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Original Research Paper

Optimal Extraction of Lipids from Microalgae, *Microcystis*

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ABSTRACT

The triacylglycerol (TAG) was extracted from *Microcystis* sp. using a mixture of chloroform and methanol (2:1). The fatty acid methyl ester compositions for each method were identified using gas chromatography-mass spectroscopy (GC-MS). Techniques such as sonication, bead beating, sonication-hexane solvent, sonication-supercritical nitrogen, supercritical nitrogen-manual, bead beating-hexane solvent and bead beating-supercritical nitrogen were explored to determine the most rapid and efficient cell disruption. Sonication, bead beating and manual methods showed 30.2%, 41.88% and 11.21% overall average dissimilarity respectively. Hierarchical cluster analysis of the fatty acid composition obtained from different methods show four clusters for sonication and three clusters for bead beating. Sonication for a period of 60 minutes yielded higher quantity of fatty acids evident from higher quantity of palmitic acid (53.89%) with sonication of 30 minutes and higher stearic acid (56.27%) when the cells were disrupted through sonication for 60 minutes. This highlights that sonication of 60 minutes is effective for disruption of microalgae *Microcystis* cells and extraction of lipids.

INTRODUCTION

Fast depletion of fossil fuel based energy sources and global warming has necessitated the transition to renewable and sustainable energy sources. Biodiesel from various renewable feed stocks of plants, microalgae and animal fat, has been attempted as viable alternative sources of energy (Vasudevan & Briggs 2008). Microalgae being primary producers in aquatic ecosystems are a viable third generation of biofuel due to abundant availability and higher accumulation of lipids. Biodiesel has dual advantages (Chisti 2008) of mitigating carbon dioxide and as a substitute for petroleum. Microalgae also have certain advantages compared to other energy crops, including a high growth rate, shorter generation time, higher biomass production (Ramachandra et al. 2009) and lower degree of unsaturation and effective accumulation of lipids in algal cells at the end of growth stage (Casadevall et al. 1985, McGinnis et al. 1997).

A standard lipid extraction process from microalgae is prioritized based on its lipid specificity, entailing minimization of co-extraction of non-lipid contaminants and selective towards desirable lipid fractions viz., neutral lipids containing mono, di, and trienoic fatty acid chains (Medina et al. 1998, Fajardo et al. 2007). The various steps involved in the biodiesel production using microalgae are isolation of species, mass cultivation, harvesting, lipid extraction (disruption of cell wall), and the conversion of the triglycerides into fatty acid methyl esters (transesterification) (Lee et al. 2010).

Cell disruption forms the most defining initial step for extraction of lipids. Various cell disruption techniques such as microwave assisted, sonication, osmotic shock, autoclaving, french press, lyophilization and bead beating have been used to extract lipids for different microalgae-*Botryococcus* (Lee et al. 1998) and *Chlorella vulgaris* and *Scenedesmus* (Lee et al. 2010). However, the consensus on optimal technique for microalgae has not yet been achieved. This study evaluates disruption methods like sonication, bead beating, sonication-hexane solvent, sonication-supercritical nitrogen, supercritical nitrogen-manual, bead beating-hexane solvent and bead beating-supercritical nitrogen for the microalgae *Microcystis* for different time intervals to evaluate the time and the disruption method required for maximum lipid yield.

METHODS

Microalgae Sample Collection

Natural population of *Microcystis* was obtained from Vaderahalli Lake situated in Bangalore. *Microcystis* formed more than 95% of the total microalgal community due to algal bloom.

Cell Disruption

An aliquot of 6.71g of the dry cell biomass was mixed with 250 mL of distilled water and disrupted using following methods.

Sonication disruption method: This includes:
a. Sonication using a sonicator (KIKA Labortechnik Staufen, Germany) for a time interval of 10 (10), 20 (20), 30 (30), 40

(40), 50 (50) and 60 (60) minutes.
b. Sonication-hexane solvent (hexane: 2-propanol, 2:3) for a time interval of 30 (VS 30) and 60 (VS 60) minutes.
c. Sonication-supercritical nitrogen for a time interval of 30 (VS 30n) and 60 (VS 60n) minutes.

Bead beating disruption method: Variants used here are:
a. Using 1mm glass beads for a time interval of 10(10), 20(20), 30(30), 40(40), 50(50) and 60(60) minutes.
b. Using hexane solvent (hexane: 2-propanol, 2:3) for a time interval of 60 minutes (VB3H).
c. Using supercritical nitrogen for a time interval of 60 minutes (VB3Hn).

