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Selection of potent microalgae for biofuel production

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Abstract
One of the most important factors in obtaining oil from microalgae is the choice of the right algal species to be used. To identify some desirable algal strains for future study and/or the production of algae-based biofuel, 05 algal strains were successfully isolated from freshwaters, and then incubated in the laboratory bioreactors for the growth and oil accumulation investigations. Five isolates (*Chlorella sp, Ulothrix, Cosmarium nitudulum, Spirulina Sp, and Botryococcus braunii*) of the microalgal cultures isolated were selected based on their purity and growth rates. During a 25 day incubation experiment, the accumulations of their biomass and total lipids, together with the lipid productivities for these algal strains were systematically investigated and compared. Results indicated that the accumulations of biomass for the 05 algal strains ranged from 0.3 g/L to 1.4 g/L during the experiments, with the highest biomass of 1.4 g/L for *Botryococcus braunii*. The lipid content for the tested algal strains varied from 8% to 40% of the dry biomass at the end of cultivation experiments. Algae *Botryococcus braunii* is one of the best oil producers based on this investigations, with the total lipid content of 40% of dry biomass. Taking the growth rates and the accumulations of intracellular lipids into the consideration, *Botryococcus braunii* was considered to have significant potential for biofuel applications. In addition, the lipid productivities of the selected strain were further investigated.

Keywords: Selection of microalgae, lipid production, biofuel.

Introduction
Algae are a group of organisms that have been generally described as photoautotrophic, simple microscopic or macroscopic, unicellular to multicellular plants and are competent converters of sun energy to useful biochemical products like oil [1]. Algae are mainly water dwelling organisms lacking complex morphological organization [2]. Algae either macro or micro, have great potential for biofuel production. Microalgae are a varied group of single-celled organisms; also they are capable to offer a range of solutions for our energy demands through a number of ways [3]. Microalgae species were isolated from mixed culture and screened them for suitability for cultivation and biofuel production. The algae were tested for their ability to grow effectively, tolerance to stress, biomass and lipid productivities and remediation ability.

For efficient use of algae as a source of biodiesel it is very important to focus on the native algal species and to select that algal species which not only has a high growth rate but has greater lipid content. Identification of local algal species, and comparison of their oil contents is the part of this work. Hence, present study is a significant step forward in utilization of algae as a source of renewable energy.

Successful algal biofuel technology is relied on choosing the right algal strain with relevant properties [4]. This is an important aspect in commercial production of algal biodiesel because it determines not only the amount of possible lipid that can be extracted but also the quality of the lipid and its suitability for fuel. Factors to consider in algal strain selection for mass culture include consistency in growth, resilience to biotic and abiotic stresses, community stability and resistance to predators present in its given habitat. Environmental conditions and cultivation methods also play important roles in determining the type of strain that can be cultivated. Selection of fast-growing, productive strains, optimised for the local climatic conditions is of fundamental importance to the success of the mass cultivation of algae species. A high growth rate can reduce the contamination risk in a continuous culture system because it can out-compete a strain which grows slower [5–6]. Autoflocculation is also desirable and could bring about cheaper extraction procedure for algal lipid [7].

Specific adaptation of a species in a stressful condition is crucial to successful cultivation of microalgae either in an open or close system.

Materials and Methods

a) **Sampling:** During the present study, total 05 samples were collected. Samples were collected in glass jars, Plastic bags and bottles and then transferred to nutrient media standardized during the present work using flasks, jars and plastic bottles.

b) **Identification:** Species were identified based on the morphological characteristics. These were studied by preparing slides and observing under by 10X and 40 X powers of Microscope.

	(G/L)
Sodium nitrate	1.500
Dipotassium hydrogen phosphate	0.0314
Magnesium sulphate	0.036
Calcium chloride dihydrate	0.0367
Sodium carbonate	0.020
Disodium magnesium EDTA	0.001
Citric acid	0.0056
Ferric ammonium citrate	0.006
pH	7.1

CHU-13 Media	**mg/L**
KNO_3	400
K_2HPO_4	80
$MgSO_4$	200
$CaCl_2.2H_2O$	107
Ferric citrate	20
Citric acid	100
$Cocl_2$	0.02
H_3BO_3	5.72
$MnCl_2.4H_20$	3.62
$ZnSo_4.7\ H_20$	0.44
$CuSO_4.5H_2O$	0.16
Na_2MoO_4	0.084
1 drop 0.072N H_2SO_4	
pH :	7.5

c) **Cultivation**

****Directions to prepare BG-11*:** Suspend 1.627 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium. It is recommended to adjust pH with 1 M NaOH or HCl if it does not achieve 7.1. Sterilize by autoclaving at 121°C for 15 minutes. Cool the medium to room temperature.

