Research Article

www.ijrap.net (ISSN:2229-3566)

STUDIES ON EXTRACTION AND ESTIMATION OF BIODIESEL PARAMETERS IN SIX CYANOBACTERIA STRAINS

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Received on: 10/02/20 Accepted on: 11/04/20

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DOI: 10.7897/2277-4343.110359

ABSTRACT

The environmental pollution due to conventional fuels could be remediated by using biofuels. The alternative source was 'biodiesel', which could be extracted from plant and animal oils and also from microalgae. *Cyanobacteria* were found to be a major source of biodiesel due to their high lipid productivity and higher growth rate. In the present work, several *Cyanobacterial* strains of different taxons were chosen and the pigments such as chlorophyll, carotenoid, and other constituents such as carbohydrate, protein and lipid were extracted and estimated. The experimental results had shown that *Oscillatoria* sp. BDUT003 and *Nostoc* sp. BDUARC10241 were potent strains in terms of growth and lipid production. The search for lipid and fatty acids will be able to serve as food and feed supplement and in pharmaceutical and fuel supplements as biodiesel.

Keywords: Alternative Fuels, Biodiesel, Microalgae, *Cyanobacteria*, Lipid Production.

INTRODUCTION

Nowadays, due to increase in population, the energy demands have also increased. To meet these needs, non-renewable energy resources like fossil fuels are exploited. The only remedy for this hitch is "biodiesel". Biodiesel is a diesel fuel derived from plant or animal oils, usually containing esters of long chain fatty acids. Biodiesel is usually prepared from oil by transesterification, where the oil is esterified by an alcohol. Biodiesel can be used in standard diesel engines, but is often blended with conventional diesel. Biodiesel is produced from different food stocks, based on the source that was used for biodiesel production. It can be classified into four major types. They are, (i) First generation or conventional biofuels are made from sugar, starch, vegetable oil and animal waste. (ii) Second generation biofuels are derived from ligno cellulosic crops. Second generation biodiesels have been developed because, the first generation biofuels have some drawbacks, i.e., they cannot produce enough biodiesel without affecting the biodiversity. Many second generation fuels are under development. They are, cellulosic ethanol, algae fuel, biohydrogen, biomethanol and wood fuel. (iii) Third generation biofuels are produced from algal and *Cyanobacterial* communities. Microalgae are microscopic aquatic organisms with high lipid productivity. Among the above different sources, Microalgae get more attention due to their higher growth rate and tolerance to varying environmental conditions. The production of biodiesel from microalgae has been considered in recent times. This is because of high lipid productivity than terrestrial plants with a higher growth rate, accumulate very large amounts of triacyl glycerides, a major feedstock in biodiesel production and no need of much land necessitates. One such microalgal community, which has a higher rate of lipid productivity, is *Cyanobacteria*.

Cyanobacteria are one of the oldest forms of life on earth. It is also known as blue-green algae and they are prokaryotic organisms¹. They are mostly found in fresh water or marine water zones and can also grow in extreme conditions like desert, hot springs, etc. They are named as *Cyanobacteria* due to their dual characteristics with algae and bacteria². *Cyanobacteria* have helped on earth in three ways by, (i) releasing oxygen (ii) fixing nitrogen and (iii) capturing sunlight as energy that other species feed on. *Cyanobacteria* have great potential as a platform for biodiesel production because of their fast growth, ability to fix carbon dioxide gas and genetic tractability. They do not require fermentable sugars or arable land for growth and so competition with crop plants would be greatly reduced^{3,4}.

Cyanobacteria are represented by a diverse group of microorganisms that, by virtue of being a part of marine and fresh water phytoplankton, significantly contribute to fixation of atmospheric carbon via photosynthesis. It is assumed that ancient *Cyanobacteria* participated in the formation of earth's oil deposits. Many *Cyanobacterial* species are easier to genetically manipulate than eukaryotic algae and other photosynthetic organisms. *Cyanobacteria* can be found in almost every terrestrial and aquatic habitat- oceans, fresh water, bare-rock and soil and even antarctic rocks⁵. They can occur as planktonic or form phototrophic biofilms. Aquatic *Cyanobacteria* are known for their extensive and highly visible blooms that can form in both fresh water and marine environments. *Cyanobacteria* can also fix atmospheric nitrogen in aerobic conditions by means of specialized cells called heterocysts. Nitrogen-fixation by *Cyanobacteria* provide rice plantations with biofertilizers. Free-living *Cyanobacteria* are also present in water column in rice paddies⁶⁻⁷.

In general, *Cyanobacteria* possess all known phycobiliproteins i.e., phycocyanin, phycoerythrin, phycoerythrocyanin and allophycocyanin. Producing suitable renewable fuel by employing biosystems is one of the priority research interests of scientific community throughout the world. *Cyanobacteria* are looked upon as a promising source of biofuel. Hence, in the present study six *Cyanobacteria* were chosen as a source of biodiesel production.

