Production of Biodiesel from Micro Algae; *Chlorella* sp. in Laboratory scale

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Abstract

Biodiesel has become a very enthusiastic topic among the global community as it is recognized to be a sustainable and clean energy source when compared with current energy sources. A variety of feedstock is used for making biodiesels. However, if first and second generation feedstock is used as the bio diesel feedstock, it would create a competition with the world food market and deforestation and land usage would also come in to play. Therefore, microalgae as a third generation feedstock, avoids major drawbacks associated with first and second generation biofuels.

In this study, the growth and lipid content of isolated *Chlorella* sp. was studied under different nutrient conditions in order to introduce an efficient growth medium. Then, the extracted algal oil was subjected to two a step conversion, which is esterification followed by transesterification and converted into biodiesel. Finally, converted biodiesel was tested according to ASTM D6751 and EN 14103 standards.

According to the results obtained, the highest specific growth rate (μ_{max}) was observed for the media containing $[NO_3^-]/[PO_4^{3-}]$ ratio of 16/20. Moreover, it was observed that media with higher nutrient concentration decelerated algal growth, but did not decrease the lipid content per unit volume of algal culture. Observed maximum lipid content per unit volume of algal culture was 0.975 g L^{-1}. Further, converted biodiesel sample contains 89.09 % total fatty acid methyl ester (FAME) mainly, methyl esters of palmitoleic (C16:1), stearic (C18:0), linolelaidic (C18:2n6t) linoleic (C18:2n6c) acids in 9.70%, 4.73%, 71.95% and 1.47%, respectively. Additionally, physicochemical parameters such as boiling point, density, flash point and viscosity of synthesized biodiesel were also consistent with the standards.

The overall study reveals the potential of using *Chlorella* sp. as a convenient biodiesel feedstock.

Introduction

With the industrial revolution, energy demand has rapidly increased in recent years with petroleum fuel making a great contribution. Due to the non-renewable nature of petroleum resources, the shadow of future energy crises is cast in several reports. Further, petroleum fuel is known to be a source of many pollutants including CO_2, CO, H_2O vapor, SO_x, NO_x, particulates and volatile organic compounds.Therefore, much attention is being paid to biodiesel which has been identified as a promising solution to meet the above mentioned crises Furthermore, they are able to facilitate efficient combustion because of its high oxygen percentage .

Biodiesel is a mixture of fatty acid methyl esters that can be synthesized *via* transesterification of renewable lipid (triglycerides -TG) feedstock in the presence of a catalyst. Methanol is usually used as the alcohol for the conversion, due to its low cost . Implementation of the biodiesel production process is also a challenge and a number of strategies are being developed. High production cost is still a problem and much effort is being directed to its reduction. In the biodiesel production process, 75% of the overall biodiesel production cost is for feed stock. Therefore, utilization of cheap, non-edible and second and third generation waste oil as feed stock sources is more meaningful than use of first generation edible feedstocks. Moreover, this will eliminate competition for food from biodiesel feed-stocks. When considering non-edible feedstocks, third generation feedstocks show significant importance, especially, because of the presence of microalgae unlike second generation non-edible terrestrial feedstocks. Even though oil yields from microalgae are strain-dependent, they are much greater than other terrestrial edible and non-edible sources. According to the review report, low oil bearing microalgae feedstocks show more than 10 times and high oil more than 25 times biodiesel productivity than the highest oil bearing terrestrial plant. Therefore, in terms of land usage, microalgae have a clear advantage because of their higher biomass productivity and oil yield. Furthermore, they have the capacity to absorb pollutants (especially N, P, K, and C containing pollutants) from water bodies in order to use for growth.

There are many reports on the feasibility of using species of microalgae *Chlorella* abiodiesel feedstock. High biomass growth rate (2 gl) and lipid productivity (54 mglday) can be obtained from *Chlorella vulgaris* under heterotrophic growth, while *C. zofingiensis gave* high specific growth rate (0.994 day) and biomass productivity (58.4 mg lday) under outdoor conditions. This species had shown higher lipid content (54.5% of dry weight) under nitrogen limiting conditions than under nitrogen sufficient conditions (27.3%). Further , It was confirmed that the lipid content of *Chlorella* species can be increased by nitrogen starvation, with the lipid content of *C. emersonii* reaching 63% of dry cell weight in low nitrogen condition. It has been shown that Fe concentration affects lipid content of *C. vulgaris* with lipid content increasing 3-7 fold by supplying 1.2 × 10 moll $FeCl_3$. .Furthermore, *Chlorella* can be cultured under autotrophic, heterotrophic or mixotrophic growth conditions, indicating its strong ability to adapt to different culture conditions. Oil yield of *Chlorella* sp. can therefore be increased by varying the nutritional modes and compositions of growth media.

