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Isolation and Characterization of Microalgae from Various Water Samples for Bio-diesel Production

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Microalgae are currently considered to be one of the most promising alternative sources for biodiesel production. In order to identify the potential microalgae for bio-diesel production, the present study was conducted. Twelve pure microalgal cultures were isolated from various water samples and were identified up to generic level. Four reference strains *Botryococcus braunii* (Ref strain1), *Neochloris oleoabundans* (Ref strain 2) Botryococcus sp. (B6) and *Scenedesmus dimorphus* (SD7) were used in this study. Further, biomass production and lipid content were analyzed. The highest biomass production was noticed in reference strain *Botryococcus braunii* 3.45 g l^{-1}, followed by *Chlorella* sp. (C3) which produced 3.2 g l^{-1}. The lipid production in *Botryococcus braunii* (Ref strain1) was 1.73 g l^{-1}, followed by *Chlorella* sp. (C3) with 1.60 g l^{-1}.

Key words: Microalgae, *Botryococcus braunii, Chlorella* sp., Bio-diesel production.

Energy is the prime mover of economic growth and is vital to sustain a modern economy and society. An economic growth combined with a rising population has led to a steady increase in the global energy demands. The continued use of fossil fuels is not sustainable, as they are finite resources and their combustion will lead to increased green house gases (GHG) viz., carbon dioxide (CO_2), sulfur dioxide (SO_2) and nitrogen oxides (NOx). Bio-energy is one of the most important components to mitigate green house gas emissions and substitute fossil fuels. Amongst primary feed stocks, microalgae are currently considered to be one of the most promising alternative sources for biodiesel. Microalgae are microscopic photosynthetic organisms that are found both in marine and fresh water environments. Several micro algae strains produces energy biomass of 30 to 100 times faster than land plants. It is interesting to note however that some algae strains or variants contain up to 50 percent lipids making them very suitable for the production of liquid fuels. Therefore, in the present study, an attempt has been made to isolate and identify the best strain producing high lipid and biomass.

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MATERIALS AND METHODS

Four cultures of microalgae were used as reference strains in the study. The first reference strain *Botryococcus braunii* (Ref. strain1) was supplied by the Department of Agricultural Microbiology, TNAU Coimbatore. The second reference strain *Neochloris oleoabundans* (Ref. strain2) was supplied by Criyagen Private Limited, IABT, UAS, Dharwad. The third and forth reference strains were *Botryococcus* sp. (B6) and *Scenedesmus dimorphus* (SD7), which were obtained from the Dept. of Agricultural Microbiology UAS Dharwad.

The individual samples (Table 1) of 10 ml were inoculated to 90 ml BG11 medium for enrichment. The cultures were observed under a microscope by wet mount. The single cell was picked up by using a Pasteur pipette and transferred to fresh BG11 medium and incubated in a growth chamber (3000 lux) for three weeks. Microscopic observations were carried out weekly to get pure cultures. The purified microalgal cultures were maintained in conical flask containing BG11 media for future study.

Identification was performed by microscopic observation. Identification characters were followed as per the descriptions[8].

The isolated cultures of microalgae were stained with nile red and observed under the fluorescent microscope. The micro algae containing high lipid in their cell showed pinkish to red fluorescence (Plate 1).

The obtained isolates were separately grown in BG11 medium in 250 ml conical flask. These isolates were incubated in growth under growth chamber with artificial light (3000 Lux) for three weeks. Biomass, chlorophyll content, and lipid were estimated for each isolate separately.

The culture was filtered in a dried and pre-weighed Whatman Filter Paper No.1 and dried in an oven at 60°C until constant weight was obtained. The biomass yield was calculated in terms of g L^{-1} [13].

Total chlorophyll was estimated by homogenizing a known volume of culture and centrifuged at 8000 rpm for 10 minutes. The pellet was treated with 10 ml of 95 per cent methanol, shook well and incubated at 60°C in water bath for 30 minutes. The supernatant was centrifuged and the absorbance was measured in the wave length of 652.4 and 665.2 nm in spectrophotometer model using 95 per cent of methanol as a blank[11].

The lipid extraction was done by using chloroform/methanol (2:1) and estimated gravimetrically[10].

