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Direct utilization of starch for heterotrophic cultivation of *Chlorella pyrenoidosa* by co-culture with immobilized *Saccharomycopsis fibuligera*

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ABSTRACT

Heterotrophic culturing of microalgae is limited by the high cost of the medium due to the addition of an extra carbon source. Starch is a natural, abundant, cheap, and renewable glucose polymer. Algal cells are incapable of hydrolyzing starch under heterotrophic conditions. To solve this issue, a co-culture system was developed using a mixed culture of *Chlorella pyrenoidosa* and immobilized *Saccharomycopsis fibuligera*. The latter is an amylolytic yeast that secretes amylase and hydrolyzes starch into glucose and other oligosaccharides after being immobilized. The co-cultured *C. pyrenoidosa* efficiently utilized the accumulated available sugar for its growth, and the highest algal biomass concentration reached 3.6 g/L, higher than that achieved by *C. pyrenoidosa* grown on an equivalent amount of glucose. The lipid content of these algal cells cultured in medium with starch was as high as that achieved when cultured in a medium with glucose. In addition, pure algal cells were able to be well separated from this system as yeast cells were immobilized on beads and could be easily separated by simple filtration. This strategy provides a new approach that allows microalgae to heterotrophically utilize starch, which will help reduce the carbon source cost in heterotrophic cultivation of microalgae.

1. Introduction

As photosynthetic microorganisms, microalgae are most commonly cultivated in the photoautotrophic mode [1]. Although the medium cost is low, the growth of algae is severely dependent on the light supply. Biomass production is greatly limited under photoautotrophic culture because of limited cell growth due to light attenuation [2]. However, some algal species are able to heterotrophically utilize exogenous organic compounds for growth. In heterotrophic culture, the light requirement is completely eliminated; the design, operation, and scale-up of the bioreactor are greatly simplified; and cell growth is significantly enhanced by the assimilation of an exogenous carbon source [3]. It has been estimated that heterotrophic culture can enhance the biomass concentration by as much as 25-fold compared to that of autotrophic culture [1]. Heterotrophic cultivation has shown good potential and has been regarded as a promising strategy for the efficient cultivation of algae to obtain an abundant algal biomass [4].

Compared with autotrophic culture, the sole difference in the medium composition is the addition of an extra carbon source, which is necessary for heterotrophic culture. Currently, glucose is the most commonly used carbon source due to its excellent culture performance [4]. However, glucose greatly increases the cost of the medium and may constitute up to 80% of the total cost of the medium, which makes heterotrophic culture of microalgae economically unfeasible [5]. To make heterotrophic culture of microalgae economically viable for the high-volume, low-cost production of commodities, developing inexpensive alternative carbon sources is required for the industrial application of heterotrophic cultures [1].

After cellulose, starch is the most abundant biomass on earth [6]. As a natural, abundant, cheap, and renewable glucose polymer, starch has been regarded as a promising substrate in the fermentation industry and has been widely used in the commercial production of biofuels, especially bioethanol [7]. There are indications that starch can be used as an alternative carbon source in heterotrophic culture of microalgae. Zhang et al. reported that starch slightly enhanced cell growth under mixotrophic culture of *Chlorella pyrenoidosa*. However, a light source was needed in this culture process and the culture performance of starch was far worse than that of glucose [8]. The use of starch hydrolysates, including corn powder hydrolysate, cassava starch hydrolysate, and potato starch hydrolysate, has also been attempted in the heterotrophic culture of algae. Nevertheless, these starch or starch-enriched materials must first be hydrolyzed using alpha-amylase and/or glucoamylase before use, which complicates the culture process and increases the cost [9–11]. To date, no feasible strategy has been reported for the direct use of raw starch in the heterotrophic culture of microalgae.

Saccharomycopsis fibuligera has attracted increasing attention as an

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important trehalose-producing strain and has been widely used in the fermentation industry. Starch, due to its high efficiency of utilization, low price, and easy availability, has been regarded as the best substrate for fermentation using *S. fibuligera* [12]. Using starch as the carbon source, *S. fibuligera* can produce high levels of various amylases, including α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3), which hydrolyze starch molecules into glucose, maltose and dextrin [12]. After immobilization, these yeast cells are able to achieve amylase activity levels that are even higher than those in free mode [13, 14].

Therefore, to enable microalgae to heterotrophically utilize starch, this study developed a co-culture system composed of *Chlorella pyrenoidosa* and immobilized *S. fibuligera* using soluble starch as the sole carbon source. In this system, starch was hydrolyzed into glucose and oligosaccharides by extracellular amylases secreted by the immobilized yeast, thus providing an adequate available carbon source for algal growth. At the end of the culture, pure algal suspension was efficiently obtained as the yeast cells were immobilized on beads that were easily separated by simple filtration. This study provides an effective strategy for heterotrophic cultivation of microalgae using starch. By using starch instead of glucose, the cost of the carbon source in the heterotrophic culture process is greatly decreased. Furthermore, the overall strategy is a promising method for large scale heterotrophic cultivation of microalgae.

2. Material and methods

2.1. Microorganism strains and inoculum culture

C. pyrenoidosa and *S. fibuligera* were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB, CAS) and the China General Microbiological Culture Collection Center (CGMCC), respectively. Before being used in the following experiments, the cells were screened and purified using streak plate combined with dilution-plate methods. The inoculum culture was developed according to the method described by Wang et al. [15].

