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Bioprospecting for High Lipid Producing Microalgae for Biodiesel Production

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ABSTRACT

Fuels based upon crude oils are become unsustainable because of draining resources and also their contribution of those fuels to the release of carbon dioxide into the environment is high. Hence, Microalgae have gained major interest as a sustainable source to replace these resources due to their high lipid content for biodiesel production on a commercial scale. This paper describes the screening of twelve microalgal species isolated from marine water resources of Kerala coastline for biodiesel production and the GC-MS/MS analysis of their fatty acid methyl esters (FAMEs). Lipid content of each isolated microalgal species has been investigated. Based on the lipid content, biodiesel was produced from Oscillatoria sp., which showed maximum lipid yield 44.8% among isolates by acid-catalyzed transesterification reaction and resulting fatty acid methyl esters (FAMEs) content was analyzed by GC/MS. Fatty acid profiling of the biodiesel showed high content of C12:1 with 43.8%, C10:0 with 14.86%, C12:3 15.5%, C18:1 with 18.44%, C19:1 with 4.57% and C20:0 with 3% respectively.

KEYWORDS: *Microalgae, biodiesel, Oscillatoria sp., transesterification, Fame analysis*

INTRODUCTION

At present, the energy crisis is the most important issue that is being discussed throughout the world. High oil prices, growing concerns towards energy security, the threat of climatic changes and depletion of energy resources due to growing populations have stimulated the need for alternative sustainable resources for energy (Markham, 2019).

The search for sustainable energy for the future is one of the most challenging tasks and is intimately linked with global constancy, economic wealth, and quality of life(Chu *et al*., 2017). According to IEA 2018, the primary sources of energy used in the world were oil (33%), coal (30%), natural gas (24%), nuclear energy (4%), biomasses (10%), hydroelectric power (7%), and new renewable energy sources (2%) (the renewable energy obtained from wind and solar energy system).

Fuels like petroleum, diesel, Kerosene, etc., were found to account 70% of the total energy requirement globally, especially in the transportation and manufacturing industries. The transport sector alone accounts for almost one-quarter of greenhouse gas emissions (Hillman and Ramaswami, 2010) and hence, there is a need an alternative source for reducing greenhouse gas emissions from automobiles. One of such alternative source is the use of biofuels; it can play an important role as a renewable source by avoiding the excessive emission of greenhouse gases, limiting the dependence on fossil fuels and ensuring the security of energy supply, in promoting environmental sustainability (Wijffels and Barbosa, 2010).

Recent years, biodiesel based fuel system has gained an important attention globally, as it is biodegradable and can be obtained from renewable resources, and provides environmental benefits like, limits the harmful emissions of greenhouse gases such as carbon monoxide, hydrocarbons and particulate matter and to the elimination of SOX emissions(Singh *et al*., 2011). Usually, Biodiesels are produced from various types of feedstocks like vegetable oils from oleaginous crops, such as rapeseed, jatropha, soybean, sunflower and palm, or from waste cooking oils, animal fats, etc., these oils are been converted to diesel with short-chain alcohols, main methanol through a chemical process called transesterification (Barnwal and Sharma, 2005). Based on the feedstocks, it has been classified as a first, second and third generation of feedstocks. Though all these generations of feedstocks are renewable, there are some disadvantages among the first and second-generation feedstocks. Subsequently, there been developing consideration towards third era feedstocks, the utilization of microalgae have turned out to be third era and one of the Earth's most significant inexhaustible fuel feedstock, as it is the most proficient natural maker of oil and be an adaptable biomass source (Makareviciene *et al*., 2011) and furthermore they are photosynthetic, have higher biomass productivities and their development rate are higher than higher plants, most astounding CO2 obsession and O2 creation, developing in fluid medium which can be taken care of effectively, can be developed in factor atmospheres and non-arable land including negligible zones unsatisfactory for horticultural purposes (for example desert and seashore lands), in non-consumable water or even as a waste treatment reason, use far less water than conventional yields and don't uproot

nourishment yield societies; their production isn't regular and can be reaped day by day (Olaizola, 2003). In actuality, normal biodiesel generation yield from microalgae can be 10 to multiple times higher than the yield got from oleaginous seeds as well as vegetable oils (Pickett *et al*., 2008).

