THIRD GENERATION BIOFUEL PRODUCTION BY USING FRESHWATER MICROALGAE

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Abstract
Increasing cost of petroleum based fuel along with its impact throughout the world are main objectives in growth and development of alternate renewable energy resources. Among the liquid fuels, biodiesel is the most promising source since it can be used with the present engines without any modification of the engine design. Microalgae is considered as the fruitful energy source of biodiesel nowadays. Inorder to improve the growth rate, biomass production and lipid accumulation, heterotrophic mode of cultivation is highly preferred. Hence the present study compared the impacts of both autotrophic and heterotrophic mode of cultivation towards microalgal growth. From the fresh water three oleaginous microalgal strains (*Chlorella sp*., *Spirulina sp*., *Scenedesmus sp*.) were screened and chosen for further analysis. While comparing both environmental conditions(Autotrophic and heterotrophic) for biodiesel production using fresh water microalgae maximum biomass(2.52±0.015 g/L) was attained by the microalgal strains on culturing under heterotrophic condition. While analyzing the lipid content of microalgal strains no significant difference was found on the two mode of cultivation.
Keywords: Microalgae, Autotrophic, Heterotrophic, FAME, Biodiesel.

1. Introduction

The global economy confides on energy which is significant for its development. The depletion of fossil fuel along with crisis of global warming are the strongest driving forces of research towards biofuel production using animal fat, crops and microalgae (Xin et al, 2009). These renewable biofuels are both economically and environmentally concerned to be the greater developing area (Marchetti et al, 2005).
Comparing with the other options exploring microalgae have dragged considerable attention as a third generation biofuel resource (Gallagher, 2011). When compared with the oil based crops, microalgae possesses high lipid content with extreme growth rate under limited nutrients and avoid competition for arable lands (Liam and Philip, 2010). These microalgae are more conservative in the sense of producing upto 10 times of biodiesel produced by oleaginous crops in a unit area of land (Chisti, 2007).
The content and composition of microalgal lipid differ considerably from one species to another (Brown et al, 1997). The lipid content of microalgae would range from 5 to 77 % weight of the dry biomass (Brown et al, 1997, Chisti, 2007). The fuel properties like heating value, cold flow, viscosity, density and flash point of microalgal biodiesel is similar to that of petro-diesel.
Before industrialization of microalgal biodiesel numerous economical and technological hurdles have to be overcome. Successful outdoor cultivation of the efficient microalgae with inclined growth rate, lipid content and resistance towards normal microbes are the major challenges of biodiesel production (Sheehan et al, 1998). Along with these, improvements in effective lipid extraction from the microalgal cells are required for effective downstream processes.
In order to develop economically feasible biodiesel using microalgae, these organisms can be grown under heterotrophic conditions by provided with organic carbons like sugars and organic acids. Culturing microalgae under the above mentioned mode will overcome the light requirement and thereby improves cell biomass, productivity (Chen, 1996), rapid growth (Xu et al, 2006) with higher oil content within the cell (Miao and Wu 2004, Xu et al, 2006).
In the present investigation comparative study has been carried out on the employment of microalgae for biodiesel under both autotrophic and heterotrophic conditions. Based on the microalgal strains, nutrients and mode of environment involved biomass production and lipid accumulation ability of the microalgal species varied. Compared to autotrophic condition heterotrophic is advantageous with shorter incubation time, greater biomass production and lipid accumulation.