Manual disruption methods: These include:
a. Supercritical nitrogen-manual for a time interval of 60 minutes (VHM).
b. Hexane solvent (hexane: 2-propanol, 2:3)-manual for a time interval of 60 minutes (VNM).

Lipid Extraction

The protocol for lipid extraction from microalgae was as follows:

i. Pre-thin layer chromatography: The extraction of lipids was done by mixing in chloroform : methanol (2:1) using a modified Bligh and Dyer's methods (Bligh & Dyer 1959). The chloroform layer was evaporated using rotary evaporator (Eppendorf Vacuum Concentrator 5301) to obtain lipids. This step was important since lipids are highly sensitive to hydrolysis and oxidation processes during storage (Sasaki & Capuzzo 1984).

ii. Thin layer chromatography: Samples were reconstituted in chloroform to make stock solutions. These were later spotted in bands onto silica gel TLC plates (Merck KGaA). The mobile phase consisted of a solvent system of hexane/diethyl ether/acetic acid (70:30:1 by volume) (Maloney 1996). The plates were developed by exposing the vapours of iodine crystals to stain the plates for visualizing neutral lipids. The samples were extracted and stored at 20°C until further analysis (Mansour et al. 2005).

iii. Gas chromatography-mass spectrometry analysis (GC-MS): After the initial thin layer chromatography (TLC) lipid screening, the extracts were converted into fatty acid methyl esters (FAME) using boron trifluoride-methanol and heated in waterbath at a temperature of 60°C for 1 hour. The methylated samples were then purified further for GC-MS. The main focus of using GC-MS was purely for lipid identification rather than quantification. The injector and detector temperatures were set at 250°C while the initial column temperature was set at 40°C for 1 min. A 1 µL sample volume was injected into the column and ran using a 50:1 split ratio. After 1 min, the oven temperature was raised to 150°C at a ramp rate of 10°C per min. The oven temperature was then raised to 230°C at a ramp rate of 3°C per min, and finally the oven temperature was increased to 300°C at a ramp rate of 10°C per min and maintained at this temperature for 2 min. The total run time was programmed for 47.667 min. The mass spectra were acquired and processed using Agilent Chem Station (5975 C; Agilent, USA).

Data Analysis

Hierarchical cluster analysis of the fatty-acid composition was performed for sonication, bead beating and hand crushing using PAST Version 2.08 (Hammer et al. 2004). Hierarchical clustering was performed based on a Bray-Curtis similarity coefficient applied to untransformed percentage composition data. The SIMPER (SIMilarity PERcentage-species contribution) in PAST (Version 2.08) was used to investigate the percentage of the fatty acids of the clusters formed. One way ANOVA was used to check the significance level for manual method, a significance level of $p < 0.05$ was used in all the tests.

RESULTS AND DISCUSSION

Sonication: Cell disruptions based on sonication methods showed overall average inter-dissimilarity of 30.2% through SIMPER analysis of the fatty acid composition. The principal fatty acids C16:0, C18:0 and C16:1n-7 contributed at least 50% of the fatty acid methyl esters in all the methods (Table 1). High percentage dissimilarity was found in the fatty acids C16:0, C18:0 and C16:1n-7 with highest mean abundance of 53.9% being in the sonication method subjected for 30 minutes, for fatty acid C16:0, 55.7% in the sonication method subjected for 60 minutes, for C18:0 and 46.1% in the sonication method subjected for 60 minutes with hexane solvent for C16:1n-7. The hierarchical cluster analysis separated the methods into 4 clusters viz., cluster 1 consisting of S10, S30 and S40; cluster 2 consisting of S30n and S60n; cluster 3 consisting of S50, S60 and S20 and cluster 4 consisting of SH60 and SH30 based on the composition of fatty acids (Fig. 1). SIMPER analysis showed these groupings had high within group similarities (Table 2). The fatty acids C16:0, C18:0 were dominant to form a single cluster of 10, 30 and 40 methods. The results show that sonication for a period of 60 minutes (60) and sonication for a period of 60 minutes with hexane solvent (SH60) contribute more amounts of fatty acids (Table 1).