Some microalgae have high oil content and can be induced to produce higher concentration of lipids (e.g. low nitrogen media, Fe3+concentration and light intensity) (Dayananda *et al*., 2006). The ability of algae to fix CO2 can also be an interesting method of removing gases from power plants, and thus can be used to reduce greenhouse gases with a higher production microalgal biomass and consequently higher biodiesel yield (Raja *et al*., 2008). Algal biomass production frameworks can be effectively adjusted to different degrees of operational and innovative abilities; some microalgae have likewise an advantageous unsaturated fats profile and an unsaponifiable part permitting a biodiesel generation with high oxidation dependability (Rani *et al*., 2011). The physical and fuel properties of biodiesel from microalgal oil in general (e.g. density, viscosity, acid value, heating value, etc.), are comparable to those of fuel diesel (Zhang *et al*., 2003). Important technical challenges include identifying the isolated strains with the highest lipid content with essential composition, which were the primary aim of this work.

MATERIALS AND METHODS

Study Area and Sample Collection

Random samples were taken from three different substrate-rocks, moist soil, and Seawater from Munnakal beach of Kochi, Kerala (10.1416^{o}N, 76.31783^{o}E). The algae samples were scraped and transferred into a sterile plastic bottle using sterile blades and forceps (Bischoff and Bold, 1963) and were transported to the Department of Biotechnology, SNMV College, Coimbatore, India for further studies. Samples were transferred to Erlenmeyer flasks containing f/2 medium. They were maintained in culture room under white fluorescence light (3000 lux), 14"10 L/D at 25 "2°C until they were examined. Microphotographs of these species were taken using a microscope with USB 3.0 digital microscope camera attached.

PHYSICO-CHEMICAL PARAMETERS OF SEAWATER

The parameters like PH, Reactive silicate, Nitrogen dioxide, Ammonia, Inorganic phosphate, Iron, Chloride, Calcium, Magnesium, Potassium, Nitrate, Sodium, Nitrite, Sulphides, bicarbonate, TSS, TDS, COD, BOD, Total alkalinity, Turbidity and Electrical Conductivity of seawater was analyzed (APHA, 2005).

HARVESTING OF ALGAL CELLS

The cells were harvested by sedimentation and centrifugation at 10,000 rpm for 10min at 4°C. Potash alum was used for sedimentation. The cells were washed with distilled water and recentrifuged (Shah *et al*., 2003). The pellet was, then, subjected to wet weight estimation and it was dried in an oven at 80°C till constant weight.

ESTIMATION OF WET WEIGHT

The microalgal cells after centrifugation were added to the pre-weighed clean crucibles (C1). The weight of the crucibles was taken (C2) and wet weight was calculated as:

Wet weight = C2 - C1

ESTIMATION OF DRY WEIGHT

The crucibles containing wet biomass of microalgal sample were pre-weighed (C2). It was kept in oven at 800C for drying. The weight of the crucibles was again measured (C3) and dry weight was calculated as :

Dry weight = C3 - C2

LIPID EXTRACTION

Lipid extraction from the dried algal biomass was done using the method of Bligh and Dyer (1959). Two ml methanol and one ml chloroform was added to one gram dried algal biomass and kept at 250C for 18h. The mixture was agitated on vortex for two min and one ml chloroform was added to the mixture. It was shaken vigorously. After that, 1 ml distilled water was added and the mixture was mixed in a vortex for 2 min. Further, they were separated by centrifugation at 2000 rpm for 10 min. The lower layer was separated and the procedure was again repeated with the pellet. The centrifuged mixture was allowed to stand for 2 h. Two layers were separated. The upper layer was discarded while the lower layer containing lipids was transferred to a clean pre-weighed Eppendorf tube (W1). Evaporation was carried out in a water bath at 80°C till constant weight was achieved. The weight of the vial was recorded after drying (W2).

Lipid content (% dry cell weight) = W2 - W1 × 100

Where,
W1 is the weight of the empty vial
W2 is the weight of vial + microalgal biomass

Lipid yield (%) = WL/WDA

Where WL is the weight of extracted lipids and WDA is the weight of dry algal biomass

CONVERSION OF LIPID TO BIODIESEL

The lipid extracted from algal biomass was further converted to biodiesel using the method of Hossain *et al*., 2008.