However, most of these non-edible oils including microalgal oil have high free fatty acid (FFA) values. Therefore, transesterification with alkali based catalyst yields a considerable amount of soap. Soaps are emulsifiers, so that separations of glycerol and ester phases become more difficult. Further, the catalyst that has been converted to soap is no longer available to accelerate the biodiesel forming reaction. Therefore high catalyst loading is required. Acid catalyzed transesterification was found to be a good solution to this problem, but. the reaction rate becomes considerably less, requiring lengthy reaction periods. Therefore, it has been well established in the literature that the best approach to produce biodiesel from non- edible oils with high FFA values is a two step process involving acid catalyzed pre-esterification followed by alkaline catalyzed transesterification.

In the present work, the growth of *Chlorella* sp. under different nutrient conditions was studied in order to identify an efficient growth medium. Qualitative and quantitative analysis of lipid extracted from algae grown in each media were also analyzed. Conversion of the extracted oil into biodiesel through a sulfuric acid followed by KOH catalyzed pathway and a study of the properties of the biodiesel produced were also performed.

Material and methods

Sampling, Isolation and Identification of algae

Water samples were collected in sterilized transparent glass bottles, from a highly polluted fresh water pond, 'Beira' lake in Colombo (6° 55' 55" North, 79° 50' 52" East) which is enriched with algae growth. The collected samples were kept below 4C under refrigeration until plate culture was done. Then, solid medium plate culturing was performed to obtain pure algal culture. Collected samples were vortexed for 5 min to obtain separated single cell colonies for isolation. The serial dilution protocol followed in sub plate culture was performed in a laminar flow. Sub plate culture was performed in petri dishes containing autoclaved (15 psi, at 121°C for 15–20 min) BG-11 medium in solidified 1.6 % (w/v) bacteriological agar. Sealed plates were incubated at 27C and for 12 h photoperiod per day using fluorescent light of intensity 9.00 kLUX for 7 days until well separated algae colonies were obtained. The species were identified under a light microscope and purity of the culture was confirmed by observing in a light microscope and by repeating plate culturing.

Medium	**[NO_3^-]/[PO_4^{3-}] ratio**	**NO_3^- concentration /ppm**
1	0	0.00
2	0.2	2.00
3	0.4	4.00
4	0.6	6.00
5	0.8	8.00
6	1	10.00
7	5	50.00
8	10	100.00
9	15	150.00
10	20	200.00
11	25	250.00
12	30	300.00
13	35	350.00
14	40	400.00
15	45	450.00
16	50	500.00

Table 1. NO_3^- concentration and [NO_3^-]/[PO_4^{3-}] ratio of media

Cultivation and Growth kinetic studies

a. Culture conditions

Glass conical flasks (500 ml) were used for the cultivation. Aeration was provided at a 10cms continuously. The condition used for cultivation was an outdoor (12 hour day) environment with temperature and light intensity during cultivation varying between 24 – 28C and 5.5 – 7.5 kLUX, respectively. Nitrate and other nutrient concentrations of each media were maintained as shown in Table 1 and Table 2, respectively, with sodium nitrate, $NaNO_3$ used as nitrate source

Component	Amount (g/L)
$NaHCO_3$	10.000
$MgSO_4.7H_2O$	0.3840
$CaCl_2.2H_20$	0.0384
$MnSO_4.5H_2O$	0.0352
$FeCl_3.6H_2O$	0.0016
Yeast extract	0.1920
KCl	0.0100
NaH_2PO_4	0.0100

Table 2. Concentrations of other nutrients in all growth media

b. Growth kinetics studies

Growth kinetics studies were conducted during the entire period of cultivation with optical density at 550 nm (OD_{550}, UV 1800 Shimadzu UV Spectrophotometer) used as the growth parameter. In optical density measurements, samples were diluted with distilled water to ensure that the measured OD_{550} values were in the range of 0.100 - 0.200. Growth rate (μ) was calculated according to Eq. 1,

$$\mu = \frac{y_c - y_0}{x_c} \qquad \text{Eq. 01}$$

Where, y_0 = initial value of growth parameter, $y_{c,}$ = value of growth parameter at maximum growth and x_c = time taken to attain the maximum growth

