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Research Article

Rapid, Cost Effective, Enhanced Lipid Extraction from Marine Cyanobacteria in A Biodiesel Perspective.

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ABSTRACT:

Rapidly depleting fossil fuels and increasing atmospheric CO_2 concentration have led to an enhanced interest in renewable energy fuels like biodiesel. Lipids and fatty acids of cyanobacteria are an ideal source of food, feed and fuels; hence their extraction becomes principally imperative. The present study investigated the nature of the biomass (wet/ dried), pre–treatment method (homogenisation, sonication, bead beating and microwave) and the solvent mixture [chloroform:methanol (2:1v/v), hexane:iopropanol (3:2 v/v), *n*–hexane (Soxhlet)] ideal for maximum extraction of lipids. Of all the tested methods, dried biomass at 40ºC (oven) increased the lipid yield by ~ 3%, where homogenised biomass with pulverised glass powder increased the recovery by 54.47 and 62.42% in marine cyanobacteria *Spirulina subsalsa* BDU141201 and *Oscillatoria willei* BDU130791 over the un-disrupted biomass. chloroform:methanol (2:1v/v) stood out as the efficient extraction solvent mixture with yield of 16.2±0.05 and 15.9±0.03% of transesterifiable lipids from *S. subsalsa* BDU141201 and *O. willei* BDU130791. The results were further evinced by the fatty acid composition of the selected strains, with enhanced recovery of oleic, linoleic and γ-linolenic acid –the pre-requisite for biodiesel when enhanced with 2:1 chloroform:methanol.

I INTRODUCTION:

In the recent past, there is renewed interest in the development of sustainable energy as petroleum based fuels are becoming unsustainable by the increasing industrialisation and atmospheric CO_2 concentrations. Currently biodiesel is one of the alternative energy sources projected to replace transport fuel without involving modification in the engines [1]. Production of biodiesel from different feedstock's namely plant, animal fat, microalgae, including cyanobacteria and diatoms is the present day focus [2].

Microalgae are miniature sunlight driven biochemical factories capable of producing significant amounts of lipid [3]. Thus is considered as a promising alternative feedstock for next generation biodiesel with low nutritional requirements and year around availability [4]. The key steps involved in microalgal biodiesel production are cultivation, harvestation, lipid extraction and transesterification [5]. Though all the steps are indispensable, the lipid extraction is principally imperative [6] as high quality biodiesel is based on complete lipid extraction.

Lipid is a biological molecule soluble in organic solvent and is broadly categorised into neutral and polar lipid. Neutral lipids are made of acylglycerols and free fatty acids, which are highly suited for biodiesel production due to their lesser degree of unsaturation [7]. Ideally, a lipid extraction method for the microalgal

system should be highly specific and selective towards acylglycerols to avoid co-extraction of non-lipid components, efficient in terms of time and energy, unreactive to lipids and cost beneficial [8]. Thus, the critical parameters of concern are method of cell disruption, solvent system, sample:solvent ratio, time of extraction and mode of drying of the biomass. The second critical prerequisite for complete lipid extraction is the choice of organic solvent [9]. Solvents namely hexane, ethanol, methanol, petroleum ether and a mixture of chloroform and methanol are conventionally used in the extraction of lipid from vegetable, flower and oil seeds [10]. However, a combination of polar and non-polar solvent system aid in better dissolution of both polar and neutral lipids [11] also the extraction method and the solvent system vary from organism to organism. Hence, present study aims to optimise an analytical procedure for maximum lipid extraction from selected filamentous marine cyanobacteria for biodiesel production, which has comparatively higher lipid content among screened 250 marine cyanobacteria.

II MATERIALS AND METHODS:

2.1 Strain selection and culture conditions:

Two filamentous non-heterocystous marine cyanobacteria *Oscillatoria willei* BDU130791 and *Spirulina subsalsa* BDU141201 were selected from the repository of the National Facility for Marine Cyanobacteria, sponsored by DBT, *Govt. of India*, Bharathidasan University, Tiruchirappalli, India. The selected strains, were grown in 250 and 500 ml Erlenmeyer flasks containing 100 and 200 ml of ASN III (Artificial Sea Nutrient) medium respectively [12] and maintained under continuous aeration and illuminated with white fluorescent light at an intensity of 20 µmol photon m^{-2} s^{-1} at 25 ± 2°C in controlled culture room. Culture was harvested on the 7^{th} day and subjected to lipid extraction.

