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Research Article

Growth, Fatty Acid, and Lipid Composition of Marine Microalgae *Skeletonema costatum* Available in Bangladesh Coast: Consideration as Biodiesel Feedstock

Tania Sharmin,[1] Chowdhury Md. Monirul Hasan,[1] Sheikh Aftabuddin,[2] Md. Atiar Rahman,[1] and Mala Khan[3]

[1]*Department of Biochemistry & Molecular Biology, University of Chittagong, Chittagong 4331, Bangladesh*
[2]*Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong 4331, Bangladesh*
[3]*Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-i-Khuda Road Dhanmondi, Dhaka 1205, Bangladesh*

Correspondence should be addressed to Chowdhury Md. Monirul Hasan; monirkyushu@gmail.com

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Among the various potential sources of renewable energy, biofuels are of most interest. Marine microalgae are the most promising oil sources for making biofuels, which can grow very rapidly and convert solar energy to chemical energy via CO_2 fixation. The fatty acid profile of almost all the microalgal oil is suitable for the synthesis of biofuel. In this research, fatty acid and lipid contents of Bangladeshi strains of marine microalgae *Skeletonema costatum* were performed. For this, the crude oil was extracted by Soxhlet extraction method, using three most common solvent systems, pure hexane and mixture of $CHCl_3$: MeOH (2 : 1) and hexane : EtOH (3 : 1) one by one. Highest oil recovery (15.37%) came from $CHCl_3$: MeOH (2 : 1) solvent system from dry biomass whereas the lowest (2.49%) came from n-hexane from wet biomass. The qualitative analysis of the extracted oil by GC/MS analysis revealed that it contained significant amount of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and palmitoleic acid (C16:1). It also indicated presence of hexadecatrienoic acid, benzenedicarboxylic acid, oleic acid, arachidonic acid, eicosapentaenoic acid (EPA), 9-Octadecenoic acid methyl ester ($C_{19}H_{36}O_2$), and so forth. The obtained fatty acid profile indicates high potentiality of *S. costatum* species to be used as promising biofuel feedstock a little improvisation and substantially it can replace diesel in near future.

1. Introduction

Energy demand worldwide is increasing continuously at a rapid rate with the increase of urbanization in developing countries. Majority of the world's energy needs are supplied through petrochemical sources, coal, and natural gases, with the exception of hydroelectricity and nuclear energy, and these sources are finite and at current usage rates will be consumed shortly [1]. Heavy dependence on petroleum-based fuels is not sustainable due to increasing fuel costs, diminishing crude oil reserves, and the environmental impact of fossil fuel usage [2–4]. Crude oil reserves are being depleted at a rate of approximately 85–90 million barrels of oil per day and it is possible that the reserve will be completely depleted within the next 50 years [5]. Therefore, alternative sources of energy will need to be researched and developed within the next 50 years. Continued research will facilitate the development and implementations of renewable fuels to help lessen the world's dependence on fossil fuels.

Biodiesel is a biofuel consisting of monoalkyl esters that are derived from organic oils, plant, or animal, through the process of trans-esterification [6]. It is also biodegradable and nontoxic and has low emission profile as compared to petroleum diesel [7]. Biodiesel can be burned in existing diesel engines with no modifications and can be blended in any proportion with petroleum diesel. This allows for its use in the existing fuel distribution infrastructure and giving it value as a fuel extender. Shay [8] reported that algae are one

of the best sources of biodiesel. In fact, algae are the highest yielding feedstock for biodiesel. It can produce 250 times more than the amount of oil per acre as soybeans and 7 to 31 time greater oil than palm oil [9]. In fact, biodiesel from algae may be the only way to produce enough automobile fuels to replace current gasoline usage. The best algae for biodiesel would be microalgae [10]. Microalgae have much more oil than macroalgae and it is much faster and easier to grow and harvest [8].

Microalgae can also provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass [11]; biodiesel derived from microalgal oil [12–17]; and photobiologically produced biohydrogen [18–22]. The idea of using microalgae as a source of fuel is not new [2, 16, 23], but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels [14].

Depending on species, microalgae produce many different kinds of lipids, hydrocarbons, and other complex oils [12, 24]. Not all algal oils are satisfactory for making biodiesel, but suitable oils occur commonly. Another important fact is that using microalgae to produce biodiesel will not compromise with the production of food, fodder, and other products derived from crops. The objective of this investigation was to identify locally available microalgal strains with sustainable lipid content for biofuel production and evaluate the biofuel productivity of that strain. For this purpose, the present work was carried out with marine microalgae, *S. costatum*, that are dominant in the coastal area, especially in Cox's Bazar coast line of Bangladesh shown in Figure 1.

