Facile and Novel Strategy for Methods of Extraction of Biofuel Grade Lipids from Microalgae- an Experimental Report

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Abstract: The structural features of microalgal cell make it too difficult to extract the total lipid content of the cell as such. Thus, the cell disruption before lipid extraction becomes mandatory and has to be cost-effective. In the present study various methods and combination of few methods were adopted for effective extraction in order to choose the most effective cell disruption method for the complete extraction of lipids from a selected indigenous freshwater isolate, *Scenedesmus* sp. NTEB03. Interestingly, we found that grinding and bead-beating method showed two fold increased lipid productivity (23.2%) than the other methods tested. Biomass and lipid productivity of *Scenedesmus* sp., was found to be 0.0418 g L^{-1} d^{-1} and 4.3 mg L^{-1} d^{-1} respectively. Fatty acid profiles revealed that oleic (C18:1) and linoleic acid (C18:2) content being higher in the lipids, which are most appropriate for the biodiesel production. A novel strategy for most effective, simple method for cell disruption in *Scenedesmus* sp., was grinding/bead-beating, which is the most suitable method for complete extraction of biofuel grade lipids.

Keywords: Biodiesel, Cell disruption, Lipid extraction, *Scenedesmus* sp. GC analysis.

1. INTRODUCTION

Microalgae are known as an economical and potential raw material for biofuels due to their efficient ability to convert sunlight and CO_2 to biomass. Of the tremendous climatic changes and world demand of fossil fuel, it is urged to find alternate fuel sources. In the world scenario, alternate and renewable fuel from bioresources would be much better than other sources. From past two decades researchers have paid attention on biofuel from plants, animals and algae and other resources [1]. Biofuels are renewable, nontoxic and biodegradable and eco-friendly in the sense that their combustion will produce little, if any, emission of harmful green house gases [2]. Due to limitation of other resources, microalgae based oil is considered to be a promising candidate for biofuel. Microalgae have diverse beneficial features such as good biomass productivity, rapid lipid accumulation, ability to grow in fresh water to marine waters including of waste waters. They are well known to accumulate maximum amount of lipids and widely used as feedstock for biodiesel production [3]. There are notable advantages of producing oil from microalgae than other higher plants, such as cultivation do not require much land, rate of photosynthetic efficiency, biomass production and growth rate are higher than the rest [4-6].

The microalgal lipid extraction is known to increase with the degree of cell disruption. During cell disruption, cells are disintegrated, intracellular lipids and cellular structures are liberated into the microenvironment [7-10]. The steps involved in biodiesel production are cultivation, harvesting, lipid extraction and the transesterification of the lipids. Even though all these steps are essential, cell disruption is particularly important, as the quantity of lipid extracted was determined by the disruption method used [9]. Therefore it is very important that the method employed for cell disruption should ensure the complete extraction of lipids.

There are several methods in practice for the extraction of lipids from diverse bioresources, such as microwaves, sonication, autoclave, osmotic shock, mechanical grinding, bead-beating, antibiotics, detergents, enzymes which could be make use in the disruption of microalgal cell for lipid extraction [9]. The microalgal lipid inclusions which are bound by membranes or cell walls are not readily available for extraction [11] and the recovery of such lipids can be

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improved upto three fold by disrupting the cells [9,12]. Although several methods have been described, a consistent method has not yet been standardized. Optimization of biomass production, harvest and extraction of lipid is mandatory for algal oil industry, which could make it cost-effective and simple. The present study analyzed various methods of cell disruption on an oleaginous microalga, *Scenedesmus* sp. and checked the suitability for feedstock to biodiesel production by comparing their biomass, lipid content and fatty acid profile. Outcome of the method can be directly utilized to oil industry for effective extraction in simple and cost-effective manner.

