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# Characterization of unicellular microgreen algae of Manipur, India falling under Indo-Burma biodiversity hotspots with special emphasis on lipid composition

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## ABSTRACT

*In the present investigation, nine (09) unicellular microgreen algae of freshwater habitats of Manipur were characterized for total lipid content and fatty acid composition. Nile red fluorescence indicated more number of lipid bodies at stationary growth phase. Maximum lipid content was observed in Chlorella sp. (BTA-3110). Profiling of fatty acid composition showed that palmitic acid (16:0) and linolenic acid (C18:3n3) were the predominant components. Chlorococcum sp. (BTA-3112) and Korshikoviella sp. (BTA-3019) showed high palmitic acid as 54.25% and 49.68% respectively. Arachidic acid (C20:0) was maximum in Chlorella sp. (BTA-3110) as 21.57%. Among the polyunsaturated fatty acid (PUFA), linolenic acid (C18:3n3) was maximum in Chlorella sp. (BTA-3063) as 37.37%. High amount of linoleic acid (C18:2n6) was observed in Chlorella sp. (BTA-3101) as 12.62%. One of the pharmaceutically potential component, eicosapentaenoic acid (C20:5n3) occurred maximum amount in Chlorella sp. (BTA-3086). Oleic acid (C18:1n9) was the most abundant monounsaturated fatty acid (MUFA) in all the strains ranging between 0.70% to 15.79%. The levels of erucic acid (C22:1n9) and palmitoleic acid (C16:1) were also present but in low quantity in most strains. Furthermore, these potent strains which showed best lipid profile may be beneficial for use in value added products and biodiesel purpose and is appropriate for exploitation for higher scale studies.*

**Keywords:** Biodiesel, Fatty acid, Hotspots, Lipid, Microgreen, Nile red

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## INTRODUCTION

Microalgae represent an exceptionally diverse but highly specialized group of microorganisms adapted to various ecological habitats. They are fast-growing unicellular or simple multicellular microorganisms, offer several advantages, including higher photosynthetic efficiency, higher growth rate and higher biomass production compared to other energy crops [1]. They are prokaryotic or eukaryotic sun light driven cell factories that coverts carbon-

dioxide ($CO_2$) and water to potential biofuel, foods, feeds and high value bio-active [2]. Among the microalgae, *Scenedesmus* and *Chlorella* species have the most desirable features for efficient and economic combination of carbon-dioxide ($CO_2$) fixation, wastewater treatment and lipid synthesis toward biodiesel production [3, 4, 5]. They are superior to traditional oleaginous crops due to higher photosynthetic efficiency, faster growth rate, higher biomass productivities, highest $CO_2$ fixation and $O_2$ production rate. The average yield of microalgal biodiesel production is 10 to 20 times higher and requires 49-132 times less land area than the other oleaginous seeds [6]. Most recently, research efforts have been aimed at identifying suitable biomass producing species which can provide high-energy outputs to replace conventional fossil fuels. Algae cultures have been principally developed as an important source of many products, such as aquaculture feeds, human food supplements, and pharmaceuticals and they have been suggested as a very good candidate for fuel production [7, 8].

Nile red staining was a high throughput, rapid screening technique to determine the neutral lipid content of many algal strains. Lipids including hydrocarbons and triglycerides were stained in yellow, while chlorophyll was stained in red [9]. It was used to screen for isoprenoid-derived hydrocarbon production in microalgae. Lipid qualification and quantification can be carried out by several means including nile red fluorescence microscopy, nile red spectrofluorometry, fourier transform infrared micro-spectroscopy (FTIR), thin-layer chromatography (TLC) and gas chromatography (GC) with mass spectrometry (MS) [10].