c. Lipid extraction

A known volume of algal culture (V ml) was removed and centrifuged at 4000 rpm for 10 min. Supernatant aqueous solution was removed and the separated biomass was dried at 40C for one day. The literature methodology[14] that consists in the Folch method[15] combined with ultrasonication was used for oil extraction with biomass/solvent ratio kept at 1/10 (v/w) and temperature maintained at solvent reflux for 6 h. The resultant mixture was agitated on a vortex for 5 min and kept in the dark for 48 h in a deep freezer (-4C). The resultant solution was vortexed for 5 min and then centrifuged at 4000 rpm for 10 min. The supernatant was transferred to a pre-weighed glass tube (w_1) and the solvent removed by heating under vacuum. Final constant weight was measured (w_2). Lipid content per unit volume of culture was calculated as $(w_2-w_1)/v$.

d. Conversion of oil extract into biodiesel.

Extracted oil was converted into biodiesel by following standard procedure[6] involving H_2SO_4 acid catalyzed pre-esterification followed by KOH catalyzed trans-esterification. The conditions applied are tabulated in Table 3.

Condition	Pre-esterification	Trans-esterification
Catalyst and catalytic dose	H_2SO_4 - 1% (v/v oil)	KOH -1% (m/m oil)
MeOH concentration	50% (v/v oil)	25% (v/v oil)
Reaction temperature /^{0}C	60	60
Reaction time/h	6	2

Table 3. Applied conditions for pre-esterification and transesterification

e. Analytical methods

Total FAME and methyl linolate content were determined using gas chromatographic methods and the calibration curve method was used in calibration techniques. Equipment: Agilent 7890B gas chromatographic system with a split inlet, flame ionization detector (FID) and Agilent J&W HP-5 30 m column. Nitrogen and hydrogen were used as carrier gas and auxiliary gas for FID. 1 μL samples were injected using a 7693 Agilent Series Injector. 37. Component mixture (Supelco # 18919) containing C4-C24 FAMEs was used as reference standard. Additionally, important physiochemical properties such as kinematic viscosity at 40C, density at 25C, flash point (closed cup) and acid number were determined using ASTM procedures.

Results and Discussion

Growth of Chlorella sp.

Growth of isolated *Chlorella* sp. in media with different NO_3 concentrations and constant 10 ppm PO_4concentration media were determined and the results (Fig. 1). *Chlorella* cells at exponential phase were inoculated into the reactor with the same initial cell concentrations, measured as optical density (OD_{550}).

