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Stato dell'arte industriale della produzione di biocarburante da microalghe

State of the industrial art of the production of biofuel from microalgae

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Sommario

Il lavoro di tesi è incentrato sullo stato dell'arte industriale della produzione di biocarburante da microalghe. Secondo molti analisti a causa degli elevati tassi di consumo delle riserve fossili del pianeta, tali riserve energetiche verranno esaurite in meno di 50 anni. Per via del fatto che le riserve fossili sono la causa maggiore dell'emissione dei gas serra, responsabili del riscaldamento globale, è estremamente necessario trovare nel breve periodo nuove alternative rinnovabili, a zero emissioni ed economicamente sostenibili per evitare l'imminente crisi petrolifera. Innanzitutto si è pensato di applicare il processo di transesterificazione all'olio ricavato dalle colture di semi oleosi con il fine di ottenere biocarburante; ma l'uso di questi semi come riserva per la produzione di biocarburante ha due importanti svantaggi: il primo consiste nella competizione del prezzo con gli altri semi venduti sul mercato alimentare, mentre l'altro svantaggio consiste nella predazione del terreno arabile per la creazione di colture intensive aggravando il processo di desertificazione. Negli ultimi dieci anni sono state investigate altre risorse che potessero superare questi svantaggi ed una soluzione è venuta proprio dall'olio dei lipidi delle microalghe. Le microalghe sono caratterizzate da una molteplicità di vantaggi: primo la resa in olio per area di coltura di microalghe può ampiamente superare la resa in olio delle migliori colture di semi oleosi, secondo le microalghe crescono in un mezzo acquatico, ma richiedono minori quantità di acqua rispetto alle colture terrestri; terzo le microalghe possono essere coltivate in acque marine o in acque salmastre in terreni non arabili, non entrando in competizione con le colture tradizionali. Quarto la produzione di biomassa di microalghe può essere combinata con la biofissazione diretta della CO_2 di scarto (1 kg di biomassa algale secca richiede circa 1.8 kg di CO_2), quinto i fertilizzanti per la coltivazione delle microalghe (specialmente azoto e fosforo) possono essere ottenuti dalle acque reflue, sesto la coltivazione delle microalghe non richiede erbicidi e pesticidi, settimo la biomassa algale residua dopo l'estrazione dell'olio può essere utilizzata come mangime e fertilizzante, o fermentata per produrre etanolo o metano. Il più importante svantaggio associata a questa nuova tecnologia consiste nella necessità di selezionare ceppi algali ricchi di lipidi altamente produttivi. Il primo capitolo di questo lavoro di tesi consiste nella raccolta di informazioni provenienti da vari articoli in cui sono stati fatti vari esperimenti di coltivazione per l'individuazione di ceppi algali altamente produttivi in lipidi. L'individuazione dei ceppi algali è stata svolta utilizzando tre importanti parametri: il contenuto di lipidi, la crescita di biomassa e infine il più importante, la produttività lipidica ovvero il prodotto del contenuto di lipidi per la biomassa prodotta. È stata posta anche attenzione alla qualità di olio ottenuto, esaminando il profilo lipidico delle specie algali ed i parametri usualmente utilizzati per la valutazione del carburante, per ottenere un biocarburante che rispettasse le normative europee ed americane. L'esame di queste informazioni ha portato ad individuare come specie adatte per la produzione di biocarburante su larga scala tre ceppi algali: Chlorella Vulgaris, Nannochloropsis e Scenedesmus, in particolare Scenedesmus si è rivelata particolarmente promettente per via della sua elevata produttività lipidica data dal prodotto del suo elevato contenuto lipidico e della sua elevata crescita di biomassa. Il secondo capitolo si incentrato su una completa visione di tutti i metodi disponibili per l'estrazione dell'olio dai lipidi delle microalghe, partendo dai primi metodi utilizzati, fino agli ultimi metodi sviluppati. I metodi adottati per l'estrazione dei lipidi vengono distinti in metodi chimici e in metodi meccanici; i metodi chimici hanno mostrato di essere efficienti per quanto riguarda l'estrazione ma hanno come svantaggio quello di utilizzare solventi dannosi per l'ambiente. I metodi meccanici hanno mostrato di essere ben adatti all'estrazione dell'olio lipidico ma il loro punto critico si è rivelato essere il calore prodotto durante il processo, contaminando

conseguentemente la qualità dell'olio ottenuto e in particolare richiedendo un elevato dispendio energetico, soprattutto per produzioni su larga scala. Dall'analisi dei vari metodi per l'estrazione il metodo di estrazione attraverso pressione osmotica e il metodo di estrazione supercritica tramite CO_2 si sono rivelati particolarmente promettenti per la loro efficienza e soprattutto per via del fatto che i processi coinvolti non siano dannosi e inquinanti per l'ambiente. Nel terzo capitolo di tesi viene trattata la conversione dell'olio algale in biocarburante con una completa schematizzazione di tutti i metodi usualmente adottati, in particolare è stata posta attenzione ai processi di transesterificazione diretta da materia algale umida, ovvero processi che non richiedessero un precedente processo di essicazione della materia prima, ovvero che non richiedessero un elevato dispendio energetico. Nel terzo e ultimo capitolo sono stati analizzati anche i metodi termochimici, che comprendono la pirolisi e la liquefazione idrotermale. Tra tutti i metodi di conversione analizzati la transesterificazione da materia algale umida e in particolare la liquefazione idrotermale si sono rivelate particolarmente promettenti. La caratteristica fondamentale della liquefazione idrotermale consiste nell'utilizzare completamente la materia prima algale, quindi non soltanto i grassi ma anche carboidrati e proteine consentendo di ottenere elevate quantità di olio e conseguentemente di biocarburante. Correlato a questo però vi è uno svantaggio secondario ovvero la presenza nell'olio di quantità di azoto e quindi a seguito della combustione del biocarburante si otterrebbero emissioni di NO_x, pertanto per ovviare a questo problema, nell'ultima parte è stata riportata una trattazione di possibili catalizzatori da poter utilizzare per incrementare la qualità dell'olio e conseguentemente del carburante.

Introduction

According to many analysts, at the present staggering rates of consumption the world fossil oil reserves will be exhausted in less than 50 years. Due to the fact that fossil fuel combustion is the major source of greenhouse gases responsible for global warming, new, renewable carbon neutral, economically viable alternatives to fossil fuels are urgently needed to avert the impending oil crisis and the dramatic consequences of climate change. The crucial point is to find in the short period an ecological substitute to the petrodiesel. It was first thought to apply the transesterification process to the oilseed crops in order to obtain biofuel. The use of the seeds as resource of oil for the prodution of biofuel is unfortunately characterized by two main effective disadavantages, the first consists in the price competition with the seeds sold in the food market. The other disadvanteage consist in the predation of the arable land in order to create intensive coltures aggravating the desertification process. In the last decades were investigated other sources that could overcome this difficulties and so a solution came from the lipid oil of microalgae. Microalgae are characterized by a lot of advantages, first the oil yield per area of microalgae cultures could greatly exceed the yield of the best oilseed crops, second microalgae grow in an aquatic medium, but need less water than terrestrial crops; third microalgae can be cultivated in seawater or brackish water on non-arable land and do not compete with conventional agriculture, fourth microalgae biomass production can be combined with direct bio-fixation of waste CO_2 (1 kg of dry algal biomass requiring about 1.8 kg of CO_2); fifth fertilizers for microalgae cultivation (especially nitrogen and phosphorus) can be obtained from wastewaters; sixth algae cultivation does not need herbicides or pesticides; seventh the residual algal biomass after oil extraction may be used as feed or fertilizer, or fermented to produce ethanol or methane. The most important disadavantage associated with this tecnology is the need to select and grow higly productive lipid-rich algal strains. The first chapter of this thesis consists in the selection from the informations of different scientific article of the main promising algal strains. The selection has been made by analizing the following different parameters: lipid content, biomass growth and the lipid productivity and founding out what species could reach the highest lipid productivity. It has been posed also attention on different methods, that could enhance the lipid productivity like nitrogen starvation. At last an important role is reserved to the lipidome profile and on how it could influences the quality of biofuel produced. In the second chapter it's reported a complete overview of the different methods for extracting algal oil from the algal paste, posing attention to the methods that doesn't require the drying of microalgae, so that doesn't consume large quantities of energy. The third chapter focuses on the main processes of lipid conversion to biofuel analysing the wet processes and the thermochemical ones.

Chapter 1

1.Definition of the main substrates of lipids involved in the production of biofuel and definition of the parameter for the selection of the algal strain

Oleaginous microorganisms synthesize various kinds of lipid classes in their cellular compartment, which, according to the polarity of their head groups, can be classified as neutral lipids that are acting as energy storage (triacylglycerols, free fatty acids, sterols, sterols esters, waxes, and hydrophobic pigments), and polar lipids that are enabling membrane integrity (phospholipids, glycolipids, polysaccharides, and lipoproteins). The major proportion of total lipids are triacylglycerides with long-chain fatty acids similar to plant oils, making them comparable to conventional vegetable oil. Triacylglycerols (TAG) are fatty acid triesters of glycerol. There are diverse types of TAG with different properties depending on their fatty acid composition. The occurrence of TAG as storage compounds is widespread among eukaryotic organisms, such as microalgae, yeast, fungi, plants, and animals, whereas the occurrence of TAG in bacteria has only rarely been described. The presence of TAGS in microalgae is extremely imporatant because they are the preferred substrate to use in the transesterification process in order to obtain the biofuel.
The most important parameters used to select the correct algal species for high lipid production are the lipid content, the biomass productivity and especially the lipid productivity. The lipid content is as it said by the name the exact content of lipid in the algae, this parameter is really influenced by different parameter, in particular by the environmental conditions. The second important parameter is represented by the biomass productivity which is extremely relevant because in the selection of right promising species it could happen that a strain of algae has a limited lipid content but a high biomass productivity resulting in an higher lipid productivity than the one of another algal strain, which has a more elevated lipid content but a minor biomass productivity. The third parameter is the lipid productivity which is represented by the product of the lipid content and of the biomass productivity. This is the main relevant parameter in the selection of specific algal species because it puts in relation the the first two parameters.

1.1.Screening of eleven species of algae

Qualities generally desirable for mass culture for lipid production include rapid growth rate, high lipid productivity, resistance to contamination (through high growth rate or growth in an extreme environment), tolerance of a range of environmental conditions (particularly those difficult to control in an outdoor environment such as temperature and changes in salinity due to evaporation or flooding), rapid CO_2 uptake and tolerance of shear force. Characteristics that facilitate harvesting and downstream processing, such as large cell size, filamentous morphology, ease of flocculation or flotation, high cell density and high product content, are also advantageous. Species selection for lipid production is challenging due to the variety of characteristics required and the limited characterisation of microalgae, particularly under comparable conditions. Lipid productivity, the product of biomass productivity and lipid content, is one of the most obvious and easily quantifiable characteristics relevant to biodiesel production. However, interspecies comparison is complicated, as the metabolism and composition of many species can vary greatly under different environmental conditions. Factors, such as temperature, light and nutrient availability, have been shown to influence lipid productivity. Nutrient limitation can increase lipid content, although this response is species-specific. Nitrogen limitation is the most frequently reported method of increasing lipid

content, as it is cheap, easy to manipulate and has a reliable and strong influence on lipid content in many species. However, stress conditions that increase lipid content, such as nitrogen limitation, also decrease the growth rate and thus may not improve lipid productivity. In particular, by careful choice of a species that can maintain a high productivity under nitrogen-limiting conditions, intermediate levels of nitrogen stress and cultivation strategy, nitrogen limitation could be used successfully to enhance lipid productivity. Another element vital to the success of large-scale algal production is the cost and energy effective harvesting of the biomass. Microalgae cells are often less than 10 µm in diameter with densities just exceeding water. This requires harvesting techniques such as disc-stack centrifugation or micro-filtration, which are energy-intensive and too expensive for large-scale fuel production. For a low-cost, high-volume product, such as lipid for biodiesel, flotation or gravity sedimentation (possibly assisted by flocculation) seem promising methods. Gravity sedimentation is particularly appealing, requiring few inputs other than transfer to a settling tank and sufficient time for the cells to settle. If the addition of chemical flocculants can be avoided (or selected appropriately), the culture medium could potentially be recycled directly back to the culture vessel. An important characteristic for any biodiesel feedstock is the suitability of the fatty acid profile for biodiesel production. Algal fatty acid composition is influenced by growth conditions such as temperature and nutrient availability. The different carbon chain lengths and number and

Table 1

Culture	Abbr.	Medium	Taxa	Origin
Ankistrodesmus falcatus	Af	3N BBM	Chlorophyte	UTEX 242
Chlorella vulgaris	Cv	3N BBM	Chlorophyte	UTEX 395
Neochloris oleoabundans	Nol	3N BBM	Chlorophyte	UTEX 1185
Scenedesmus sp.	Sc	3N BBM	Chlorophyte	Own isolate, Upington, South Africa
Cylindrotheca fusiformis	Cf	Walne's	Diatom	UTEX B2087
Isochrysis C4	Iso	Walne's	Haptophyte	WITS culture collection, Johannesberg, South Africa
Nannochloropsis sp.	Nan	f/2	Eustigmatophyte	University of Hawaii culture collection
Pavlova sp.	Pav	Walne's	Haptophyte	MCM culture collection, Cape Town, South Africa
Phaeodactylum tricornutum	Pt	Walne's	Diatom	University of Hawaii culture collection
Tetraselmis suecica	Ts	f/2	Chlorophyte	UTEX LB 2286
Spirulina platensis	Spir	Zarrouk's	Cyanobacteria	Own isolate, abandoned tannery treatment pond, Wellington, South Africa

position of unsaturated bonds found in fatty acids influence the cetane number (CN), iodine value, oxidative stability, cold flow properties and viscosity of the fuel. Biodiesel quality is governed by different regulations in different countries (the ASTM D6751 in the United States and the EN 14214 in Europe). Fuel that does not meet these specifications could still be used if blended with petroleum diesel; however, this would be undesirable for the goal of producing sustainable, carbon-neutral fuel. CN is a dimensionless parameter related to the tendency of the fuel to ignite in the engine.

An adequate CN is required for good engine performance, cold start properties and minimisation of exhaust emissions. The CN of methyl esters decreases with increasing unsaturation (more double bonds) and shorter chain length. The iodine value is a measure of the total unsaturation of a mixture of fatty acids. It is expressed as grams of iodine required to react with 100 g of a sample. Oxidative stability is a measure of how long a fuel can resist oxidative degradation. Oxidative stability decreases with an increase in the number of double bonds present in the fatty acid methyl esters (FAMEs). This is a major issue affecting biodiesel, due to the common occurrence of fatty acids with more than one double bond. Oils rich in linoleic and linolenic acids tend to have poor oxidative stability. Biodiesel made from most common raw materials requires the addition of antioxidants. Biodiesel generally has relatively poor cold flow properties, and wax settling and plugging of filters and fuel lines can occur at overnight temperatures routinely reached in many parts of the world. Regulatory requirements for cold filter plug point (CFPP) differ by country. For Spain, a country with

Table 2

	μ_{MAX} (day^{-1})	$T_{D\ MAX}$ (days)	X_{MAX} (g L^{-1})	$Q_{X\ MAX}$(g L^{-1} day^{-1})	P_{MAX} (% DW)	$P_{VOL\ MAX}$ (mg L^{-1})	$Q_{P\ MAX}$ (mg L^{-1} day^{-1})
AfH	0.99	0.70	1.12	0.25	12	96	13
AfL	0.95	0.73	1.48	0.23	30	397	55
CvH	1.34	0.52	1.65	0.28	14	231	27
CvL	1.30	0.53	1.05	0.24	57	597	67
NolH	1.56	0.45	1.13	0.29	13	140	29
NolL	1.69	0.41	0.98	0.29	44	428	50
ScH	1.15	0.60	2.61	0.30	9	242	29
ScL	1.20	0.58	1.73	0.36	43	649	106
CfH	0.72	0.96	1.52	0.35	27	357	55
CfL	0.69	1.00	1.39	0.29	32	366	52
IsoH	1.32	0.52	2.48	1.23	7	165	24
IsoL	0.79	0.88	1.72	0.34	15	235	42
NanH	0.76	0.91	1.74	0.24	24	413	42
NanL	0.73	0.95	1.32	0.21	35	471	63
PavH	0.60	1.15	1.00	0.17	11	105	13
PavL	0.58	1.19	0.94	0.18	14	122	12
PtH	0.68	1.02	1.74	0.34	18	308	30
PtL	0.69	1.00	1.51	0.32	28	406	46
TsH	1.20	0.58	2.32	0.49	9	177	37
TsL	1.32	0.53	2.10	0.49	13	259	48
SpirH	0.43	1.63	2.74	0.29	4	95	14
SpirL	0.68	1.02	1.48	0.34	2	23	5

a relatively mild climate, a CFPP of below 0 and −10°C is specified for summer and winter, respectively. CFPP is increased by the presence of saturated fatty acids, particularly those with a long chain length, corresponding to a relatively high melting point. The properties of fatty acids that produce a favourable CN (long, saturated carbon chain) also cause poor cold flow properties and viceversa; therefore, a mixture of saturated and unsaturated fatty acids is required. In the following pharagraphs are investigated the extent and utility of information available in the literature for the comparison of 55 microalgae species according to characteristics relevant for biodiesel production. Direct comparisons were confined to growth rate and biomass productivity under nutrient replete conditions, and lipid content under nitrogen and silicon limited conditions. Lipid productivity and growth rates under nutrient limitation were seldom reported. This study builds on the previous work by comparing, under similar culture conditions in airlift photobioreactors, the lipid productivity, settling potential and fatty acid profile of 11 microalgae species, under both nitrogen replete and limited conditions. The aim was to assist in identifying the most promising species for lipid production and to test whether the trade-off between lower biomass productivity and higher lipid

content under nitrogen limitation would lead to higher lipid productivity and yield. Microalgae were grown in batch culture in airlift photobioreactors. The glass reactors were 60-cm high with an external diameter of 10-cm, a draft tube of 5-cm diameter and a working volume of 3.2 L. Air enriched with 0.29% CO_2 was sparged at 2 L min^{-1}. Light (250 µmol photons m^{-2} s^{-1} at the reactor surface) was provided by three cool white 18 W fluorescent light bulbs (Osram). Culture temperature was monitored daily and remained constant at 25±1°C. Starter cultures were grown in 500 mL glass bottles for 7–10 days before being used to inoculate the airlift reactors at a starting concentration of 0.05 g L^{-1}, except for the diatoms and haptophytes which required a starting concentration of between 0.1 and 0.2 g L^{-1}. Four freshwater species (grown in 3 N BBM medium), six marine species (grown in either f/2 or Walne's medium, depending on their nutrient requirements) and one halophilic species (grown in Zarrouk's medium) were tested (Table 1). The NO_3 concentration in all the media was 1,500 mg L^{-1} (nitrogen replete, H) or 150 mg L^{-1} (nitrogen-limited, L). At 1,500 mg L^{-1}, all the cultures still had sufficient nitrate for unlimited growth at the end of 14 days, whereas at 150 mg L^{-1}, the nitrate had been completely utilised by day 3 to 5 in all cultures. Biomass was measured by optical density at 750 nm. Dry weight (DW) measurements, taken once a day throughout the growth cycle, were used to construct a standard curve of DW relative to optical density. Samples (5–20 mL) were filtered through pre-weighed 0.45 µm cellulose nitrate filters (Sartorius Stedim) and dried at 80°C overnight. The weight of any media components was eliminated by filtering equivalent amounts of sterile culture media. The filters were dried and their weight subtracted from the sample weight. The fatty acid content and profiles were measured by direct transesterification and gas chromatography. For the purposes of this work, the lipid content was taken as being equivalent to the fatty acid content. At the end of the growth curve, cell shape was noted and cell size estimated using an Olympus BX40 microscope equipped with a digital camera and AnalySIS software (manual function, approx. 30 cells per culture). At the end of the 14-day growth period, 1 L of each culture, diluted in medium to 1 g L^{-1} (except for Neochloris oleoabundans, which had a biomass concentration of less than 1 g L^{-1} at the end of 14 days and was settled at its final concentration of 0.33 and 0.56 g L^{-1} for the nitrogen replete and limited cultures, respectively), was settled in a conical settling funnel for 24 h. The supernatant and the pellet were sampled and the DW of each was estimated by optical density at 750 nm. The settling rate was determined by monitoring the volume of the sedimented cell pellet over time and multiplying the volume

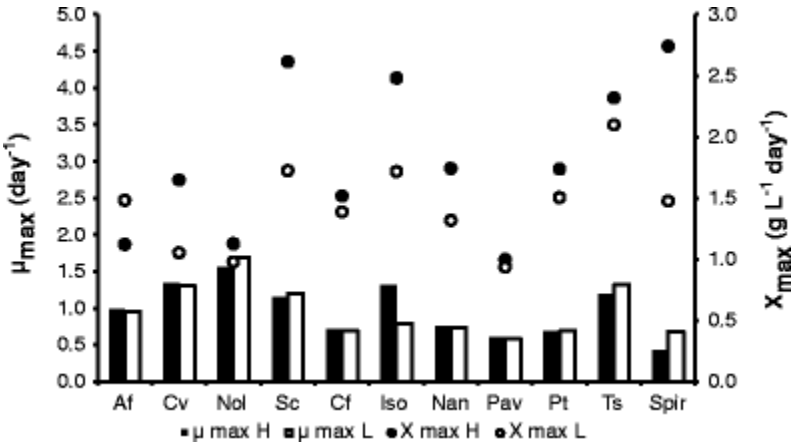

Figure 1

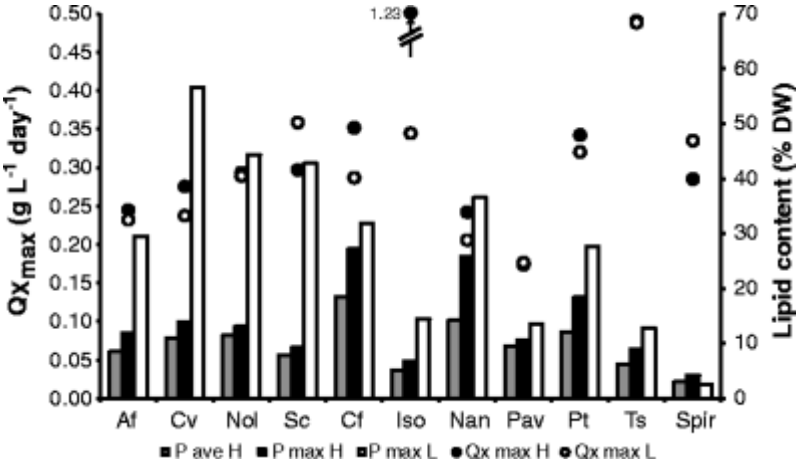

Figure 2

by the concentration of the cell pellet to calculate the biomass settled out as a function of time. The biomass recovery in the pellet after 24 h was calculated as a percentage of the original biomass present in the culture. The concentration factor was calculated as the concentration of cells in the pellet after 24 h, divided by the concentration of cells in the culture before settling. Instantaneous biomass productivity (Q_X) was calculated as the change in biomass concentration (*X*) per unit time between two consecutive sampling times. Specific growth rate (μ) was determined from the slope of the natural logarithm of biomass concentration as a function of time across each pair of sample points. Doubling time (T_d) was calculated as the natural logarithm of 2 divided by μ. Volumetric lipid content (P_{VOL}) was calculated as the product of biomass concentration (*X*) and lipid content (*P*; Eq. 1). Lipid productivity (Q_P) was calculated as the change in P_{VOL} as a function of time (Eq.2). Maximum parameters were defined as the highest value reached within the 14-day culture period. For the calculation of μ_{MAX}, $Q_{X\,MAX}$ and $Q_{P\,MAX}$, three consecutive instantaneous values of μ, Q_X and Q_P were averaged across the time course to provide a rolling average, and the maximum of each determined.

Eq.1:

$P_{VOL} = X \times P$ (1)

Eq.2:

$$Q_P = \frac{P_{VOL2} - P_{VOL1}}{t_2 - t_1} \quad (2)$$

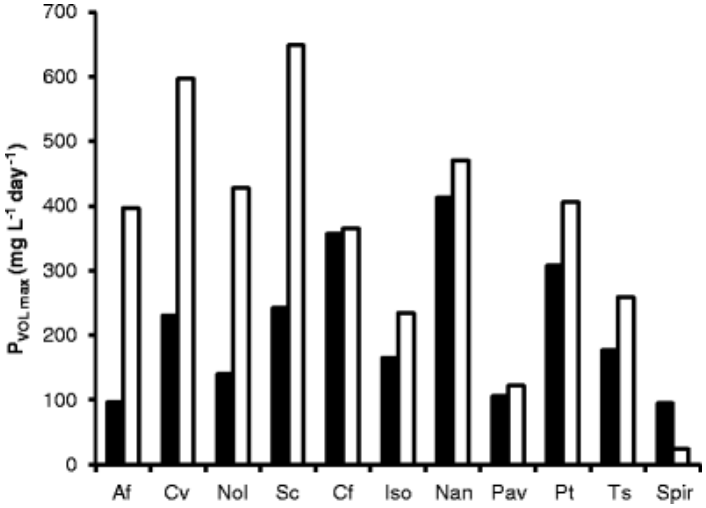

Figure 3

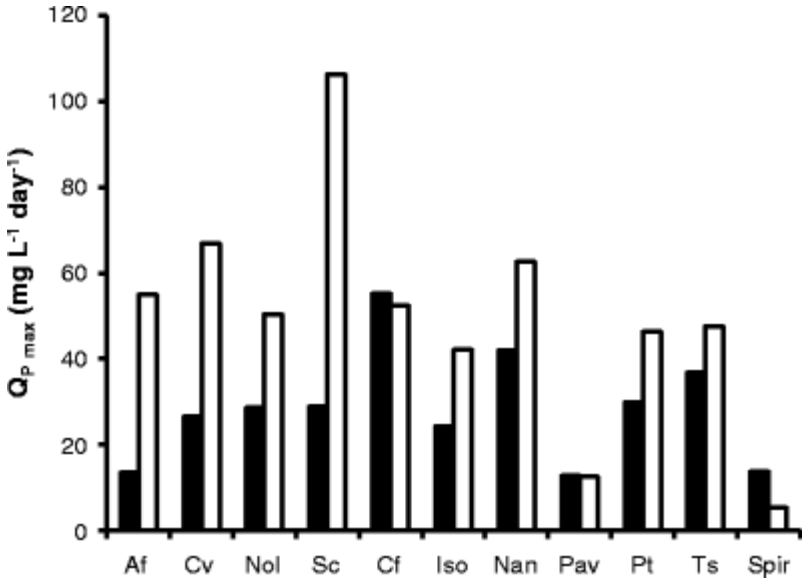

Figure 4

The FAME profile was used to calculate the CN and CFPP, the iodine value was calculated from the degree of unsaturation.

Table 3

	μ_{MAX} (day^{-1})	$T_{D\ MIN}$ (days)	X_{MAX} (g L^{-1})	$Q_{X\ MAX}$ (g L^{-1} day^{-1})	P_{MAX} (% DW)	$P_{VOL\ MAX}$ (mg L^{-1})	$Q_{P\ MAX}$ (mg L^{-1} day^{-1})
Cv	2.5	2.5	4.4	4.3	3.6	9.2	9.5
Sc	3.5	3.5	4.7	11.9	3.6	8.1	19.8

The biomass concentration and lipid content, under nitrogen replete (H) and limited (L) conditions, were reported as a function of time for each species. The growth rates, lipid content and productivities for each species under nitrogen replete and limited conditions, over a 14-day growth curve, are summarised in Table 2. The experimental error was investigated using five replicate cultures of Chlorella vulgaris. The relative error in biomass concentration throughout the growth curve across the five cultures was less than 5%. The variation in the key parameters presented in Table 2 was further investigated using duplicate cultures of C. vulgaris and Scenedesmus under both nitrogen replete and limited conditions. The average relative errors for the different growth and lipid characteristics are shown in Table 3. The error in μ_{MAX}, $T_{d\ MAX}$, X_{MAX} and P_{MAX} were all less than 5%. The error was larger (between 4.3 and 19.8%) in $Q_{X\ MAX}$, P_{VOL} and $Q_{P\ MAX}$ due to the propagation of error in calculations involving more than one measurement. Figure 1 shows the maximum specific growth rate and biomass concentration during 14 days of batch growth for each species under nitrogen replete and limited conditions. N. oleoabundans had the highest μ_{MAX} (day^{-1}; 1.56 and 1.69 for nitrogen replete and limited, respectively), followed by C. vulgaris (1.34 and 1.30), Isochrysis (1.32 and 0.79), Tetraselmis suecica (1.20 and 1.32) and Scenedesmus (1.15 and 1.20). The cultures that reached the highest X_{MAX} (g L^{-1}) were Spirulina platensis H (2.74), Scenedesmus H (2.61), Isochrysis H (2.48) and T. suecica H (2.32). In all species, except for A. falcatus, the nitrogen replete culture reached a higher final biomass concentration than the nitrogen-limited culture. The maximum biomass productivity ($Q_{X\ MAX}$) and lipid content (P_{MAX}) for each species under nitrogen replete and limited conditions, as well as the average lipid content (P_{AVE}) across the growth curve for nitrogen replete conditions, are shown in Figure 2. The maximum $Q_X$was similar for nitrogen replete and limited cultures, except for Isochrysis H which was very much higher than any other culture (1.2 vs. 0.2– 0.35 g L^{-1} day^{-1}). The highest $Q_{X\ MAX}$ was reached by Isochrysis H, followed by T. suecica H and T. suecica L. Some of the nitrogen replete cultures began to increase in lipid content towards the end of 14 days. This can be seen in the difference between the average lipid content (Figure 2, grey bars) and the maximum lipid content (Figure 2, black bars) reached during the culture period. Nannochloropsis (Nan), Cylindrotheca fusiformis (Cf) and P. tricornutum (Pt), in particular, showed an increase in lipid content with culture age, despite having sufficient nitrogen. In all cases, except for S. platensis, nitrogen-limited conditions resulted in a higher lipid content than nitrogen replete conditions. Volumetric lipid content (P_{VOL}) is a measure of the lipid yield per volume of culture at a particular time point. Figure 3 shows that, in all cases other than S. platensis, the maximum P_{VOL} was higher for nitrogen-limited than for nitrogen replete cultures. The highest P_{VOL} were achieved by Scenedesmus L (649 mg L^{-1}) and C. vulgaris L (597 mg L^{-1}). Lipid productivity (Q_P), calculated as the rate of change in P_{VOL}, shows the rate of lipid accumulation. The maximum Q_P achieved by each species during 14 days of cultivation is shown in Figure 4.

As with P_{VOL}, the Q_P was higher for nitrogen-limited than nitrogen replete cultures in all cases except C. fusiformis (Cf), Pavlova (Pav) and S. platensis (Spir). Scenedesmus L showed the highest lipid productivity (106 mg L^{-1} day^{-1}), more than 50% greater than the next highest species. This was followed by C. vulgaris L (67 mg L^{-1} day^{-1}) and Nannochloropsis L (63 mg L^{-1} day^{-1}).