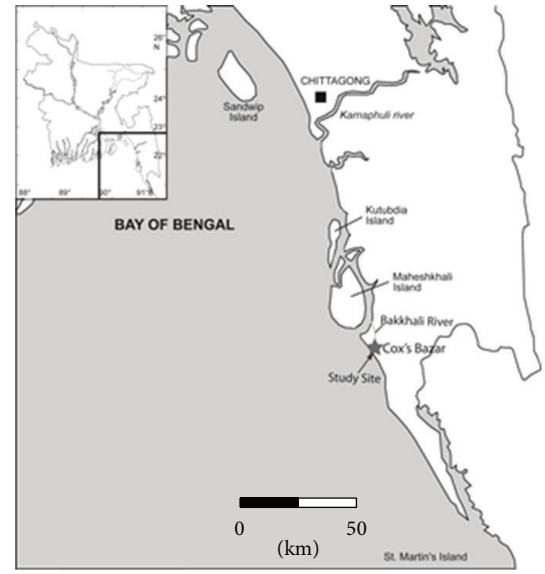

Figure 1: Location of the *S. costatum* sample collection area in the southeastern region, Bangladesh.

2. Materials and Methods

The present investigation was carried out in the Centre for Research Excellence (CRE), Department of Biochemistry and Molecular Biology, University of Chittagong, Chittagong, Bangladesh.

2.1. Sample Collection. *S. costatum* sample water was collected from Bakkhali River of Cox's Bazar (Figure 1), Bangladesh, in January 2014. Microscopic isolation and identification were done at Institute of Marine Sciences and Fisheries, University of Chittagong.

2.2. Culture of Skeletonema costatum. For isolation, standard f/2 medium [25] nutrients were added with the sample water and left under light until diatom bloom. Bloomed *S. costatum* was then isolated and purified by serial dilution and micropipette method [26, 27]. In micropipette method, single cells or filaments were picked up under a dissecting microscope from the enriched sample water, by using 5 mL syringe. The individual cell was transferred to fresh sterile f/2 medium containing 100 mL flasks and kept under light at 25°C for 1 week. For serial dilution, 5 sterilized test tubes, filled with 9 mL sterilized seawater enriched with f/2 medium, were marked as 10^{-1} up to 10^{-5} serially and 1 mL of sample containing bloomed diatom was transferred to 10^{-1} test tube, then 1 mL from 10^{-1} to 10^{-2}, and so on. The clones were kept in 24 hours photoperiod to develop for 10 to 15 days as stock culture. For large scale *S. costatum* production 200 liters FRP tanks were used according to the procedure of Aftab Uddin and Zafar [28]. Cell counts were observed by a compound microscope and concurrently salinity (25–28 ppt), temperature (25–27°C), and pH (7.6–8.2) were maintained during the entire culture period and sufficient aeration was provided.

2.3. Biomass Processing. At first, the biomass was dewatered by filtration with a Whatman filter paper. Then the wet algal biomass was collected from the filter paper with a spatula and taken in Petri dishes. The biomass was then dried at 80°C for 2 hours and kept in the desiccator for whole night so that there is no water left in the sample. The dry mass was then grounded properly and weighed immediately. The drying step was skipped in case of wet extraction process.

2.4. Soxhlet Extraction of Fatty Acid and Lipids. Extensive study and research were conducted earlier to determine the best extraction method over wet and dry algae biomass. In this experiment, the effect of three different solvents, that is, n-hexane [29], the mixture of chloroform and methanol at 2 : 1 v/v ratio [30], and n-hexane and ethanol at 3 : 1 v/v ratio [31], had been studied over wet and dry biomass of *S. costatum* by using Soxhlet apparatus [32, 33]. For this, 5 g of dry weight and 10 g of wet fresh algae paste were taken into the extraction chamber separately. Both ends of the chamber were enclosed with cotton balls to withstand any solid algae discharge. The bulb was filled with 180 mL of one of the three organic solvents and heated at 80°C. The solvent gradually started to vaporize and after condensation, it fell into the extraction chamber containing the algae and release lipids into the chamber. The solvent-lipid mixture was then reached to a critical height within the chamber and the siphoning process

was initialized; that is, the mixture was drawn back to the bulb. The system recirculates the solvent by constantly boiling and condensing it. At the end of this extraction process, a mixture of solvent and oil was left in the bulb and the algae biomass was left in the chamber. The processing before evaporation was performed as described by Kumar et al. [34]. The crude extract was taken into a separating funnel and washed with 1% aqueous sodium chloride solution (50 mL) twice. The aqueous layer was removed and the solvent layer was passed through a layer of anhydrous sodium sulfate by taking it in a glass funnel, blocked with cotton plug.