RESULTS AND DISCUSSION

Twelve pure microalgae cultures obtained (Table 1) were identified up to generic level by referring the keys[8]. *Botryococcus braunii* were isoliated from the water sample collected from Brar Shola Falls at Kodaikanal[4]. The green colonical hydrocarbon rich unicellular microalgae *Botryococcus braunii* was obtained in the brackish water, fresh water and ponds[1,15]. The green microalgae strain such as *Scenedesmus acutus* and *Quadrigula subsala* were isolated from the natural water samples collected from Thailand[12]. This indicates that green microalgae are ubiquitous in nature.

The confirmation of the microalgal isolates for lipid production was performed by using Nile red stain. The nile red is a red phenoxazine dye which is used to screen the lipid producing microalgae. The isolated cultures of microalgae were stained with nile red and observed under the fluorescent microscope. The micro algae which are containing high lipid in their cell showed pinkish to red fluorescence (Plate 1). Nile Red (9-diethylamino-5 H-benzo (α) phenoxazine-5-one) used to determine the neutral lipid in microalgal cells[3]. There is a increase in fluorescence of lipid accumulating algal cells was due to the increased synthesis of neutral lipid[7]. The dye has been used for the detection of intracellular lipid droplets using fluorescent microscope[7].

Such red fluorescence was observed in four reference cultures *Botryococcus braunii* , *Neochloris oleoabundans*, *Scenedesmus dimorphus* and *Botryococcus* sp. The intense fluorescence was observed among the twelve local isolates also. But, *Chlorella* sp. (C3) showed more intense fluorescence (Plate 1). The Nile Red staining assay was a rapid method for the determination of the lipid in the green algae *Botryococcus braunii* and was as good as the gravimetric method commonly used for lipid determination[10]. Lyophilized *Chlorella vulgaris* culture and found that the lipid content was linearly correlated to relative intensity of fluorescence[7]. The modified technique of using the solvent Dimethyl Sulfoxide (DMSO) was used for rapid screening of naturally occurring algal strains[2]. In another study, Nile red assay was performed to screen the *Nannochloropsis* sp. and found that the cells grown under nitrogen deficient conditions exhibited 6-7 times more fluorescence than the cells grown under nitrogen rich condition[5].

The growth of the isolates was measured in terms of biomass production, estimation of chlorophyll and lipid production from microalgae comparing with reference strains.

From the Table 2, significantly the highest biomass production was noticed in reference strain *Botryococcus braunii* (Ref strain1) 3.45 g l^{-1}, followed by the next significantly superior microalgae *Chlorella* sp. (C3), which produced 3.2 g l^{-1} of biomass. The biomass obtained from *Botryococcus braunii* (CFTRI-Bb1) was 0.78 g l^{-1} [4]. The biomass estimation in other algae like *Oedogonium* sp. and *Spirogyra* sp. were 3.5 g l^{-1} and 3.8 g l^{-1}, respectively[8]. *Chlorella* sp. (C3) isolated from Gokarna sea water was efficient biomass producers as compared with reference

