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Optimization of lutein production with a two-stage mixotrophic cultivation system with *Chlorella sorokiniana* MB-1

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ABSTRACT

The major purpose of this study was to investigate the effects of operational factors and bioprocess strategies on the mixotrophic cultivation of a microalgae *Chlorella sorokiniana* MB-1 for lutein production. Aeration with CO_2 showed the highest biomass productivity and lutein productivity of 0.89 g/L/d and 3.49 mg/L/d, respectively. Semi-batch operation performed with 80% medium replacement ratio resulted in the highest biomass productivity and lutein productivity of 1.55 g/L/d and 5.51 mg/L/d, respectively. A two-stage strategy was developed to enhance the biomass production of the MB-1 strain in stage 1 with semi-batch mixotrophic culture and to optimize lutein accumulation in stage 2 under photoautotrophic conditions. The maximum biomass productivity and lutein productivity was 1.98 g/L/d and 7.62 mg/L/d, respectively, with a medium replacement ratio of 80% in stage 1. Compared with batch cultivation, the lutein productivity was enhanced by 32.7% for semi-batch operation alone and 85.9% for the semi-batch-integrated two-stage process.

1. Introduction

Lutein is a primary carotenoids contained in plants or microorganisms extensively, and is classified as a xanthophyll because of the presence of two hydroxyl functional groups in the structure (Bowen et al., 2002; Chen et al., 2018; Ho et al., 2015). Based on plant physiology, lutein acts as an accessory light harvesting pigment aiding in the collection of light energy for enhanced photosynthesis efficiency and prevents photo-damage via quenching of the chlorophyll triplets and heat dissipation (Chen et al., 2017; Ruban et al., 2007). Moreover, lutein also acts as an antioxidant metabolite and protects the cells from the oxidative damage caused by free radicals, either of environmental origin or those produced by the inherent metabolic activities (Peng et al., 2013). With outstanding properties as an antioxidant, lutein can scavenge free radicals and reduce the damage to DNA molecules, proteins, and membranes (Yang et al., 2018). The existing commercial production of lutein is primarily from marigold petals (Lin et al., 2015a). However, it is still challenged by the low lutein content (as low as 0.03%) and the high labor demand for the harvesting and separation of the marigold petals (Lin et al., 2015b; Utomo et al., 2013). Also, lutein primarily exists as mono- or di- ester forms in the marigold flowers which needs to be de-esterified before intestinal absorption in animals, hence the bioavailability is significantly lower than the free form of lutein (Bowen et al., 2002). Therefore, there is an ongoing search for an alternative feedstock for the commercial production of lutein (Chiu et al., 2016; Lin et al., 2015b).

In recent years, microalgae have been increasingly recognized as a source of natural pigments (such as lutein and astaxanthin) (Chiu et al., 2016). Some microalgal species are rich in lutein and thus microalgae have been investigated as a prospective source of lutein in recent years (Del Campo et al., 2001). Photoautotrophic cultivation of microalgae has been primarily applied for the production of lutein, since pigment synthesis is the cell's physiological response for high light stress. Mixotrophic cultivation, where both organic and inorganic carbon can be assimilated in the presence of light by microalgae by aerobic respiration and photosynthesis, combines the advantages of both phototrophy and heterotrophy (Velea et al., 2017)). Mixotrophic cultivation is superior to phototrophy in case of the carbon footprint (Chen et al., 2018; Xiong et al., 2010), reduction in photo inhibitory effect and night biomass loss, reduction in photo-oxidative damage form the metabolic oxygen accumulation in the culture, and the flexibility to shift to heterotrophic or phototrophic modes (Velea et al., 2017). Mixotrophic cultivation has been successfully applied for production of lutein from microalgae, with considerable lutein content and productivity (Hu et al., 2018).

Nowadays, numerous types of fermentation operating strategies are widely used for microorganism cultivation to improve the cells growth rate, production of biomass and yield of target products (Fu et al., 2014; Utomo et al., 2013). Therefore, it is important for selecting a suitable

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operation strategy for cost effective biochemical production from various microorganisms. Batch, semi-batch, fed-batch, continuous processes are considered as fundamental operation modes which have been commercially used in industrial fermentation (Gharibzahedi et al., 2014; Ho et al., 2015). Semi-batch cultivation system is often used for periodic harvesting, which involves partial replacement of fresh medium with flexibility of adding more as time passes. The advantages of using semi-batch process includes (Peng et al., 2013): (1) overcomes substrate inhibition and maintains high biomass concentrations; (2) periodic replacement of fresh medium to sustain growing and bleeding of microorganism; (3) decreasing the lag phase with higher initial inoculum size; (4) removing excessive extracellular products to avoid product inhibition.

This study focused on the optimization of lutein production from an indigenous microalgae *Chlorella sorokiniana* MB-1 under mixotrophic cultivation. Aeration, light intensity and semi-batch mode of cultivation were evaluated for enhanced lutein content and productivity from *C. sorokiniana* MB-1. A novel two-stage approach for the cultivation of *C. sorokiniana* MB-1 was developed, performing biomass production and lutein accumulation in two separate stages with a semi-batch operation mode.