2.5. Evaporation. The solvent-oil mixture was placed into a preweighed flask for evaporation. In the laboratory setup, the solvent and oil mixture was exposed to a vacuum and then heated at 60°C by using a vacuum pump Rotary Evaporator (RE 200, Bibby Sterling, UK) in order to remove all the solvent. After evaporation, the flask was weighed again and compared with the first weight. In this way, the weight of the oil content was calculated. The oil was recovered by using dichloromethane and was collected into a small vial. The percentage of oil extraction was calculated by the following formula:

$$\%\ \text{of total oil recovered} = \frac{\text{Weight of crude lipid extracted}}{\text{Weight of dry algae biomass}} \times 100. \quad (1)$$

2.6. Quantitative Analysis of Algal Oil

2.6.1. TLC Analysis. The components of extracted lipids were separated by modified thin-layer chromatography (TLC) method [35]. This was done by TLC silica gel plates (50 mm × 100 mm in size) and was diffused by two mixed solvents [36]. The first eluent was the mixture of ethyl acetate, isopropanol, chloroform, methanol, and KCl (0.25% solution) in a ratio of 25 : 25 : 25 : 10 : 9 (v/v/v/v/v) running to a height of 10 cm from the origin and the second eluent was the mixture of hexane, diethyl ether, and acetic acid in a ratio of 80 : 20 : 2 (v/v/v) to a height of 18 cm from the origin. After dried, the plate was kept in a TLC jar with iodine powder. The individual lipid bands became detectable as yellow bands within 5 min. In iodine vapor exposure, four individual bands were found for $CHCl_3$: MeOH (2 : 1) extract, six bands for hexane : EtOH (3 : 1) extract, and a single band for pure hexane were found. For the recovery of individual lipid component, the corresponding silica gel was removed from the plates and washed it with chloroform/methanol (2 : 1 v/v) [36]. The lipid component, separated by TLC as described above, was then kept for the fatty acid composition analysis by GC/MS.

2.6.2. Sample Preparation for GC/MS. The fatty oils were esterified with methanol prior to Gas Chromatography-Mass Spectroscopy (GC/MS) analysis to make the fatty oils more volatile and to avoid the acidic attack to the stationary phase/column. For this, 10 mg (2-3 drops) of fatty oil was taken in a screw capped glass tube and 1 mL of Boron trifluoride methanol complex was added. The mixture was then heated at 100°C for 1 hr using water bath and cooled at 25°C. Then 1 mL of deionized water and 2 mL hexane were added and vortexed for 1 min. Then the mixture was centrifuged at low rpm for 2 min, and upper layer was collected, diluted with hexane, and filtered for GC/MS analysis.

2.6.3. GC/MS Analysis. Thin-layer chromatographic (TLC) separation was analyzed by GC-MS with electron impact ionization (EI) method on a gas chromatograph (GC-17A, Shimadzu Corporation, Kyoto, Japan) coupled to a mass spectrometer (GC-MS QP 5050A, Shimadzu Corporation, Kyoto, Japan). A fused silica capillary column (30 m × 2.5 mm; 0.25 m film thickness) is coated with DB-1 (J&W). The inlet temperature was set at 260°C and the oven temperature was programmed as 70°C (0 min); 10°C, 150°C (5 min); 12°C, 200°C (15 min); 12°C, 220°C (5 min). The column flow rate was 0.6 mL/min He gas at a constant pressure of 90 KPa. The aux (GC to MS interface) temperature was set to 280°C. The MS was set in scan mode with a scanning range of 40–350 amu; the ionization mode was EI (electron ionization) type. The mass range was set in the range of 50–550 *m/z*. The prepared sample was then run for GC/MS analysis. One μL sample was injected in spilt fewer modes. Vial containing lipid was used for GC/MS analysis to make lipid profile. This lipid profile indicates the fatty acids, which are important to evaluate the biomass for biofuel production.

2.7. Statistical Analysis. All the data were expressed as mean ± SD and they were analyzed by using Microsoft Excel 2007. The significance of difference was calculated by Student's *t* test and the values $P < 0.05$ were considered to be significant.