Table 4

	Cell shape	Average cell length (μm)	Average cell width (μm)	Maximum settling rate (g L^{-1} h^{-1})	Biomass recovery after 24 h (%)	Biomass concentration factor at 24 h
AfH	Spindle	50	4.0	0.6	54	18
AfL	Spindle	50	3.0	0.008	18	14
CvH	Sphere	2.5	2.5	–	25	52
CvL	Sphere	2.5	2.5	–	2	15
NolH	Sphere	4.0	4.0	0.07	23	35
NolL	Sphere	3.5	3.5	0.2	24	65
ScH	Pointed oval	9.0	3.8	0.3	86	167
ScL	Pointed oval	9.1	4.7	0.04	79	103
CfH	Spindle	30	4.0	16	96	23
CfL	Spindle	30	4.0	24	94	19
IsoH	Sphere	5.2	5.2	–	15	12
IsoL	Sphere	5.2	5.2	0.005	7	50
NanH	Sphere	3.0	3.0	0.01	59	36
NanL	Sphere	3.0	3.0	–	59	15
PavH	Tapering sphere	5.0	5.0	0.005	40	19
PavL	Tapering sphere	5.6	5.6	0.001	41	17
PtH	Spindle	18.5	5.2	0.004	42	31
PtL	Spindle	11.5	5.0	0.08	42	6
TsH	Oval	12.5	8.3	0.4	80	102
TsL	Oval	12.2	9.5	0.07	94	109
SpirH	Filament	60–500	10	11	95	15
SpirL	Filament	60–90	10	29	95	136

Ease of harvesting was compared by measuring the rate and efficiency of gravity sedimentation of each culture after 14 days of growth (Table 4). The rate of accumulation of the cell pellet, and the proportion of the suspended biomass that had settled into a recoverable pellet after 24 h were used as crude indicators of the potential of a species to be harvested by this method. Figure 5 shows the biomass recovery after 24 h for each species under nitrogen replete and limited conditions. The species which settled best in a conical funnel at a dilution of 1 g L^{-1} (measured as % recovery in 24 h) were S. platensis (95%), C. fusiformis (94–96%), T. suecica (80–94%) and Scenedesmus (79–86%). S. platensis and C. fusiformis settled very quickly with C. fusiformis H and L cultures reaching maximum sedimented pellet volume within 5 min, while S. platensis H and L cultures took 3 h and 10 min, respectively. A. falcatus H and Nannochloropsis H also reached recoveries of over 50% within 24 h, while all other cultures had recoveries of less than 43%. The size and shape of cells in the different cultures at the end of the growth period were also recorded (Table 4) as this could influence the choice of harvesting technique.

The properties of biodiesel are heavily influenced by the fatty acid composition of the feedstock oil. To investigate the suitability of their oil for biodiesel production, the fatty acid profile of each species under nitrogen replete and limited growth conditions was analysed. FAME profiles at 5, 10 and 14 days were very similar, indicating that the relative proportions of fatty acids remained similar throughout the growth cycle. The FAME profiles after 14 days of growth are shown in Table 5. The most common FAMEs found in the microalgae tested were C16 and C18:1, with several species having high proportions of C16:1, C18:2 and C18:3 and some of the marine species C14:0 and C20:5. There were significant differences between species and between growth conditions. All the freshwater Chlorophyta showed a large increase in C18:1 with nitrogen limitation, along with a decrease in C16:0, C18:2 and C18:3. S. platensis showed an increase in C14:1 and C18:2 with nitrogen limitation. The fatty acid profile of the marine species was less influenced by nitrogen limitation, although Nannochloropsis showed an increase in C18:1 and a decrease in C14:0, C16:0

and C20:5. C. fusiformis showed an increase in C16:1 and polyunsaturated C20 with a decrease in C16 and C18:1. The biodiesel properties calculated from the fatty acid profile of each species under *Table 5*

	C14:0	C14:1	C15:0	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3	C18:4	C20:1	C20:3	C20:4	C20:5
AfH	0.6	1.2		25.7	2.4	3.2	0.8		15.9	18.7	22.8					
AfL	0.2			10.5	1.2	2.8	0.3	2.5	52.6	13.5	12.0		1.3			
CvH	0.5	0.6	0.6	23.1	0.2	7.4	5.8	5.2	16.1	20.9	18.0					
CvL	0.1	0.2		16.9	0.6	2.0	5.1	6.5	48.2	8.5	11.6					
NolH				29.8	2.0	5.1	9.7	2.3	1.6	29.0	17.3					
NolL	0.3	0.6		25.6	1.1	1.9	1.6	4.0	42.4	22.4						
ScH	0.3	1.0		24.5	2.1	2.3	6.0	4.9	19.8	34.2	2.5			0.8		
ScL	0.2	0.1		24.3	1.9	3.3	1.0	4.1	46.2	15.9	1.0					
CfH	6.6	0.3	0.7	42.8	29.0		0.3	0.5	16.4	1.2				0.6	1.1	0.6
CfL	5.7	0.3	0.6	34.9	35.3		1.0		9.2	2.8				2.4	4.9	2.8
IsoH	25.5	1.0	0.8	10.8	4.7		0.8	0.6	35.2	5.1	4.9	6.9				1.7
IsoL	25.4		0.5	17.2	3.0		0.4	1.0	34.1	2.9	3.0	9.9				1.4
NanH	8.4	0.4	0.5	40.9	26.3			1.0	7.0	1.5					3.3	10.0
NanL	6.5	0.1	0.4	36.1	27.6		0.1	1.1	19.7	1.2					2.3	4.5
PavH	32.7			17.7	11.0				3.7	0.6						28.9
PavL	31.8			19.9	16.2				3.8	0.6						23.4
PtH	6.3			23.7	45.5	0.8	3.2		5.7	0.9						14.0
PtL	5.8			26.3	48.8	0.7	1.8		7.1	0.5						9.0
TsH	0.7	0.4		31.7	5.4	1.9	0.3	0.8	40.2	8.8	4.7		0.5	0.9	1.0	
TsL	0.7	0.1		35.0	4.4	1.4	0.2	1.0	42.9	6.9	3.5		0.5		0.6	0.8
SpirH		1.2		51.8	3.9				7.4	23.7						
SpirL	2.6	5.5		30.8	6.2				2.1	48.1						

nitrogen replete and limited conditions are shown in Table 6. None of the nitrate replete freshwater cultures (CN of 43–50) satisfied the EN 14214 criteria of a CN greater than 51, although C. vulgaris L, Scenedesmus L and N. oleoabundans L did. A. falcatus L, Pavlova H and S. platensis L also did not make the cutoff. The iodine values calculated for all cultures were within the EN 14214 requirement. C. vulgaris H, N. oleoabundans H and A. falcatus H and L had a linolenic acid content above the upper limit of 12%, and all the seawater species, except T. suecica H, had a content of PUFAs that was outside of the EN 14214 limit of 1%. All the freshwater species, as well as S. platensis, had high linoleic or linolenic contents, particularly under nitrogen replete conditions (Table 5).
The CFPP varied from −2°C (Isochrysis H) to 19°C (S. platensis H).
The maximum specific growth rate (μ_{MAX}) gives an indication of the biological potential with respect to doubling time of the species, under a particular growth environment (for example temperature). It occurs very early in the growth curve, when the biomass concentration is low and before any nutrients or light become limiting. The μ_{MAX} was very similar under conditions of high and low nitrate supply (Figure 1), as it occurred prior to nitrogen depletion in the nitrogen-limited media. The species with the highest μ_{MAX} were all green algae (N. oleoabundans, C. vulgaris, Scenedesmus and T. suecica), other than the haptophyte Isochrysis. The biomass concentration achieved by nitrogen replete cultures was higher than that under nitrogen limitation in all species tested except A. falcatus (Figure 1). This difference may have resulted from the settling behaviour of A. falcatus H in the airlift reactor. Despite daily re-suspension to ensure appropriate sampling, settled cells experienced a reduced mass transfer and light environment, potentially causing the maximum biomass concentration, and lipid productivity, of A. falcatus H to be underestimated. The nitrogen-limited cultures reached between 54% (S. platensis) and 94% (Pavlova) of the $X_{MAX}$of the nitrogen replete cultures, with an average across all species of 84%. T. suecica L had a relatively high X_{MAX} (2.10), indicating that the biomass concentration reached by this species was not significantly

influenced by nitrate deficiency. The lipid content of T. suecica was also not significantly affected by nitrogen limited conditions. Maximum biomass obtained is important not only for calculating maximum product yield; a high biomass concentration can also facilitate harvesting. The species that obtained the highest X_{MAX} were those with the highest μ_{MAX}, with the exception of N. oleoabundans, which had a surprisingly low X_{MAX} that reached a maximum at day 6 and decreased from day 8. Growth of N. oleoabundans has been shown to be inhibited at a nitrate concentration above 900 mg L^{-1}. The maximum biomass productivity ($Q_{X\,MAX}$) gives an indication of the productivity that could be maintained in continuous culture. As with the μ_{MAX}, the $Q_{X\,MAX}$ occurred early in the growth curve, before the influence of nitrate limitation had become apparent, hence $Q_{X\,MAX}$ is very similar under nitrogen replete and limited conditions for all species except Isochrysis (Figure 2). Lipid content was higher in all species (except S. platensis) under conditions of low nitrate supply. The highest lipid content occurred in C. vulgaris L, N. oleoabundans L and Scenedesmus L (all above 40% DW). The lipid content of C. vulgaris (57%) and Scenedesmus (43%) were still increasing after 14 days and may have reached higher values if cultivation had been continued. Lipid content was particularly enhanced by nitrate limitation (between 2.5 and 4.6 times larger under nitrogen-limited conditions) in the freshwater green algae (C. vulgaris, Scenedesmus, N. oleoabundans and A. falcatus), while the marine species (Nannochloropsis, T. suecica, C. fusiformis, P. tricornutum, Pavlova and Isochrysis) showed less effect (lipid content in nitrogen-limited cultures was 1.3–2.1 times that in nitrogen replete cultures). The only cyanobacterium tested (S. platensis) had a very low lipid content under both conditions. Previous studies have also shown that cyanobacteria have a relatively low lipid content and that nitrogen limitation has less impact on lipid accumulation than for freshwater algae. Volumetric lipid content was consistently higher in nitrogen-limited than nitrogen replete cultures, with the exception of S. platensis (Figure 3). For 8 of the 11 cultures, the difference was significant. For 4 of the 11 cultures, more than a twofold increase was observed.

Table 6

	CN	IV (g I_2/100 g)	Lin (%)	PUFA (%)	CFPP (°C)
AfH	43	98	22.8	–	10
AfL	48	100	12.0	–	−1
CvH	47	107	18.0	–	8
CvL	52	92	11.6	–	4
NolH	45	111	17.3	–	11
NolL	55	86	0.0	–	9
ScH	50	102	2.5	–	8
ScL	54	82	1.0	–	8
CfH	59	50	–	1.7	15
CfL	55	66	–	7.7	12
IsoH	52	72	4.9	1.7	−2
IsoL	54	66	3.0	1.4	4
NanH	56	59	–	13.3	14
NanL	57	59	–	6.7	13
PavH	50	67	–	28.9	4
PavL	51	63	–	23.4	6
PtH	51	80	–	14.0	7
PtL	53	72	–	9.0	9
TsH	54	74	4.7	1.0	12
TsL	56	68	3.5	1.4	13
SpirH	51	56	–	–	19
SpirL	48	98	–	–	11
EN 14214	>51	<120	<12%	<1%	

This higher P_{VOL} was found throughout the growth period after the onset of nitrogen limitation and was caused by the higher lipid content of nitrogen-limited cultures, despite their lower biomass concentrations. This proved that nitrogen-limited microalgae cultures could reach a higher lipid yield than nitrogen replete cultures, despite the trade-off between increased lipid content and

decreased biomass concentration. Lipid productivity is the parameter which ultimately determines the rate of oil production. It has been suggested as the most appropriate kinetic parameter for the comparison of species for biodiesel production. The species which obtained the highest lipid productivity and volumetric lipid content (Scenedesmus L and C. vulgaris L) were those that showed the greatest lipid accumulation (43% and 57% DW) and a rapid growth rate (μ of 1.2 and 1.3 day^{-1}), despite an intermediate final biomass concentration (1.7 and 1 g L^{-1}). The high lipid productivity of Scenedesmus L was due to its high lipid content under nitrogen-limited conditions, and its ability to maintain a high biomass productivity (equivalent to Scenedesmus H) until day 7, when the lipid content of Scenedesmus L had already accumulated to nearly 30% DW. C. vulgaris L had the second highest average lipid productivity, due to a rapid accumulation of lipid under nitrogen limitation, to 39% DW by day 7 and 57% by day 14. The relatively high lipid productivity of N. oleoabundans L was also due to a rapid accumulation of lipids. Different mechanisms lead to high lipid productivity in different species. For example, in C. fusiformis H and L, it was due to a combination of reasonably high biomass productivity and an intermediate lipid content. The lipid productivity of Nannochloropsis H and L was due to a relatively high lipid content, while that of T. suecica H and L was due to rapid growth and high biomass concentrations, despite relatively low lipid content. In particular, in this study, all nitrogen-limited cultures except for S. platensis showed a consistently higher lipid productivity and volumetric lipid content than nitrogen replete cultures throughout the growth curve. This was because the increase in lipid content (1.3–4.6 times that of the nitrogen replete culture) was greater than the decrease in biomass concentration (0.54–0.94 times that of the nitrogen replete culture). Harvesting the nitrogen-limited culture at any point in the growth cycle gave a higher lipid yield than that from the nitrogen replete culture. A large variation in settling rates and biomass recovery was observed. Gravity sedimentation is a simple and inexpensive harvesting technique and appears promising for some species, particularly C. fusiformis, T. suecica and Scenedesmus. However, it is not the optimal method of biomass concentration for other species such as Chlorella and Isochrysis, which showed poor settling. There are several alternative harvesting methods, including centrifugation, flotation, flocculation and microfiltration, as well as techniques for enhancing settling, such as bioflocculation, addition of chemicals or changes in pH. The parameters provided in this study assist in assessing of the suitability of a subset of these to algal recovery. In addition, the dimensions of the settling vessel, concentration of the culture and environmental conditions may significantly influence the settling rates recorded here. An important and under-recognised property of algal oil is the suitability, for biodiesel, of the fatty acid profile. The fatty acid profiles reported in this study correlated well with lipid profiles reported in the literature. Various biodiesel properties can be predicted from the fatty acid profile, including the viscosity, iodine value, CN and CFPP. All species examined had an iodine value that conformed to EN 14214 specifications (<120 g I_2 100 g^{-1}). Of the 22 cultures tested, seven had CNs too low to meet EN 14214 standards. The low CNs in algal oils are largely related to the proportion of PUFAs such as C18:2 and C18:3. C. Vulgaris H, Scenedesmus H, N. oleoabundans H and A. falcatus H have a high proportion of polyunsaturates, leading to a CN below the EN 14214

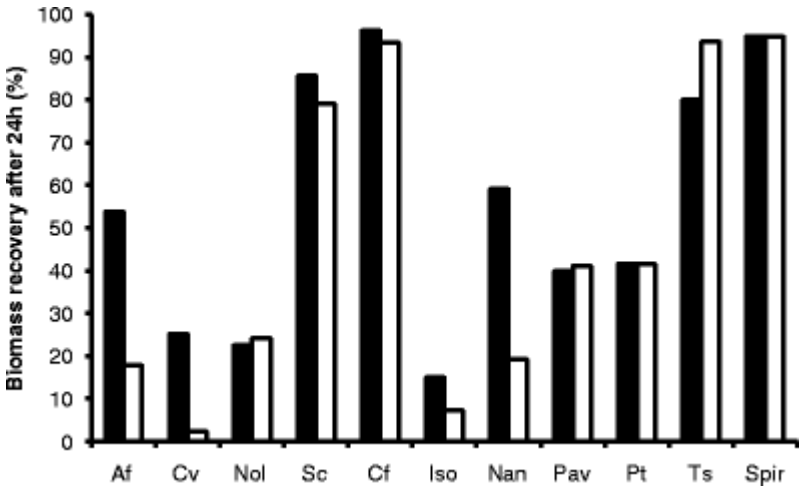

Figure 5

specification of 51. The decrease in C18:2 and C18:3, along with a significant increase in C18:1 was responsible for the nitrogen-limited Chlorophyta cultures reaching the required specification. Oils rich in C16:0 and C18:0, such as those from C. fusiformis, Nannochloropsis and T. suecica, have a higher CN and theoretically better combustion. The CFPP predicted from the fatty acid profile of the algal species investigated here were relatively poor. None of the species examined would meet the winter specification, even for a relatively warm country such as Spain (−10°C) or South Africa (−3°C). Only two would meet the Spanish summer standard (0°C) and five the South African summer standard (4°C). Cold flow properties depend mostly on the saturated fatty acid content, as well as the chain length of the saturated esters. Longer chain lengths have higher melting points; hence, those species with a lower content of saturated fatty acids, or those with mostly shorter chain length saturated fatty acids (for example: C14:0), are predicted to have better lowtemperature properties (for example: A. falcatus L, C. vulgaris L, Isochrysis and Pavlova). There are no models relating the oxidative stability of a fuel to the fatty acid composition. However, fuels with a high proportion of PUFA and linolenic acid (C18:3) are likely to have poor oxidative stability due to the large number of double bonds susceptible to autoxidation. This includes A. falcatus, C. vulgaris H, N. oleoabundans H, Pavlova, P. tricornutum and Nannochloropsis H. Species with a low proportion of highly unsaturated fatty acids include N. oleoabundans L and Scenedesmus H and L. Oxidative stability is a significant problem in biodiesel fuels and most will require the addition of antioxidants. None of the species were predicted to meet all the EN 14214 requirements for biodiesel quality, but this does not mean that they should be ruled out for biodiesel production. C. vulgaris L, N. oleoabundans L, Scenedesmus L, T. suecica H and S. platensis H were excluded only due to CFPP. Of these, C. vulgaris L had the lowest CFPP (4°C), which met the summer specification for South Africa. Additionally, Isochrysis was excluded only due to a PUFA content just above the cutoff point. If this limitation could be overcome, the lower C18:2 and C18:3 content of this species suggest a more favourable oxidative stability compared to the freshwater species. It should be noted that the biodiesel fuel specifications calculated only approximate the actual measured qualities. As evidenced by the different conditions of nitrogen availability, the fatty acid profile of microalgae can be adjusted through modification of growth conditions. Several properties, particularly the oxidative stability and cold flow properties, may be readily overcome by the use of additives or blending. Aside from the fatty acid profile, the most promising species tested were the freshwater Scenedesmus and C. vulgaris. Scenedesmus had an outstanding lipid productivity under nitrogen deprivation and a good biomass recovery after 24 h settling. C. vulgaris had the highest lipid content under nitrogen deprivation. It is known to be resilient and easy to grow (as evidenced by the fact that it is one of a handful of microalgae species that have already been cultivated on a commercial scale). Both species also met all of the EN 14214 requirements, except for CFPP, under nutrientlimited conditions. Among the most promising marine species were Nannochloropsis, C. fusiformis and P. tricornutum, due to their high lipid productivities and potential for settling. C. fusiformis was particularly easy to harvest by sedimentation and had a relatively high lipid productivity, despite the fact that it did not respond to nitrogen deprivation. T. suecica was also notable due to its high growth rate and biomass concentration, although it had a low lipid content and did not respond to nitrogen starvation. S. platensis has many promising qualities such as high biomass concentration, ability to grow in an extreme environment and it can also be harvested by flotation or filtration, due to its filamentous morphology. It is a well-characterised species and has been grown commercially. However, due to its low lipid productivity, it is unlikely to be used for biodiesel production.

1.2.Screening of algae based on fatty acid profile

In this study a total of 12 strains, identified and maintained by LABIOMAR (IBL-Microalgae Collection), at the Federal University of Bahia, Brazil, were used. Nine strains of Chlorophyceae were tested: Ankistrodesmus falcatus (IBL-C113), Ankistrodesmus fusiformis (IBL-C111), Kirchneriella lunaris (IBL-C118), Chlamydomonas sp. (IBL-C108), Chlamydocapsa bacillus (IBL-C103), Coelastrum microporum (IBL-C119), Desmodesmus brasiliensis (IBLC106), Scenedesmus obliquus (IBL-C110), and Pseudokirchneriella subcapitata (IBL-C112). Additionally, three species of Trebouxiophyceae were tested: Chlorella vulgaris (IBL-C105), Botryococcus braunii (IBL-C117), and Botryococcus terribilis (IBL-C115). The organisms were collected from a eutrophic lagoon located at Salvador City, Bahia, Brazil (12°93'26.38" S–38°39'09.41" W and 12°94'56.02" S–38°21'29.40" W). Trebouxiophyceae and Chlorophyceae strains were maintained respectively in a modified CHU 13 and LC Oligo media, described as nutrient-sufficient media.

Table 7

Local strain	Specific growth rate $\mu = \ln\left(\frac{N_y}{N_x}\right)/(t_y - t_x)$ (day^{-1})	Biomass productivity P_{dwt} ($g\ l^{-1}\ day^{-1}$)	Lipid content L_c % dwt	Volumetric lipid productivity P_{dwt} x L_c x 10^{-3} ($mg\ l^{-1}\ day^{-1}$)
Ankistrodesmus falcatus	0.57	0.34	16.49±0.44	56.07±1.75
Ankistrodesmus fusiformis	0.39	0.24	20.66±2.07	49.58±5.74
Kirchneriella lunaris	0.25	0.14	17.30±1.12	24.22±1.81
Chlamydomonas sp.	0.30	0.24	15.07±0.95	36.17±2.61
Chlamydocapsa bacillus	0.75	0.32	13.52±0.65	43.26±2.40
Coelastrum microporum	0.13	0.11	20.55±0.99	22.61±1.26
Desmodesmus brasiliensis	0.28	0.13	17.99±0.42	23.39±0.63
Scenedesmus obliquus	0.21	0.16	16.73±1.37	26.77±2.53
Pseudokirchneriella subcapitata	0.27	0.08	28.43±5.40	22.74±4.97
Chlorella vulgaris	0.53	0.73	28.07±4.31	204.91±6.37
Botryococcus braunii	0.14	0.25	44.97±4.00	112.43±11.52
Botryococcus terribilis	0.13	0.20	49.00±1.48	98.00±3.42

The choice was to have the results under standard growth conditions to provide comparison between the tested strains, without the risks of variations in the proportion of the resultant lipid classes, due to variation on experimental conditions. The trials were carried out in triplicate using Erlenmeyer flasks containing 600 ml of standardized medium and a 10 % volume of algal inoculums in the exponential growth phase. The flasks were kept under constant temperature and agitation (25±2°C and 90 rpm, respectively); the aeration rate was maintained at 0.50 vvm of atmospheric air enriched with 2% of CO_2. Cells were incubated at a neutral pH range (6.8±0.6), and light (140 µE m^{-2} s^{-1}) was provided within a photoperiod of 12:12 h light and dark cycles.

Growth was monitored every 48 h, by haemocytometer cell counting and by optical density (OD). OD was determined at 680 nm using a UNICAM®Spectrophotometer model Helios Epsilon.

Counting of cells per ml and/or OD680 measurements were plotted against time and used to estimate the growth kinetics. Growth kinetic parameters were obtained in triplicates for the 12 distinct strains and data were compared using ANOVA test. The growth curves were adjusted using Boltzman sigmoid model described in Origin software version 7 (Origin Lab Data Analysis and Graphing Software®), which was also tested for the model validity ($p \geq 0.05$).

The analyzed parameters were:

1. Specific growth rate (μ), based on the equation:

$\mu=\ln(N_y/N_x)/(t_y-t_x)$ (Eq.3)

where N_y and N_x are the numbers of cells (N) at the start (t_x) and the end (t_y) of the logarithmic growth phase.

2. Biomass productivity (P_{dwt}), as the dry biomass produced (in grams per liter per day), during the exponential growth phase. For P_{dwt} determination, samples were collected at the end of the exponential phase and cells were harvested by centrifugation for 5 min at 5,000×g at 4°C (Sorvall ultracentrifuge ®, Evolution RC). Supernatant discarded pellets were washed with distilled water, freeze-dried, and the dry weight was determined gravimetrically.

3. Total lipids content (L_c), reported as percentage of the total biomass (in % dwt), determined by using the chloroform/methanol approach.

4. Volumetric lipid productivity (L_p), calculated according to the equation: $L_p=P_{dwt}\times L_c$ (Eq.4) and expressed as milligrams per liter per day. The results were compared using ANOVA and multiple-range test, based on GraphPad Software Inc.

Fatty acids profile was determined by the capillary column gas chromatographic method applied to the oil methyl esters. The amount of total fatty acids (sum of free and bounded fatty acids) of each microalgae species was obtained by transesterification into the corresponding methyl esters (fatty acid methyl esters (FAME)), through saponification with NaOH in methanol, followed by methylation with BF_3 catalyst (12 % in methanol).

The FAME were extracted with iso-octane and stored in an inert atmosphere (N_2) in freezer at −18°C. The FAME separation was performed on a gas chromatograph (Varian ® 3800) equipped with a flame ionization detector and a fused silica capillary column Elite-WAX (30 m×0.32 mm×0.25 mm). The analysis parameters were: injector temperature of 250°C and detector temperature of 280°C. The following thermal program was used: 150°C for 16 min, then increasing 2°C/min up to 180°C, held for 25 min, following an increase of 5°C/min up to 210°C, held for 25 min more. Helium was used as carrier gas at 1.3 ml min^{-1}. Nitrogen gas was used as make up gas (30 ml min^{-1}); flow of hydrogen gas and synthetic air were provided at 30 and at 300 ml min^{-1}, respectively. The injections were performed in duplicate for each extraction in volume of 1 μl. FAME were identified by comparing their retention times with those of authentic standards (189-19, Sigma-Aldrich ®, USA). The quantification of fatty acids, expressed in milligrams per gram of lipids, was performed by adding internal standard (C23:0 Sigma®, USA) and calculating the extracted lipids according to Eq.5. Reported yields were averaged from three replicate extractions:

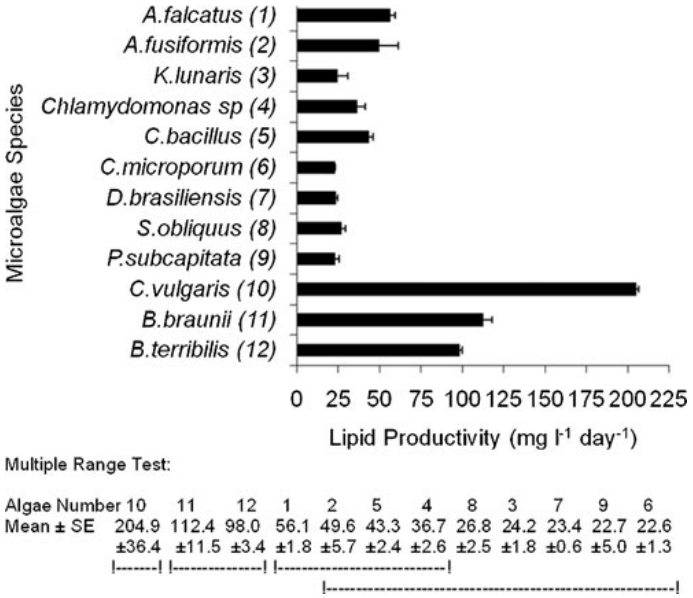

Figure 6

Concentration (mg g^{-1}) = $(A_x \times W_{is} \times CF_x)/(A_{is} \times W_s \times CF_s) \times 1000$ Eq.5

Ax: Area of methyl ester fatty acid peek in the chromatogram of the sample.

Wis: Weight (in milligrams) of internal standard added to the sample.

CFs: Conversion factor of fatty acid methyl ester to fatty acid.

Ais: Area of internal standard methyl ester of fatty acid peek in the chromatogram of the sample.

Ws: Sample weight (in milligrams).
CFx: Correction factor response of each fatty acid methyl ester ionization detector, relative to C23:0.
The parameters attesting for the quality of the biodiesel were estimated in relation to the molecular structures of FAME, which may vary according to carbon chain sizes and the amount and/or position of double bonds. These molecular characteristics greatly influence the main parameters of biodiesel quality such as cetane number, iodine value (IV), the cold filter plugging point (CFPP), and the oxidation stability. The cetane number (CN) is indicative of the time delay in the ignition of fuel, for diesel cycle engines. The higher the CN, the shorter is the ignition time. CN increases with the length of the unbranched carbon chain of the FAME components. Thus the hexadecane (cetane) is set to the default value of 100 on the cetane scale, where the minimum value is 15. The higher the carbon chain length of the methyl esters, the higher is the density and viscosity of the biodiesel, characteristics that will decrease with the increasing number of double bonds. Both characteristics are also related to the CFPP. Range of required CN for a quality biodiesel is usually 40–50; American standards American Society for Testing and Materials (ASTM) D675 (minimum CN of 47) and European standards EN 14214 (minimum CN of 51) differ from the one in Brazil, where Resolution Brazilian National Agency for Petroleum, Natural Gas and Biofuels (ANP) 07/2008 requires a minimum CN of 45.

Table 8

Microalgae species	Total lipids		Saturated	Monounsaturated	Polyunsaturated
	Total fatty acids (mg/g lipid)	Total lipids in Biomass (% dwt)	Fatty acids in Biomass (%dwt)	Fatty acids in Biomass (%dwt)	Fatty acids in Biomass (% dwt)
Ankistrodesmus falcatus	106.36	16.49	41.39	28.41	30.20
Ankistrodesmus fusiformis	131.96	20.66	37.33	22.43	40.24
Kirchneriella lunaris	176.07	17.30	32.06	23.11	44.83
Chlamydomonas sp	93.19	15.07	78.61	14.63	6.76
Chlamydocapsa bacillus	141.79	13.52	35.68	23.58	40.74
Coelastrum microporum	239.05	20.55	45.87	38.03	16.10
Desmodesmus brasiliensis	205.65	17.99	34.54	44.08	21.38
Scenedesmus obliquus	26.53	16.73	70.83	21.71	7.46
Pseudokirchneriella subcapitata	128.90	28.43	35.39	47.36	17.25
Chlorella vulgaris	270.56	28.07	52.15	37.51	10.33
Botryococcus braunii	130.97	44.97	9.85	79.61	10.54
Botryococcus terribilis	34.00	49.00	43.15	44.29	12.56

The IV refers to the tendency of biodiesel to react with oxygen at near ambient temperature. This characteristic depends on the number and the position of the double bonds in the carbon chains of the alkyl esters. The higher the IV (the mass of iodine, in grams, that is consumed by 100 g of a chemical substance), the higher the possibility of oxidation, deposits formation and deterioration of the biodiesel lubricity. The maximum IV accepted in Europe is 120 g I_2/100 g. The IV for soybean oil, in the range of 120 to 141 is indicative of a higher susceptibility to oxidative attack.
The CFPP is usually used for the prediction of the flow performance of biodiesel at low temperatures.
ANP standardizes a maximum of 19°C for this parameter. At lower temperatures, the crystallization of the FAME molecules grow and agglomerate, clogging fuel lines and filters. The larger the size of the carbon chains or the higher degree of saturation of FAME molecules composing biodiesel, the higher will be the value of CFPP, and the worse their low temperature properties. However,

additives can be used to inhibit the crystals agglomeration. The standards do not mention a low-temperature parameter in their lists of specifications.

Table 9

Microalgae strains	Cetane number	Saponification value	Iodine value	Degree of Unsaturation (wt %)	Long-chain saturation Factor (wt %)	Cold filter plugging point (°C)
Ankistrodesmus falcatus	50.52	201.97	101.33	88.81	1.69	−10.43
Ankistrodesmus fusiformis	48.00	199.85	113.81	102.91	1.78	−10.14
Kirchneriella lunaris	42.47	202.21	136.97	112.77	1.94	−9.62
Chlamydomonas sp	64.94	220.17	27.34	28.15	1.11	−12.23
Chlamydocapsa bacillus	48.38	197.50	113.95	105.06	1.77	−10.17
Coelastrum microporum	52.95	205.63	88.42	70.23	1.98	−9.52
Desmodesmus brasiliensis	53.28	205.46	87.05	86.84	1.97	−0.55
Scenedesmus obliquus	63.63	216.04	35.38	36.63	1.23	−11.87
Pseudokirchneriella subcapitata	53.94	207.68	82.83	81.86	1.95	−9.60
Chlorella vulgaris	61.83	199.37	52.63	58.17	1.57	−10.81
Botryococcus braunii	52.67	197.36	94.60	100.69	2.47	−7.96
Botryococcus terribilis	59.50	193.22	66.90	69.41	1.74	−10.26

The necessity of specialized apparatus, not always available, besides a high amount of oil to directly measure those critical biodiesel properties, lead to the use of predictive models that allow preliminary evaluation of potential feedstocks, if their FA composition is known. In this work, the estimation of algae-biodiesel properties follows an empirical correlative model (CN = a+b/x+cy), whose predictive capacity was previously defined, based on diverse vegetable oils.