MATERIALS AND METHODS

Six *Cyanobacterial* strains such as *Oscillatoria* sp (BDUT 001), *Oscillatoria* sp (BDUT 002), *Phormidium* sp (BDUT 003), *Phormidium* sp (BDUT 004), *Nostoc* sp (BDU ARC12501) and *Nostoc* sp (BDU ARC10241) were collected from the germplasm of NFMC, Bharathidasan University, Tiruchirappalli, for the present work. Of these, 4 were thermophilic and 2 were psychrophilic species. The strains were observed under microscope to identify the morphological features of cultures. The cultures were prepared in BG 11(+) (Table 1 and 2) medium for thermophilic species and BG 11(-) medium for psychrophilic species. In BG 11 (-) medium all the components were used as followed in BG 11 (+) medium except NaNO₃. The thermophilic cultures were grown under white fluorescent lamp at a temperature of 40°C and the psychrophilic cultures were grown at a temperature of 15°C.^{8,9}

Table 1: BG 11 (+) Medium composition for thermophilic species

S. No.	Compound	Weight
1.	NaNO ₃	1.5 g
2.	K ₂ HPO ₄	0.04 g
3.	MgSO ₄ .7H ₂ O	0.075 g
4.	CaCl ₂ .2H ₂ O	0.036 g
5.	Citric acid	0.006 g (2 ml)
6.	Ferric Ammonium Citrate	0.006 g (2 ml)
7.	EDTA (disodium salt)	0.001 g (2 mL)
8.	Na ₂ CO ₃	0.02 g
9.	Trace metal mix A ₅	1.0 ml
10.	Agar (if needed)	10.0 g
11.	Distilled water	11 ml

Note: pH should be maintained at 7.1 after sterilization

Table 2: Trace metal mixing composition for BG 11 (+) Medium

S.No.	Compound	Weight
1.	H ₃ BO ₃	2.86 g
2.	MnCl ₂ .4H ₂ O	1.81 g
3.	ZnSO ₄ .7H ₂ O	0.222 g
4.	NaMoO ₄ .2H ₂ O	0.39 g
5.	CuSO ₄ .5H ₂ O	0.079 g
6.	Co(NO ₃) ₂ .6H ₂ O	49.4 mg
7.	Distilled water	11 ml

Estimation of Biofuel parameters

The selected strains were grown in a 250 ml conical flask in their respective media. After incubation, the cultures were harvested through centrifugation. Obtained pellet was washed once with the medium and then with distilled water to remove the impurities in the pellet. The washed pellet was subjected for various estimations. The quantitative determination of Carotenoid, Chlorophyll, Carbohydrate and Protein were estimated using UV-visible spectrophotometer [HITACHI (U-2000), Japan].

Chlorophyll by Mackinney method (1941)

The culture was centrifuged at 5000 rpm for 10 minutes and the pellet was washed twice with distilled water. The pellet was suspended in 4 ml of methanol and vortexed thoroughly. The test

tube was covered with aluminum foil to prevent evaporation and the test tube was incubated in water bath at 60°C for 1 hour with occasional shaking. The test tube was cooled and centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and 4 ml methanol was added again and the pigment was extracted. The supernatant was pooled and made up to 10 ml volume with methanol. The absorbance was read at 663 nm in a UV-Visible spectrophotometer, taking methanol as a blank solution. The amount of chlorophyll present was calculated using the equation, $A_{663} \times 12.63$, expressed as $\mu\text{g chl a g}^{-1}$ dry wt, where A_{663} is the absorbance, 12.63 is the correction factor¹⁰.

Carotenoids by Jensen method (1978)

The washed culture was suspended in 3 ml of acetone and incubated in dark for 45 minutes. The contents were centrifuged at 5000 rpm for 5 minutes. The supernatant was stored in the refrigerator and the extraction was continued till acetone was colourless. The supernatants were pooled and made up to 10 ml with acetone¹¹. The absorbance was read at 450 nm in a UV-Visible spectrophotometer against acetone blank. Total carotenoids calculated by following formulae.

$$\text{The amount of carotenoids} = A_{450}/2500$$

Carbohydrates Estimation

The pellet was washed with distilled water. To 1 ml of *Cyanobacterial* suspension, 4 ml of anthrone reagent was added. This was kept in water bath for 15 minutes and after cooling, absorbance was read using an UV-Visible spectrophotometer at 625 nm. The amount of carbohydrate was estimated from the standard curve prepared using standard glucose solution.

