting solvent system. Shin et al. (2014) reported variation in the lipid yield from 2.4 to 4.1% at different time intervals with hexane as an extraction solvent for *S. obliquus* sp. For dry biomass, the lipid yield varies in order of hexane < isopropanol < isopropanol/hexane (2:1) < chloroform/ethanol (1:1) < DCM/methanol (2:1) < chloroform/methanol (2:1). The last three solvents

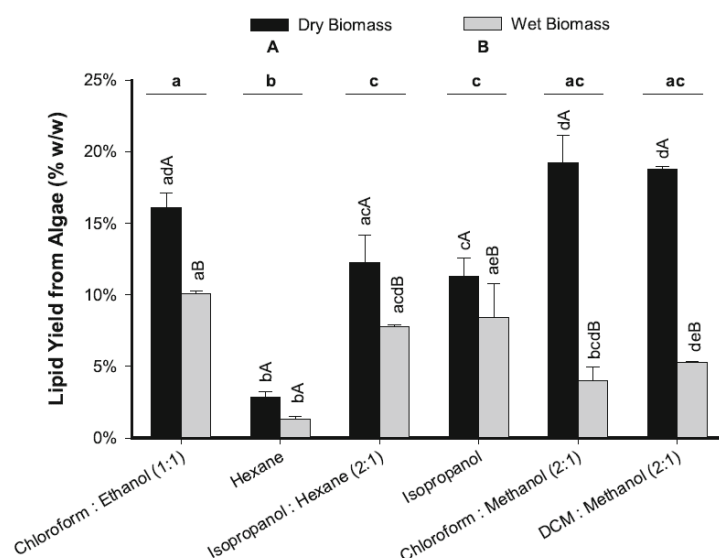


Fig. 1 Effects of different biomass dryness conditions and lipid-extracting solvents on the lipid yields of *S. obliquus*. *Small normal face letters* signify level of significance between different lipid-extracting solvents for an individual biomass condition. *Small bold face letters* signify significance level between different lipid-extracting solvents for overall dryness condition. *Capital normal face letters* signify level of

significance between different biomass dryness conditions for an individual lipid-extracting solvent. *Capital bold face letters* signify significance level between different biomass dryness conditions for overall lipid-extracting solvents. Processes followed by *same letters* have no significant difference (two-way ANOVA, Tukey's test, $p < 0.05$)

showed better yields without significant difference. There is no significant ($p = 0.99$) difference in lipid yields in chloroform/methanol and DCM/methanol. The results also showed that there is no significant difference ($p = 0.15$) in lipid yield using chloroform/ethanol and chloroform/methanol. Similarly, chloroform/ethanol and DCM/methanol do not show any significant ($p = 0.29$) difference in lipid yield. The physical and chemical properties of DCM are similar to chloroform but it is less hazardous. The chloroform is ten times more toxic than DCM; thus, the use of DCM/methanol is better than the use of chloroform reducing the risk of accidental hazards (Chen et al. 1981).

In case of wet algal biomass, lipid yield was significantly lower ($p < 0.05$) in comparison to the dried biomass and ranged from 1.35% with hexane to 10.08% with chloroform/ethanol solvent mixtures (1:1 v/v). The lipid yield in wet biomass was noticed are as follows: hexane < chloroform/methanol (2:1) < DCM/methanol (2:1) < isopropanol/hexane (2:1) < isopropanol < chloroform/ethanol (1:1). Taher et al. (2014) reported the lipid yield of 10% in wet biomass of *Scenedesmus* sp. using hexane as lipid-extracting solvent with acid pretreatment (Table 1), whereas the total lipid yield with chloroform/methanol extraction of the wet biomass of *Nannochloropsis* sp. ranged from 5.43 to 38.3% and 1.9 to 23.01% using microwave and water bath as cell disruption technique, respectively (Wahidin et al. 2014). The maximum lipid yield observed in dry biomass is 15.25% more than that observed in wet biomass for the same

solvent system (chloroform/methanol 2:1). Maximum lipid yield in wet biomass was observed in chloroform/ethanol system (Fig. 1) which was 6.02% less than dry biomass for the same solvent system. Lower lipid yields in wet biomass can be attributed to the water content (>80%) in biomass which hinders interaction of solvent and lipids. There was no significant difference found in lipid yields using chloroform/ethanol, isopropanol/hexane and isopropanol as lipid-extracting solvents for wet biomass. The chloroform/ethanol is a better option for lipid extraction if wet biomass is used for the lipids only. Other two solvent systems (isopropanol/hexane and isopropanol) could be suitable option if LEA biomass is used for further application (feed, food, or energy) because of less toxic nature. During the solvent extraction, the non-polar solvent interacts with neutral lipids and forms an organic solvent and neutral lipids complex due to weak Van der Waals forces. This mixture diffuses across the cell membrane by driven gradient; therefore, neutral lipids get extracted by non-polar solvents from the algal cells. However, some neutral lipids which are in complex form and polar lipids present in the cytoplasm are strongly linked to proteins by hydrogen bonds. The Van der Waals forces developed by neutral lipids and the non-polar solvent are insufficient to break such lipid-protein bonds (Halim et al. 2012). Therefore, use of a solvent system with both polar and non-polar organic solvents is important to ensure complete extraction of all types of associated lipids. An organic solvent such as chloroform extracts neutral lipids and ethanol for extraction of

Table 1 Comparison of lipid yields in different wet slurries of microalgae

Microalgae	Pretreatment	Solvent/condition	Lipids	LEA biomass		Reference
				Protein	Carbohydrates	
Mixed culture	Hot water	1 M H ₂ SO ₄ and then 5 M NaOH (90 °C for 30 min)	10.9	–	–	Sathish and Sims 2012
<i>Scenedesmus</i> sp.	Hydrolysis	<i>n</i> -Hexane	10	–	–	Taher et al. 2014
<i>D. salina</i>	–	Soxhlet/ <i>n</i> -hexane	8.31	–	–	Tanzi et al. 2013
<i>S. obliquus</i>	Microwave	Chloroform/ethanol	10.08	25.95	24.67	Present study

polar lipids. Therefore, the highest lipid yield was observed in chloroform/ethanol (2:1 v/v) extracts whereas the lowest yield was observed for hexane extracts.

The interaction between and within these two-factor dry/wet biomass and different solvents was analyzed with two-way ANOVA at 95% confidence level, and the results are shown in Table 2. Variation in the biomass condition significantly affects ($p < 0.001$) the lipid yield. Similarly, changes in solvents significantly affect lipid yield ($p < 0.001$). The interaction between these two factors was significantly observed ($p < 0.001$). Post-hoc analysis with Tukey's test was carried out to investigate pairwise comparisons within each group to find out their impact on lipid yield. The dried and wet biomass was compared to the different solvents for lipid extraction. Least square means were calculated to be 13.417 for dry biomass and 6.172 for wet biomass with a standard error of 0.343. These comparisons are summarized in Fig. 1.

Extraction of proteins from dry and wet biomass of lipid-extracted algae

LEA contains several valuable components such as proteins, carbohydrates, and other bioactive compounds, even after most of the lipid soluble components have been removed during lipid extraction (Ju et al. 2009). After lipid extraction from algal biomass, protein content (% w/w) increases in LEA due to the extraction of the majority of lipids and a small portion of mass removed along with lipid-extracting solvent (Ansari

et al. 2015). Therefore, it is very important to investigate and compare the effects of different solvents on the reduction of protein yield of lipid-extracted algae (LEA) to establish the economical feasibility of the processes. Figure 2 presents the reduction in protein yield in lipid-extracted algae by different solvents employing microwave as cell disruption technique. The yield of proteins in whole algae was noticed as 37.83%. Becker (2007) also reported around 50–56% proteins content in whole *S. obliquus*. Protein yield in LEA was investigated in both wet and dry algal biomass.

For dry algal biomass, protein yields ranged from 27.04 to 39.12% with isopropanol and hexane as lipid-extracting solvent systems, respectively (Table 3). The yield of proteins varies in dried LEA biomass as follows: isopropanol < chloroform/ethanol < isopropanol/hexane < DCM/methanol < chloroform/methanol < hexane. There was no significant difference in protein yield in all six solvent systems except chloroform/ethanol vs isopropanol/hexane ($p < 0.92$) and chloroform/methanol vs DCM/methanol ($p < 0.654$). The reduction in protein yield from whole algae due to lipid-extracting solvents is as follows: isopropanol > chloroform/ethanol > isopropanol/hexane > DCM/methanol > chloroform/methanol. In case of hexane, protein yield increased (1.45%). Isopropanol reduced protein yield significantly (10.62%) in comparison to other solvent system used for lipid extraction.

The selection of the appropriate solvent system is very crucial as LEA biomass is mostly used for feed, food, and other value added products. The solvent system DCM/methanol gave 0.5% less lipid and 1.60% less protein yield, respectively, than chloroform/methanol lipid-extracting solvent systems. DCM/methanol had a 1.56% reduction in protein yield as compared to chloroform/methanol from whole algae. Thus, DCM/methanol solvent system could be selected over chloroform/methanol system because of minimal negatively effect on LEA biomass, its less toxic and inexpensive nature.

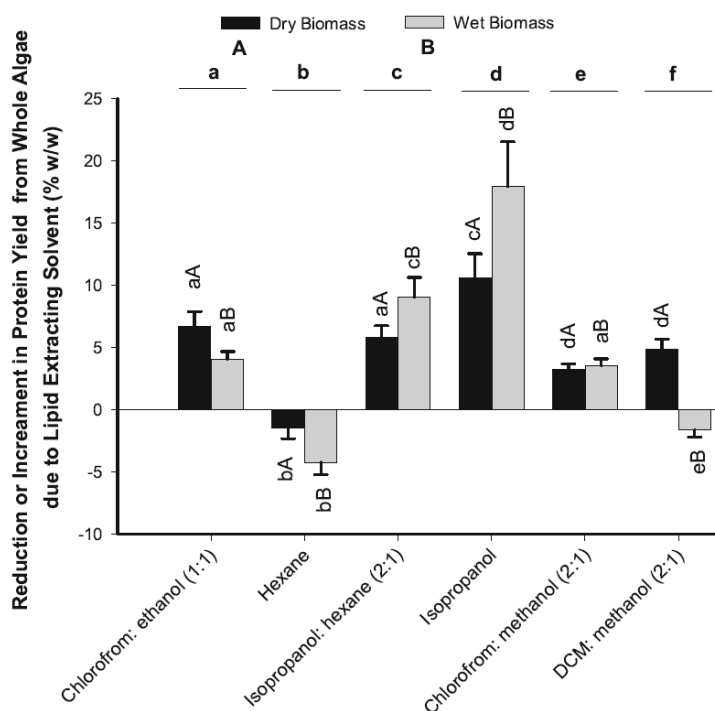
The protein yield in whole wet algal biomass was observed as 7.63% lesser than dry whole algal biomass. In the wet biomass LEA, protein yield ranged from 12.08% with isopropanol to 34.57% with hexane. The variation in the protein yields of wet biomass LEA was as follows: isopropanol < isopropanol/hexane < chloroform/ethanol < chloroform/

Table 2 Results of two-way ANOVA for different biomass dryness conditions and lipid-extracting solvents on lipid yield from *S. obliquus*

Source of variation	DF	SS	MS	F	p
Biomass condition	1	314.940	314.940	223.576	<0.001
Extracting solvent	5	314.288	62.858	44.623	<0.001
Biomass × solvent	5	163.443	32.689	23.206	<0.001
Residual	12	16.904	1.409		
Total	23	809.575	35.199		

DF degree of freedom, SS sum of square, MS mean of square, F likelihood ratio, p probability

Fig. 2 Effects of different biomass dryness conditions and lipid-extracting solvents on reduction of protein yield between whole algae and lipid-extracted *S. obliquus*. *Small normal face letters* signify level of significance between different lipid-extracting solvents for an individual biomass condition. *Small bold face letters* signify significance level between different lipid-extracting solvents for overall dryness condition. *Capital normal face letters* signify level of significance between different biomass dryness conditions for an individual lipid-extracting solvent. *Capital bold face letters* signify significance level between different biomass dryness conditions for overall lipid-extracting solvents. Processes followed by *same letters* have no significant difference (two-way ANOVA, Tukey's test, $p < 0.05$)



methanol < DCM/methanol < hexane. The difference between maximum protein yields in dry and wet biomass LEA was 7.48% in a different solvents (hexane, DCM/methanol) while the minimum protein yield for both biomass condition observed in isopropanol (Fig. 2). The reduction in protein yield from whole wet algae due to lipid-extracting solvents was as follows: isopropanol > isopropanol/hexane > chloroform/ethanol > chloroform/methanol. Moreover, protein was increased using hexane (4.21%) and DCM/methanol (1.61%). Similar to dry algal biomass, isopropanol also reduced protein yield (17.95%) significantly in wet algal biomass.

Table 3 Results of two-way ANOVA for different biomass dryness conditions and lipid-extracting solvents on reduction of protein yield between whole algae and lipid-extracted *S. obliquus*

Source of variation	DF	SS	MS	F	p
Biomass condition	1	374.310	374.310	264.482	<0.001
Extracting solvent	5	1179.741	235.948	166.718	<0.001
Biomass \times solvent	5	1218.765	243.753	172.232	<0.001
Residual	24	33.966	1.415		
Total	35	2806.781	80.194		

DF degree of freedom, SS sum of square, MS mean of square, F likelihood ratio, p probability

The appropriate solvent could only be selected on the basis of lipid yield and toxicity. The three solvent systems (chloroform/ethanol, isopropanol/hexane, and hexane) which gave better lipid yield in wet biomass and protein yield in LEA biomass did not show any significant difference in protein yield. Isopropanol/hexane resulted in 8.89% higher protein yield and 0.6% higher lipid yield than isopropanol alone. In the case of wet biomass, isopropanol/hexane was a preferable solvent if LEA biomass is directed towards aquaculture feed or other food application based on protein yields.

The interaction between and within these two factor dry/wet biomass and different solvents was analyzed with two-way ANOVA at 95% confidence level, and results are presented in Table 3. A variation on biomass conditions (dry/wet) significantly ($p < 0.001$) affects the protein yield of LEA biomass. We also noticed that variation in solvent systems used for lipid extraction also significantly affects ($p < 0.001$) the protein yield of LEA biomass. The interaction between these two factors was observed at the significance level of $p < 0.001$. Post-hoc analysis with Tukey's test was carried out to investigate pairwise comparisons within each group to find out their impact on protein yield. The protein yields of dried and wet biomass were compared for the different solvents. Least square means were calculated to be 11.24 for dry biomass and 4.97 for wet biomass with a standard error of 0.28. These comparisons are summarized in Fig. 2.

Extraction of carbohydrates from dry and wet biomass lipid-extracted algae

The third major constituent of microalgae is carbohydrates. Due to its high content of carbohydrates (enriched cellulose) and no lignin and hemicellulose content, algae can be used as an ideal feedstock for bioethanol production (Chen et al. 2013). Both starch and cell wall polysaccharides enriched in cellulose could be easily converted to bioethanol by a fermentation process (Wang et al. 2011). The total amount of carbohydrates in LEA was not reduced significantly with only 7.2% losses during lipid extraction process in *Chlorella vulgaris* (Lam et al. 2014). It has been also reported that *S. obliquus* has sufficient amount of carbohydrates left after lipid extraction of which glucose is a major sugar fraction (Miranda et al. 2012). The presence of carbohydrates in residual biomass of algae after lipid extraction was also investigated to explore the concept of the algal biorefinery. The yield of carbohydrates in whole algae was 29.23% (dry cell weight (DCW)). The yield of carbohydrates in LEA biomass depends upon the biomass quality (dry or wet) and organic solvent used for lipid extraction. It ranged from 20.53% for dry biomass using hexane to 26.29% for dry biomass employing DCM/methanol as organic solvents. The yields of carbohydrates that vary in LEA biomass (dry) are as follows: hexane < chloroform/ethanol < isopropanol/hexane < isopropanol < chloroform/methanol < DCM/methanol. Due to different lipid-extracting solvent systems, the reduction in

carbohydrate yield in whole algae was observed as hexane > chloroform/ethanol > isopropanol/hexane > isopropanol > chloroform/methanol > DCM/methanol.

