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Effects of soybean curd wastewater on the growth and hydrocarbon production of *Botryococcus braunii* strain BOT-22

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ABSTRACT

A laboratory study was conducted to evaluate the possibility of using wastewater from a soybean curd manufacturing plant as a growth promoter of *Botryococcus braunii* strain BOT-22. Soybean curd wastewater (SCW) were added to AF-6 medium to set the final concentration to 0% (control), 1%, 2%, 5%, and 10% (v/v). The growth and hydrocarbon production observed in the cultures with 1% and 2% SCW were significantly higher than that observed in the control. It was postulated that proteins and/or reducing sugars in SCW could enhance the growth. A major finding was a shift in the chemical composition of hydrocarbons from $C_{34}H_{58}$ to $C_{32}H_{54}$ in association with increased concentrations of SCW. Considering the inorganic ions in SCW, it was presumed that a mixture of nitrate, 1–2% SCW, and secondarily treated SCW can be applied for mass cultivation of *Botryococcus*.

1. Introduction

Microalgae have the potential to generate significant quantities of biomass and oil suitable for conversion to biodiesel. *Botryococcus braunii*, a colonial green microalga, produces large amounts of hydrocarbons (Brown et al., 1969; Largeau et al., 1980), which have high potential to be used as renewable energy sources and industrial ingredients; however, if its hydrocarbon production is to be economically viable and sustainable, further optimization of mass cultivation is necessary. Commercial production of hydrocarbons by *B. braunii* is not achieved mainly because of economic and technical barriers. Further studies are required to determine how organic materials can be cost-effectively obtained, particularly since the slow growth of *Botryococcus* has been dramatically improved by an addition of low concentration of glucose to the cultures (Tanoi et al., 2011). To resolve this issue, it is important that algal biofuel production is in concert with municipal and industrial wastewater treatment (Pittman et al., 2011). It was previously reported that secondarily treated wastewaters derived from municipal and industrial activities potentially provide a cost-effective and sustainable means of *Botryococcus* growth for hydrocarbon production (An et al., 2003; Órpez et al., 2009; Sawayama et al., 1992); however, little is known of the effects of untreated wastewaters on the growth of this species.

This study focuses on soybean curd wastewater (SCW) because soybean curd is a highly digestible and nutritive product (Schroder et al., 1973) that has been widely used in a variety of dishes, particularly in Asia, for many centuries; furthermore, SCW is readily available. It was reported that SCW contains polysaccharides (Sonda et al., 2002); however, their effect on *B. braunii* has never been evaluated.

The present study was conducted to evaluate the possibility of using SCW obtained from a soybean curd manufacturing plant as a promoter of growth and hydrocarbon production of *B. braunii*.

2. Methods

2.1. Preparation of SCW culture media

SCW, the byproduct of soybean curd production, was provided by Shinanoyuki Co. Ltd. (Nagano Prefecture, Japan). SCW was filtered through No. 2 filter paper (Tokyo Roshi Kaisha, Ltd., Japan) and was frozen at −20 °C until required for this study. AF-6 medium (Kasai et al., 2004) was used as a control. The experimental media containing 0% (control), 1%, 2%, 5%, and 10% (v/v) SCW were prepared by diluting each one with equal amounts of AF-6 medium. In this case, all the experimental media were prepared with the same amount of components present in AF-6 medium. The pH was adjusted to 6.6 with 10 N NaOH and 0.1 N HCl prior to autoclaving (121 °C, 2 atm, 30 min).

2.2. Algal strain and culture conditions

BOT-22, a strain of *B. braunii,* was isolated from the Okinawa prefecture, Japan. This strain produces botryococcene ($C_{34}H_{58}$), a main component of hydrocarbons (Ishimatsu et al., in press). In

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this experiment, an axenic strain of BOT-22 was cultivated in 300 mL conical flasks containing 200 mL of SCW media with 0%, 1%, 2%, 5%, and 10% SCW at 25 °C under continuous white fluorescent light (70 μmol photon m^{-2} s^{-1}), and aerated with sterile air containing 1% (v/v) CO_2 through a polytetrafluoroethylene filter (0.2 μm, Tokyo Roshi Kaisha, Ltd., Japan). The experiments were conducted twice (first test, $n = 3$; second test, $n = 4$). The initial concentrations of algal cells were adjusted to 4 and 52 mg dry weight L^{-1} for the first and second tests, respectively.

2.3. Determination of algal growth

Growth was monitored based on the algal dry weight. All samples were filtered using a dry glass fiber filter (GF/C 25-mm diameter, Whatman) at 60 °C for 24 h. Weights of the filtered samples were measured after drying at 60 °C for 48 h.

The specific growth rate (μ, day^{-1}) was calculated using the following formula: $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_2 is the algal dry weight at t_2, N_1 is the algal dry weight at t_1, and t_1 and t_2 are times at the exponential phase, respectively.

2.4. Chemical analysis

Dissolved protein content (DPC) and total reducing sugar content (TRSC) in the cultures were analyzed using the Lowry (Lowry et al., 1951) and Anthrone methods (Roe, 1955), respectively. The samples were filtered using a glass fiber filter and immediately measured for protein content. The filtered samples used to determine total sugar content were frozen at −60 °C until they were measured.

