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Enhanced biomass and oil production from sugarcane bagasse hydrolysate (SBH) by heterotrophic oleaginous microalga *Chlorella protothecoides*

Jinxiu Mu, Shitian Li, Di Chen, Hua Xu, Fangxin Han, Bo Feng, Yuqin Li *

School of Chemical Engineering, Xiangtan University, Xiangtan 411105, Hunan, PR China

HIGHLIGHTS

- Sugarcane bagasse hydrolysate (SBH) is a cost-effective and efficient carbon source.
- SBH performed better than glucose for lipid accumulation by *C. protothecoides*.
- Xylose and arabinose involved in PPP was significant contributor for oil synthesis.
- XYLB, ACAD, and DLD are the metabolic checkpoints in SBH-induced *C. protothecoides*.

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ABSTRACT

The potential use of sugarcane bagasse hydrolysate (SBH) for microalgal oil in a heterotrophic mode and the oil accumulation mechanisms by SBH-induced *Chlorella protothecoides* cells were investigated in this study. Results demonstrated that SBH performed better than glucose for cell growth and lipid accumulation under the same reducing sugar concentration. The lipid productivity of 0.69 g/L/d was accomplished at 40 g/L of reducing sugar by batch culture. Under the fed-batch culture condition, the maximum biomass and lipid productivity were 24.01 g/L and 1.19 g/L/d, respectively. Metabolic pathway analysis results indicated that xylose and arabinose involved in pentose phosphate pathway might be predominant over sole glucose involved in glycolysis for lipid accumulation in cells. Three metabolic checkpoints in the proposed metabolic network, including xylulose kinase, acyl-CoA dehydrogenase, and dihydrolipoyl dehydrogenase reveal new possibilities in developing genetic and metabolic engineering microalgae for desirable lipid productivity.

1. Introduction

Biodiesel (fatty acid methyl esters) is usually obtained from plant oils, such as rapeseed oil, soybean oil, and palm oil. However, booming biodiesel production has put it into competition with food supplement and agricultural land. It is apparently pivotal to develop unconventional crop-based feedstock for lipid production. Microbial oils produced by oleaginous microorganisms have been considered as alternatives for biodiesel production due to similar fatty acid compositions to traditional plant oils, short production period, cheaper labor required and easiness to scale up (Li et al., 2008; Easterling et al., 2009). Among oleaginous microorganisms (e.g. bacteria, fungi, yeasts and microalgae), microalgae have elicited a great deal of attention for high light-to-biomass conversion efficiency and high levels of lipids accumulation (Mata et al., 2010; Huang et al., 2013).

The lipid productivity of microalgae is a crucial factor in making algal biodiesel economically viable (Davis et al., 2011). A proposed heterotrophic cultivation regime for microalgae could effectively convert organic carbon into algal lipid and provide favorable lipid productivity (Xiong et al., 2010). However, the high cost of heterotrophic carbon substrate (e.g. glucose) remains to be the main barrier for algal lipids. Many researchers are concentrating on finding waste or low-grade biomass as carbon and energy source instead of glucose for the heterotrophic microalgae. For instance, cassava starch hydrolysate (CSH), corn powder hydrolysate (CPH), sorghum juice (SJ), and *Cyperus esculentus* waste hydrolysate (CEWH) have been used for high-quality lipids production by *Chlorella* (Xu et al., 2006; Gao et al., 2010; Cerón-García et al., 2013; Wang et al., 2013). Moreover, the lipid productivity of heterotrophic microalgae is ultimately dependent on the efficiency

* Corresponding author. Tel.: +86 731 58629863; fax: +86 731 58298172.
E-mail addresses: yuqinli2004@126.com, yuqinli2004@xtu.edu.cn (Y. Li).

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of carbon utilization rate and the downstream cellular processes that drive carbon into useful lipid precursors. Therefore, a cost-effective and efficient carbon source is essential to enhance lipid accumulation in heterotrophic microalgae.

Sugarcane bagasse is an important biological resource that has received increasing interest due to high biomass productivity and multiple carbohydrate compositions (Huang et al., 2013). Sun et al. (2004) reported that the major constituents from hydrolyzed sugarcane bagasse are xylose, arabinose, and glucose, which are just promising carbon sources in the preparation of valuable microbial products by heterotrophic fermentation. For instance, sugarcane bagasse hydrolysate (SBH) as the carbon source for succinate production by engineered *Escherichia coli* (Liu et al., 2013) and xylanase production by *Bacillus circulans* D1 (Bocchini et al., 2005). In fact, these sugar compositions from SBH have different transport and metabolic pathways in microbial cells. Some literatures suggested that arabinose erected great contribution to intracellular biochemical components accumulation because of uptake more efficiently than xylose or glucose (Liu et al., 2013; Tsigie et al., 2011). Therefore, sugarcane bagasse hydrolysate received special attention because of multiple sugar components than sole glucose. However, considerable efforts related with microalgal lipids by SBH are very limited.