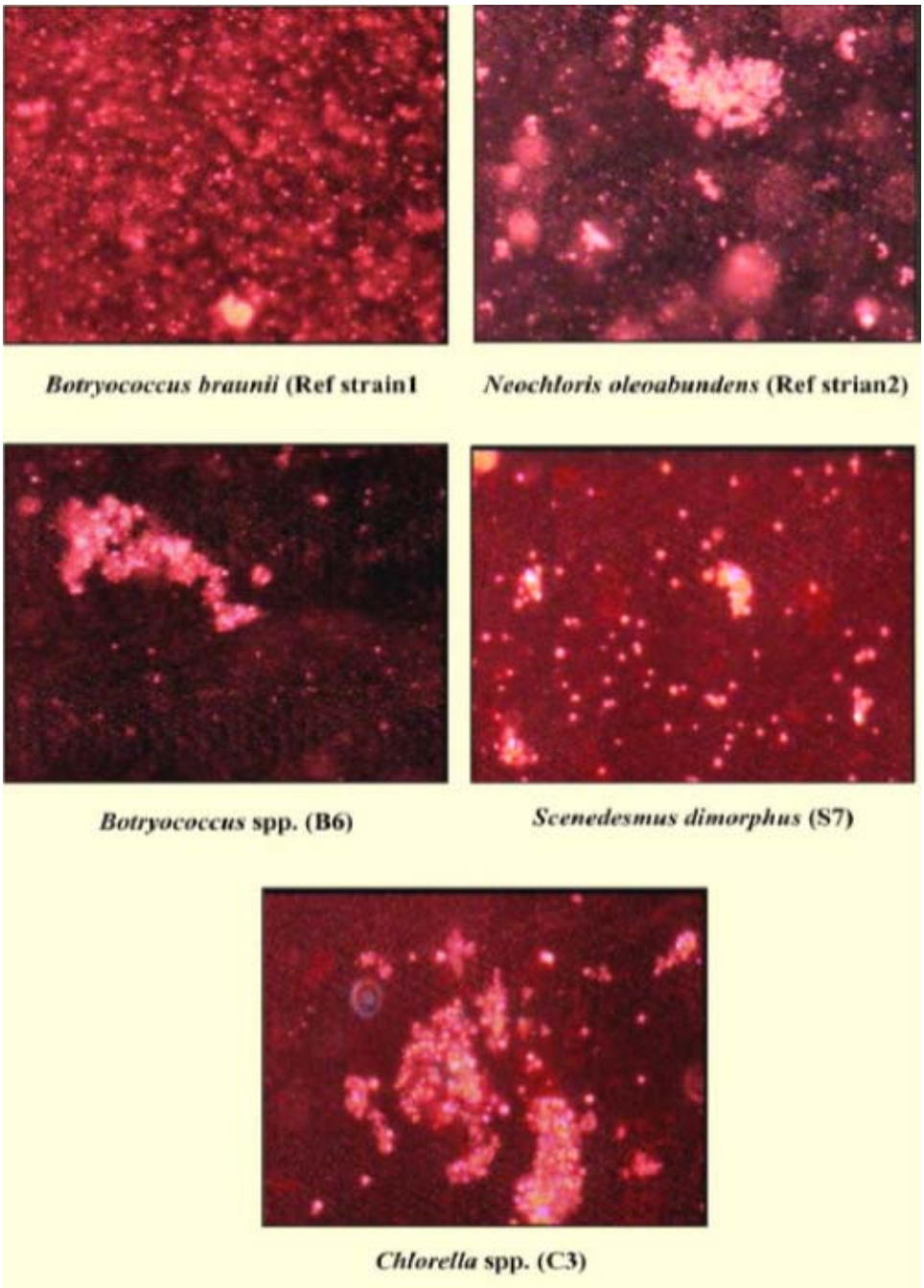

Plate 1. Confirmation of lipid producing microalgae from nile red staining

strains *Neochloris oleoabundans* (ref strain2), *Scenedesmus dimorphus* (SD7) and *Botryococcus* sp. (B6) which produced biomass of 2.2 g l^{-1}, 2.81 g l^{-1} and 2.35 g l^{-1}, respectively.

Microalgae *Botryococcus* sp. (B2) was on par with reference strains *Botryococcus* sp. (B6) and *Neochloris oleoabundans* (Ref strain2). The least biomass was produced by *Neochloris* sp. (N1) and *Chlorella* sp. (C5), which produced biomass yield of 1.12 g l^{-1}.

From the Table 2, the chlorophyll content recorded was significantly higher in *Botryococcus braunii* (ref strain2) (10.38 ìg ml^{-1}), followed by *Chorella* sp. (C3) (8.89 ìg ml^{-1}). Local isolate *Chorella* sp. (C3) showed significantly superior to reference strains *Scenedesmus dimorphus* (SD7) and *Botryococcus* sp. (B6), recorded 7.845 ìg ml^{-1} and 6.52 ìg ml^{-1} respectively. Besides it is highly significant over *Neochloris oleoabundans* (ref strain2) which recorded 4.49 ìg ml^{-1}. The increase in the chlorophyll content from 2 ìg L^{-1} to 10 ìg L^{-1} was observed when the biomass production was increased (Daynanda *et al*., 2007), which shows that the correlation exists between biomass and chlorophyll.

There was no significant difference

Table 1. Microalgae isolated from various water samples

S. No.	Source of sample	Code	Tentative identification of the Microalgae
1.	Mundagod lake (Karvar Dt.)	B2	*Botryococcus* sp.
2.	Saligaav lake (Karvar Dt.)	C1	*Chlorella* sp.
3.	Kator lake (Karvar Dt.)	C4	*Chlorella* sp.
4.	Dasanakoppa lake (Karvar Dt.)	C5	*Chlorella* sp.
5.	Niralagi lake (Karvar Dt.)	B1	*Botryococcus* sp.
		N1	*Neochloris* sp.
6.	Gokarna sea water (Karvar Dt.)	C3	*Chlorella* sp.
		N2	*Neochloris* sp.
7.	Ranebennur lake (Haveri Dt.)	C2	*Chlorella* sp.
8.	Nukapur lake (Haveri Dt.)	S2	*Scenedesmus* sp.
9.	Shingavi lake (Haveri Dt.)	S1	*Scenedesmus* sp.
10.	Sorab gaddi (Shimoga Dt.)	N3	*Neochloris* sp.