The carbohydrate yield in whole wet algae biomass was 28.92% (DCW). The yield of carbohydrates in wet LEA biomass ranged from 20.67 to 26.29% when hexane and isopropanol were used as lipid-extracting solvents (Fig. 3). The yield of carbohydrates in wet biomass of LEA varies as follows: hexane < isopropanol/hexane < chloroform/ethanol < chloroform/methanol < DCM < methanol < isopropanol. The maximum carbohydrate yield in wet biomass was 2.54% higher than dry biomass for the same solvent (isopropanol) system. The maximum carbohydrates in dry biomass are 0.25% higher than the wet biomass for the same solvent (chloroform/methanol) system. The difference in maximum carbohydrate yield in dry and wet LEA biomass was found to be 0.40%. The reduction of carbohydrate yield from wet whole algae due to lipid-extracting solvent was observed in the following order: hexane > isopropanol/hexane > chloroform/ethanol > chloroform/methanol > DCM/methanol > isopropanol.

Carbohydrates left in LEA biomass could be used for energy (biomethane, bioethanol, and biohydrogen) production. Chloroform/methanol shows high lipid yield but residual biomass left after lipid extraction still contained chloroform content which negatively affect biomethane production (Yun et al. 2014). Chloroform inhibits methanogenic and acidogenic bacteria as well as sulfate reducing bacteria essential for biomethane production at

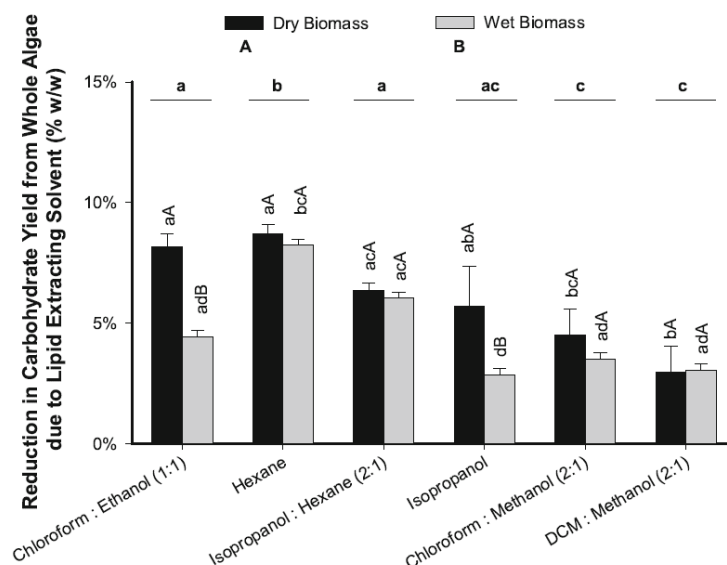


Fig. 3 Effects of different biomass dryness conditions and lipid-extracting solvents on reduction of carbohydrate yield between whole algae and lipid-extracted *S. obliquus*. *Small normal face letters* signify level of significance between different lipid-extracting solvents for an individual biomass condition. *Small bold face letters* signify significance level between different lipid-extracting solvents for overall dryness

condition. *Capital normal face letters* signify level of significance between different biomass dryness conditions for an individual lipid-extracting solvent. *Capital bold face letters* signify significance level between different biomass dryness conditions for overall lipid-extracting solvents. Processes followed by *same letters* have no significant difference (two-way ANOVA, Tukey's test, $p < 0.05$)

Table 4 Results of two-way ANOVA for different biomass dryness conditions and lipid-extracting solvents on reduction of carbohydrate yield between whole algae and lipid-extracted *S. obliquus*

Source of variation	DF	SS	MS	F	p
Biomass condition	1	16.944	16.944	11.853	0.002
Extracting solvent	5	119.360	23.872	16.700	<0.001
Biomass \times solvent	5	18.045	3.609	2.525	0.057
Residual	24	34.307	1.429		
Total	35	188.655	5.390		

DF degree of freedom, SS sum of square, MS mean of square, F likelihood ratio, p probability

very low concentrations (1.67 μ M) (Yun et al. 2016). Therefore, LEA biomass needs to be used for anaerobic digestion after evaporating solvents or washing of biomass. But these methods have limitations such as the requirement of high energy and are difficult to scale up (Yun et al. 2014).

In case of dry biomass, if microalgae were to be utilized for lipid and carbohydrate only, DCM/methanol would be the most appropriate solvent system. Similarly, only 2.94% reduction in carbohydrate yield was observed when DCM/ethanol solvent system was used. For carbohydrate based applications of residual microalgae biomass, hexane was found as suitable solvent for lipid extraction, as the reduction in carbohydrate yield was only 8.7%. Hexane is single solvent system and less toxic than other solvent/solvent systems. While in the case of wet biomass, if biomass is oriented either for oil or for carbohydrates, then in both cases, isopropanol/hexane is a suitable solvent for lipid extraction and carbohydrates utilization in LEA biomass. As in such case, only 6.04% reduction in carbohydrate yield was observed.

The interaction between and within these two-factor dry/wet biomass and different solvents was analyzed with two-way ANOVA at 95% confidence level, and results are shown in Table 4. Variation on biomass condition (dry/wet) and lipid-extracting solvent ($p < 0.014$ and $p < 0.001$, respectively) significantly affects carbohydrate yield in LEA biomass (dry/wet). The interaction between these two factors were not found to be significant ($p = 0.057$). Post-hoc analysis with Tukey's test was carried out to investigate pairwise comparisons within each group to find out their impact on carbohydrate yield. The dried and wet biomass was compared to the different solvent for carbohydrates analysis. Least square means were calculated to be 6.06 for dry biomass and 4.68 for wet biomass with a standard error of 0.282. These comparisons are summarized in Fig. 3.

Conclusion

The different solvent systems were investigated for lipid extraction from microalgae from dry and wet biomass, while having minimal effect on metabolites left in residual biomass.

The amount of lipids extracted from dry and wet biomass depends on the solvent or solvent mixture system. Wet algal biomass could be a better for lipid extraction option with respect to the time and energy; however, the water content in algal biomass directly affects the lipid yields. Lipid yield was found to be lower in wet biomass compared to dry biomass, while protein and carbohydrate yields were comparable in both biomass conditions. The results showed that chloroform/ethanol (1:1 v/v) was most effective from among six solvent systems for wet biomass if utilized for lipids. To explore the biorefinery concept, isopropanol/hexane is the most appropriate solvent system because it is less toxic and gave comparable protein (20.07%) and carbohydrate (22.87%) yields in LEA biomass. For dry biomass, chloroform/methanol (2:1 v/v) is an appropriate solvent system if biomass is used solely for lipid (19.25%). If LEA biomass is to be used for energy or feed or food application, DCM/methanol was found to be a suitable solvent which gave 32.79% protein and 26.92% carbohydrate yields. The choice of solvent system and biomass condition whether dry or wet is directed by the nature of microalgal biomass application.

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A comparative study on biochemical methane potential of algal substrates: Implications of biomass pre-treatment and product extraction



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HIGHLIGHTS

- Four different pretreated microalgae biomass were used for biomethane production.
- CH₄ production was nearly 2.5 times higher in lipid and protein extracted algae.
- Among all, LEA biomass was most suitable substrate for biomethane production.
- Cumulative CH₄ production was higher in pretreated whole algae samples.

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ABSTRACT

Dried powdered algae (SDPA), heat treated algae (MHTA), lipid extracted algae (LEA) and protein extracted algae (PEA) were digested to determine biomethane potential. The average CH₄ production rate was ~2.5-times higher for protein and lipid extracted algae than for whole algae (SDPA and MHTA) whilst the cumulative CH₄ production was higher for pre-treated algae. Highest cumulative CH₄ production (318.7 ml CH₄ g⁻¹ VS) was observed for MHTA followed by SDPA (307.4 ml CH₄ g⁻¹ VS). CH₄/CO₂ ratios of 1.5 and 0.7 were observed for MHTA and LEA respectively. Pre-treatment processes disrupted the algal cell wall, exposing intracellular material which remained intact as opposed to product extraction processes which broke down the intracellular compounds resulting in changes in elemental composition and decreases the cumulative gas yield and CH₄/CO₂ ratio. Comparative analysis determined that the most profitable route of biomass utilisation was protein extraction followed by biogas production giving ~2.5-times higher return on investment.

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1. Introduction

Algae research has brought forth its potential in wastewater treatment (Gupta et al., 2016, 2017), biofuels application (Chisti, 2007; Rashid et al., 2013) and several other products such as nutraceuticals, speciality chemicals, animal and fish feed supplements etc. (Becker, 2007; Ju et al., 2012). The cost of production of most of the algae based products especially biodiesel severely limits scale up of the process to commercial level (Lakaniemi et al., 2013; Rashid et al., 2013). Quantitatively the economically important intracellular biochemical compounds are only a minor fraction of algal biomass, consequently the majority of the biomass

constituents remains unutilized (Ju et al., 2012). Lipid extracted algal biomass (from a biodiesel production process) and protein extracted biomass (from animal or fish feed production) contains ~30–60% of residual carbon (Ansari et al., 2015). Therefore, the lipid or protein extracted biomass (or residual carbon) of an algal product manufacturing process must be utilized for optimum process yield and improved overall economic viability. As the algal biomass is free of lignin, lipid and protein extracted algal biomass can be further be utilized for biofuels production through anaerobic processes.

Anaerobic digestion of organic biomass is a well-researched technology, and can easily be adapted to digestion of algal biomass. Several studies have shown potential of biomethane production from whole algae and product extracted biomass (Ehimen et al., 2011; Alzate et al., 2014; Tartakovsky et al., 2015). However, a comprehensive evaluation of biomethane production from whole cell and product extracted algae, and its dependence on sludge

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(inoculum) to algae biomass ratio has been reported rarely. Improved understanding of these important aspects could prove decisive in devising the strategies for optimum biomass use. The pre-treatment of algal biomass improves biomethane production. However pre-treatment is an energy intensive process which directly corresponds with the biomethane production rate and thus the economic feasibility of the process. Extraction of lipids or proteins from algae also affects the composition of residual biomass which in turn can affect the yield and quality of biomethane produced (Yun et al., 2014). Therefore, it is important to analyse and understand how the various product extraction procedures contribute to the changes in the biochemical composition of the algal biomass as well as the effects of pre-treatments on the biochemical methane potential (BMP).

In this study, we present a comprehensive evaluation of BMP of: a) dried powdered algal biomass (SDPA); b) heat treated algal biomass (MHTA); c) lipid extracted algal biomass (LEA); and d) protein extracted algal biomass (PEA). The changes in the biochemical composition of the biomass in specific pre-treatment processes and product extractions were observed along with their effects on the BMP of specific substrate type. Additionally, the BMP was also compared at two different sludge to algae (SAR) ratios. Such important aspects will improve the current understanding about the energy and costs for both cases i.e. when algal substrate is digested alone or co-digested (in equal ratio) with the sludge. Finally, energy and cost balance were determined in order to understand how an anaerobic digestion of algal biomass (or residual biomass) can be incorporated for optimum and economic process yield in a biorefinery concept.

2. Materials and methods

2.1. Algae cultivation

The algal strain, *Scenedesmus obliquus* (Genbank accession number: FR751179.1) used in present study was isolated from the Durban region, KwaZulu Natal, South Africa. Algal biomass was cultivated in a 300kL raceway pond using BG11 nutrient medium defined by Lee et al. (2010) under natural sunlight ($400\text{--}1200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) and temperature ($18\text{--}27\ ^\circ\text{C}$) (Guldhe et al., 2014; Ansari et al., 2015; Gupta et al., 2016). Algal cells were pre-concentrated using gravitational settling and harvested as a thick slurry (15–20% solids content) using centrifuge at 1059g (Thermo electron Corporation Multifuge 4KR). The thick slurry was sun dried and powdered using mortar and pestle. The powdered biomass was stored in a sealed container at $4\ ^\circ\text{C}$ till further processing.

2.2. Algae substrate for biochemical methane potential (BMP)

Two pre-treated and two product extracted algae samples: a) whole cell sun dried powdered algae (SDPA) biomass, b) milled and heat treated algae (MHTA) biomass, c) lipid extracted algal (LEA) biomass, and d) protein extracted algal (PEA) biomass. SDPA obtained by sun drying and grinding and used as the base biomass for further treatments, therefore, it was considered as treatment control while evaluating the effect of different treatments on BMP. For the preparation of MHTA, the biomass was heat treated in oven at $100\ ^\circ\text{C}$ for 8 h and powdered (Ward et al., 2014). The LEA was prepared by lipids extraction from SDPA using hexane as extracting solvent following the method of Stephenson et al. (2010). The LEA was stored in a sealed container at $4\ ^\circ\text{C}$ till further use. The PEA was prepared by extraction of protein from SDPA by using ethanol (95%) and methanol (5%) following Kale, 2012. The

PEA was separated by decanting the supernatant and dried at room temperature.

2.3. Experimental Setup for BMP and analyses

The BMP tests were conducted with 0.5% solids and two different sludge to algae ratio (SAR). The compositions were as follows, sludge control with no algae (SC), dried powdered algae, SAR = 1:4 (S1D4), dried powdered algae, SAR = 1:1 (S1D1), heat treated algae, SAR = 1:4 (S1H4), heat treated algae, SAR = 1:1 (S1H1), lipid extracted algae, SAR = 1:4 (S1L4), lipid extracted algae, SAR = 1:1 (S1L1), protein extracted algae, SAR = 1:4 (S1P4), and protein extracted algae, SAR = 1:1 (S1P1). For SAR 1:4, precisely 70 mg of sludge and 280 mg of algal substrate while for SAR 1:1, 175 mg of sludge and 175 mg of algal substrate were added to the culture media (Owen et al., 1979). 350 mg of sludge devoid of algal biomass was used as substrate control. The nutrient medium for this experiment was prepared following Owen et al., 1979. All the tests were performed in triplicate, in 120 ml serum bottle with recommended 70 ml volume of mixture of nutrient medium, a, sludge and algal substrate. Serum bottles were purged with helium for 15 min (to create anaerobic environment), closed tightly with butyl septum and sealed with aluminium caps after filling them with nutrient-substrate mixture and the pH was adjusted to 7.1. Sealed serum bottles were then placed in incubator shaker (Model TU-454, mrc Ltd., Israel) at 60 rpm at constant temperature ($35\ ^\circ\text{C}$) for 45 days.

The biogas analysis in each sample was done with gas chromatography (Agilent 7820A, USA) fitted with thermal conductivity detector (TCD), GS-Gaspro (30 m x 0.32 mm ID) packed column. The nitrogen was used as carrier gas and the oven temperature kept $80\ ^\circ\text{C}$ whilst TCD temperature was $250\ ^\circ\text{C}$ and the injection port temperature was set $120\ ^\circ\text{C}$. For the analysis of CH_4 and CO_2 , the gas samples were drawn from the headspace of BMP test bottles following the method by Donoso-Bravo et al. (2011). Biogas analysis was conducted every fifth day up to day 45. The mixture contents of serum bottles were analysed for total and dissolved chemical oxygen demand (COD), total and dissolved nitrogen (N) and ammonia-N following standard method (APHA, 2005). These tests were carried out before and after completion of the BMP test. Both the contents of serum bottles and substrate (algal biomass and inoculum) were also tested for total solids (TS), volatile solids (VS), and ash content following the standard methods (APHA, 2005). The carbon (C), hydrogen (H), and nitrogen (N) contents of algal biomass and inoculum was quantified using CHNS analyser (PerkinElmer 2400, USA).

Two-way ANOVA was used to test for statistical differences in methane production of different algal samples with different SAR and treatments as independent fixed factors. A significance level of $p < 0.05$ was applied throughout.

2.4. Energy and cost analysis

For energy and cost analysis of the BMP test were extrapolated to the quantity of biomass generated at Kingsburgh pilot plant. The cost and energy involved in preparation of each algal substrate was calculated using results from past studies with similar pre-treatments and product extraction procedures. It was assumed that an extra 5% biomass was lost during each product extraction process. Since lipids and proteins were extracted through solvent extraction, a product loss of 10% was assumed for each of lipid and protein extraction process (i.e. product extraction efficiency of 90%). Also, 5% extra biomass loss was assumed in each of product extraction procedure due to unintended concomitant losses (Lam et al., 2014). Revenue generated from primary products i.e. biodiesel from extracted lipids and animal feed obtained by protein

extracted biomass were added to the revenue generated from methane production in case of product extracted algal substrates (LEA and PEA). For SDPA and MHTA only the revenue generated from biomethanation of the biomass is considered. To estimate the revenue from biogas, it was assumed that the biomass obtained after pre-treatment and product extraction was digested with two different SAR i.e. half of the biomass was digested with SAR of 1:4 and the other half with SAR of 1:1."