2. MATERIALS AND METHODS

2.1. Culture Isolation and Growth

Scenedesmus sp., NTEB03 was isolated from a stagnant fresh water body near Mathur, Tiruchirappalli district, Tamilnadu, India. The collected strain was maintained in the microalgal repository of the Department of Microbiology, Bharathidasan University, India. Culture was grown in Chu 10 medium under white photo-fluorescent light at a rate of 45μEm^{-2}s^{-1} with a photoperiod of 12/12h light /dark at 24°C for 20 days. The Chu 10 medium comprised of 0.232g of Calcium nitrate, 0.01g of Dipotassium hydrogen phosphate, 0.025g of Magnesium sulphate, 0.02g of Sodium carbonate, 0.044g of Sodium silicate, 3.5 mg of Ferric ammonium citrate, 3.5 mg of Citric acid, and 1000ml of distilled water. 1 ml of trace metal mix comprising Boric acid (2.4g), Manganeous chloride (1.4g), Zinc chloride (0.4g), Calcium chloride (0.02g) and Copper chloride (0.1g) in 1000ml was also added to the medium.

2.2. Measurement of Growth and Harvesting

Growth rate of *Scenedesmus* sp. was determined by measuring the optical density. Briefly, about 2 ml of well-shaken culture was drawn at an interval of two days. During every sampling, 2 ml of fresh Chu 10 medium was added in order to compensate the acquired volume. Optical density was measured at 600 nm using UV-vis spectrophotometer (Cary 60, Agilent, USA). The biomass was harvested by centrifugation and was lyophilized for 24 hrs under vacuum.

2.3. Disruption of Cells

An aliquot of 500 mg of the cell biomass was resuspended in 100 ml of distilled water and the mixture was subjected to disruption using 8 different methods as follows: 1) Autoclaving at 121°C for 5 minutes 2) bead-beating using a bead beater (bead diameter 0.1 mm) at a high speed of 3000 rpm for 10 min. 3) microwave method using a microwave oven at a high temperature for 5 min 4) sonication using an ultra sonicator (JY92-IIN,USA) at a resonance of 15 kHz for 5min 5) osmotic shock using a 10% NaCl solution with a vortex for 5 min and incubated for two days 6) mechanical grinding with a mortar and pestle for 5 min 7) keeping in water bath at 70°C for 1 hour and 8) a combination of mechanical grinding and bead beating.

2.4. Lipid Extraction

The total lipids were extracted by mixing methanol:chloroform (1:2 v/v) with the samples in a proportion of 1:1 using the modified method of Bligh and Dyer [13]. The mixture were transferred into a separatory funnel and shaken well for 20 minutes. Finally the extracted lipid was collected by evaporating the solvent under rotary vacuum evaporator. The total lipids collected from the sample were measured using an electronic balance.

2.5. Fatty Acid Composition Analysis by Gas Chromatography

Fatty acid methyl esters (FAME) were prepared by using a modified protocol [9]. Briefly, 50 mg of samples were taken in a reflux tube, it was saponified with 1 ml of saturated potassium hydroxide/methanol (KOH-CH_3OH) solution at 75°C for 10 min, and then it was subjected to methanolysis with HCl (5%) in methanol at 75°C for another 10 min. Thereafter the aqueous phase was extracted twice with ethyl acetate. The organic layer containing the FAME was then collected, dried over anhydrous sodium sulphate to remove any excess moisture content. The FAME was analyzed by Gas chromatography. Fatty acid composition analysis was performed by using the Gas chromatograph (Shimadzu GC 2014, Japan) with flame ionization detector (FID). 1 μl of sample was injected into FAME WAX column (Restek,USA) (30mx32mmIDx25μm film thickness). The temperature program was as follows: initial 140°C with 5min hold; ramp 2°C/min to 230°C with a 5min hold. Column flow was set at 22.2ml /min. The instrument condition was as follows: carrier gas nitrogen: FID set at 260°C, and split ratio of 10:1. The run time for a single sample was 55min. The components were identified by comparing with the standard fatty acids used.

