

## Mixotrophic Cultivation of *Botryococcus Braunii* for Biomass and Lipid Yields with Simultaneous CO<sub>2</sub> Sequestration

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### ABSTRACT

In the present study an attempt has been made to utilize glucose as a carbon source to cultivate *Botryococcus braunii* mixotrophically with CO<sub>2</sub> inputs to achieve biomass and lipid yields along with CO<sub>2</sub> reduction. Experiments were carried out in laboratory culture flasks using different glucose concentrations (1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L, 3.5g/L, 4g/L, 4.5g/L and 5g/L) with 6% CO<sub>2</sub> inputs at a flow rate of 0.1vvm at different time intervals of CO<sub>2</sub> aeration (1hr/d, 2hr/d, 4hr/d, 6hr/d, 8hr/d, 10h/d, 12h/d and 24hr/d). The maximum biomass and lipid yields of 2.43g/L and 1.29g/L respectively were obtained at the glucose concentration of 2.5g/L. On the other hand the CO<sub>2</sub> removal efficiency reached upto 75%. Hence in the present study it was observed that the microalgae *B. braunii* grew efficiently in the mixotrophic cultivation mode utilizing the glucose and CO<sub>2</sub> as carbon source for its growth to achieve CO<sub>2</sub> reduction and in turn it produced biomass and lipids yields efficiently.

**Keywords:** Flue gas, *Botryococcus braunii*, Open raceway pond, Biomass, Lipid.

### I. INTRODUCTION

The scarcity of conventional fossil fuels, growing emissions of combustion-generated pollutants, and their increasing costs will make biomass sources more attractive. Petroleum-based fuels are limited reserves concentrated in certain regions of the world. These sources are on the verge of reaching their peak production. The fossil fuel resources are shortening day by day and fossil fuel resources are decreasing daily. The scarcity of known petroleum reserves will make renewable energy sources more attractive.

The production of biodiesel has recently received much attention worldwide. Because of the world energy crisis, many countries have started to take a series of measures to resolve this problem. Finding alternative energy resources is a pressing mission for many countries, especially for those countries lacking conventional fuel resources. In the 1930s and 1940s, vegetable oils have been used as diesel fuels in the emergency situation. With the rapid development of the modern industry, the demand for energy has been greatly increased in recent years, and therefore alternative energy sources are being explored. Thus, the term “biodiesel” has appeared very frequently in many recent reports[1].

Algae can grow practically anywhere where there is enough sunshine. Some algae can grow in saline water. All algae contain proteins, carbohydrates, lipids and nucleic acids in varying proportions. While the percentages vary with the type

of algae, there are algae types that are comprised up to 40% of their overall mass by fatty acids. The most significant distinguishing characteristic of algal oil is its yield and hence its biodiesel yield. According to some estimates, the yield (per acre) of oil from algae is over 200 times the yield from the best-performing plant/vegetable oils. Microalgae are the fastest-growing photosynthesizing organisms. They can complete an entire growing cycle every few days. Approximately 46 tons of oil/hectare/year can be produced from diatom algae. Different algae species produce different amounts of oil. Some algae produce up to 50% oil by weight (Table. 1).

Oil production from microalgae is one promising alternative to complement and eventually replace fossil fuels in next decades. Microalgae, in fact, are photosynthetic unicellular organisms which can grow at a much faster rate than plants and reach higher productivities [2]. In addition, microalgae can reduce carbon dioxide emissions, by adsorbing CO<sub>2</sub> from combustion gases. In fact, atmospheric CO<sub>2</sub> concentration is limiting for algal growth and flue gas can be used as a cheap source with the double goal of supporting algal growth and reducing carbon dioxide released into the atmosphere, although SO<sub>2</sub> and NO<sub>x</sub> might cause microalgae growth inhibition [3]. If organic compounds supporting growth are derived from industrial or agricultural wastes, an increased productivity is also achieved at low cost. Additional potential environmental benefits are also to be considered if wastewaters from

municipal, agricultural or industrial activities are exploited as sources for organic molecules [4].