The two independent variables, x and y, represented the chain length and the degree of unsaturation (DU) of each component ester. They were expressed, respectively, in terms of saponification value (x=SV, in milligrams of potassium hydroxide required to saponify 1 g of oil, which is inversely related to the esters' molecular weight), and the IV (y=IV, directly related to the DU or number of double bonds in the oil). The values of the three constants (a, b, and c) required three independent equations that have been generated based on the SV and IV values from the vegetable oils of palm, peanut and soybean. The equation precision has been ascertained by a correlation, which indicates that the increase of each unit of IV lowers the CN by 0.225. Thus, the CN, SV, and IV for each microalgae biodiesel were estimated by using the derived Eq. 6–8. The final values for these estimated properties for each microalgae oil-based biodiesel were calculated as the average of the products of these values for each FAME and its percent in the mixture.

CN = 46.3 + (5,458/SV) – (0,225 × IV) (Eq.6)

SV and IV were calculated respectively by using Eq. 7 and 8, where D is the number of double bonds, M is the FA molecular mass, and N is the percentage of each FA component of the microalgae oil.

SV = $\sum(560 \times N)/M$ (Eq.7)

IV = $\sum(254 \times DN)/M$ (Eq.8)

The DU was calculated based on Eq.9, as the amount of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA; in weight percent) present in the microalgae oil. Eq.10 has been generated by correlating the value of the CFPP with a factor related to chains saturation and length (long-chain saturated factor (LCSF)). The LCSF value was estimated through Eq.11, applied to several oils sources, by weighing up values of the longer chains (C16, C18, C20, C22 and C24 are the weight

percentages of each of the fatty acids) to reproduce their impact on the fuel cold flow properties. A regression, based on data of these two properties (LCSF and CFPP), defined the cetane response for different levels of saturation, with a good correlation (R^2=0.966).

DU = MUFA + (2 × PUFA) (Eq.9)
CFPP = (3.1417 × LCSF) – 16.477 (Eq.10)
LCSF = (0.1 × C16) + (0.5 × C18) + (1 × C20) + (1.5 × C22) + (2 × C24) (Eq.11)

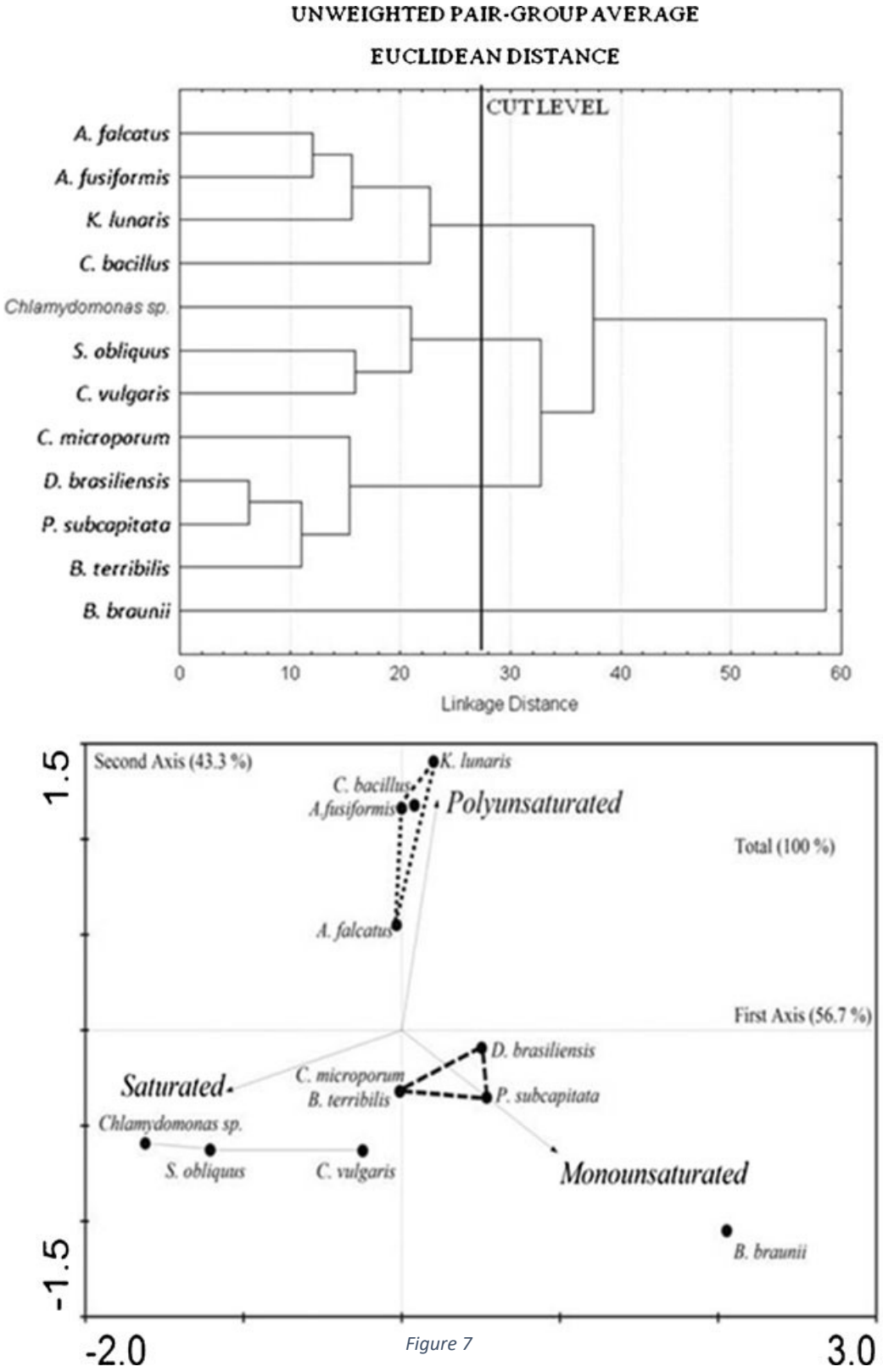

Figure 7

All these equations have been previously used to estimate the quality of algal biodiesel in comparison to biodiesel from different vegetable oils.

For a more accurate selection of microalgae species for biodiesel production, the observed data were first organized in a meaningful way through a cluster analysis representing similarities in algae fatty acid composition. A Euclidian distance matrix based on FA percentages for all the algae strains was used in an unweighted pair group average algorithm to construct dendrograms. This procedure was done in R PACKAGE VEGAN2.1–3. This same analysis was done to represent the algae similarities based on FA saturation and unsaturation levels, in relation to the lipid productivity and the estimated properties (CN, IV, CFPP, and OS) of biodiesel generated from algal oil.

The influence of each fatty acid in the formed groups was synthesized by a principal components analysis (PCA).

Samples envelopes were constructed based in the cluster analysis results and superimposed in the PCA ordination diagram. This procedure was done in CANOCO 4.5®. The PCA analysis was used to reduce dimensionality and to identify which components were more closely related to each microalgae group in terms of the strains able to generate oils and biodiesels with similar characteristics.

Growth rate and oil content (in % dwt) have been the two most studied parameters in the search for the success of large-scale cultivation of microalgae for biofuels production. However, fast growth only rarely correlates with high lipid productivity. Lower growth rates and/or small cell size contribute to lower the biomass productivity, even when the lipid content is high. Therefore, biomass yields may be considered as an adequate criterion for biodiesel production only when associated with lipid productivity (L_p).

Generally, high specific growth rate depends on cell proliferation and it does not reflect the microalgae specific capacity for producing and storing lipids. The production of lipids is commonly observed during the stationary phase, when the cells have most of their biosynthetic capacities redirected to the production of triacylglycerols or hydrocarbon lipids. For this reason, the lipid volumetric productivity and the qualitative lipid composition should be considered as the most appropriate parameters to facilitate decision making on species selection for biodiesel.

The obtained data on biomass productivity (Table 7) are in agreement with previous findings in the literature for some of the studied strains. It should be highlighted that lipids bioprospection of some of the local studied strains has not yet been referred elsewhere previously. However, the local strain C. vulgaris, showed a higher P_{dwt}(0.73 g l^{-1} day^{-1}) than previously reported (0.25 to 0.31 gl^{-1} day^{-1}). For other six strains (Table 7), biomass productivity varied between 0.20 and 0.34 gl^{-1} day^{-1}. Nevertheless, the top biomass producers in the present study did not correspond to the top lipid producers, what is in agreement with the fact that biomass productivity did not correlate (r^2=0.018) with lipid content (in % dwt). Two Chlorophyceae species (C. bacillus and A. falcatus) and one Trebouxiophyceae (C. vulgaris) showed the highest specific growth rates (above 0.50 day^{-1}) and biomass productivities (above 0.30 g l^{-1} day^{-1}), while their lipid contents have not comparatively been the most conspicuous (Table 7). On the other hand, the two Botryococcus strains showed the highest lipid contents, while presenting the lowest specific growth rates, even though with biomass productivities above 0.20 gl^{-1} day^{-1}. Similar performance has also been observed with B. braunii grown in photobioreactors under nitrogen-sufficient condition.

Literature data on volumetric lipid productivity for most of the species focused in the present research are relatively scarce. However, recently, this characteristic was averaged for 55 microalgae species, as 50 mg l^{-1} day^{-1}, even though some of them showed outstanding values (for example, A. falcatus, 109 mg l^{-1} day^{-1}); this species showed a similar lipid productivity (56.07 mg l^{-1} day^{-1}).

Trebouxiophyceae strains presented the highest volumetric lipid productivities (corresponding to daily productions of 204.91, 112.43, and 98.00 mg l^{-1} of oil, respectively for C. vulgaris, B. braunii,

Table 10

Fatty acids	Names	Ankistrodesmus falcatus	Ankistrodesmus fusiformis	Kirchneriella lunaris	Chlamydomonas sp.	Chlamydocapsa bacillus	Coelastrum microporum
C4	Butyric	1.52	0.74	n.d.	3.93	n.d.	0.60
C6	Caproic	0.91	0.94	1.21	1.79	0.63	0.69
C8	Caprylic	n.d.	0.09	n.d.	0.18	0.24	n.d.
C10	Capric	0.48	0.51	n.d.	0.77	0.50	0.52
C11	Undecanoic	n.d.	n.d.	n.d.	0.11	0.80	n.d.
C12	Lauric	n.d.	n.d.	n.d.	0.14	0.60	n.d.
C13	Tridecanoic	n.d.	0.14	n.d.	n.d.	n.d.	n.d.
C14	Myristic	1.07	2.02	1.53	1.61	1.44	0.80
C15:1	Pentadecenoic-cis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C16	Palmitic	30.23	26.95	25.28	50.77	24.51	25.66
C16:1	Palmitoleic	0.47	0.25	n.d.	0.28	0.84	1.00
C17	Margaric/ Heptadecanoic	0.48	0.20	n.d.	1.81	1.31	n.d.
C17:1	Heptadecenoic	1.52	1.31	1.89	n.d.	3.19	0.73
C18	Steararic	2.72	2.10	2.01	11.54	2.96	2.91
C18:1c	Oleic	24.12	18.80	18.50	7.82	7.01	44.24
C18:1t	Elaidic/ Octadecenoic	0.67	0.81	2.06	5.95	11.12	1.07
C18:2w6	Linoleic	2.00	12.23	4.50	3.93	13.33	8.58
C18:3w6	Linolenic	0.37	0.22	n.d.	0.82	4.49	11.12
C18:3w3	α-linolenic	26.49	26.28	39.66	1.94	20.96	n.d.
C20:1	Eicosenoic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:1w9	Gadoleic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Fatty acids	Names	Desmodesmus brasiliensis	Scenedesmus obliquus	Pseudokirchneriella subcapitata	Chlorella vulgaris	Botryococcus braunii	Botryococcus terribilis
C4	Butyric	0.43	1.25	1.65	n.d.	n.d.	n.d.
C6	Caproic	0.61	2.23	n.d.	n.d.	n.d.	n.d.
C8	Caprylic	n.d.	0.68	n.d.	n.d.	n.d.	n.d.
C10	Capric	0.41	1.91	n.d.	n.d.	n.d.	n.d.
C11	Undecanoic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C12	Lauric	n.d.	n.d.	n.d.	0.10	n.d.	0.62
C13	Tridecanoic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C14	Myristic	0.66	1.06	1.01	0.63	0.73	n.d.
C15:1	Pentadecenoic-cis	n.d.	n.d.	n.d.	0.44	n.d.	n.d.
C16	Palmitic	27.61	52.07	28.00	40.31	7.17	35.22
C16:1	Palmitoleic	n.d.	n.d.	n.d.	3.16	n.d.	n.d.
C17	Margaric/ Heptadecanoic	n.d.	n.d.	n.d.	0.51	n.d.	n.d.
C17:1	Heptadecenoic	1.37	n.d.	0.42	0.82	1.26	n.d.
C18	Steararic	3.34	7.48	2.85	8.01	1.59	3.12
C18:1c	Oleic	42.42	21.46	46.15	29.29	76.29	39.74
C18:1t	Elaidic/ Octadecenoic	n.d.	n.d.	0.38	0.60	0.93	n.d.
C18:2w6	Linoleic	12.03	4.60	7.49	8.54	5.16	5.02
C18:3w6	Linolenic	0.97	2.83	0.60	n.d.	n.d.	n.d.
C18:3w3	α-linolenic	8.46	n.d.	9.27	1.57	5.34	7.22
C20:1	Eicosenoic	n.d.	n.d.	n.d.	1.95	n.d.	n.d.
C20:1w9	Gadoleic	n.d.	n.d.	n.d.	n.d.	n.d.	2.81

and B. terribilis), which differed significantly ($p<0.05$) from all the other native strains (Figure 6). Species from the class Trebouxiophyceae have been extensively referenced in the literature as promising for biofuel production. It is believed that this group is capable of supporting high oil production systems. In spite of this, some species from this taxonomic group may not be the best choice for biodiesel production.

Microalgae FA profiles are shown on Table 10. Palmitic acid (C16:0) was the predominant fatty acid in most of the algal lipid extracts. The highest percentage was obtained with S. obliquus and Chlamydomonas sp. Exceptions were observed with K. lunaris, which showed the highest percentage of α-linolenic, and with C. microporum, D. brasiliensis, P. subcapitata, and both Botryococcus strains, which showed high percentages of oleic acid (a maximum of 76.29 % for B. braunii). Isomers of the monoenoic acids C16 and C18 were detected in low quantities except for K lunaris, A falcatus and C bacillus, where α-linolenic was found above 20 % (Table 10).

The results show that Botryococcus strains are mostly represented by saturated or MUFA, with chain lengths from C12 to C32. Fatty acids with C21 to C32 were in very low quantities (0.4 to 2.4 %). As opposed to fuels composed mostly by polyunsaturated FAME, the high proportion of saturated and MUFA in microalgae oils of Botryococcus strains, may incur in less problems with fuel polymerization during combustion.

The FA concentrations (Table 8) varied from 26.53 to 270.56 mg g^{-1} of total lipid for S. obliquus and C. vulgaris, respectively. The averaged value for the total lipid extracted from all the studied strains, was 140.42 mg g^{-1}.

The total lipids ratios (as percent of dry biomass) and the percentages of saturated, MUFA and PUFA in the dry biomass are shown in Table 8. Comparatively, Chlamydomonas sp. and S. obliquus produced values above 70 % of saturated FA and the lowest percentages of PUFA (6.76 and 7.46 %, respectively). The highest percentages of PUFA were for K. lunaris, C. bacillus, and A fusiformis (44.83, 40.74, and 40.24, respectively). The remaining strains showed higher concentrations of saturated (A. falcatus, C. microporum, C. vulgaris, and B. terribilis) or MUFA (D. brasiliensis, P. subcapitata, and B. braunii). If saturated and monoenoic FA are considered in combination, all Trebouxyophyceae species show very similar values for these lipids contents (around 89 %). The highest values for the sum of saturated and MUFA, however, were for Chlamydomonas sp. (93.24 %) and S. obliquus (92.54 %).

Several types of lipids, such as phospholipids, glycolipids, mono-, di-, and triglycerides, among others, are produced by microalgae and their ratios depend on each species and the growing conditions applied (14, 23, 29, 30, 33, and 50). Free fatty acids are generally only about 1–2 % of the lipids in microalgae. Most of the fatty acids are bounded to glycerol molecules forming the acylglycerols. Among these, only triglycerides are easily converted into biodiesel by the transesterification method. Thus, for pure ASTM grade biodiesel, it is the FA composition rather than the lipid content that must be considered for potential biodiesel production.

The most important properties of the biodiesel potentially produced from the obtained microalgae oils were empirically estimated in this research (Table 9). This estimation allowed a comprehensive assessment of biodiesel quality according to the properties specifications. The estimated CN for microalgal biodiesels varied between the 12 microalgae strains from 42.47 to 64.94, with an average value of 54.34. The ANP Resolution specifies a minimum of 45 for CN. In the present study, except for K. lunaris (CN042.47), all the strains showed CN values in a similar range (between 40 and 65) as most of the biodiesels originated from vegetable oils, such as 49 for sunflower, 52.9 for rapeseed, 50.9 for soybean, and 61.0 for palm oil.

Nevertheless, different oil extraction methods and transesterification approaches may cause some variation in the resultant biodiesels viscosity and, consequently, in the CN values. The SV estimated for biodiesels from all the strains examined, except for Chlamydomonas sp. (whose SV was 220.17)

varied within the same range observed for vegetable oils (196–202 for palm oil, 189–195 for soybean oil, and 188–194 for sunflower oil).

This parameter, however, is highly variable because it is also directly associated to the technology used for biodiesel production.

The IV is a parameter not included in ASTM or Brazilian standards, even though it represents the DU, involving the weighted sum of the masses of MUFA and PUFA, important for the biodiesel

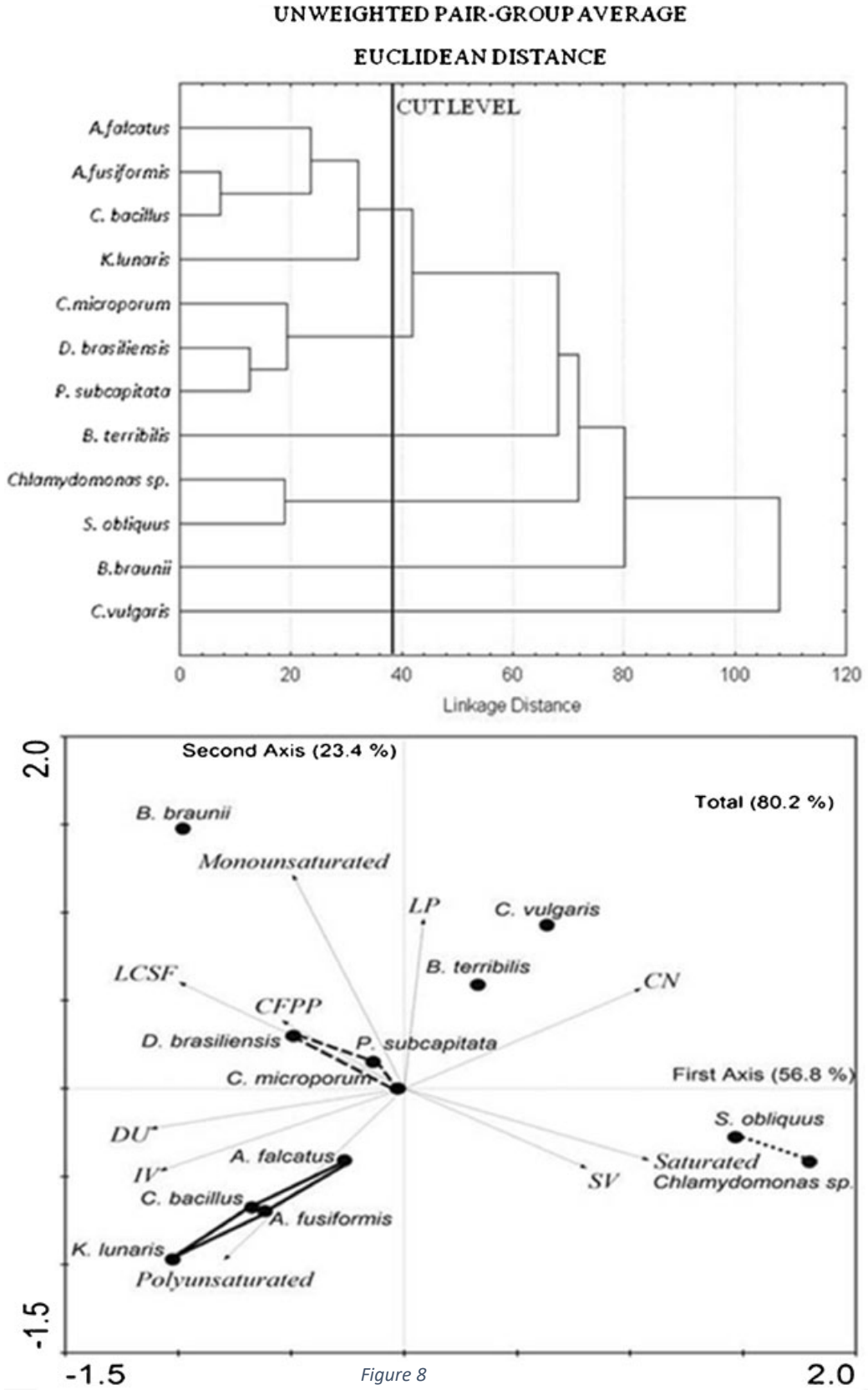

Figure 8

oxidative stability. High unsaturation levels may result in polymerization of glycerides and formation of deposits. In comparison to biodiesel from vegetable oils most of the estimated IV for the biodiesels from the microalgae strains (Table 9) were lower than for soybean oil (120–141) and sunflower oil (110–143). For some microalgae such as Chlamydomonas sp., S. obliquus, and C. vulgaris, the IV (Table 9) were lower than, or similar to palm oil (48–56), indicative of a lower susceptibility to oxidative attack.

Saturated FA have higher melting points than unsaturated FA compounds. When most saturated molecules of FA esters are present in oils, crystallization may occur at temperatures within the normal engine operation range, what gives biodiesel poor CFPP properties.

Biodiesel rich in stearic and palmitic acid methyl esters have a tendency to present a poor CFPP (equivalent to a higher temperature of plugging point), because when a liquid biodiesel is cooled, these FAME are the first to precipitate. In these examined strains, the levels of Stearic acid (Table 10) were generally very low (below 3.34 %), except for Chlamydomonas sp. C. vulgaris and S. obliquus (11.54, 8.10, and 7.48 %, respectively). These low values of stearic acid may have contributed for the lower temperatures of CFPP for the majority of the studied strains. The CFPP values of the strains estimated for biodiesel, ranged from −0.55 (D. brasiliensis) to −12.23 (Chlamydomonas sp.).

The CFPP obtained for different microalgae oils varied from −12.3 to 20.8°C. Peanut has the highest CFPP value (19°C) among the vegetable oils.

When algae species were compared based on FA saturation and unsaturation levels, three groups were identified by cluster analysis. In the PCA plotting (Figure 7), the two axis explained 100 % of total variation in algae fatty acid composition. The first axis showed the effect of variations on MUFA, mostly associated with D. brasiliensis and P. subcapitata and to a less extent, C microporum and B. terribilis. The left side of diagram was associated with the increase in saturated-FA and involved Chlamydomonas sp., S. obliquus, and C. vulgaris. The second axis was related with the increase in composition of PUFA. The species K. lunaris, C. bacillus, A. fusiformis, and, to a less extent, A. falcatus, were associated with this pattern. The bottom part of diagram was associated with the decrease of PUFA (Figure 7).

The microalgae B. braunii appeared isolated in the dendrogram highlighting its different composition of fatty acid types, in particular, the highest percentage (76.29 %) of oleic acid. Among the studied strains, Botryococcus have characteristically long chain lipids, which included chains longer than C30 while most oils currently used for biodiesel production are mainly composed of C16 and C18. These long chains would generate a biodiesel with higher density and viscosity that would contribute to increase the CN and the CFPP, influencing negatively the motor combustion process.

The statistical approach applied to microalgae strains based on FAME profiles in relation to the estimated biodiesel characteristics (CN, SV, DU, IV, LCSF, and CFPP) and the volumetric lipid productivity (Figure 8) is indicative that the degree of saturation/unsaturation represents an important discriminatory factor. The two PCA axis explained 80.2 % of the total variation. The first axis, explaining 56.8 %, was related to CN and SV increase and associated with Chlamydomonas sp. and S. obliquus, richer in saturated fatty acids. These species will generate biodiesel of higher CN, lower IV and higher oxidation stability. In the left side of the diagram, K. lunaris, A. fusiformis, C. bacillus, and A. falcatus, characterized by generating the highest percent of polyunsaturated FAME (higher DU), compose a group able to generate biodiesel more prone to oxidation, with the lowest CN, and the highest IV. The second axis explained 23.4 % of total variation and was related with increases of CFPP and LCSF values. The strains C. microporum, D. brasiliensis, and P. subcapitata were associated with this pattern. These species, having oils with higher percentages of MUFA, generate biodiesel of intermediate CN (52.95 to 53.94), IV (82.83 to 88.42), and also, intermediate susceptibility to oxidation. PCA analysis (Figure 8) discriminated, in isolation, the Trebouxyophyceae

species (B. braunii, B. terribilis, and C. vulgaris), as a result of their high lipid yields when compared with the other studied strains. C. vulgaris was found to be very productive, but not comparatively the best among the studied species as feedstock for biodiesel.

In this study, qualitative analysis of FAME showed this species has a predominance of saturated (52.15 %) and MUFA (37.51 %), tending to generate a biodiesel with a good oxidative stability (Table 11) but not the best in terms of ignition quality and lubricity (CN of 61.83, and IV of 52.63 g I_2/100 g). As previously recommended, a good quality biodiesel should be composed of a 5:4:1 mass ratio of C16:1, C18:1, and C14:0, with a low oxidative potential while retaining good cold flow characteristics and high CN. These characteristics were not found in any of the studied microalgae oils. Thus, the desirable quality of biodiesel may be achieved by selecting an appropriate mixture of oils from different organisms.

1.3.Experiment made on the microalgae Scenedesmus

As underlained by the precedent studies, the most promising algae for biofuel production is represented by the strain of Scenedesmus. The following study underlines the caracteristics of this algae, shown by a growth experiment in order to put a more detailed focus on the possibility to use Scenedesmus as the foundamental source for the production of biodiesel on large scale.

In this study, pure strains of the microalgae Scenedesmus dimorphus and Selenastrum minutum were grown in untreated municipal wastewater for six days under mixotrophic conditions. The algae strains were subjected to different stresses such as nutrient deprivation, and 5% (w/v) salinity to trigger lipid production and to study effect on FAME composition. The highest lipid concentrations were found in S. dimorphus (35 and 34%) and in S. minutum (40 and 39%) under nutrient deprivation and 5% salinity, respectively. On the one hand, salt stress decreased biomass production; on the other hand in both S. dimorphus and S. minutum salt stress significantly increased the concentration of saturated fatty acid (SFA) and it decreased the concentration of poly-unsaturated fatty acid (PUFA) contents, which are desirable for the production of good quality biofuel such as biodiesel.

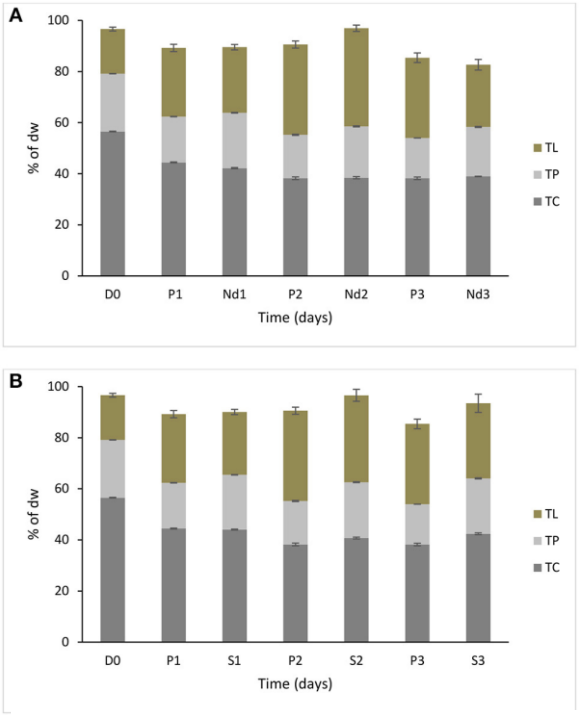

Figure 9 Biochemical composition of S. dimorphus over time (three days) (A) under nutrient deprivation stress and (B) 5% salt stress.
Total lipid (TL), total protein (TP) and total carbohydrate (TC) concentrations are reported as % of biomass dry weight.

Most of the essential nutrients of algae can be supplied by wastewater and atmospheric CO_2, leading to high productivity and an associated high lipid content, making them an attractive option. For the commercial production oil-derived biodiesel from microalgae biomass, high lipid productivity of dominant, fastgrowing algal strains is a major prerequisite. However, when large amounts of algal biomass are produced under optimal growth conditions, often the lipid content is relatively low; while slow growing species have typically high lipid contents. However, it seems clear that there are great potential benefits in using environmental stress as a tool to induce lipid biosynthesis. Different stresses can be used to improve lipid productivity in microalgae, and many studies have evaluated the effects of various stressors on algae, such as carbon dioxide, pH, UV treatment, temperature, higher salt content, and limited nitrogen availability. Salinity stress increases neutral lipid content of algal cells

Municipal untreated (raw) wastewater was obtained from the nearby wastewater treatment plant in Umeå (VAKIN, Umeå, Sweden). Initial pH of the wastewater was 7.5±0.08. Pure strains of S. dimorphus (417) and S. minutum (326) were bought from UTEX, The Culture Collection of Algae at the University of Texas at Austin (in parenthesis is the strain UTEX id).

The two algal strains were separately cultured in a 16 L working volume photobioreactor consisting of a cylindrical transparent glass flask with a top drivemotor that stirs an impeller (Wheaton, USA). The algal strains grown in the photobioreactor received municipal untreated wastewater filtered with two layers of towel paper (100% cellulose) with a water filtration velocity of about 1.3ml cm^{-2} min^{-1} to remove the largest particles. The algae were grown at room temperature (22±2°C) under

Table 11

Treatment	Total lipid	Total carbohydrate	Total protein	Biomass
S. dimorphus nutrient dep	+++	+++	+++	+++
S. dimorphus prolonged	+++	+++	+++	---
S. dimorphus 5% salt	+++	+++	+++	+++
S. minutum nutrient dep	--+	--+	+++	+++
S. minutum prolonged	---	+++	+++	+++
S. minutum 5% salt	---	-++	+++	-++

+++ represents statistically significant differences for all three days (1,2,3);- - - represents no statistically significant difference at any of the three treatment days; the + and – represent a combination of statistically significant and not differences at different days, from 1 to 3.

16:8 h lightdark cycle with an irradiance of 150 µmol m^{-2} s^{-1} at the photobioreactor surface. The culture was grown up to six days with continuous agitation without CO_2 addition.

To investigate a possible role of bacterial growth on nutrients removal and biomass production a non-inoculated control experiment was performed in 1 L Erlenmeyer flask totally covered with aluminum foil to avoid exposing the wastewater to light. Non inoculated controls were kept for six days at room temperature (22±2°C) similarly as above mentioned for growth in the photobioreactor.

Ammonium and total phosphorus were measured in untreated wastewater and after six days of algal growth in the photobioreactor and after six days for non-inoculated control. Analyses were performed in duplicate with a spectrophotometer (DR 3900 Hach Lange, Germany) following the manufacturer instructions (Hach Lange, Germany).

