➔DOI : 10.15740/HAS/AJBS/11.1/42-48
e ISSN-0976-8343 | ■ Visit us : *www.researchjournal.co.in* ■ – AFBSAH-2016 ASIAN JOURNAL OF BIO SCIENCE
Volume **11** | Issue 1 | April, 2016 | 42-48

RESEARCH **P**APER

Optimization studies for algae biofuels production

PRATEEK SINGH[1], DHEEBAN CHAKRAVARTHI KANNAN [2], VINEETA GURNANI[1], ARADHANA IRENE CHARAN[1] **AND** AMIT ALEXANDER CHARAN[1]

[1]Department of Molecular and Cellular Engineering, Sam Higginbottom Institute of Agriculture, Technology and Sciences, ALLAHABAD (U.P.) INDIA
[2] The Energy and Resources Institute, NEW DELHI, INDIA
Email : prateek0783@gmail.com

Article Info : Received : 05.12.2015; Revised : 08.01.2016; Accepted : 07.02.2016

Continuous use of fossil fuel is globally considered as unsustainable because of depleting supplies and these fuels also account for accumulation of green house gases in the environment. Renewable, carbon neutral, transport fuels are necessary for environmental and economic sustainability. Microalgae feedstocks are gaining interest in the present day energy scenario due to their fast growth potential coupled with relatively high lipid, carbohydrate and nutrients contents. All of these properties render them an excellent source for biofuels such as biodiesel, bioethanol and biomethane; as well as a number of other valuable pharmaceutical and nutraceutical products. It's a carbon neutral fuel. But high production cost is still a big hurdle in its commercialisation. Various optimisations are discussed in this research for the commercialization of algal biofuels. The production cost of algal biofuel is still quite high, so a lot of optimisation studies for growth parameters, lipid productivity and lipid extraction process are needed, which are discussed in this study. Commercialization of microalgae for biodiesel is technically feasible. Studies had shown that algae biofuel has the potential to completely displace liquid fuels derived from petroleum. Economics of producing microalgal biodiesel need to improve substantially to make it competitive with petrodiesel, but the level of improvement necessary appears to be attainable.

Key words : Microalgae, Biofuels, Nutraceutical, Biomass, *D. tertiolecta, D. salina*

How to cite this paper : Singh, Prateek, Kannan, Dheeban Chakravarthi, Gurnani, Vineeta, Charan, Aradhana Irene and Charan, Amit Alexander (2016). Optimization studies for algae biofuels production. *Asian J. Bio. Sci.*, **11** (1) : 42-48 [Special Issue of AFBSAH-2016].

INTRODUCTION

Algae are widely regarded as most efficient candidate for biofuel production. Microalgae appear to be the only source of renewable biofuel that is capable of meeting the global demand for transport fuels (Mata *et al.*, 2010 and Terry and Raymond, 1985). Studies have shown algae as an excellent source for biodiesel, bioethanol and biomethane production. Like plant, microalgae use sunlight to produce oil by the process of photosynthesis but has higher oil yield as compared most of the fuel crops.

Microalgae are accepted by the international experts and policy makers to play a crucial role in a clean environmentally sustainable future (Brennan and Owende, 2010; Chisti, 2007 and Greenwell *et al.*, 2009). More significantly they have a higher yield per hectare than most of fuel crops and can be cultivated non-arable land, thereby reducing the competition with food crops for land (Richmond, 2004 and Schenk *et al.*, 2008). Several studies have been conducted to calculate the cost of algae oil production from large scale farms. Huntley and Redalje (2006) estimated algae oil production costs to be $84 US/bbl. This scenario was based on the infrastructure cost assumptions by Benemann and Oswald but utilized a hybrid system (Combination of

raceway pond and photobioreactor) with an aerial productivity of 70.4 gm^{-2} day^{-1} and 35 per cent algal lipid yield. Microalgal oils can potentially completely replace petroleum as a source of hydrocarbon feedstock for the petrochemical industry but the cost of production needs to further optimization (Fukuda *et al.,* 2001; Huntley and Redalje, 2006). Studies have shown that the desired levels of cost reduction are substantial, but attainable. The objective of the present work was to compare the growth of *Dunaliella tertiolecta* and *Dunaliella salina* for biofuel production and furthermore optimization of various physical parameters is needed for cost effective biofuel production.