Procedure

Potassium hydroxide (0.25g) was mixed with 24 mL methanol and stirred properly for 20 min. The mixture of potassium hydroxide and methanol was poured into the algal oil in a conical flask.

The following reaction and steps were followed:

A. **Transesterification:** The conical flask containing the mixture was shaken for 3 h by rotary shaker at 300 rpm.
B. **Settling:** After shaking, the solution was kept for 16 h to settle the sediment and biodiesel layers clearly.

The biodiesel was carefully separated from sediments by separating funnel. Biodiesel was washed with 5% distilled water until it became clear. Biodiesel was kept under the running fan for 12 h and measured using measuring cylinder.

GCMS ANALYSIS

The chemical composition of the samples was performed using Shimadzu Gas with a direct capillary column TG WAX-MS. The components were identified by comparison of their retention times and mass spectra with those of NIST 05 mass spectral database (Halim, 2013).

RESULTS

ISOLATION OF MICROORGANISM

The sample was collected from the Munakkal beach, Kochi and they were enriched in the F/2 medium. The enrichment was done by inoculating ten ml of the sample in 250 ml Erlenmeyer flasks. The algal isolates were grown at 23^{0}C temperature with 50-55% humidity and 16:8 hours light: dark photoperiod for three weeks to obtain the growth. Aeration was provided manually by shaking the flasks daily to avoid sticking of biomass to the surface of the flasks.

MICROSCOPIC EXAMINATION OF MICROALGAE

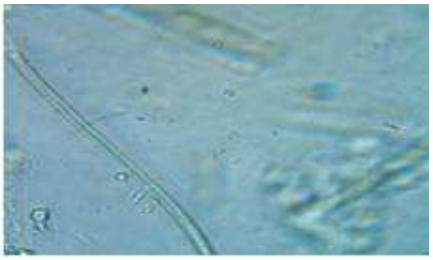
The cultivated microalgae (M30-M41) were primarily screened by direct wet mount technique. Under the microscope, long filamentous cells were observed which reveals that these species Table 1 & Fig.1.

Table.1 Morphological Identification of Isolated Strains

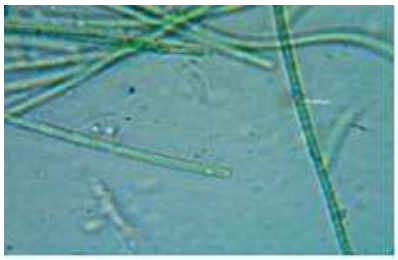
Strain	Morphology	Strain Name
m30	Filamentous	Phormidium sp.
m31	Filamentous	Anabaena sp.,
m32	Filamentous	Phormidium sp.
m33	Filamentous	Oscillatoria sp.,
m34	Filamentous	Tetrasporidium sp.,
m35	Filamentous	Tetrasporidium sp.,
m36	Filamentous	Tetrasporidium sp.,
m37	Filamentous	Pseudanabena sp.,
m38	Filamentous	Pseudanabena sp.,
m39	Filamentous	Navicula sp
m40	Filamentous	Geitlerinema sp
m41	Filamentous	Oscillatoria sp.,