Both the media BG-11 & CHU-13 were tested for the algal growth and support. Two growth medium are mostly adapted to freshwater algae. Growth experiments were performed in conical flasks of 500 ml. Each flask was filled with 200 ml of media and initially 3 g of algal sample was added to each flask. Every experiment was performed in duplicates and both the media were compared for each culture conditions.

d) **Optimization of different culture conditions:** The effect of different parameters on the growth rate of algae was assessed by following completely randomized experimental design. In these experiments only one factor was variable while all other conditions were kept constant. The culture conditions which were controlled for the algal growth were nutrients, light, temperature, pH and constant mixing or aeration.

e) **Culture medium/Nutrients:** For monitoring the best growth of algae both the media (BG11 & Modified CHU-13) were used for culturing algal cells and the best growth rate was estimated after a required period of time has been passed. The first calculation was made on 5th day and the rate was estimated on the basis of the rate of increase in fresh weight of biomass. The cell mass was separated through filtration and then weighted after blotting the excess water.

f) **Aeration/mixing:** Different means of mixing were used for this purpose. For mixing orbital shaker OS 5 was used and the flasks were kept over the shaker at 300 rpm. Aeration was supplied through aerating pumps. These cultures were placed indoor where constant sunlight was given. The growth results were then compared and cultures with maximum growth were continued.

g) Five algae strains were selected and screened for fast growth and high oil content. Each algal was grown in a BG11 & CHU-13 medium [8] and incubated under 30µE m-2 S -1 light intensity, 23 ± ºC and 16/8 light dark cycle. Air was provided to the flask to homogenize the medium and supply carbon dioxide for photosynthesis process. The rate of algal growth and oil content were measured in order to select the algal with high growth rate and oil content. Algal concentration was monitored more often by measuring the culture turbidity and/or cell count. Similarly, algal oil content was monitored at the same frequency by adding Nile red dye to algal solution sample. The Nile red would stain oil in the algae; one strain oil has a characteristic of fluorescence that is measured using a spectrofluorometer.

h) **Growth and lipid accumulation experiment:** To measure growth, 5 mL of the algal culture was collected and filtered using a pre-weighed GF-C filter paper (Whatman, USA.) and dried in a 105 °C oven overnight. Algal growth was expressed as the increase in dry algal biomass as a function of time (day) on a volumetric basis. For the determination of lipid content, around 50 mg of the dried algal samples as used for lipid accumulation measurements. For all the growth and lipid accumulation experiment, duplicates were used.

i) **Lipid extraction:** The total lipids were extracted from microalgae biomass using a modified method of Bligh & Dyer, 1959 [21]. The lipids were extracted using a mixture of chloroform/methanol (1:2 v/v) and then separated into chloroform and aqueous methanol layers by the addition of methanol and water to give a final solvent ratio of chloroform: methanol: water of 1:1:0.9. The chloroform layer was washed with 20 ml of a 5% NaCl solution, and evaporated by rotary vacuum evaporator (Rotavapor R-210, Buchi). The weight of the crude lipid obtained from each sample was measured using an electronic scale.

j) **Calculations and statistical analysis:** Lipid content (*C*) was reported as percentage of dry mass (% of DW), while biomass (*B*) was reported as grams of dried biomass in per liter of culture.

Lipid productivity (*P*) was reported as milligrams per liter per day using the following equation for calculation:

P=1 000CB/D

where P is lipid productivity (mg/(L·d)); C is lipid content (% of DW); B is biomass (g/L); D is cultivation time (d).

Results and Discussion

The Objective of this study was to collect and to analyze the growth conditions and determine the oil contents of different algal strains. During the present work 05 samples were collected. These samples were identified based on morphological characters.

Cell physiology and morphology

Microscope image of *Botryococcus braunii* show that they exhibit a typical morphology (Fig.1), characterized by a botryoid organization of individual pyriform shaped cells held together by a refringent matrix containing lipids. Droplet can be squeezed from the matrix by the pressure of a coverglass. Ultrastructural studies reveal that the matrix surrounding the basal part of the cells consists of outer walls originating from successive cellular divisions (Fig.2). Furthermore, the bulk of *Botryococcus braunii* hydrocarbons are stored in these outer walls [9].