Bead-beating: An overall average dissimilarity of 41.88% was observed within the bead beating disruption methods through SIMPER analysis. C16:0, C18:0 and C12:0 were the principal fatty acids contributing at least 50% of the difference between the fatty acid methyl esters in all the methods. High percentage dissimilarity was found in the fatty

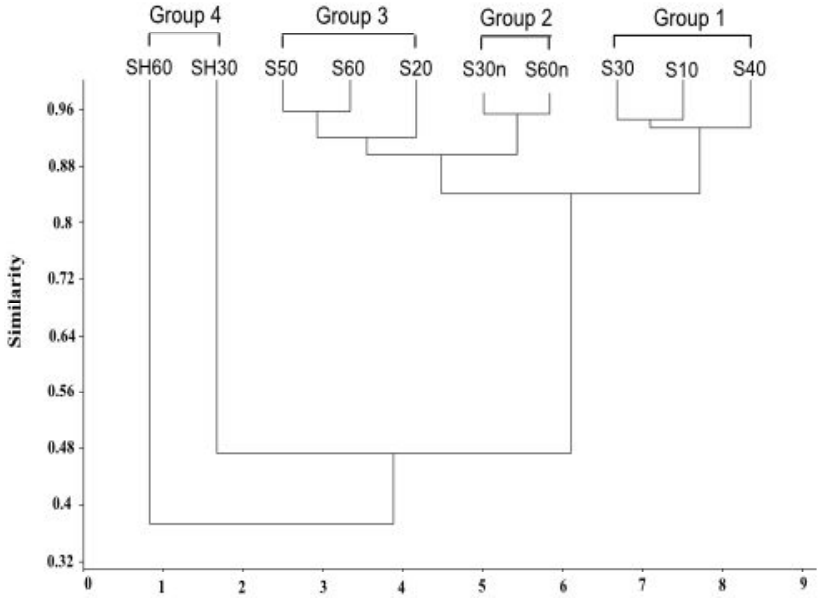

Fig. 1: Hierarchical cluster analysis of fatty acid composition (%) of the triacylglycerol extracted through sonication method from *Microcystis*.

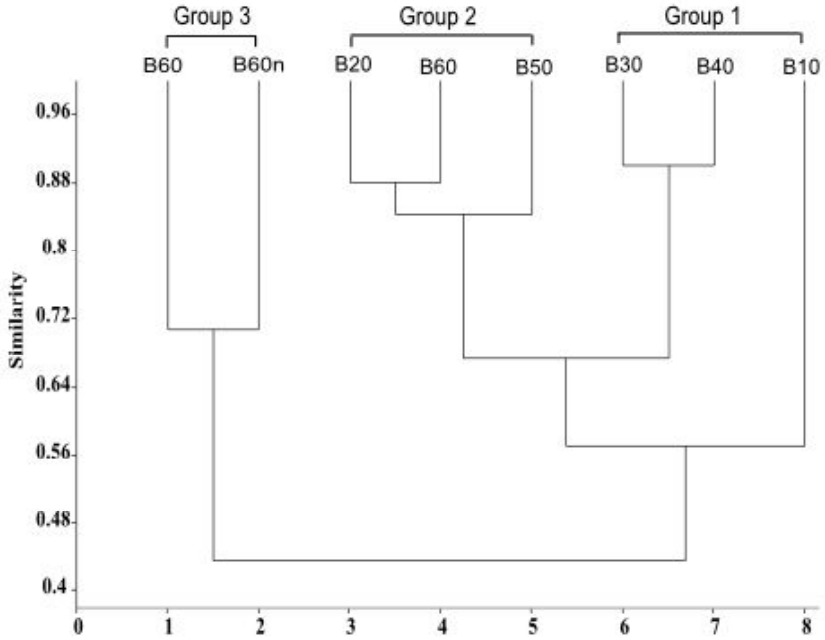

Fig. 2: Hierarchical cluster analysis of fatty acid composition (%) of the triacylglycerol extracted through bead beating method from *Microcystis*.

acids C16:0 and C18:0 with highest mean abundance for the former fatty acid of 57.3% being in the bead beating method with hexane solvent subjected for 60 minutes and the latter having 54.6% in the bead beating method with hexane solvent and liquid nitrogen subjected for 60 minutes. The hierarchical cluster analysis separated the methods into 3 clusters viz., cluster 1 consisting of B10, B30 and B40; cluster 2 consisting of B20, B60 and B50 and cluster 3 consisting of BH60 and BH60n based on the composition of fatty acids (Fig. 2). SIMPER analysis showed these groupings had within group similarities except for cluster 1 (Table 4). The fatty acids C18:0 was dominant to form a single cluster of

Table 1: SIMPER analysis of fatty acid extracted through sonication method from *Microcystis*. 0.53% of total fatty acids was contributed by other* fatty acids which were negligible hence not included in the table.