M30

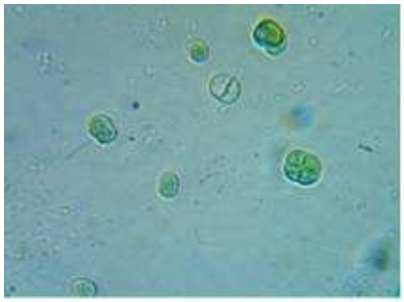
M31



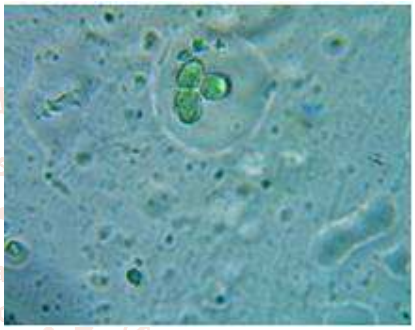
M32



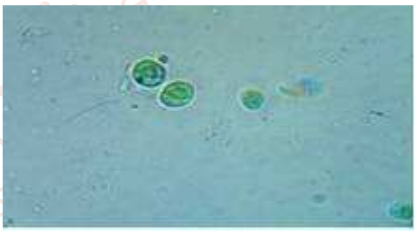
M33



M34

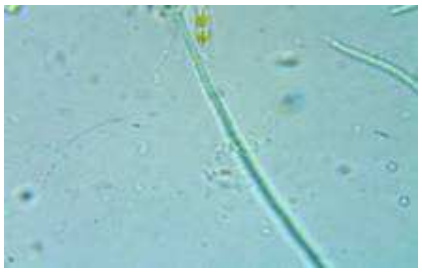


M35

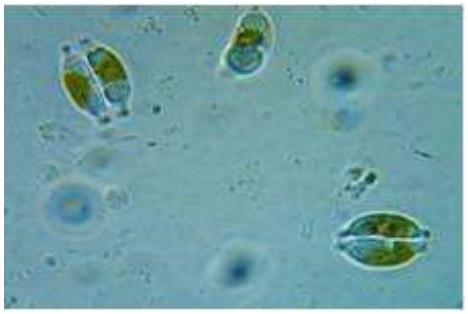


M36

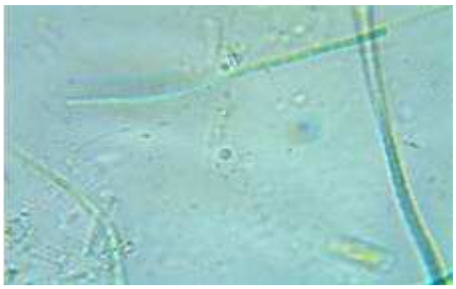
M37



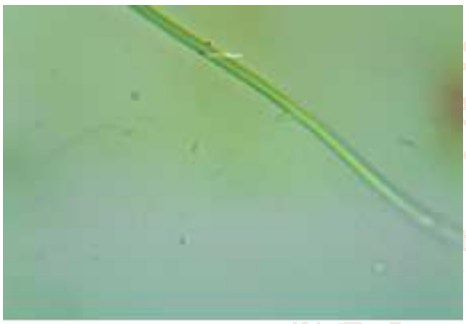
M38



M39



M40



M41

Fig.1 Microscopic images of Isolated Strains (M30-M41)

PHYSIOCHEMICAL PARAMETERS OF DIFFERENT SAMPLES:

The physiochemical parameters of marine water were determined and noted below (Table. 2).

Table2: Physiochemical parameters of Sea Water

S. No	Parameters	Marine water
1	Turbidity	59 NTU
2	Electrical conductivity	30674µs/cm
3	Total alkalinity	1819mg/L
4	TSS	4271.3 mg/L
5	TDS	12300 mg/L
6	COD	1983.8 mg/L
7	BOD	1166 mg/L
8	Reactive silicate	0.235 mg/L
9	NO_2	0.052 mg/L
10	Ammonia	0.022 mg/L
11	Nitrate	0.67 mg/L
12	Nitrite	0.69 mg/L
13	Iron	1.3 mg/L
14	Chloride	2776.09 mg/L
15	Calcium	170.9 mg/L
16	Magnesium	3251 mg/L
17	Potassium	816.3 mg/L
18	Sodium	3986.6 mg/L
19	Sulfides	1.99 mg/L
20	Ph	8.1
21	Bicarbonate	144 mg/L

LIPID PRODUCTION

Total lipids were extracted from the microalgal biomass using a slightly modified version of Bligh and Dyer's method (1959). One gram dried algal biomass was mixed with the solvent mixture, methanol: chloroform (2:1) and kept at 25^0C for 18 hours. The organic layer was separated and the extraction procedure was repeated.

On quantitative estimation, the isolated microalgal culture's (Table.3) of M32 and M40 resulted in 32% lipid accumulation whereas, among the microalgal isolates, M33 was found to produce the maximum lipid content of 44.8%. The isolate M37 had 30% lipid content respectively.