2.2 Optimization of the drying method:

The two selected high lipid yielding strains were subjected to drying at different temperature regimes namely drying in room (27°C), sun drying (35°C) and oven drying (50°C). The fresh biomass served as control.

2.3 Optimization of the cell disruption/ pre – treatment method:

One gram of dry cyanobacterial biomass of seven day old culture was disrupted following homogenization methods with pulverised glass powder (30min), bead beating (3500 rev min^{-1} for 5min), sonication (40 Hz for 15min) and microwave methods (2450 MHz for 5min) following the methods of [6] were tested against the lipid yield from the undisrupted biomass as control. Lipid was extracted from one gram of disrupted dried biomass using the conventional Bligh and Dyer, (1959) [13] – 2:1 chloroform:methanol.

2.4 Optimization of the solvent system for lipid extraction:

To standardize the ideal solvent system for maximum lipid recovery from cyanobacterial system, three different solvent mixtures [chloroform:methanol (2:1) [13]; *n*-Hexane (Soxhlet) [14]; hexane:isopropanol (3:2) [15] were evaluated, with a known volume of dried cyanobacterial biomass (1g). Total lipid was extracted and the results compared by gravimetric estimation.

2.5 Fatty acid profile analysis:

A known amount of the lipid extracted by all of the solvent systems were transesterified by a modified two-step process [16]. Initially, the lipid samples were saponified with 3.75M NaOH at 100ºC for 30min. Then the lipid samples were subjected to methanolysis using 5% methanolic hydrochloride at 80ºC for 20min. Methanolysed contents were then cooled and a mixture of freshly prepared hexane: diethyl ether (1:1) was added to it. This mixture was then vortexed for 1min and allowed to stand for 10min to enable phase separation for removal of the aqueous phase FAME containing hexane layer was washed with 300mM sodium hydroxide. The washed FAME was analysed using Clarus 500 gas chromatograph (Perkin Elmer, USA) equipped with a flame ionization detector using SP 2650 (100m) column (Supelco St. Louis, MO, USA). The carrier gas used was helium. The temperature at the injector port and the detector was 260°C. The initial column

temperature was set at 140°C for 1min and increased to 260°C at the rate of 4°C min^{-1} by injecting 2µL of the sample. The fatty acid methyl esters (FAMEs) of the test organisms were identified by comparing the retention times (*RT*) of the sample peaks with that of the standard fatty acids (Supelco 37 component FAME mix, St. Louis, MO, USA).

III RESULTS AND DISCUSSION:

The prime objective of the study is to enhance the extraction efficiency or recovery of transesterifiable lipids from two filamentous marine cyanobacteria which had comparatively higher lipid. Extensive studies of researchers [15], [6], [17], [18], and [19] postulated the significance of solvent mixture, method of mechanical disruption, the nature of the sample, solvent ratio, particle size of organism etc. The first phase of the experiment aimed to identify the nature of the biomass to be used. Hence the selected two strains were dried at different temperatures *viz*., [room temperature (27°C), sun drying (35- 37°C) and microwave oven drying (50°C)] were extracted for lipid using the conventional solvent system chloroform:methanol (2:1). Method of Bligh and Dyer, (1959) [13] using chloroform:methanol (2:1 v/v) is the oldest known and most widely used methods for extracting lipids, from any tissue and hence was used as the default solvent system to standardise the nature of the biomass and the pre-treatment methods. However, the effect of other solvent systems on lipid recovery from marine cyanobacteria have also been evaluated in the later part of our experiments.

Of the two organisms, *S. subsalsa* BDU141201 yielded a maximum of 16.20±0.03% in microwave oven dried biomass and 14.76±0.05 % in room dried condition while *O. willei* BDU130791 yielded 15.80±0.03% of lipid in both oven and room dried biomass respectively (Fig 1). Fresh biomass presented lower lipid recovery of 13.22±0.03 and 11.80±0.02% in *S. subsalsa* BDU141201 and *O. willei* BDU130791 respectively. In the case of sun dried biomass, a 1% reduction in lipid over the microwave oven and room dried conditions were recorded (Fig 1). Drying of the cyanobacterial pellets appeared to increase the lipid yield by 3% over the fresh biomass, with minor variation in oven, room and sun dried biomass. Use of dried biomass over the wet/fresh biomass warrants detailed investigation, however several theories suggest dried biomass for lipid extraction [8]. In wet biomass, presence of residual water acts as a barrier for effective lipid mass transfer to the extraction solvent [20] and lesser lipid in wet biomass is understandable. However, studies of Sathish et al., (2015) [21] attained 75% of total lipids from wet biomass of *Chlorella sp.*, and *Scenedesmus sp.* Of the three treatments, as both the organisms' yielded higher lipid in oven dried biomass (50ºC) it was selected for further studies.