2. Materials and Methods

2.1. Isolation of microalgal isolates

The microalgal strains were isolated from the algal rich freshwater pond of Thirupparangundram, Madurai, South India. About 10ml of the water sample was aseptically transferred to 90ml of freshly prepared BG11 medium [$NaNO_3$-1500 mg/L, $K_2HPO_4.3H_2O$- 40mg/L, $MgSO4.7H_2O$ - 75 mg/L, Na_2CO_3 -20mg/L, $CaCl_2$ -27 mg/L, citric acid monohydrate - 6 mg/L, ammonium ferric citrate -6mg/L,Na_2 EDTA -1 mg/L , Trace element solution- 1ml(H_3BO_3 -2.86 g/L, $MnCl_2.2H_2O$ – 1.81g/L, $ZnSO_4.6H_2O$- 0.222g/L, $CuSO_4.5H_2O$ -0.079 g/L, $CoCl_2.6H_2O$ – 0.050 g/L, $Na_2MoO_4.2H_2O$ – 0.39g/L)] (pH 6.8). The inoculated flasks were incubated under illumination (1500-2000 Lux) of 12 hours/day for 14 days at 25°C (Henrard et al, 2013).

2.2. Screening of oleaginous property of the microalgal isolates

The oleaginous ability of the microalgal isolates were screened by nile red staining. About 250 µlof the microalgal cultures were vortexed under 10,000 rpm for 10minutes.The obtained pellet was suspended in freshly prepared 0.2 M Phosphate buffer solution (pH 7.0) and 25% DMSO. Then the suspension was mixed with 25 µl of freshly prepared nile red solution (0.5mg of nile red dissolved in 1ml of acetone) and incubated in dark for 10 minutes under room temperature (Lee et al, 1998). The lipid particles present within the cells were observed under fluorescent microscope (Nikon, Japan) with the excitation range between 450-490 nm and emission range of 520 nm.

2.3. Identification of microalgal strains

The microalgal strains which were confirmed to be oleaginous were identified based on the cell morphology. About 2 drops of axenically grown microalgal isolates were placed on a clean glass slide and covered with a cover glass. Then the slide was observed under microscope and observed with 100X magnification power.

2.4. Enrichment of the microalgal isolates

The oleaginous microalgal isolates were enriched by inoculating 5ml of the strains in 95ml of sterile freshly prepared BG11 medium. The inoculated flasks were incubated under illumination for 7 days as per the above mentioned method.

2.5. Production of biodiesel under autotrophic and heterotrophic condition

The oleaginous microalgal strains were employed for the biodiesel production under both autotrophic and heterotrophic condition. For autotrophic mode of cultivation about 5ml of enriched microalgal isolates were inoculated onto 95ml of sterile BG 11 medium [$NaNO_3$-1500 mg/L, $K_2HPO_4.3H_2O$- 40mg/L, $MgSO4.7H_2O$ - 75 mg/L, Na_2CO_3 -20mg/L, $CaCl_2$ -27 mg/L, citric acid monohydrate - 6 mg/L, ammonium ferric citrate -6mg/L,Na_2 EDTA -1 mg/L , Trace element solution- 1ml(H_3BO_3 -2.86 g/L, $MnCl_2.2H_2O$ – 1.81g/L, $ZnSO_4.6H_2O$-0.222g/L, $CuSO_4.5H_2O$ -0.079 g/L, $CoCl_2.6H_2O$ – 0.050 g/L, $Na_2MoO_4.2H_2O$ – 0.39g/L)].The inoculated flask were incubated at 24ºC under fluorescent light(1500-2000 Lux) with systemic light/dark cycle of 12/12 hours for 14 days.

For heterotrophic mode of biodiesel production 5ml of the enriched microalgal cultures were inoculated onto the 95ml of BG11 medium supplemented with 2% D-glucose. The flask were wrapped with aluminium foil to maintain darkness and incubated under shaking incubator for 7 days. The growth profiles of the selected organisms under the above mentioned optimum temperature and growth conditions were analyzed periodically.

2.6. Biomass analysis

The growth profiles of the microalgal isolates under both autotrophic and heterotrophic conditions were analysed at the end of incubation. Dry weight of all the isolates were measured gravimetrically in triplicates. The microalgal biomass which were collected by centrifuging 10ml of the cultures at 5000 rpm for 10 mins. The obtained pellets were suspended in distilled water and recentrifuged. Then the pellets were dried at 60ºC and the microalgal biomass was calculated by gravimetric measurement. All the measurements were statistically analysed to determine their mean value with standard error values by using Graph pad Prism software version 6.0.