RESEARCH METHODOLOGY

Sample collection, isolation and identification :

Algae samples were collected from the sea coasts of Tamil Nadu and Karnataka. Ponds, lakes and back waters having stagnated water are well suited for algal growth. Ponds or lakes with a green tint have the maximum probability for find algae colonies. The samples were collected in 50 ml vials. The samples were inoculated on agar plate with different screening media composition. The plates were then kept in culture room and frequently analysed for morphological studies using microscope. The colonies of *D. tertiolecta* and *D. salina were* identified and then isolated and inoculated on a new agar plate. The colonies were successively screened and isolated to obtain a pure colony.

Media preparation :

Defined medium was used for the culture of marine strains of *D. tertiolecta and D. salina*. 1 L of defined medium was prepared by mixing 100 ml of macronutrients stock solution, 2 ml of micronutrient 50 g of NaCl, 5 ml of iron chloride solution and 10 ml of KH_2PO_4 in 880 ml of distilled water in the following order, respectively. pH of medium was adjusted to 7.5 using 1N HCl after addition of macronutrient stock solution.

Growth comparison of *D. tertiolecta* and *D salina* for biofuel production :

Medium was prepared as per the defined media composition. The prepared medium was then inoculated separately with the 20 per cent of *D. tertiolecta* and *D. salina* seed cultures. The experiment was conducted in triplicates in 18:6 light and dark cycle at 20° C. 3 ml of sample was collected and analysed using spectrophotometer on every alternate day. The obtained optical density (OD) was then plotted against number of days to get the corresponding growth curves for *D. tertiolecta* and *D. salina.*

Comparison of sea salt medium with the defined medium for cost effective algae culture :

Defined medium is the commonly used medium for the cultivation of marine microalgae species. Defined medium is composed of expensive salt which can be used for lab scale studies. But for the large scale cultivation and biofuel production it cannot be used, as it tends to increase the production cost. So using the raw sea salt for the preparation of artificial sea water is a cheap method to replace the defined medium salts in cultivation of marine algal strains in non-coastal regions, thus, lowering the overall production cost.

Three different media composition was prepared for the growth comparison in each media. First medium was the sea salt media formed by mixing of 36.84g/lit. raw sea salt into water. The quantity of sea salt to be added was molar equivalent to the concentration of NaCl in the defined media. Second medium was a control media which was prepared as per the defined media composition except the amount of NaCl added was 31.314 g/lit., which was equivalent to salt composition of 85 per cent NaCl as in sea water. Third medium was an extra control which was prepared as per the defined media composition by mixing 50g/lit. of NaCl.

Each of these Medias were prepared in duplicate batches and were inoculated with 25 per cent *D. tertiolecta* seed culture after autoclaving, to make the final volume to 800 ml. Zero day OD was recorded. The zero day concentration was also recorded from inoculum itself. After inoculation, the flasks were kept in shaker to avoid settling of cells and for the proper distribution of light. ODs were recorded on few days interval and the first harvest (semi-continuous harvest 25%) was made after the batches have attained high OD value. OD was taken at 680 nm. Three sets of readings were taken for each batch to minimize the error.

Harvest analysis :

Once the experimental batches attained suitably high OD value, 200 ml of culture was harvested (semi-continuous harvest) and the removed volume was replaced accordingly with new 200 ml media having 3

different compositions as mentioned earlier. The obtained 200 ml cultures were centrifuged at 10,000 rpm. The supernatant was discarded and the pellet was left for drying overnight. The weight of plates was pre- recorded. On the next day the dry mass was scraped from the plate surface and the plates were weighed again. The weight of dried biomass was obtained by subtracting the initial from the final weights of the plate.

Effect of nitrogen starvation for lipid enhancement in *Dunaliella tertiolecta* :

A number of factor have been shown to influence the lipid content of algae, such as nitrogen deficiency, phosphate limitation, salt stress, temperature fluctuation, light intensity and iron content of the medium. The amount of lipid produced in algae is inversely proportional to the conc. of nitrogen in the media while the number of cell is directly proportional to the conc. of nitrogen in the medium. Algae exhibits restricted cell division, increase in cell size and increase in lipid:amide I and carbohydrate:amide I ratios over time in response to N-limitation in the growth media (Dean *et al.,* 2010). Lipid tends to accumulate in the algae cell as a response to the nutrient stress and the cell ceases to multiply.