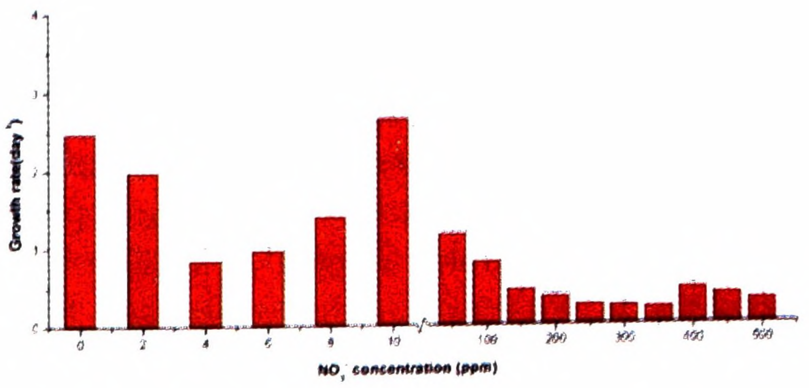

Fig. 1- Growth rates for *Chlorella* sp. at different NO_3^- concentrations

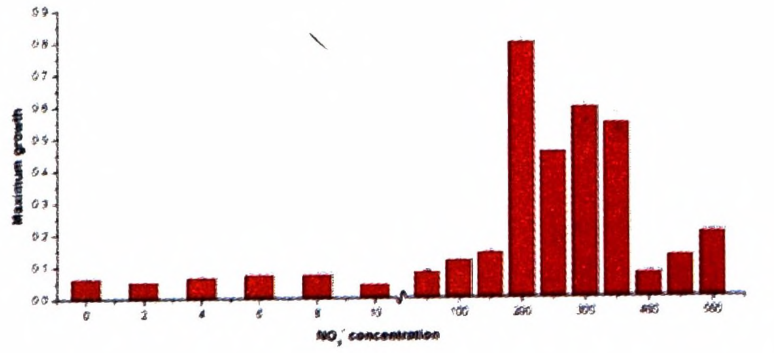

Fig. 2. Maximum growth for *Chlorella* sp at different NO_3^- concentrations

The results showed that the maximum growth was observed (Fig. 2) when $[NO_3]/[PO_4]$ ratio was kept at 16-20. Low maximum growth was observed in low $[NO_3]/[PO_4]$ media, but was effected in a short time period. Therefore, even though growth was less, a fairly high μ value can be obtained. High growth is observed with high $[NO_3]/[PO_4]$ media, but the time required to achieve maximum growth was high. Therefore, high μ values cannot be observed, even though it has a high nutrient concentration. According to Equation 1, μ is a parameter which indicates algal growth as well as time required to achieve maximum growth. Therefore, a high μ value is an indication of rapid growth and high biomass outcome. Nitrogen as NO_3 or NH_4 is the next important nutrient for biomass production after carbon and its main function is to produce chlorophyll which is essential for photosynthesis. It is also the basic element of plant proteins, including genetic material, and is important in periods of rapid plant growth. Although nitrogen plays a significant role, excess of it in the growth medium can stress nutrient intake and thereby decelerate bio mass production. According to the growth studies, algal cells in high N containing media took a longer time to achieve maximum growth than those in low N containing media. Similar results have been reported when the highest production rates for *Scenedesmus*, *Chlorella* and *Monoraphidium* species were measured in media with N and P concentrations exceeding 25 and 2 ppm, respectively. Therefore, the results indicate the requirement of an optimum nutrient level for maximum growth to take place rather than having a very high nutrient concentrations.

Lipid content of Chlorella sp.

The effect of nutrient concentration, especially N, for lipid content is more significant than operational factors such as shear produced by mixing, dilution rate, growth depth and harvest frequency. Since carbon intake flows towards fatty components rather than chlorophyll in nitrogen deficient photosynthesis, lipid content in a single algal cell is higher under a nitrogen deficient condition than in a nitrogen sufficient condition. Additionally, with nitrogen limitation, the available nitrogen is exhausted quickly by any carbon source to be converted into lipid or carbohydrate rather than protein or chlorophyll. Therefore, high lipid content per unit dry bio mass weight can be observed under N starvation. In addition to that, population of algal cells increases under N sufficient condition as chlorophyll promotes for the photosynthesis.

The three modes of product formation in terms of its relationship with microorganism growth have been classified as a. growth associated' products arising directly from the energy metabolism of carbohydrates supplied b. indirect products of carbohydrate metabolism and c. products apparently unrelated to carbohydrate oxidation. The effects of operating variables on the primary kinetic processes, growth, sugar utilization and antibiotic formation, in the penicillin process, illustrate the special nature of indirect product formation mode. A linear and growth associated relationship has been shown between lipid formation and micro algal growth for *Chlorella minutissima.* Therefore high lipid content per unit volume of algal culture can be observed under N sufficient conditions than in nitrogen deficient conditions as cell population is high, as shown in Fig. 3

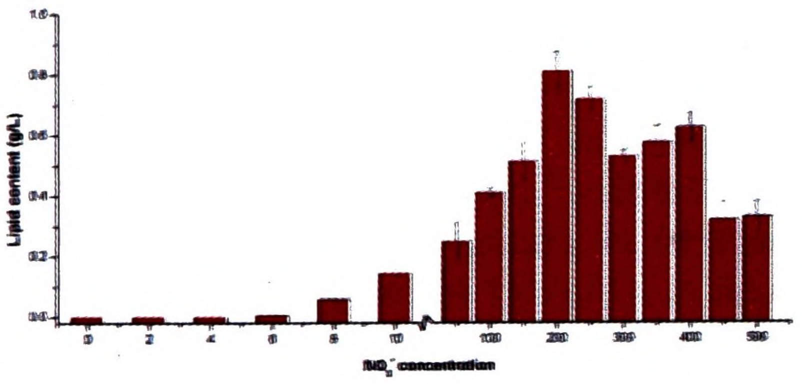

Fig. 3- Lipid content of *Chlorella* sp. at different NO_3^- concentrations

Biodiesel characterization

To assess the potential of biodiesel as a substitute of diesel fuel, values of some physicochemical properties of biodiesel should meet the international standard values. As other fuel types, behavior of biodiesel is totally controlled by its chemical composition. In there, FAME composition of biodiesel is the key factor and mainly, unsaturation level and length of the carbon chain are considered. The composition of the algae B100 produced is shown in Fig. 4A and 4B and Table 4. The results indicated total FAME content of 84.03%, which approximately meets the EN 14103 standard value of more than 90 % FAME content. Physical properties such as kinematic viscosity at 40 C, density at 25 C, flash point (closed cup) and acid number also met the required standard values. Therefore it is feasible to use this bio diesel as an energy source.