2.7. Extraction and direct transesterification of biodiesel

The lipids were extracted and transesterified from the microalgal isolates by a one step extraction method proposed by Cheng et al., 2013. About 1 gm of the dried biomass was mixed thoroughly with 8ml of freshly prepared chloroform :methanol (1:1) and 0.2ml of H_2SO_4.The obtained mixture was heated using microwave for 40 seconds and then cooled for 20 mins. The mixture was then added with 15 ml of distilled water and centrifuged at 10,000 rpm for 10 mins. The amount of lipid seperated was quantified gravimetrically. The lipid concentration and lipid yield of the isolates were quantified by using the following equation.

$$C_L = \frac{Weight of lipid(g)}{Volume of fermentation broth(L)} \quad (1)$$

$$Y_L = \frac{Weight of lipid(g)}{Weight of carbon substrate consumed(g)} \quad (2)$$

2.8. Characterisation of biodiesel

The absorption peaks present in the obtained biodiesel was characterised by FT-IR analysis using SHIMADZU-IR spectrometer (Japan) at room temperature. The spectra were analysed between the wave number 4000–400 cm^{-1}.

The fatty acid profiles of the FAME (Fatty acid methyl ester) was quantified by Gas Chromatography SHIMADZU – (01224) gas chromatograph (Japan) with FID (Flame ionization detector). The DB5 capillary column was set at temperature of 100ºC and the injector and detector temperatures were maintained to 220ºC and 250ºC. Nitrogen was provided as carrier with pure air and hydrogen. The respective components present in FAME were detected and quantified as per the retention time and peak area.Which was done by correlating to a standard pattern using Schimadzu C-R4Achromatopac integrator without correction factor.

3. Results and Discussion

3.1. Isolation, screening and Identification of microalgal isolates

Ten different microalgal isolates were isolated from Thirupparangundram lake. Morphologically differerent colonies were separated and were identified by microscopic observation. The ability of oil production differs among the varied microalgal species. Other than the species the factors influencing oil production are the cultivation parameters like light intensity, temperature, pH, salinity, temperature, nitrogen and mineral sources. As per the observation of Illman et al, the nitrogen limiting environment would increase oil content in the strains of *Chlorella* (Illman et al, 2000). As per Liu et al, the concentration of Fe3+ and its time of addition are to some extent responsible for lipid accumulation (Liu et al, 2007).

Eventhough huge number of tests are available to detect the oleaginous property of the algal cells, Nile red fluorescence is the promising test for the rapid quantification of cellular lipid. Among the isolated microalgal strains, the cells which exhibited yellow to red fluorescence under microscope were screened for further analysis. The fluorescence exhibited by the microalgal cell is proportional to the amount of lipid bodies present within the cell. Hence organism with great fluorescence can be confirmed to the highly oleaginous strain.

The microalgal cell with globular shape, dense chloroplast and lack flagella was identified as *Chlorella sp*. (A1).The helical microalgal cell with transverse cross walls was identified as *Spirulina sp*. (A2). Two to three oval cells coalesce together was identified as *Scenedesmus sp*. (A3).

3.2. Enrichment and Lipid production

The microalgal cultures were enriched under phototrophic condition. BG11 medium is widely used for the enrichment of several microalgal species collected from water samples. The medium is useful for culturing microalgae under both photoheterotrophic and chemoheterotrophic conditions. BG11 medium was applied as the standardized medium screening facultatively heterotrophic algal strains (Zhou et al, 2011). The enriched microalgal samples were employed for lipid production under both autotrophic and heterotrophic environment.