Inorganic ions in SCW were analyzed using capillary electrophoresis (Agilent 7100). The operation conditions of the cations were as follows: running buffer, commercial buffer (Agilent inorganic anion buffer); pH, 4.5; injection time, 3 s; column temperature, 25 °C; separating potential, 25 kV; column length, 64.5 cm; signal wavelength, 280/40 nm; and reference wavelength, 210/10 nm. The operation conditions of the anions were as follows: running buffer, 10 mM imidazole, 5 mM lactic acid, 0.5 mM 18-crown-6; pH, 4.5; injection time, 5 s; column temperature, 20 °C; separating potential, −30 kV; column length, 80.5 cm; signal wavelength, 350/20 nm; reference wavelength, 275/10 nm.

2.5. Hydrocarbon analysis

Algal dry weight was gravimetrically measured using a freeze-dried sample. A test tube was prepared containing 10 mg freeze-dried algal cells, 1 mL H_2O, and 0.2 mg triacontane as an internal standard. The sample was shaken and then suspended in 2 M KOH ethanol solution (1 mL) at 35 °C for 2 h. Methanol (1 mL) and hexane (5 mL) were added after the reaction. Two milliliter of the organic layer was dried and measured. The hexane fraction was analyzed using a gas chromatography flame ionization detector (GC-FID) (Shimadzu GC-2010), GC with chemical ionization mass spectrometry (Agilent 7890A and JEOL Q-1000) with a fused silica DB-5MS (30 m × 0.25 mm inside diameter [ID], film thickness 0.25 μl; J&W Scientific, Inc.), and an InertCap 1 MS (30 m × 0.25 mm ID, film thickness 0.25 μl; GL Sciences, Inc.) capillary column. The GC operation conditions were as follows: column temperature, 130 up to 270 °C (20 °C min^{-1}), 270 up to 300 °C (2 °C min^{-1}) and held for 8 min; FID port temperature, 320 °C; carrier gas (He) flow rate, 2.25 ml min^{-1}; FID H_2 flow rate, 40 ml min^{-1}; and air flow rate, 400 ml min^{-1}.

3. Results and discussion

3.1. Effects of SCW on growth of B. braunii

The growth of *B. braunii* was facilitated by the addition of a suitable concentration of SCW (Fig. 1, Table 1). The final biomasses compared to the control were 2.19 times (2.92 g L^{-1}) heavier in 2% SCW, 1.66 times (2.21 g L^{-1}) in 1% SCW, and 1.36 times (1.81 g L^{-1}) in 5% SCW in the first test. Similar results were obtained also in the second test (2.17 times heavier in 2% SCW, 2.12 times in 1% SCW, and 1.61 times in 5% SCW: data not shown in figure and table). The growth rates in 1% and 2% SCW were comparable to or slightly higher than those in the control; however, in the 5% SCW, it took 40 days for growth to reach the stationary phase. In the 10% SCW, all the algae changed color from green to white within 1 week and did not grow further (data not shown). These findings indicate that SCW contains both growth promoter(s) and inhibiter(s) for the strain BOT-22.

Dissolved protein content (DPC) in the control gradually increased with increasing algal growth and reached 29.4 mg L^{-1} by day 35 (Fig. 2a), suggesting that protein compounds and/or agents (e.g., enzymes) were excreted from the cells of the BOT-22 strain. In 1% and 2% SCW, the DPC amounts showed little change by day 22 in spite of the exponential and early stationary phases, and then increased to 35.7 and 39.5 mg L^{-1}, respectively, by day 42 (late stationary phase) (Fig. 2a). Since the proteins are excreted from the cells in the control, we subtracted DPCs in control from DPCs in the SCW additions. Subtracted DPCs in 1% and 2% SCW gradually decreased from day 14 when cell growths in 1% and 2% SCW surpassed that of the control (Fig. 2b). This is suggestive of the fact that the some proteins in SCW could be absorbed into the cells to promote the growth of strain. On the other hand, in the 5% SCW, the DPCs decreased rapidly from 95.2 mg L^{-1} (day 28) to 60.5 mg L^{-1} (day 42) with increasing biomass (Fig. 2a); this is a strong indicator of the fact that some proteins in SCW inhibited growth at the early stages.

Total reducing sugar content (TRSC) gradually increased from 12.3 to 57.0 mg L^{-1} in the control and from 55.6 to 160.1 mg L^{-1} in 1% SCW by day 35 (Fig. 3a). In 2% SCW, the sugar content remained unchanged until day 14, and then increased to 179.4 mg L^{-1} by day 35 (Fig. 3a). As in the case of DPC, we sub-

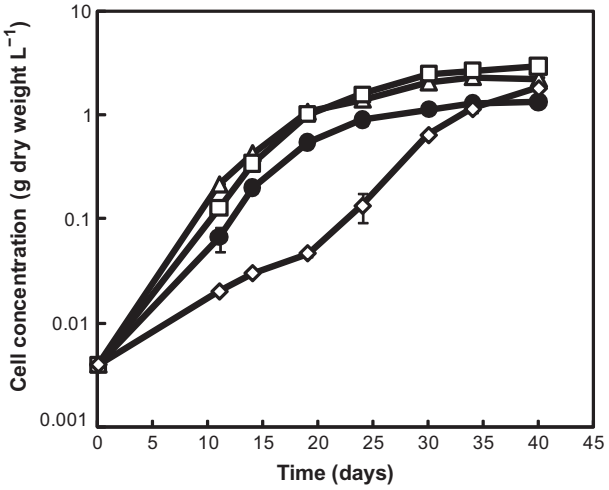

Fig. 1. Growth curves of *B. braunii* strain BOT-22; control (filled circles), 1% soybean curd wastewater (SCW) (blank triangles), 2% SCW (blank squares), and 5% SCW (blank diamond shaped). All initial cell concentrations were adjusted to 4 mg L^{-1}. Error bars indicate means and standard errors (SEs) ($n = 3$).