Six-day-old S. dimorphus and S. minutum cultures grown in the photobioreactor were subjected to treatments such as nutrient deprivation and 5% (w/v) NaCl in 1–3 day time course. It has been chosen 5% salt because it is the concentration found in some chemical industries wastewater effluent. The samples were kept in the growth cabinet (Conviron, Canada) under 16 h of light at 22°C and 8 h of dark at 16°C for three days under the optimal light intensity of 150 µmol m^{-2} s^{-1} as PAR (photosynthetic active radiation). Nutrient deprivation stress was created using tap water, pH 8.1, ammonium, nitrate, and phosphate concentrations were < 0.01 mg/l (below the instrument detection limit). For tap water treatment, sterile 50ml centrifugal plastic tubes received 25ml of six-day-old algal cultures from the photobioreactor; samples were centrifuged at 3,520 g for 5min and supernatants were discarded. Subsequently, the remaining pelleted cells of algae were washed with distilled water, resuspended in 25ml of tap water and kept for up to three days. For the prolonged growth on original wastewater, sterile 50ml centrifugal plastic tubes received 25ml of six-day-old algal cultures from the photobioreactor. Then the algae were kept to grow in the same wastewater used in the photobioreactor. This treatment was used as reference treatment when compared to the nutrient deprivation or the salt stress treatment. For the 5% salt treatment, NaCl was added to the required volume of six-day-old culture withdrawn from the photobioreactor. Fifteen replicates of six-day-old cultures of S. dimorphus and S. minutum grown in the photobioreactor were used as the day 0 reference to compare with the treated samples. Treated samples were collected daily in fifteen replicates at each time point. Each sample consisted of 25ml of culture volume in a 50ml tube.

All treated cultures were harvested by centrifugation at 3,520 g for 5min and the cell pellets were washed with distilled water. The dry weight of algae biomass was determined gravimetrically after overnight oven drying at 65°C. Algae biomass was expressed as dry weight (mg/l).

All biomass determination was performed in triplicate.

Total lipids were extracted from fresh microalgae biomass using the simplified Folch method. The lipids were extracted using a mixture of chloroform/methanol (2:1 v/v) and 0.73% NaCl water solution. The quantity of total lipids was measured gravimetrically and expressed as dry weight percentage. Total lipid extraction was performed in five replicates.

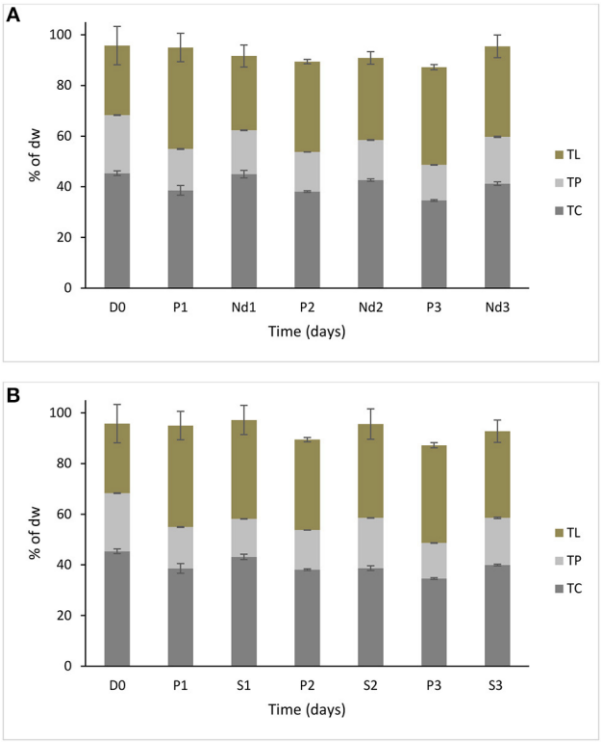

Figure 10 Biochemical composition of S. minutum over time (three days) (A) in nutrient deprivation stress and B) 5% salt stress.

Algae were harvested by centrifugation at 3,520 g for 5min and the cell pellets were washed with distilled water. Pelleted cells were boiled immediately in 1–2ml of isopropanol at 80°C for 10min and stored at −20°C. All samples were brought to room temperature before total lipid extraction. The extracted lipids were dried using a multievaporator under vacuum (Büchi, Switzerland). Once dry, the samples were flushed with N_2 at 30°C. Transesterification of fatty acids (FA) into FAmethyl esters (FAME) was carried out as follows: 1ml of 5% H_2SO_4 in dry methanol was added to the dried samples and flushed briefly with N_2 to completely remove air and boiled at 80°C for 2 h, it was added 1ml of distilled water and 2ml of pure petroleum ether, vigorously vortexed for 30 s and centrifuged at 1,250 g for 2min. The upper phase was removed using a long Pasteur pipette and the supernatant was transferred into a clean glass vial. This step was repeated twice, adding only 2ml of petroleum ether. The samples were completely dried using the multievaporator (Büchi, Switzerland). Dry samples were blown with N_2 at room temperature. All the reagents used were of analytical grade (Sigma Aldrich). Dry samples were dissolved in 50–60 µl hexane and 1 µl was injected and analyzed using a gas chromatograph CP 3800 (Varian AB, Stockholm, Sweden) equipped with a flame ionization detector and a split injector and fitted with a 50m length x 0.22mm i.d. x 0.25µm film thickness BPX 70 fusedsilica capillary column (SGE, Austin, TX, USA). The temperatures of the injector and detector were 230 and 250°C, respectively. Fatty acids were identified by comparing their retention times with those of the standard mixture GLC-461 (Nu-check Prep, Elysian, USA). Peak areas were integrated using Galaxie chromatography data system software version 1.9 (Varian AB, Stockholm, Sweden).

The differences were analyzed using one-way analysis of variance, setting the level of significance of <0.05 (Minitab 18).

After six days, non-inoculated controls showed a limited ammonium and total phosphorus reduction of 12.8 and 9.4% respectively. After six days, control biomass had a mean ±SE value of 7 ± 1 mg/L. There was a clear biomass reduction considering that the biomass at experiment start had a mean ± SE value of 16 ± 2.8 mg/L. While after six days of algal growth in the photobioreactor the two algae could reduce ammonium by 79%

and total phosphorus by 93.7%. Biochemical composition is shown in Figure 9; the contents of total lipids, proteins and carbohydrates were analyzed in the prolonged growth in the original wastewater, nutrient deprivation and 5% salt for 1–3 days' time course. Tap water had very limited amounts of nutrients, so it was regarded as a nutrient deprivation stress condition. In nutrient deprivation, the total carbohydrate content of S. dimorphus showed a dramatic reduction over time (Figure 9A). Total carbohydrate content was 56.5% of the dry weight at day 0, 42.2, 38.4, and 38.9%

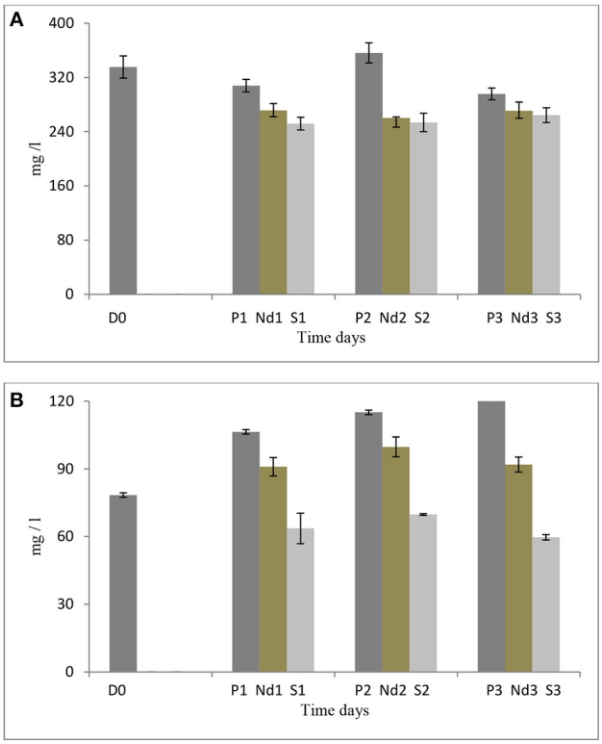

Figure 11 (A) Biomass production of S. dimorphus over time (three days) in prolonged growth, nutrient deprivation and 5% salt stress. (B) Biomass production of S. minutum over time (three days) in prolonged growth, nutrient deprivation, and 5% salt stress.

at day 1, day 2 and day 3, respectively (Figure 9) this reduction was statistically significant (Table 11). In nutrient deprivation stress treatment, total protein content had a limited reduction from 22.6% of the dry weight at day 0 to 21.7, 20.1, and 19.4% at day 1, day 2, and day 3, respectively. However, even total protein reduction was statistically significant (Table 11). In particular, it is interesting to observe that lipid content was significantly increased in nutrient deprivation from 17.4% of the dry weight at day 0 to about 24.3, 38.2, and 29.6 at day 1, day 2, and day 3, respectively (Figure 9A, Table 11). Most highly triggered lipid production was observed in nutrient deprivation at day 2. In the prolonged growth in original wastewater samples of c1, c2, and c3, both total carbohydrate and total protein contents were decreased from day 0 to day 3 (Figure 9A, Table 11). Total carbohydrate content decreased from 56.5% at day 0 to 44.4% at day 1, 38.2% at day 2, and 37.7% at day 3, while total protein content dropped from 22.6% at day 0 to 17.9% at day 1, 17.0% at day 2, and 15.8 % at day 3. Total lipid content remarkably and significantly increased from day 0 to day 2 in both nutrient deprivation and prolonged growth in original wastewater samples from 17.4 to 38.2% and from 17.4 to 35.1%, respectively (Figure 9A). During the 5% salt stress, the algae biomass pigmentation changed from green to yellow at day 1. Later, the color of the algal biomass turned to white or mostly colorless with progressing stress reaction in day 2 and 3. We observed an increased lipid content in day 1, day 2, and day 3 as 24.5, 33.8, and 28.9%, respectively, compared to17.4% in day zero (Figure 9B, Table 11). Total carbohydrate content showed a drastic reduction from 56.5% at day 0 to 44.0 % at day 1. We could observe a more limited reduction of total protein content of algal biomass during salt stress from 22.6% at day 0 to 21.4, 21.9, and 21.6 at day 1, 2, and day 3, respectively (Figure 9B); however this reduction was statistically significant (Table 11). Salt stress clearly negatively affected S. dimorphus biomass production (Table 11). During salt stress, S. dimorphus had a biomass of 335.4 mg/L at day 0 to 251.9 mg/L at day 1, 253.6 mg/L at day 2, and 264.4 mg/L at day 3 (Figure 11A). As shown in Figure 12, S. dimorphus showed a significant reduction of biomass during salt stress of 22.7, 24.4, and 21.2% at day 1, 2, and 3, respectively. It was possible to observe algal cells creating aggregates during the salt treatments as a result of cells excreting mucilage as a response to the unfavorable growth conditions caused by the high salinity. S. dimorphus shows a reduction in algal biomass not only in 5% salt conditions, but also during nutrient deprivation as 271.4, 260.2, and 270.7 mg/L at day 1, 2, and 3, respectively (Figure 11A; Table 11). However, under prolonged growth in original wastewater conditions, the reduction was more limited and not significant (Table 11). As shown in Figure 11A, biomass production of c1, c2, and c3 was 307.9, 356.2, and 295.7mg/L, respectively, while at day 0, biomass production was 335.4 mg/L. It was interesting to observe that under nutrient deprivation, S. minutum showed a reduction of total carbohydrate content statistically significant only at day 3 (Table 11) and a considerably higher reduction of total protein content compared to S. dimorphus (compared Figures 9A, 10A). Total carbohydrate content was less reduced than total protein, varying from 45.4% of the dry weight at

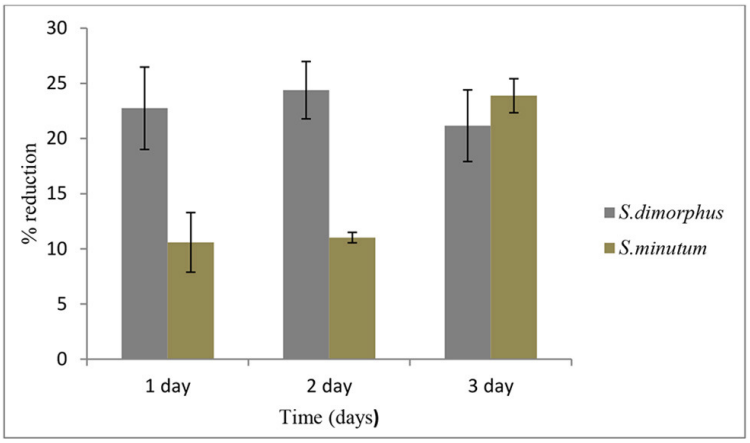

Figure 12 Biomass reduction of S. dimorphus and S. minutum over time (D1, D2, and D3) in 5% salt stress. Biomass reduction values are presented as % reduction with the reference to day 0. Bars represent mean ±SE of five replicates.

day 0 to 45.0, 42.7, and 41.3% at day 1, day 2, and day 3, respectively (Figure 10A). Total protein content was significantly reduced from 22.9% of dry weight at day 0 to 17.3% at day 1 and 15.8% at day 2 in nutrient deprivation samples (Figure 10A, Table 11). Even though we could observe a substantial increment of total lipid content at day 1, 2, and 3 as 29.0, 32.6, and 35.5%, respectively (Figure 11), this increase was not statistically significant (Table 11). It was found that biomass of S. minutum significantly increased over time with nutrient deprivation (Figure 11B, Table 11). Algal biomass increased from 78.4 mg/L at day 0 to 91.0 mg/L at day 1, 99.8 mg/L at day 2, and 92.0 mg/L at day 3 (Figure 11B). The behavior of S. minutum and S. dimorphus showed two different patterns of biomass accumulation during nutrient deprivation (Figures 11A, B). The prolonged growth in original wastewater treatments c1, c2, and c3 had a stimulating effect on total lipid content of S. minutum (Figure 10A). Total lipid contents increased at day 1, 2, and 3, respectively; however the increase was not statistically significant (Table 11). It has been observed a statistically significant reduction of both total carbohydrate and total protein contents in the prolonged growth in original wastewater treatments (Figure 10A, Table 11). Hereby, total protein content was reduced from 22.9% of dry weight at day 0 to 16.3% at day 1, 15.6% at day 2, and 14.0% at day 3, while total carbohydrate content was reduced from 45.4% at day 0 to38.6% at day 1, 37.4% at day 2, and 34.6% at day 3 (Figure 10A). As shown in Figure 11B, algal biomass of S.minutumin prolonged growth in original wastewater treatment significantly increased from 78.4 mg/L at day 0 to 106.4 mg/L at day 1, 115.0 mg/L at day 2, and 122.0 mg/L at day 3 (Table 11). It is possible to confirm that in S. minutum, both nutrient deprivation and prolonged growth in original wastewater had a similar pattern in terms of lipid accumulation, biomass gain and total protein reduction during the 3 days' time course. Total lipid content of S. minutum was influenced by the 5% salt stress (Figure 10B) at day 1, 2, and 3 but the differences were not statistically significant (Table 11). According to Figure 12, total carbohydrate content was reduced from 45.4% at day 0 to 43.1% at day 1, 38.7% at day 2, and 39.9% at day 3; while total protein content displayed the highest reduction from 22.9% at day 0 to 15.0% at day 1. It was clear that during the 5% salt stress, S. minutum biomass was reduced, from 78.4 mg/L at day 0 to 63.6 mg/L at day 1, 69.8 mg/L at day 2, and 59.7 mg/L at day 3 (Figure 11B). The biomass reduction of S. minutum was reported as 10.6, 11.0, and 23.9% in day 1, 2, and 3, respectively, in 5% salt stress (Figure 12) and it was statistically significant for day 2 and 3 (Table 11).

The extracted lipids from both S. dimorphus and S. minutum in prolonged growth, nutrient deprivation and 5% salinity consisted of C16 and C18 groups (Table 12) that accounted approximately for 80–88% of total fatty acids. Relative percentage of saturated fatty acids (SFA) was higher than that of unsaturated fatty acids, such as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), in both S. dimorphus and S. minutum under 5% salt stress (Table 12). The results revealed that relative percentage of unsaturated fatty acids, such as MUFA and PUFA, was higher than that of SFA in both S. dimorphus and S. minutum in prolonged growth and nutrient deprivation (Tables 11,12). As shown in Table 12, in S. dimorphus, palmitic acid contents

were 21.6, 20.6, and 40.5% in prolonged growth, nutrient deprivation and 5% salinity, respectively. Fatty acids belonging to the C18:1 group had a concentration of 23.3, 21.2, and 13.7% in S. dimorphus in prolonged growth, nutrient deprivation, and 5% salt stress samples for the 3 days' time period, respectively (Table 12). Polyunsaturated fatty acids, such as C18:3, were accumulated at 24.1% in prolonged growth, 29.7% in nutrient deprivation and 4.16 in 5% salt stress (Table 12). Under salt stress, stearic acid (C18:0) was similar in prolonged growth and nutrient deprivation at 2.5 and 2.7%, respectively, while at 11.2% under salt stress (Table 12). Hence, stearic acid content was remarkably higher in 5% salinity, which is 4.5 and 4.1 fold higher than in the prolonged growth

Table 12

Algae strain	Fatty acid methyl ester	Relative content %		
		Prolonged	Nutrient dep	5% salt
S. dimorphus	C15:1 total	5.8 ± 0.15	5.6 ± 0.46	4.9 ± 0.48
S. dimorphus	C16:0 palmitic acid	21.6 ± 0.46	20.6 ± 1.30	40.5 ± 1.97
S. dimorphus	C16:1 total	4.1 ± 0.08	4.0 ± 0.06	8.2 ± 0.42
S. dimorphus	C18:0 stearic acid	2.5 ± 0.14	2.7 ± 0.29	11.2 ± 0.24
S. dimorphus	C18:1 total	23.3 ± 0.07	21.2 ± 0.10	13.7 ± 0.43
S. dimorphus	C18:2 (n-6) Cis-9, 12-Linoleic acid	5.3 ± 0.30	5.3 ± 0.29	3.8 ± 0.36
S. dimorphus	C18:3 total	24.1 ± 0.58	29.7 ± 0.12	4.16 ± 0.48
S. minutum	C15:1 total	3.6 ± 0.07	3.2 ± 0.18	2.3 ± 0.27
S. minutum	C16:0 palmitic acid	29.3 ± 0.54	31.2 ± 0.90	44.6 ± 0.87
S. minutum	C16:1 total	3.6 ± 0.13	3.9 ± 0.27	3.8 ± 0.88
S. minutum	C18:0 stearic acid	3.3 ± 0.14	4.5 ± 0.44	11.2 ± 1.14
S. minutum	C18:1 total	25.2 ± 0.66	26.5 ± 0.74	20.4 ± 0.34
S. minutum	C18:2(n-6) Cis-9; 12-Linoleic acid	3.6 ± 0.24	4.0 ± 0.22	4.1 ± 0.13
S. minutum	C18:3 total	18.7 ± 0.71	15.8 ± 0.52	5.2 ± 1.39

and in nutrient deprivation samples. Extracted lipids from S. dimorphus were reported to have cis-9,12-linoleic acid at 5.3, 5.3, and 3.8%, respectively, in prolonged growth, nutrient deprivation and in 5% salt stress. However, the lipids extracted from both S. dimorphus and S. minutum in all three treatments had less polyunsaturated methyl esters than saturated and monounsaturated methyl esters (Table 12). According to the lipid profile of S. minutum (Table 12), palmitic acid concentration was 29.3, 31.2, and 44.6% in prolonged growth, nutrient deprivation and 5% salinity, respectively. Stearic acid levels were 3.3% in prolonged growth, 4.5% in nutrient deprivation and 11.2% in salt 5% condition. As a consequence, stearic acid content in salt stress samples were 3.4 fold higher than in prolonged growth samples and 2.5 fold higher than in nutrient deprivation samples (Table 12). Based on Table 12, the fatty acid distribution of both S. dimorphus and S.minutumin all three treatments showed more palmitic acid (C16:0) and oleic acid (C18:1 group) and less linoleic (C18:2 n-6) and linolenic acid (C18:3 group). The ratio SFA/PUFA and MUFA/PUFA (Table 13) reveals a higher value of saturated fatty acids and monounsaturated fatty acids than of polyunsaturated fatty acids in S. minutum in all three treatments.

However, for S. dimorphus, the ratios SFA/PUFA and MUFA/PUFA (Table 13) displayed the highest value in 5% salinity, while nutrient deprivation and prolonged growth treatment obtained lower ratio values.

Based on the literature, neutral lipids accumulation begins at the end of the log phase or in the stationary phase, when the nutrient content of the growth medium is limited.

In conclusion, in S. dimorphus nutrient and salt stress stimulated a reduction of carbohydrates and protein and an increase in lipids. Nitrogen limitation or deprivation can generate also a significant accumulation of saturated and monounsaturated fatty acids on Nannochloropsis sp as well as decreased percentages of polyunsaturated fatty acids in the total fatty acids. These fatty acids are mainly associated to TAG storage, the preferred substrate for biodiesel production. The color change at 5% salinity was a good indicator of pigment degradation, followed by cell death and

triggered lipid production in S. dimorphus. Under nutrient deprivation, total protein content was significantly reduced; this could be ascribed to the fact that under nitrogen starvation, the carbon dioxide fixed is converted into carbohydrates or lipids rather than proteins due to unavailability of nitrogen.

Lipid production of microalgae can be grouped into two categories: storage lipids (non-polar lipids) and structural lipids (polar lipids). However, during nutrient stress, 80% of the accumulated lipids are comprised of TAGs storage lipids, which can be transesterified to produce biodiesel. The results show that both under nutrient deprivation and prolonged growth in original wastewater, total lipid

Table 13

Ratio of FAMES						
Ratio between FAMES		S. dimorphus			S. minutum	
	Prolonged	Nutrient dep	5 % salt	Prolonged	Nutrient dep	5 % salt
SFA/PUFA	0.82	0.67	6.50	1.46	1.80	6.0
MUFA/PUFA	0.93	0.72	2.75	1.29	1.54	2.60

content especially of S. dimorphus was remarkably high (Figure 9A). Interestingly, lipid production increased even if related to biomass production (Figures 9, 10**,** figures 11A,B).

However, concerning the relationship between biomass and lipid production under salt stress the two algal strains behaved differently as underlined by the fact that S. dimorphus even with a clear reduction of biomass still accumulated lipid when compared to day 0; while in S. minutum the reduction of biomass was not balanced by a large lipid production when compared to day 0. Lipids produced from microalgal strains usually consist of fatty acids comprising mainly C16 and C18 fatty acids, which are very similar to those of vegetable oils; C16 and C18 fatty acids are suitable for biodiesel production. The results show that the dominant fatty acids of S. dimorphus and S. minutum in prolonged growth, nutrient deprivation and 5% salinity were C16 and C18 fatty acids (Table 12). Therefore, the extracted oils can be used as basic material for the production of biodiesel. Particularly interesting was the effect of salinity stress on the fatty acids quality of both algal strains, as shown by the clear reduction of the unsaturated fatty acids 18:3 and 18:1 and the increase of the saturated 16:0 and 18:0 (Table 12). Hence the salinity stress could generate lipid suitable for warmer climates where cold-flow properties are less important. The presence of polyunsaturated methyl esters in algal oil greatly affects the oxidative stability of biodiesel. The susceptibility to oxidation of the double bonds during storage, reduces the acceptability of microalgal oil for production of biodiesel. The increased relative percentage of C16:0 and the decreased percentage of C18:3 group fatty acids will result in a fatty acid profile that is most likely to yield biodiesel with poor cold-flow properties and good oxidative stability. The C18:1 group of the fatty acid content of S. minutum was 25.2% in prolonged growth, 26.5% in nutrient deprivation, and 20.4% in 5% salinity (Table 12). A considerably higher level of oleic acid (C18:1 group) is desirable for a good quality biodiesel. Both of the algal strains we studied had high values of SFA/PUFA and MUFA/PUFA ratios in 5% salinity (Table 13), which is desirable for good quality biodiesel as they guarantee oxidative stability of the fuel. The extracted lipids from both S. dimorphus and S. minutum in in all three treatments showed satisfying overall properties suitable for biodiesel production. From an application point of view the salt water present in industrial effluent and of course in sea water can be successfully used to induce lipids accumulation but especially fatty acids saturation in fresh water microalgae.

1.4.Screening of 30 strains of algae through three different techniques

In the context of this work, a differentiation between nutrient deficiency (also called nutrient starvation or deprivation) and nutrient limitation is necessary. Nutrient deficiency is achieved when the exogenous supply is exhausted and the cell is forced to use endogenous reserves. If nutrient deficiency is prolonged, growth will be eventually halted. Differently, under nutrient limitation, the culture is growing in, and generally adapts to, an environment of constant, but insufficient supply of the limiting nutrient. The intracellular level of the limiting nutrient will determine growth rate and biomass composition. Productivity is generally reduced in comparison with nutrient-sufficient growth. In any process aimed at oil production by photosynthesis, the key objective is a high areal oil productivity or, in other words, a high photosynthetic efficiency of lipid production. The target of this work is to maximize areal lipid productivity with a biomass containing about half of the fixed energy in the form of lipids (from 35% to 40% of the dry weight). The best conditions to obtain maximal biomass productivity,were investigated both in the laboratory and outdoors as well as the induction of lipid synthesis through nutritional limitation/deprivation. Three different strategies were compared: **(1)** nutrient-sufficient cultures having a relatively low lipid content, but able to attain high biomass productivity (one-phase strategy in nutrient-sufficient medium); **(2)** nutrient-limited cultures of lower productivity, but with a lipid content high enough to compensate for the productivity loss (one-phase strategy in nutrient-limited medium); **(3)** a two-phase strategy, with a first nutrient-sufficient biomass production phase followed by a lipid induction phase under nutrient deprivation.

Thirty microalgal strains, listed in Table 14 along with their origin, were screened for their growth characteristics and lipid content. The 30 strains were cultivated in nutrient replete medium in 250-mL Erlenmeyer flasks (100 mL of culture) maintained in an orbital incubator flushed with air/CO_2 (95/5, v/v) at a temperature of 25°C, under continuous illumination (100 mmol PAR photons/m²/s) provided by daylight fluorescent tubes. Each strain was cultivated in a single batch for about 2 weeks. Some of the most promising strains identified from the screening were cultivated at laboratory scale in 0.6-L glass bubbled tubes (4.5 cm in diameter) to investigate the induction of lipid synthesis through nitrogen deprivation. The 0.6-L tubes, containing 0.5 L of culture, were bubbled with a sterile air/CO_2 mixture (97/3, v/v) to support growth and maintain pH within the desired range (7.5–8.1). The tubes were immersed in a water bath thermoregulated at 25°C. Continuous artificial illumination (about 200 mmol PAR photons/m²/s) was provided by daylight fluorescent tubes on both sides of the water bath. A semicontinuous harvesting regimen (50% of the culture volume harvested daily) was adopted for 4 days, after which the cultures were maintained in batch for 3–5 days. The cultures were carried out in duplicate. Starting from a culture grown in replete medium, N-deprivation was achieved by replacing the daily harvested culture volume with the same volume of N-depleted medium. Scale-up experiments with the eustigmatophyte Nannochloropsis sp. F&M-M24 were carried out in 20-L FAP photobioreactors under artificial illumination to evaluate the influence of light intensity, and nitrogen and phosphorus deprivation on culture productivity and fatty acid accumulation. The culture system used in these experiments consisted of six removable 20-L FAP placed back to front and 24-cm apart. Illumination was provided by banks of daylight fluorescent tubes placed between the panels. The reactors and the illumination system were contained in a thermoregulated cabinet. Compressed air was bubbled at the bottom of the panels through a perforated plastic tube, for mixing and gas exchange. For experiments carried out in the FAP, air was filtered through 1-mm Polycap HD encapsulated filters (Arbor Tech, Ann Arbor, MI). Air-flow rate was maintained at 0.5 L/L/min and gas-hold-up was about 3.3%. CO_2 was continuously added to the air stream (3% v/v) to maintain pH at 7.5±0.2. The cultures were kept at 25°C, the optimal temperature for Nannochloropsis sp. F&M-M24. The influence of

Table 14

Algal group	Strain	Culture collection	Freshwater/marine
Diatoms	Chaetoceros calcitrans CS 178	CSIRO Collection of Living Microalgae, Australia	Marine
	Chaetoceros muelleri F&M-M43	F&M Culture Collection, Italy	Marine
	Phaeodactylum tricornutum F&M-M40	F&M Culture Collection, Italy	Marine
	Skeletonema costatum CS 181	CSIRO Collection of Living Microalgae, Australia	Marine
	Skeletonema sp. CS 252	CSIRO Collection of Living Microalgae, Australia	Marine
	Thalassiosira pseudonana CS 173	CSIRO Collection of Living Microalgae, Australia	Marine
	Chlorella sp. F&M-M48	F&M Culture Collection, Italy	Freshwater
	Chlorella sorokiniana IAM-212	IAM Culture Collection, University of Tokyo, Japan	Freshwater
	Chlorella vulgaris CCAP 211/11b	Culture Collection of Algae and Protozoa, UK	Freshwater
	Chlorella vulgaris F&M-M49	F&M Culture Collection, Italy	Freshwater
	Chlorococcum sp. UMACC 112	University of Malaya Algae Culture Collection, Malaysia	Freshwater
	Scenedesmus quadricauda	ISE—CNR Culture Collection, Italy	Freshwater
	Scenedesmus sp. F&M-M19	F&M Culture Collection, Italy	Freshwater
	Scenedesmus sp. DM	Istituto di Biofisica—CNR, Italy	Freshwater
	Tetraselmis suecica F&M-M33	F&M Culture Collection, Italy	Marine
	Tetraselmis sp. F&M-M34	F&M Culture Collection, Italy	Marine
	Tetraselmis suecica F&M-M35	F&M Culture Collection, Italy	Marine
Eustigmatophytes	Ellipsoidion sp. F&M-M31	F&M Culture Collection, Italy	Marine
	Monodus subterraneus UTEX 151	UTEX, Culture Collection of Algae at University of Texas, USA	Freshwater
	Nannochloropsis sp. CS 246	CSIRO Collection of Living Microalgae, Australia	Marine
	Nannochloropsis sp. F&M-M24	F&M Culture Collection, Italy	Marine
	Nannochloropsis sp. F&M-M26	F&M Culture Collection, Italy	Marine
	Nannochloropsis sp. F&M-M27	F&M Culture Collection, Italy	Marine
	Nannochloropsis sp. F&M-M28	F&M Culture Collection, Italy	Marine
	Nannochloropsis sp. F&M-M29	F&M Culture Collection, Italy	Marine
Prymnesiophytes	Isochrysis sp. (T-ISO) CS 177	CSIRO Collection of Living Microalgae, Australia	Marine
	Isochrysis sp. F&M-M37	F&M Culture Collection, Italy	Marine
	Pavlova lutheri CS 182	CSIRO Collection of Living Microalgae, Australia	Marine
	Pavlova salina CS 49	CSIRO Collection of Living Microalgae, Australia	Marine
Red algae	Porphyridium cruentum	Istituto di Biofisica—CNR, Italy	Marine

irradiance and one-side versus twoside illumination was studied at two different irradiance levels (115±25 and 230±51 mmol PAR photons/m²/s) continuously provided by daylight fluorescent tubes. In the deprivation experiments, one-side illumination of 115 mmol PAR photons/m²/s was adopted. To induce deprivation the cultures were grown in batch until complete N or P consumption, at which time a semicontinuous harvesting regimen (30% of the culture volume harvested every 3 days) with nutrient depleted medium started. Outdoor Experiments in GWP Experiments aimed at maximizing lipid production outdoors were carried out with Nannochloropsis sp. F&M-M24 in 110-L GWP photobioreactors during the summer 2006, at Maricoltura di Rosignano Solvay S.r.l. (Livorno, Italy; latitude: 4382308100N; longitude: 1082505200E). The GWP, patented in 2004 (Tredici and Rodolfi, 2004), comprises a culture chamber made of a 0.3-mm thick flexible LDPE film enclosed in a

rectangular metal frame. The modules used in the experiments were 1 m high, 2.5 m long and, on average, 4.5 cm thick, with a culture volume of 110 L. For mixing, compressed air was bubbled at the bottom of the reactor through a perforated plastic tube. CO_2 was injected into the culture through a gas diffuser placed in an un-aerated zone, as carbon source and for pH regulation. A control unit provided temperature regulation of the cultures by automatically activating water spraying on the reactor surface, when temperature exceeded the preset value. At the beginning of August, to study the influence of N and P deprivation, four south-west facing GWP modules were placed side by side in a single row on a platform. In one reactor the culture was grown under nutrient sufficient conditions (control culture), in the other three the cultures were grown under nutrient deprivation by replacing the daily harvested culture volume (40%) with N-, P- or both N- and P-deficient medium. At the beginning of September, the influence of N-limiting conditions was investigated using four GWP modules which were placed facing south in 1-m apart parallel rows so as to simulate a full-scale plant. The reactors were operated at a daily harvest rate of 40%. Three different N-limiting regimes were compared with the N-sufficient control culture. For the outdoor experiments, air-flow rate was maintained at 0.3 L/L/min, gas-hold-up was about 2.5% and air was filtered through 1-mm filters. CO_2 was injected during daylight hours to maintain pH in the range 7.5–8.0. The cooling system prevented the culture temperature to exceed the value of 30°C. During the night, the culture temperature was allowed to equilibrate to ambient. In the deprivation experiment, nutrient deficiency was attained by replacing the daily harvested culture volume with nutrient (N, P or both N and P) depleted medium. In the N-limitation experiment, four different N levels were tested. A culture was considered as Nsufficient when the N level allowed to obtain a biomass with a N content of 10%. In this culture nitrogen was added in amounts equal to 10% of productivity. N-limited cultures were obtained by adding nitrogen in amounts equal to 5%, 2.5%, or 1.25% of their productivity. The experiment started with a N-sufficient culture which was distributed in equal volumes in four GWP and then diluted with fresh medium containing the different N levels. In all the experiments a 40% daily harvest rate was adopted.