3.3. Biomass & Lipid analysis

Heterotrophic growth conditions could provide significant increase in growth rate and cell density of microalgal cells than autotrophic conditions (Xiong et al, 2008). It has been reported that the algal cultivation under heterotrophic condition would provide huge biomass productivity as well as extreme oil content (Xu et al, 2006). Similarly in our present study greater biomass and lipid content were attained by the microalgal strains grown under heterotrophic conditions (Fig. 1 & Table 1).

Greater biomass of 2.52±0.015 g/L was attained by *Spirulina sp.* While culturing under heterotrophic condition which was followed by *Chlorella sp.* (2.33±0.036 g/L) under heterotrophic environment. While analysing the lipid content greater value was attained by *Chlorella sp*. (0.663 g/L) under both autotrophic and heterotrophic condition. Highest lipid concentration of 28.78±0.233% and 28.48±0.458 was attained by the *Chlorella sp*. under autotrophic and heterotrophic environment (Fig. 2 & Table 2).

3.5. Lipid extraction and transesterification

The lipids were extracted and transesterified to FAME in a single step assisted with microwave treatment (Fig. 3). After addition of respective solvents with acid catalyst, the transesterified FAME was collected from the upper organic phase. As per the observation of Gao et al, microwave treatment will probably increase the ratio of methyl esters in oleaginous microbes while analysed by GC analysis (Gao et al, 2012).

3.6.Characterisation of Biodiesel

3.6.1. FTIR analysis

As per the observation of Stuart the CH_2 stretching vibrations between the range of 3100-2800 cm^{-1}denotes the presence of lipid (Stuart, 2004). The absorption peaks at 2928 and 2860 cm^{-1} signifies the symmetric stretching in the lipids (Patel et al, 2015). In our present investigation, similar peaks like 2962.66, 2926.01 and 2858.51 cm^{-1} were observed (Fig. 4).

3.6.2. GC Analysis:

The presence of saturated, mono-unsaturated and poly-unsaturated fatty acids were determined by the GC analysis. The FAME produced by *Spirulina sp*. in our present work consisted of five major fatty acids like Cis-8,11,14-Eicosatrienic acid(C20:3), Cis-10-Heptadecanoic acid(C17:1), Pentadecanoic acid (C15:0), Caproic acid(C6:0) and Butyric acid (C4:0). It has been reported by Knothe et al, that increasing amount of saturation of FAMEs with fewer double bonds and increased number of carbon atoms in the fatty acid chain will contribute the increased cetane number of FAMEs (Knothe et al, 1998). The monounsaturated fatty acid such as Cis -10-Heptadecanoic acid (50.0940% & 16.6492%) was present in predominant amount. The saturated fatty acid like Caproic acid, Butyric acid and Pentadecanoic acid contributed 16.0995%, 7.7548% and 6.2323% of FAME. The polyunsaturated fatty acid, Cis- 8,11,14 - Eicosatrienic acid contributed 3.1702% of FAME (Fig. 5).

Conclusion

In the present investigation the freshwater microalgal strains were employed for biodiesel production. The biomass and lipid content produced during biodiesel production were studied under both autotrophic and heterotrophic environment. Among the two mode of production higher biomass was attained by *Spirulina sp*. under heterotrophic condition. But no significant difference was observed on the environmental condition on lipid accumulation. The GC analysis proved the presence of both saturated and unsaturated fatty acids in the needed a mount. This confirmed the efficient fuel quality of the biodiesel obtained.

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Figure 1 –Biomass analysis of the microalgal isolates under phototrophic and heterotrophic condition