Many microalgae have the ability to produce substantial amounts (20-50% dry cell weight) of triacylglycerols (TAG) as a storage lipid under photo-oxidative stress or other adverse environmental conditions. Microalgae are rich source of protein, carbohydrates and especially essential fatty acids. The growth of phytoplankton is a function of many factors, including light, nutrients, temperature, pH, and salinity. Microalgae with high oil productivities are desired for producing biodiesel. Depending on the species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils [11, 12]. Identifying a good strain for oil production, which could feature high lipid content, high biomass and tolerance to extreme environment, remains a difficult prospect. Under adverse growth conditions such as nitrogen limitation, low temperature, high light intensity, high salt concentration and high iron concentration the lipid content in some of microalgae increased [13, 14]. In selecting algae strains for biomass conversion into the energy, attention was focused on those which were robust, highly productive, etc. [15]. Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and fatty acid composition. Strains with relatively high lipid content are very attractive for biodiesel fuel production [16]. Microalgae have been attracting attention as a source of high-lipid material to produce biofuel because the biofuel they produce are biodegradable, renewable, non-toxic fuel and do not compete with food crops. The aim of the present study was to investigate microgreen algal species with high lipid content and suitable fatty acids where the potent strains could be used for the production of biodiesel and commercially important products.

## MATERIALS AND METHODS

### Microalgal strains and growth conditions

Microgreen algal strains were obtained from National Repository for Cyanobacteria and Microgreen algae (Freshwater), a facility created by the Department of Biotechnology, Government of India with vide order no. BT/PR11863/PBD/26/424/2014 dated 23-03-2015 at Institute of Bioresources and Sustainable Development (IBSD), Imphal, Manipur, India. These strains were previously isolated from fresh water habitat from different location of Manipur, the Indo-Burma biodiversity hotspot in the north-eastern region of India.

Microgreen algal strains were inoculated in Hoffmann's flasks containing BG-11 broth medium [17]. The flasks were kept in the culture room under light:dark cycles of 14:10 h conditions maintained at 28±2ºC under illumination of 54-67 µmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. The flasks were shaken manually for 5 mins daily to prevent cell clumping.

### Nile red staining for lipid determination

Nile red (9-diethylamino-5H-benzo [α] phenoxazin-5-one) staining was carried out to detect intracellular lipid droplets [18]. Nile red solution (0.1 mg $ml^{-1}$ in acetone) was added to cell suspensions and incubated for 10 mins. Nile red stained cells were observed in a Fluorescence microscope. Stained cell fluorescence was measured after 30 mins using the excitation wavelength as 460 nm by using fluorescence microscope Axio Scope A1 coupled with Carl Zeiss Imaging Systems 32 software Axio Vision 4.7.2.

**Lipid extraction and transesterification**

The total lipids were extracted using [19]. Dried biomass (250 mg) was taken into 100 ml round bottom flask and added 15 ml of methanolic-sulphuric acid. The flask along with the content was refluxed for 4h at 60°C, the content was filtered and collected into a separating funnel. The filtrate was separated using ethyl acetate followed by washing with distilled water until pH of the filtrate shows neutral. Once pH becomes neutral, the organic phase was collected and anhydrous $Na_2SO_4$ was added to remove the impurities. Solvents were removed by vacuum rota evaporator (Buchi Rotavapor R-215) at a pressure of 175 mbar with temperature of 60°C. Then 400-500 µl of dichloromethane was added to dissolve the organic phase. 1 µl of the sample was injected by a syringe (Hamilton 701N) in GC for fatty acid profiling.

**Gas chromatography of FAME**

Profiling of fatty acids was carried out using GC-FID having SGE forte capillary column (60 m × 0.32 mm I.D. × 0.25 µm film thicknesses). During analysis, temperature of the injector and detector were kept at 240°C and 250°C respectively. On the other hand, oven temperature was adjusted to 140°C at 5 mins and raised to 240°C at 4°C $min^{-1}$ and later kept to 240°C at 52 mins. Nitrogen gas was used as carrier gas which was maintained at the flow rate of 1.0 ml $min^{-1}$. Sample of 1 µl was used for analysis with a split ratio of 100:1. Supelco$^{TM}$ 37 component FAME mix (Sigma Aldrich) was used as standard. Retention time was recorded for each sample from which each component of fatty acid can be known. Each concentration of different FAMEs was calculated by the percentage area method comparing the peak areas of their corresponding concentrations of standard using Chemito Chrom-card software version 2.6.