For calculation of selling price of biogas it is assumed that 1 m³ of biogas with 60% methane content is equivalent to 0.6 L of diesel fuel and sold at 60% of the cost of diesel fuel (Fran et al., 2011). To calculate the revenue generated from the biogas output of each algal substrate, amount of methane found in CH₄ + CO₂ analysis was considered to be the representative of amount of methane present in the biogas produced by that particular algal substrate. Each output from the algal samples from current study where normalized to 60% methane content in the biogas and the revenue generated were calculated.

3. Results and discussion

3.1. Composition of algal biomass and different samples used for BMP test

Mechanical size reduction of dried algal biomass as in case of SDPA ruptures algal cell wall and make the intracellular products available for anaerobic digestion, however, it is likely to have minimal or no effect on the composition of algal biomass (Ehimen et al., 2013). In case of thermal treatment of algal biomass (MHTA), disintegration of intracellular compounds into smaller compounds takes place due to application of heat (González-Fernández et al., 2012; Schwede et al., 2013). The heat treatment could also be accompanied by some change in elemental composition due to the loss of volatile organic compounds (Rodríguez et al., 2015). Processes such as lipid and protein extraction not only alters the elemental composition in residual biomass LEA and PEA respectively but also significantly affects the relative ratios of different elements in the biomass (Zhao et al., 2014; Parimi et al., 2015). All of these factors contribute substantially to biogas yields and methane production during biomethanation of algal biomass.

The intracellular composition of untreated algal biomass and physicochemical characteristics of different samples used for BMP test is presented in Table 1. Since lipids and proteins make up 20% and 48% of algal biomass respectively, the extraction of these intracellular compounds also significantly affects the elemental composition of the residual biomass. Due to the unique composition and properties of the intracellular products, the extraction of any of these products results in varying the biochemical composition as well as C/N ratio of the residual biomass. (Zhao

et al., 2014; Bohutskyi et al., 2015). Table 1 showed that samples containing lipid extracted algae (S1L1 and S1L4) have lowest C/N of all samples due to the removal of high C containing compound i.e. lipids. While samples containing protein extracted algae (S1P1 and S1P4) showed highest C/N ratio due to the removal of high N containing compound, i.e. protein. Since the C/N ratio in the sludge sample was comparatively higher than any of the algal substrate, the samples with higher sludge content (SAR = 1:1) contained a higher C/N ratio compared to those with lower sludge content (SAR = 1:4) for same algal substrate.

The VS/TS ratio is an important parameter in predicting the digestibility of a substrate and overall biogas production in anaerobic digestion. The samples with higher VS/TS are expected to be more digestible and produce greater amount of biogas in an anaerobic digestion processes (Zhao et al., 2014). The results (Table 1) clearly revealed that the samples comprising of product extracted algal biomass (LEA and PEA) had lower VS/TS ratio compared to the samples comprising of whole algal biomass (SDPA and MHTA). The changes in VS/TS ratio can be attributed to the removal of lipids and proteins resulting in the residual biomass containing lower organic content (volatile solids) compared to whole algal biomass (Zhao et al., 2014).

3.2. Effect of SAR and treatment on different algal samples

The interaction between SAR and treatment (pre-treatments applied to whole algal cells in SDPA and MHTA, and product extraction steps in case of LEA and PEA) on methane production by different algal substrates has been plotted in Fig. 1. The effect of SAR and treatment was found to be more prominent in samples containing pre-treated SDPA and MHTA than LEA and PEA. Samples containing whole algal biomass but with lower SAR i.e. 1:4 showed higher methane production, whereas the samples containing product extracted algal biomass with higher SAR i.e. 1:1 showed higher methane production. However, such differences in the cumulative methane production at two different SARs were found to be significant only in case of MHTA containing sample. The quantitative difference in the methane production in the two samples containing MHTA biomass, S1H4 and S1H1 was 13%, which was much higher than the samples containing SDPA, LEA, and PEA where such differences were 4.5%, 0.38%, and 4.5% respectively.

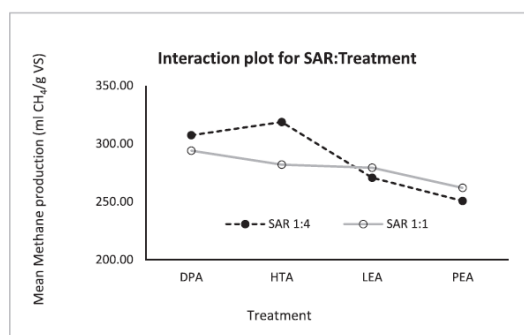
3.3. Influence of treatment on BMP of different algal substrate

Variances in VS/TS and C/N ratios have been attributed to different pre-treatments and product extraction processes changing the elemental composition of the biomass (Zhao et al., 2014). These treatments (pre-treatments and product extractions) can have

Table 1
Characteristics of Algal biomass and samples used for BMP test.

Untreated algal biomass composition	Lipids (%)	Carbohydrates (%)	Protein (%)	Ash/others (%)	Moisture Content (%)
	20 ± 4	18 ± 3	48 ± 8	11 ± 3	3 ± 1
Samples for BMP test					
Sample name	Description		VS/TS	C/N	
SC	Sludge control with no algae		0.78 ± 0.08	25.8 ± 0.30	
S1D4	Dried powdered algae, SAR = 1:4		0.66 ± 0.04	6.5 ± 0.20	
S1D1	Dried powdered algae, SAR = 1:1		0.72 ± 0.10	10.9 ± 0.70	
S1H4	Heat treated algae, SAR = 1:4		0.64 ± 0.08	6.2 ± 0.10	
S1H1	Heat treated algae, SAR = 1:1		0.68 ± 0.02	10.3 ± 0.5	
S1L4	Lipid extracted algae, SAR = 1:4		0.54 ± 0.05	5.2 ± 0.20	
S1L1	Lipid extracted algae, SAR = 1:1		0.62 ± 0.06	10.1 ± 0.40	
S1P4	Protein extracted algae, SAR = 1:4		0.50 ± 0.04	6.9 ± 0.50	
S1P1	Protein extracted algae, SAR = 1:1		0.62 ± 0.05	11.9 ± 0.40	

^aSAR = Sludge to algae ratio. All data are Mean ± SD of triplicate determination.



SAR 1:4 conditions DPA (304.33), HTA (318.67), LEA (207.67) and PEA (250.67)

SAR 1:1 conditions DPA (294.00), HTA (282.00), LEA (279.33) and PEA (262.00)

Fig. 1. Effect of interaction of Sludge to algae ratio and treatment on production of methane by different algal substrates.

varying effects on biogas potential of algal substrate. Certain treatments may disintegrate the algal biomass constituents from complex organic compounds into simpler compounds thus allowing intracellular components to be readily available for anaerobic digestion (Rodríguez et al., 2015). Other treatments can detrimentally affect the digestion of biomass by altering the composition and relative ratios of different elements in the biomass (Ward et al., 2014). Fig. 2 illustrates CH₄ and CO₂ production profiles of different substrates over the course of BMP experiment. Product extracted algal biomass produced CH₄ at a higher rate than the samples containing whole algal biomass at the start of the BMP test. The average CH₄ production rate (50 ml CH₄ g⁻¹ VS) in samples S1L4, S1L1, S1P4, and S1P1 was found approximately 2.5 times higher than the average CH₄ production (22 ml CH₄ g⁻¹ VS) in samples S1D4, S1D1, S1H1, and S1H4 on the 5th day of the BMP test. Similar trends were observed for CO₂ production rates in the samples. The lowest CO₂ production rate of 18 ml CO₂ g⁻¹ VS was recorded in S1D4 whereas the highest of 50 ml CO₂ g⁻¹ VS in S1P1.

The production of both CH₄, CO₂ gases in samples containing product extracted algal biomass began almost immediately as the BMP test was initiated. While in case samples containing whole algal biomass, a lag period in the production of gases was observed. The higher rate of production of CH₄ and CO₂ in samples with product extracted algal biomass was likely due to the availability of readily digestible organic compounds (Alzate et al., 2014). Previous studies have also reported that the pre-treatment and extraction procedures involved in lipid and protein extraction from algal biomass not only tend to disrupt the cell wall and release the intracellular material but also disintegrate the cellular material (Ansari et al., 2015; Maurya et al., 2016). The finding of this study also revealed that pre-treatment would have only resulted in disruption of the cell wall only in SDPA and MHTA pre-treated biomass. González-Fernández et al. (2012) reported that most of the cellular material is likely to remain intact rendering the intracellular organic compounds unavailable for digestion by anaerobic bacteria. The resultant effect is lower initial rate of gas production due to anaerobic bacteria having to mineralize complex intracellular compounds into simpler compounds. In samples containing LEA and PEA, it is possible that due to high C/N ratio and easily digestible C in LEA and PEA containing samples, most of N was exhausted in the initial phase and this lack of N could have halted anaerobic digestion. The findings of the present study is in accordance with the observations of Parimi et al. (2015).

Samples containing whole algal biomass produced gases for longer period of time, which resulted in higher cumulative CH₄

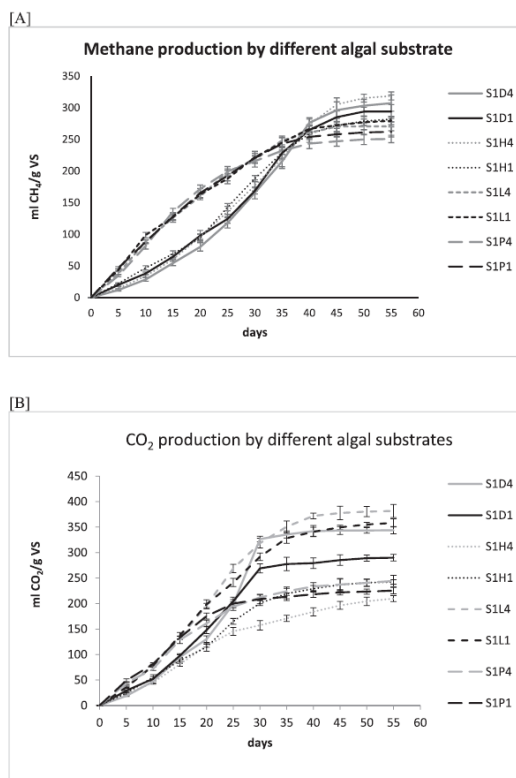


Fig. 2. Cumulative CH₄ and CO₂ production by different samples over the course of BMP experiment.

production than those with product extracted algal biomass. Highest cumulative CH₄ production of 318.7 and 282 ml CH₄ g⁻¹ VS was seen in MHTA containing samples followed by 307.4 and 294 ml CH₄ g⁻¹ VS in SDPA containing samples (Fig. 2). This was due to higher availability of digestible-C g⁻¹ VS in samples containing whole algal biomass than in product extracted algal biomass. Table 2 shows initial and final COD and % reduction in COD in different algal samples during the BMP test. It can be seen that SDPA and MHTA containing samples on average have 20–25% higher initial COD than lipid and protein extracted samples. Also, the % reduction in COD through anaerobic digestion was about 10% higher in whole algal biomass containing samples than in product extracted algae. This could be attributed to the difference in C/N ratio among two types of samples. A product extraction process also involves various treatments and down-streaming steps that can alter the overall elemental composition of algal biomass and in turn affect the C/N ratio also (Ward et al., 2014; Zhao et al., 2014), which can negatively affect methane yield and digestion efficiency of biomass (Yun et al., 2014). Therefore, in almost all the samples containing product extracted algal biomass the methane yields and digestion efficiency was found comparatively lower than whole algal biomass.

3.4. Effect of treatment on CH₄/CO₂ and cumulative gas production in algal samples

Fig. 3 shows the comparison of gas production and CH₄/CO₂ ratio in various algal samples. The findings clearly showed lowest CH₄/CO₂ ratio in LEA containing samples S1L4 and S1L1 with ratio

Table 2

Reduction (%) in COD in sludge control and different algal samples during the BMP test.

Sample	COD _{before}	COD _{after}	Digestion efficiency (%)
SC	3.14 ± 0.18	0.45 ± 0.10	85.7
S1D4	2.74 ± 0.09	0.56 ± 0.11	79.6
S1D1	2.92 ± 0.08	0.46 ± 0.06	84.2
S1H4	2.58 ± 0.14	0.63 ± 0.02	75.5
S1H1	2.70 ± 0.09	0.56 ± 0.02	79.4
S1L4	2.24 ± 0.10	0.63 ± 0.07	71.9
S1L1	2.39 ± 0.12	0.53 ± 0.05	78.0
S1P4	2.09 ± 0.11	0.59 ± 0.08	71.8
S1P1	2.14 ± 0.13	0.51 ± 0.04	76.1

All data are Mean ± SD of triplicate determination.

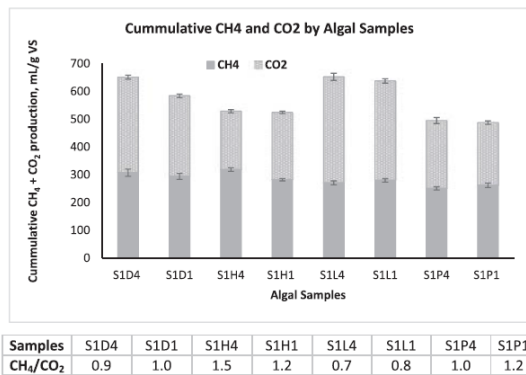


Fig. 3. Comparison of gas production and CH₄/CO₂ ratio in different algal samples.

of 0.7 and 0.8 in respectively. Yun et al., 2014, demonstrated that CH₄/CO₂ ratio of gasses produced in anaerobic digestion of biomass containing high energy compounds such as lipids is higher than non-lipid containing biomass. The results showed that anaerobic digestion of lipid extracted algal biomass had least amount of CH₄ content in the emanating gases among all the samples (Fig. 3). The highest CH₄/CO₂ ratio was recorded in samples containing MHTA with a ratio of 1.52 and 1.16 in S1H4 and S1H1. This result was expected as the intracellular components (lipids, protein and carbohydrates) remained somewhat intact in MHTA containing samples but readily available for anaerobic digestion due to disintegration of cell wall as a result of heat treatment.

Table 3A

Cost incurred in different pre-treatment and product extraction processes applied to algal biomass to produce different algal substrates and revenue generated from the extracted products. (MODIFIED).

Algal substrate	Cost incurred in pretreatment or product extraction per year	Quantity of biomass processed for product extraction	Quantity of product generated	Revenue generated from the product = quantity of product * unit price of product	Quantity of biomass left after product extraction and losses
Lipid extracted algae	1. Cost incurred in size reduction = \$805.72. Cost incurred in lipid extraction = \$4292.73. Cost incurred in transesterification of oil = \$70.6 Total = \$5669.0/yr	8640 kg/yr	(0.9 * 0.20 * 8640) = 1555.2 kg/yr	1555.2 * 1.6 = \$2488.32/yr	8640 - {(0.9 * 0.20 * 8640) + (0.05 * 8640)} = 7084.8 kg/yr
Protein extracted algae	1. Cost incurred in size reduction = \$805.72. Cost incurred in solvent extraction of protein = \$307.2 Total = \$1112.9/yr	8640 kg/yr	(0.9 * 0.48 * 8640) = 3732.5 kg/yr	3732.5 * 0.6 = \$2239.5/yr	8640 - {(0.9 * 0.48 * 8640) + (0.05 * 8640)} = 4907.5 kg/yr

1. Dried & powdered algae (Cost incurred in size reduction of biomass = \$805.7 and total cost = \$805.7/yr).

2. Heat treated algae (a. Cost incurred in size reduction of biomass = \$805.7; b. cost incurred in thermal treatment of biomass = \$1900.8 and total cost = \$2706.5/yr).

From Fig. 3, total cumulative gas (CH₄ + CO₂) production (g⁻¹ VS) was found to be highest for LEA sample followed by SDPA, MHTA samples, whilst the lowest gas production was observed for PEA samples. Higher production of CH₄ + CO₂ in LEA can be attributed to the highest CO₂ production for LEA containing samples (Figs. 2 and 3). It has been shown in past studies, that during anaerobic digestion, the presence of higher protein content in algal biomass comparably increases CO₂ production over CH₄ (Ehimen et al., 2011; Parimi et al., 2015). From the various samples used in this study, the protein fractions were expected to be highest in LEA biomass, which correlated positively with the higher CO₂ production recorded. SDPA and/or MHTA results showed that the higher presence of carbohydrates in the algal biomass corresponds higher fraction of volatile fatty acids (VFAs) in the gases produced in anaerobic digestion of biomass. Therefore, it is likely that anaerobic digestion of samples containing PEA would have produced higher amounts of VFAs (not measured in this study) than either of CH₄ and CO₂, which resulted in lowest production of (CH₄ + CO₂) g⁻¹ VS in these samples.