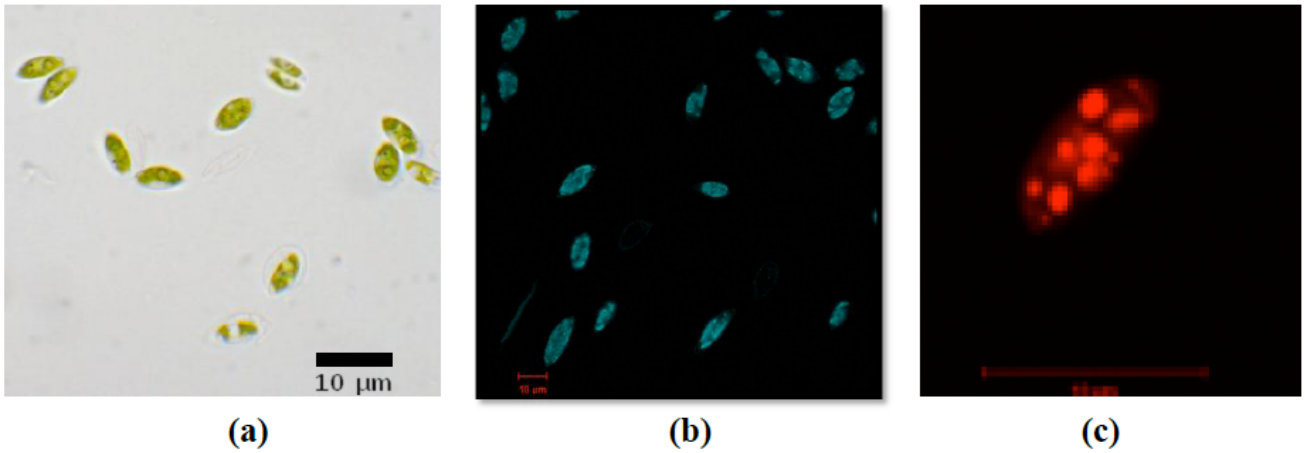

Figure 1: Microphotograph of *Scenedesmus* sp. NTEB03: Bright field (**a**); Confocal (**b**) and showing lipid inclusions stained by Nile Red (**c**).

3. RESULTS AND DISCUSSION

3.1. Cell Growth and Harvest

Microalgal sample was collected from a stagnant fresh water body near Mathur, Tiruchirappalli, Tamilnadu, India. In total five strains were isolated and morphologically identified using microphotographic system (Micros Austria) and documented namely *Scenedesmus* NTEB03, *Chlorococcum* NTEB09, *Chlorella* NTEB10, *Spherocystis* NTEB11, *Scenedesmus* NTEB12. All the isolated strains were deposited in the microalgal repository. Among the isolates *Scenedesmus* NTEB03 was found to be dominant and producing considerable amount of biomass. It was then chosen for further studies. According to our previous studies on the optimization of growth medium for *Scenedesmus* sp., Chu 10 was found to support well by significant increase in biomass. All the experiment and storage of the microalgae was done with Chu 10 medium [14]. The confocal microscopic image showing the lipid inclusions inside the strain using Nile red staining was shown in Figure **1**. The growth curve of the isolate was studied from the optical density determined every two day interval from the day of inoculation (Figure **2**). A clear idea about different phases of growth was obtained from the growth curve and the culture reached a stationary phase on 12th day of incubation. On the 16th day of incubation the strain was found to enter the decline phase.

3.2. Comparison of Cell Disruption Methods and Lipid Extraction

Different types of disruption methods were used for the extraction of lipids and they are broadly divided into four categories; namely, mechanical, physical,

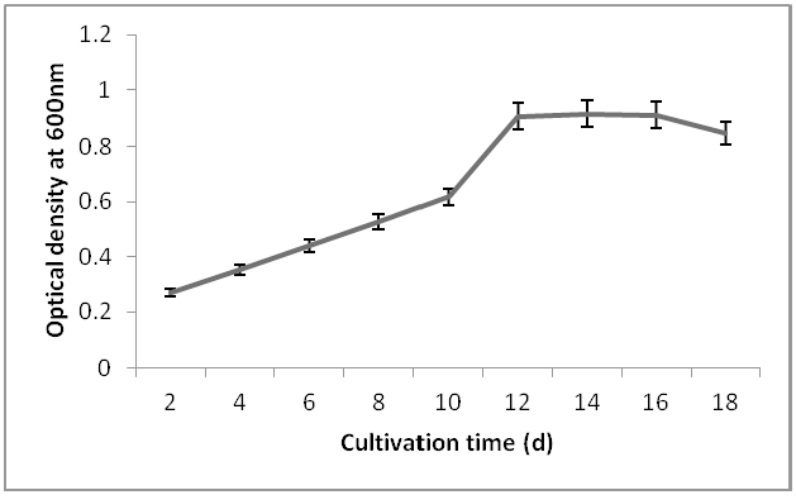

Figure 2: Growth curve of *Scenedesmus* sp. NTEB03, cultured in Chu 10 medium: stationary phase starts at the 12th day. Data were the mean values and standard deviations of three replicates (n=3).