Growth media used included BG11 for the freshwater microalgae and f medium for all the marine species. Sodiummetasilicate was added (45 mg/L) as a silica source for diatoms. The f medium was prepared with artificial seawater (Adriatic Sea Aquarium & Equipment, Rimini, Italy) at 30 g/L salinity for laboratory cultures, whereas for outdoor cultures UV-treated natural seawater diluted at 25 g/L salinity was used. For laboratory experiments in flasks and bubbled tubes artificial seawater was autoclaved, and after cooling, sterile nutrient solutions were added. The artificial seawater for cultures in FAP and the natural seawater for cultures in GWP were filtered through 10- and 1.5-mm polypropylene filters (Domnick Hunter) and then added with sterile nutrient solutions. Except for the N-limited and N- and P-deprived cultures, $NaNO_3$ and NaH_2PO_4 were added to the cultures according to algal growth assuming that N and P represent 10% and 1% of the biomass, respectively. Culture growth was estimated by measuring the dry biomass concentration. In the strain selection experiment, the growth curve was followed by measuring the optical density at 750 nm and daily biomass productivity was calculated dividing the difference between the dry weights at the start and at the end of the experiment by its duration (days). When a culture entered the stationary phase before the end of the experiment, the difference between the dry weights was divided by the time elapsed between the start of the experiment and the onset of the stationary phase. Lipid content was determined at the end of the culture period in the strain selection experiment and daily or every 2 days in all the other experiments. In the experiments carried out in the FAP, fatty acid analysis was performed on samples collected at the start and every 2–3 days during the deprivation period. For lipid and fatty acid analyses, the collected samples were centrifuged and frozen. Lipid content was determined spectrophotometrically after carbonization of the material extracted with a 2:1 methanol/chloroform solution.

Lipid concentration was calculated from dry biomass concentration and lipid content. Daily lipid productivity was calculated from the difference in lipid concentration in two consecutive days. In the selection experiment, lipid content was determined only at the end of the culture period, and daily lipid productivity was calculated from daily biomass productivity and lipid content at the end of the experiment. For fatty acid determination, the lyophilized biomasses were extracted and methylated according. The methyl esters were analyzed with a GC 8000 Fisons gas-chromatograph (Fisons, Milan, Italy). For lipid class analysis, lyophilized biomass was extracted and analyzed as reported, with the sole modifications that dichloromethane was used instead of chloroform and the solvent mixture used for lipid separation was hexane/diethyl ether/acetic acid (60:10:0.1). Nitrate and phosphate were measured according to APHA. In outdoor experiments, all the samples were collected at the end of the dark period. The daily global solar radiation on the horizontal was obtained from La.M.M.A.-Regione Toscana Laboratory for Meteorology and Environmental Modeling (Livorno, Italy). PAR was measured by a LI-190SB cosine quantum sensor connected to a LI-185B quantum/radiometer/photometer (Li-Cor, Inc,Lincoln, NE).

Thirty microalgal strains were tested for their lipid production potential by evaluating biomass productivity and lipid content in 250-mL flask laboratory cultures. The best biomass producers were four marine microalgae: Porphyridium cruentum (with a productivity of 0.37 g/L/day) and three Tetraselmis strains (with productivities ranging from 0.28 to 0.32 g/L/day). Lipid content in these algae was, however, rather low (below 15% of the dry biomass) and thus lipid productivities were not among the highest. Lipid content of marine strains was highly variable (from 8.5% to 39.8%). Lipid content of freshwater microalgae was near 20%, and the best biomass producers, Chlorococcum sp. UMACC 112 and Scenedesmus sp. DM (0.28 and 0.26 g/L/day, respectively), were also the best lipid producers (54 mg lipid/L/day). The highest lipid content (40%) was found in the marine Chaetoceros calcitrans CS 178, which was the least productive of all the strains tested. In general, productivity and lipid content were inversely related, a fact that has its rationale in the high metabolic cost of lipid biosynthesis. The best lipid producers, that is, the strains showing the best combination of biomass productivity and lipid content, were three members of the marine genus Nannochloropsis (out of the six tested), with a lipid content of 30% or higher and a lipid productivity ranging from 55 to 61 mg/L/day. The marine genus Nannochloropsis emerged from the screening as one of the best candidates for algal oil production. Two freshwater, Chlorella sp. F&M-M48 and Scenedesmus sp. DM, and two marine, T. suecica F&M-M33 and Nannochloropsis sp. F&MM24, strains, which were among the best producers in terms of biomass or lipid and had shown in previous experiments to perform well in outdoor conditions, were selected for the subsequent trials. Induction of Lipid Synthesis Through Nitrogen Deprivation in Small Scale Bubbled Tubes In the two freshwater microalgae selected, N-deprivation led to a progressive reduction of productivity and eventually to growth cessation after four (Chlorella sp. F&M-M48) or seven (Scenedesmus sp. DM) days. The two marine strains behaved differently. In N-deprived medium, productivity of T. suecica F&MM33 remained high for 2 days, then decreased until growth ceased on the 7th day. Lipid content increased slowly during the first days, then more markedly with growth cessation, a behavior which was also observed, although to a much lower extent, in the N-sufficient medium. The result was that N-deprivation did not bring about a substantial increase of lipid production in this microalga. Under N-deprivation, productivity of Nannochloropsis sp. F&M-M24 declined slowly in the first 3 days, then more markedly. On the second day, when nitrogen in the medium had been completely exhausted and the lipid content of the biomass had raised to more than 45%, biomass productivity was still about 75% of that of the N-sufficient control culture. On the fourth day, the lipid content reached 60%, but biomass productivity declined to less than 15% of that measured in the control. This eustigmatophyte, which responded to nitrogen deprivation with a considerable increase of its lipid content and a limited loss of productivity, was chosen for further study. When

Nannochloropsis sp. F&M-M24 was grown in the FAP with one-side illumination, an increase of irradiance from 115 to 230 mmol photons/m²/s brought biomass productivity from 0.61 to 0.85 g/L/day and FA from 14.7% to 19.6%. With two-side illumination, productivity and FA increased from 0.97 g/L/day and 24.3% at 115 mmol photons/m²/s to 1.45 g/L/day and 32.5% at 230 mmol photons/m²/s, respectively. The FA increase was due to the increase of saturated (14:0 and 16:0) and monounsaturated (16:1n7 and 18:1n9) fatty acids, mainly associated with storage lipids.
It is worth noting that with two-side illumination at an irradiance of 115 mmol photons/m²/s, both the culture productivity and the FA were higher than with one-side illumination at an irradiance of 230 mmol photons/m²/s, despite the fact that the same amount of photons impinged on the culture in the two different conditions. As observed in the small-scale tubes, productivity decreased following N-deprivation. It took about 2 weeks, however, to halt growth completely. FA increased after about 5 days of N-deprivation and reached, at the end of the experiment, about 50% of the dry biomass. The increase was due to the increase of four fatty acids: 14:0 (from 0.7% to 2.9%), 16:0 (from 4.8% to 19.8%), 16:1n7 (from 3.9% to 15.1%) and 18:1n9 (from 0.7% to 6.8%), which represented about 90% of the FA of the N-starved biomass at the end of the experiment. Lack of phosphorus elicited a similar response, but it took longer to decrease productivity (about 1 week) and halt growth (20 days;). The FA increased from 13.2% to 50.1% and the fatty acids responsible for the increase were the same as those under N-deprivation, although the synthesis of 18:1n9 was further stimulated (from 0.5% to 10.6%;). Upon resuming phosphate supply, growth immediately restarted. Very likely this quick recovery was supported by the breakdown of stored fatty acids, which in 1 week reassumed the initial value. While the main lipid classes in the P-sufficient biomass (start of the experiment) were polar lipids and sterols, TAGs constituted the bulk (67%) of the lipid fraction of the P-starved biomass.
Three different strategies for lipid production were compared outdoors in two experiments. The experiment under nutrient deprivation (two-phase strategy) was carried out during 1 week in the summer with an average solar radiation of 15.9±2.9 MJ/m²/day. The nutrient sufficient control culture (one-phase strategy in nutrient sufficient medium) attained a biomass productivity of 0.36±0.10 g/L/ day, a lipid content of 32.3±1.0% and a lipid productivity of 117±28 mg/L/day (averages of the whole experimental period). Biomass productivity of the starved cultures declined slowly in the first 4 days, then more markedly. The lipid content of the P-deprived culture increased only after 4 days of shortage and was substantially balanced by the decrease in biomass productivity, with no beneficial effect in terms of lipid productivity. Differently, the N-deprived culture increased regularly its lipid content since the first day, reaching 60% after 3 days of deprivation. During the first 4 days the biomass productivity of the N-starved culture remained relatively high. The high biomass productivity (on average 0.30±0.05 g/L/day) and the regular increase of lipid content in the first 3 days of deprivation (from 30 to more than 60%) brought average lipid productivity to 204±47 mg/L/day. The culture depleted of both N and P showed a similar behavior to the N-deprived culture. The experiment under nitrogen limitation (one-phase strategy in nutrient limited medium) was carried out during 1 week at the end of the summer with an average solar radiation of 15.4±2.6 MJ/m²/day. Generally, the lower the nitrogen in the medium, the lower the biomass productivity and the higher the lipid content. In the nutrient sufficient control culture, the average biomass productivity and lipid content were 0.30±0.04 g/L/day and 28.0±2.4%, respectively, and lipid productivity averaged 81±15 mg/L/ day. Lipid content increased substantially only under severe N-limitation (2.5% and 1.25% N), but because of the biomass productivity reduction, lipid productivity was not enhanced, with the exception of the second day, when three of the four cultures showed a marked increase. The strong enhancement of lipid productivity observed in the second day in the cultures maintained at 2.5% and 1.25% N was very likely due to

the combined effect of nitrogen limitation and a sudden increase of solar irradiance (from 10 to 17 MJ/m²/day).

In a microalgae-based oil production process, a high lipid yield per area (the result of biomass areal productivity and lipid content) is one of the main goals, since it greatly influences production costs. A high lipid content is also desirable per se as it improves the efficiency of biomass processing (oil extraction). The areal productivity mainly depends on climatic conditions (above all temperature and solar radiation) and culture management, but a suitable (selected) organism is also a fundamental requirement. The selected microalgal strain must be highly productive in outdoor culture, with a constitutional high lipid content and/or able to respond with a substantial accumulation of lipids to nutrient deficiency, robust enough to withstand mixing generated shear-stress, and flexible so as to adapt to the unavoidable changes in physico-chemical parameters of an outdoor environment. Locally isolated strains may be useful, but do not necessarily lead to increased stability of the culture, since even a native strain may have difficulties in dominating year round in the highly variable environment of outdoor mass cultures. In this study, 30 microalgae were tested in the laboratory for their oil production potential. As expected, lipid-rich strains showed in general lower biomass productivity. This confirms what was known for some time: that high productivity and high lipid content, the desired traits for biodiesel production from microalgae, are mutually exclusive. However, some promising candidates, both freshwater and marine, were found and investigated more in depth. Selection was guided not only by productivity and lipid content of laboratory cultures, but also by the strain robustness and capacity to dominate for relatively long periods in outdoor culture. After studying the induction of oil synthesis under nitrogen deprivation in the laboratory with two freshwater and two marine strains, the focus was put on the marine eustigmatophyte Nannochloropsis sp. F&M-M24, a microalga endowed with a high constitutional lipid content (about 30%) and able to respond with oil accumulation to nitrogen deprivation. Nannochloropsis spp. are widely used in aquaculture and have been investigated as a potential source of eicosapentaenoic acid. Its small cell size (2–5 mm), which makes harvesting very expensive, the hard cell wall and the presence of polyunsaturated fatty acids must be taken into account when proposing this organism for biodiesel production. Another marine strain, T. suecica F&M-M33, very productive and robust, was also considered of high potential for oil production. However, the basal lipid content of T. suecica F&M-M33 is rather low (8.5–20%) and the alga responded to nitrogen deficiency with a delayed lipid accumulation, which did not bring about a substantial increase of lipid production in comparison with the N-sufficient control. With the exception of Nannochloropsis salina, a general trend has emerged of an increased lipid storage (mainly TAGs) at increasing irradiances and as a consequence of nutrient (particularly nitrogen) deprivation. In this study, using a 20-L alveolar panel under artificial illumination, it was confirmed that high irradiances and particularly nutrient (N or P) shortage can lead to significant accumulation of saturated and monounsaturated fatty acids, which represented up to 90% of the FA in the nitrogenstarved biomass. These fatty acids are mainly associated with storage TAGs, the preferred substrate for biodiesel production by trans-esterification. To enhance oil yield of algae cultures the cell lipid content should be increased over the basal value without significant losses of productivity. This appears a rather difficult task given the high specific caloric value of lipids (38.9 kJ/g) compared with proteins (24 kJ/g) and carbohydrates (15.6–17.5 kJ/g). Generally, under nutrient shortage, although the fraction of lipids may increase, cellular growth declines with the overall effect being a decrease of lipid productivity. The genus Nannochloropsis appears as a glaring exception: it was observed in laboratory trials that lipid synthesis may continue in this organism during N-starvation not only at the expense of other cellular components, but starting from newly fixed carbon, and this generally is coupled with enhanced lipid productivity. The enhanced lipid biosynthesis resulted primarily from de novo CO_2 fixation. In this work with Nannochloropsis sp. F&M-M24, higher lipid

content and productivity were achieved in batch cultures when, during the early stages of nitrogen deficiency, a light shift was also applied and, for the first time, enhancement of both lipid content and lipid productivity was demonstrated in outdoor cultures. This is an important finding, since it is commonly believed that the production of lipids will be more economical with nitrogen-sufficient cells, despite their lower lipid content, because of their much higher biomass productivity. N-limitation did not appear as effective as N-deprivation in maximizing lipid production, although when severe nitrogen limitation was associated with an increase of sunlight availability, lipid productivity increased. In outdoor cultures under nutrient replete conditions, the lipid content of Nannochloropsis sp. F&M-M24 varied between 28% and 32% and lipid productivity between 81 (end of the summer) and 117 (mid summer) mg/L/day. The lipid production potential for cultures under nutrient sufficient conditions can thus be projected to about 50 kg/ha/day. This value was calculated as follows. By placing the 1-m high, 2.5-m long, 110-L GWP in east-west oriented (facing south) parallel rows at a distance of 0.9 m, 4,444 reactors can be accommodated in one hectare and shading of direct sunlight between rows can be prevented from mid March to mid September. In this arrangement there will be some reduction of diffuse and reflected radiation at the reactors' surface, but direct illumination will not be reduced in comparison with the isolated unit and productivity will not be significantly affected. Besides, since solar radiation in the 2 weeks of the experiments was quite low (15.9 and 15.4 MJ/m²/day against a typical radiation for August in Tuscany of more than 20 MJ/m²/day) and, differently from the horizontal, vertical south-facing surfaces receive a relatively stable daily solar radiation from March to October, a lipid productivity of about 100 mg/L/day (average of the two experiments) can be adopted for the whole 6-month period during which mutual shading between rows was avoided. This productivity is equivalent to 11 g per reactor per day, that is, about 50 kg/ha/day or about 9 tons per hectare in 6 months. Under N-deprivation, the increased lipid synthesis brought lipid productivity to more than 200 mg/L/day (average of the first 3 days of N-deprivation), that is to 22 g per reactor per day or about 100 kg of lipid/ha/day. This is not the true oil production potential of an industrial plant, since a two-phase strategy will require that, before the culture is N-starved for oil synthesis, enough biomass (to be used as inoculum) is produced under N-sufficiency. A two-step process is therefore here envisaged, according to which 22% of the plant is dedicated to biomass production under N-sufficiency and the rest is devoted to oil production under N-deprivation. The lipid productivity achievable with this two-phase strategy is equivalent to about 90 kg of lipid/ha/day (10 and 80 kg of lipid/ha/day in the first and second phase, respectively) or, in 6 months, to more than 16 t/ha. For a 10-month production period, even considering the much lower productivities achievable in the early spring and late autumn, an annual lipid yield of about 20 t/ha can be projected. For many tropical areas of the world, with an average annual solar radiation of 20 MJ/m²/day or more, it is estimated a potential production of 30 tons of oil per hectare per year. For an energy generation system to be sustainable, its NER must be greater than 1 and as high as possible. The NER is the ratio, calculated for the lifetime of the system, between the energy output (in this case the energy content of the algal oil and of the residual biomass) and the energy content of all the materials (their embodied energy) with which the plant is constructed, plus the energy needed for all the operations. In a microalgae plant, the reactor energy content, and the energy expenditure for mixing and harvesting will have a major influence on the NER. Although the embodied energy of the GWP is low compared to other PBR and has been reduced further in the GWP of second generation, the NER of algae cultivation in this system is not sufficiently higher than 1 as required, mainly because of the large expenditures for mixing and harvesting. Reduction of aeration rates (especially at night) without significantly decreasing productivity might be possible due to the high buoyancy of the small Nannochloropsis cells, but needs to be proven. In the laboratory, ''autoflocculation'' or pH induced flocculation can be successfully used to concentrate the algal suspension and significantly reduce

the energy cost of harvesting, but again, this is an issue that requires testing at large scale. A possibility to significantly reduce capital and operating costs of microalgae cultivation would be using raceway ponds. Plastic lined, paddle-wheel mixed raceway ponds are much less expensive to build and operate than PBR. Raceway ponds were developed in the 1950s, first in Germany and Japan, then in the USA and extensive experience exists on their operation. These open-air systems, of maximum size of a third of a hectare, may be built in concrete or compacted earth and lined with cheap plastic. Both cooling, achieved by evaporation, and mixing by paddle wheels require low energy inputs per unit area, and a recent life cycle analysis suggests that microalgae cultures in raceway ponds might have a largely positive energy balance. However, open systems suffer from a severe limitation, contamination with unwanted algae and other organisms is inevitable in long-term operation and translates into the impossibility of maintaining the desired microalga in culture long enough, unless species that tolerate extreme conditions, such as Dunaliella or Arthrospira, are cultivated. With the exclusion of these few examples, microalgae cultures in open ponds are very unstable ecosystems, affected by large diurnal and seasonal variations of physicochemical and biological parameters that lead to frequent changes in the community population structure. The only solution seems to be enclosing the culture in the tightly controlled, more expensive PBR, which, although does not provide sterility, guarantees a relatively long and stable cultivation. Combining closed and open systems may provide a reasonable solution to obtain culture stability at relatively low cost. The coupling of PBR and open ponds might be well adapted to the two-phase strategy successfully experimented in this work. The first N-sufficient phase could be carried out in the PBR to produce the inoculum for the N-starved phase leading to oil accumulation in the open ponds. This strategy presents the advantage that, since the cultivation in the open pond lasts only few days, there will be not time for contaminants to prevail. Finally, it would be advisable to operate so that, when the pond is inoculated, the culture is also diluted to attain, besides N-starvation, a light increase per cell leading, as demonstrated in this study, to increased lipid productivity.

Chapter 2

2.Definition of cells extraction methods

Cell disruption is the process of breaking indehiscent bacterial cells and cell wall structures of eukaryotic microorganisms, such as yeast, algae, and fungi. The structure of the cell wall varies with the type of microorganism and the given growth conditions. Knowledge of the cell wall structure of a microorganism helps with the selection of a suitable treatment method to disrupt its cellular integrity. Disruption of yeast cell walls is more straightforward when compared to bacterial cells due to their larger cell size and a unique cell wall structure. The cell wall of yeasts contains mainly glucans, mannans, and proteins and the overall structure is thicker than in gram-positive bacteria. Older cells have thicker cell walls than younger ones. Microalgae are also characterized by a thicker cell wall structure made up of complex carbohydrates and glycoproteins.
Some researchers divide the cellular disintegration into thermal treatment methods and non-thermal treatment methods, whereas others categorize them as mechanical methods, such as oil expeller, ultrasonication, and microwave-assisted extraction or chemical methods, such as Soxhlet extraction, supercritical fluid extraction, and accelerated solvent extraction. All of these treatment methods have been extensively utilized for the efficient lipid extraction from various oleaginous microorganisms, such as yeast, microalgae, fungi, and bacteria.

2.1.Folch method

Various organic solvents or combination of different solvents have been suggested to selectively extract lipids from a complex mixture of organic compound. The Folch method employs the use of chloroform–methanol (2:1 by volume) for extraction of lipids from endogenous cells. Briefly, the homogenized cells were equilibrated with one-fourth volume of saline solution and mixed well. The resulting mixture was allowed to separate into two layers and lipids settle in the upper phase. This method is one of the oldest initiatives in lipid extraction, which formed the basis for development of future extraction procedures with improvements. The above method with some modification is still used for the estimation of algal lipids spectrophotometrically. Rapid and easy processing of large number of samples is the major advantage of this method. However, it is less sensitive when compared with other latest procedures.

2.2.Bligh and Dyer method

Lipid extraction and partitioning are performed simultaneously in the Bligh and Dyer method, wherein proteins are precipitated in the interface of two liquid phases. The Bligh and Dyer method is one of the widely practised methods for lipid extraction. It is very similar to the Folch method, but mainly differs in sol- vent/solvent and solvent/tissue ratios. This procedure is performed by extracting lipids from homogenized cell suspension using 1:2 (v/v) chloroform/methanol. The lipids from the chloroformphase are then extracted and processed by various procedures. The above gravimetric method is still widely used for the estimation of lipids by algal technologists, and the same procedure is also followed for pilot-scale and large-scale extraction processes. In order to improve the above basic method, many modifications have been adopted by researchers. The most common modification is the addition of 1M NaCl instead of water, to prevent binding of acidic lipids to denatured lipids. The addition of 0.2M phosphoric acid and HCl to the salt solution improves lipid recovery with a shorter separation time in comparison with the earlier extraction methods.

Similarly, addition of 0.5% (v/v) acetic acid to the water phase increased the recovery of acidic phospholipids.

2.3.In Situ lipid hydrolysis and supercritical in situ transesterification

In the in situ lipid hydrolysis and supercritical in situ transesterification method, wet algal biomass is processed for the extraction of lipids followed by transesterification to obtain biodiesel, and therefore, it gains significance in the field of algal biofuel. In brief, wet algal biomass is kept in a stainless steel reactor immersed in a pre-heated isothermal fluidized sand bath for the desired time and then promptly removed and cooled in water. Hydrolysis reaction is carried out simultaneously at each condition in two other reactors. Then, the dried algal biomass (1g) was mixed with 4 g of water in a large reactor (10ml), and the reaction was continued for 15, 30, 45, and 60 min at 250°C. In this method, simultaneous drying and dehydration converts the wet algal biomass into a solid, and this process facilitates precise loading of solids in hydrolysis reactions. Upon cooling, the aqueous phase and solids were separated under light vacuum condition using an appropriate filter (934-AH filter paper; Whatman). However, the above method has to be tested for its commercial feasibility in a large-scale cultivation facility.

2.4.Mechanical approach to algal oil extraction

Many mechanical methods are being used to extract lipids from microalgae both at pilot-scale and commercial levels. Mechanical methods present an effective approach because of less dependence on the type of microalgae species to be processed and they are also less likely to cause contamination of the extracted lipid product. However, the above methods usually require higher energy inputs than the chemical or enzymatic methods. Moreover, heat generation during mechanical disruptions can cause damage to the end products and a cooling system becomes vital during extraction of heat sensitive products. The energy and equipment costs for installation and functioning of a cooling system will add up to the process costs. Some of the mechanical extraction processes that do not necessarily require solvent assistance, include bead mills, expeller press procedure, microwave-assisted extraction as well as ultrasound-assisted extractions and pulsed electric field.

2.5.Expeller press

Expeller press or oil press is one of the simplest and oldest methods used for extracting oil from oil seeds. The simple, yet effective, mechanical crushing method is also being used in the extraction of oil from algal biomass. Dried algal biomass retains oil content, which then can be pressed out using an oil press. The principle underlying this technique is to apply high mechanical pressure for crushing and breaking the cells, and to squeeze out the oil from the algal biomass. Application of pressure in a particular range improves the extraction efficiency, but too much of pressure will result in decreased lipid recovery, increased heat generation, and choking problems. Algal biomass characteristics vary widely, particularly in their physical attributes, based on the morphological differences of different strains, and various tailor-made press configurations (screw, expeller, piston, etc.) are required. Usually, the oil recovery is in the range of 70–75%. Sometimes for enhanced oil recovery, mechanical crushing is used in conjunction with chemical methods. However, press methods are expensive and involve prolonged processing times. In addition, mechanical pressing generally requires input materials with very low-moisture content, and drying of the algae biomass, which is an energy intensive process, can account for up to 30% of the total

production costs. Unlike vegetable oils, which can be easily extracted by crushing the seeds accompanied by a solvent extraction, releasing oil from algal cells is hindered by the rigid cell wall structure. The major technical drawback is the presence of pigments along with oil. Before conversion to oil, the pigments have to be removed either by solvent extraction or by activated carbon adsorption, which again adds up to the cost. Other major drawbacks of this method include high-maintenance cost, requirement of skilled labor, and less efficiency compared to other methods.

2.6.Bead beating

Bead beating is a mechanical method for the disruption of cells, where a direct damage to the cells is caused by the concept of high-speed spinning of the biomass slurry with fine beads. In bead mills, the cells are disrupted by the impact of grinding beads against the cells. All types of cells including those of microalgae can be processed by the above method. Shaking vessels and agitated beads are the two common types of bead mills. In the shaking vessel type, the cells are damaged by shaking the entire culture vessel. Usually, multiple vessels are shaken on a vibrating platform and this type of bead mill is suited for samples requiring similar disruption treatment conditions. Hence, this set-up can be exclusively used on a laboratory scale. Better disruption and extraction efficiencies can be obtained with the second type, where the beads are agitated along with the cell culture. As the rotating agitator inside the culture vessel generates heat, the vessels are provided with cooling jackets to protect the heat-sensitive biomolecules. Unquestionably, the combined effect of agitation, collision, and grinding of the beads produces a more effective disruption process. Similarly, dewatering of algal slurry is not required unlike in the expeller press method and this contributes to reduction in processing costs. Various beads are used for different types of cells; the optimal bead diameter for microalgae cells is 0.5 mm and the optimal volume fraction of bead loading is about 0.5mm. Beads made of zirconia-silica, zirconium oxide, or titanium carbide can enhance the disruption rates and extraction efficiency of microalgal cells, presumably because of their greater hardness and density.

2.7.Ultrasonic-assisted extraction

Ultrasound-assisted extraction of lipids is an alternative technique, which is devoid of the difficulties associated with the conventional mechanical disruption methods. The process is simple with easy working set-up conditions, imparting higher purity to the final product and eliminating treatment of wastewater generated during the process. Furthermore, the technique is more economical and eco-friendly and can be completed in a very short time with high reproducibility. The energy input is very little when compared to that in conventional methods, and can be operated at lower temperatures. When liquid cultures are used, there are two major mechanisms by which ultrasound can cause damage to the cells, namely, cavitation and acoustic streaming. Cavitation is the production of microbubbles as a result of the applied ultrasound, which in turn can create pressure on the cells to break up, and acoustic streaming facilitates mixing of the algal culture. The ultrasonic waves generate transient and stable cavitation due to the rapid compression/decompression cycles occurring during the treatment. Unsteady oscillations will result in transient cavitation, which will ultimately implode. A cavitation implosion produces extremely localized heat shock waves,which disrupt the microalgal cells. Thus, sonication cracks the cell wall and membrane due to the cavitation effect. Microstreaming and heightened mass transfer resulting from cavitation and bubble collapse are the two critical steps to determine the lipid yield extraction efficiency. Horn and bath are the two basic types of sonicators and both processors are commonly employed in batch operations but

can be adapted for continuous operations as well. Piezoelectric generators made of lead zirconate titanate crystals are used in horns, which vibrate with an amplitude of 10–15 mm, whereas sonicator baths use transducers, which are placed at the bottom of the reactor to generate ultrasonic waves. In the bath type, the capacity and shape of the reactor determine the number and arrangement of transducers. The major advantage of the sonication process is that it generates relatively low temperatures when compared to microwave reactors and autoclaves, thereby leading to less thermal denaturation of biomolecules. Furthermore, it does not require the addition of beads or chemicals, which have to be removed later in the process, which in turn will incur more cost.

2.8.Microwave assisted extraction

Earlier, the applications of microwave radiation were limited to the digestion of samples for measuring trace metals and extraction of organic contaminants. The feasibility of extracting lipids using microwave irradiation was first reported in the mid-1980s. It was developed a microwave extraction technique for isolating lipids and pesticides from seeds, foods, feeds, and soil, which was more effective than the conventional procedures. Thus, microwave technology has allowed the development of rapid, safe, and economical methods for extracting lipids and does not require dewatering of algal biomass. Similarly, use of microwave remains the most simple and most effective method among the other tested methods for microalgal lipid extraction. A dielectric or polar material introduced in a rapidly oscillating electric field, such as that produced by microwaves, will generate heat because of the frictional forces arising from inter- and intra-molecular movements. Intracellular heating results in the formation of water vapor, which disrupts the cells from within. This in turn leads to the electroporation effect, which further opens up the cell membrane, thereby rendering efficient extraction of intracellular metabolites. Thus, rapid generation of heat and pressure within the biological system forces out compounds from the cell matrix, resulting in the production of good-quality extracts with better target compound recovery. Microwaves can also be used to extract and transesterify the oils into biodiesel. Microwave heating consumes almost two to three times less energy than that involves in the conventional heating.
Moreover, lipid extraction using a single-step microwave-assisted extraction is more convenient and effective than the multistep, time consuming traditional Bligh & Dyer method. Furthermore, In a study conductedby Lee et al, it was stated that the lipid extraction yield was higher for the microwave method when compared to autoclaving, bead beating, ultrasonication, and 10% NaCl solution extraction methods.
Microwaves are the pick of the options at present because of the economics involved in the above process; it is expected to be attractive due to short reaction time, low-operating costs, and efficient extraction of algal oils. In particular the recovery of biodiesel from the reaction mixture in a microwave-assisted process is approximately 15–20 min, which is far quicker when compared to the 6-h period in the conventional heating method. However, the disadvantage with the microwave-assisted process is the formation of free radicals, the increased temperature and the maintenance cost involved, particularly on a commercial scale.

2.9.Algal oil extraction using electroporation

Electroporation is a technique that is diversely used in molecular biology research to enable transportation of chemical, drug, and foreign DNA products into the cell. Nowadays, this technique is also used for lipid recovery from microalgal cells. The applied electrical field on wet algal biomass creates aqueous pores in the cell walls, which enhances both membrane permeability and conductivity, hence mass transfer across the cell membrane is also increased.