2.2. Preparation of immobilized yeast beads (IYBs)

Yeast cell immobilization was performed as described by El-Dalatony et al. [16]. Briefly, after determining the cell density using a hemocytometer and a microscope (Olympus BX53, Japan), cells were collected at a concentration of $8.9 \times 10^7\ mL^{-1}$ by centrifugation at 4000 rpm for 10 min. After mixing the sludge with a sterilized Na-alginate solution (2%, w/v), the mixture was extruded dropwise through a 10 mL disposable plastic syringe into a $CaCl_2$ (2%, w/v) solution to prepare uniform spherical beads. The beads were hardened at 4 °C for 2 h and then washed with sterile distilled water three times to remove unbound impurities. The diameters of the IYB were from 2–3 mm as measured using a digital Vernier caliper. The yeast cell concentration was approximately 1.4×10^6 cells per bead.

2.3. Comparison of cultures of free and immobilized yeast

The same numbers of free and immobilized yeast cells were inoculated in liquid BG-11 medium with 10 g/L soluble starch (Sinopharm, China). Starch was produced from potato and consisted of approximately 20% amylose and 80% amylopectin. The cultures were incubated at 25 °C in an orbital shaker set at 150 rpm. Samples were taken to measure the cell concentration, pH, and extracellular amylase activity.

2.4. Co-culture of C. pyrenoidosa and IYBs

In 250 mL Erlenmeyer flasks, 100 mL of liquid BG-11 medium with 10 g/L soluble starch was used in following experiments. Various numbers of IYBs (0, 30, 60, 90, and 120) were simultaneously added to each algal cell culture. Pure cultures growing in BG-11 medium with 10 g/L glucose were used as controls. All cultures were incubated at 25 °C in an orbital shaker set at 150 rpm in a dark environment. At the end of the co-culture process, the used IYBs were collected. After washing the beads three times using sterile distilled water, the IYBs were reused in the following co-culture process two additional times as described previously. The culture performance was evaluated.

2.5. Analytical methods

2.5.1. Biomass concentration

The biomass concentration of microalgae was determined gravimetrically [17]. The kinetic parameters of cell growth were calculated as described by Wang et al. [15].

2.5.2. Amylase activity

Amylase activity was measured according to the method of Gen et al. [13] with modifications. Briefly, the culture was centrifuged at 9000 ×*g* and 4 °C for 10 min, and the supernatant was taken as the crude amylase extract. The enzymatic reaction mixture consisted of 200 μL of crude amylase extract, 100 μL of acetate buffer (0.1 M, pH 5.0), and 700 μL of a 1% (w/v) soluble starch solution. This mixture was reacted at 50 °C for 10 min and stopped by heating at 100 °C for 10 min. After adding 2 mL of dinitrosalicylic acid (DNS) reagent to the mixture, the resulting mixture was incubated in boiling water for 5 min and then cooled on ice to room temperature. Added 9 mL distilled water to the mixture and the absorbance was determined at 540 nm in a 722 N visible spectrophotometer (Shanghai P&S instrument CO., LTD, China). Blank controls were prepared with inactivated crude enzyme extracts treated by heating at 100 °C for 10 min. One unit of amylase activity (U) was defined as the amount of enzyme responsible for the production of 1 μmol of reducing sugar per minute under the measurement conditions.

2.5.3. Sugar content measurement

The total sugar content was measured according to the method described by González et al. [18]. In detail, the culture was centrifuged at 8000 rpm and 4 °C for 10 min, and 180 μL of the supernatant was mixed with 180 μL of 1 N HCl. After being boiled for 45 min, the mixture was neutralized with 1 N NaOH. The released reducing sugars were colorimetrically measured using the DNS method. For the measurement of reducing sugars, the supernatant was directly measured using the DNS method.

2.5.4. Lipid analysis

Lipids were extracted and gravimetrically quantified using the method described by Morales-Sánchez et al. [19]. In detail, the cell pellet was collected by centrifugation at 4 °C and 14,000 ×*g* for 10 min. The cell pellet was resuspended in methanol/dichloromethane (2:1, v/v) containing 0.5 mg of butylated hydroxytoluene and stored at 4 °C overnight. The supernatant was transferred to another tube after centrifugation at 4 °C and 14,000 ×*g* for 10 min. The residue was re-extracted twice using 4 mL of methanol/dichloromethane (1:1, v/v) for 10 min in an ultrasonic cleaner. After removing the solvents form the combined organic phase using an evaporator at 40 °C and then drying under a nitrogen atmosphere, the lipids were gravimetrically quantified.

Extraction and transesterification of the fatty acids were performed according to the method described by Wang et al. [15]. Briefly, the collected algal biomass was first lyophilized and then ground for 10 min. Afterwards, the powder was dispersed in 3 mL of a 7.5% (w/v) KOH/CH_3OH solution, and the mixture was incubated at 70 °C for 4 h. After heating, 2 mL of HCl/CH_3OH (1:1, v/v) and 2 mL of 14% BF_3/CH_3OH (ANPEL, China) were added, and transesterification was allowed to proceed at 70 °C for 1.5 h. After centrifugation at 2000 rpm for 3 min at 4 °C, 1 mL of 0.9% NaCl and 4 mL of n-hexane were added to