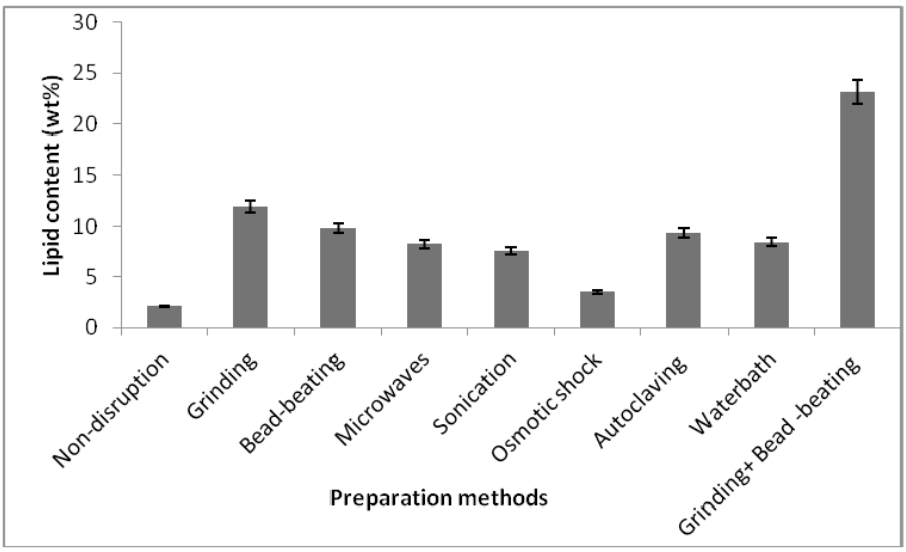

Figure 3: Lipid extraction efficiency according to different cell disruption methods for the *Scenedesmus* sp. NTEB03; grinding and bead-beating showed high lipid extraction of about 23.5%. Data were the mean values and standard deviations of three replicates.

chemical and enzymatic but, an optimized disruption method has not yet been developed [15]. It was reported that a method that works well in one organism may not be the method of choice for another organism [16]. Thus, we attempted to standardize an efficient extraction method using a moderate lipid containing *Scenedesmus* sp. as a model organism. As reported earlier dry biomass of microalgae contains about 5 to 77 percentage of lipids, which has been found to vary in consistency from one species to another [3,17].

In the study the quantity of lipid extracted by different cell disruption methods were found to be different for the same strain. The total lipid content of *Scenedesmus* sp. was found to vary 2% to 24% depending upon different cell disruption methods adopted (Figure **3**). The lipid productivity of the *Scenedesmus* sp. was found as 4.3 $mgL^{-1}d^{-1}$by grinding and bead-beating method. Very interestingly, combined method of grinding and bead-beating yielded two fold increased quantity (23.16%) than those done separately. The method of osmotic shock using 10% NaCl solution was found to be 3.54% and all other methods extracted less than 9.5%. Bead-beating and microwave oven method was reported as good in lipid extraction for *Botryococcus* sp. It has been reported that the microwave oven method was best method for the extraction of lipids from *Scenedesmus* sp. Furthermore, sonication method was better than microwaves, bead-beating, osmotic shock and autoclave for *Scenedesmus* sp. [18]. Bead beating that causes direct mechanical damage to cells where it was based on high-speed spinning with fine beads has been used both on a laboratory as well as in an industrial scale [19]. In this study it was found that a combination of mechanical grinding and bead-beating was the most effective method for lipid extraction from microalga, *Scenedesmus* sp NTEB03. It could be the method of choice for the lipid extraction and it can be scaled-up easily.