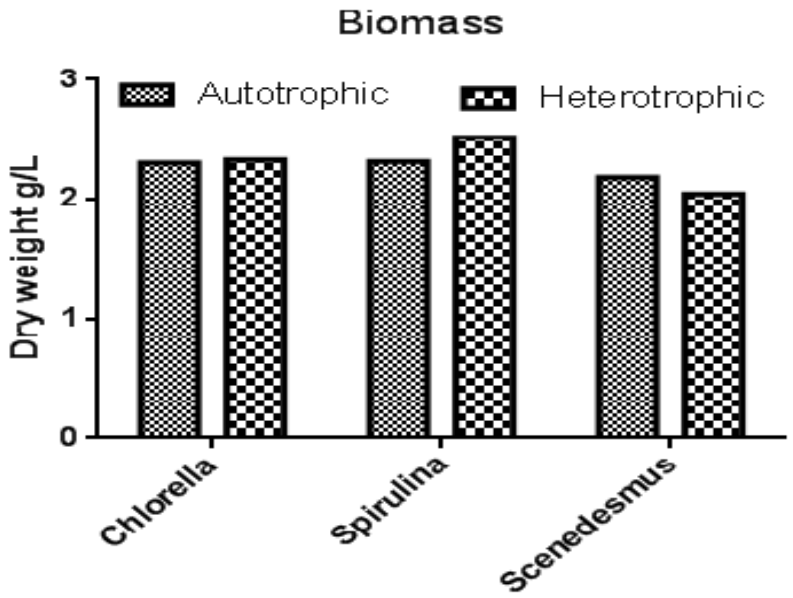


Figure 2 – Lipid analysis of the microalgal isolates under autotrophic and heterotrophic condition.

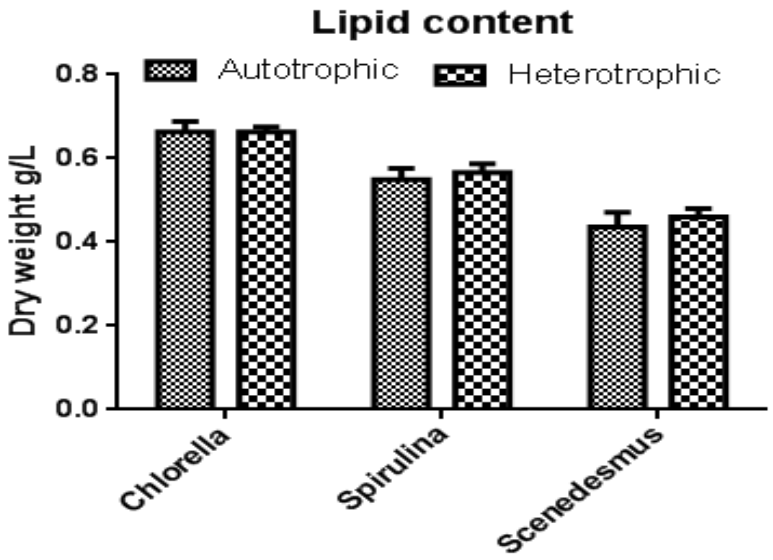


Figure 3 - Lipid extraction from the microalgal isolates.

Figure 4- FTIR analysis of the FAME extracted from Microalgal isolate *Spirulina sp.*