3.1 Optimisation of cell disruption / pre-treatment method of extraction of lipid from marine cyanobacteria:

Having identified the best drying method for higher lipid extraction yet another inevitable parameter is, to identify the ideal pre-treatment method that aids maximum lipid extraction. Of the different homogenisation methods, sonication, microwave, bead beating against the un-disrupted biomass, homogenisation of the cyanobacterial biomass with glass powder showed a maximum lipid yield of 16.08±0.08 and 15.57±0.04 in *S. subsalsa* BDU141201 and *O. willei* BDU130791 respectively, followed by sonication which yielded 13.96±0.02 and 12.25±0.07. The lipid extracted from uninterrupted biomass was only 7.32±0.03 and 5.85±0.08 in *S. subsalsa* BDU141201 and in *O. willei* BDU130791 the yields were 54.47 and 62.42 % lesser than that of the homogenisation method (Fig 2). The efficacy of lipid extraction is dependent on the cell disruption method [8]. It may be observed that most cell disruption methods namely bead beating, microwave and sonication requires a certain amount of water content, wherein; homogenisation using pulverised glass powder enables cellular disintegration and complete extraction of lipids with no water requirement [22]. Ryckebosch et al., 2011 [18], evaluated the impact of different pre-treatments on microalgal lipids and suggested that solvent composition ration were more decisive than the

pre-treatment methods while Sheng et al., (2012) [23] reported that high pressure homogenisation was the ideal method for *Synechocystis sp.* PCC6803 which yielded a maximum of 20.6 g L^{-1} lipid.

Bead beating method yielded 10.52±0.08 and 9.72±0.02 lipid in marine cyanobacteria *S. subsalsa* BDU141201 and *O. willei* BDU130791 respectively, while the lipid extracted by the microwave method decreased by 43.28 and 54.27 % respectively (Fig 2). Interestingly, microwave extraction of lipids supported higher total lipid in *Botryococcus sp., Chlorella sp., and Scenedesmus sp.*,[8]; Prabakaran et al., (2011a) [19] reported sonication and microwave methods to result in maximum yield in *Nostoc* sp. Microwave aided solvent extraction employs electromagnetic radiation to deliver large amount of thermal energy and thereby cause cellular rupture and result in a higher yield of lipids [24]. Inspite, it is a rapid method, and not cost effective for large scale operations. The plausible explanation for homogenisation being the ideal method for extracting maximum lipid in our studies could be the presence of thick cyanobacterial cell membranes, which needed sheer pressure for its disruption and interaction with solvent systems as compared to other methods.

3.2 Optimization of solvent system for lipid extraction

Although an appropriate method of cell disruption is a prerequisite; usage of solvent is still another important criterion. Efficient extraction is highly dependent on the solvent mixture as given the large diversity of the microalgae it is paramount to standardise a strain specific analytical procedure for extraction of lipid. The high lipid yielding two marine cyanobacterial strains were subjected to lipid extraction using different solvent systems. A maximum of 16.20±0.05 % and 15.9±0.03 % of lipid was obtained from *S. subsalsa* BDU141201 and *O. willei* BDU130791 respectively in 2:1 chloroform:methonal [13], from the oven dried homogenised biomass, followed by n-hexane:isopropanol (3:3 v/v) with yields of 13.22±0.07 and 11.56±0.03 of lipid from *S. subsalsa* BDU141201 and *O. willei* BDU130791 (Table 1). Studies of Ryckebosch et al., 2011 [18] clearly suggested the use of a mixture of a polar and nonpolar solvent will ensure complete extraction of both polar and neutral lipids from microalgal cells, and reported a maximum of 76.5 % from *Arthrospira platensis* over the other tested solvent systems. Halim et al., (2012) [8] identified *n*-hexane:isopropanol (3:2 v/v) as the most suited solvent and that it works analogous to that of chloroform:methanol systems in bi-phasic lipid extraction which corroborates with our findings.