In this experiment the appropriate amount of the salts except $KNO_{3,}$ were added as per the defined media composition and autoclaved. A separate 100 x solution of KNO_3 was prepared by adding 2.5g of KNO_3 in 10 ml of sterilized RO water. The autoclaved medium was then divided into 6 different batches having triplicates of each batch, representing the 6 different conc. of KNO_3 as per the Table A and the media was then inoculated with 20 per cent *D. tertiolecta* inoculums and 3 sets of samples were taken from the inoculated culture for taking O.D. 3 sets of 20ml samples were taken from original inoculum for finding the concentration of each batch by multiplying with the dilution factor.

Table A: Amount of KNO_3 per ml and the corresponding concentration

Conc. of KNO_3 (g/lit.)	Amt. of KNO_3 added from the 10ml stock (ml)
0.05	0.2
0.1	0.4
0.2	0.8
0.35	1.4
0.5	2
1	4

Lipid analysis protocol :

The lipid was analysed by harvesting from each of the n-starved batch. The collected sample was centrifuged at 5000 rpm for 15 minutes and the supernatant was discarded and the pellet was retained and washed, using R.O. water. The pellet was transferred to pre weighed vials (w_1). The vials were placed in hot air oven at 100ºC for overnight to dry the water and weighed on the next day (w_2). Biomass was then calculated by subtracting w_1 from w_2. The biomass obtained was then analysed for the lipid. For every 1g of algal biomass 2 ml of methanol and 1 ml of chloroform was added and the solution was kept at 25ºC for 18 hrs. The mixture thus obtained was then agitated for 2 minutes. 1 ml of chloroform was again added to the mixture and was vigorously agitated for 1 minute. 1ml of distilled water was added and the solution was agitated for 2 minutes. The obtained solution was centrifuged for 10 minutes at 2000 rpm. The supernatant was stored separately and the whole sequence of process was repeated with the pellet. The two supernatant obtained were collected and were allowed to stand for 2 hrs. The lower organic layer with the lipid was transferred to a clean pre weighed vial (w_3). The vials were kept in hot air oven at 80ºC for 50 minutes to evaporate the solution. The vials were again weighed after drying. (w_4). Lipid content was then calculated by subtracting w_3 from w_4. Lipid was extracted using Folch method (Folch *et al.,* 1957).

Transesterification of algae biodiesel :

0.13g of lipid was extracted using 0.148119798 ml methanolic NaOH (Methanol + NaOH) for the extraction process. NaOH was used as catalyst for the reaction. The conical flask containing the mixture was kept on stirrer at 60° C (as boling point of methanol is 65°C) for 90 minutes for the transesterification step. The mouth of the conical flask was sealed with the help of aluminium foil to avoid any kind of evaporation loss. After 90 minutes, the flask was removed from the stirrer and hexane was added to the cooled transesterified lipids in the flask. Hexane tends to dissolve the methanol and lipid separating it from the pigments and glycerine, produced in the transestrification reaction. The hexane layer was then collected carefully in a glass vial. The conical flask was rinsed twice with hexane to avoid any lipid loss. The lipid was then concentrated by evaporating the excess hexane. The lipid sample can be analysed directly using GC-MS.

RESEARCH FINDINGS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Growth comparison of *Dunaliella tertiolecta* and *Dunaliella salina* :

D.tertiolecta and *D.salina* are two marine strains commonly used for lipid production. Both are comfortably grown in defined media under similar conditions and so they have similar cultivation cost. So a growth study is needed for selection of strain having high commercialization potential. Result has shown that both *Dunaliella tertiolecta* and *Dunaliella salina* have an equal growth rate. Both the strains grew at an equal rate for the first 6 days, having a similar OD vs day's trend. Then after 6 days *D. salina* has shown slight higher growth but on the 20th day they have an equal concentration. Both of these strains have comparable growth rate. Thus, the study shows that *Dunaliella tertiolecta* and *Dunaliella salina* had an equal potential for algal biofuel production (Fig. 1).