**Table 1. Lipid content of different Algal strains**

Strains	Lipid content %
<i>Chroococcus minutus</i>	25-86
<i>Scenedesmus obliquus</i>	12-14
<i>Scenedesmus quadricauda</i>	1.9
<i>Scenedesmus dimorphus</i>	16-40
<i>Chlamydomonas reinhardtii</i>	21
<i>Chlorella vulgaris</i>	14-22
<i>SChlorellapyrenoidosa</i>	2
<i>Spirogyra sp.</i>	11-21
<i>Dunaliellabioculata</i>	8
<i>Dunaliellasalina</i>	6
<i>Euglena gracilis</i>	14-20
<i>Prymnesium parvum</i>	22-38
<i>Tetraselmis maculata</i>	3
<i>Porphyridium cruentum</i>	9-14
<i>Spirulina platensis</i>	4-9
<i>Spirulina maxima</i>	6-7
<i>Synechococcus sp.</i>	11
<i>Anabaena cylindrical</i>	4-7

Beside the benefits in terms of biomass accumulation, the addition of an organic carbon source was also reported to stimulate lipids accumulation [5]. It is worth mentioning, however, that all studies analysing mixotrophic biomass and lipid production were performed in laboratory conditions and the possibility that the reported biomass yield can be maintained over long cultivation periods remains undemonstrated [4]. Especially at the industrial scale, mixotrophy is likely to increase bacterial and fungal contaminations, a problem that can be controlled only in closed and strongly controlled systems such as photobioreactors [6]. The use of chlorination, antibiotics and herbicides could potentially reduce contamination, but in many cases they also inhibit microalgal growth.

Heterotrophic and mixotrophic cultures of microalgae have been reported using different carbon sources. Glucose is most commonly used for sustaining microalgae growing in the dark [7] and was employed as carbon source in heterotrophic or mixotrophic culture of several microalgal species [8] reaching high production of both biomass and lipids [9], [10]. From an industrial perspective, however, glucose is not a suitable choice because of its high cost and alternative carbon sources, derived as byproducts from other industrial processes, are preferable: this is the case for acetate [11], ethanol

[12] and glycerol [5]. Heterotrophic and mixotrophic growth using glycerol has been demonstrated for several algae, although the knowledge of glycerol metabolism is still limited, as reviewed by Perez-Garcia *et al.*, 2011 [7].

In this work, the capability of *Botryococcus braunii* of using organic substrate glucose as carbon source was tested, by comparing maximum biomass concentration and lipid accumulation under autotrophic and mixotrophic conditions. Particular attention was dedicated to the effect of supplying CO<sub>2</sub> during mixotrophy. In fact, carbon dioxide is a major limiting factor for algal growth and its excess strongly enhances photosynthetic productivity [13], and therefore, CO<sub>2</sub> supply is needed to achieve the best overall productivity even in mixotrophic conditions. Interestingly, results show that with excess CO<sub>2</sub> concentration microalgae do not consume the organic substrate. Thus, CO<sub>2</sub> and organic compounds supply need to be finely optimized to achieve the best productivities in mixotrophy conditions.

## II. METHODOLOGY

### 2.1 Microalgae culture and cultivation

The presence of *B. braunii* was identified in a local freshwater body by microscopic examinations under Olympus CX21 light microscope according to morphological properties. Pure colonies of *B. braunii* were then isolated and purified by micro-capillary pipetted method, serial dilution and plating techniques (Fig. 1) accordingly.

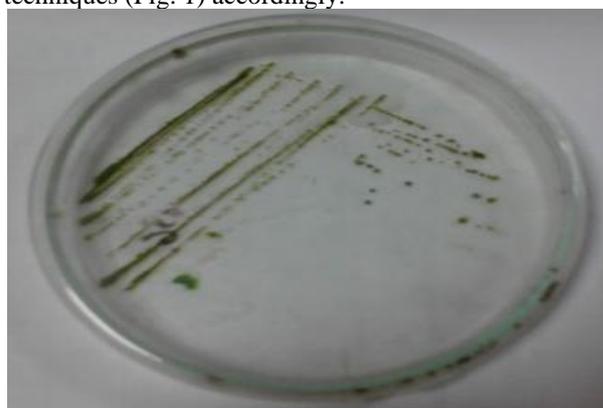


Fig. 1 quadrant streak plates of *b.braunii*

The isolated colonies were then cultured in BG-11 medium whose composition was Citric acid 6.0 mg/L, Ferric ammonium citrate 6.0 mg/L, EDTA 1.0 mg/L, NaNO<sub>3</sub> 1.5 g/L, K<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O 0.051 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.075 g/L, CaCl<sub>2</sub> 0.024 g/L, Na<sub>2</sub>CO<sub>3</sub> 0.02 g/L, H<sub>3</sub>BO<sub>4</sub> 2.86 g/L, MnCl<sub>2</sub>·4H<sub>2</sub>O 1.81 g/L, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.222 g/L, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.391 g/L, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.079 g/L, Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O 0.049 g/L in a 250mL Erlenmeyer flask with 100mL working volume. The culture was maintained at 27°C temperature and 99µmol.photons m<sup>-2</sup> s<sup>-2</sup> light

intensity in a culture rack. The initial pH was measured which ranged between 7.2–7.5.