Soxhlet extraction of the cyanobacterial pellets in *n*-hexane could extract only 3.40 ± 0.04 % and 4.7 ± 0.02 % of lipid from *S. subsalsa* BDU141201 and *O.willei* BDU130791 respectively. The resulting cyanobacterial pellet when re-extracted using chloroform:methanol (2:1), yielded 11.30 ± 0.15% and 10.02 ± 0.02 % from *S. subsalsa* BDU141201 and *O. willei* BDU130791 respectively (Table 1). Li et al., (2014) [11] tested the efficacy of *n*-hexane and *n*-hexane:ethanol (3:1 v/v) by soxhlet extraction in *Tetraselmis* sp. M8 and concluded that with *n*-hexane solvent alone the yield got reduced by ~60 %. Lipid extraction by Soxhlet method employs heat reflux leading to minimal penetration in to the cell and also is time consuming [25]. Given its ability to extract maximum lipid, chloroform:methanol (2:1v/v) is the identified solvent suitable for maximum lipid recovery from marine cyanobacteria by homogenisation.

3.3 Fatty acid profile of marine cyanobacteria in different solvent systems:

The lipid extracted under different solvent systems were further transesterified for FAME analysis, as the composition of individual fatty acid signifies the efficiency of the extraction and quality of the biodiesel produced. Qualities including ignition, oxidative stability, viscosity and cold filter plugging point are dependent upon fatty acids like oleic, linoleic and gamma linolenic acids respectively [26]. Long chain, saturated and unsaturated fatty acids were extracted eminently with chloroform:methanol (2:1v/v) extracted lipids and were minimally identified or not detected in *n*-hexane soxhlet system (Fig 3a and b). The inefficiency of *n*-

hexane in extracting the transesterifiable lipid implies the appropriate use of a polar and nonpolar solvent and in the present study, *n*-hexane alone lead to a threefold decreased yield of lipid and fatty acids [11] or thermal degradation of PUFA in prolonged heated conditions [27].

Fatty acid percentage of C18:1n 9c, C18:3n3, C 18:3n6, C 20:0 and C20:4n6 were 9.45, 6.94, 11.45, 3.98 and 4.78 in *S. subsalsa* BDU141201 and 11.87, 8.45, 10.97, 1.36 and 2.56 in *O. willei* BDU130791 from the lipid extracted using chloroform:methanol (2:1 v/v). *n*-hexane:isopropanol (3:2) extracted lesser lipids of 8.78, 6.11, 9.1, 2.56 and 5.45 % in *S. subsalsa* BDU141201 and *O. willei* BDU130791 showed 9.23, 8.12, 7.07, 0.57 and 1.1 % of respective fatty acids. Selected two marine cyanobacterial strains revealed the dominance of oleic and γ-linolenic fatty acids along with other unsaturated fatty acids, including *cis*-8,11,14 eicosatrienoic acid and arachidonic acid (Fig 3a and b). The results acknowledge the findings of Prabakaran et al., (2011b) [28] in *Nostoc* sp., extracted using chloroform:methanol (2:1v/v). Given the enhanced recovery of lipid and the fatty acid profile, the obtained results thus evidently suggested the use of conventional Bligh and Dyer method using chloroform:methanol (2:1 v/v) for homogenisation of the oven (40ºC) dried cyanobacterial biomass using pulverised glass powder as the plausible better method for extraction and gravimetric estimation of lipids.

IV CONCLUSION:

An optimised analytical procedure for the enhanced recovery of lipid from marine cyanobacteria has been developed. Among the myriad of methods tested, the study identified that the amount of extractable lipids is highly dependent on the pre-treatment method and the solvent mixture. The results postulate that for efficient lipid extraction from marine cyanobacterial system–dried biomass homogenised using pulverised glass powder in chloroform: methanol (2:1v/v) could not only lead to near total extraction of lipids but better recovery of Fatty Acids Methyl Esters (FAME).