3.3. Fatty Acid Composition

The important properties of biodiesel including its ignition quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity and lubricity which are highly dependent on the fatty acid composition [20]. The fatty acid profile of strain, *Scenedesmus* sp. NTEB03 was determined by gas chromatographic analysis. It was observed that Cis-10-Heptadecanoic acid (C17:1); Oleic acid (C18:1) and Linoleic acid (C18:2) were found to be abundant in the extracted lipids (Table **2**). Previous report mentioned that fatty acid profile of *Scenedesmus* sp. have about 36.8% polyunsaturated fatty acids (PUFA) containing

Table 1: Biomass, Lipid Contents and Productivity of *Scenedesmus* sp. NTEB03

Item	*Scenedesmus* sp.,
Incubation days	14
Dry weight (gL^{-1})	0.5853
Biomass productivity ($mgL^{-1}d^{-1}$)	41.8
Average lipid content (mgL^{-1})	59.6
Lipid productivity ($mgL^{-1}d^{-1}$)	4.3

Table 2: Fatty Acid Composition of *Scenedesmus* sp. NTEB 03 under Two Different Cell Disruption Methods

Sample No.	Fatty acid Methyl Esters		Nature	% composition under different conditions	
				Grinding + Beads beating	Grinding
1	Pentadecanoic	C15:0	SFA	0.20	ND
2	Cis-10-pentadecanoic	C15:1	MUFA	0.43	ND
3	Palmitic	C16:0	SFA	0.61	0.41
4	Cis-10-Heptadecanoic	C17:1	MUFA	27.41	20.55
5	Elaidic	C18:1	MUFA	2.59	ND
6	Oleic	C18:1	MUFA	11.60	10.04
7	Linolelaidic	C18:2	PUFA	0.98	6.29
8	Linoleic	C18:2	SFA	34.23	32.72
9	Linolenic	C18:3	PUFA	10.37	5.29
10	Cis-11,14 Eicosadienoic	C20:2	PUFA	0.89	9.22
11	Behenic	C22:0	SFA	8.25	ND
12	Erucic	C22:1	MUFA	0.49	5.39
13	Arachidonic	C20:4	PUFA	0.76	ND
14	Lignoceric	C24:0	SFA	ND	7.92
15	Nervonic	C24:1	MUFA	1.00	2.63
16	Cis-4,7,10,13,16,19-Decosahexaenoic	C22:6	PUFA	0.28	ND

SFA- Saturated Fatty Acid, MUFA-Monounsaturated Fatty Acid, PUFA- Polyunsaturated Fatty Acid, ND- Not detected.

linoleic acid (21.1%) and oleic acid (17.5%) [21]. Whereas palmitic and linoleic acid were reported as the major fatty acids in the *Scenedesmus* sp. [22].

When the GC profiles from the grinding and grinding/bead-beating methods were compared it was found that there is significant difference among the fatty acid profiles. Fatty acids (C20:2, C24:0 and C22:1) were abundant from grinding method but barely detectable by the grinding/bead-beating method. Likewise the fatty acids C22:0 abundant in grinding/bead-beating method is not detected from grinding method, which implicates that the mode or methods used for lipid extraction influences the fatty acid profile also. So, further optimization in this regard could pave a way for exploring the complete biofuel potential of microlage.

Apart from the biofuel properties microlagal FAME from *Scenedesmus* sp. has extensively studied for antimicrobial property due to consistency of fatty acid [23]. There are some reports emphasizing that palmitic, stearic, oleic, linoleic acids are most common and important fatty acids in biodiesel [24, 25]. It is essential that the biodiesel must have high levels of saturated and monounsaturated fatty acids with low levels of polyunsaturated fatty acids [26]. According to the European standard EN14214 for biodiesel production the level of C18:3 content has to be less or equal to 12%. Here also it was seen to be less than 12%. FAME profile showed a major content of fatty acids which is having high oxidative stability, thus can be stored for a longer time. High levels of saturated and monounsaturated fatty acids with low levels of polyunsaturated fatty acids favors the production of good quality biodiesel [2]. There are a good number of reports on different lipid induction methods for microlage [27]. Nutrient starvation (mainly nitrogen and phosphorous), salinity, pH, UV irradiation etc being some of the important factors among them. There are specific reports on lipid induction in *Scenedesmus* sp. by nitrogen and phosphorous limitation [28-30]. Thus combining any suitable lipid induction method with the discussed grinding/bead beating extraction protocol, microalgae *Scenedesmus* sp. can be presented as a good biofuel feedstock. From a comparative investigation of previous reports, *Scenedesmus* sp. NTEB03 has got all the desirable features in agreement with biodiesel standards, was thus considered to be most suitable for the production of high quality biodiesel.