**Statistical analysis**

All analysis was performed in triplicates. Results are presented as the mean ± standard deviation.

## RESULTS AND DISCUSSION

Geographical data and cultural studies of the microgreen algae isolated from different habitats of Manipur were recorded (Table 1). Thallus behaviour of in-situ germination was studied (Fig. 1). Nile red staining technique was carried out for the nine (09) studied strains for characterization of lipid composition and photomicrographs were also taken (Fig. 2). Percentage of total lipid content of the nine (09) strains at stationary phase were analysed (Fig. 3).

The fatty acid profile of the strains was summarized in (Table 2). In the present analysis, the concentrations of fatty acids were found to vary from organism to organism. Nitrogen is an important macronutrients for growth and metabolism of algal cells. It is an essential constituent of the cell, needed for algal growth, either in combined or in molecular form. Chemical composition of microalgae may change largely when the species are cultured under contrasting conditions, as well as in different growth phases [20]. Many algal species exhibit rapid growth and high productivity and several microalgal species can be induced to accumulate substantial quantities of lipid, often greater than 60% of their dry biomass [21]. A high throughput method using nile red to detect neutral lipid in microalgae was also described [22] and we have also incorporated nile red technique in our study to detect the lipid.

The emission and excitation maxima of nile red in lipid suspensions depend upon the concentration of the dye. Intracellular lipid droplets of microalgae were observed by nile red staining in a fluorescence microscope with blue light as the excitation light having wavelength of 460 nm. Hydrophobic lipid fluoresces orange-yellow with blue excitation. Both yellow and red fluorescence cells were observed and showed a large number of lipids inside the cell and within the gelatinous matrix, as indicated by the yellow colour under blue light during the stationary phase in our study.

Results showed that the highest lipid content was observed as 16.2% at the stationary phase of 18$^{th}$ day. Accumulation of lipid though started at the early phase of growth but maximum accumulation was observed at the late stationary phase. Total lipid ranged between 4.4% to 16.2%. Maximum lipid percentage was observed in *Chlorella* sp. BTA-3110 followed by *Chlorella* sp. BTA-3063 and *Chlorococcum* sp. BTA-3106 while *Korshikoviella* sp. BTA-3019 showed minimum. Similar finding was reported by [23] that an increase in total lipid content in the stationary phase usually predominates due to an accumulation of neutral lipids in the form of triglycerides rather than polar lipids located in the cell membrane. There is linear correlation between nile red fluorescence and the total lipid content of microalgae, where an increase of lipids in aging algal cells was due primarily to neutral lipids rather than glyco or phospholipids [24]. Some microalgal strains produce large quantities