Fatty acid methyl esters	% Contribution	% Cumulative	S10	S20	S30	S40	S50	S60	SH30	SH60	S30n	S60n
C16:0	37.74	37.73	51.8	50.4	53.9	46.6	36.3	34.1	0	0	36.5	34.8
C18:0	18.28	56.02	34.8	42.8	38.5	36.9	56.3	55.7	41.5	30.8	49.6	48.4
C16:1n-7	16.89	72.91	0	0	0	0	0	0	0.59	46.1	1.24	1.65
C10:0	9.92	82.84	0	0	0	0	0	0	27.5	0.11	0	0
C8:0	4.26	87.19	0.11	0.1	0.14	0.1	0.06	0.4	1.2	0.05	0.06	0.14
C18:1n-7	2.97	91.45	0	0	0	0	0	0	1.07	11.3	0	0
C18:1n-8	4.35	94.42	6.08	1.05	1.9	1.06	1.77	0.7	0	0	0.61	0.42
C12:0	2.16	96.57	0	0	0	0	0	0	2.27	1.77	2.56	2.23
C14:0	1.15	97.72	3.02	3.55	3.3	4.54	2.71	2.4	3.02	3.5	3.3	3.65
C18:2n-6	0.94	98.66	0	0	0	0	0	0	0	0	1.03	1.9
C15:0	0.81	99.47	0	0	0	0	0	0	0.6	0.65	0.81	1.13

*12-tetradecanoic acid, methyl ester and 14-hexadecanoic acid, methyl ester

Codes: S10-sonication for 10 mins, S20-sonication for 20 mins, S30-sonication for 30mins, S40-sonication for 40 mins, S50-sonication for 50 mins, S60-sonication for 60 mins, SH30-sonication with hexane solvent for 30 mins, SH60-sonication with hexane solvent for 60 mins, S30n-sonication with liquid nitrogen for 30 mins, S60n-sonication with liquid nitrogen for 60 mins

Table 2: Results of SIMPER analysis: Within-group similarity (% in parenthesis) and average dissimilarity (%).

Average dissimilarity	Group 1 (81.235)	Group 2 (97.091)	Group 3 (79.641)	Group 4 (41.6)
Group 1	-	13.96	10.71	43.82
Group 2	-	-	8.605	45.92
Group 3	-	-	-	43.20
Group 4	-	-	-	-

Table 3: SIMPER analysis of fatty acid extracted through bead beating method from *Microcystis*. 0.38% contribution by other fatty acids* was found negligible hence not included in the table.

Fatty acid methyl esters	% Contribution	% Cumulative	B10	B20	B30	B40	B50	B60	BH60	B60n
C16:0	35.74	35.74	0	0	0	0	0	0	57.3	34.3
C18:0	34.41	70.15	20.9	30.9	16.7	19.2	34.4	39.4	26.4	54.6
C12:0	21.89	92.04	29.9	1.71	0.76	1.54	11.1	1.76	3.28	1.55
C14:0	3.12	95.16	5.09	2.57	1.9	2.55	2.7	3.03	5.3	2.56
C18:1n-7	1.53	96.69	0	0	0	0	0	0	1.85	2.16
C16:1n-7	1.32	98.00	1.54	0.6	0.32	0.8	1.11	1.27	0.49	0.58
C20:0	0.71	98.72	0	0	0	0	0	0	0.76	1.08
C15:0	0.46	99.18	0	0	0	0	0	0	0.72	0.46
C18:1n-8	0.44	99.62	0	0	0	0	0	0	0.41	0.71

*Decanoic acid, methyl ester, Octanoic acid, methyl ester and 12-tetradecanoic acid, methyl ester

Codes: B10 - bead-beating for 10 mins, B20 - bead-beating for 20 mins, B30 - bead-beating for 30mins, B40 - bead-beating for 40 mins, B50 - bead-beating for 50 mins, B60 - bead-beating for 60 mins, BH60 - bead-beating with hexane solvent for 60 mins, B60n - bead-beating with liquid nitrogen for 60 mins

B30 and B40; C18:0 (<30.00%) was dominant to form a cluster of B20, B50 and B60 and C16:0, C18:0 was dominant to form a separate cluster of BH60 and BH60n. The results show that bead beating for 60 minutes of hexane solvent (BH60) and hexane solvent with liq. nitrogen for a period of 60 minutes (BH60n) contribute more amounts of fatty acids (Table 3).