3. Results

The result of total oil recovery by three different solvents is shown in Table 1. It showed that every solvent system gives maximum oil recovery for dry sample and relatively low recovery for wet sample. The total oil recoveries obtained from dry and wet biomass by using pure hexane were 11.24% and 2.49%, by $CHCl_3$: MeOH were 15.37% and 6.70%, and by hexane : EtOH were 11.67% and 5.32%, respectively. The result showed that, in both wet and dry conditions, $CHCl_3$: MeOH (2 : 1) solvent system gives the highest yield.

The comparison of oil content and % oil contents from dry and wet sample of different solvent extracts is shown in Figure 2. It is clearly evident that both oil content and % oil content of dry wet from $CHCl_3$: MeOH extract are significantly ($P < 0.05$) different from those of two other solvent systems. Similarly, their contents in wet sample are also higher in $CHCl_3$: MeOH although the values were not statistically significant (Figure 2).

The fatty acid methyl ester (FAME) composition analysis of the extracted oil was very important for the evaluation of a biofuel feedstock. For this purpose, the extracted algae oil was analyzed by GC/MS to obtain the fatty acid profile of *S. costatum*. The result showed the fatty acid and lipid profile from pure n-hexane extract presented in Table 2, $CHCl_3$: MeOH extract in Table 3, and hexane : ethanol extract in Table 4.

Table 1: Percentage of total oil recovered by the three different solvents.

Solvent	Physical condition	Sample wt. (g)	Oil content (g)	% of oil content
n-Hexane	Dry	5.00 ± 0.00	0.563 ± 0.052	11.24 ± 1.20
	Wet	10.00 ± 0.00	0.247 ± 0.115	2.49 ± 1.29
$CHCl_3$: MeOH (2 : 1)	Dry	5.00 ± 0.00	0.77 ± 0.054	15.37 ± 1.23
	Wet	10.00 ± 0.00	0.67 ± 0.094	6.70 ± 1.06
n-Hexane : EtOH (3 : 1)	Dry	5.00 ± 0.00	0.58 ± 0.089	11.67 ± 1.98
	Wet	10.00 ± 0.00	0.53 ± 0.151	5.33 ± 1.68

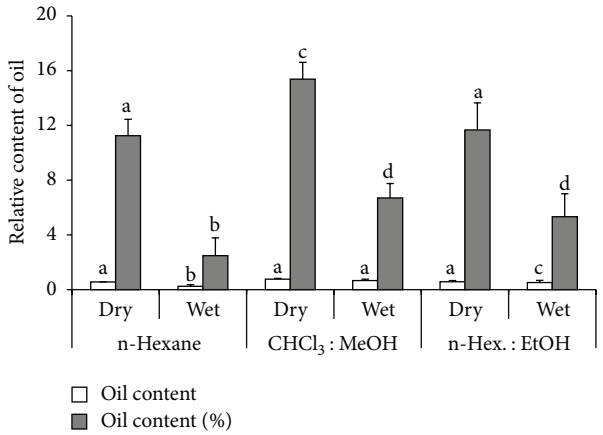

Figure 2: Comparative oil contents in dry and wet sample of *S. costatum* due to solvent variation. Data are shown as mean ± SD (a, b, c, and d) with different superscript letters over the bars for both dry and wet samples are significantly different from each other (paired t-test, $P < 0.05$).

Most importantly, the fatty acids suitable for biodiesel production such as myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), and stearic acid (C18:0) were the most frequent entities encountered in *S. costatum* profile.

Table 2 showed that myristic acid (C14:0) and palmitic acid (C16:0) were the dominants in pure hexane extract about 35.93% and 27.21%, respectively. The fatty acid profile of $CHCl_3$: MeOH extract (presented in Tables 3 and 4) showed that the main constituents of this extract are myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0) up to 36.39%, 25%, and 27.72%, respectively. Palmitoleic acid, oleic acid, arachidonic acid, pentadecanoic acid, eicosapentaenoic acid, and other fatty acids were also present in low content.

4. Discussion

Bioenergy is one of the most important sources that are concerning the scientists and industrial sector. In view of ever increasing global demand for energy, there has been substantial interest in developing renewable biologically produced fuel. Marine algae are one such emerging resource considered as an alternative for biofuel production. Oil extracted from algal biomass can be turned into biodegradable and carbon neutral biodiesel through transesterification reaction [2, 37].