of lipid as a storage product, regularly achieving 50 to 80% of their dry weight as lipids [25]. Most common algae like *Chlorella*, *Crypthecodinium*, *Cylindrotheca*, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum*, *Porphyridium*, *Schizochytrium*, *Tetraselmis*, *Botryococcus braunii* and *Scenedesmus* have oil levels between 20 and 50% but higher productivities can be reached [26, 27, 28, 29, 30, 31].
Fatty acids play a key role in biodiesel and nutraceuticals. Because of this, some microalgal species may be potentially indicated as useful, while others showed to be suitable as food-species, if the essential PUFA is present in them. The result showed that seven types of saturated fatty acid (C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0), three MUFA (C16:1, C18:1n9, C22:1n9) and four types of PUFA (C18:2n6, C18:3n3, C18:3n6, C20:5n3) were observed. In this study, most of the strains had C8:0, C14:0, C16:0 and C20:0 as the dominating saturated fatty acids. Unsaturated fatty acids like C18:1n9, C18:2n6, C18:3n3 and C18:3n6 were also present. The content of C16:1, C22:1n9 and C20:5n3 were minimum. *Chlorococcum* sp. BTA-3112 showed high content of palmitic acid (C16:0) as 54.25%. Stearic acid (C18:0) content was maximum in *Chlorella* sp. BTA-3101 (10.55%). Lauric acid (C12:0) content was maximum in *Chlorella* sp. BTA-3023 (8.45%) and maximum percentage of arachidic acid (C20:0) was found in *Chlorella* sp. BTA-3110 (21.57%). Among the polyunsaturated fatty acids, high amount of linolenic acid (C18:3n3) was present only in *Chlorella* sp. BTA-3063 (37.37%). Linoleic acid (C18:2n6) was observed highest in *Chlorella* sp. BTA-3101 (12.62%). *Chlorella* sp. BTA-3086 was the only strain which showed highest eicosapentaenoic acid (C20:5n3) with 1.79% while other strains showed less amount. This fatty acid has a great value in pharmaceutical applications. The levels of SAFAs were also high in all the strains except capric acid (10:0) and caprylic acid (C8:0). Palmitic acid (C16:0) and arachidic acid (C20:0) were the most abundant saturated fatty acids which were present in all the strains. Oleic acid (C18:1n9) was the most abundant MUFAs in all the strains ranging between 0.62% to 15.79% and for PUFA, linoleic acid (C18:2n6) was present in moderate amount in all the strains. The function of fatty acids in algae is related to cell membrane, energy storage and metabolic processes [32].

**Table 1: Cultural studies of microgreen algae isolated from different habitats of Manipur**

| SN | Name of the strains and code no. | Date of collection | Habitat | Location | Colour | Cell shape |
|---|---|---|---|---|---|---|
| 1 | *Korshikoviella* sp. BTA-3019 | 11-06-2010 | Water sample, Chandel, Manipur, India. | 195m N 24˚15´08.1´´ E094˚17´59.9´´ | Light green | Sickle and elongated |
| 2 | *Chlorella* sp. BTA-3023 | 11-06-2010 | Moist soil, Imphal East, Manipur, India. | 793m N 24˚49´23.3´´ E093˚58´29.3´´ | Dark green | Spherical |
| 3 | *Chlorella* sp. BTA-3038 | 11-06-2010 | Dry soil, Imphal East, Manipur, India. | 793m N 24˚49´30.1´´ E094˚06´23.1´´ | Light green | Oval |
| 4 | *Chlorella* sp. BTA-3063 | 11-06-2010 | Muddy soil, Thoubal, Manipur, India. | 775m N 24˚38´07.3´´ E093˚59´51.8´´ | Light green | Spherical |
| 5 | *Chlorella* sp. BTA-3086 | 02-07-2010 | Epiphytic, Chandel, Manipur, India. | 213m N 24˚15´11.1´´ E094˚17´50.5´´ | Light green | Oval |
| 6 | *Chlorella* sp. BTA-3101 | 12-09-2011 | Periphery of Loktak Lake, Bishnupur, Manipur, India. | 820m N 24˚30´58.9´´ E093˚48´01.5´´ | Dark green | Spherical |
| 7 | *Chlorococcum* sp. BTA-3106 | 12-09-2011 | Loktak Lake, Bishnupur, Manipur, India. | 825m N 24˚31´08.2´´ E093˚48´46.7´´ | Dark green | Oblong |
| 8 | *Chlorella* sp. BTA-3110 | 12-09-2011 | Periphery of Loktak Lake, Bishnupur, Manipur, India. | 820m N 24˚30´58.9´´ E093˚48´01.5´´ | Dark green | Spherical |
| 9 | *Chlorococcum* sp. BTA-3112 | 12-09-2011 | Rice fields, Bishnupur, Manipur, India. | 773m N 24˚42´09.6´´ E093˚48´22.3´´ | Dark green | Spherical |