## 2.2 Mixotrophic cultivation of *B. braunii* in culture flasks

Cultivation of *Botryococcus braunii* has been mainly reported in photoautotrophic mode which has some disadvantages such as low cell growth rate and low cell density. For high density and high productivity, this study attempts to cultivate *B. braunii* in mixotrophic mode. Effects of different glucose concentrations (1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L, 3.5g/L, 4g/L, 4.5g/L and 5g/L), CO<sub>2</sub> inputs (6% (v/v) CO<sub>2</sub>) and time intervals of CO<sub>2</sub> aeration (1hr/d, 2hr/d, 4hr/d, 6hr/d, 8hr/d, 10h/d, 12h/d and 24hr/d) were investigated in 500mL Erlenmeyer flasks containing 200mL of algae medium (BG 11). The entire setup was made in a laboratory culture rack. A series of 8 flasks were arranged in each shelf within a five-stairs culture rack (Fig. 2) equipped with fluorescent lights delivering 132 $\mu$ mol. Photon m<sup>-2</sup> S<sup>-2</sup> light intensity. All the vials were connected to gaseous flow meters through which the CO<sub>2</sub> gas and air enter the vials through PU tubing with valves and joints. The different percentages of CO<sub>2</sub> were purged into each vial through adjustments on the flow meters and the gas-air mixture was purged into the vials for 2 minutes in every hour for 8 hours photoperiod with a flow rate of 1vvm. The culture flasks were exposed to light at 132 $\mu$ mol.photons m<sup>-2</sup> s<sup>-2</sup> delivered by four cool fluorescent lights adjusted to every shelf on the top. The light intensity was measured at the base of the flask using an indigenous photon meter. The incubation temperature was maintained at 28-32°C. All the experiments were conducted in duplets.



Fig. 2 culture rack

## 2.3 Determination of algal growth

Algal growth was analysed in terms of optical density (absorption) which was daily read at 680nm using a spectrophotometer. A growth curve was generated based on the readings. Alternatively the

biomass growth was determined by measuring the cell dry weight, obtained after filtering a sample of microalgal cells, drying and weighing on a pre-weighed whatman No.1 filter paper. Both the dry weights and absorbance were correlated to determine the biomass growth.

## 2.4 Determination of total algal biomass yield

Algae were harvested after 13 days of cultivation. Cultures were filtered through pre weighed Whatmann No.1 filter papers. The papers with attached cells were dried under natural sun light, cooled to room temperature in desiccator, and the dry weights were measured.

## 2.5 Determination of substrate (glucose) concentration

The initial and final organic substrate concentrations were measured in terms of chemical oxygen demand (COD) according to Standard methods for examination of water and waste water (APHA 20<sup>th</sup> edition)

## 2.6 Determination of CO<sub>2</sub> concentration

The CO<sub>2</sub> concentrations in original CO<sub>2</sub>-air mixtures of different percentages and exhaust gas outlets from laboratory culture flasks were measured accurately using a gas chromatography (GC). Dissolved free carbon dioxide was measured in the three culture modes by titrimetric method according to Standard methods for examination of water and waste water (APHA 20<sup>th</sup> edition).

## 2.7 Extraction of lipids

The weighed biomass was ground with a mortar and pestle to form a powder. Then the lipids were extracted from powdered biomass with benzyl alcohol. For each extraction the mixture was sonicated at 50MHz in an ultrasonicator at room temperature for an hour. The extraction mixture was filtered through a Buchner funnel with a Whatmann No.1 filter paper disc placed on the top to collect the waste biomass. The filtration was repeated twice. The filtrate collected at the bottom was poured into a round bottom flask and subjected to distillation to separate solvent and algal fatty acid (Fig 3). The temperature was set to 210°C as benzyl alcohol boils off at 205°C which was collected as distillate and reused. Whereas the remaining extract in the flask was found to be algae lipids. The oil was collected and weighed. The lipid content was calculated and expressed as dry weight percentage based on total biomass obtained.