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Fig: 1. Optimization of biomass drying conditions for marine cyanobacteria *Spirulina subsalsa* BDU141201 and *Oscillatoria willei* BDU130791

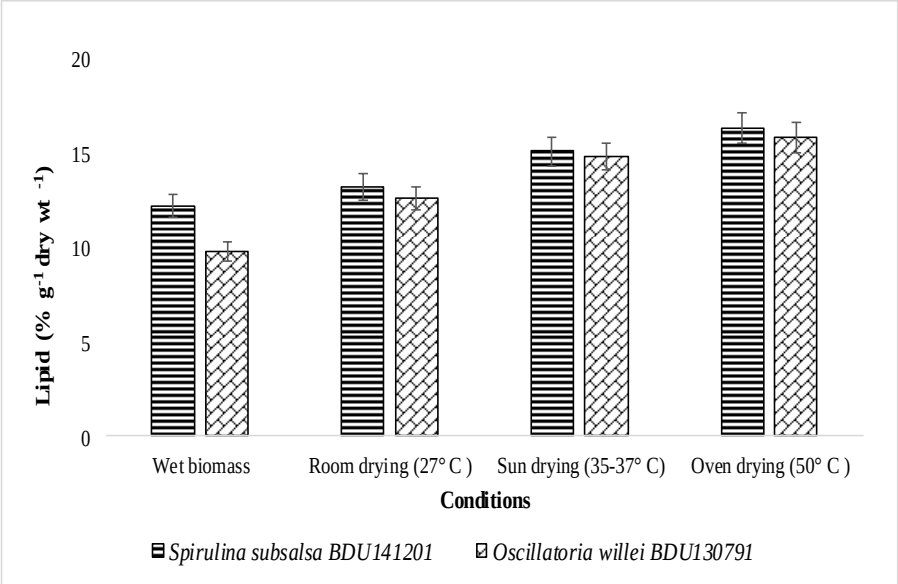

Fig 2: Lipid yield of marine cyanobacteria under different pretreatment conditions

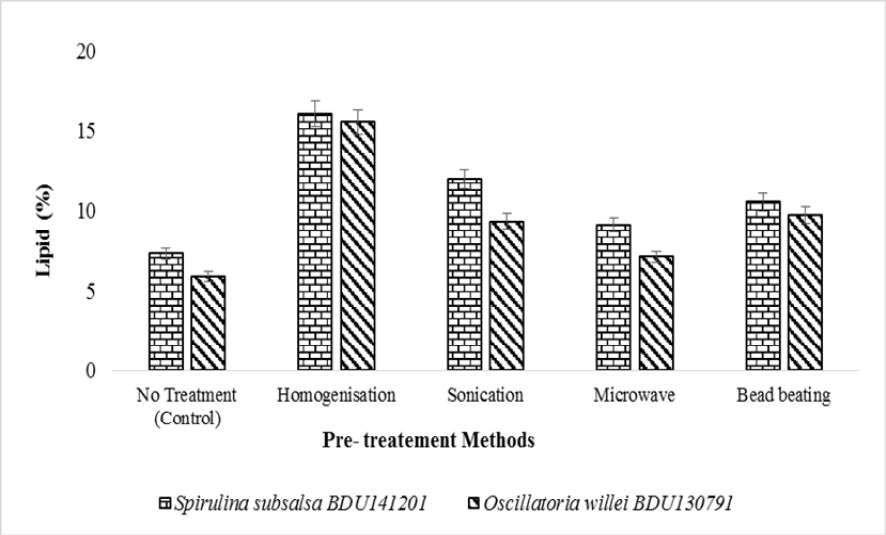

Table 1. Optimization of solvent mixture for enhanced lipid recovery from marine cyanobacteria

Solvent mixtures	***Spirulina subsalsa* BDU141201**	***Oscillatoria willei* BDU130791**
Chloroform:Methanol (2:1 v/v)	16.2 ± 0.05	15.9 ± 0.03
n -hexane:Isopropanol (3:2)	13.02 ± 0.05	11.26 ± 0.03
n- hexane (Soxhlet)	3.4 ± 0.04	4.7 ± 0.08
Re extracted from pellet using Chloroform:Methanol (2:1 v/v)	11.3 ± 0.15	10.2 ± 0.02

Fig 3 (a.) Comparison of FAME compositions of marine cyanobacteria *Spirulina subsalsa* BDU141201 and (b) *Oscillatoria willei* BDU130791in different solvent systems.