In a previous study on microalgae by [33], the most commonly synthesized fatty acids were chain lengths that range from C16 to C18, similar to those of higher plants. In another previous report by [34] palmitic, stearic, oleic and linolenic acids were recognized as the most common fatty acids contained in biodiesel. In the present investigation, among saturated fatty acids, palmitic acid (C16:0) and unsaturated fatty acids, linolenic acid (C18:2n6c) was the most dominant fatty acid. The total highest saturated fatty acid was found to be 65.84% in *Chlorella* sp. BTA-3110 and had C16:0 (42.33%) and C20:0 (21.57%) as principal saturated fatty acids. Highest total amount of unsaturated

fatty acid 48.94% was found in *Chlorella* sp. BTA-3063 with C18:1n9c (10.71%) and C18:3n3 (37.37%). Biodiesel from highly unsaturated sources oxidizes more rapidly than conventional diesel, resulting in forming insoluble sediments to interfere with engine performance. However, in our finding the content of saturated fatty acid in the studied strains was found to be more than the unsaturated which could prove to be a potential feedstock for biodiesel.

On the contrary, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) contents were generally moderate. PUFAs play an important role in regulating cell membrane properties, precursors for production in animal hormones, maintaining high growth, survival and reproductive rates, aquaculture studies [35]. PUFA was found to produce in greater quantity than MUFA in most of the studied strains. Microalgae have been applied for the production of a range of value-added pharmacological and industrial desired products. Therefore, the proper ratio of saturated and unsaturated fatty acid is very important to microalgae as a biodiesel feedstock. Microalgae strains with high oil or lipid content are of great interest in search for a sustainable feedstock for the production of biodiesel [36]. This study provides basic information on the chemical composition of microalgae and the possible uses of this knowledge are wide and could stimulate other studies.

**Table 2: Fatty acid composition (% fatty acid) from microgreen algae of Manipur, India**

| Strains | Saturated fatty acid content (% fatty acid) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $C_{8:0}$ | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | $C_{20:0}$ |
| *Korshikoviella* sp. BTA-3019 | 4.21 | 0.73 | 2.98 | 2.00 | 49.68 | nil | nil |
| *Chlorella* sp. BTA-3023 | 1.37 | 1.78 | 8.45 | 1.39 | 32.62 | nil | 5.56 |
| *Chlorella* sp. BTA-3038 | 5.73 | 0.08 | 0.01 | 0.06 | 1.53 | 8.56 | 0.09 |
| *Chlorella* sp. BTA-3063 | 0.32 | nil | 0.03 | 1.09 | 23.29 | nil | 19.16 |
| *Chlorella* sp. BTA-3086 | 0.41 | 0.18 | 2.09 | 1.00 | 30.94 | 3.75 | 2.53 |
| *Chlorella* sp. BTA-3101 | 0.28 | nil | 0.09 | 1.29 | 26.36 | 10.55 | 0.10 |
| *Chlorococcum* sp. BTA-3106 | 0.52 | nil | 0.04 | 1.09 | nil | nil | 6.97 |
| *Chlorella* sp. BTA-3110 | 0.81 | nil | nil | 0.82 | 42.33 | 0.31 | 21.57 |
| *Chlorococcum* sp. BTA-3112 | 1.29 | 0.13 | 0.19 | 3.49 | 54.25 | 1.68 | 0.12 |
| **Strains** | **Monounsaturated and polyunsaturated fatty acid content (% fatty acid)** | | | | | | |
| | $C_{16:1}$ | $C_{18:1n9}$ | $C_{22:1n9}$ | $C_{18:2n6}$ | $C_{18:3n3}$ | $C_{18:3n6}$ | $C_{20:5n3}$ |
| *Korshikoviella* sp. BTA-3019 | 0.21 | 1.09 | 0.38 | 7.43 | 6.56 | 5.45 | 0.07 |
| *Chlorella* sp. BTA-3023 | 0.60 | 1.89 | 0.31 | 0.12 | 1.57 | 1.61 | 0.53 |
| *Chlorella* sp. BTA-3038 | 1.16 | 7.18 | 0.07 | 7.18 | 0.24 | 13.66 | 0.14 |
| *Chlorella* sp. BTA-3063 | 0.13 | 10.71 | 0.11 | 0.36 | 37.37 | 0.12 | 0.14 |
| *Chlorella* sp. BTA-3086 | 1.32 | 0.70 | 2.15 | 0.37 | 1.47 | 1.57 | 1.79 |
| *Chlorella* sp. BTA-3101 | 1.21 | 0.62 | nil | 12.62 | 0.09 | 0.23 | 0.92 |
| *Chlorococcum* sp. BTA-3106 | 0.19 | 14.55 | 0.02 | 6.97 | nil | 0.25 | 0.26 |
| *Chlorella* sp. BTA-3110 | 0.08 | 3.13 | 0.20 | 0.04 | 8.30 | nil | 1.26 |
| *Chlorococcum* sp. BTA-3112 | 0.08 | 15.79 | 0.42 | nil | nil | 0.40 | 0.92 |