Manual disruption: The differences between the amounts of fatty acid compositions obtained by manual methods were less than 3% for any fatty acid. The analysis of variance (one way ANOVA) showed that the differences were not statistically significant for any fatty acid ($P > 0.05$). An overall average dissimilarity of 11.21% was observed within the manual method through SIMPER analysis where, C12:0,

Table 4: Results of SIMPER analysis: Within-group similarity (% in parenthesis) and average dissimilarity (%).

Average dissimilarity	Group 1 (0.29)	Group 2 (56.59)	Group 3 (70.6)
Group 1	-	30.96	51.18
Group 2	-	-	36.87
Group 3	-	-	-

Table 5: SIMPER analysis of fatty acid composition of triacylglycerol extracted through manual method from *Microcystis*. Percentage contribution of 0.7 %* is not included in the table.

Fatty acid methyl esters	% Contribution	% Cumulative	M60	MH60
C12:0	39.54	39.25	38.5	42.5
C18:0	23.07	62.15	34.5	30.5
C16:0	11.92	73.98	11.2	13.7
C16:1n-7	6.73	80.66	2.8	1.23
C18:1 n-8	6.07	86.69	2.07	0.86
C15:0	5.32	91.97	7.5	8.07
C10:0	2.91	94.87	0.81	0.5
C8:0	2.75	97.6	0.0008	0.007
C14:0	1.71	99.29	0.00809	0.00434

*14-hexadecanoic acid, methyl ester; Codes: M60-manual with liquid nitrogen for 60 mins, MH60-manual with hexane solvent for 60 mins

C18:0 and C16:0 contributed at least 50% of the difference between the fatty acid methyl esters in all the methods. High percentage dissimilarity was found in the fatty acids C12:0 and C18:0 with highest mean abundance for the former fatty acid of 42.5% being in the manual method subjected for 60 minutes with hexane solvent and liq. nitrogen and the latter having 38.5% in the manual method subjected for 60 minutes with hexane solvent (Table 5). Method MH60 recovered slightly greater quantities of almost all identified fatty acids (Table 5).

Optimal cell disruption technique: Sonication and bead beating methods are efficient for the extraction of *Microcystis* as the manual method is complicated, time-consuming and difficult to scale up. Comparison of bead beating and sonication methods for lipid extraction, show that sonication is the most efficient method due to cavitation effects (Lee et al. 1998, Engler 1985). Lee et al. (1998) have demonstrated higher lipid content from *Botryococcus braunii* through bead-beating method. However, efficiency of the bead-beating method is not easy to scale-up (Lee et al. 2010). Even though sonication with liquid nitrogen is as efficient as in the absence of liquid nitrogen, the latter method can be optimized as liquid nitrogen is dangerous to handle. Hence, this study confirms that the sonication method was the most simple, easy and efficient method for the tested microalgae. In conclusion, the sonication method for 60 minutes was found to be the most applicable for large-scale lipid extraction from a microalgal biomass.

Fatty acid composition: The major fatty acid composition of the tested microalgae *Microcystis* for all the methods was determined using GC-MS (Table 1, Table 3 and Table 5). In the three methods tested, palmitic acid (C16:0) and stearic acid (C18:0) were commonly dominant. Palmitic acid was higher (53.89%) in the sonication method for 30 minutes, while stearic acid was highest (56.27%) in the sonication method for 60 minutes, further supporting that sonication for 60 minutes can be used as an effective method for disruption of microalgae and hence extraction of lipids. Palmitic, stearic, oleic, and linolenic acid were observed as the most common fatty acids contained in biodiesel (Knothe 2008).

CONCLUSION

In an endeavour towards achieving optimal efficacy in extraction, the results reveal that sonication is the most efficient compared to other methods apart from the advantages such as easy to scale up, less complicated and reasonable time. These parameters directly depend upon the fatty acid composition of the biodiesel fuel during the transesterification reaction or storage conditions. However, the physical properties of the fuel which include ignition quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity and lubricity, have to be determined to assess the complete potential of *Microcystis* for biofuel production.

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