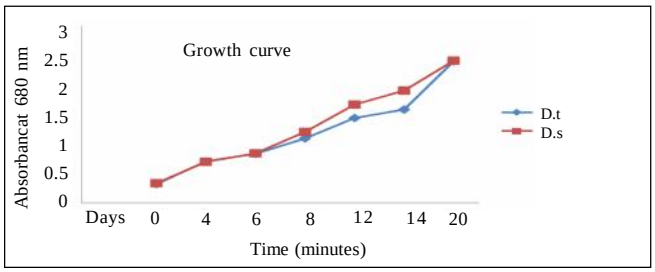

Fig. 1: Growth comparison between *Dunaliella tertiolecta* and *Dunaliella salina* marine algae strains

Comparison of sea salt media with the defined media for mass algal culture :

Marine algae are grown in the defined media for lab studies. Using defined media salt for pilot scale algae cultivation is very costly venture in non-coastal areas. So for the large scale production of algae for biofuel, minimizing the media cost is the first and most important step. *Dunaliella tertiolecta* was selected for the media replacement experiment. The results obtained from the Fig. 2 have shown that the sea salt batch culture has higher growth rate and is capable to be used as replacement for the expensive defined media salts. So it can be concluded that the use of sea salt for culturing of marine algal strains is a cost effective step and has potential to lower the overall production cost.

Effect of nitrogen deprivation on algae lipid production :

Dunaliella tertiolecta was grown autotrophically in defined medium with different KNO_3 concentration. The source of nitrogen as per the defined medium is

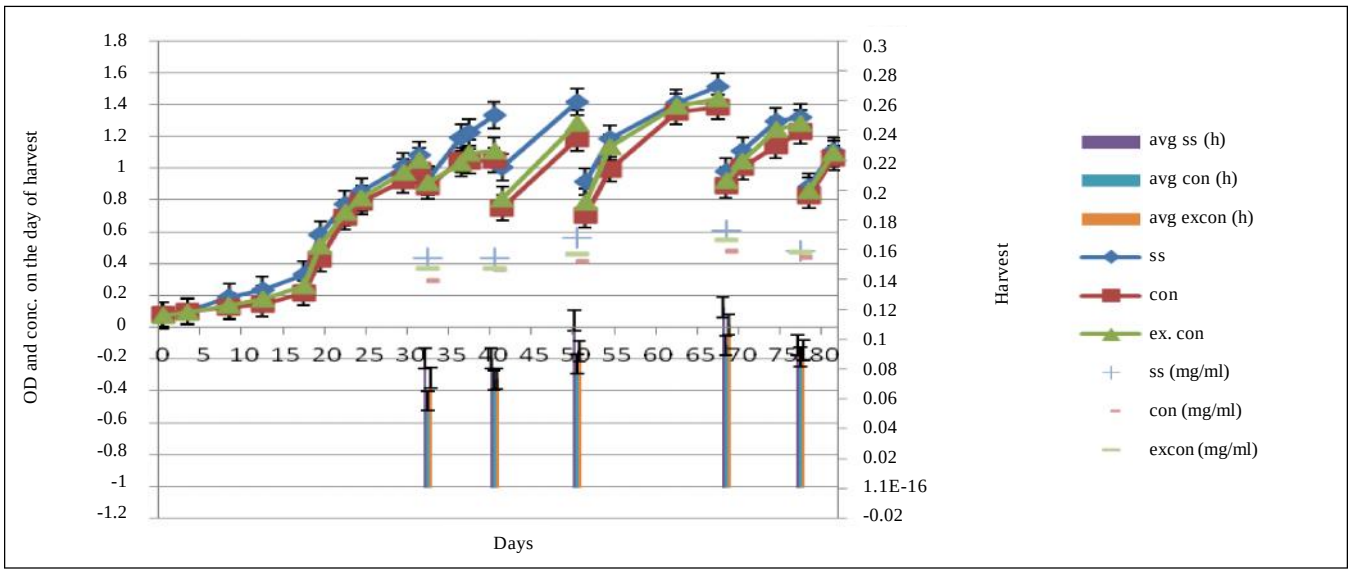

Fig. 2: ss, con, ex.con indicates the OD of culture at corresponding days. avg ss (h), avg con (h), avg excon (h) indicates the amount of harvest (g/200 ml). ss (mg/ml), con (mg/ml), excon (mg/ml) indicates the concentration on the day of harvest for the sea salt, control and extra control media respectively