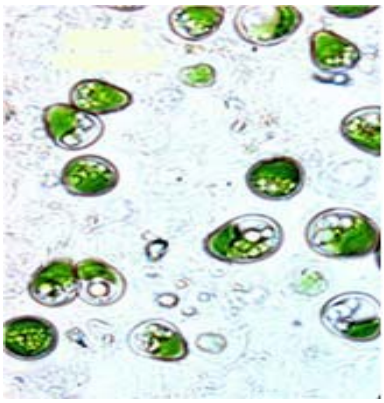

Fig 1: Microscope observation of *B.braunii.*

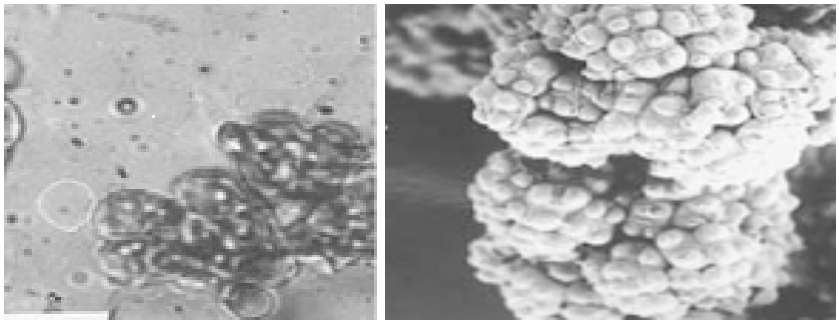

Fig. 2: Light microscopy of *Botryococcus braunii.*

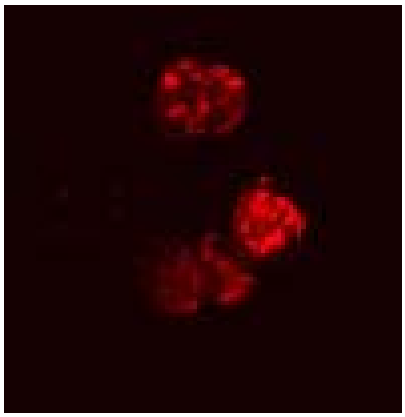

Fig.3 Microscopic image of Nile red stained cell. (Nile red specifically binds to lipids molecules inside the cells).

Nile red stained cell image under confocal microscope is shown in (Fig.3). While Nile red specifically binds to neutral lipids in the cells, it shows a high fluorescent signal at 525nm under an excitation at 485nm. The rubbery matrix containing oils shows a high lipid content, as well as the cytoplasm. During cell division, each cell secretes new matrix containing oil within the extracellular matrix of the mother cell. Thus, the rubbery matrix saturating with oils grows while the successive matrices gradually accumulate. The cytoplasm is filled with lipid globules [10-11].

In the linear phase, *Botryococcus braunii* produce various types of carotenoids. During the linear phase, lutein is the

primary carotenoid. Secondary carotenoids produced during the stationary phase are canthaxanthin and echinenone [12]. Echinenone constitutes a substantial portion in extracellular carotenoids. *Botryococcus braunii* undergoes a color change during stationary phase because of an accumulation of ketocarotenoids, mainly echinenone, which are overproduced under stress conditions such as nitrogen depletion and high light intensity. Another possible explanation is the simultaneous decrease in the amount of intracellular pigments in the plastids [13].

In this study, the five different microalgal strains were collected and the microalgal cultures were purified and maintained in pure culture. Only five isolates (*Chlorella sp, Ulothrix, Cosmarium nitudulum, Spirulina Sp, and Botryococcus braunii)* were selected based on their purity and growth rates. Many algal species exhibit rapid growth and high productivity, and certain microalgal species can be induced to accumulate substantial quantities of lipids, often greater than 60% of their dry biomass [14]. After mass multiplication, the microalgae was harvested and used for oil extraction. The total lipid contents for the microalgae cultured in this study ranged from 8% to 40% of the dry weight. Among the isolated microalgae, *Botryococcus braunii, Chlorella sp, & Spirulina Sp*. produced high lipid content of 40±0.3%, 28±0.28% and 14±0.25% of dry weight (Fig. 4 & 5) respectively, *Cosmarium nitudulum* recorded lowest lipid content of 8±0.42%.

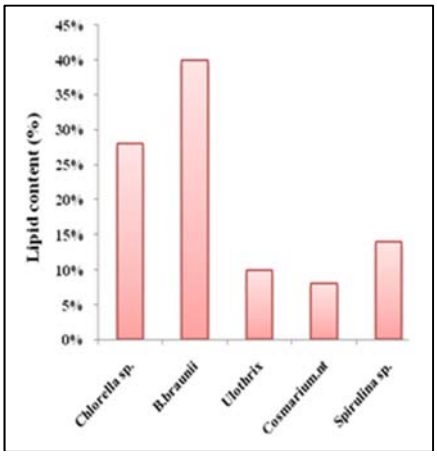

Fig 4: Amount of lipid content produced after two weeks of culture for 5 different algal strains.

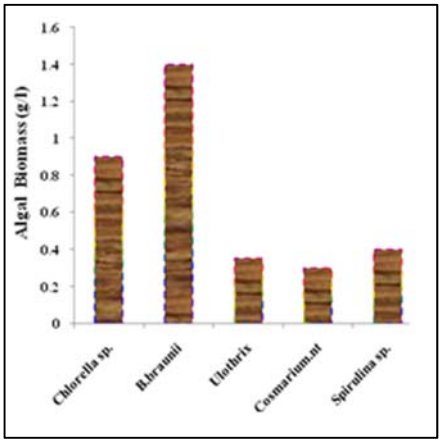

Fig 5: Concentration of mature culture (dry algal biomass per unit volume of algal broth) after two weeks for 5 different algal strains.