| | | |
|---|---|---|
| *Korshikoviella* sp. BTA-3019 | *Chlorella* sp. BTA-3023 | *Chlorella* sp. BTA-3038 |
| *Chlorella* sp. BTA-3063 | *Chlorella* sp. BTA-3086 | *Chlorella* sp. BTA-3101 |
| *Chlorococcum* sp. BTA-3106 | *Chlorella* sp. BTA-3110 | *Chlorococcum* sp. BTA-3112 |

**Fig. 1: Thallus behaviour of microgreen algae strains**

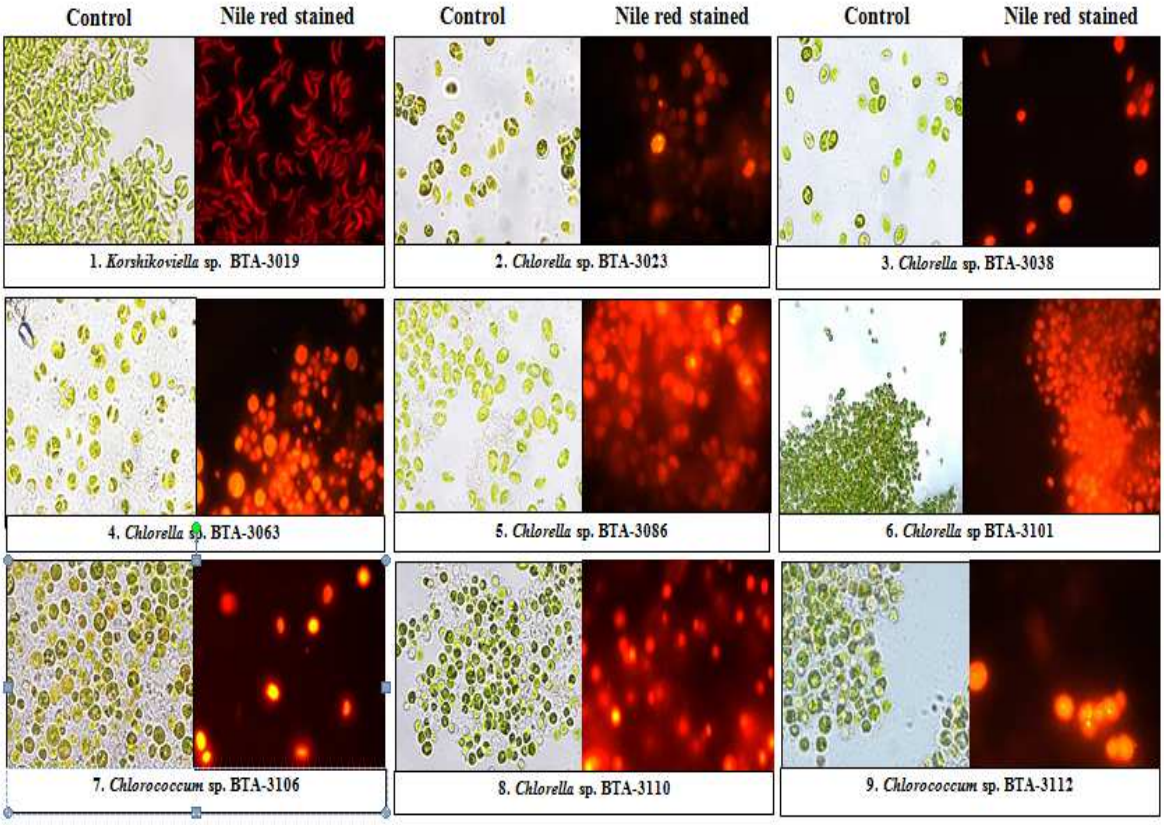


**Fig. 2: Photomicrograph images of control and nile red stained- Chlorophyll emits red fluorescence and lipid granules appeared as yellow**

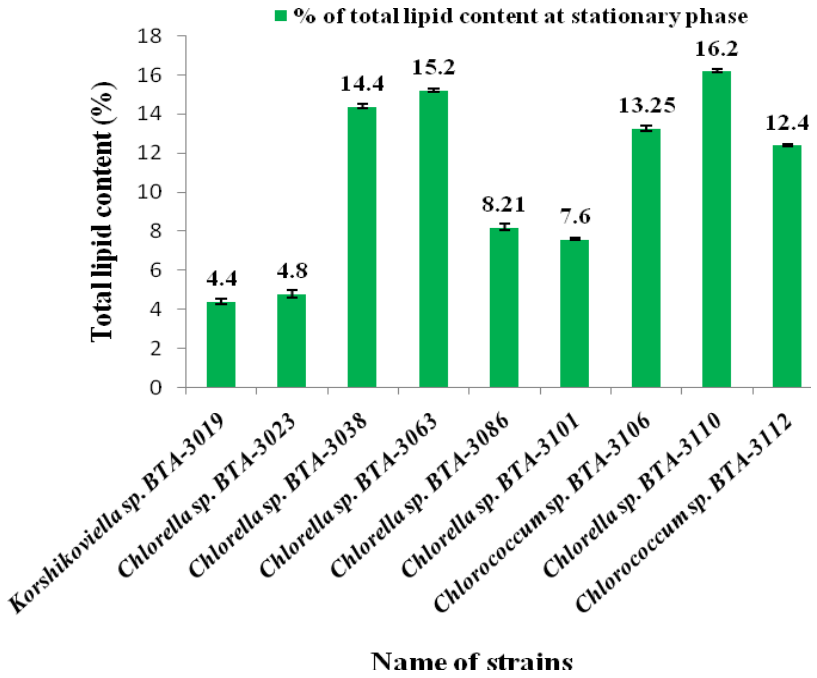


**Fig. 3: Total lipid content of microgreen algae strains**

## CONCLUSION

The experimental result suggests that the characterized strains were found to be the best candidate due to its easy growth in a relatively low-priced media without the necessity of utilizing very specific compounds and its significant lipid content. In conclusion, the qualitative analysis of fatty acids showed high value of palmitic acid along with maximum amount of saturated fatty acids. The results also indicated that the naturally isolated microgreen algal strains showed the presence of essential fatty acids like PUFAs which are also valuable for use in nutraceutical and of biotechnological importance. Further, process control and optimization are worth studying to develop an economically feasible biodiesel production protocol and for upscaling purpose.

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