3.5. Energy and cost calculations

To understand the application of biomethanation of algal biomass as part of a biorefinery or as a standalone biomethane production operation, the input and output costs were calculated. The cost incurred in different pre-treatment and product extraction processes to produce different algal substrates and revenue generated from the extracted products have been tabulated in Table 3A. It can be seen that the input cost involved in producing SDPA from dried algal biomass is less than one-third of cost of producing MHTA. In case of product extracted algal substrates, the cost incurred in extraction and transesterification of lipids is approximately five times than the cost of extraction of proteins from biomass. Nevertheless, the revenue generated from lipids (as biodiesel) is only 10% more than that of proteins (as animal feed). This gives the difference in input and output cost of -\$3180.7 yr⁻¹ for LEA and +\$1116.6 yr⁻¹ per ton for PEA. Therefore, it can be concluded that the cost incurred as operational expenditures for product extraction and revenue generated from the products, the algal protein based products are more commercially viable than algal lipid based products.

The quantity of biomass represented in last column of Table 3A shows that nearly 18% and 43% of biomass was lost in lipid and protein extraction process respectively during product extraction and concomitant losses. Table 3B represents the revenue generated from anaerobic digestion of different algal substrates which

Table 3B

Revenue generated from anaerobic digestion of different algal substrates.

Algal substrate	Quantity of biomass digested for biogas	Biogas yields from different algal samples normalized to 60% CH ₄ content	Revenue generated from selling biogas = quantity of algal biomass digested (kg) * biogas yield (m ³ /kg) * selling price of biogas (\$/m ³)	Overall return on investment = Total revenue generated – total input cost (\$/yr)
Dried powdered algae	4320 kg/yr	S1D4: 0.51 m ³ /kg	\$793.2/yr	\$[(793.2 + 762.0) – 805.7]/yr = \$749.5/yr
	4320 kg/yr	S1D1: 0.49 m ³ /kg	\$762.0/yr	
Heat treated algae	4320 kg/yr	S1H4: 0.53 m ³ /kg	\$824.3/yr	\$[(824.3 + 730.9) – 2706.5]/yr = –\$1151.3/yr
	4320 kg/yr	S1H1: 0.47 m ³ /kg	\$730.9/yr	
Lipid extracted algae	3542.4 kg/yr	S1L4: 0.45 m ³ /kg	\$573.9/yr	\$[(2488.3 + 573.9 + 599.4) – 5669]/yr = –\$2008.3/yr
	3542.4 kg/yr	S1L1: 0.47 m ³ /kg	\$599.4/yr	
Protein extracted algae	2453.8 kg/yr	S1P4: 0.42 m ³ /kg	\$371.0/yr	\$[(2239.5 + 371 + 402.1) – 1112.9]/yr = \$1899.7/yr
	2453.8 kg/yr	S1P1: 0.44 m ³ /kg	\$402.1/yr	

revealed that the overall return on investment (ROI) is positive only in the case of SDPA and PEA while MHTA and LEA had negative ROI. The negative ROI for MHTA and LEA was due to high cost of heat treatment and lipid extraction respectively. Also, revenue generated from biogas production was comparably less in PEA than SDPA as after extraction of proteins, less biomass remained available for biomethanation. The overall ROI from PEA substrate was nearly five times higher than SDPA mainly because of high revenue generated from the primary product i.e. proteins for animal feed. The production of SDPA required the lowest input cost compared to all other algal substrates used in this study. Moreover the revenue generated from biomethanation of SDPA was almost double the input cost. Therefore, biomethanation of SDPA can be looked as a low investment revenue generating option as it requires minimum amount of operating cost with positive ROI.

It should be noted here that all calculations presented in Tables 3A and 3B are done based on the consumables (e.g. solvents, reagents, and other chemicals) and operational costs (e.g. electricity and heat) only. Capital costs such as purchase of land for algal biomass cultivation and machines and instruments for processing and analyses of the biomass have been omitted for the sake of simplicity. Please also refer to the attached appendices for further clarifications.

4. Conclusions

This study determined that whilst cumulative biogas production was significantly higher in pre-treated whole algae samples (SDPA and MHTA), and the rate of biogas production was comparatively higher in product extracted algal samples (LEA and PEA). This is due to the extent of the breakdown of intracellular components caused by pre-treatment and product extraction. The overall cost analyses was done based on the input and output costs determined that revenue generated from biogas production that protein extraction for aquaculture feed and anaerobic digestion of the residual biomass is most commercially lucrative option giving nearly 2.5 times higher returns on investment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.03.068>.

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4. CRITICAL OVERVIEW

4.1 Microalgae cultivation in wastewater as nutrient medium

Biodiesel production from microalgae remains uneconomical commercially because of high cultivation cost, energy intensive harvesting and drying processes, and inefficient technologies for conversion of algal lipids into biodiesel (Ren *et al.*, 2017). Microalgae synthesize different types of metabolites, which can be used for different purposes. Cultivation at demonstration scale requires huge amounts of water and nutrients, which makes commercial scale microalgae biomass production an economically inefficient process (Ebrahimian *et al.*, 2014). Microalgal cultivation in wastewater is an economical way for the comprehensive treatment and simultaneously production of biomass. The choice of wastewater depends on the type of algal biomass based end product. The most important characteristics of algae for the use in wastewater treatment and biofuel production include high growth rate, lipid content and productivity. High tolerance to inorganic nutrients and robust growth properties with high resistance to varying environmental conditions are vital. In **paper I** two microalgae species were selected for study of growth and treatment potential in wastewater and BG11 medium. **Paper I** briefly discusses the applicability of raw sewage as a growth medium and comprehensive wastewater treatment that includes nutrient removal, pathogen removal and organic carbon removal and lipid yield in biomass. From this study *S. obliquus*, was selected as desirable microalgae for large-scale open pond cultivation (300, 000 L) and utilization of biomass for energy and feed applications. It has good characteristics for large-scale cultivation such as robustness and tolerance to varying cultivation conditions and biomass production containing higher major metabolites. *S. obliquus* accumulate high amounts of protein (<40% DCW), lipid (<17% DCW) and carbohydrate (<15% DCW) and their large cell size have self-flocculating properties, which allow more economical harvesting methods.

4.1.1 Cultivation of *S. obliquus* and *C. sorokiniana* in different ratio of domestic raw sewage and post-chlorinated wastewater

Microalgae, *S. obliquus* and *C. sorokiniana* are cultivated in raw sewage and post-chlorinated wastewater (PCW) as nutrient medium to reduce the cultivation cost. The details of wastewater analysis done are presented in **Figure 1 of Paper 1**. The characteristics of raw wastewater and PCW are tabulated in **Table 1 of Paper 1**. Cultivation of microalgae in wastewater is regarded as an ideal scenario to obtain microalgal biomass for biofuel purposes mainly. *S. obliquus* and *C. sorokiniana* both were predominantly grown in domestic wastewater and showed excellent nutrient removal.

Four dilutions (25, 50, 75 and 100%) of raw sewage were made with post-chlorinated wastewater and used for the cultivation of both of the species. All dilutions, showed reasonable growth of both species, except in 25% dilution due to lower concentration of the nutrients. *C. sorokiniana* achieved maximum growth with 75% (75% raw sewage and 25% PCW) and maintained a higher biomass level for the duration of the experiment (**Figure 2a of Paper I**). The nutrients content in the 75% dilution was sufficient for the cultivation. It was observed that diluted raw sewage provided more favourable growth conditions compared to the raw sewage (100%). In raw sewage, the lag phase was of two days due to the acclimatization of culture in new medium. The other dilution levels i.e. 75, 50 and 25% provided comparably favourable cultivation conditions and showed no lag phase for acclimatization (**Figure 2a Paper I**). Growth of *C. sorokiniana* in 25% wastewater was comparably very low due to the nutrients being exhausted very quickly, whereas, 75% dilution was found to be suitable to cultivate *C. sorokiniana*.

In case of *S. obliquus*, results up to 6 days were comparable for 100, 75 and 50% dilutions, but further into the trial, growth was declined in 100% than 75 and 50% dilutions. This may be due to

higher sustained physiological stress exerted by use of raw sewage (see section 3.2 and Figure 2b of Paper I). The 50% (W-50) wastewater was found to be optimal cultivation medium for *S. obliquus* for the overall experimental duration, while 75% (W-75) provided marginally sub-optimal conditions. In contrast, 25% was found to be nutrient limited and supported inadequate growth. The effects of using raw sewage as a substrate on physiological conditions of *C. sorokiniana* and *S. obliquus* were observed by determination of the quantum efficiency (Fv/Fm) of reaction centres in PS-II of chlorophyll by non-invasive fluorescence measurements. The value of $Fv/Fm > 0.5$ attributes the suitability of microalgae cultivation medium (Figure 3a and b of Paper I).

4.1.2 Nutrient and bacterial removal efficiency

Nitrogen present in the raw sewage was predominantly in a reduced form, as ammonia, whilst in the post-chlorinated effluents, nitrate was the dominant nitrogen form (Table 1 of Paper I). Dilution resulted in changing levels of different nitrogen species with different wastewaters. In raw wastewater ammonia concentration was dominant as the nitrogen whose contribution declined from 99.24% for WW-100 to 81.65% for WW-25; and the nitrate levels increased with dilution of raw wastewater from 0.76% for WW-100 to 16.81% for WW-25.1. (Figure 6a of paper I) and (Figure 7d, j and j of Paper I). *C. sorokiniana* and *S. obliquus* were able to remove 43.46 and 63.64% ammonia, respectively from wastewater due to good nitrogen uptake efficiency. *S. obliquus* showed marginally better potential for removal (Figure 6a and 7 of Paper I) in comparison to *C. sorokiniana* for all wastewater dilutions (Figure 6d of Paper I).

Phosphorus remains in the form of orthophosphate in wastewater. Various studies have demonstrated removal of phosphorus from wastewater by microalgae (Caporgno *et al.*, 2015; Cuellar-Bermudez *et al.*, 2017; Delgadillo-Mirquez *et al.*, 2016; Rasoul-Amini *et al.*, 2014). Phosphate removal was compared for both the algae in different ratios of WW and PCW (**Figure 8 of Paper I**). The *C. sorokiniana* showed lower phosphate removal from the 100% wastewater due to stress. Phosphate removal increased at reduced stress levels of culture and favoured the growth. Highest phosphorus removal was obtained in 50% and lowest was in 25%, which could be due to stressed growth of *C. sorokiniana* with low nutrient content. *S. obliquus* demonstrated similar phosphate removal efficiency with all combination of WW and PCW but comparatively better phosphorus removal efficiencies to *C. sorokiniana* (**Figure 8 of Paper 1**).

COD removal potential varied with the influent COD and the growth conditions for the *C. sorokiniana*, and achieved highest COD removal (77.89%) in WW-75 dilution, but declined in WW-50 (70.49% and WW-25 (55.15%) wastewater. Lower COD removal of 69.38% was recorded for raw sewage (100%). *S. obliquus* achieved 76.13% COD removal with raw wastewater 69.31% in WW-75, 70.56% in WW-50 and 71.10% in WW-25 (**Figure 4 and 5, table 2 of Paper 1**). These results indicate the better performance of *S. obliquus* for COD removal with variable loading despite stressing culture conditions, possibly due to their higher mixotrophic growth potential (Kim et al., 2013; Mandal and Mallick, 2009)

Both algal species showed significant removal of coliforms from the wastewater (Fig. 9). *C. sorokiniana* resulted $99.78 \pm 0.12\%$ removal efficiencies of total coliforms in 100 to $99.93 \pm 0.12\%$ in 25% (Table 2). In comparison, application of *S. obliquus* achieved total coliforms removals from $99.93 \pm 0.12\%$ in 100% wastewater and $99.97 \pm 0.12\%$ from 25% (**Figure 9a of Paper I**). Complete removal of faecal coliforms was achieved for all wastewaters with both species (**Figure**

9b of Paper I). These results indicate the applicability of both species to achieve pathogen removal during wastewater treatment without any additional process(s) such as chlorination. *S. obliquus* showed marginally better potential for pathogen removal. Cultivation of *S. obliquus* and *C. sorokiniana* in wastewater resulted in the increase in pH to values above 10 (**Figure 10 of Paper I**). The removal of coliforms can be correlated to high levels of pH, which is antibacterial on pathogens.

4.1.3 Lipid production from microalgae

Microalgae utilize nutrients for metabolic processes to produce different types of metabolites (lipids, proteins, carbohydrates). The unfavourable conditions could change the biomass composition of algae, their growth rate and productivity (Sajjadi *et al.*, 2018). The lipid yields of both *C. sorokiniana* and *S. obliquus* grown in all wastewaters are presented in **Figure 11 (Paper I)**. Due to lower nitrogen in 25% dilutions, the lipid accumulation was almost two times higher than control grown in BG11. The lipid yield in *C. sorokiniana* and *S. obliquus* grown on BG11 in medium were 14.17 and 15.82 %, respectively. While maximum lipid yield was obtained with 25 % dilution for both *C. sorokiniana* (27.68%) and *S. obliquus* (28.36%). Since, the nutrient levels in BG11 are higher than wastewater, the cultivation of algae with wastewater provided relatively nutrient deprived conditions and resulted in nutrient stress conditions. The lipid accumulation potential was comparatively higher for *S. obliquus* and may be a consequence of increased stress levels in the cell compared to *C. sorokiniana*. In stress, microalgae changed their metabolic pathway by producing more lipid to survive in unfavourable conditions. The overall results showed importance of selecting algal species with better stress resistance to extend their applicability for comprehensive wastewater treatment.

4.2 Microalgae downstream processing

LEA is good source of reducing sugar and protein with good minerals content, which can be further used as feedstock for energy and feed (Maurya *et al.*, 2016). High input and low energy recovery negatively affect the microalgae biofuel production at commercial scale. The effective utilization of LEA has great potential to improve the overall economics of microalgae biodiesel production at commercial scale. It is important to determine the most suitable route for LEA utilisation (Rashid *et al.*, 2013). **Paper II** focussed on the effects of the different drying and cell disruption processes on lipid yield in whole microalgae and their effect on protein, and reduced sugar in LEA. The extraction and application of other valuable production from LEA would improve economics of the algal biofuels. This is an important component of integrated microalgal biorefinery approach for the production of algal biofuels.

4.2.1 Effect of drying and cell disruption on lipid recovery

After harvesting, the thick algal slurry was dried to improve the stability and viability of the biomass for downstream processes. Most common drying techniques used in algal technologies are sun drying, oven drying, freeze drying, drum drying and spray drying (Guldhe *et al.*, 2014; Show *et al.*, 2015). After drying, cell disruption is required to enhance lipid recovery. This section emphasized on effect of drying and cell disruption techniques on lipid recovery from algal biomass.

In **Paper II** microalgae biomass was dried using three different techniques (oven, sun and freeze drying) and their effects on biochemical composition were analysed. Four different cell disruption

techniques (microwaving, sonication, osmotic shock and autoclaving) were employed for lipid extraction. The effect of different drying and cell disruption techniques on lipid recovery were compared and effects of drying and cell disruption on LEA metabolites have been discussed.

Lipid recovery from *S. obliquus* depends significantly on the different drying and cell disruption techniques employed during the lipid extraction process (**Table 1 of Paper II**). The effect of drying and cell disruption on lipid yields are presented on **Figure 1 (Paper II)**. Microwave assisted extraction resulted in significantly improved lipid yields for all dried samples (Sun drying, freeze drying and oven drying) in comparison to other employed methods. Osmotic shock with 10% NaCl solution showed lowest lipid yield. In addition, sun drying resulted insignificantly decreased lipid yields, compared to oven drying ($p = 0.44$) and freeze drying ($p = 0.005$), while no significant difference ($p = 0.588$) in yields was observed between oven dried and freeze-dried samples. Similarly, comparisons for different cell disruption techniques were also employed for all drying processes (**see section 3.1 of Paper II**). These findings showed that the sun drying resulted significantly decreased lipid yields when compared to oven drying ($p = 0.044$) and freeze-drying ($p = 0.005$), while no significant difference in yields was found for oven dried and freeze-dried algal biomass. On demonstrate scale sun drying technique is economically feasible compared to other techniques.