Fig. 3 oil extraction/distillation

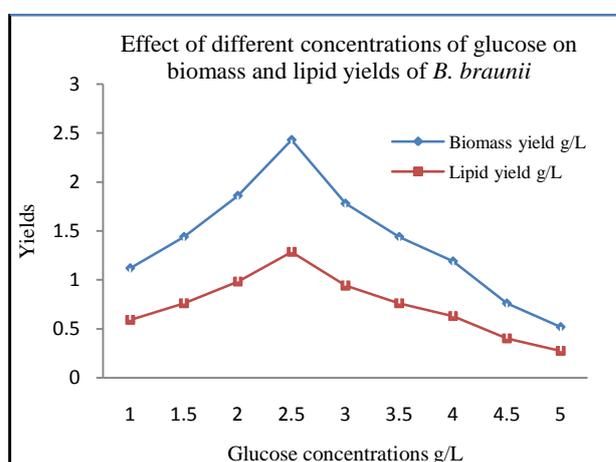
### III. RESULTS AND DISCUSSION

#### 3.1 Effect of organic substrate (glucose) on growth and hydrocarbon yields of *B. braunii*

The mixotrophic medium was BG 11 with different concentrations of glucose added (1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L, 3.5g/L, 4g/L, 4.5g/L and 5g/L). *B. braunii* grew into exponential phase firstly with the lowest initial glucose concentration (1 g/L), and lastly with the highest one (5 g/L), which indicated that high initial glucose concentration can inhibit the growth of *B. braunii* at early culture phase. However, *B. braunii* grew faster with higher initial glucose concentration than that with lower one in exponential phase, and the growth rate of *B. braunii* was the highest with the initial glucose concentration of 5g/L. The glucose consumption was measured in terms of chemical oxygen demand (COD). The consumption of glucose was very low at the lag period in the mixotrophic culture of *B. braunii*, and it increased fast when the cell growth reached the exponential phase. The biomass and lipid yields of *B. braunii* cultured mixotrophically with different initial glucose concentrations were shown in Graph 1. With the increase of initial glucose concentration, the average growth rate of cells increased, whereas the total biomass yield of cells decreased. However, the biomass and lipid yields of *B. braunii* were 2.43g/L and 1.29g/L on 7<sup>th</sup> day with 2.5g/L glucose, which were higher than that with other doses of glucose (Graph 1). These results were in accordance with Zhang *et al.*, 2011 [14]. Also the substrate consumption was high up to 94% at 2.5g/L (Table. 2). Therefore, 2.5 g/L glucose was selected to be the optimum under flask conditions.

Table. 2 Initial and final COD values of different glucose substrate concentrations

S.No	Glucose concentrations	Initial COD mg/L	Final COD mg/L
1.	1g/L glucose	1120	480
2.	1.5g/L glucose	1600	320
3.	2g/L glucose	2080	240
4.	2.5g/L glucose	2560	160
5.	3g/L glucose	3120	560
6.	3.5g/L glucose	3680	720
7.	4g/L glucose	4080	880
8.	4.5g/L glucose	4560	1200
9.	5g/L glucose	4960	1440