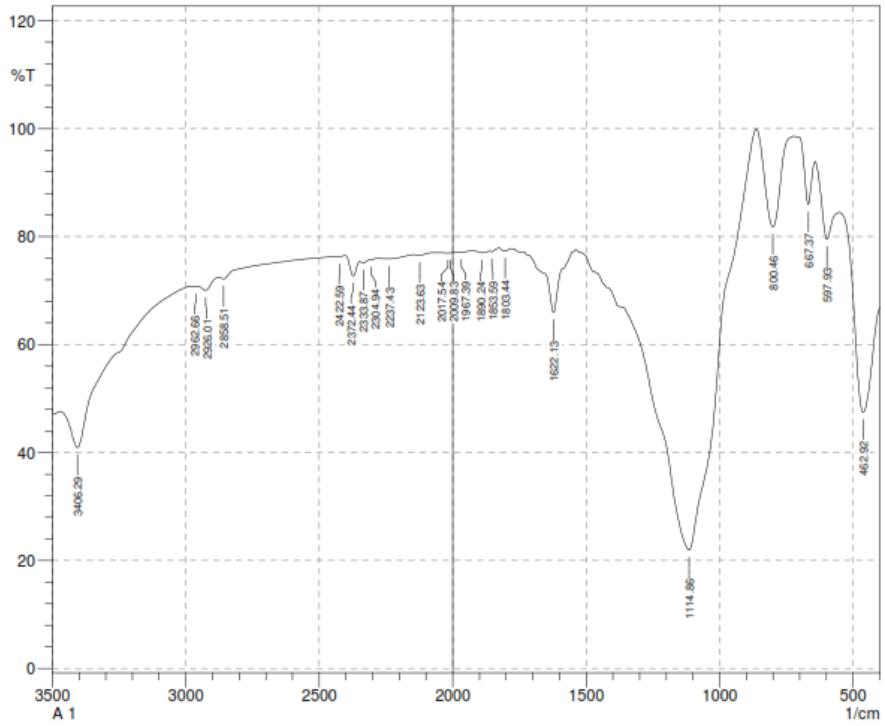
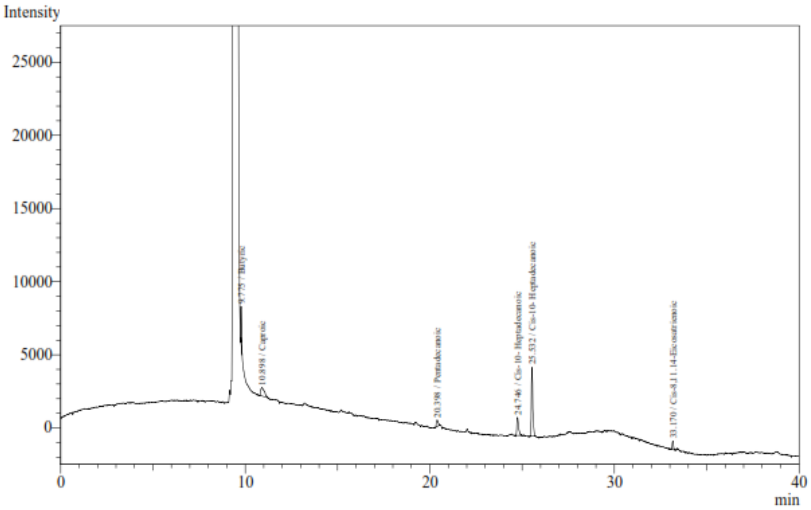


Figure 5 - Gas chromatography analysis of FAME produced by *Spirulina sp.*



Peak#	Ret.Time	Area	Height	Cmpd Name	Area%
1	9.775	3735	2484	Butyric	7.7548
2	10.898	7755	580	Caproic	16.0995
3	20.398	3002	433	Pentadecanoic	6.2323
4	24.746	8020	1230	Cis-10- Heptadecanoic	16.6492
5	25.532	24129	4706	Cis-10- Heptadecanoic	50.0940
6	33.170	1527	541	Cis-8,11.14-Eicosatrie	3.1702
Total		48168	9974		100.0000

Table 1- Biomass analysis of the fresh water microalgae under autotrophic and heterotrophic condition.

S.No.	Isolate	Autotrophic condition (g/L)	Heterotrophic condition (g/L)
1.	*Chlorella sp.*	2.30±0.038	2.33±0.036
2.	*Spirulina sp.*	2.32±0.012	2.52±0.015
3.	*Scenedesmus sp.*	2.18±0.009	2.05±0.029

Table 2- Lipid content and lipid concentration of the microalgal isolates.

S.No.	Isolates	Lipid content (g/L)		Lipid concentration %	
		Autotrophic	Heterotrophic	Autotrophic	Heterotrophic
1.	*Chlorella sp.*	0.66±0.015	0.66±0.007	28.78±0.233	28.48±0.458
2.	*Spirulina sp.*	0.55±0.015	0.57±0.012	23.75±0.771	22.52±0.587
3.	*Scenedesmus sp.*	0.44±0.020	0.46±0.012	20±0.904	22.47±0.251