4.2.2 Effect of drying and cell disruption on protein in LEA

LEA are rich in proteins and reduced sugars which can be further utilized for feed applications or energy production (Ehimen *et al.*, 2013; Ju *et al.*, 2012). Theoretically, the protein content of LEA residue should increase compared to whole algal biomass because of their increased fraction to account for the removed mass of extracted lipid. During organic solvent assisted lipid extraction,

some protein was also extracted with lipid, which resulted in loss of some protein in LEA. The effect of different lipid extraction processes on protein yield are compared in whole algae and LEA and presented in **Table 2 and Figure 2 (Paper II)**. Sun drying techniques showed comparably higher protein recovery in whole algae biomass. The effects of drying and cell disruption techniques on protein availability in LEA shown in **section 3.2 of Paper II**.

LEA obtained after sonication showed significantly higher ($p < 0.001$) protein yield than whole cell algae for oven dried sample. Protein yield was significantly reduced in microwave ($p < 0.001$) and autoclave ($p < 0.001$) assisted LEA compared to the yields of intact whole cell algae. LEA had similar protein yields to whole cell algae during osmotic shock with 10% NaCl. From all the cell disruption procedures, sonication favoured highest protein yields after lipid extraction, while yields were reduced for both microwave and autoclave assisted cell disruption (**section 3.2 of Paper II**). In some drying and cell disruption techniques, the protein yield in LEA increased as well. Microwave and autoclaved assisted lipid extraction showed higher lipid yield than ultrasonic and osmotic shock (**Figure 1 of Paper II**). Cell disruption techniques such as ultrasonication and osmotic shock showed lower cell disruption efficiency than microwave and autoclave hence lower lipid yields were obtained but protein yields increased in respective LEA. Despite the loss in LEA for different cell disruption procedures, sufficient protein yields were obtained which established the feasibility of extraction of these co-products from algae

4.2.3 Effect of drying and cell disruption on reduced sugar in LEA

Microalgae are rich in carbohydrate, which can be used for energy production. There are ample amount of carbohydrates present in *S. obliquus* biomass and after reduction, glucose was the major fraction followed by other monosaccharides (mannose, galactose, xylose and arabinose (Miranda *et al.*, 2012)).

LEA has shown comparable yield of reduced sugar obtained after different drying and cell disruption techniques. It ranging from 12.37% for freeze dried with sonication to 19.51% for oven dried sample with autoclaving as cell disruption techniques (**Figure 3 of Paper II**). No significant effect on the yields of reduced sugar were noticed due to variability in either drying or cell disruption methods for lipid extraction (**Table 3 of Paper II**). Similarly, no significant ($p = 0.981$) interaction was observed between these two treatments on the yield of reduced sugar. These comparable yields of reduced sugar for whole cell algae and LEA establish the feasibility of LEA as reduced sugar feedstock.

4.3 Effect of different solvents/solvent mixtures on lipid recovery from dry and wet microalgal biomass

The energy required to produce 1 kg of algal biodiesel from dried biomass is approximately 4000 times higher than the wet biomass (Lardon *et al.*, 2009). Previously, many studies have been done to minimize the energy consumption and utilization of wet algal slurry for biodiesel production purposes (Cheng *et al.*, 2014, Taher *et al.*, 2014; Wahidin *et al.*, 2014). The lipid extraction from wet algal slurry followed by direct transesterification to make biodiesel as an energy saving approach (Cheng *et al.*, 2014; Cheng *et al.*, 2013; Sathish and Sims, 2012; Wahidin *et al.*, 2014). The selection and optimization of lipid extracting solvent/solvent mixtures and extraction process

are vital. Easily available, less toxic and inexpensive solvents are preferable for lipid extraction. The LEA generated, can further be potentially utilized for energy, feed and other purposes to offset the overall microalgal biodiesel production costs (Alzate *et al.*, 2014; Ju *et al.*, 2012; Patterson & Gatlin, 2013; Quinn *et al.*, 2014). **Paper III** is based on selection of appropriate solvent/solvent mixtures for lipid extraction from wet algal slurry and dry biomass having minimal effects on metabolites left in residual LEA. The results showed that choice of solvent system and biomass conditions (wet slurry or dry) are determined by microalgal biomass application. Microalgae *S. obliquus* was cultivated in BG11 medium in 3000 L pond under natural light condition. After harvesting, biomass was divided in two parts, a portion was used as wet slurry whilst the other was sun dried.

Microwave cell disruption and six common solvent/solvent systems were used to extract lipids from wet and dry biomass. (**Figure 1 of Paper III**). Lipid recovery from dry biomass was higher than wet biomass. In the case of dry biomass, chloroform: methanol was observed to be a suitable solvent system if algae used only for lipids. If LEA is to be used for other purposes (feed, biomethane production etc.) then DCM: methanol was found suitable solvent. The physical and chemical properties of chloroform is similar to DCM but it is 10 times more toxic than the DCM. Thus, the use of DCM: methanol is a prospective option to reduce the risk of accidental hazards (Chen *et al.*, 1981).

In the case of wet biomass, lipid yield was significantly lower than the dry biomass. Higher lipid yield was obtained using chloroform: ethanol solvent system as compared to hexane, chloroform: methanol, DCM: methanol, isopropanol: hexane and isopropanol (**Figure 1 of Paper III**). Other solvent/ solvent systems such as isopropanol, hexane could be suitable options if LEA is used for

feed and energy applications. The main reason for lower lipid recovery from wet biomass is, the presence of water content (>80 %) which hinders interaction between solvent and lipid.

4.3.1 Effect of different lipid extracting solvent on (dry and wet biomass) protein and carbohydrates in LEA

Solvent systems employed to extract the lipid from wet and dry biomass also affects the protein, carbohydrate yield and biomass quality (**Figure 2 and 3 of Paper III**). It is important to investigate and compare the effects of different solvents on the reduction of protein and carbohydrate to established the economical feasibility of the overall process. Protein contents were analysed in whole algae and LEA obtained from dry and wet biomass. The protein yield varies in dried LEA, in the cases of isopropanol, chloroform: ethanol, isopropanol: hexane, DCM: methanol and chloroform: methanol. In some cases, no significant differences were observed in protein yields obtained either in between chloroform: ethanol and isopropanol: hexane or chloroform: methanol and DCM: methanol (**Figure 2 of Paper III**). DCM: methanol is suitable for lipid extraction if LEA would be further used for energy or feed applications. Because it extracts 0.55% less lipid and 1.6% less protein than chloroform: methanol solvent system. The other benefit to select DCM: methanol over chloroform: methanol, is less toxic in nature, inexpensive and left minimal toxic effect on LEA. So, LEA could be further utilized as potential feedstocks for feed, biomethane, biohydrogen, biobutanol etc.

In case of whole wet algae biomass, the protein yield was recorded up to 7.63% lesser than dry whole algal biomass. In wet biomass of LEA, protein yield ranged from 12.08% with isopropanol to 34.57% with hexane (**Figure 2, Paper III**). Moreover, protein recovery is increased using

hexane (4.21%) and DCM/methanol (1.61%). Suitable solvent/systems were identified according to lipid yield and toxicity. The three solvent systems (chloroform: ethanol, isopropanol: hexane, and hexane) resulted better lipid yields from wet biomass and protein yield in LEA, where protein yields were comparable to whole algal biomass. Isopropanol: hexane resulted in 8.89% higher protein yield and 0.6% higher lipid yield than isopropanol alone. In the case of wet biomass, isopropanol/hexane and hexane are the preferable solvents if LEA to be used for aquaculture feed application or biomethane production.

The carbohydrate contents of LEA are also affected by lipid extracting solvents. The yield of carbohydrates in LEA depends upon the biomass drying condition and solvent system used for lipid extraction process. The carbohydrate content ranged from 20.53% to 26.53% when hexane and DCM: methanol was used for lipid extraction. The reduction in carbohydrate yield in whole algae was recorded with different lipid-extracting solvent systems in following order, hexane > chloroform/ethanol > isopropanol/hexane > isopropanol > chloroform/methanol > DCM/methanol (**Figure 3 of Paper III**).

The carbohydrate content in whole wet algal biomass was 28.92% and the yield of carbohydrate in LEA ranged from 20.67% to 26.29% using hexane and isopropanol, respectively as lipid extracting solvent (**Figure 3 of Paper III**). Carbohydrates left in LEA in both cases (dry and wet) could be used for biomethane, bioethanol, biohydrogen etc. production. Chloroform: methanol showed higher lipid yields but LEA obtained was not found to be suitable for biomethane production because a small proportion of chloroform remains in the LEA, which negatively affect the biomethane productivity. Hexane extracted LEA biomass is suitable for biomethane production because it is single solvent, less toxic and more cost effective than other solvent systems.

4.4 Biochemical potential for biomethane production using microalgae as a substrate

To minimize the cost of overall algal biomass and biodiesel production, maximum utilization of LEA is imperative (Maurya *et al.*, 2016). The LEA is good source of carbon, which can be used as renewable substrate for biomethane production through anaerobic digestion (Table 6). The cell wall of *S. obliquus* is constituted by glucose, mannose and galactose (Miranda *et al.*, 2012). Being high in sugar contents, the anaerobic biodegradability should be high, but its linkage forming cellulose and hemicellulose together in the presence of some other cell components (sporopollenin-like biopolymer confers the cell wall a high resistance to bacterial attack) (Passos *et al.*, 2014). Even though the anaerobic biodegradability of sugars is high, their availability for hydrolysis and subsequent anaerobic degradation is limited by the structure of the cell wall. In order to improve the hydrolysis, pre-treatment is required to make the algal cell wall prone to the anaerobic degradation (Ehimen *et al.*, 2013; Ward *et al.*, 2014). Biomass pre-treatment increases the surface area allowing the microorganisms to easily take up the carbon from the algal biomass. Product extracted algae and pretreated algae have potential to improve algal anaerobic digestion primarily due to cell disruptive effect and higher solubilization of organic matter.

In **Paper IV** the application of different pretreated and product-extracted algae with varying sludge to substrate ratios on biomethane potential were evaluated. The change in biochemical composition of the biomass in specific pre-treatments and product extractions were observed with their effects on the BMP as specific substrate type. Energy and cost balance were also calculated in order to understand how an anaerobic digestion of algal biomass can be incorporated for optimum and economic process yields in algal biorefinery.

Four types of algae biomass were used as substrates in which two were whole algae (SDPA and MHTA) and two were product-extracted algae (LEA and PEA). The intracellular composition of

untreated biomass and physiochemical characteristics of different samples used for BMP test is presented in **Table 1 (Paper III)**. To improve biomethane production, pre-treatments are required (**discussed in literature review**). The BMP test was conducted with 0.5% solid and two different sludge ratio (SAR 1:1 and SAR1:4) as per literature reported (Alzate *et al.*, 2012; Alzate *et al.*, 2014) (**section 2.3 of Paper IV**). The nutrient medium for BMP used as per Owen *et al.* (1979). All the test were conducted in 120 mL serum bottles with 70 mL volume including media and algae sludge substrate.

4.4.1 Effect of sludge to inoculum ratio on biomethane production

Proteins and lipids are major components in the algal biomass, the extraction of these major metabolites from microalgae significantly affects the elemental composition of LEA and PEA (Parimi *et al.*, 2015; Zhao *et al.*, 2014). The relation between SAR (sludge to algae ratio) and pre-treated biomass (SDPA and MHTA) and product-extracted biomass (LEA and PEA) shown in **Figure 1 of paper IV**). Due to suitable chemical and elemental composition of SDPA and MHTA than PEA and LEA, the sludge to algae ratio (SAR) were more prominent. Samples containing whole algae biomass (SDPA or MHTA) but with lower SAR such as 1:4 gave higher methane production. In case of product-extracted algae, higher methane production yields were achieved with higher SAR such as 1:1 ratio. The quantitative difference in the methane production in the two samples containing MHTA biomass, S1H4 and S1H1 was 13%, which was much higher than the samples containing SDPA, LEA, and PEA where such differences were 4.5%, 0.38%, and 4.5%, respectively (**section 3.2, Figure 1 of Paper IV**).

4.4.2 Effect of pre-treatment on biomethane production using different algal substrate

Different downstream processing algal biomass changes the elemental and metabolites compositions. **Figure 2 of Paper IV** shows CH₄ and CO₂ production profiles of different substrates over the course of BMP experiment. The rate of biomethane production from LEA and PEA was higher than whole algae at the start of the experiment. The average CH₄ production rate (50 ml CH₄ g⁻¹ VS) from samples S1L4, S1L1, S1P4, and S1P1 was found approximately 2.5 times higher than the average CH₄ production (22 ml CH₄ g⁻¹ VS) from samples S1D4, S1D1, S1H1, and S1H4 on the 5th day of the BMP test. In the case of products extracted algae (PEA & LEA) the production of CH₄ and CO₂ starts as soon as BMP test was initiated. In case of whole algae biomass (SDPA and MHTA), a lag phase was observed. Product extraction was itself a pre-treatment in which high C/N ratio and digestible organic compounds were made easily available for microorganisms. Whole algal biomass produces gas for longer period, which result in higher cumulative CH₄ production than PEA and LEA (**Figure 2, A and B of Paper IV**). This is due to higher amount of digestible carbon that was present in whole algae compared to LEA and PEA. The initial COD in whole algae was higher than the LEA and PEA (Table 2). Overall biomethane production was higher in whole algae while biomethane production rate was higher in products extracted algae.

The pre-treatment also affected the CH₄/CO₂ ratio. **Figure 3** shows the comparison of gas production and ratio of CH₄/CO₂ in different algal biomass (**section 3.4 of paper IV**). The highest CH₄/CO₂ ratio was obtained algal samples containing MHTA with a ratio of 1.52 and 1.16 in S1H4 and S1H1, respectively. This was due to the intracellular components (lipids, proteins and carbohydrates) remaining intact in MHTA containing samples but being readily available for anaerobic digestion due to disintegration of cell wall caused by heat treatment. Figure 3 shows the

total cumulative gas ($\text{CH}_4 + \text{CO}_2$) production is found to be highest in LEA>SDPA>MHTA samples. LEA biomass was found to be a suitable feedstock for biomethane production to improve economic aspects of microalgae biodiesel production. The overall process showed that PEA could be a good source for biomethane production.

4.5 Aquaculture feed and challenges

Due to fast growth of aquaculture food industry, the aquaculture feed market has grown exponentially in the last decade and this growth expected to continue (Shah *et al.*, 2018b). Due to increased health awareness, the consumption of fish has been increased globally, which required the aquaculture industry to increase productivity and provide quality fish to consumer (Yaakob *et al.*, 2014). There are huge requirement of aquaculture feed to fulfil the gap developed between demand and supply. Fishmeal (FM) is one of the best protein sources which is predominantly used in aquaculture feed due to high protein content, balanced amino acids profile and high digestibility (Jasour *et al.*, 2018). To reduce the feed production cost lower amount of FM used. Reduction of the FM content in aquaculture feed may lower the feed efficiency, growth performance, palatability nutrient uptake, digestion, absorption etc. Despite availability of many conventional aquaculture feed feedstocks, there is no single ingredient which can replace FM in fish diets. Replacement feedstocks lack essential amino acids, essential fatty acids, natural pigment etc. which makes use of conventional aquaculture feed more challenging (Olsen *et al.*, 2012). By-products or waste products from many process industries are used as constituents of conventional feed. These constituents include feedstuffs from plants (soya bean whole, soya bean oil extracted, wheat middling, ground nut cake, palm kernel cake, rice brans, maize, sorghum etc.), brewery (brewery dried yeast, brewery dried grains), and animal by-products. Soybean and FM are the most

important constituents in aquaculture feed, which were used as a source of proteins and oil. Plant-based proteins tend to be deficient in essential amino acids such as methionine, lysine, tryptophan and threonine (Li *et al.*, 2009). Methionine is the amino acid, which is involved in the initiation of peptide synthesis required for proteins. Thus, it is important to provide methionine in soybean-based fish feed in order to get optimum growth, health and nutritional quality in fish flesh. Soybean meal in aquaculture feed have been reported to induce intestinal inflammation and reduce survival rate of fish (Bravo-Tello *et al.*, 2017). Soybean has high-fat content and trypsin inhibitors, hemagglutinin and antivitamin whilst brewery dried yeast has limited methionine and cysteine (Nguyen, 2008). Soybean meal contains high amounts of crude protein and appropriate amino acid profiles for the fish growth from amongst plant-based protein sources, but it also contains anti-nutritional compounds such as phytic acid (Huang *et al.*, 2017).