To be an ideal source of sustainable biodiesel, selected microalgal species should contain sufficient lipid with suitable fatty acids for good biodiesel properties. Several works were carried out to extract algal oil for biofuel production, commonly using different organic solvents, and the lipid contents were often reported as the weight ratio of the crude extract and the dry biomass. In this study, the algal strains were cultured and their oil extraction was done by using three different solvent systems, pure hexane [29], $CHCl_3$: MeOH [35], and hexane : EtOH [31] to compare their oil recovery. The result showed that every solvent system recovered their highest yield from dry biomass and in both dry and wet conditions, $CHCl_3$: MeOH (2 : 1) and n-hexane : EtOH (3 : 1) were comparatively more effective than pure n-hexane recovering about 15.367% and 11.673% oil, respectively. However, the oil content in the dry sample through $CHCl_3$: CH_3OH is significantly ($P < 0.05$) different from that of two other solvent systems. This may be because $CHCl_3$: MeOH (2 : 1) and n-hexane : EtOH (3 : 1) both are the mixture of polar (MeOH, EtOH) and nonpolar ($CHCl_3$, n-hexane) solvents; thus both neutral and polar lipids could be extracted by these two solvent systems. On the other hand, the nonpolar solvent n-hexane could preferably dissolve only nonpolar lipids in the microalgae. However, it could not dissolve all the oil content from the cells alone.

A systematic analysis of the fatty acid methyl ester (FAME) composition and comparative fuel properties is very important for species selection for biodiesel production. It has been suggested that the higher the degree of unsaturation of the FAMEs of a biodiesel, the higher the tendency of the biodiesel to oxidize. There are, however, other parameters which also define the oxidation stability of the fuel, for example, natural antioxidant and free fatty acid content [38–40]. The most common fatty acids of microalgae are palmitic-(hexadecanoic-C16:0), stearic (octadecanoic-C18:0), oleic (octadecenoic-C18:1), linoleic-(octadecadienoic-C18:2), and linolenic-(octadecatrienoic-C18:3) acids [41]. Most algae have only small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6); however, in some species of particular genera, these PUFAs can accumulate in appreciable quantities depending on cultivation conditions [42]. Consistently the oil extract of *S. costatum* showed the prevalence of methyl palmitate, methyl stearate, and methyl myristate when methyl myristate, methyl palmitate, and methyl 6,9,12-hexadecatrienoate were 35.93%, 27.21%, and 12.36% in the n-hexane fraction respectively. While $CHCl_3$: MeOH fraction showed methyl palmitate (22.41),

Table 2: Fatty acid profile of pure n-hexane extract.

S. number	Retention time	Area%	Compound name
1	16.699	35.93	Methyl myristate
2	18.045	8.73	trans-2-(3-Methoxyphenyl)-3-phenyl oxirane
3	19.225	12.36	Methyl 6,9,12-hexadecatrienoate
4	19.418	7.94	Methyl palmitoleate
5	19.860	27.21	Methyl palmitate
6	21.964	1.92	5H-Benzofuro[3,2-e]imidazo[1,2-a]pyrazin-4-one
7	22.585	1.12	(trans)-2-(3′-Chlorophenyl)-1-nitroethylene
8	24.903	3.38	Methyl stearate

Table 3: Fatty acid profile of lipid from $CHCl_3$: MeOH extract.

S. number	Retention time	Area%	Compound name
1	9.523	11.53	1,2-Benzenedicarboxylic acid, dimethyl ester
2	10.731	3.72	Phenol, 2,4-bis(1,1-dimethylethyl)-
3	10.869	2.19	Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-
4	15.461	4.38	Methyl myristate
5	16.841	2.46	Pentadecanoic acid, methyl ester
6	16.855	3.47	2,2-Dimethyl-6,7-dimethoxychromanone
7	16.862	2.34	2,6-Dimethoxy-3-(3′-methyl-2′-butenyl)-1,4-benzoquinone
8	17.113	1.46	1-(4-Fluorophenyl)-2-[(4-hydroxy-6-methylpyrimidin-2-yl)thio]ethan-1-one
9	17.365	1.68	1-(4-Fluorophenyl)-2-[(4-hydroxy-6-methylpyrimidin-2-yl)thio]ethan-1-one
10	17.510	2.04	2-Cyclohexen-1-one, 4,4-dimethyl-
11	17.655	9.96	Methyl 6,9,12-hexadecatrienoate
12	17.762	1.39	(E)-2,4-Phytadiene m-Menth-1(7)-ene, (R)-(−)-
13	17.772	6.91	Methyl palmitoleate
14	17.810	8.35	9,12-Octadecadien-1-ol, (Z,Z)-
15	17.983	22.41	Methyl palmitate
16	19.201	3.24	Methyl(5R)-4-hydroxy-5,6-(isopropylidenedioxy)-2-methylenehexanoate
17	19.294	1.39	Methyl 3-methylquinoxaline-2-carboxylate
18	19.318	14.99	Methyl N-[(1-naphthyl)methyl]carbamate
19	19.698	2.30	Methyl oleate
20	19.860	24.74	Methyl stearate
21	20.967	1.68	Ethoxylate ester of methyl oleate
22	21.005	3.48	Methyl arachidonate
23	21.084	7.72	*cis*-5,8,11,14,17-Eicosapentaenoic acid, methyl ester (EPA methyl ester)
24	22.464	3.40	3-*cis*-Methoxy-5-*cis*-methyl-1R-cyclohexanol
25	28.253	2.40	Propane, 1,1-dibromo-2-chloro-