KNO_3. So different sets of nitrogen concentrations 0.05, 0.1, 0.2, 0.35, 0.5 and 1 g/lit. was studied. Results have shown that by increasing the KNO_3 concentration, growth and biomass production is enhanced. Biomass concentration was found to be highest in the batch having 1g/lit. KNO_3 concentration. The cell concentration was found to be decreasing with the decrease KNO_3 concentration. So KNO_3 concentration was found to have a direct effect on the biomass concentration. For economical production of biofuel from microalgae, biomass as well as lipid content plays an important role. In the experiment, the results obtained are shown in Fig. 3 and 4 which indicated that high concentration of nitrogen source supported high biomass production but lipid content of cell was hampered by high nitrogen concentration. Growth of *Dunaliella tertiolecta* is directly proportional to the concentration of nitrate in the medium and the lipid productivity is inversely proportion to the nitrate content of the media.The present experiment suggest, the most effective approach to enhance lipid in *Dunaliella tertiolecta* is to grow it autotrophically in growth medium with concentration of 0.05g/lit. KNO_3 as per Fig. 4, which has given the highest lipid productivity among the other experimental batches.

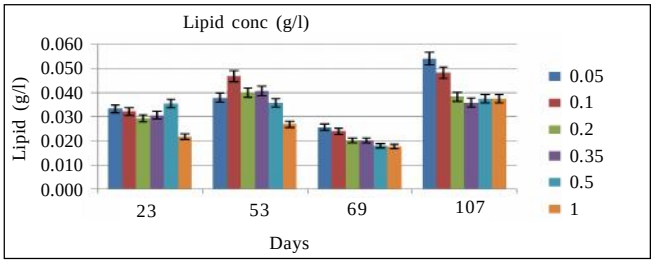

Fig. 4: Comparison of lipid content of *Dunaliella tertiolecta* grown on defined medium with different KNO_3 concentration (0.05-1 g/lit.)

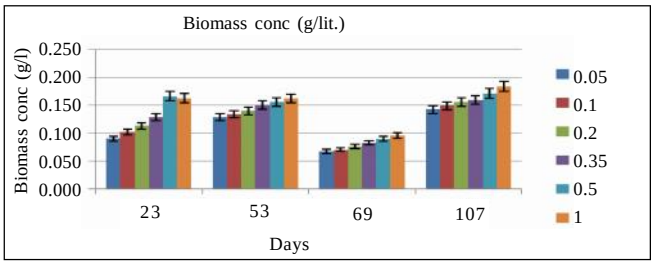

Fig. 3: Biomass production of *Dunaliella tertiolecta* grown on defined medium with different KNO_3 concentration (0.05-1 g/lit.)

FAME analysis :

The fatty acid profile was obtained for the algal sample from GC (Fig. 5). The obtained sample profile was then compared with the standard sample for identification of the unknown peaks. The concentration and percentage of the fatty acids methyl esters in the lipid sample is listed and analysed in Table 1. The algal sample is composed of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) in different proportion.

Table 1 : Concentration and percentage of FAME in the algal lipid sample

Fatty acids	Concentration (mg/ml)	Percentage	Fatty acids2	Concentration (mg/ml)	percentage
C4	112.38178	98.1629	C17	4.96E-02	0.0434
C10	1.79E-02	0.0156	C17:1	4.79E-02	0.0418
C11	3.11E-02	0.0272	C18	5.56E-01	0.4857
C12	3.79E-02	0.0331	C18:1	1.22992	1.0743
C14	4.56E-02	0.0398	C18:2 TANS	1.78797	1.5618
C14:1	1.00E-01	0.0876	C18:2 CIS	1.1714	1.0232
C15	6.59E-02	0.0575	C20	2.32E-01	0.2029
C15:1	2.43E-01	0.2126	C20:01	7.09E-02	0.0619
C16	2.37E-01	0.2071	C18:1	5.85E-02	0.0511
C16:1	1.32397	1.1565	C20	7.73E-02	0.0676
			C22:2	5.36E-02	0.0469
			Total	114.4849482	
SFA				94.92%	
MUFA				2.51%	
PUFA				2.51%	
				For aviation fuel	
				For biofuel	