In the absence of good aquaculture feed ingredients, a review of the existing feedstock, with a critical assessment of the replacement of FM with various feedstock inclusion level may help in the selection of better feedstock for proper growth of fish for sustainable aquaculture industry.

Paper V (ready to submit) is a current review of literature available in this field. The paper focuses on conventional aquaculture feed and its limitations and potential application of microalgae in aquaculture. Microalgae have been used in aqua feed (live microalgae, whole microalgae and LEA) to improve the feed quality. Different types of ingredient have been used in aquaculture feed, such as waste product, poultry meal, chicken feather meal, fish waste such as head tail skin etc. Other feed ingredients include plant-based products such as soybean for protein and fatty acids, maize, corn meal, barley etc. All these ingredients have own advantages and disadvantages based on its application in aquaculture feed. The review also deals with potential substitution of various conventional constituents by microalgae in aquaculture and focuses on the

main ingredients used in aquaculture feed. The manuscript also gives emphasis to microalgae, aquaculture based integrated biorefinery approach, challenges, and recommendation for future works.

4.5.1 Application of whole and LEA in Nile tilapia (*Oreochromis niloticus*) feed experimental set up

Microalgae have shown potential to reduce the dependency on conventional feedstocks in aquaculture (Shah *et al.*, 2018b). The use of microalgae showed significant beneficial effects on many species of fishes. Microalgae can be used as a protein source, additionally it also provides fatty acids, pigments, vitamins, minerals etc. (**discussed in literature review**) (Becker, 2007). Inclusion of whole algae and LEA as protein sources have been discussed in detail in **Paper V (review paper, ready to submit)**. The cultivation of *S. obliquus* at pilot scale biomass harvesting, different feed production, fish cultivation etc. shown in appendix III-VI. Microalgae inclusion level depends on nutrient value of algae and fish species.

Previously many researcher used microalgae as aquaculture feed supplement but most of the studies done for certain weeks or months or a particular time period which were significantly shorter than full cultivation period to observe the different characteristics behavior in fish and carcass compositions (Fadl *et al.*, 2017; Gbadamosi & Lupatsch, 2018; Ju *et al.*, 2012, Lu *et al.*, 2006; Mahmoud *et al.*, 2018). Despite these studies, there is lack of information available regarding rearing of the tilapia from juvenile to finisher stage using microalgae of *S. obliquus* (whole and LEA) supplemented feed as protein source at demonstration scale.

Application of microalgae in aquaculture feed can be grouped in two parts (i) whole algae in aquaculture feed as a protein source (ii) LEA as a protein source in aquaculture feed.

In this manuscript (**undergoing preparation, Paper VI**), application of microalgae in aquaculture has been discussed in detail. In the 1st trial, the optimization of whole algae used as a protein source with four different inclusion levels (2.5%, 5%, 7.5% and 10%) in Nile tilapia (*Oreochromis niloticus*) diets were carried out. Commercial feed was used as a control. Five fish tanks were installed (3 m³ each) have carrying capacity 15-20 kg fish biomass m⁻³ at Northdene aquaculture research facility, Durban, South Africa. Each tank was stocked with juvenile 100 tilapia of similar size (average 5 cm and weight 10 g. Each diet (commercially feed as control and four algae supplemented feed) was randomly assigned to five tanks. In the first four tanks, algae supplemented as protein source (2.5%, 5%, 7.5% and 10%) feed were used and, in the fifth tank commercially available (without algal) feed was fed to Nile tilapia. Prior to start of the feeding trials the tilapia were reared to 24 h without feed to acclimatize to the experimental environment. Initially all Tilapia were fed by hand 4 times daily at 8:00 am, 10:00 am, 2:00 pm and 7:00 pm). The feeding rate to Tilapia was adjusted according to body weight (6-10% biweekly) obtained using weekly morphometric analysis at the site (Ju *et al.*, 2012).

4.5.2 Application of whole and LEA in Nile tilapia (*Oreochromis niloticus*) feed and growth performance

The application of whole cell algae and LEA in tilapia feed trials were conducted for 44 weeks (juvenile to finisher stage). Five fishes were randomly collected from each tank by cast net every week during course of study. Total length (cm) and weight (g) were measured and recorded using

scale and digital balance. The results showed that tilapia feed 7.5% (whole algae) had better growth performance compared to control and other inclusion levels. The results showed 7.5% has better specific growth rate (1.57%), daily body weight gain (1.1 g), body weight (427.16 g) as compared to other algae supplemented feed. 10% whole algae supplemented feed and control feed showed better results than 2.5% and 5%. The growth performance of the 10% whole algae inclusion into tilapia feed and the control were specific growth rate (1.5 and 1.5%), daily body weight gain (0.86 and 0.85 g) and body weight gain (331.48 and 330.48 g), respectively). In contrast, 2.5% whole algae supplemented feed showed comparatively lower growth performance: body weight gain 207.19 g, specific growth rate 1.38, and daily weight gain 0.54 g. Similarly, 5% whole algae supplemented feed also showed lower growth performance: body weight gain 216.11 g, specific growth rate 1.39% and daily weight gain 0.56 g. After 44 weeks of experiment (both trials), no mortality was recorded in any of the experimental feed. The higher value of feed efficiencies were found in 10% whole and LEA supplemented feed, which were 74 and 65.3, respectively. The hepatosomatic index was found highest (2.01%) in 7.5% whole algae supplemented feed as compared to other algae supplemented feeds. The carcass index (%) was similar in whole algae supplemented feed but lower in LEA supplemented feed. Condition factor was found highest (18.81%) in 7.5% whole algae supplemented and lowest (8.98%) in 2.5% whole algae supplemented feed (Table 10). The results showed that whole and LEA of *S. obliquus* (7.5%) could be incorporated in to tilapia feed at commercial level.

Table 9 Growth performance of Nile tilapia fed the whole algae, LEA supplemented, and control feed

Parameter	Whole algae				LEA		Control
	2.5%	5%	7.5%	10%	7.5%	10%	
Body weight gain (g)	207.2	216.11	427.16	331.48	357	331.78	330.08
Specific growth rate %	1.38	1.39	1.57	1.5	1.21	1.17	1.5
Daily wt. gain	0.54	0.56	1.1	0.86	0.88	0.86	0.85
Feed conversion ratio (FCR)	2.92	1.86	1.35	1.69	1.52	1.60	1.71
Feed efficiency % (FE)	34.85	53	74	59	65.3	51	58
Hepatosomatic index %	1.4	1.63	2.01	1.47	1.8	1.85	1.9
Carcass Index %	0.85	0.87	0.86	0.88	0.78	0.77	0.84
Condition Factor %	8.98	9.45	18.81	12.74	13.2	12.01	12.53
Survival rate %	100	100	100	100	100	100	100

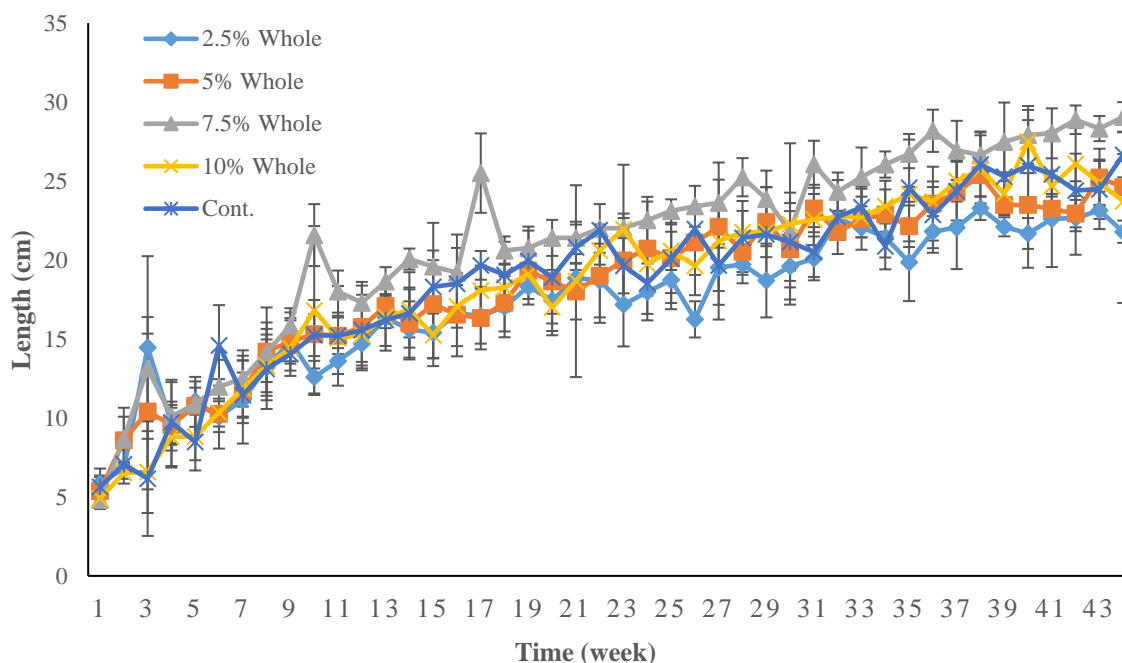


Figure 3 Total length gain (cm) of Nile tilapia fed the whole algae supplemented and control feed

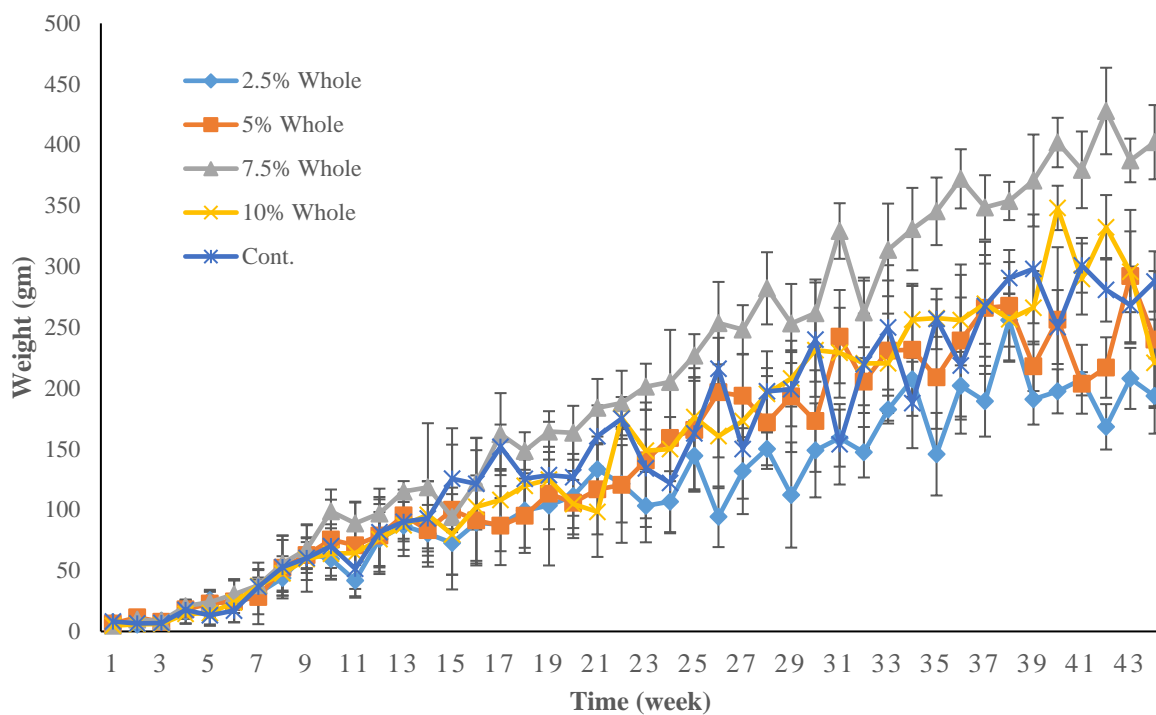


Figure 4 Total body weight (g) of Nile tilapia fed the whole algae supplemented and control feed

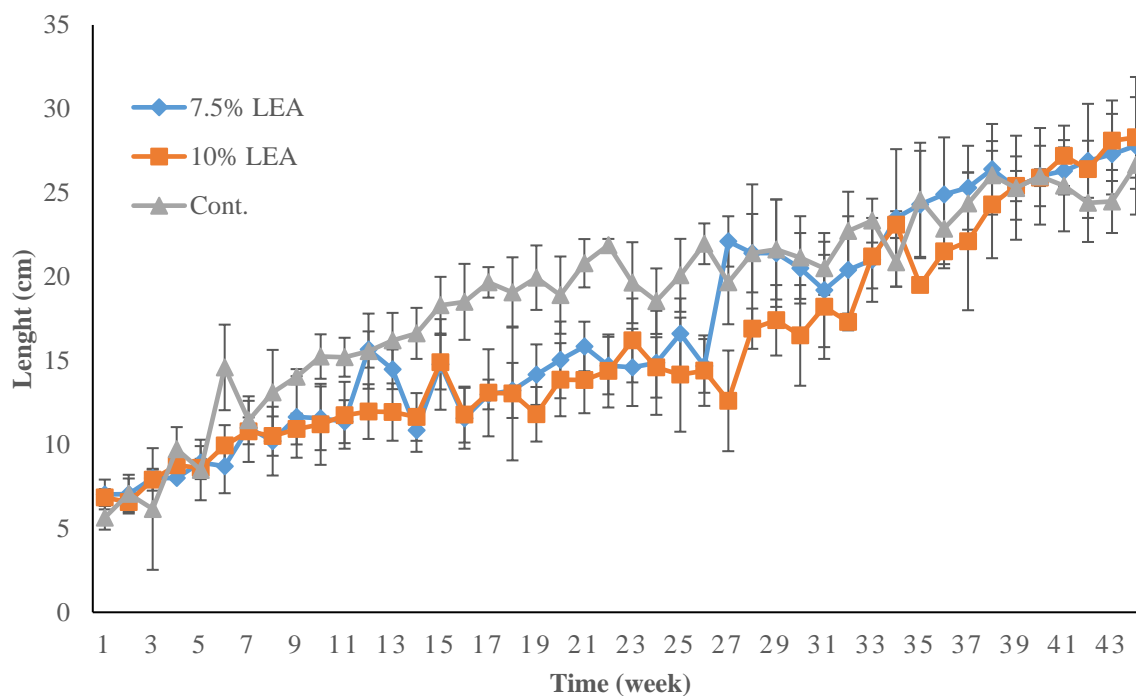


Figure 5 Total length gain (cm) of Nile tilapia fed the LEA supplemented and control feed

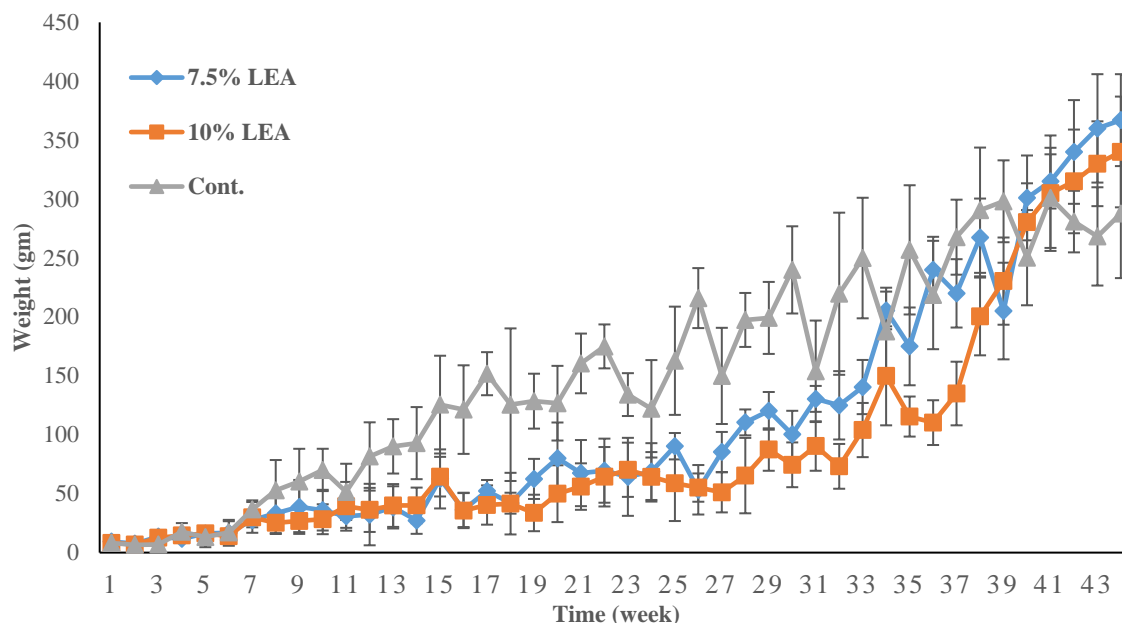


Figure 6 Total body weight (g) of Nile tilapia fed the LEA supplemented and control feed

4.5.3 Application of whole and LEA in Nile tilapia (*Oreochromis niloticus*) feed and body composition

The body composition (%) of tilapia is shown in table 10. The highest carcass protein was measured in fish feed with 7.5% whole algae (43.96%), followed by 7.5% LEA supplemented feed (42.2%) followed by 10% whole algae (40.46%). The highest carcass lipid was recorded in control feed (19.94%) followed by the 7.5% whole algae supplemented feed (18.0%). The carbohydrate content in tilapia carcass was higher in LEA supplemented feed than whole algae and control. There was not much difference found in carcass ash content between different algae supplemented feed and control. The highest moisture content (73.4%) of tilapia was found in 7.5% whole algae supplemented feed and lowest in (69.2%) in control. The finding of this study conclude that whole biomass or LEA of *S. obliquus* can be used as protein source in Nile tilapia (*Oreochromis niloticus*). The 7.5% whole and LEA were optimal inclusion levels as protein source to improve

the growth performance and biochemical composition). Application of LEA in Nile tilapia feed can make biodiesel from microalgae more competitive to fossil fuel.