Proteins by lowry's method (1951)

To the washed pellet, 5 ml of 10% TCA was added and left in water bath for 30 minutes. The contents were cooled and centrifuged at 5000 rpm for 5 minutes. The pellet was dissolved in 1N NaOH by boiling in water bath. An aliquot of 0.1 ml was taken from this and made up to 1 ml. Then 4 ml of alkaline reagent was added and incubated for three minutes at 37°C. To the same was added 0.5 ml of Folin-Ciocalteu's phenol reagent and allowed to stand for 30 minutes at room temperature. The absorbance was read at 750 nm. The amount of protein was calculated using standard curve prepared using BSA solution¹².

Lipids Estimation

The pellet was washed with distilled water and dried completely. The pellet was ground in a mortar and pestle with a known volume of 2:1 chloroform: NaCl in a glass powder. The extract was centrifuged and the supernatant was collected in the fresh tube. The extraction was repeated. 1% NaCl was added to the extract and vortexed thoroughly. A little amount of sodium sulphate was added to remove moisture. The extract was transferred in to a pre-weighed bottle and dried in vacuum evaporator. The bottle was re-weighed and the amount of lipid was estimated¹³⁻¹⁴.

RESULTS AND DISCUSSION

Cultures grown in BG 11 (+/-) medium at the pH of 7.1 at 40° ± 5°C under 1500 lux, exhibited the maximum growth. Microscopy studies showed the clear variations in the morphology of *Cyanobacteria* of different taxons which is shown in Figure 1 and 2.

Carotenoid estimation

From the graph 3,4 and Table 3, we get to know that, among the four thermophilic strains, *oscillatoria sp.* BDUT003 has higher amount of carotenoid (0.00089 $\mu\text{g}/0.508\text{ g}$) content. Similarly, among the two psychrophilic strains, BDUARC 11101 has got a higher carotenoid content (0.00166 $\mu\text{g}/0.0020\text{ g}$).

Chlorophyll estimation

From the Figure 5, 6 and Table 4 it can be seen that, amongst the four thermophilic strains, *phormidium sp.* BDUT004 shows higher chlorophyll content of 10.91 μg per 0.024 g. Similarly, among the two psychrophilic strains, BDUARC10241 shows higher chlorophyll content of 5.0494 μg per 0.0016 g.

Carbohydrate estimation

The carbohydrates present in the culture was extracted and estimated. The amount of carbohydrate present in each sample is tabulated in Table 5. The Figures 7 and 8 reveal that among the thermophilic strains, *oscillatoria sp.* BDUT001 has got higher carbohydrate content and among the two psychrophilic strains, BDUARC11101 has got higher carbohydrate content.

Protein estimation

The amount of protein present in each sample is tabulated in Table 6. Figure 9 and Figure 10 show that *oscillatoria sp.* BDUT001 has higher value of protein (1.02 mg/g) among the thermophilic samples and BDUARC11101 has got higher value (0.7 mg per g) of protein among psychrophilic species.

Lipid estimation

The total amount of lipid present in each sample is tabulated in Table 7 and Figure 11 and 12. The main idea of the study is to look into the lipid yield of thermophilic *Cyanobacteria* isolated from Manauli and Psychrophilic strains which is initially isolated from Arctic polar regions. Both the organisms belong to two extreme climatic conditions. Interestingly both thermophilic *Oscillatoria sp.* showed appreciable amounts of lipid yields, which ranged up to 13%. *Oscillatoria sp.* BDUT 003 accumulated a maximum of 12.8% of lipid followed by *Oscillatoria sp.* BDUT 001 with 12.3% followed by *Phormidium sp.* BDUT 002 with 7.1 and *Phormidium sp.* BDUT 004 with 6.5%. Psychrophilic strains exhibited lipid range in between 7-8%, where *Nostoc sp.* BDUARC 10241 exhibited a highest of 8.5 % among the studied psychrophilic strains. *Cyanobacteria* lipids content differed from strain to strain.

Figure 1: Microscopic image of four thermophilic *Cyanobacteria* strains

Figure 2: Microscopic image of two psychrophilic *Cyanobacteria* strains

Table 3: Amount of carotenoids present in each strain

S. No.	Sample	Amount of carotenoid
1	<i>Oscillatoria sp.</i> BDUT001	0.00043 µg per 0.030 g dry wt
2	<i>Phormidium sp.</i> BDUT002	0.00028 µg per 0.05 g dry wt
3	<i>Oscillatoria sp.</i> BDUT003	0.00089 µg per 0.508 g dry wt
4	<i>Phormidium sp.</i> BDUT004	0.00019 µg per 0.136 g dry wt
5	<i>Nostoc sp.</i> BDUARC10241	0.000163 µg per 0.0044 g dry wt
6	<i>Nostoc sp.</i> BDUACR11101	0.00166 µg per 0.0020 g dry wt