According to Sommerfeld et al. (2008), electroporation/electropermeabilization altered the cellular membranes and cell walls of tested algal cells and improved lipid extraction efficiency in terms of time and solvent use without affecting the composition and quality of extracted fatty acids. They also report that 92% of the total lipid was extracted from the algal biomass after a single electroporation treatment, while only 62% of the total lipid was extracted from the same amount of algal biomass without the electroporation treatment.

2.10.Solvent-free extraction methods for algal biomass

Lipid extraction from algal biomass is typically carried out using organic solvents such as hexane, chloroform, petroleum ether, acetone, and methanol. At present, no other potential alternatives are available, which can overcome the fire hazards and huge costs involved in the utilization of organic solvents. Although organic solvent-based extraction works fairly well with some algal strains, it is not widely applicable for all algal strains and it also consumes time and labor. Similarly, mechanical approaches may prove costly and cause damage to the end products. All the methods discussed above have their advantages and disadvantages, but none of them has been confirmed as a suitable extraction method for algal fuel production. An innovative efficient eco extraction/fractionation process technique apart from mechanical/solvent extraction approaches could result in the reduction or control of the production costs. The solvent-free extraction is often an ecologic and more economic process; indeed, it needs no supplementary energy to separate phases and elimination of the solvent is not necessary if no final product recirculation system exists.

2.11.Osmotic Pressure method

An innovative and alternate approach of using osmotic pressure is considered an ecological and cost-effective way to compete with other extraction methods. Osmotic pressure can disturb algal cell walls through a hasty increase and decrease in the salt concentration of the aqueous media; this can disturb the balance of osmotic pressure between the interior and exterior of the algal cells. Algal cell damage can occur by two osmotic stresses: hyper-osmotic and hypo-osmotic. When the salt concentration is higher in the exterior, the cells suffer hyper-osmotic stress. As a result, the cells shrink as fluids inside the cells diffuse outwards, and damage is caused to the cell envelopes. In contrast, hypo-osmotic stress occurs when the salt concentration is lower in the exterior; the fluid flows into the cells to balance the osmotic pressure, and the cells swell or burst if the stress is too high. Hypo-osmotic shock is a procedure commonly used for the extraction of intracellular substances from microorganisms. According to some authors, positive results could be achieved by using the osmotic pressure method for the extraction of oil from various microalgal biomasses such as those of Chlamydomonas reinhardtii, Botryococcus sp., Chlorella vulgaris, and Scenedesmus sp. Thus, it was concluded that the osmotic pressure method would appear to be the most simple, easy, and efficient method for lipid extraction from microalgae.

2.12.High pressure homogenization

This method is suitable for the stabilization of emulsification processes in cosmetic, pharmaceutical, and food industries, however, it has also been extensively utilized for the microbial cell disruption of microalgae, bacteria, and yeast. The cell disruption efficiency varies according to the valve seat configurations of the homogenizer. High disruption efficiency is usually achieved through shear forces of highly pressurized fluids on the stationary valve surface and hydrodynamic cavitation from the shear stress induced by pressure drop. High-pressure homogenization has many advantages as

it is a simple continuous operating system and can be applied for wet biomass, where the processing fluid is pressurized in intensifiers and passed through a homogenization chamber. The energy is accumulated in the fluid by the pressure and released into the passage through an orifice valve, where the velocity of the fluid increases to up to 200–400 m/s.
Increased velocity generates mechanical stress, such as shear and elongational forces, turbulence, and cavitation, which are responsible for disruption of cells.

2.13.High speed shearing Homogenization

High-speed shearing homogenization (HSH) is usually utilized to prepare foams, emulsions, and suspensions. It is a very effective method to disrupt cells, where a slurry of biomass is stirred in a specific device consisting of a stator–rotor assembly with a small gap (100–3000 μm). The cells are disintegrated due to hydrodynamic cavitation and the shear forces that are caused by stirring at high rpm, which creates high shear rates (20,000–100,000 s^{-1}).
The performance of the high-shear mixer is quite similar between wet and dry biomass of microalgae when extracted with different solvents, such as hexane, hexane-isopropanol, and ethanol. The mixtures developed a strong shear stress and cavitation effect when stirred at 15,000 rpm for 10 min, which was enough to extract all the esterifiable lipids from the microalgae. The most important feature of this method is that it can be directly used for high moisture containing samples, thus reducing the water footprint and downstream process costs.
However, extensive heat generation and high energy consumption during the operation are the major drawbacks when it comes to scale-up processes.

2.14.Autoclaving

Autoclaving is usually utilized for the sterilization of laboratory equipment and media prior to the growth of microorganisms. Various microalgal species, such as Haematococcus pluvialis, Botryococcus sp., C. vulgaris, and Scenedesmus sp. were disrupted by autoclaving at 121 °C and 1.5 MPa for 5 or 30 min
Similarly, Florentino de Souza Silva et al. (2014) suggested that autoclaving is a more efficient technique than ultrasonication but not as efficient as microwaving and electroflotation by alternating current (EFAC), when mixed cultures of microalgae were treated with different methods.

2.15.Pulsed electric field

Pulsed electric field (PEF) treatment works based on electroporation phenomena, including electromechanical compression and electric field-induced tension, where an external electric field is used to induce the critical electrical potential across the cell membrane. The increase in membrane porosity is directly proportional to the strength of the applied electric field and pulses and the pore formation in the membrane can be reversible or irreversible, depending on the size and number of pores in comparison to the total surface area of the membrane or cell wall.

2.16.Laser

Laser treatment is a well-known technique to disintegrate the cellular membrane without damaging the compartments of the cell factory or other interior compounds. Most importantly, laser treatment is free of the use of any organic solvent, fast, and requires no laborious effort.

Previously, researchers have studied the efficiency and mechanism of this method of cell lysis in static mode for different microorganisms, like Escherichia coli, Saccharomyces cerevisiae, and microalgae at various wavelengths and energy inputs.
In a study, oleaginous microalgae N. oculata cells were disrupted by using various pretreatment methods, such as microwave, water bath, blender, ultrasonic, and laser treatment, and it was revealed that the highest disruption efficiency was achieved with laser treatment (96.53%), followed by microwave treatment (94.92%).
However, the number and scope of these studies are limited and further investigations, especially in the continuous system, are required in order to examine the potential applications of this cell disruption method.

2.17.Isotonic extraction method

Use of ionic liquid for algal lipid extraction is an innovative and emerging alternative treatment technology. Intensive studies on ionic liquid extraction in microbes have been carried out by various researchers and it is more bio-attuned regardless of detailed procedures. The idea is to replace toxic organic solvents with ionic liquids, the so called “green” designer solvent. Ionic liquids are non-aqueous solution of salts that could be maintained at liquid state at moderate temperatures ranging between 0 and 140°C. They are composed of a large asymmetric organic cation and an inorganic or organic anion. These ionic liquids allow synthetic flexibility by the distinct combination of the anion and cation so that one can design the solvent’s specific polarity, hydrophobicity, conductivity, and solubility according to needs. However, only few studies have been performed on microalgal species such as Chlorella vulgaris to extract lipid through eco-friendly ionic liquid extraction method. The economic and technical viability has not been worked out so far and it is too early to predict that this method is one of the better methods for algal oil extraction. However, this method appears to be promising, as it can be an eco-friendly alternative for organic solvents.

2.18.Acid-catalyzed hot water

Hot water treatment is a well-known technique to disintegrate the crystalline nature of cellulosic biomass.
Hot water requires high pressure at an elevated temperature in order to remain in liquid form.
It is to be noted that this treatment under acidic conditions is applicable for the extraction of lipids from biomass in wet condition ensuring the cost-effectiveness since no extra energy input is required for dewatering processes. During acid-catalyzed hot-water treatment, cells are disrupted along with the degradation of other cellular components, leading to excess acid loading and devaluation of co-products.
In a study lipids with high free fatty acid content were extracted from C. vulgaris by using acid-catalyzed hot-water treatment and the anionic surfactant sodium dodecyl benzene sulfonate (SDBS). The lipid extraction yield was 266.0 mg/g of cell weight from a total fatty acid content of 296.0 mg/g of cell weight, when the concentration of sulfuric acid and SDBS were 2.0% and 0.2%, respectively.

2.19.Enzyme-assisted extraction

The extraction of lipids using enzymatic treatment completely depends on the cell wall characteristics of the subjected oleaginous microorganism. This technique includes various cell wall degrading enzymes, such as xylanase, cellulase, amylase, papain, pectinase, and hemicellulase.

Enzymatic treatment is a well-known technique in the vegetable oil industry to degrade the structural polysaccharides of the cell wall of oily seeds. It constitutes a favourable cell disintegration method due to its specificity and mild operating temperature, as well as its low time and energy requirements. Furthermore, the method is devoid of harmful solvents and harsh physical conditions, such as shear forces.

Moreover, this method has also been applied to extract lipids from oleaginous microalgae. The microalga C. vulgaris was treated with cellulases for 72 h and the hydrolysis efficiency of the cell wall carbohydrates was 85.3%.

After enzymatic hydrolysis, the lipid extraction efficiency by solvent extraction was higher than without hydrolysis.

In another study, the oleaginous microalga Scenedesmus sp. was treated with various enzymes, such as cellulase, xylanase, and pectinase under varying conditions, including enzyme concentration, temperature, pH, and incubation time. The results demonstrated that the combination of cellulase, xylanase, and pectinase for 190 min improved the lipid extraction yields by 96.4% when compared to the untreated microalga.

Another oleaginous marine microalga, Nannochloropsis sp., was treated with cellulase and mannanase and the results revealed the improvement of lipid extraction yields from 40.8% to over 73%. Treatment of the same microalga with similar enzymes under different conditions significantly improved the recovery of lipids from Nannochloropsis sp. biomass. Hence, enzymatic treatment can improve the lipid extraction from various oleaginous microorganisms and scaling up the process is relatively easy. However, long processing times and high capital costs hinder the scale-up of enzymatic treatment for lipid extraction in the biorefineries.

2.20.Supercritical fluid extraction

Although supercritical fluid extraction (SFE) has been known of for more than 100 years for its solvent and abilities; however, its commercial application was not feasible due to the lack of focused research in this area. Incessant research in the field of extraction evolved this technique to a new level with lipid extraction from microalgae in the presence of supercritical fluids like ethylene, ethane, methanol, benzene, ethanol, toluene, CO_2, and water.

The basic principle behind SFE is to achieve a condition in which the meniscus separating the liquid and vapor phases disappears,which is obtained beyond the critical point of a fluid. Among the different choices of supercritical fluids, CO_2 is gaining increasing attention in the extraction of pharmaceutical and health-related products. Due to the non-polar nature of CO_2, this shows high selectivity towards neutral lipids, especially TAGs, which together with its inability to solubilize phospholipids, makes the process highly specific towards TAGs. Along with these properties, low critical temperature (31.1°C) of SFE provides an excellent solution to extract thermally sensitive lipids avoiding thermal degradation; however, supercritical CO_2 (SC-CO_2) extracted products also contain a small amount of impurities of free fatty acids, sterol, and pigments. The efficiency of the supercritical CO_2 extraction processes depends on the chosen operating conditions, in which different conditions of several physical parameters such as pressure (20–60MPa), temperature (303.15–333.15K), and CO_2 flow rate (0.06–30g/min) have been tested, and it has been suggested that up to 100% extraction yield can be achieved only by increasing the pressure at a constant temperature. Often the extraction efficiency can present variations between dried and wet microalgal biomass and it has been proposed that high extraction yield can be obtained with biomass of low moisture content, hence drying of biomass is required prior to SC-CO_2 extraction to enhance the lipid yield. As such, this technique can serve as a suitable alternative for the extraction technique.

The incredible properties of supercritical CO_2 eventually increase the levels of lipid extraction compared to other conventional methods. This process overcomes one of the biggest disadvantages of other methods, which is the degradation of extracts, by providing a non-oxidizing environment and the low critical temperature (around 31°C) also prevents the thermal degradation of extract. Other advantages include non-toxicity, simple and easy downstream process as a result of the easy separation of CO_2, and the high diffusivity and low surface tension increase the penetration of pores that are too small for chemical solvents. SFE can be a promising technique with several distinct properties:

(1) Prior discussion highlights that SFE involves different parameters like temperature and pressure that are easy to modify and provide high selectivity, which allows easy extraction of complex samples.

(2) Extraction and quantification process of highly volatile compounds is another advantage of SFE, which becomes possible due to the direct coupling with a chromatographic method.

(3) It can be applied on a wide range of sample amounts ranging from a few grams to kilograms and even up to tons, which is very helpful for both lab and pilot levels of production.

(4) Quick and selective extraction reduces the separation cost.

(5) SFE is the latest technique to provide more information about extraction and purification. One can use this information for optimization purposes and to evaluate the extraction efficiency.

(6) Low viscosity and high diffusivity enhance porosity, which enables penetration into solid material very efficiently, resulting in faster, quantitative, and complete extraction.

(7) Proper elimination of polluting organic solvents and flexibility of process encourages its worldwide application.

(8) Supercritical CO_2 is more advantageous as compared to other organic solvents, it is considered as a safe and non-flammable solvent.

(9) SC-CO_2 demonstrates enormous capabilities to separate less volatile and high molecular weight compounds; as pressure rises, more polar molecules also can be separated.

(10) Use of CO_2 as compared to other solvents makes the extraction cheaper because of the reusable properties of CO_2. Its easy availability and contamination-free products are the major advantages of the process.

A list of microalgal species that have been tested for lipids extraction with SC-CO_2 under optimal conditions and their advantages and disadvantages are listed in Table 15.

Tang et al. isolated lipids from the microalga Schizochytrium limacinum by using SC-CO_2 and under the optimum pressure (35MPa) and temperature (40°C) conditions; 33.9% of lipid yield was achieved while high-purity of docosahexenoic acid (DHA) was processed by a urea complexation method.

In another study, it was revealed that bead milling of microbial biomass enhances the lipid extraction from Chlorella vulgaris when using SC-CO_2. The lipids and pigments from the oleaginous microalga Nannochloropsis sp. were extracted with SC-CO_2 at the optimized conditions of pressure and temperature, while ethanol was used as co-solvent.

It was reported that a maximum of 45 g of lipids per 100g dry microalgal biomass and 70% of the total pigment can be extracted after optimized condition by this method.

In a study, various extraction techniques were evaluated for the fatty profiles of marine microalga Tetraselmis sp. M8, where it was reported that SC extraction method was the most effective method among all tested techniques for the lipid extraction especially for long-chain unsaturated fatty acids.

Solana et al. compared three microalgae, Scenedesmus obliquus, Chlorella protothecoides, and Nannochloropsis salina for the oil rich in α-linolenic acid as essential fatty acids by supercritical fluid extraction. The highest extraction yield was reported at 60°C and 30MPa with 0.4 kg/h of CO_2 and

5% of ethanol as co-solvent. Among all tested microalgae strains, Scenedesmus obliquus showed the highest amount of total ω-3 fatty acid and α-linolenic acid compared to the other species.
Andrich et al. used supercritical CO_2 for the extraction of bioactive lipids with a high proportion of polyunsaturated fatty acids (PUFA) from unicellular microalga Nannocloropsis sp.
SFE is one of the oldest methods for lipid extraction and, on the analytical and preparative scale, it is one of the most widely used techniques, which enables the efficient lipid recovery from microalgae.
SFE is an excellent method to overcome these problems as inactive CO_2 does not interact with the desired product and, due to its gaseous nature, it is easy to separate, allowing a good product quality.

Table 15

Microalgae species	SC-CO_2 condition for lipid extraction e.g. pressure (MPa), temp (°C), CO_2 flow rate and/or co-solvents	Lipid concentration, LC (%, w/w) or extraction yield, EY (%)	Remarks
Chlorella protothecoides	30MPa, 70°C	LC, 21%	High efficiency
Nannochloropsis	55MPa, 55°C	LC, 44%	CO2 is highly selective and no chance of polar substances forming polymers exists
Hypnea charoides	37.9MPa, 50°C	LC, 6.7%	
Chlorella vulgaris	35MPa, 55°C	LC, 13%	Recycling of CO_2 minimizing waste generation
Crypthecodinium cohnii	30.0MPa, 49.85°C	LC, 50%	Selective extraction of the specific compound; Energyintensive due to use of high pressure
Botryococcus braunii	25MPa, 50°C	LC, 17.6%	Ideal technique to study thermally labile compounds
Arthrospira (Spirulina) maxima	25MPa, 49.95°C	LC, 40%	
Chlorella vulgaris	35.0MPa, 54.95°C	LC, 52%	

Chapter 3

3.1.Characterisation of the methods for the lipid conversion

The oil extraction step from algae includes cell disruption by mechanical, chemical, or biological methods and then the conversion of lipids into biofuel. The techniques generally used for the conversion of lipids are transesterification and the thermochemical methods.

3.2.Transesterification processes

Several studies have been conducted using dry microalgal biomass to investigate direct transesterification processes. Dry biomass reacts with sulfuric acid and methanol. Methanol acts both as an extraction solvent and an esterification reagent. The use of an additional solvent such as hexane or chloroform helps the easy extraction of oils within microalgal cells and enhances the contact of microalgal oils with the esterification reagent.

Sathish et al. (2014) discussed the effects of water inhibition on direct transesterification and concluded that: **(1)** the formation of fatty acid methyl ester (FAME) is a reversible reaction and water can hydrolyze biodiesel back to methanol and free fatty acids, **(2)** water contained within the biomass can shield oils from the extracted solvent and prevent oils from being brought into the reaction, and **(3)** the acid catalyst can be deactivated owing to water competing for available protons in the reaction. Although water inhibits the transesterification of wet microalgal biomass, an economic process using wet biomass should be developed.

Several wet oil extraction technologies have been developed recently and these methods can be combined with a direct process to overcome water inhibition. Transesterification of Chlorella sp. and N. oculata was performed at moisture contents of 0%, 1.5%, and 10% using acid and alkaline catalysts (sulfuric acid, sodium hydroxide, and sodium methoxide).

Sulfuric acid as a catalyst showed a higher FAME yield of 73% for N. oculata and 92% for Chlorella sp., and the yield was not dependent on the salinity of the biomass and decreased with the increase of moisture. Direct transesterification of mixed microalgal biomass for biodiesel production was reported with an increased biodiesel yield obtained because of esterification of fatty acids from membrane phospholipids as well as transesterification of triglycerides. Biodiesel conversion yield was proportional to methanol loading and inversely proportional to water content.

Im et al. (2014) reported direct transesterification of wet microalgae, N. oceanica, with a 65% moisture content and obtained a high conversion yield of 91.1% under 0.2 g cell, 0.3 g H_2SO_4, 2 ml chloroform, and 1 ml methanol at 95 °C for 90 min. To enhance the yield, a chloroform–methanol mixture (2:1, v/v) was applied as a solvent and reaction reagent.

Cao et al. (2013) carried out direct biodiesel production from C. pyrenoidosa (90% moisture content).

Hexane was used as an additional solvent and the maximum biodiesel yield was 92.5% under 0.1 g cell, 0.5 M H_2SO_4, 8 ml hexane, and 4 ml methanol at 120 °C for 180 min.

For direct transesterification, a high level of methanol and sulfuric acid is required compared with a commercial biodiesel process.

The amount of methanol and sulfuric acid should be reduced to avoid the need for a large reactor and reactor corrosion by sulfuric acid. Solvents such as pentane and diethyl ether have been used to reduce the volume of methanol by enhancing the reaction yield. These solvents assist in the extraction of microalgal oils in conjugation with methanol by improving the diffusion of the microalgal oils across the cell walls. This is facilitated by increasing the selectivity and solubility of

the extraction media, thereby providing greater availability of the oils for the transesterification process. In a one-step reaction using S. limacinum (80% moisture content), chloroform (4 ml)-methanol (3.4 ml) showed a higher biodiesel yield compared with methanol (7.4 ml) only (Johnson and Wen, 2009). Although this indicates the possibility of reducing the amount of methanol, further development is necessary to reduce the amount of solvent. Through a combination of sonication and co-solvent using Chlorella sp., the molar ratio of oil to methanol decreased markedly. After 2 h ultrasound agitation, the FAME conversion yield was the same at a molar ratio of 1:105 as at 1:210 and 1:315. After 8 h mechanical stirring with diethyl ether, there was a good conversion yield at a molar ratio of 1:79. After 2 h with a combination of the above two methods, there was a conversion yield of 99.9% at a molar ratio of 1:52 (Ehimen et al., 2012).

To enhance the conversion yield of direct transesterification, the application of microwave or ultrasound that can enhance the mass transfer rate between immiscible phases, simultaneously diminishing the reaction time, was suggested (Hidalgo et al., 2013). In microwave-assisted transesterification, methanol absorbs microwave radiation, quickly redirecting its dipole. This rearrangement allows the destruction of the methanol-oils interface. The microwaves transfer energy in an electromagnetic form, and the oscillating microwave field tends to move continuously to the polar ends of molecules or ions. Consequently collisions and friction between the moving molecules generate heat. Heat is transferred directly into the reaction media with a rapid temperature increase throughout the sample (Hidalgo et al., 2013). Rapid heating leads to localized high temperature and pressure gradients, which assist in cellular wall degradation and enhance mass transfer rates (Patil et al., 2011a). Microwave with 800 W power was applied to Nannochloropsis sp. and a FAME conversion yield of 80.1% was obtained (Patil et al., 2011a). C. pyrenoidosa (moisture content 80%) was mixed with methanol, chloroform, and sulfuric acid; and the biodiesel production yield through a one-step process using microwave were 6-fold and 1.3-fold higher respectively than with a two-step process using conventional heating (Cheng et al., 2013). Ultrasound is an effective method to enhance the mass transfer rate between immiscible phases (Hidalgo et al., 2013); therefore, it improves transesterification yields and reduces reaction times.

The oil-alcohol phase boundary is disrupted because of the collapse of ultrasonically induced cavitation bubbles (Ehimen et al.,2012). The cavitation bubbles produced by ultrasound also attack the microalgal cell walls enhancing oil extraction from cells.

Through a combination of sonication and co-solvent using Chlorella sp., the FAME conversion yield reached 99.9% (Ehimen et al., 2012).

Although these processes enhance the FAME yield or reaction rate, it is still necessary to decrease the costs of these technologies including equipment installation.

To enhance the transesterification yield, the application of supercritical conditions was suggested (Patil et al., 2011b). Supercritical methanol without catalyst and with additional solvent produced FAME from Nannochloropsis sp. (moisture content 90%) with the conversion yield increased to 85.8% and polar phospholipids were converted to FAME as well as free fatty acid and triglyceride (Patil et al., 2011b). In another approach, it was shown that when triglycerides were hydrolyzed to free fatty acids by an anionic surfactant, SDBS, at low pH during the microalgal oil extraction process, the amount of sulfuric acid used as an esterification catalyst could be reduced markedly (Park et al., 2014b). For direct transesterification, the application of a surfactant as an additive can reduce the dosage of sulfuric acid.

Some pretreatments, except for conventional oil extraction using solvents, were performed before direct transesterification to enhance the conversion yield. After cell walls of C. vulgaris ESP-31 (moisture content 86–91%) were disrupted by sonication, direct enzymatic transesterification was performed with methanol, hexane, and immobilized lipase and the yield of FAME increased to 95.7% (Tran et al., 2013). Levine et al. (2010) applied a supercritical method to the system: first, C. vulgaris

(80% moisture content) was hydrolyzed by supercritical water at 250 °C; second, supercritical direct transesterification was performed using ethanol without catalyst; and, at 325 °C, the biodiesel yield increased to 100%.

In addition, wet microalgal biomass was pretreated with a short chain alcohol in order to remove excess water, which inhibited the transesterification. During the second step, an alcohol and a catalyst were added to the pretreated biomass for esterification at mild conditions less than 120 °C. Water was removed from the alcohols used in both steps, after which both the solvent and catalyst could be reused (Yoo et al., 2012, 2014).

In most studies, the transesterification catalyst was sulfuric acid because microalgal oils contain high free fatty acid content.

Koberg et al. (2011) used SrO as a solid alkaline catalyst with Nannochloropsis for biodiesel production and showed a high biodiesel conversion yield. Patil et al. (2011a) also used an alkaline catalyst, potassium hydroxide (KOH), for direct transesterification of Nannochloropsis sp., and the FAME conversion yield was 80.1% with 2% catalyst. Free fatty acids form soap with alkaline catalysts, and this makes difficult the separation of biodiesel and alcohol layers. Consequently, acid catalysts are typically used for microalgal oil that contains at least 1% free fatty acid (Huerga et al., 2014); however, alkaline catalysts may be suitable for the direct transesterification of microalgae because oils structured in microalgae are mostly triglyceride form.

Since the majority of the research results are derived from small laboratory scale experiments and from batch mode reactions rather than continuous process, it is rather difficult to calculate and compare the economics of various procedures. However, it is clear that the energy requirement is still substantial for the majority of the processes that employ dry biomass.

Lardon et al. (2009) reported an analysis of the potential impact of biodiesel production from microalgae. They compare nominal fertilizing or nitrogen starvation and dry or wet extraction.

For C. vulgaris, by nitrogen starvation, oil content increased to 38.5% from 17.5% although the growth rate decreased. Wet oil extraction significantly reduces heat requirements, but the lower extraction yields erode slightly the benefit of this technique. The total energy consumptions for 1 kg biodiesel production were 106.4, 41.4, 48.9, and 19.9 MJ for normal-dry, normal-wet, low N-dry, and low N-wet respectively. Net energy balances were -2.6, 105, 12, and 66 MJ for normal-dry, normal-wet, low N-dry, and low N-wet respectively; therefore, wet extraction is the desirable direction for commercialization. If the advanced wet oil extraction methods mentioned above are used in the analysis, there will be a remarkable increase in net energy due to the increased oil extraction yield from wet biomass.

Xu et al. (2011) compared the energy balance of the dry and wet routes for biofuel production from microalgae. The drying process in the dry route and the oil extraction process in the wet route consume a significant amount of energy. The analytical results indicate that the wet route has more potential for producing biofuel. Sills et al. (2013) also agreed with the need for wet oil extraction. For economic feasibility, they suggested the recovery of nutrients from waste streams and the production of high energy co-products such as methane derived by anaerobic digestion.

In the following study, the direct transesterification approach using micro-scale samples of microalgal biomass and fresh microalgal cells without dehydration is investigated in detail. The effects of temperature, catalyst concentration, and water content on the transesterification efficiency were investigated using eight species of microalgae.

Chromatographic grade nhexane and methanol were purchased from J&K Scientific Ltd. (China).

Chloroform and sulfuric acid (purity, 95–98%) was analytical grade and obtained from Tianjin Kemiou Chemical Reagent Co., Ltd. (China). Methyl heptadecanoate (C17-ME) and glyceryl trioleate (C18-TAG) were purchased from Sigma–Aldrich (USA).

Microalgal cells were harvested by centrifugation at 4000 rpm for 10 min once reaching a stationary phase, except for Spirulina, which were harvested by two centrifugation steps. After centrifugation, the fresh microalgal cells, which are referred to as microalgal paste, were obtained by discarding the supernatant.

Microalgal pastes from marine species and fresh water species were washed twice with 0.5 mol L^{-1} NH_4HCO_3 or distilled water respectively, and dried at 60 °C until the weight of samples remained constant. Samples were then ground into a powder.

Total lipids from 100 mg microalgae were extracted using 2 mL chloroform/methanol (v/v: 2/1) (Bligh and Dyer, 1959), ultrasonic treatment for 10 min and centrifugation at 4000 rpm for 5 min. The supernatants were then collected into pre-weighted centrifuge tubes. This process was repeated three times. The collected supernatants were dried under nitrogen flow and then at 60 °C until the weight of samples remained constant. The samples were weighed and moved to 10 mL flasks. Then, 5mL H_2SO_4–methanol (v/v H_2SO_4/methanol) was added, and the flask was stirred at a specific temperature for a specific amount of time with refluxing. After the specific time period, the flask was cooled to room temperature.

Next, 2 mL of hexane and 0.75 mL of distilled water were added to the flask and mixed for 30s on a vortex mixer. The mixture formed two phases, and the upper hexane layer contained the fatty acid methyl esters (FAMEs). The hexane layer was transferred to a new vial and mixed with the internal standard C17-ME for analysis by gas chromatography (GC).

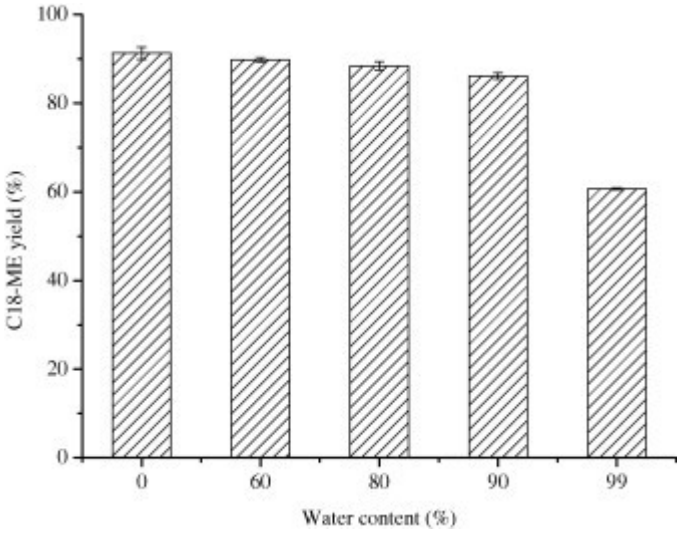

Figure 13

Micro-scale samples for transesterification were prepared following a different procedure from that described above. After adding hexane and distilled water and vortex mixing the solution for 30 s, precisely 1.5 mL of the upper layer was transferred to a 2 mL vial and dried completely under a nitrogen flow. The FAMEs were re-dissolved in 120 µl of hexane for GC analysis.

FAME analyses were carried out by an Agilent 6890 GC instrument was equipped with a flame-ionization detector (FID) and a DB-23 capillary column (Agilent Technologies, USA, 30 m × 0.32 mm × 0.25 µm). FAME yield was calculated using the following equation:

FAME Yield(%) = (FAME mass)/(oil mass) × 100%

All the measurements of the values used in the tables and figures represent the average ± SD of four individual replicates during the whole experiment.

The effects of temperature, reaction time, and concentration of H_2SO_4 on the transesterification efficiency were studied using a substrate of C18-TAG standard. The optimal transesterification conditions were determined to be a temperature of 70 °C, a catalyst concentration of 2% H_2SO_4 in methanol, and a reaction time of 1 h.

In consideration of the effect of the volume of methanol on the conversion of TAG, the volume of methanol was in large excess and thus did not have effect on the transesterification in the present study. These conditions were applied in subsequent experiments.

Transesterification of extracted microalgal oil, 5 mg samples of dry cells, and micro-scale samples of dry cells was carried out.

According to our experience in the analysis of microalgal fatty acids, the ±10% variation in the percentage of each fatty acid is acceptable. The oil samples, 5 mg samples of dry cells, and micro-

Table 16

Fatty acid	Extracted lipid	5 mg dry cells	300 μg dry cells	Fresh cells (paste)	±10% variation range
C14:0	16.4 ± 0.1	16.2 ± 0.1	15.0 ± 0.4	16.0 ± 0.3	14.6–17.8
C16:0	10.4 ± 0.2	11.0 ± 0.2	11.3 ± 0.3	11.2 ± 0.3	9.9–12.1
C16:1n7	8.3 ± 0.1	8.3 ± 0.2	8.1 ± 0.1	8.1 ± 0.1	7.5–9.1
C18:1n9	10.1 ± 0.2	9.6 ± 0.1	8.9 ± 0.1	10.0 ± 0.1	8.6–10.6
C18:2n6	9.7 ± 0	9.6 ± 0.2	9.6 ± 0.2	9.6 ± 0.1	8.6–10.6
C18:3n3	15.4 ± 0.1	15.5 ± 0.4	15.2 ± 0.3	15.3 ± 0.2	13.9–17.1
C18:4n3	15.3 ± 0.2	14.8 ± 0.1	15.7 ± 0.3	14.8 ± 0.1	13.3–16.3
C18:5n3	2.8 ± 0.1	3.1 ± 1.1	3.4 ± 0.4	2.3 ± 0.1	2.8–3.4
C20:5n3	2.9 ± 0	2.8 ± 0.3	2.6 ± 0.3	3.2 ± 0.4	2.5–3.1
C22:6n3	8.8 ± 0.1	9.2 ± 0.3	10.1 ± 0.3	9.5 ± 0.2	8.3–10.1
Total	100	100	100	100	100

scale samples of dry cells were prepared from Isochrysis zhangjiangensis biomass. The results obtained for the oil samples, 5 mg samples, and micro-scale samples of 300 μg are shown in Table 16. Relative fatty acid percentages within ±10% variability were obtained from all three microalgal forms. However, 300 μg of dry I. zhangjiangensis cells was found to be the minimum quantity required for this approach. When the amount of dry cells was less 300 μg, the relative fatty acid percentages varied dramatically, and the analysis results differed greatly from the results obtained with samples containing more than 300 μg of dry cells. Thus, a sample of less than 300 μg led to non-negligible whole process error, including error related to the fatty acid content of the specific microalgal species.