Table.3: Lipid Percentage of Isolated Microalgae

Isolate	wet weight (g)	Dry weight (g)	Lipid concentration	Lipid (%)
M30	1.323	1.122	1.111	21
M31	1.380	1.181	1.120	16
M32	1.451	1.253	1.154	32
M33	1.333	1.133	1.119	44
M34	1.480	1.281	1.140	19
M35	1.426	1.227	1.123	13
M36	1.425	1.329	1.163	25
M37	1.415	1.317	1.170	30
M38	1.425	1.321	1.128	9
M39	1.552	1.341	1.154	20
M40	1.490	1.292	1.167	32
M41	1.323	1.122	1.111	21

FAME ANALYSIS

According to our gas chromatography results, C33 had methyl ester that containing carbon chains such as (12:1) with 43.8%, (10:0) with 14.86%, (12:3) 15.5%, (18:1) with 18.44%, (19:1) with 4.57% and (20:0) with 3%. Detailed chromatogram of C33 was given in Fig.2 and Table 4.

Table.4 Fame content of Microalgae M33

Peak	Name	R. Time	I. Time	F. Time	Area %	Height %
1	$C_{12}H_{34}O_4$	3.099	3.008	3.167	43.87	27.96
2	$C_{10}H_{30}O_5$	5.843	5.750	5.917	14.86	14.60
3	$C_{14}H_{44}O_6$	9.855	9.792	9.933	15.25	20.69
4	$C_{17}H_{50}O_7$	13.676	13.617	13.750	18.44	28.74
5	$C_{20}H_{40}O_5$	17.070	17.025	17.133	4.57	6.19

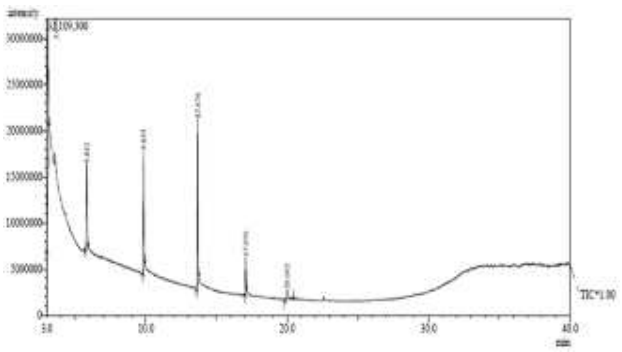


Fig. 2 FAME Analysis of M33 by GC-MS

DISCUSSION

The energy requirements of the global community are rising year by year. Currently, fossil fuels are a prominent source of transportation fuels and energy. The world's demand fossil is expected to rise by 60% from the current level by 2025 (Khan *et al*., 2009). In view of the increasing oil demand and the depleting oil reserves, development of innovative techniques for the production of biofuels from novel inexhaustible biomass feedstock sources are gaining importance all over the world. No single feedstock is likely to solve the supply issues facing the biodiesel industry. Microalgae could be a future of fuel production. Biomass, whether terrestrial or aquatic, is considered a limitless energy source. Relative to alternative energy sources, the aquatic biomass represents the strategy that is almost ready to be executed on a large scale without any economic or environmental penalty (Aresta *et al*., 2005). Among these, algae are endowed with unique adaptability to grow in diverse habitats, either in marine or freshwaters. As such, large scale microalgal cultivation should pose minimal interference with valuable agricultural resources (Halim, 2013).

Microalgae have high photosynthetic rates and can accumulate a substantial amount of lipids in their biomass (up to 77% of dry cell mass in some species). At present, it is very important to screen the indigenous hyper-lipid producing microalgae for biofuel application and cultivates native microalgal strains that adapt to their local environmental conditions (Sathya and Srisudha, 2013). The primary objective of this study was to isolate lipid generating microalgal isolates for biodiesel production, to fulfill this objective the microalgal samples were collected from Munnakal beach, Kochi, Kerala, India and seawater was analyzed for pH, EC, salinity, turbidity and total P. The pH of the water samples varied from acidic to alkaline. The electrical conductivity (EC) that mainly indicates the presence of the total soluble salts in the sample was more than 1 DSM-1 indicating that all the water samples contained more salts. The microalgae have a unique feature of growing under such alkaline and high EC conditions. Therefore, such extreme water bodies can be a good source for the production of microalgal biomass and in turn biodiesel production (Rani and Shimalli, 2011).