4. CONCLUSION

Achieving a high quality and quantity of lipid from microalgae for the production of biodiesel greatly depends on the extraction in general and cell disruption in particular. In our study the *Scenedesmus* sp., was found to have a high lipid productivity of about 4.3 $mgL^{-1}d^{-1}$. When using the combined method of grinding and bead-beating two fold increased lipid yield was obtained apart from using them separately. The quantity and quality of oleic (C18:1) and linoleic (C18:2) fatty acids also confirms that these methods can be used to extract the lipids from microalgae effectively. It is strategized that the combined method of grinding and bead-beating was most efficient, cost effective and simple method for extraction of algal lipids for biofuel production.

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REFERENCES

[1] Macedo N, Brigham CJ. From Beverages to Biofuels: The Journeys of Ethanol-Producing Microorganisms. Int J Biotech Well Indus 2014; 3(3): 79-87. http://dx.doi.org/10.6000/1927-3037.2014.03.03.1

[2] Praveenkumar R, Johncy K, Mubarak Ali D *et al*. Demonstration of increased lipid accumulation potential of *Stigeoclonium* sp., Kütz. BUM11007 under nitrogen starved regime: A new source of lipids for biodiesel production. J Biol Mater Bioenergy 2012; 6(2): 209-13 http://dx.doi.org/10.1166/jbmb.2012.1200

[3] Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007; 25: 294-306. http://dx.doi.org/10.1016/j.biotechadv.2007.02.001

[4] Milne TA, Evans RJ, Nagle N. Catalytic conversion of microalgae and vegetable oils to premium gasoline, with shape-selective zeolites. Biomass 1990; 21: 219-32. http://dx.doi.org/10.1016/0144-4565(90)90066-S

[5] Dote Y, Sawayama S, Inoue S, *et al*. Recovery of liquid fuel from hydrocarbon-rich microalgae by thermochemical liquefaction. Fuel 1994; 73: 1855-7. http://dx.doi.org/10.1016/0016-2361(94)90211-9

[6] Minowa T, Yokoya SY, Kishimoto M, *et al*. Oil production from algae cells of *Dunaliella Tereiolata* by direct thermochemical liquefaction. Fuel 1995; 74: 1731-8. http://dx.doi.org/10.1016/0016-2361(95)80001-X

[7] Chisti Y, Moo-Young M. Review: disruption of microbial cells for intracellular products. Enz Microb Technol 1986; 8: 194-204. http://dx.doi.org/10.1016/0141-0229(86)90087-6

[8] Gouveia L, Nobre BP, Marcelo FM, *et al*. Functional food oil colored by pigments extracted from microalgae with supercritical CO_2. Food Chem 2007; 101: 717-23. http://dx.doi.org/10.1016/j.foodchem.2006.02.027

[9] Lee JY, Yoo C, Jun SY, *et al*. Comparison of several methods for effective lipid extraction from microalgae. Bioresour Technol 2010; 101: 75-7. http://dx.doi.org/10.1016/j.biortech.2009.03.058

[10] Mendes-Pinto MM, Raposo MFJ, Bowen J, *et al*. Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. J Appl Phycol 2001; 13: 19-24. http://dx.doi.org/10.1023/A:1008183429747

[11] Simon D, Helliwell S, Extraction and quantification of chlorophyll a from freshwater green algae. Water Res 1998; 32; 2220-3. http://dx.doi.org/10.1016/S0043-1354(97)00452-1

[12] Boldor D, Kanitkar A, Terigar BG, *et al*. Microwave assisted extraction of biodiesel feedstock from the seeds of invasive Chinese tallow tree. Environ Sci Technol 2010; 44: 4019-25. http://dx.doi.org/10.1021/es100143z