Table 2. Screening of the microalgal strains for biomass (g l^{-1}), chlorophyll (µg ml^{-1}) and lipid (g l^{-1}) production

S. No.	Isolates	Biomass (g l^{-1})	Total Chlorophyll (µg ml^{-1})	Lipid (g l^{-1})
1.	*Botryococcus braunii* (Ref. strain1)	3.45^{a}	10.38^{a}	1.73^{a}
2.	*Neochloris oleoabundans* (Ref. strain2)	2.20^{d}	4.49^{fg}	1.09^{cd}
3.	*Botryococcus* sp. (B6)	2.35^{d}	6.52^{d}	1.17^{c}
4.	*Scenedesmus dimorphus* (SD7)	2.81^{c}	7.85^{c}	1.41^{b}
5.	*Scenedesmus* sp. (S2)	1.30^{h}	2.87^{ij}	0.65^{gh}
6.	*Chlorella* sp. (C1)	1.61^{g}	3.83^{h}	0.81^{ef}
7.	*Scenedesmus* sp. (S1)	1.75^{fg}	4.10^{gh}	0.87^{ef}
8.	*Botryococcus* sp. (B2)	2.20^{d}	4.56^{f}	1.09^{cd}
9.	*Neochloris* sp. (N1)	1.12^{h}	2.61^{j}	0.58^{h}
10.	*Chlorella* sp. (C3)	3.20^{b}	8.89^{b}	1.60^{a}
11.	*Botryococcus* sp. (B1)	1.57^{g}	3.26^{i}	0.78^{fg}
12.	*Chlorella* sp. (C2)	2.14^{de}	5.37^{e}	1.07^{cd}
13.	*Neochloris* sp.(N2)	1.22^{h}	2.65^{j}	0.64^{gh}
14.	*Chlorella* sp. (C5)	1.12^{h}	2.66^{j}	0.57^{h}
15.	*Chlorella* sp. (C4)	1.90^{ef}	4.23^{fgh}	0.95^{de}
16.	*Neochloris* sp. (N3)	1.81^{fg}	4.17^{fgh}	0.91^{ef}
	S.Em±	0.06	0.10	0.035
	CD at 1%	0.24	0.40	0.15

between the isolates B2, Ref strain2, C4, N3, C1, S1, B1, S2, C5, N2, and N1 in terms of chlorophyll content as compared to other cultures. The least lipid production was observed in *Neochloris* sp. (N1), which recorded 2.61 ìg ml^{-1}.

The lipid production in *Botryococcus braunii* (Ref strain1) culture was 1.73 g l^{-1} and was highly significant as compared to other isolates. This was followed by *Chlorella* sp. (C3), which was showing 1.60 g l^{-1}, this *Chlorella* sp. (C3) was on par with the reference strain *Botryococcus braunii* (Ref strain1). The *Chlorella* sp. (C3) was significantly superior to reference strains *Scenedesmus dimorphus* (SD7), *Botryococcus* sp. (B6) and *Neochloris oleoabundans* (ref strain2), which recorded lipid yield of 1.41 g l^{-1}, 1.17 g l^{-1} and 1.09 g l^{-1} respectively. As shown in the table 2. The highest hydrocarbon production in *Botryococcus braunii* grown in BG11 media obtained was 30 per cent of hydrocarbon per cent (w/w) where the biomass production was 2.10 g L^{-1}[4]. The lipid content of *Botryococcus* sp. is 160.3 mg L^{-1}, in *Chlorella vulgaris* it is 77.9 mg L^{-1} and in *Scenedesmus* sp. it is 71.4 mg L^{-1}[10]. The accumulation of lipid was maximum {12.7 per cent dcw (Dry Cell weight)} in *Scenedesmus obliquus*[13].

Therefore *Chlorella* sp. (C3) was shown efficient lipid production. There was no significant difference among other cultures, B2, C2, C4, N3, S1, C1, B1, S2, N2, N1 and C5. The lipid produced from *Chlorella* sp. C5 (0.57 g l^{-1}) was least significant with respect to other lipid producing algae. As shown in the table

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