Liu *et al*. 2008 [15] reported that total lipid contents representing 20-50% of the dry biomass weight were found to be quite common, and some microalgae even exceeded 80%. The oil content of *Chlorella* typically ranges between 28 and 32% dry weight but can reach 46% dry weight under stress [16]. Chisti 2007 [17] reported that oil content of microalgae is usually between 20-80%. Results in terms of growth and lipd content have shown similar pattern of results in both the media i.e BG-11 & CHU-13.

A best algae strain was selected among a five of screened algae. *B.braunii* resists to contamination from bacteria, viruses and unknown microorganisms that destroy algal cell. It has a high growth rate, which enhance reaching high yield lipid due to high microalgae biomass yield per unit volume of the broth. This strain has been selected for the rest of the study based on high lipid yield. Figure 4 shows lipid content of the five strains samples. Hence, based on the lipid content and good biomass *B.braunii* is selected for the growth evaluation.

Analysis of lipid content

B.braunii algal strains showed the lipid contents began to increase after the lag phase of their growth stage, and reached the maximum values after cultivation for 15 day (Fig.6 & Fig. 7).

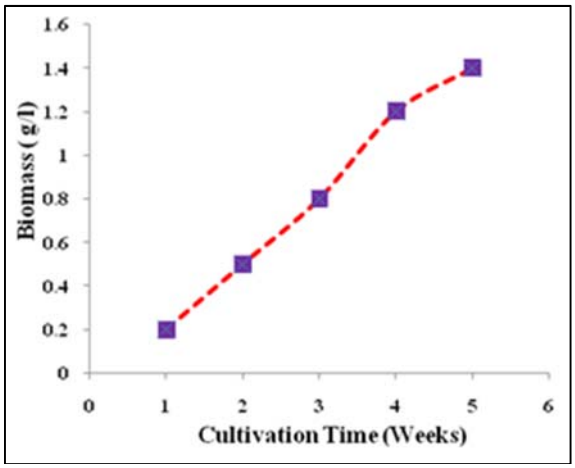

Fig 6: Growth curve of *B.braunii* strain over an five weeks culture period.

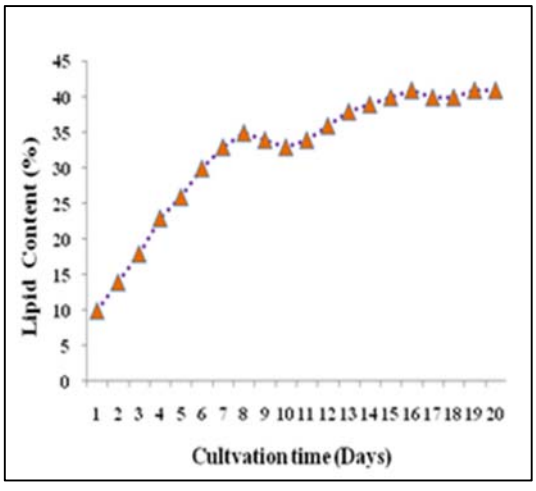

Fig 7: Lipid accumulation curve for *B.braunii* strain

As the starting point for algal biodiesel production, isolating and screening of high neutral lipid-producing microalgae is very crucial to the commercial success of algae-based biofuel production. The expected algal strain that is selected should satisfy the following requirements: 1) high growth rate; 2) high cell density at the end of stationary growth stage; 3) high total lipid content; and 4) high lipid productivity. The best oil producer and fast-growing algae (e.g *Botryococcus braunii).*

The above results demonstrated great potentials of the selected candidate strains for future biodiesel production, as the lipid productions for these strains could be further increased after changing the culture conditions. On the other hand, concerning the lipid productivities, the average value for these five strains were around 200 mg/(L·d), which were a little higher than the average lipid productivities reported previously with the range of 97 to 160 mg/(L·d) [18, 19–20]. In addition, some species showing high lipid contents but poor biomass productivities were not taken into consideration based on our criteria. At the same time, the enhancement of both lipid content and lipid productivity could also be achieved by changing the culture condition a little. Over 25 days of cultivation period, termed *Botryococcus braunii* out of five showed a high growth rate and less risk of contamination. *Botryococcus braunii* has been selected for the rest of the study.

Conclusion

From the present work it can be suggested that *Botryococcus braunii* is a common species. The comparison of oil contents indicate that *Botryococcus braunii* has a higher oil content as compared to other algal strains tested during the present work. Thus a suitable algal strain as *B.braunii* has been selected for high growth rate and contamination resistance for biofuels production.

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