[13] Bligh EG, Dyer WJ, A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1956; 37: 911-7. http://dx.doi.org/10.1139/o59-099

[14] Ilavarasi A, Mubarakali D, Praveenkumar R, *et al*. Optimization of various growth media to freshwater microalgae for biomass production. Biotechnology. 2013; 10: 540-5. http://dx.doi.org/biotech.2011.540.545

[15] Middelberg APJ, Process-scale disruption of microorganisms. Biotechnol Adv 1995; 13: 491-551. http://dx.doi.org/10.1016/0734-9750(95)02007-P

[16] Ranjitha K, Kaushik BD, Purification of phycobiliproteins from *Nostoc muscorum*. J Sci Indus Res 2005; 64: 372-5.

[17] Brown MR, Jeffrey SW, Volkman JK, *et al*. Nutritional properties of microalgae for mariculture. Aquaculture. 1997; 151: 315-31. http://dx.doi.org/10.1016/S0044-8486(96)01501-3

[18] Pandian P, David Ravindran A. A study on effective lipid extraction methods from certain fresh water microalgae. Elixir Bio Tech 2011; 39: 4589-91. http://dx.doi.org/10.1111/j.1472-765X.2011.03082.x

[19] Geciova J, Bury D, Jelen P, Methods for disruption of microbial cells for potential use in the dairy industry-a review. Int Dairy J 2002; 12: 541-53. http://dx.doi.org/10.1016/S0958-6946(02)00038-9

[20] Knothe G, Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 2005; 86: 1059-70. http://dx.doi.org/10.1016/j.fuproc.2004.11.002

[21] Jena J, Nayak M, Panda HS, *et al*. Microalgae of Odisha Coast as a Potential Source for Biodiesel production. World Environ 2002; 2: 12-17. http://dx.doi.org/10.5923/j.env.20120201.03

[22] Wu YH, Yu Y, Li X. Biomass production of a *Scenedesmus* sp. under phosphorous-starvation cultivation condition. Bioresour Technol 2002; 112; 193-8. http://dx.doi.org/10.1016/j.biortech.2012.02.037

[23] Mubarak Ali D, Praveenkumar R, Shenbagavalli T, *et al*. New reports on anti-bacterial and anti-candidal activities of fatty acid methyl esters (FAME) obtained from *Scenedesmus bijugatus* var. bicellularis biomass. RSC Adv 2012; 2: 11552-6. http://dx.doi.org/10.1039/C2RA21130K

[24] Knothe G, Designer biodiesel: optimizing fatty esters composition to improve fuel properties. Energy Fuel 2008; 22: 1358-64. http://dx.doi.org/10.1021/ef700639e

[25] Rashid U, Anwar F, Moser BR, *et al*. Moringa oleifera oil: a possible source of biodiesel. Bioresour Technol 2008; 99; 8175-79. http://dx.doi.org/10.1016/j.biortech.2008.03.066

[26] Baldev E, MubarakAli D, Ilavarasi A, *et al*. Degradation of synthetic dye, Rhodamine B to environmentally non-toxic products using microalgae. Colloids Surf B 2013; 105; 207-14. http://dx.doi.org/10.1016/j.colsurfb.2013.01.008

[27] Sharma K K, Schuhmann H, Schenk PM, High Lipid Induction in Microalgae for Biodiesel Production. Energies 2012; 5: 1532-53. http://dx.doi.org/10.3390/en5051532

[28] Gardner R, Peters P, Peyton B, *et al*. Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the chlorophyta. J Appl Phycol 2011; 23: 1005-16. http://dx.doi.org/10.1007/s10811-010-9633-4

[29] Dean AP, Sigee DC, Estrada B, *et al*. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioresour Technol 2010; 101: 4499-507. http://dx.doi.org/10.1016/j.biortech.2010.01.065

[30] Xin L, Hong-ying H, Ke G, *et al*. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga, *Scenedesmus* sp. Bioresour Technol 2010; 01: 5494-5500. http://dx.doi.org/10.1016/j.biortech.2010.02.016

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