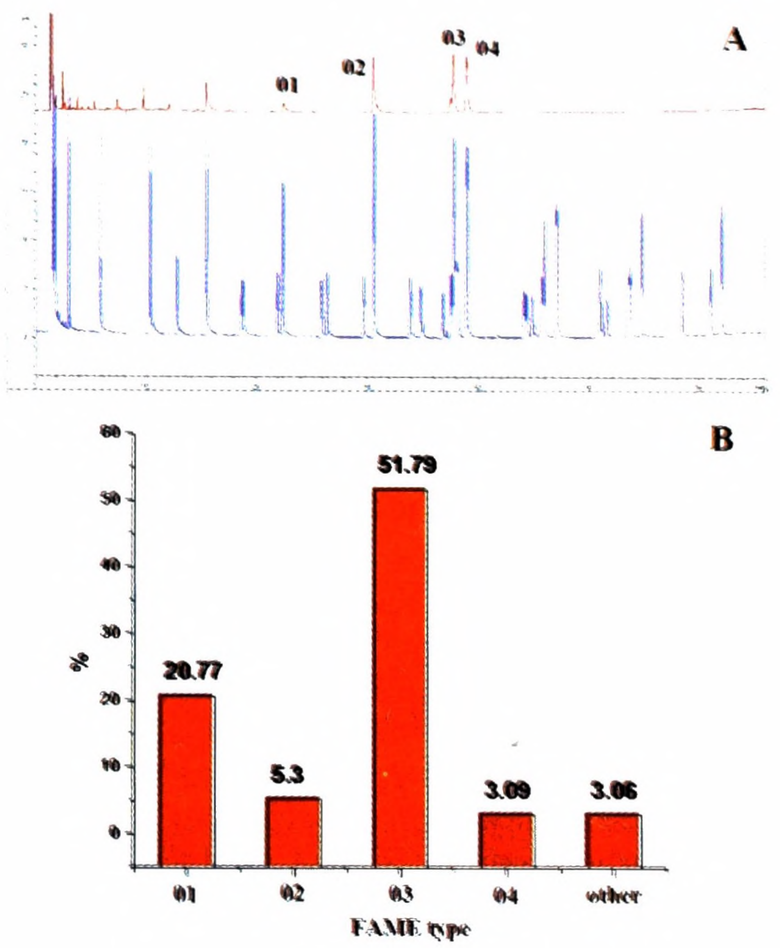

Fig. 4A. Gas chromatograms (Algae B-100 /Reference) Fig. 4B. Percentages FAME in B-100.
01 - Palmitoleic Acid Methyl Ester (C16:1),
02,- Stearic Acid Methyl Ester (C18:0),
03 - Linolelaidic Acid Methyl Ester (C18:2n6t)
04 Linoleic Acid Methyl Ester (C18:2n6c).

Conclusion

The present study showed the possibility of biodiesel production from oil extracted from *Chlorella* sp. cultivated under the heterotrophic condition with HCO_3 as carbon source). The effect of nitrate concentration on growth and lipid content were studied and nitrate concentration 150-200 ppm under 10 ppm phosphate was observed as the best values for maximum growth and high lipid content. At this concentration, 0.975 glof lipid could be extracted. The extracted lipids were converted into biodiesel using the two step method which is H_2SO_4 acid catalyzed pre-esterification followed by KOH catalyzed trans-esterification. The biodiesel produced was in consistence with international standards. The study therefore showed the possibility of utilization *Chlorella* sp. for production of biodiesel under laboratory conditions, which requires scale up to industrial scale for commercial production.

The authors are very grateful to the National Research Council, Sri Lanka for financial support (NRC grant No. 13-15 and to Dr. Gehan Jayasooriya, Department of Botany, University of Peradeniya for laboratory facilities for isolation of algal species.

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Table 4 - Physicochemical properties of Algae B-100 produced

Parameter	Test method	Algae B100	B100 limits		Unit
			min	max	
FAME content	EN 14103	84.03	90.0	96.5	% (m/m)
Linolenic Methyl Ester content	EN 14103	0.0	-	12.0	% (m/m)
Kinematic viscosity at 40 °C	ASTM D445	3.9572	3.5	5.0	mm^2/s
Density at 25 °C	ASTM D1298	876.9	860	890	kg/m^3
Flash point (closed cup)	ASTM D93	210	120	-	°C
Acid number	ASTM D5555	0.0692	-	0.80	mg KOH/g

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