2. Materials and methods

2.1. Microorganism and microalgae culture

The acetate-tolerant strain used in this study (*Chlorella sorokiniana* MB-1) was isolated from freshwater located in Taiwan (Chen et al., 2016). The BG-11 medium was used for the cultivation of microalgae which consisted of: $NaCH_3COO$, 4.88; $NaNO_3$, 1.83; NaCl, 0.218; K_2HPO_4, 0.03; $MgSO_4{\cdot}7H_2O$, 0.075; Citric acid anhydrous, 0.006; Na_2CO_3, 0.02; $CaCl_2{\cdot}2H_2O$, 0.03855; Ammonium iron (III) citrate, 0.006; EDTA·2Na, 0.001; H_3BO_3, 0.00286; $MnCl_2{\cdot}4H_2O$, 0.00181; $ZnCl_2{\cdot}7H_2O$, 0.000222; $Na_2MoO_4{\cdot}2H_2O$, 0.00039; $CuSO_4{\cdot}5H_2O$, 0.000079; $Co(NO_3)_2{\cdot}6H_2O$, 0.000049. After 4–5 days pre-culture with continuous lighting, the microalgae were grown in a 1-liter glass bottle at room temperature (26–28 °C) with 2.0% CO_2 supply at an aeration rate of 0.1 vvm and continuous illumination with TL5 tungsten filament lamp at a light intensity of 100 μmol/m^2/s. The light intensity was measured by Li-250 Light Meter with a Li-190SA pyranometer sensor (Li-COR Inc., Lincoln, Nebraska, USA). The centrifuged microalgal biomass was freeze dried by a freeze-dryer (FDU-1100, EYELA, Tokyo, Japan) and used for further analysis.

2.2. Analysis of microalgal growth parameters

2.2.1 Microalgal biomass concentration

Microalgae biomass concentration in the photo-bioreactor (denoted as PBR) was determined by measuring the optical density (OD) of samples at a wavelength of 688.6 nm (denoted as $OD_{688.6}$) using a UV/Vis spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). The $OD_{688.6}$ were converted to biomass concentration by means of appropriate calibration, and the conversion factor of MB-1 between $OD_{688.6}$ and dry cell weight was determined as follows: 1.0 $OD_{688.6}$ equals to 0.21 ± 0.02 g DCW/L.

2.2.2 Determination of nutrient concentration

The nitrogen and acetate concentration in the samples was measured by ion chromatography (ICS-3000, Thermo Scientific DIONEX, USA) equipped with a carbonate eluent anion-exchange column (IonPac AS12A, Thermo Scientific DIONEX, USA) and a conductivity detector.

2.2.3 Determination of lutein content and productivity

Lutein was extracted using the modified method reported previously (Ceron et al., 2008; Chen et al., 2016). A 1 mL of 60% w/w KOH solution was added to 10 mg dry microalgal biomass to hydrolyze the lipids and promote cell disruption. After cell disruption, the mixture was placed in hot water bath at 40 °C for 40 min to react completely, followed by extraction with diethyl ether and supernatant phase was collected until it was clear. The organic solvent was then purged with nitrogen gas, and the precipitate was dissolved in acetone for high-performance liquid chromatography (HPLC) measurements equipped with photodiode array detector (PDA) (Taylor et al., 2006). An YMC Carotenoid RP-30 column with the particle size 5 μm and column size of 4.6 × 250 mm (YMC Schermbeck, Germany) was used for separation. The binary mobile phase included: (a) 3% ddH_2O in methanol containing 0.05 M ammonium acetate and (b) 100% TBME (tert-butyl methyl ether). Both mobile phases contained 0.1% (w/v) BHT and 0.05% triethylamine (TEA). The extracts were eluted at a flow rate of 1 mL/min and lutein was detected by measuring absorbance at the wavelength range of 220–750 nm. Three absorbance wavelengths were used (i.e., 450, 652, and 478 nm) and accurate quantification of lutein extracts was determined by the maximal absorbance (450 nm). The lutein standards used for quantification were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.3. Two-stage mixotrophic cultivation system

The two-stage cultivation system consisted of two tanks for cultivation and one smaller bottle as buffer tank. The first stage (Tank 1) was operated under mixotrophic condition on semi-batch mode to achieve better growth of the microalgal strain (Chen et al., 2018) using acetate as the organic carbon source and 2.5% CO_2 as the inorganic carbon source. Next, the culture in the first stage was transferred into the second stage (Tank 2), which was operated under phototrophic condition (illumination at 150 μmol/m^2/s; aeration with 2.0% CO_2 at 0.1 vvm) to trigger lutein accumulation. By operating microalgal growth and lutein accumulation in two separate stages under their own preferable conditions, higher lutein productivity and CO_2 fixation efficiency of the whole process could be achieved.

2.4. Statistical analysis

Statistical analysis of the data was conducted using the one-way ANOVA with Prism 5 software. The variables analyzed included gas type, light intensity, replacement ratio and two-stage cultivation system. The effects of these parameters on microalgal biomass and lutein content were of significant difference when the p-value was lower than 0.05.

3. Results and discussion

3.1. Effect of different aeration strategy on lutein content and lutein productivity of C. sorokiniana MB-1

Aeration is an important parameter when it comes to microalgal cultivation. In this study, the microalgae culture was continuously fed with two different gases: CO_2, air, and a control experiment was performed without aeration. 1.0 g/L of sodium nitrate and 3.0 g/L of sodium acetate were used as nitrogen and carbon sources, respectively, in the culture medium. The microalgae were grown at 25 °C with a light intensity of 100 μmol/m^2/s and the results are summarized in Fig. 1. Biomass growth, lutein content and lutein productivity were significantly higher when the culture was supplied with CO_2 aeration. Maximal biomass was obtained with CO_2 aeration, with a higher lutein content of 3.5 mg/g, which is almost double the lutein content obtained with ambient air aeration. Lutein productivity was also the highest while using CO_2, reaching a value of 3.49 mg/L/d. Without aeration, the performance of cell growth in term of biomass concentration and productivity was very poor. Lutein content was significantly lower at 1.26 mg/g biomass. These results can be expected since carbon dioxide is essential for photosynthesis, and they also clearly indicate that *C.*