Figure 3: Graph of carotenoids estimation in thermophilic strains

Figure 4: Graph of carotenoids estimation in psychrophilic strains

Table 4: The amount of chlorophyll present in each sample

S. No.	Sample	Amount of Chlorophyll
1	<i>Oscillatoria sp.</i> BDUT001	10.9 µg per 0.05 g dry wt
2	<i>Phormidium sp.</i> BDUT002	10.91 µg per 0.024 g dry wt
3	<i>Oscillatoria sp.</i> BDUT003	10.919 µg per 0.177 g dry wt
4	<i>Phormidium sp.</i> BDUT004	13.70 µg per 0.086 g dry wt
5	<i>Nostoc sp.</i> BDUARC10241	5.0494 µg per 0.0016 g dry wt
6	<i>Nostoc sp.</i> BDUACR11101	3.0956 µg per 0.0002 g dry wt

Figure 5: Chlorophyll estimation for thermophilic strains

Figure 6: Chlorophyll estimation for psychrophilic strains

Table 5: The amount of carbohydrate present in each sample

S. No.	Sample	Amount of carbohydrates
1	<i>Oscillatoria sp.</i> BDUT001	3 mg per g
2	<i>Phormidium sp.</i> BDUT002	2.98 mg per g
3	<i>Oscillatoria sp.</i> BDUT003	2.96 mg per g
4	<i>Phormidium sp.</i> BDUT004	2.88 mg per g
5	<i>Nostoc</i> BDUARC1024	1.54 mg per g
6	<i>Nostoc</i> BDUACR11101	2.96 mg per g

Figure 7: Carbohydrate estimation for thermophilic species

Figure 8: Carbohydrate estimation for psychrophilic species

Table 6: The amount of protein present in each sample

S. No.	Sample	Amount of Protein
1	<i>Oscillatoria sp.</i> BDUT001	1.02 mg per g
2	<i>Phormidium sp.</i> BDUT002	0.6 mg per g
3	<i>Oscillatoria sp.</i> BDUT003	0.68 mg per g
4	<i>Phormidium sp.</i> BDUT004	0.58 mg per g
5	<i>Nostoc</i> BDUARC10241	0.64 mg per g
6	<i>Nostoc</i> BDUACR11101	0.7 mg per g

Figure 9: Protein estimation for thermophilic samples

Figure 10: Protein estimation for psychrophilic samples

Table 7: The amount of lipid present in each sample

S. No.	Sample	Amount of lipids
1	<i>Oscillatoria sp.</i> BDUT001	12.3%
2	<i>Phormidium sp.</i> BDUT002	7.1%
3	<i>Oscillatoria sp.</i> BDUT003	12.8%
4	<i>Phormidium sp.</i> BDUT004	6.5%
5	<i>Nostoc</i> BDUARC10241	8.5%
6	<i>Nostoc</i> BDUACR11101	7.8%

Figure 11: Lipid content of thermophilic samples

Figure 12: Lipid content of psychrophilic samples

CONCLUSION

The above study is a view point on marine *Cyanobacteria* as potential source for biodiesel production. This work was targeted to identify those potent strains with high lipid content for pilot scale production. Six marine *Cyanobacteria*, from various genera was chosen from the germplasm of NFMCC, Bharathidasan University, Trichy and different parameters such as carotenoid, chlorophyll, carbohydrate, protein and lipid are estimated. Among the studied thermophilic strains *Oscillatoria* sp BDUT 003 showed higher chlorophyll content of 10.9 µg/ml and 12.8 % of lipid. Among the two psychrophilic strains *Nostoc* sp BDUARC 10241 exhibited 5.04 µg/ml chlorophyll content and 8.5 lipid percentage. This study throws light upon the comparative study of thermophilic and psychrophilic *Cyanobacteria* taking growth and lipid percentage into consideration. This work also suggests *Oscillatoria* sp BDUT 003 and *Nostoc* sp BDUARC 10241 are potent strains in terms of growth and lipid production. Marine biotechnology is an enterprising area, of many countries. The tropical Indian sub continent is one of the 12 mega diversity hotspots of the world. Exploring marine *Cyanobacteria* has a number of advantages in view of their limited need of only sea water, minerals and sunlight and can be grown at zero budgets.

Due to rise in human population, the need for food, feed, fine chemicals, pharmaceutical and fuel has also risen. Any research finding in this direction will add a new dimension to science and have a say in Indian future economy. Hence, an attempt was made to screen the marine *Cyanobacteria* in the germplasm for their lipids, pigments chlorophyll, protein estimation. The project has looked into lipid content of six *Cyanobacterial* strains.

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Cite this article as:

J. Amala Infant Joice et al. Studies on extraction and estimation of Biodiesel parameters in Six *Cyanobacteria* Strains. *Int. J. Res. Ayurveda Pharm.* 2020;11(3):52-57 <http://dx.doi.org/10.7897/2277-4343.110359>

Source of support: Nil, Conflict of interest: None Declared

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