Table 10 Body composition of tilapia fed the whole algae, LEA supplemented and control feed

Proximate composition (%)	Whole algae				LEA		control
	2.5%	5%	7.5%	10%	7.5%	10%	
Protein	33.53	37.53	43.96	40.46	42.2	41.3	36.1
Lipid	15.07	14.65	18.0	17.56	15.1	14.8	19.94
Carbohydrate	15.75	14.69	13.69	14.11	17	18	13.63
Ash	8.1	7.6	8.5	7.4	9.3	9.8	7.6
Moisture	72.2	69.8	73.4	71.4	70.3	73.2	69.2

4.6 Standing of journals and receptions of publications

Paper	5 Year Impact factors	Total download	Total read	Total citation
Paper I	6.2	>600	>700	65
Paper II	6.1	>600	>700	44
Paper III	2.74	>500	>500	5
Paper IV	6.1	>300	>400	2

(Source: google scholar and research gate)

Paper I: This paper was published in Journal of Cleaner Production, a journal with a 5-year impact factor of 6.2. It has received 63 citations as at 9th April, 2019.

Paper II: Published in Bioresource Technology, a high impact peer reviewed journal with a 5-year impact factor of 6.1. It has currently >600 downloads and received 44 citations as at 9th April 2019.

Paper III: This paper was published in Environmental Science and Pollution Research with a 5-year impact factor of 2.74. It has currently >600 downloads and received 5 citations as at 9th April, 2019

Paper IV: This paper was also published in Bioresource Technology journal. It has currently >300 downloads and received 2 citations as at 9th April, 2019.

5.0 CONCLUSIONS

The major conclusions based on the objectives of the thesis are as follows:

Cultivation of microalgae *S. obliquus* and *C. sorokiniana* were achieved successfully in raw sewage and different mixture with PCW, which can be further used for wastewater treatment and biomass production. The cultivation of microalgae in wastewater is economical and simultaneously it can be used for the treatment of various types of wastewater. The mixture of raw sewage with PCW play important role in improving the growth, nutrient removal efficiency, biomass production and lipid yield.

- This study presents a comprehensive investigation of applicability of two microalgae for the complete wastewater treatment.
- *S. obliquus* showed overall better nutrient and pathogen removal efficiency and lipid yield than *C. sorokiniana*.
- *C. sorokiniana* showed better adaptability to physiological stress due to varying nutrient loading than *S. obliquus*.
- *S. obliquus* showed greater potential for removing COD, nutrients and comparable pathogens removal in comparison to *C. sorokiniana*

The LEA biomass remains after the lipid extraction, account for 70% of the total biomass, which are rich in protein, carbohydrates as reduced sugar and other value-added product. Therefore, the LEA can be used for other purposes such as energy production or feed generation to make microalgal biofuel economically sustainable.

- The LEA biomass of *S. obliquus* showed an excellent source of protein and reduced sugar.
- Microwave cell disruption technique was found to be most efficient for lipid extraction while osmotic shock was least
- LEA biomass obtained after lipid extraction using ultra-sonication as cell disruption technique showed higher protein yields than whole algae. While LEA biomass obtained from microwave and autoclaved cell disruption techniques have lower protein yield than whole algae.
- Oven and freeze-drying techniques were found to be efficient for lipid and protein recovery but these techniques are energy intensive in nature and challenging to scale up.
- Sun drying is a natural drying process, required least energy, easy to scale up but weather dependent and time taking.

To improve the lipid recovery from wet and dry microalgal biomass, selection of suitable lipid extracting solvent and its right proportion is crucial. It is also important that selected solvent should not have deteriorating effect on the protein and carbohydrate in LEA so that the LEA can be further used.

- Water content in wet microalgal biomass significantly affect the lipid recovery.
- The lipid extraction efficiency depends highly on types of biomass (wet and dried) and solvent/solvent mixtures used.
- Chloroform: ethanol was observed to be the most appropriate solvent from amongst all those tested, if biomass used only for lipid extraction purpose. Whereas, Isopropanol:

hexane or hexane only are good option due to their low toxicity, hence less deleterious effect on the LEA metabolites.

- In case of dry biomass, chloroform: methanol proved an appropriate solvent system if the biomass used solely for lipids.
- If dried LEA biomass further used for energy production such as biomethane, bioethanol, biohydrogen etc. or animal feed or food applications, DCM: methanol is the suitable solvent system.
- For lipid extraction, choice of solvent systems depends on the algal biomass (dry or wet) and further application of LEA.

LEA contains high proportions of proteins and carbohydrates thus it can be used in energy production and/ or feed preparation. Selection of the solvent used to extract lipid is vital when the subsequent use of LEA is for biomethane production. Pre-treatment of biomass improves the production of biomethane. Lower C/N ratio due to high protein content in LEA biomass negatively affects biomethane potential.

- Being less toxic, hexane extracted LEA is suitable for biomethane production.
- The lipid extraction process is in itself an affective pre-treatment process to enhance the rate of biomethane production.
- The average CH₄ production was ~ 2.5 times higher for protein and lipid extracted algae than for other pre-treated whole algae (SDPA and MHTA).
- The cumulative CH₄ production was higher in sun dried and mild heat-treated whole cell algae than LEA and PEA biomass.
- The highest cumulative CH₄ production was found in mild MHTA than SDPA

- The biomass having higher C/N ratio, has higher biomethane production potential. The protein extracted biomass showed approximately 2.5 times higher biomethane potential

Aquaculture is one of the fastest growing food industries in world. For aquaculture feed production, different ingredients are added to accomplish the dietary requirements of the fish. Application of microalgae in aquaculture feed showed great potential in terms of improved biomass production and flesh quality.

- Whole and LEA of *S. obliquus* has potential to be used as a protein source in tilapia feed.
- Overall whole algae supplement feed has shown better growth performance than the control when fed to tilapia.
- The results revealed that whole and LEA supplemented feed were well utilized by the tilapia.
- The results showed that supplementation of 7.5% *S. obliquus* (whole or LEA) improved the proximate carcass composition.
- Results of this research contribute to reduce the dependence on FM traditionally used in commercial feed
- Application of LEA in tilapia can improve the economics of algal biodiesel production

6.0 RECOMMENDATIONS

Based on the findings from this research, recommendations for future studies on LEA obtained after lipid extraction are as follows:

- Wastewater cultivation of microalgae needs scaled up trials for successful implementation of this technology.
- Drying and solvent extraction studies needs techno-economic evaluation for their industrial application
- Co-digestion studies are needed with different feedstock for biomethane production using microalgae
- Aquaculture feed application of microalgae needs life cycle assessment studies to establish its commercial application.
- Feed trials are needed with other commercially important fish species.

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8.0 APPENDIXES

Appendix I

BG11 nutrient medium

Component	Quantity
NaNO ₃	1.5 g
K ₂ HPO ₄	0.04 g
MgSO ₄ .7H ₂ O	0.075 g
CaCl ₂ .2H ₂ O	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
Na CO ₃	0.02 g
Trace metal mix A5	1 ml
Agar (if needed)	10.0 g
Distilled water	1.0 L

Trace metal mix A5

Component	Quantity
H ₃ BO ₃	2.86 g
MnCl ₂ .4H ₂ O	1.81 g
ZnSO ₄ .7H ₂ O	0.222 g
NaMoO ₄ .2H ₂ O	9.39 g
CuSO ₄ .5H ₂ O	0.079 g
Co(NO ₃) ₂ .6H ₂ O	49.4 mg
Distilled water	1.0L

Appendix II

Wastewater test physiochemical test

Chemical oxygen demand

Closed Reflux titrimetric method.

Procedure

2.5 ml sample was digested with digestion reagents consist of 1.5 ml $K_2Cr_2O_7$ and 3.5 mL sulphuric acid reagent. The mixture was then put in COD digestion apparatus for 2 hours at a temperature of 150 °C. After hours, the samples were not disturbed and allowed to cool to ambient temperature. After sufficient time digestion, tubes were shaken and left for another 30 minutes to let the precipitate and settle.

Digested sample was titrated against ferrous ammonium sulphate until the end achieved (wine red color). Two blanks solutions were prepared using distill water instead of sample and digested.

Calculation

The COD was calculated by following equation

$$[(A-B)*8000*(0.1M\ FAS)]/Volume\ of\ sample\ taken$$

Where, A= Titrant used for blank

B= Titrant used for sample

Alkalinity

10 ml of the sample was taken in a flask and 2-3 drops of phenolphthalein was added. Since there was no carbonate, so the color did not come. Then 2-3 drops of methyl orange were added and was titrated against the standardized acid (H_2SO_4) added from the burette. The end of titration was given by the color change from yellow to pinkish yellow.

$$Alkalinity = A * N * 5000 / mL\ of\ sample$$

A=Volume of acid used

N=Normality of acid used

Total solids

Clean porcelain crucibles are taken and ignited at 550 °C for one hour in a muffle furnace. They were allowed to cool and weighed just before used. Fixed volumes of well-mixed samples were taken in pre-weighed crucibles. The crucibles were kept in an oven at 98 °C overnight. The

evaporated sample was then dried at 103°C C to 105 °C in an oven for one hour. The crucibles are cooled to room temperature and then weighed. Total solids were then calculated as follows:

$$\text{Mg of total solids} = (A - B) * 1000 / \text{sample volume, (mL)}$$

Where, A= weight if dried residues + crucible (mg)

B= weight of crucible (mg)

Total suspended solids

Total suspended solids are determined by subtracting total solids from dissolved solids.

Appendix III Pilot scale microalgae cultivation



Seed culture pond

Raceway pond



Appendix IV Microalgae harvesting at pilot scale



Appendix V Aquaculture facility at Northdene, Durban, South Africa and different size of feed



Appendix VI Fish growth performances and dissection





Appendix VII

Formulae used in fish growth performance analysis

$$(a) \text{ Weight gain (\%)} = \frac{(\text{Final body weight (gm)} - \text{initial body weight (gm)})}{\text{Initial body weight (gm)}} \times 100$$

$$(b) \text{ Feed efficiency ratio (FER)} = \frac{\text{Fish weight gain (gm)}}{\text{Protein feed (gm)}}$$

$$(c) \text{ Feed conversion ratio (FCR)} = \frac{\text{Feed weight as dry (gm)}}{\text{Weight gain (gm)}}$$

$$(d) \text{ Feed conversion rate (FCR)} = \frac{\text{Feed intake (gm)}}{\text{Weight gain (gm)}}$$

$$(e) \text{ Feed conversion efficiency \%} = \frac{\text{Biomass (gm)}}{\text{Total feed intake (gm)}}$$

$$(f) \text{ Hepatosomatic index \% (HSI)} = \frac{\text{Liver weight (gm)}}{\text{Body weight gain (gm)}} \times 100$$

$$(g) \text{ Carcas index (CSI)\%} = \frac{\text{Carcas weight (gm)}}{\text{Body weight gain (gm)}} \times 100$$

$$(h) \text{ Protein retention (PR)\%} = \frac{\text{Final fish body protein (gm)} - \text{Initial fish body protein (gm)}}{\text{Protein fed (gm)}} \times 100$$

$$(i) \text{ Energy retention (ER)\%} = \frac{\text{Final fish body energy (MJ)} - \text{Initial fish body energy (MJ)}}{\text{Energy fed (MJ)}} \times 100$$

$$(j) \text{ Survival rate} = \frac{\text{Number of live fish}}{\text{Number of fish introduced initailly}} \times 100$$

$$(k) \text{ Weight gain (gm /kg} = \text{Final weight (gm)} - \text{Initial weight (gm)}$$

$$(l) \text{ Condition factor (CF)\%} = \frac{\text{Final weight (gm)}}{\text{Final length (cm}^3)} \times 100$$



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Evaluation of various cell drying and disruption techniques for sustainable metabolite extractions from microalgae grown in wastewater: A multivariate approach

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ABSTRACT

This study attempted to determine the yield of extractable metabolites and the cost of oil production from *Scenedesmus obliquus* cultivated in municipal wastewater. The microalgae achieved a biomass concentration of 1.64 g L^{-1} as well as pollutant removal efficiencies of 87.86% COD, 86.16% $\text{NH}_4\text{-N}$, and $\approx 100\%$ $\text{PO}_4^{3-}\text{-P}$. The harvested microalgae were subjected to different drying and cell disruption techniques for lipid extraction, and the residual biomass was used to recover proteins and carbohydrates. Principal component analysis was employed to evaluate the metabolic yields obtained from sun-, freeze-, and oven-drying methods and microwave-, sonication-, autoclaving-, and osmotic shock-disruption techniques. The lipid yield varied between $4.90 \pm 0.42\%$ for sun-dried biomass subjected to osmotic shock and $25.39 \pm 1.08\%$ for freeze-drying with microwave-assisted extraction. Protein yield of the whole microalgae cells ($31.26 \pm 3.76\%$) was comparable ($p > 0.05$) to that resulting from lipid-extracted microalgae by either autoclaving or osmotic shock. Carbohydrate yield of the intact microalgae cells ($19.80 \pm 1.49\%$) was comparable ($p > 0.05$) to that of lipid-extracted microalgae from amongst all the cell disruption methods. Results of a techno-economic analysis indicated that the cost of oil production from microalgae varied between \$0.883 and \$2.088 per liter. These results revealed the feasibility of using a sequential extraction of lipids followed by proteins and carbohydrates.

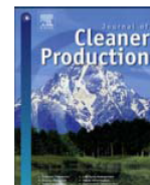
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Sustainable dewatering and drying of self-flocculating microalgae and study of cake properties



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ABSTRACT

Efficient dewatering and drying at lower cost have been a requirement in reducing the cost of microalgal biomass production. The self-flocculation capacity of *Scenedesmus obliquus* and the hydrophobic nature of polypropylene non-woven fabric membrane (PNM) are explored in this study. Formation of 'algal bio-filter layer' due to natural forces showed the efficiency of the process. Compared to the conventional methods, the cost of harvesting reduced substantially. The physico-chemical and mechanical properties of the harvested cake were studied. To the best of our knowledge, this is the first report on PNM usage for dewatering and drying as well as algal cake properties. The membrane showed high biomass recovery efficiency (94%) for *Scenedesmus* sp. but very low (58%) for *Chlorella sorokiniana* (non-self-flocculating). Dewatering on sand-bed followed by drying on wire-gauge (10.5 m.c) found to be the best arrangement. Solar drying of 1–2 cm thick slurry was achieved within 34 ± 2 h. Based on drying period, true density, bulk density, inter-cake porosity, breaking strength, grinding and selective physical properties, obtaining a 2-mm-thick cake was comparatively more energy efficient for post-harvest operations. The estimated cost of pilot-scale dewatering-drying was \$ 0.048 per kg of dry algal biomass. Dehydration using PMN followed by natural drying was found to be energy efficient and comparatively very cost effective for pilot scale harvesting.