The sensitivity of transesterification to water was evaluated by adding different amounts of water to the system for TAG transesterification. Figure 13 shows that the yield of C18-ME decreased with increasing water content. The yield was 91% when no water was added to the TAG feed. With TAG samples containing 80% water, the yield was reduced to 88%, and with TAG samples containing 90% water, the yield was further reduced to 86%, which was still 94% of the yield achieved with no water added. The yield of FAMEs decreased sharply to 61% with a 99% water content in the TAG samples. These results demonstrate that transesterification of TAG proceeds under the conditions used in this study with a sample water content less than 90% is accredited. As shown in Figure 13, with TAG samples containing no more than 90% water, the transesterification conditions used in this study provided yields of greater than 85%, which are considered acceptable transesterification yields.

The water content of microalgal paste obtained after centrifugation was within 60–80% for all species except Spirulina, for which a water content of 90% was achieved by filtration in the study. Therefore, typical microalgal paste samples meet the moisture content requirements for direct transesterification without drying.

The feasibility and efficiency of direct transesterification of dry microalgal cells and cells without dehydration were investigated and compared. The fatty acid profiles and compositions determined by transesterification of microalgal paste are presented in Table 16. The results achieved by transesterification of microalgal cells without dehydration were in agreement with those obtained for dry microalgal cells.

In addition, the yield of FAMEs per milligram of equivalent dry microalgal biomass from microalgal paste reached 94% of that obtained from dry microalgal cells.

The yield of C18-ME achieved with a TAG sample water content of 90% also reached 94% of that obtained for TAG without water.

Direct transesterification was applied to I. zhangjiangensis and an additional seven species of microalgae, namely, Nannochloropsis, Tetraselmis subcordiformis, Arthrospira (Spirulina) platensis, Dunaliella salina, Chlamydomonas reinhartii, Synechocystis sp. 6803, and Chlorella pyrenoidosa. The amounts of FAMEs per milligram of equivalent dry microalgal biomass and the fatty acid profiles and compositions of the microalgal pastes and powders of each microalgal species determined by

transesterification are shown in Table 17. The amount of FAMEs per milligram of equivalent dry microalgal biomass obtained from microalgal paste was, on average, 94% of that obtained from dry microalgal cells.

In the fatty acid profiles of paste samples of all eight microalgal species, the percentages of each fatty acid are comparable to those obtained from the corresponding dry cell samples based on the criteria of ±10% variation described above. In exception, both the deviations of C20:5n3 percentage in T. subcordiformis and C18:0 in A. platensis are larger than 10%, partly because their percentages are so small that a tiny change will bring a huge deviation.

In addition, C20:5n3 in T. subcordiformis and C18:0 in A. platensis make up only a small portion of the whole fatty acids (less than 5%) and are not the major ingredient. Therefore the method of direct transesterification of microalgal paste is reliable.

Table 17

Fatty acid	Nannochloropsis			Tetraselmis subcordiformis			Arthrospira platensis		
	Fresh	Dry	Deviation	Fresh	Dry	Deviation	Fresh	Dry	Deviation
C14:0	5.5 ± 0	5.4 ± 0	1.9						
C16:0	34.6 ± 0.2	34.8 ± 0	-0.6	23.6 ± 0.3	23.2 ± 0.1	1.7	39.3 ± 0.1	40.8 ± 0.1	-3.7
C16:1n7	27.8 ± 0.2	27.6 ± 0	0.7				4.9 ± 0.4	4.7 ± 0	4.3
C16:3n4									
C16:4n4				16.9 ± 0.3	16.9 ± 0.1	0.0			
C18:0	1.4 ± 0.1	1.3 ± 0	7.7				2 ± 0	1.7 ± 0.1	17.6
C18:1n9	7.6 ± 0.2	7.8 ± 0	-2.6	6.2 ± 0	6.7 ± 0.3	-7.5	5.5 ± 0	5.3 ± 0.2	3.8
C18:1n7				4.8 ± 0	4.7 ± 0.4	2.1			
C18:2n6	1.6 ± 0	1.6 ± 0	0.0	10.2 ± 0.1	9.9 ± 0.1	3.0	19.8 ± 0.2	18.6 ± 0.1	6.5
C18:3n6							28.6 ± 0.3	29 ± 0.1	-1.4
C18:3n3				29.7 ± 0.1	28.7 ± 0.1	3.5			
C18:4n3				4.9 ± 0	4.8 ± 0	2.1			
C18:5n3									
C20:4n6	4.4 ± 0.1	4.5 ± 0	-2.2						
C20:5n3	17 ± 0.4	16.9 ± 0	0.6	3.8 ± 0.2	4.9 ± 0	-22.4			
C22:6n3									
Total	100	100		100	100		100	100	
FAME (μg/mg)	9.5 ± 0.3	9.8 ± 0.2		9.5 ± 0.3	9.8 ± 0.2		2.3 ± 0.3	2.6 ± 0.2	

Fatty acid	Dunaliella salina			Chlamydomonas reinhartii		
	Fresh	Dry	Deviation	Fresh	Dry	Deviation
C14:0						
C16:0	24.1 ± 0.4	22.5 ± 0.3	7.1	26.5 ± 0.3	27.9 ± 0.1	-5.0
C16:1n7				1.0 ± 0	1.0 ± 0	0.0
C16:3n4	3.7 ± 0.1	3.9 ± 0.1	-5.1	2.0 ± 0	2.0 ± 0	0.0
C16:4n4	11.8 ± 0.2	13 ± 0.3	-9.2	11.7 ± 0.1	10.9 ± 0.1	7.3
C18:0				3.1 ± 0	3.3 ± 0	-6.1
C18:1n9	12.5 ± 0.4	12.9 ± 0.2	-3.1	10.1 ± 0.5	9.7 ± 0.1	4.1
C18:1n7				5.2 ± 0.1	5.3 ± 0	-1.9
C18:2n6	17.0 ± 0.1	16.4 ± 0.1	3.7	15.4 ± 0.1	15.7 ± 0.1	-1.9
C18:3n6						
C18:3n3	30.9 ± 0.4	31.2 ± 0.1	-1.0	24.9 ± 0.1	24.3 ± 0.1	2.5
C18:4n3						
C18:5n3						
C20:4n6						
C20:5n3						
C22:6n3						
Total	100	100		100	100	
FAME (μg/mg)	3.9 ± 0.2	3.9 ± 0.4		5.7 ± 0.5	6.1 ± 0.4	

Fatty acid	Synechocystis sp.6803			Chlorella pyrenoidosa		
	Fresh	Dry	Deviation	Fresh	Dry	Deviation
C14:0				0.5 ± 0.2	0.5 ± 0	0.0
C16:0	49.3 ± 0.2	50.9 ± 0	_3.1	22.7 ± 0.2	23.2 ± 0.1	_2.2
C16:1n7	6.4 ± 0	6.8 ± 0.2	_5.9	1.7 ± 0	1.6 ± 0	6.3
C16:3n4				10.4 ± 0	9.7 ± 0	7.2
C16:4n4				2.7 ± 0	2.5 ± 0	8.0
C18:0				1.9 ± 0	2.0 ± 0.1	_5.0
C18:1n9				9.2 ± 0.1	9.9 ± 0.2	_7.1
C18:1n7				2.6 ± 0.1	2.7 ± 0.1	_3.7
C18:2n6	15.6 ± 0	15.2 ± 0.1	2.6	30.5 ± 0.1	30.7 ± 0.1	_0.7
C18:3n6	24.4 ± 0.2	23.1 ± 0.2	5.6			
C18:3n3	4.2 ± 0.1	4.1 ± 0	2.4	17.7 ± 0.1	17.3 ± 0.1	2.3
C18:4n3						
C18:5n3						
C20:4n6						
C20:5n3						
C22:6n3						
Total	100	100		100	100	
FAME (µg/mg)	2.6 ± 0.1	3.1 ± 0.4		6.2 ± 0.5	6.5 ± 0.2	

3.3.Thermochemical methods

Thermochemical methods namely pyrolysis, gasification/hydrothermal gasification, combustion, hydrothermal liquefaction and hydrothermal carbonization are widely practiced to convert algal biomass into fuels. Among the methods, pyrolysis, and hydrothermal liquefaction are most commonly practised to convert numerous algal biomasses into bio-oil and/or biochar to substitute crude oil in petroleum refinery.

3.4.Pyrolysis

Pyrolysis, an emerging thermochemical technique, utilizes numerous and different kinds of biomasses for char or bio-crude production.
Pyrolysis or dry pyrolysis is an endothermic reaction that decomposes the biomass at extremely high temperature
in the absence of oxygen and under atmospheric pressure, resulting in solid (bio-char), liquid (bio-oil) and gaseous (syngas) products.
Apart from the conventional dry pyrolysis process, the pyrolysis technology can also be carried out under subcritical or supercritical conditions. Under these conditions water acts as the reaction medium at higher temperature and pressure, usually above 374 °C and 22.1 MPa respectively. Pyrolysis, in supercritical condition, is much favoured than subcritical condition because of the following reasons: **(i)** water remains polar in subcritical and non-polar in supercritical conditions, and **(ii)** dielectric constant of water in supercritical condition is low. For these reasons, under supercritical condition water behaves as an excellent organic solvent and aid in solubilizing the non-polar organic compounds. At present many technological developments in bio-oil production from several consistent feedstock have emerged to replace the existing fossil

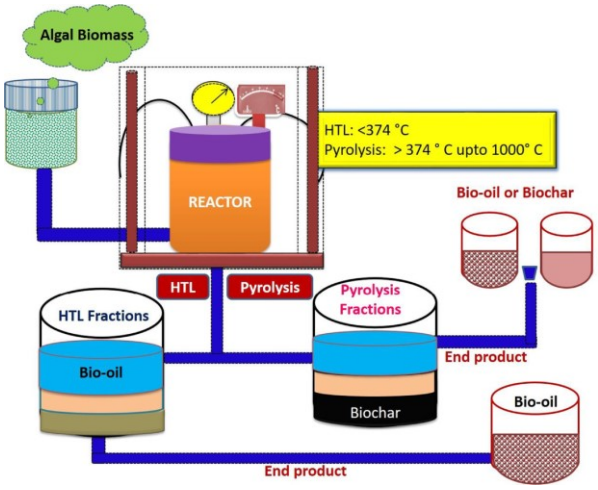

Figure 14

fuels. Aquatic biomass have been well established as the consistent feedstock for biomass conversion by pyrolysis owing to its properties such as, **(i)** lack of complex polymers such as lignin, cellulose, hemicellulose, etc, thereby facilitating the degradation at relatively low temperatures, **(ii)** low O/C ratio leading to high calorific value, **(iii)** low ash content, and **(iv)** less energy consumption ratio. The schematic illustration of typical pyrolysis processing of algae and the pyrolysis end products is given in Figure 14. The liquid product of pyrolysis contains phenolic ethers, alkyl phenolics, heterocyclic ethers, polyaromatic hydrocarbons while, the solid char comprises of carbon, hydrogen, oxygen and gaseous products comprising of CO_2, CO, CH_4 and H_2.

Table 18

Microalgal strains	Temperature (°C)	Bio-oil yield (%)	Atomic ratios of Bio-oil	Biochar yield (%)
Chlorella protothecoides	500	17.5	1.72 (H/C) 0.24 (O/C)	–
Microcystis aeruginosa	500	23.7	1.71 (H/C) 0.27 (O/C)	–
Chlorella protothecoides	500	52	–	–
Spirulina platensis	500	28.5	1.68 (H/C) 0.06 (O/C)	57.29 as Fixed carbon (FC)
Chlorella protothecoides	450	57.9	–	–
Chlorella vulgaris	400	72	–	~22
Chlorella vulgaris	500	53	–	31
Isochrysis sp.	475	41.32	–	–
Tetraselmis sp.	500	25.86	1.56 (H/C) 0.15 (O/C)	~18
Isochrysis sp.	500	25.49	1.55 (H/C) 0.09 (O/C)	~25
Pavlova sp.	500	23.2	–	~34
Spirulina sp.	550	45	–	~31
Scenedesmus dimorphus	500	39.6	1.49 (H/C) 0.08 (O/C)	~27
Isochrysis sp.	475	49.36	–	–
Scenedesmus sp.	480	55	–	15.9 as FC
Microcystis sp.	500	59	0.34 (O/C)	25.6
Desmodesmus sp.	750	42.25	–	–
Scenedesmus sp.	440	41.54	1.68 (H/C) 0.27 (O/C)	–
Macroalgae				
Saccharina japonica	470	37.9	1.4 (H/C) 0.17 (O/C)	–
Porphyra tenera	500	47.4	–	~37
Undaria pinnatifida	500	45.8	–	~26
Saccharina japonica	450	47	–	33
Laminaria digitate	500	17	–	~30
Fucus serratus	500	11	–	~31

Temperature is the fundamental and crucial factor in pyrolysis which ranges from 200 to 1000 °C for complete degradation of biomass and various research works have been undertaken to assess the influence on pyrolysis temperature on bio-oil or solid char yield and quality (Table 18). In consideration with the thermal decomposition of algae, different ranges of temperature favour the stepwise decomposition reactions. For example, temperature below 200 °C is enough for dehydration, which is followed by devolatilization (for example: the actual process of pyrolysis) in the temperature range of 200–550 °C and solid decomposition at above 550 °C. In the dehydration process, water bound moieties are removed from algae and in the devolatilization process, volatile

compounds are removed and biomolecules are decomposed. Carbohydrates and proteins are decomposed at temperature below 400 °C and lipids decompose at 550 °C. At temperature above 550 °C, solid decomposition occurs which involve: **(i)** secondary cracking of larger molecular weight hydrocarbons into smaller molecular weight components which leads to a decrease in the bio-oil content and **(ii)** recombination reaction occurs when the residence time of volatile compounds in the reactor is too long.

Pyrolysis process is classified into different categories: **(i)** slow pyrolysis, **(ii)** fast pyrolysis, **(iii)** flash pyrolysis, and **(iv)** catalytic pyrolysis depending on the operating conditions such as temperature, heating rate, vapor and solid residence times, catalyst types, etc.

In slow pyrolysis, the temperature is gradually increased at a slow rate for the degradation of biomass, typically ranging from 5 to 7 °C/min characterized by long vapor residence times, usually for 10–30 s and the particle size for degradation ranging between 5 and 50mm. However, this process is quite useful for biochar production since the other end products namely liquid and gaseous products are produced at low quantities compared to solid products (char).

Few studies have been reported for slow pyrolysis of microalgal biomass for bio-oil production. For instance, bio-oil yield from Spirulina sp. was 45% when the temperature is maintained at 550 °C for 60 min with a heating rate of 8 °C/min. The energy consumption ratio was observed to be 0.49 which is less than unity indicating a positive net energy output. Similarly, bio-oil yield from Spirulina platensis was 28.5% when the temperature is maintained at 500 °C with a heating rate of 7 °C/min for the total reaction time of 60 min. Energy consumption ratio for the above mentioned operating conditions was found to be 1.56 which is slightly higher than unity, indicating the reaction as net energy consumer.

In fast pyrolysis, temperature is increased at a faster rate in the absence of oxygen leading to fast heating of biomass for thermal degradation.

Rapid heating rate at 300–600 °C/min, a short vapor residence time and the particle size less than 1mm are generally adopted for obtaining high grade bio-oil. Many microalgal species have been reported to yield bio-oil in the range of 17 to 72 wt% from fast pyrolysis process. Microalgal species such as Chlorella vulgaris, Arthrospira platensis, Nannochloropsis oculata, and Schizochytrium limacinum have been examined under fast pyrolysis in the range of 400–700 °C with a heating rate of 80 °C/min. However when considering the bio-oil yield, 18% and 24% were obtained for Chlorella protothecoides and Microcystis aeruginosa from fast pyrolysis with the operating parameters of 500 °C, 600 °C/s heating rate and vapor residence time of 2–3 s.

Harman-ware et al. (2013) reported the bio-oil yield of 55% from fast pyrolysis of Scenedesmus sp. at 500 °C with vapor residence time of 2 s.

In another study, Chlorella vulgaris was reported to yield 53% bio-oil at a pyrolysis temperature of 500 °C.

The biomass composition also poses significant effect on the bio-oil yield which is substantiated by Belotti et al. (2014), where the nitrogen starved C. vulgaris yielded 72% bio-oil at 400 °C with an energy consumption ratio of 0.53 and high heating value. Also, it is noteworthy to mention that, nitrogen upon combustion releases NO_x as atmospheric pollutants and its presence in bio-oil as heteroatomic (N) compounds succumbs the refinery process from direct upgradation.

In the flash and catalytic pyrolysis, as the name suggests, biomass is heated within a fraction of seconds and the process is operated at around 20 Mpa in a hydrogen rich atmosphere, with less residence time in the reactor, heating rate greater than 1000 °C/s, which very rapid than fast pyrolysis and the particle size less than 0.2mm. Flash pyrolysis is generally employed for yielding higher bio-oil content from biomass with a residence time of less than 1 s. Bio-oil yield also depends on the type of reactor vessel. For example, flash pyrolysis of Chlorella vulgaris in an entrained flow reactor yielded 60.22 wt% of bio-oil at the pyrolysis temperature of 800 °C. However, flash pyrolysis

of algal biomass in fluidized bed reactor by CFD modelling is expected to yield 0.27 for bio-oil and 0.53 for non-condensable gases. Similarly, flash pyrolysis of macroalgae in pyrolysis centrifugal reactor yielded 78% energy recovery in bio-oil with a residence time of 0.8 s, at a reaction temperature of 550 °C. Concerning the composition of bio-oil, the presence of high oxygen content in bio-oil creates problematic issues during the production process wherein its direct use in refineries for transportation is not recommended. Therefore to enhance the bio-oil quality with less oxygenic compounds, several catalysts have been used for the pyrolysis of biomass.

3.5.HTl (hydrothermal liquefaction)

HTL is performed at subcritical temperature of water where water acts as a lucrative and environmental friendly reaction medium for the conversion of biomass into fuels. HTL, in general, performed in the temperature and pressure between 280–370 °C and 10–25 MPa, respectively, produces bio-oil and also solid residue (s), gases, and aqueous phase products. The typical HTL processing of algal biomass into various products is illustrated in Figure 15. The key aspect of HTL is the ability to use wet biomass without subjecting it to the drying process. Water as the reaction medium under the above conditions has two major advantages in HTL: **(i)** it initiates the interaction with biomass and aid in breakage of chemical bonds between them, and **(ii)** the ease of separation of bio-oil from other components. HTL entails a series of structural and chemical transformation processes in the water medium, and broadly, it involves three steps; depolymerization, decomposition and recombination. Bio-crude or bio-oil is highly dense: 0.97–1.04 kg L^{-1}, highly viscous: 3.27–330 mPas, and a dark liquid fuel obtained from various biomasses. Biocrude comprises of mainly phenolics, aromatic hydrocarbons, nitrogen containing compounds, amides, fatty acids and esters. With reference to the gaseous products obtained from HTL, hydrogen, carbon dioxide, carbon monoxide, and methane are present in the gaseous products in the percentage of 23.7%, 19.1%, 22.8%, and 25.8%, respectively.

Regarding the experimental reactors or systems for HTL, it is carried out mostly in batch autoclaves (10–100 mL), large batch reactors, and continuous reactors.

Among the different reaction parameters, the physical and chemical features of bio-oil rely on biochemical composition of algae and reaction temperature. In HTL of biomass, the reaction temperature affects the production of bio-crude and its composition as the ionic characteristics of water changes with respect to reactor temperature that accelerates various reactions. The two competitive reactions such as hydrolysis and repolymerization dominate one another based on the temperature regime, for example, hydrolysis dominates repolymerization at low reaction temperature whereas repolymerization takeover or competes for hydrolysis at high temperature.

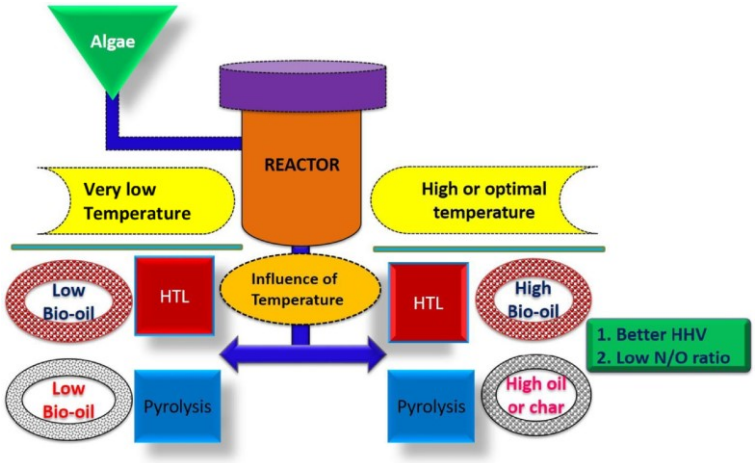

Figure 15

Decarboxylation of amino acids yields amino compounds and carbon dioxide by removing oxygen from the biomass and at the same time, deamination of some amino acids produce carboxylic acids and ammonia by removing the nitrogen from biomass. On the other hand, decarboxylation of fatty acids produces alkanes and alkenes, and the degradation of reducing sugars produce cyclic

oxygenates in the same 100 to 200 °C temperature regime. However, according to another report, lipids are composed of three fatty acids held by a glycerol backbone. When the temperature is increased to values>300 °C, lipids are broken into fatty acids and hydrophilic glycerol and then, the produced fatty acids could be decomposed into hydrocarbons (alkanes).

Table 19

Strains	Temperature (°C)	Bio-oil (%)	Atomic ratios of bio-oil
Chlorella vulgaris	300	29.37	–
Scenedesmus almeriensis	400	60.9	1.516 (H/C) 0.021 (O/C)
Nannochloropsis gaditana	400	51.7	1.622 (H/C) 0.025 (O/C)
Chlorella	350	94.8	–
Nannochloropsis sp.	350	43	1.63 (H/C) 0.089 (O/C) 0.044 (N/C)
Nannochloropsis oceanica	290	54	0.13 (H/C) 0.08 (N/C) 0.15 (O/C)
Galdieria sulphuraria	350	27.5	–
Phaeodactylum tricornutum	350	39	–
Tetraselmis sp.	350	65	1.6 (H/C)
Nannochloropsis sp.	600	66	–
Cyanobacteria sp.	325	21.10	–
Bacillariophyta sp.	325	18.21	–
Arthrospira platensis	350	35	–
Tetraselmis sp.	350	40	–
Nannochloropsis sp.	260	55	–
Chlorella sp.	220	82.9	–
Arthrospira platensis	310	45	–
Chlorella sp.	350	27.3	–
Nannochloropsis sp.	250	20.2	1.68 (H/C) 0.11 (O/C) 0.061 (N/C)
Nannochloropsis sp.	350	48.67	–
Pavlova sp.	350	47.05	–
Dunaliella tertiolecta	360	25.8	–
Spirulina sp.	260	43.05	–
Nannochloropsis salina	350	46	–
Nannochloropsis sp.	300	48.23	–
Enteromorpha prolifera	290	28.4	–
Chlorella pyrenoidosa	280	50	–
Chlorella pyrenoidosa	280	57.3	–
Macroalgae			
Oedogonium sp.	350	26.2	–
Derbesia tenuissima	350	19.7	–
Enteromorpha prolifera	300	23.0	1.43 (H/C)
Mixed culture algal biomass	300	50	0.050 (N/C) 0.30 (O/C)

As described by Kumar & Gupta, at ambient temperature, water is a polar solvent with a boundless network of H-bonds and it does not solubilize organic compounds. When the temperature is increased to the vicinity of the subcritical zone, the H-bonds of the water start weakening; the ionization constant (kW) of water begins to increase at about 3 orders of magnitude higher than the water at ambient temperature. Hence, acidic hydronium ions (H_3O^+) and basic hydroxide ions (OH^-) are usually dissociated, which makes subcritical water an acidic medium for hydrolyzing biomass. On the other hand, low dielectric constant allows the solubility of certain salts considerably, for example: the dissolution of NaCl is higher in subcritical water followed by Na_2SO_4. The amide compounds are produced from the long chain fatty acids by replacing their hydroxyl groups by

ammonia (from deamination of amino acids), and esters are produced from the reaction between the fatty acids with the alcohols (from the reduction of an organic acid). Besides, N&O-heterocyclic compounds such as pyrrole, pyridine, pyrrolidinone, pyrrolidinedione, imidazole, thiazole, and their derivatives are formed from the Maillard reaction between amino acids, and reducing sugars and further, certain nitrogen and oxygen heterocyclic compounds might react with long chain fatty acids to form pyrrolidinone derivatives of fatty acids.

There are several research works that have discussed the impact of reaction temperature on the yield and composition of bio-oil (Table 19).

HTL reaction temperature of 300 °C was found to yield higher biocrude from Nannochloropsis, at about 48.23% with 89.28% HTL conversion efficiency in the presence of Ni/TiO_2 catalyst. In another recent investigation, 300 °C was identified to be optimal and produced 29.37% bio-oil from Chlorella vulgaris with other conditions such as 60 min, 3% nano ZnO catalyst and 15 g/200 mL biomass loading.

During CO_2-HTL, the CO_2 addition elicits the hydrolysis rate of biomolecules owing to the formation of carbonic acid, which acts as an acid catalyst. Further, rapid heating of reactor is preferable to avert the depolymerization reaction and the degradation of certain biopolymers prior to the reach suitable temperature. For example, the hydrolysis of cellulose is tenfold higher in the vicinity of 240 and 310 °C, and specifically at 280 °C, 2 min residence time, 100% cellulose conversion was observed. However, better decomposition of glucose was observed at 250 and 270 °C. In Scenedesmus almeriensis, the higher bio-oil content of 60.9% was observed at 400 °C HTL temperature with H/C and O/C ratios of 1.516 and 0.021 respectively.

In a very recent study, the effect of temperature, time, and catalyst concentration of HTL process on the yield and composition of bio-oil from Spirulina was evaluated and a reaction temperature of 260 °C increased the bio-oil yield up to 43.05% in 30 min time and 5% catalyst with 27.28–36.01 MJ/kg higher heating value (HHV).

In addition to the exploitation of microalgal species for bio-oil production, six outdoor cultivated macroalgal species were converted into bio-oil in a batch reactor via HTL. The impact of biochemical composition of algae on the yield and composition of bio-oil was analysed. Among the freshwater macroalgae, Oedogonium yielded greater bio-oil at about 26.2%, whereas Derbesia yielded higher bio-oil of 19.7% amongst marine macroalgae. However, the elemental composition of bio-crudes obtained from both fresh and marine macroalgae showed similar pattern with 33–34 MJ kg^{-1} HHV.

With reference to the effect of temperature on the elemental composition of bio-oil, reaction temperature between 270 and 330 °C yielded bio-oil with 4.7% nitrogen and it is lowered to 4% when the temperature was increased to 370 °C. Similarly, extending the reaction temperature of HTL of Spirulina Platensis from 250 °C to 350 °C reduces the nitrogen content in bio-oil from 3.8% to 1.61% and also decreases the content of nitrogen containing compounds to 10%.

In general, GC–MS analysis of bio-oils produced from the HTL of algal biomasses carried out under different temperature showed cyclic oxygenates, N & O-heterocyclic compounds, straight & branched amides, esters, ketones, hydrocarbons, alcohols, and fatty acid derivatives.

Similarly, composition of bio-oil obtained from Spirulina indicates the presence of amides, esters, nitriles, hydroperoxide and alkanes.

From the literature, it is perceived that the content and profile of biocrude depends on the temperature and increasing temperature was found to increase the bio-crude yield in microalgae. Maximal bio-oil content under high reaction temperature is owing to two key phenomena, i.e., a high ionic product of water and low dielectric constant.

At high water temperature, the high ionic product of water ($K_W=[H^+][OH^-]$) provides dissociated H^+ and OH^- ions, which drives acid and base-catalyzed reactions, and during this process, biomass components undergo isomerization, reforming, depolymerization and repolymerization to yield bio-

oil. In HTL temperature region, the ionic product of water (Kw) is high at about 10^{-12} compared to 10^{-14} for ambient water. This property of water favours the formation of oil products by having ionic bond between water and biomass, thereby reducing the formation of solid char or gas by means of blocking the radical reactions. At the same time, the dielectric constant (ε) of water decreased from 80 to 20 in the subcritical temperature. More specifically, the dielectric constant of water drops down from 78 Fm^{-1} to 14.07 Fm^{-1}, when the temperature and pressure are raised from 25 °C and 0.1 MPa to 350 °C and 20 MPa, respectively. The low dielectric constant makes water a hydrophobic solvent, allowing to dissolve hydrophobic organic compounds like free fatty acids.
On the other hand, conditions that enhance the yield of liquefaction products are achieved at supercritical conditions. Also, attaining supercritical condition is cost effective due to its variability in specific heat capacity and low compressibility of liquids. With respect to product yield from HTL, temperature greatly influences the formation of various products such as biochar, bio-oil and syngas.
For yielding high amount of bio-oil, intermediate temperature of 300–350 °C is advantageous because of the following facts: **(i)** at temperature higher than the activation energy of bond dissociation, secondary decompositions and boudouard reaction (reaction of solid char with CO_2 to produce carbon monoxide) occurs which leads to the formation of more gaseous products, **(ii)** at higher temperature, there is an increased char formation because of condensation of free radicals, **(iii)** at lower temperature, the decomposition of biomass is incomplete which does not yield sufficient bio-oil, and **(iv)** at higher temperature, operational cost is high and bio-oil yield is low. This implies the importance of temperature in HTL reactions for yielding the desired liquefaction products.

3.6.Catalytic thermochemical conversion of algae

3.6.1.Catalytic pyrolysis of algae

The extraction–transesterification of algae only utilizes the lipid fraction of algae, leaving other parts (for example: saccharides and proteins) as waste. Thermochemical conversion of algae is a viable method to fully utilize algae.
The quality of bio-oil can be improved by using catalysts in pyrolysis. The oxygen content of pyrolytic bio-oil needs to be reduced in order to improve the stability and heating value. In addition, the high proportion of N atoms in the bio-oil should also be removed to meet the standard of combustion fuel. With the aid of an appropriate catalyst, the pyrolytic pathway can be changed, and consequently the selectivity of products is influenced. Generally, catalysts such as zeolite are frequently applied in catalytic pyrolysis, due to the high activity in deoxygenation, cracking, and dehydration.
Moreover, metal oxides and supported metal catalysts have also been explored for catalytic pyrolysis of algae. Therefore is reported an overview of the performance of different types of catalysts on catalytic pyrolysis of algae.