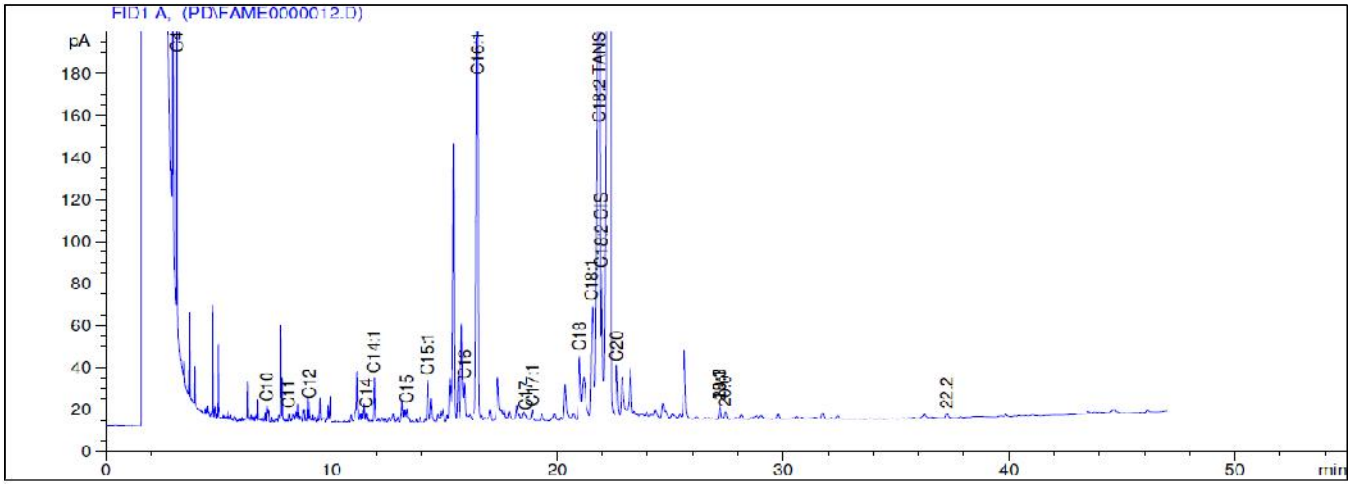

Fig. 5: Gas chromatogram of sample prepared

Based on analysis from the Table 1, it is seen that the lipid has an equal percentage of PUFA and MUFA content. The monounsaturated fatty acids between C12 to C15 are suited for their use as aviation fuels so algal biofuel can be a cheaper source of aviation fuel replacing the current expensive jet fuels. The monounsaturated fatty acids in the range between C15 to C20 are considered to be well suited for being used as biodiesel. From the chromatogram it was found that the algal biodiesel has a great opportunity and scope for being used as an alternative source of biodiesel. The high percentage of saturated fatty acid may be a problem in cold countries as the oil will freeze at low temperature. So this problem is needed to be solved by decreasing the percentage of SFA in the biodiesel in the refining step.

Conclusion :

Algae are considered as the most suitable candidate for biofuel production and for the replacement of the unsustainable fossil fuels. But still lot of research and development is needed for the successful commercialization of algae biofuel. Study of algal biology is the first step for the commercialization of algal biofuel. Selection and screening of algal stains is done based on these studies. High growth rate and high lipid and biomass productivity are the few desired characteristics of the strains selected for commercial biofuel production. Growth comparison for the *Dunaliella tertiolecta* and *Dunaliella salina* has shown that both of these marine strains have a comparable growth rate and are equally suited for biofuel production. Robust and stable strains are best suited for commercial scale biofuel production.