The raw samples (10ml) were inoculated into 100ml of seawater for the enrichment and grown at 23^{0}C with 16:8 h light; dark photoperiod, for 21 days in the growth chamber and three isolates were obtained in the same medium on the basis of morphology. Various researchers obtained and isolated the green microalgae *Botryococcus braunii* from the brackish water, freshwater and ponds (Banerjee *et al.,* 2002, Metzger and Largeau, 2005). The microalgal strains of *Scenedesmus acutus* and *Quadrigula subscale* have been isolated from the natural water samples collected from Thailand by Pokthitiyook *et al.,*2009. Metzger and Largeau (2005) cultivated most of the *Botryococcus* strains in the laboratory from wild samples collected from lakes. These reports suggest that green microalgae are ubiquitous in nature.

Microalgal strains have been analyzed for lipid production by various researchers (Metzger and Largeau, 2005; Dayananda *et al*., 2007; Samori *et al*., 2010) and are reported to produce 5 to 42% of lipids and 0.1 to 61.0% hydrocarbons on their dry weight basis. Therefore, there is an increasing quest to isolate newer species and strains of the microalgae to produce lipids and other chemicals of industrial importance.

For microalgal oil extraction, although an appropriate technique of cell disruption is a prerequisite (Burja *et al*., 2007; Lee *et al*., 2010), the efficient extraction of lipids is highly dependent on the polarity of the organic solvent or solvent mixture used. In general, solvent mixtures containing a polar and a non-polar solvent could extract a greater amount of lipids. For example, a combination of chloroform (non-polar), methanol (polar) and water, known as the Bligh & Dyer method, has been used for lipid extraction from a wide range of biological samples (Ryckebosch *et al*., 2012). Once the oil is extracted, it is refined using fatty acid chains through a process called transesterification.

Transesterification is the most common method and leads to monoalkyl esters of algal oil, vegetable oils and fats (Demirbas, 2010). The production of biodiesel from microalgae oil by transesterification process has previously been demonstrated in the literature using the conventional methods (Nagle and Lemke, 1990; Miaoa and Wu, 2006) and then algal oil is converted to fatty acid alkyl esters (FAAE) and glycerol. The transesterification reaction can be catalyzed by alkali (Gryglewicz, 1999; Chitra et al.,2005), acid (Furuta *et al*., 2004), or enzymes (Du *et al*., 2004; Noureddini *et al*., 2005).

Algal oil from microalgal isolate was extracted for biodiesel production and strain M33 found to produce high lipid yield of about 44.8% comparing to other Strains. Transesterification of algal oil of microalgal isolate M33 using KOH as catalyst and methanol resulted in the production of biodiesel. Hossain et al. (2008) conducted similar type of investigation to know the proper transesterification, amount of biodiesel production (ester) and physical properties of biodiesel using the two common species, *Spirogyra* and *Oedogonium*. The algal oil and biodiesel (ester) production was higher in *Oedogonium* than *Spirogyra sp*.

Transesterification of algal oil was performed by Ahmad *et al*. (2013) using sodium methoxide as a catalyst and yield of biodiesel from extracted oil was calculated 95%for *Chlorella Vulgaris*, 92% for mixed algal culture and 91% for *Rhizocoloniumhiero glyphic*. (Shimi *et al*.,2013) conducted experiments to determine the effect of variations in the volume of reacting methanol, the concentration of an acid catalyst, time, temperature and stirring effect on the biodiesel yield of *Spirulina platensis*. Fatty acid methyl ester (FAME) yield of 84.7% was obtained at 100% (wt./wt.oil) catalyst concentration, 80ml methanol volumes, 8 h reaction time and 65°C reaction temperature with continuous stirring at 650 rpm.

The biggest challenge over the next few years in the biodiesel field will be to reduce costs for cultivation and to further improve the biology of oil production. New materials and designs for cultivation in closed bioreactors and the use of cutting-edge metabolic engineering and screening/selection techniques are thought to provide the biggest promises.

CONCLUSION

To our knowledge, the present study is the first report demonstrating biodiesel production from green microalgae isolated from Munnakal beach, Kerala, Kochi. These organisms are found to be a promising source for biodiesel by consisting of rich lipid content and different fatty acid groups. Future studies can reveal how all three isolates perform in different and large reactor systems. Additionally, Lipid contents of all three isolates can be increased by genetic engineering methods for obtaining of the huge amount of biodiesel production.

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