methyl N-[(1-naphthyl)methyl]carbamate (14.99), and 1,2-Benzenedicarboxylic acid as prevalent fatty acids. However, in hexane : EtOH fraction, methyl stearate (20.64%) is only the fatty acid present in considerable quantity.

The abovementioned FAs of marine microalgae *S. costatum* found from the lab-scale examination are dominant FAs which are known to be most common fatty acids with good biofuel properties [39]. Other FAs detected were hexadecatrienoic acid, benzenedicarboxylic acid, oleic acid, arachidonic acid, and eicosapentaenoic acid (EPA). Indeed, most of the microalgae investigated have similar fatty acid profile, but the percentage content of fatty acid for each microalga is very different. This mainly depends on the strain used and culture conditions [43, 44]. All of the microalgae have the same fatty acid profile in chain C16 and C18. Palmitic fatty acid (C16:0) is a predominant fatty acid in

Table 4: Fatty acid profile of lipid band no. 1 of Hexane : EtOH extract.

SN	Retention time	Area%	Compound name
1	10.728	3.40	Phenol, 2,4-bis(1,1-dimethylethyl)-
2	10.862	3.20	Butylated Hydroxytoluene
3	10.866	2.29	Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-
4	11.007	1.54	Methyl laurate
5	15.199	1.43	Cyclohexanecarboxylic acid, 2,2-dimethylpropyl ester
6	15.461	4.08	Methyl myristate
7	16.858	1.62	10-Ethyl-3,3,6,9,9-pentamethyl-2,10-diazabicyclo[4..0]-1-decene
8	17.979	1.77	Methyl palmitate
9	19.856	20.64	Methyl stearate
10	20.967	1.44	Methoxylate ester of methyl oleate
11	17.082	1.14	2-Pentadecanone, 6,10,14-trimethyl-
12	17.800	1.71	Methyl palmitoleate
13	18.773	1.14	(E)1-Allyl-2-methylcyclohexanol
14	19.232	1.07	Sulfurous acid, di(2-ethylhexyl) ester
15	19.297	6.00	1,4-Dihydropyrano[3,4-b]indol-3-on
16	19.459	1.14	Methyl 4-oxododecanoate
17	19.698	2.40	Methyl oleate
18	21.043	1.21	Butyl-2-ethylhexyl phthalate

most microalgae research. Some critical fuel parameters like oxidation stability, cetane number, iodine value, and viscosity were correlated with the methyl ester composition and structural configuration. From the current literature, it was found that the FAs indicated by the fatty acid profile of *S. costatum* are of better fuel properties and thus it is likelihood to be a viable fuel source for internal combustion engines.

The present study offers a scientific basis of the use of this microalga as a feedstock for biofuel. Still it is in a preliminary stage that requires further study on other parameters. It also indicates that it may be possible in the future to improve the properties of biodiesel by means of genetic engineering of the parent oils, which could eventually lead to a fuel enriched with a combination of improved fuel properties.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Chowdhury Md. Monirul Hasan and Tania Sharmin have designed the study and performed the experimental works in laboratory. Sheikh Aftabuddin has assisted in the collection and identification of microalgae strains and prepared their growth environment. Mala Khan has provided her laboratory facilities in analyzing the compounds. Chowdhury Md. Monirul Hasan, Sheikh Aftabuddin, Md. Atiar Rahman, and Tania Sharmin drafted the paper. Md. Atiar Rahman has endeavored in data analysis and interpretation of the results. All authors read and approved the final version of the paper.

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