Cultivation of algae is the next step after strain selection. Land, water, and nutrient should be utilized sustainably and in a cost effective manner. In an experiment for testing the use of sea salt as nutrient for *Dunaliella tertiolecta*, it was found that the sea salt gave comparable growth as compared to the expensive defined media salts. It is, thus, recommended based the result obtained that sea salt is a cheaper and more suitable source of nutrition for *Dunaliella tertiolecta* in the regions distant from the sea coast. It was observed in the study that sea salt can be used as a replacement for expensive salts that are used in the defined medium for the cultivation of marine strains in non coastal areas.

Several biotic and abotic stresses during algae cultivation are found to cause lipid enhancement in algae. Study for nitrogen stress was done on *Dunaliella tertiolecta* and it was found that the stress condition tends to increase the lipid content inside the nitrogen stressed cells. So nitrogen stress could be one of the available options for increasing the lipid productivity. Research is needed in the extraction process for the selection of most efficient method. Now day's sonication, microwave, solvent systems, supercritical fluid, subcritical water, selective extraction, and secretion are the few extraction methods used. But the method having the best lipid yield should be selected for the commercial scale. Furthermore the by product formed in lipid production should also be evaluated and can be sold for reducing the overall production cost.

From the FAME (Fatty Acid Methyl Ester) analysis it was found that the transesterified lipid has an equal percentage of mono-unsaturated fatty acid methyl esters

(MUFA) and poly-unsaturated fatty acid methyl esters (PUFA). Based on FAME analysis it can be concluded that the algal lipid has capacity to be used as aviation fuel. Replacing the current expensive aviation fuel with algal fuel is a promising area of research and has got bright future.

LITERATURE CITED

Abu, G.O., Ogbonda, K.H. and Aminigo, R.E. (2007). Optimization studies of biomass production and protein biosynthesis in a *Spirulina* sp. Isolated from an oil polluted flame pit in the Niger delta. *Afr. J. Biotechnol.*, **6**: 2550–2554.

Brennan, L. and Owende, P. (2010). Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. *Renew. Sust. Energ. Rev.*, **14**: 557–577.

Chen, W., Zhang, Q. and Dai, S. (2009b). Effects of nitrate on intracellular nitrite and growth of Microcystis aeruginosa. *J. Appl. Phycol.*, **21:** 701–706.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnol. Adv.* **25** (3): 294–306.

Dean, A.P., Sigee, D.C., Estrada, B. and Pittmann, J.K. (2010). Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresour. Technol.*, **101**: 4499–4507.

Folch, J., Lees, M. and Stanley, Sloane G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Bio.Chem.*, **226** : 497.

Fukuda, H., Kondo, A. and Noda, H. (2001). Biodiesel fuel production by transesterification of oils. *J. Biosci. Bioeng.*, **92**: 405–416.

Greenwell, H.C., Laurens, M.L., Shields, R.J., Lovitt, R.W. and Flynn, K.J. (2009). Placing microalgae on the biofuels priority list: a review of the technological challenges. *J. Roy. Soc. Interface*. doi: 10.1098/rsif.0322.

Huntley, M. and Redalje, D. (2006). CO_2 mitigation and renewable oil from phytosynthetic microbes: A new appraisal report, University of Hawaii, University of Mississippi. Mitigation and Adapatation strategies for global change. United States.

Kacka, A. and Donmez, G. (2008). Isolation of *Dunaliella* spp. from a hypersaline lake and their ability to accumulate glycerol. *Bioresour. Technol.*, **99**: 8348–8352.

Mata, T.M., Martins, A.A. and Caetano, N.S. (2010). Microalgae for biodiesel production and other applications: A review. *Renew. & Sust. Energy Rev.*, **14**: 217-232.

Richmond, A. (2004). *Handbook of microalgal culture: biotechnology and applied phycology*. Blackwell Science Ltd. Iowa State Press, A Blackwell Publishing Company, 2121 State, Avenue, Ames, IOWA, U.S.A.

Schenk, P., Thomas-Hall, S., Stephens, E., Marx, U., Mussgnug, J., Posten, C., Kruse, O. and Hankamer, B. (2008). Second generation biofuels: High efficiency microalgae for biodiesel production. *Bio Energy Res.*, **1**: 20-43.

Terry, K.L. and Raymond, L.P. (1985). System design for the autotrophic production of microalgae. *Enz. Microb. Technol.*, **7**: 474–87.