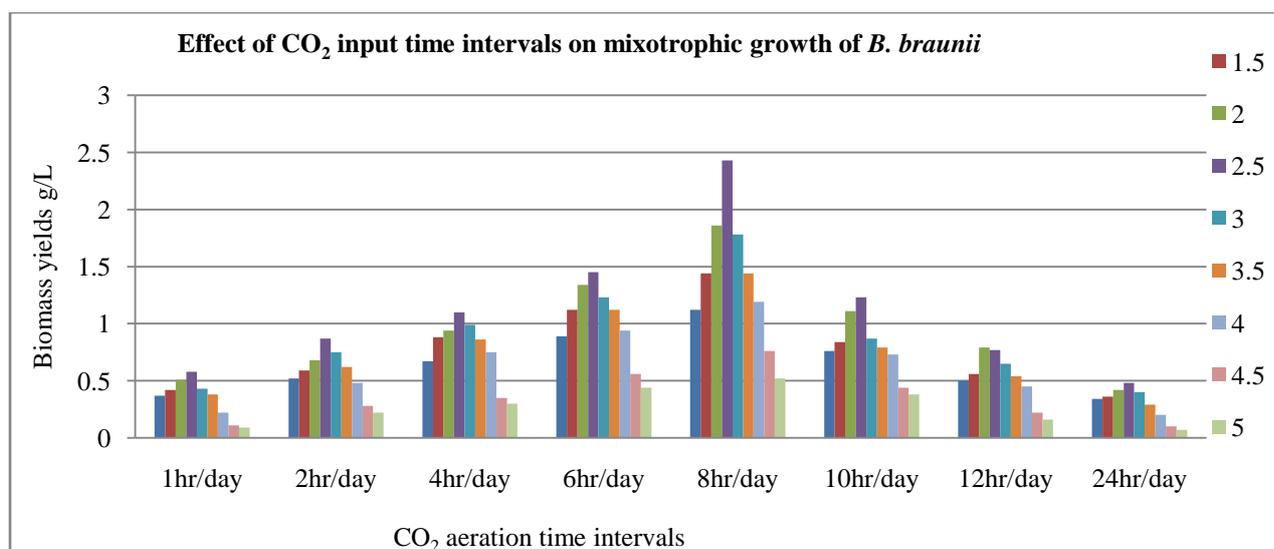
Graph 1: Representing the growth curve

#### 3.2 Effect of CO<sub>2</sub> on mixotrophic growth of *B. braunii*

Results obtained showed that addition of CO<sub>2</sub> stimulated autotrophic growth, while it was well known that carbon dioxide in excess strongly enhances biomass productivity in algae autotrophic photobioreactors. The improvement of photosynthetic efficiency would lead to a further improvement of growth rate and productivity and for this reason *B. braunii* was cultivated with air enriched with CO<sub>2</sub> (6% v/v) at different time intervals CO<sub>2</sub> aeration (1hr/d, 2hr/d, 4hr/d, 6hr/d, 8hr/d, 10h/d, 12h/d and 24h/d). *B. braunii* in CO<sub>2</sub> enriched experiments, however, showed lesser yields in the absence (autotrophic) of glucose when compared to mixotrophic yields. When compared between the CO<sub>2</sub> input time intervals the highest biomass and lipid yields were noted at 8hr/day (Graph 3) however more time of exposure caused excess of CO<sub>2</sub> accumulation which stimulated microalgal photoautotrophic growth, maximizing photosynthetic reactions, but reduced the efficiency of organic

substrate uptake, which remained in the culture medium. This was because organic substrate supported growth during the night, but only if CO<sub>2</sub> supply was not provided. These results were in accordance with Sforza *et al.*, 2012 [13]. The amount of CO<sub>2</sub> removed was estimated from the deference of inlet and outlet gases using GC and determination of free CO<sub>2</sub> in the medium. *B. braunii* was able to remove up to 75% with unutilized CO<sub>2</sub> being 20% in the gases stream and 5% from aqueous medium. Interestingly the CO<sub>2</sub> input time had also played a vital role in CO<sub>2</sub> removal. *B. braunii* cultures exposed

to CO<sub>2</sub> inputs for longer times above 8hrs/day have removed CO<sub>2</sub> up to only 25%. This was in accordance with the argument of Sforza *et al.*, 2012 [15] who reported that cultures bubbled with CO<sub>2</sub>-free air during the night showed better growth rates than those continuously provided with 5% CO<sub>2</sub> due CO<sub>2</sub> inhibitory effect on respiration which reduced biomass accumulation since respiration should consume organic carbon molecules fixed during the day.



Graph 3: Representing the effect of carbon dioxide

#### IV. CONCLUSIONS

From the above studies, some conclusions can be drawn as follows: The cell density in mixotrophic cultivation was obviously higher than that in photoautotrophic mode, so mixotrophic mode is the optimum for culturing *B. braunii*. 2.5g/L Glucose is the optimal carbon source for the mixotrophic cultivation of *B. braunii* among all the doses experimented, including 1g/L, 1.5g/L, 2g/L, 3g/L, 3.5g/L, 4g/L, 4.5g/L and 5g/L. High concentration of initial glucose above 2.5g/L could not enhance the growth of *B. braunii* whereas the low from 1g/L to 2.5g/L concentrations induced better yields. The organic substrate i.e., glucose was consumed up to 94% by *B. braunii* with highest biomass and lipid yields at 2.5g/L dosage. CO<sub>2</sub> experiments (autotrophic) without glucose however showed lesser yields up to 40% compared to mixotrophy. Excess CO<sub>2</sub> exposure has limited the mixotrophic growth of *B. braunii*. Hence optimum conditions were found to be CO<sub>2</sub> aeration for 8hrs/day.

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