3.6.2.Zeolites

Zeolites are regarded as highly efficient catalysts for upgrading bio-oil from algae, due to the suitable acidity, resistance of carbon deposition, and the ability to eliminate oxygen atoms without hydrogen. Of the zeolite catalysts, ZSM-5 is commonly used because of its adjustable acidity and high performance in deoxygenation, decarboxylation, and decarbonylation. Its acidity can be controlled by varying the Si/Al ratio. High Si/Al ratio results in low acidity of the zeolite. The acid

sites on zeolites make the macromolecules of algae degrade to compounds with small molecular size.
Subsequently, the formed compounds pass through deoxygenation or aromatization forming reduced compounds such as hydrocarbons.
The performance of a catalyst on catalytic pyrolysis of algae can be evaluated by the bio-oil yield, the oxygen and nitrogen content of bio-oil. Pyrolysis of macroalgae Enteromorpha clathrata over metal modified Mg-Ce/ZSM-5 catalysts at 550 °C produced bio-oil with high quality. The 1 mmol Mg-Ce/ZSM-5 showed the ability to increase bio-oil yield from 33.77% (without catalyst) to 37.45% and decrease the acid content.
In addition, the average molecular weight of bio-oil obtained over such catalyst seemed to decrease, with the content of gasoline-like (C_5-C_7) compounds increased.
Primary cracking and decarboxylation might occur due to the presence of 1 mmol Mg-Ce/ZSM-5.
Catalytic fast pyrolysis of spirulina sp. over different types of zeolites (ZSM-5, zeolite-β and zeolite-Y) was performed. The HHV of pyrolysate ranged over 30-37 MJ kg^{-1}, which was much higher than that of algae feedstock.
All types of zeolites facilitated the formation of aromatics (monoaromatics, PAHs, and indoles). Cycloalkanes were formed over ZY and Zβ, while C_2-C_4 nitriles formed over high acidity zeolites.
Anash et al. studied the pyrolysis behavior of Chlamydomonas debaryana with and without β-zeolite or activated carbon (AC). The yields of total hydrocarbons were highest over β-zeolite than that over AC and without catalyst.
The combination of hydrothermally carbonized pretreatment and catalytic pyrolysis could effectively reduce nitrogen content of bio-oil, and produce more hydrocarbons, including aromatics. It was found that AC catalyst was more likely to form coke than β-zeolite.
To summarize, catalysts applied for pyrolysis of algae usually have high activity in deoxygenation. The deoxygenation performance of zeolites can be adjusted by changing the Si/Al ratio of the zeolite.
In addition, aromatization of pyrolytic products can be observed, generating abundant aromatics in bio-oil.
The increasing acidity of catalysts (low Si/Al ratio) results in promotion of aromatization.
After catalytic pyrolysis with zeolites, bio-oil with high HHV and low O/C ratio is obtained, but the nitrogen content could hardly be reduced to meet the standard of commercial transportation fuel.
The bio-oil obtained from catalytic pyrolysis contained about 5% of N content.
To produce high-quality biofuel with low N content, catalysts with the ability to remove N in bio-oil need to be developed. The bio-oil obtained from catalytic pyrolysis needs to be further upgraded by catalysts such as sulfide $CoMo/Al_2O_3$ or $NiMo/Al_2O_3$, which have high activity in hydrodenitrogenation (HDN).

3.6.3.Other catalysts

Except zeolites, other catalysts such as metal oxides and supported metal catalysts are applied in catalytic pyrolysis.
Transition metal such as nickel has high activity in C-C and C-O bonds cleavage, resulting in high performance for decarbonylation and decarboxylation. Furthermore, reducible metal oxides are considered as a favorable support or catalyst because of their superior redox properties and low carbon deposition rate.
It was found that the pyrolysis of Tetraselmis sp. and Isochrysis sp. over $Ni\text{-}Ce/Al_2O_3$ and $Ni\text{-}Ce/ZrO_2$ produced a higher yield of bio-oil (26 wt %).

The catalysts exhibited strong deoxygenation and denitrogenation ability, with only 9–15% oxygen remained and removal of 15–20% nitrogen from bio-oil. In addition, pyrolysis of Pavlova sp. over Ce/Al_2O_3-based catalysts produced bio-oil with a low O/C ratio (0.1–0.15). MgCe/Al_2O_3 exhibited the best performance on the reduction of oxygen content from 14.1 to 9.8 wt %, while NiCe/Al_2O_3 produced the highest hydrocarbon fraction.
Catalytic fast pyrolysis of Nannochloropsis oculate over Co-Mo/γ-Al_2O_3 was carried out in an analytical micropyrolyzer coupled with a gas chromatograph/mass spectrometer (py-GC-MS). It was found that aliphatic alkanes and alkenes, aromatic hydrocarbons, and long-chain nitriles were the main products in bio-oil.
Co-Mo/γ-Al_2O_3 catalyst could promote the formation of 1-isocyanobutane and dimethylketene with 35% selectivity. In addition, catalytic pyrolysis over Co-Mo/γ-Al_2O_3 produced pyrolysates with higher calorific value (33–39 MJ kg^{-1}) compared with that of algal feedstock (18 MJ kg^{-1}).

3.7.Catalytic HTL of algae

3.7.1.Catalytic HTL with homogeneous catalysts

Because HTL is conducted in aqueous phase, water-soluble homogeneous catalysts can be used. They commonly have the ability to improve biocrude yield or produce target compounds with high selectivity. Generally, the homogeneous catalysts include acid catalysts (HCl, H_2SO_4 and other organic acids), alkali catalysts (Na_2CO_3), or other inorganic salts. These catalysts usually promote bonds cleavage, so the degradation of components in algae to small molecular compounds is facilitated.
In the presence of homogeneous catalysts, the dissolution of components from algae is facilitated and hence the biocrude yield enhances.
However, the homogeneous catalysts can hardly improve the quality of biocrude. In other words, they have a weak influence on deoxygenation and denitrogenation of algal biocrude.
In general, the homogeneous catalysts used in HTL are catalysts with proper acidity or basicity.
Koley et al. studied the catalytic and non-catalytic HTL of wet Scenedesmus obliquus (Table 20). The optimizations of HTL temperature, pressure, and residence time were conducted on both catalytic and non-catalytic HTL. They found that 300 °C, 200 bar, and 60 min was the optimum condition for obtaining the maximum yield of biocrude (35.7 wt %). By adding catalysts, the biocrude yield increased and followed the order: acidic catalyst CH_3COOH (45%) > HCOOH (40%) > HCl (39%) > H_2SO_4 (38%) > H_3BO_3 (37%), and basic catalyst Na_2CO_3 (40%) > NaOH (38%) > $Ca(OH)_2$ (37%) > KOH (37%) > K_2CO_3 (36%). In addition, the acetic acid had the ability to reduce the oxygen content of biocrude, resulting in a considerable HHV of 40.2 MJ kg^{-1}.
However, the composition of biocrude in the presence of CH_3COOH was still complex, containing fatty acids, phenols, indoles, monoaromatics, and N-heterocycles.
The effect of acidic, neutral and basic catalysts on the conversion of microalgae (Spirulina platensis) by HTL at various temperatures was studied by Zhang et al.
HCl and acetic acid were used to create acidic condition, while KCl for neutral, and K_2CO_3 and KOH for basic conditions.
Among these catalysts, only acetic acid and KOH are found to positively influence the biocrude yield, which was more obvious at lower temperatures. The acid and base catalysts promoted the degradation of components in microalgae and suppressed the condensation reaction, resulting in lower average molecular weight of biocrude.
However, the distribution of compounds detected by GC-MS showed little change even with the aid of acid or base catalysts.

Typically, Na_2CO_3 is a commonly-used homogeneous catalyst for HTL because of its ability to enhance the biocrude yield.

Shakya et al. studied the effect of temperature on the HTL of three kinds of algal species Nannochloropsis, Pavlova, and Isochrysis over Na_2CO_3.

Table 20

Catalyst	Algae Feedstock	Condition	Biocrude Yield (wt %)	Performance
Without catalyst	Scenedesmus obliquus	300 °C, 200 bar, 1 h	35.7	HHV: 39.4 MJ kg^{-1}
CH_3COOH	Scenedesmus obliquus	300 °C, 200 bar, 1 h	45.1	HHV: 39.1 MJ kg^{-1}, O%: 8.9%
Na_2CO_3	Scenedesmus obliquus	300 °C, 200 bar, 1 h	40.2	HHV: 40.2 MJ kg^{-1}, N%: 4.7%
KOH	Spirulina Platensis defatted	300 °C, 35 min	30.1	Positive effect on biocrude yield, more ketone and amides formed, lighter volatiles generated
K_2CO_3	Cryptococcus Curvatus defatted	350 °C, 1 h	68.9	The highest biocrude yield, HHV: 38.2 MJ kg^{-1}
K_2CO_3	Cryptococcus curvatus	300 °C, 1 h	63.9	The best biocrude quality, HHV: 36.9 MJ kg^{-1}, lowest nitrogen (0.77%)
KOH	Cyanidioschyzon merolae	300 °C, 0.5 h, 120 bar	22.67	HHV: 33.66 MJ kg^{-1}
CH_3COOH	Cyanidioschyzon merolae	300 °C, 0.5 h, 120 bar	21.23	HHV: 33.36 MJ kg^{-1}
KOH	Ulva prolifera	290 °C, 10 min	26.7	Biocrude yield increased from 12.0 wt % to 26.7 wt % with KOH; Higher HHV: 33.6 MJ kg^{-1}
Na_2CO_3	Green macroalgal blooms	270 °C, 45 min	20.1	The highest bio-oil yield was achieved over Na_2CO_3

It can be concluded that the biocrude yield increased with the rise of temperature from 250 to 350 °C. The maximum biocrude yields from HTL of three algae species followed the order: Nannochloropsis (48.67 wt %) > Isochrysis (40.69 wt %) > Pavlova (39.96 wt %). When using Na_2CO_3 as the catalyst, the biocrude yield changed to the order of Pavlova > Isochrysis > Nannochloropsis. The biocrude yields of algae with high carbohydrates content (Pavlova and Isochrysis) increased at higher temperatures (300–350 °C) with the aid of Na_2CO_3, whereas the high-protein-containing algae (Nannochloropsis) showed higher yield of biocrude at lower temperature (for example: 250 °C).

However, the conversion of algae with Na_2CO_3 did not significantly improve biocrude properties. The biocrudes obtained were still not suitable for application in transportation.

Overall, homogeneous catalysts have a strong impact on the products yields, especially enhancing the biocrude yields.

This is probably ascribed to the degradation ability of these catalysts to create an acid or basic condition. There were few reports about the deoxygenation and denitrogenation ability of homogeneous catalysts. Thus, the quality of biocrude from HTL can hardly be improved by homogeneous catalysts.

The biocrude obtained cannot meet the standard for transportation fuel.

Furthermore, the acidity and basicity of catalysts might influence the pH value of biocrude and lead to corrosion to the equipment. The homogeneous catalysts can hardly be recovered after reaction, and this leads to further expense.

Therefore, finding catalysts with good reusability and highly efficient for deoxygenation and denitrogenation is pressingly required.

3.7.2.Catalytic HTL with heterogeneous catalysts

The heterogeneous catalysts, which exist in different phases with the reaction media, are usually solid catalysts.
Heterogeneous catalysts can be easily recovered after reaction, therefore reducing the cost of the process.
Conversion of algae in the presence of heterogeneous catalysts might cover the deficiency of homogeneous catalysts.
Commonly, heterogeneous catalysts include zeolites (for example: H-ZSM-5), supported metal catalysts (Pt/C), and other metal oxide supported catalysts (for example: sulfide $CoMo/Al_2O_3$ and Ni/TiO_2).
These materials have strong activity for bonds cleavage, resulting in facilitation of macromolecule degradation and conversion of oxygenates and nitrogenates to high-grade hydrocarbons. As a result, biocrude with low viscosity, high HHV, and low N content is produced in the presence of heterogeneous catalysts.
The effects of heterogeneous catalysts on the yield and quality of biocrude are summarized in Table 21.
After screening, the majority of the catalysts listed in the table are supported metal catalysts and can be recycled several times. For example, magnetic nanoparticles (MNPs) were synthesized for microalgae separation and catalytic HTL by Egesa et al. Firstly, the MNPs were used for separation of algae from the culture medium, with a separation efficiency of 99% achieved. Then, the MNPs were applied in catalytic HTL for the production of biocrude from microalgae. The biocrude yield significantly increased from 23.2% (without catalyst) to 37.1% in the presence of Zn/Mg-ferrite MNPs.
Moreover, the percentage of hydrocarbons increased by 26.4%, and the percentage of heptadecane increased by 27.8%, while the percentage of oxygenates and N-containing compounds decreased. This indicated the catalysts had activity in deoxygenation and denitrogenation. In addition, the MNPs could be easily recovered and recycled several times.
Additionally, noble metal catalysts such as commercial Pd/C, Ru/C and Pt/C are widely used in algae conversion and show excellent catalytic performance.
Liu et al. reported a two-step catalytic conversion of algae (Spirulina) via solvent extraction followed by catalytic HTL of the extracted residue. In the extraction process, ethanol was found to be the best solvent with the highest extraction efficiency, and the introduction of $MgSO_4$ could produce ethyl esters from fatty acids. The lipid extracted residue was treated by HTL in the presence of commercial Pd/C, Pt/C, Ru/C, Rh/C, and Pd/HZSM-5. Among all catalysts, Rh/C exhibited the best performance in catalytic conversion of algae, producing 50.98% yield of biocrude with 30.7 MJ kg^{-1} HHV.
The O and N content of biocrude obtained from HTL over Rh/C decreased significantly from 32.2% and 7.1% to 23.6% and 4.4%, respectively. In addition, the percentage of hydrocarbons in biocrude obtained over Rh/C based on GC-MS results was 55.7%.
Xu et al. studied the catalytic effects of Pt/C, Ru/C and Pt/C + Ru/C on the HTL of Chlorella in the presence of H_2.
They divided the biocrude into water-soluble biocrude (WSB) and water-insoluble biocrude (WISB). The addition of catalysts could decrease the fraction of WSB but increase WISB fraction.
At optimized conditions, Pt/C and Ru/C led to the highest carbon (63.6% and 74.2%) and hydrogen (7.3% and 8.4%) contents but lowest oxygen (14.1% and 9.2%) and nitrogen (12.2% and 7.1%) contents, and the highest HHV (29.7 and 35.6 MJ kg^{-1}) for WSB and WISB fraction, respectively.

Table 21

Catalyst	Algae Feedstock	Condition	Biocrude Yield (wt %)	Performance
Co/CNTs	Dunaliella tertiolecta	320 °C, 30 min, catalyst/algae = 0.1	40.25	Higher percentage of hydrocarbons, lower content of fatty acid and lower N-compounds
nano-Ni/SiO_2	Nannochloropsis sp.	250 °C, 60 min, catalyst/algae = 0.05	30.5	Bio-oil with lower O and N content, catalyst recovery 2–3 times
Pd/HZSM-5@MS	Spirulina	380 °C, 2 h, HCOOH, catalyst/algae = 0.02	37.3	Promotion of HDO for bio-oil, HHV: 32.65 MJ kg^{-1}, catalyst recovery for 5 times
Ni/TiO_2	Spirulina	250 °C, 30 min, catalyst/algae = 0.05	43.1	Promoting the formation of hydrocarbons (14%) and esters (15%), and decreasing oxygenates and nitrogenates.
Ru/C	Nannochloropsis sp.	350 °C, 20 min, H2, catalyst/algae = 0.2	43.5	Increasing the yield of water-insoluble biocrude, decreasing O (7.95%) and N (4.95%) content
Ru/C	Chlorella	350 °C, 30 min, 0.3 MPa H2, catalyst/algae = 0.2	27	Water insoluble biocrude with highest C (74.2%) and H (8.4%) contents, the lowest O (9.15%) and N (7.1%) and highest the HHV (35.6 MJ kg^{-1})
ZSM-5	Ulva prolifera	280 °C, 10 min, catalyst/algae = 0.15	29.3	HHV of biocrude was 34.8MJ kg^{-1} (non-catalytic was 21.2 MJ kg^{-1});
Ni/TiO_2	Nannochloropsis	300 °C, 30 min, catalyst/algae = 0.1	48.23	Biocrude with lower viscosity, more light fractions, HHV: 35 MJ kg^{-1}, reproduction for at least 10 times
Fe/HZSM-5	Nannochloropsis	365 °C, 60 min	38.1	Increase of carbon into biocrude, nitrogen into aqueous phase
Zn/Mg-ferrite MNPs	Scenedesmus obliquus	320 °C, 60 min, catalyst/algae = 0.12	37.1	The percentage of hydrocarbons (46.5%), heptadecane (37.8%), HHV: 35.4 MJ kg^{-1}

The water insoluble biocrude obtained from HTL of algae over Pt/C contained amides (48.2%), hydrocarbons (17.7%), acids (12.8%), and phenols (7.7%).

In addition, catalytic HTL produced biocrude with more low boiling point fractions.

Apart from noble metal catalysts, the application of non-noble metal catalysts in algae HTL has drawn lots of attention due to their low cost and high activity in bonds cleavage.

Among the non-noble metals, nickel, cobalt, iron, and molybdenum are proved to be active in deoxygenation and denitrogenation.

Kohansal et al. conducted the HTL of Scenedesmus obliquus in the presence of Ni-based catalysts (Ni/AC, Ni/AC-CeO_2 nanorods and Ni/CeO_2 nanorods). The optimum condition for the catalytic HTL of microalgae over the catalysts was to set at 324.12 °C, 43.52 min, and 19.90 wt % feedstock. With the addition of heterogeneous catalysts, the biocrude yields over three catalysts were higher than that from a non-catalytic process. The highest biocrude yield of 41.87% was achieved over Ni/AC-CeO_2 nanorods, with the HHV of 38.57 MJ kg^{-1}.
In the presence of Ni-based catalysts, the percentage of hydrocarbons in biocrude was higher than that of the non-catalytic biocrude, but the content of nitrogen-containing compounds was also higher.
Overall, heterogeneous catalysts perform better than homogeneous catalysts in terms of the improvement of algal biocrude quality. In addition, the yield of biocrude can be improved in the presence of heterogeneous catalysts. The catalysts can be easily recovered and reused after HTL, but the coke formation is still the major problem during catalytic HTL.
However, in the previous literature, the contents of oxygen and nitrogen in the obtained biocrude are still too high to satisfy the standard of transportation fuel. Therefore, the technologies for production of high-quality liquid fuel with extremely low O and N content from algae need to be further developed.

3.8.Catalytic conversion of oil derived from algae

3.8.1.Catalytic hydroprocessing of extracted algal oil

Generally, the oil recovered from algal cell consists of different types of triglycerides. The fatty acids fraction of triglycerides usually contains palmitic, palmitoleic acid, stearic acid, and oleic acid. Some algal species also contain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA), arachidonic acid (AA), and docosahexaenoic acid (DHA), which are value-added health care products. The algae derived triglycerides can be hydrotreated by catalysts to fuel-like hydrocarbons with the aid of a proper catalyst. Since hydrogen could be obtained from a wealth of sources, including water splitting, especially by electrolysis of water with renewable electricity such as wind power or solar power, the consumption of hydrogen in the hydroprocessing can be ignored.
Conventional NiMo sulfide catalyst has been widely used in hydrogenation of natural oil into fuel-ranged hydrocarbons.
Liu et al. investigated the hydrocracking of algal oil from Botryococcus braunii (C_nH_{2n-10}, n = 29–34) with sulfide NiMo into fuel-ranged hydrocarbons. The support effect on the selectivity of products was studied under the conditions of 300 °C for 6 h under 4 Mpa H_2. For the hydrotreating of the model compound (squalene $C_{30}H_{50}$), the main product was squalane ($C_{30}H_{62}$) over NiMo/SiO_2, C_1-C_4 gas hydrocarbons over NiMo/HZSM-5, C_5-C_9 gasoline-ranged hydrocarbons over NiMo/HY and NiMo/SiO_2-Al_2O_3, and C_{10}-C_{15} aviation fuel-ranged hydrocarbons over NiMo/Al_{13}-Mont, respectively. The hydrocracking of algal oil over NiMo/Al_{13}-Mont gave aviation fuel-ranged hydrocarbons (C_{10}-C_{15}) with a yield of 52%. The sulfide NiMo catalyst acted as a bifunctional catalyst for hydrogenation of squalene to squalane followed by cracking of the formed squalane to shorter-chain hydrocarbons.
Zhao et al. explored the hydrotreating of extracted algal lipids from Nannochloropsis for the production of aviation fuel. The effect of hydrotreating temperature (270–350 °C) and catalyst loading (10-30%) was investigated. The optimum condition for hydrotreating reaction was 350 °C and 30% catalyst loading.
The main components of biofuel were C_8-C_{16} hydrocarbons and aromatics. The two-step hydrotreating process obtained biofuels with oxygen content below 0.3% and nitrogen content below 0.007% and HHV of 46.24 MJ kg^{-1}.

In addition, noble metal catalysts such as Pt, Pd and Ru, which have high activity in hydrodeoxygenation, are also suitable for conversion of algal oil into high-grade hydrocarbons.
Xu et al. explored a technology for selective extraction of neutral lipid from algae Scendesmus dimorphus and subsequently conversion into jet fuel. Hexane and ethanol solvent mixture was used for selective extraction of neutral lipids. Then, the extracted lipid was hydrogenated over the Pt/Meso-ZSM-5 catalyst. The obtained product oil (38%) mainly contained branched paraffin with C_9-C_{15} chain length. The jet fuel product satisfied the ASTM 7566 standard with the desired freeze point (-57 °C), flash point (42 °C), heating value (45 MJ kg^{-1}), and aromatics content (<1%).
Although metal sulfides and noble metals are highly active in deoxygenation of algal lipid/oil, the sulfur leaching of metal sulfides and high-cost noble metal make these process not environmentally and economically friendly.
Except for metal sulfides and noble metal catalysts, the sulfur-free non-noble metal catalysts are promising in heterogeneous catalysis.
It is found that non-noble metals (for example Ni, Co, Cu) are active in deoxygenation of fatty acids and natural oil to hydrocarbons.
Santillan-Jimenez et al. investigated the continuous catalytic hydrogenation of model compound and algal lipids to fuel-like hydrocarbons using Ni-Al layered double hydroxide. In addition, Ni/Al_2O_3, Ni/ZrO_2, and Ni/La-CeO_2 were applied for the comparison experiments. Of all Ni-based catalysts, Ni-Al LDH showed the best results for conversion of tristearin to C10-C17 hydrocarbons at 260 °C. Higher temperatures favored the cracking reaction to form lighter alkanes, while lower H_2 pressure favored the formation of heavier hydrocarbons. For the hydrogenation of algal oil, ~50% yield of hydrocarbons was obtained over Ni-Al LDH. Generally, extracted algal oil/lipids have the potential to be converted to high-grade, fuel-like hydrocarbons. The nitrogen and oxygen content of algal-lipid derived fuel are low enough to satisfy the standard for transportation fuel due to the low nitrogen content and easily editable oxygen of algal lipids. However, due to the limited lipid content of algae, the yield of algal lipids derived green fuel based on the whole algal cell is also low.

3.8.2.Catalytic upgrading of biocrude oil from thermochemical conversion of algae

Another way for production of fuel-ranged hydrocarbons from algae is the upgrading of the biocrude oil from thermochemical conversion. The biocrude oil obtained at high temperatures contains the components derived from saccharides and proteins apart from lipid.
Some of the oxygenates and nitrogenates cause the undesired properties of biocrude oil.
Therefore, the large proportion of oxygen and nitrogen of bio-oil needs to be removed for obtaining high-quality biofuel. This process involved the use of a proper heterogeneous catalyst with high activity in deoxygenation and denitrogenation.
Generally, the upgrading process also needs H_2 to remove the heteroatoms (O, N, and S), for improving the heating value and reducing the O, N, and S content of product oil.
The resulted biofuel should have low viscosity, high stability, and high HHV.

3.8.3.Catalytic upgrading of pyrolysis bio-oil

As mentioned in the previous section, the bio-oil from direct pyrolysis of algae contains high proportion of O and N due to the degradation of the whole algae cell at high temperatures. The quality of pyrolytic bio-oil needs to be upgraded via catalytic process before utilization as transportation fuel.
Elkasabi et al. investigated one-step hydrotreating and aqueous extraction of O and N-containing compounds, for the production of fuel-range hydrocarbons from Spirulina pyrolysis bio-oil.

The catalytic hydrodeoxygenation (HDO) and hydrodenitrogenation (HDN) were conducted over commercial Ru/C catalyst. The upgrading at 385 °C resulted in organic oil with low N and O content (<1 wt %). The selectivity of products could be controlled by varying reaction conditions.
More paraffins were obtained at higher temperature (~400 °C), while lower temperature (350 °C) resulted in more phenolics. The remaining oxygen and nitrogen-containing compounds in the upgraded oil could be removed through aqueous extraction with HCl.
Guo et al. reported an approach to get high-quality liquid fuel from catalytic HDO of pyrolysis oil from Chlorella and Nannochloropsis by using Ni-Cu/ZrO_2 bimetallic catalysts. The highest HDO activity was obtained over 15.71 wt % Ni 6.29 wt % Cu supported on ZrO_2, with the HDO efficiency of 82% for the upgrading of bio-oil from Chlorella. The Ni-Cu/ZrO_2 catalyst showed excellent stability after reaction, with low sintering and coking.
In addition, the heating value, viscosity, and the water content of the upgraded oil were improved. Particularly, the cetane number of the product oil from Nannochloropsis satisfied the standard of EN 590-09.
Overall, catalytic upgrading of pyrolytic oil from algae can successfully remove the O and N content to a low level. In addition, the remaining N and O can possibly be removed using physical adsorption or the extraction method.
The resulting liquid biofuel can meet the standard of transportation fuel.
However, the mentioned works did not study much on the recyclability and regeneration of the catalysts, and the mechanism of upgrading process for a better understanding of catalyst design.

3.8.4.Catalytic upgrading of HTL biocrude

Like pyrolysis, direct HTL of algae produces biocrude oil with poor quality, especially for its high O and N content, and the physical properties that do not suit the fuel standards.
Patel et al. Explored a method for catalytic upgrading of biocrude from fast HTL of algae over Pt, Pd, Ru supported on C and Al_2O_3, and sulfide NiMo/Al_2O_3. The highest oil yield (60 wt %) and highest denitrogenation ability (2.05 wt %) were obtained over NiMo/Al_2O_3, but the effect of deoxygenation was poor.
The oxygen content of the upgraded biocrude ranged from 1.60-6.07 wt %, while the nitrogen content ranged from 2.05-3.47 wt %. The decrease of O content resulted in the increase of HHV to 38.36-45.40 MJ kg^{-1}.
The boiling point distribution of upgraded biocrude decreased from the gas oil fraction (271–343 °C) to the kerosene fraction (<271 °C).
In addition, the abundant components in upgraded biocrude were branched alkanes and straight-chain alkanes.
Shakya et al. studied the catalytic upgrading of biocrude oil from HTL of Nannochloropsis.
Five different catalysts (Pt/C, Ru/C, Ni/C, ZSM-5 and Ni/ZSM-5) were used as the upgrading catalysts. Upgrading at 300 °C showed higher oil yield, while higher temperature at 350 °C resulted in bio-oil with higher quality. The maximum upgraded oil yield was obtained over Ni/C at 350 °C, whereas upgraded oil with higher HHV, lower acidity, and nitrogen content was achieved over Ru/C and Pt/C. The HHVs of upgraded biocrude ranged from 40–44 MJ kg^{-1}, which were highly improved compared with biocrude feedstock (36.44 MJ kg^{-1}).
The catalytic upgrading produced upgraded oil with a 65–75% decrease in nitrogen content, and 95-98% decrease in oil acidity.
Biller et al. investigated the hydroprocessing of biocrude on sulfide CoMo and NiMo catalysts from continuous HTL of Chlorella.

In the non-catalytic HTL step, 40 wt % yield of biocrude was obtained with 6% nitrogen, 11% oxygen, and HHV of 35 MJ kg^{-1}. The upgrading of biocrude over both NiMo and CoMo catalysts was conducted at 405 °C and 350 °C.

The two catalysts showed similar performance on the improvement of hydroprocessed oil. The upgraded oil with the highest HHV (45.4 MJ kg^{-1}) was achieved over CoMo catalyst at 405 °C, with the oil yield of 69.4 wt %, nitrogen content of 2.7%, and oxygen content of 1.0%. Hydroprocessing at high temperature (405 °C) resulted in upgraded oil with higher gasoline and diesel fractions. Moreover, hydrocarbons (C_9-C_{26}) were the main products in upgraded bio-oil.

In the previous section, catalytic HTL can improve the quality of biocrude, but the characteristics of biocrude still cannot meet the ideal standard. In comparison to one-step catalytic HTL, the upgrading of biocrude from HTL obtained biofuel with higher quality, with low boiling point, low viscosity, high HHV over 40 MJ kg^{-1}, and low oxygen and nitrogen content, which is more preferable than the one-step catalytic HTL.

Conclusions

The overall lifecycle emissions of CO_2 from the use of 100% biodiesel is 78.45% lower than those of petroleum diesel and a blend with 20% biodiesel fuel can reduce the net CO_2 emissions by 15.66%; another adavantage is that biofuel at any blend ratio can be directly used in existing diesel engine withouth the need of mechanic modifications. Biodiesel is less volatile than petroleum diesel, and it is also safer for transportation, having a flash point greater than 150°C.
The target of this technology to be utilized at large industrial scale is to find a source for the production of biodiesel that doesn't require the oilseeds of the landcrops cultures, avoiding the price competition in the food market and the desertification associated with intensive cultures.
The elaborated informations of this thesis pointed out microalgae as the best solution for the raw material source for the production of biodiesel.
This organism has many really interesting characteristics compared with oil crops, infact microalgae have higher photosynthesis efficiency, faster growth and can synthetize and accumulate larger quantities of lipids, infact oil yield per area of microalgae can largely exceed the yield of oilseed crops.
Typically biodiesel from seed oils (rapeseed or soybean) produces 37 MJ kg^{-1}, comparable to the energy density of petroleum diesel (higher heating value of 42.7 MJ kg^{-1}), while biodiesel derived from microalgae yields 41 MJ^{-1} kg.
The main disadavantage associated to this technology relies in the selection of a fast growing species of algae with high lipid productivity and resistant to the environmental conditions. Through the informations of the first chapter the most promising species came out to be: Chlorella Vulgaris, Nannochloropsis and Scenedesmus. In particular Scenedesmus has the highest lipid productivities due to its biomass growth and especially for its elevated lipid content. Really importatnt is also the capacity of this algae to enhance the lipid content, especially the TAGS, which are the best substrate for biodiesel production, through the nitrogen starvation, that could be obtained through the utilization of water industrial effluent. Especially Scenedesmus has an outstanding lipid productivity under nitrogen deprivation and a good biomass recovery after 24 h settling. Really importatnt is the fact that this algal strain has a really good oil quality, thanks to this, the biodiesel made from this algal oil meets almost all the parameters of the European standards for biofuel production.
Another important element vital to the success of the selection of an algal species is its suitability for the lipid extraction methods. The most promising methods shown in the second chapter of this thesis are the ones, that doesn't utilize the chemical toxic solvent, respecting the environment and especially that doesn't degrade the oil quality or use elevated quantities of energy. The extraction methods that satisfied all this requirements came out to be: the osmotic pressure and the supercritical fluid extraction. In particular osmotic pressure is the most simple, safer, efficient and environmentally safe method. On the other hand also supercritical fluid extraction is a promising extraction method, because of its several advantages: it uses a safe and non flammable solvent and also because it allows easy, quick and selective extraction of complex samples.
The last importatnt aspect of microalgae biofuel is the lipid conversion technique, the best have been analyzed in the third chapter of this thesis and they are represented by the ones that doesen't require the use of dried algal paste, which are the wet transesterification and the hydrotermal liquefaction. The wet transesterification permits to avoid the waste of energy for drying the algal paste and has turned out to be a viable technique, with which it's possible to obtain in water sample content of 90%, yield of TAGs of 94% of that sample withouth water.
Hydrotermal liquefaction came out to be a really efficient and performing technique, because it allows to use not only the lipid content but also the carbohidrates and the proteins to obtain higher amounts of oil and then of biofuel from wet algal paste. The only disadvantage consists in the

presence of quantities of nitrogen which leads to the emissions of NO_x as a consequence of the combustion of the biofuel.
The analysis of different catalysts especially the heterogeneous has shown to be a really important and efficient way in order to reduce the amount of N-atoms in the bioalgal oil, resulting in a biofuel with high quality.
At last the selection of the algal Scenedesmus with its high lipid productivity associated with the extraction methods as osmotic pressure or supercritical fluid extraction and then the conversion technique such as the hydrotermal liquefaction, can be a real and effective solution in the short period to obtain a good quality biofuel in order to reduce the use of petroleum diesel in the transportation sector and the consequently environmental pollution.

Sitography

Chapter 1

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