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Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation



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ABSTRACT

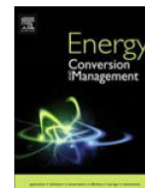
Cultivation of microalgae utilizing wastewater substrate could form a sustainable biorefinery with double benefit of biomass generation and nutrient remediation. In this study potential of aquaculture wastewater is evaluated for cultivation of *Chlorella sorokiniana* in heterotrophic mode for generation of high value biomass. Nutrient removal potential is also assessed. Aquaculture wastewater with 400 mgL⁻¹ sodium nitrate supplementation resulted in biomass productivity of 498.14 mgL⁻¹ d⁻¹. The biomass generated showed lipid productivity of 150.19 mgL⁻¹ d⁻¹, carbohydrate productivity of 172.91 mgL⁻¹ d⁻¹ and protein productivity of 141.57 mgL⁻¹ d⁻¹. The nutrient removal efficiencies were 75.56% for ammonium, 84.51% for nitrates, 73.35% for phosphates and 71.88% for COD (chemical oxygen demand). The findings of this study underline the potential of aquaculture wastewater for production of valuable microalgal biomass which can be utilized for biofuels or feed application. This biorefinery concept also polished aquaculture wastewater which can be effectively reused.

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Energy Conversion and Management

journal homepage: www.elsevier.com/locate/enconmanEvaluating the potential of cytokinins for biomass and lipid enhancement in microalga *Acutodesmus obliquus* under nitrogen stressNirmal Renuka, Abhishek Guldhe, Poonam Singh, Faiz Ahmad Ansari, Ismail Rawat, Faizal Bux^{*}

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ABSTRACT

Despite the significant reduction in biomass production, nitrogen stress is the most feasible strategy used for lipid enhancement in microalgae. The present study evaluates the potential for application of exogenous cytokinins to enhance the lipid accumulation and overcome the biomass production constraint of microalgae under nitrogen stressed conditions. This study was undertaken to investigate the effect of exogenously supplied cytokinins viz. kinetin and zeatin at various stages of growth on biomass and lipid productivities of *Acutodesmus obliquus* under nitrogen stress (ON). The effect of cytokinins on photosynthetic performance and biochemical composition of biomass was also evaluated. Enhanced biomass and lipid productivities were observed upon supplementation of the microalgae with selected cytokinins. Supplementation with kinetin (1 mg L^{-1}) and zeatin (0.1 mg L^{-1}) resulted in increased biomass productivity up to 50% and 60.7% respectively as compared to ON control. The highest biomass productivity of $176.79 \text{ mg L}^{-1} \text{ d}^{-1}$ was obtained with the addition of 0.1 mg L^{-1} zeatin (ON-Z_i) at the initial log phase. Cytokinin supplementation resulted in improvement in photosynthetic performance and exhibited higher rETR (relative electron transport rate) values as compared to ON control. The addition of kinetin (ON-K_m) at mid log phase and Zeatin (ON-Z_i) at initial log phase resulted in 64.95% and 63.06% increase in lipid productivity respectively, compared to ON control. Carbohydrate productivity was also found to be increased with zeatin (ON-Z_i) supplementation. The supplementation of cytokinins is an easy and scalable strategy for biomass and lipid enhancement in *Acutodesmus obliquus* under nitrogen stress.

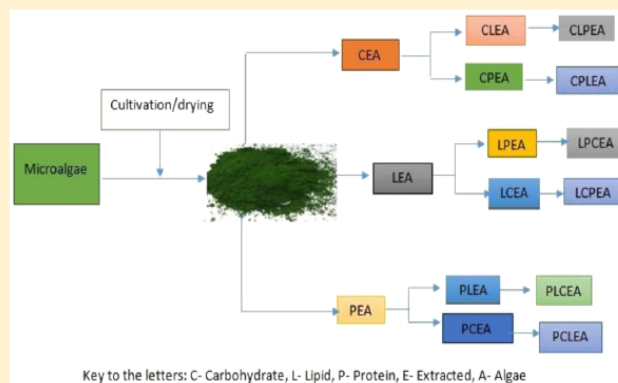
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Exploration of Microalgae Biorefinery by Optimizing Sequential Extraction of Major Metabolites from *Scenedesmus obliquus*

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ABSTRACT: The effects of six different sequential extractions of proteins, lipids, and carbohydrates on their yields and subsequent biomass recoveries was investigated. The maximum yields of lipids, proteins, and carbohydrates were $26.50 \pm 1.32\%$, $28.14 \pm 1.97\%$, and $16.40 \pm 0.43\%$, respectively, in primary extraction of biomass. Compared to the primary extractions, lipid yields were significantly lowered by 20–22% in secondary extractions. The maximum loss of proteins in secondary (post lipid extraction) and tertiary extractions was 34.79% and 56%, respectively. The most significant loss (38–44.5%) in carbohydrates was recorded after tertiary extractions. Among all of the extraction sequences, the sequence of proteins–lipids–carbohydrates extracted algae (PLCEA) showed optimum recovery of individual metabolite. For this extraction sequence, the yields of proteins, lipids, and carbohydrates were found to be 28.14%, 22%, and 10.17%, respectively. It was also characterized by the highest residual biomass available for second (80%) and third (61%) steps of extraction. Finally, the cumulative yields of these metabolites were converted into net value gains. The extraction sequence PLCEA could result in 66.5% net value gain overcoming the cost of biomass generation.





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Bioresource Technology

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Adaptability of growth and nutrient uptake potential of *Chlorella sorokiniana* with variable nutrient loading



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HIGHLIGHTS

- *C. sorokiniana* can adapt N and P uptakes according to levels in external medium.
- It can maintain uniform growth rates and productivity despite variable uptakes.
- Evidences of stresses were observed on quantum efficiencies and chlorophyll.
- Increased nitrite excretion was observed with high nitrate levels in the feed.

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Chlorella sorokiniana

Nutrient uptake potential

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Quantum efficiencies

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ABSTRACT

Chlorella sorokiniana can sustain growth in conditions hostile to other species, and possesses good nutrient removal and lipid accumulation potentials. However, the effects of variable nutrient levels (N and P) in wastewaters on growth, productivity, and nutrient uptake by *C. sorokiniana* have not been studied in detail. This study demonstrates the ability of this alga to sustain uniform growth and productivity, while regulating the relative nutrient uptake in accordance to their availability in the bulk medium. These results highlight the potential of *C. sorokiniana* as a suitable candidate for fulfilling the coupled objectives of nutrient removal and biomass production for bio-fuel with wastewaters having great variability in nutrient levels.

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Full Length Article

Biodiesel synthesis from microalgal lipids using tungstated zirconia as a heterogeneous acid catalyst and its comparison with homogeneous acid and enzyme catalysts



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HIGHLIGHTS

- Tungstated zirconia used for conversion of *S. obliquus* lipids.
- Tungstated zirconia showed 94.58% FAME conversion and reuse up to 3 batches.
- Heterogeneous catalyst showed comparable conversion with homogeneous catalyst.
- Heterogeneous catalyst showed higher conversion than enzyme catalyst.
- Time requirement for tungstated zirconia catalysis was lowest.

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ABSTRACT

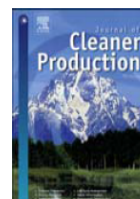
Downstream step of catalytic conversion is scarcely investigated area in microalgal biodiesel production process. In this study a heterogeneous acid catalyst, tungstated zirconia (WO_3/ZrO_2) is evaluated for conversion of *S. obliquus* lipids. Catalytic efficiency of tungstated zirconia catalyst was compared to the homogeneous acid catalyst and enzyme catalyst in terms of conversion efficiency, reaction parameters, energy consumption and reusability. Tungstated zirconia catalyst showed maximum biodiesel conversion of 94.58% at 100 °C temperature, 12:1 methanol to oil molar ratio and 15% of catalyst amount based on oil weight in 3 h. Tungstated zirconia showed comparable biodiesel conversion to homogeneous catalyst and higher conversion than the enzyme catalyst. The time requirement for heterogeneous catalyst was lowest, while, the energy consumption was highest among the selected catalysts. Most of the fuel properties of biodiesel synthesized by tungstated zirconia catalyzed conversion of *S. obliquus* lipids comply with the specifications set by ASTM and EN standards.

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Cultivation of *Chlorella sorokiniana* and *Scenedesmus obliquus* in wastewater: Fuzzy intelligence for evaluation of growth parameters and metabolites extraction



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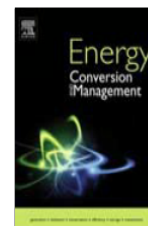
Metabolic yields

Physiological health

ABSTRACT

This study attempted to investigate the reuse potential of flocculated wastewater for the growth of microalgae species and to understand the relationship between microalgae growth parameters and metabolites extraction using a fuzzy logic model. For these purposes, batch experiments were conducted using two microalgae species, namely, *Chlorella sorokiniana* and *Scenedesmus obliquus*, cultivated in different types of flocculated wastewater, viz., BlueGreen (BG11), polymer (P_{ww}), chitosan (CH_{ww}) and alum (A_{ww}). Results indicated that a logistic model fitted well the biomass profiles with r^2 -values >0.95 . The carrying capacities of *C. sorokiniana* (K : 45.13 mg L^{-1}) and *S. obliquus* (K : 71.59 mg L^{-1}) grown in the CH_{ww} medium were significantly ($p < 0.01$) higher than other flocculants. Additionally, the two microalgae species cultivated in CH_{ww} showed a good physiological health throughout the growth period. It was found that higher extraction yields of lipids: 16.0–19.8% and carbohydrates: 19.98–20.43% (w/w) of the dry weight were obtained when the microalgae species were grown in the CH_{ww} wastewater. Further, a fuzzy inference system confirmed the experimental data by predicting the yields of lipids, carbohydrates, and proteins at particular inputs of logistic model parameters and physiological health condition (r^2 -values > 0.98). It was concluded that CH_{ww} could be used as an efficient flocculated wastewater for the growth of microalgae species and that the suggested modeling technique was promising.

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Design and development of polyamine polymer for harvesting microalgae for biofuels production[☆]



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ABSTRACT

Research findings of the past few decades on the cultivation of microalgae for biodiesel production from laboratory to pilot scale microalgal cultivation have translated into empirical hope of developing an eco-friendly biofuel from algae. As far as economic sustainability is concerned, harvesting of microalgae is one of the most energy extensive processes and thus a major challenge, being faced by this industry. In our study, we designed and developed a quaternary ammonium salt based cationic polymer and evaluated its effectiveness for freshwater microalgae harvesting. An epichlorohydrin-*n,n*-diisopropylamine-dimethylamine polymer with high viscosity (1040 cps) was synthesized. The flocculation performance of this polyamine polymer was evaluated in terms of biomass recovery efficiency of microalgae (*Scenedesmus* sp.), its effect on lipid yield and composition. The results revealed that due to high molecular weight, the biomass recovery efficiency of the polymer was achieved >90% at a very small dose of 8 mg/L whereas similar biomass recovery efficiency of chitosan and alum were achieved at 80 and 250 mg/L respectively. The presence of functional quaternary amine and hydroxyl groups played an important role in electric charge neutralization of microalgal cells, hence the improved microalgal flocculation performance in comparison to the natural flocculants but not affecting the lipid yield and its composition. The approximate cost of harvesting 1 kg of *Scenedesmus* biomass is approximately 0.5 USD for the polyamine polymer whereas 50 USD for chitosan. Therefore, polymer based harvesting of microalgae for low valued products such as biodiesel, polyamine based polymers would be preferred over the natural polymer.

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Research article

Techno-economic estimation of wastewater phycoremediation and environmental benefits using *Scenedesmus obliquus* microalgae



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ABSTRACT

This study investigated the dual application of *Scenedesmus obliquus* for wastewater phycoremediation and biochemical component accumulation in microalgal cells. The microalgae grown in wastewater showed microelements uptake and removal efficiencies of $71.2 \pm 3.5\%$ COD, $81.9 \pm 3.8\%$ NH_4^+ , $\sim 100.0\%$ NO_3^- , and $94.1 \pm 4.7\%$ PO_4^{3-} . The growth profile of *Scenedesmus obliquus* indicated a specific growth rate of $0.42 \pm 0.02 \text{ d}^{-1}$ and carrying capacity of $0.88 \pm 0.04 \text{ g L}^{-1}$. The lipid, protein, and carbohydrate yields (w-w^{-1} of dry weight) were $26.5 \pm 1.5\%$, $28.5 \pm 1.5\%$, and $27.5 \pm 1.6\%$, respectively. The de-oiled biomass was subjected to biochemical extraction, achieving protein and carbohydrate yields of $25.3 \pm 1.4\%$ and $21.4 \pm 1.2\%$, respectively. Fourier transform infrared spectroscopy showed several functional groups (e.g., N–H, CH_3 , CH_2 , C=O, C–N, P=O, and Si–O) on the biomass surface, confirming the accumulation of biochemical elements in microalgae. The thermal analysis of microalgal biomass depicted sequential stages of dehydration ($60\text{--}190^\circ\text{C}$), devolatilization ($200\text{--}490^\circ\text{C}$), and solid residue decomposition ($490\text{--}600^\circ\text{C}$). The cost-benefit analysis of microalgae cultivated in wastewater was derived regarding amortization and operating costs and energy and environmental benefits. The net profit of phycoremediation was $16885 \text{ US}\$\text{y}^{-1}$, resulting in a payback period of 14.8 years (i.e., shorter than the project lifetime). Accordingly, the proposed phycoremediation process was economically viable.

Microalgae for Biofuels: Applications, Process Constraints and Future Needs

Faiz Ahmad Ansari, Ajam Yakub Shekh, Sanjay Kumar Gupta,
and Faizal Bux

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Chapter 6

Harvesting of Microalgae for Biofuels: Comprehensive Performance Evaluation of Natural, Inorganic, and Synthetic Flocculants

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Abstract Microalgal biomass is considered as one of the most suitable alternative feedstocks for the renewable biofuels. Microalgae have several advantages such as ability to grow in harsh environment, comparatively very high productivity, and high lipid contents. Due to such potentials, microalgal biomass is preferred over the convention biofuel feedstocks. The concentration of microalgal biomass typically ranged between 0.5 and 1 kg/m³ in the raceways or open pond type cultivation systems and around 5–10 kg/m³ in the closed photobioreactor-type cultivation systems. The bottleneck of the algal biofuels is the harvesting of microalgae biomass from diluted culture media. Irrespective of the density of the algal biomass, the water content in microalgal culture exceeds 99% that makes the separation process lengthy and energy intensive. This largely determines the economic viability of microalgae-based biofuels and by-products. Among various techniques used for the harvesting of microalgal biomass, coagulation and flocculation have been found very effective and inexpensive; however, the choice of the coagulant depends on the use of harvested biomass for desired end products. The success of microalgae har-

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Phycoremediation: An Eco-friendly Algal Technology for Bioremediation and Bioenergy Production

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Abstract

Substantial amount of the refractory organics; inorganic nutrients, mainly nitrogen and phosphorus; heavy metals; etc. is discharged in conventional wastewater treatments. The concentration of such contaminants in the discharged wastewater depends on the performance and maintenance of the wastewater treatment plants (WWTPs). Though further reduction in such contaminants is possible with an aid of some of the advance technologies and skilled manpower, it makes wastewater treatment more expensive. More importantly, the running and maintenance of WWTPs are uncommon in economically weaker countries especially in the rural areas. This leads to the hunt of economically viable and environmentally sustainable alternative wastewater treatments. The truism nowadays is to recognize the emergence of phycoremediation as an alternative. Algae-based bioremediation has been found excellent for the nutrient, organic, pathogen, heavy metal, etc. removal from various types of wastewater. Green microalgae possess the unique potential of high photosynthetic activity compared to food crops and terrestrial plants. Therefore, such systems are capable of high biomass

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Techno-Economic Perspectives of Bioremediation of Wastewater, Dewatering, and Biofuel Production From Microalgae: An Overview

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19.1 INTRODUCTION

Water quality and energy are major issues faced by humans in the 21st century due to the rapid growth in population, urbanization, and industrialization, leading to global warming. To overcome such issue carbon dioxide (CO₂) emissions need to be diminished by 50%–80% for human welfare (Salama et al., 2017). Wastewater and industrial effluents are rich in nutrients, when released into the natural water bodies, cause severe environmental problems. Therefore, it is important to remove the

nutrients and toxic metals from the wastewater to a permissible level prior to discharge or reuse (Cai et al., 2013). There are numerous techniques available in the market to remove excess nutrients and various other toxicants from wastewater. However, the conventional wastewater treatment technologies are not sustainable, not economical. Biomass left after lipid extraction is known as lipid extracted biomass that can be used as feedstock for energy generation (biohydrogen, syngas, biomethane, etc.), in aquaculture feed, and in fertilizer to enhance crop productivity

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