This study is aimed to explore heterotrophic microalgal oil production from SBH by oleaginous *Chlorella protothecoides*. The effects of the initial reducing sugar concentrations (RSCs) in SBH on the biomass and lipid productivity were investigated. A classical culture strategy-fed-batch regime was also employed to further enhance biomass and lipid productivity by utilizing SBH as feed supplement. Additionally, it is continued to pay attention to find key intermediates for lipid biosynthesis and reveal lipid accumulation mechanism by SBH-induced *C. protothecoides* cells. It is expected to have advances in developing the heterotrophic regime for microalgae-based biodiesel production.

2. Methods

2.1. Materials

Sugarcane bagasse was supplied by Changsha Sugar Industry Co. Ltd., Hunan province. Cellulase enzyme and β-glucosidase were purchased from Advanced Enzyme Technologies Co. Ltd., India (http://www.enzymeindia.com). The green microalgae *C. protothecoides* was obtained from FACHB-collection, Wuhan, China. All other chemicals were of the highest purity available.

2.2. Preparation and carbohydrate compositions analysis of sugarcane bagasse hydrolysate

50 g of sugarcane bagasse (SB) was washed 3–5 times with distilled water to remove the residual sugar and then dried in drying oven at 80 °C for 48 h. The dried SB was broken to pieces that could pass 20-mesh (0.90 mm) sieve. The SB powder was mixed with $C_6H_8O_7$–Na_2HPO_4 buffer (1:4, w/v) and the mixture was then incubated in a thermostated water bath shaker (50 °C) for 30 min. Subsequently, the pH of the power slurry was adjusted to 4.8 with 0.1 M HCl and cellulase enzyme (500 mg) was introduced and kept at 50 °C for 6 h in an oscillator. Afterwards, β-glucosidase (50 mg) was added to the mixture and incubated at 50 °C for 48 h. The enzyme was deactivated with microwave boiling method. The hydrolysate was collected by centrifugation and the supernatant was filtered to obtain the sugarcane bagasse hydrolysate (SBH).

The carbohydrate profiles in SBH were analyzed by High Performance Liquid Chromatography. SBH samples (20 μL) were injected into an Agilent Zorbax column (4.6 mm × 250 mm) operating on Waters 600 Controller machine with a Waters 2414 RID detector. The operating temperature was 90 °C, and the flow rate was 1.2 mL/min. The mobile phase consisted of EDTA-Ca^{2+} (0.05 g/L) and 0.01 mol/L oligosaccharide standards from Sigma (St. Louis, MO).

2.3. Algae strain and heterotrophic cultivation regime

C. protothecoides seed liquid was prepared in the improved Basal medium (Wei et al., 2009), which was supplemented with 1.25 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 83.75 mg $CaCl_2$, 49.8 mg $FeSO_4{\cdot}7H_2O$, 15.7 mg $CuSO_4{\cdot}5H_2O$, 38.2 mg $ZnSO_4{\cdot}7H_2O$, 0.5 g EDTA, 114.2 mg boron, 14.4 mg $MnCl_2{\cdot}4H_2O$, 7.1 mg MoO_3, 4.9 mg $CoNO_3{\cdot}6H_2O$, 3 g urea, and 10 g glucose.

To compare the effects of glucose, artificial mixture sugars (AMS), and SBH as carbon source for cell growth and lipid content, the same initial sugar concentration (10 g/L) was used in Basal medium for batch culture. And six levels (5, 10, 20, 30, 40, 50 g/L) of reducing sugar concentrations in SBH were used in Basal medium to investigate the effects of initial reducing sugar concentration on biomass and lipid accumulation of *C. protothecoides*.

Fed-batch culture was conducted to further enhance the lipid productivity. In fed-batch culture, the initial reducing sugar concentration was 20 g/L. The concentration of reducing sugar was controlled at the range of 5–15 g/L by feeding sterilized and concentrated SBH. The culture conditions were the same with batch culture as described above. The biomass and the residual reducing sugar concentration were determined at 7–30 h interval. The lipid productivity was measured by the feeding time.

All the culture medium were adjusted to pH 6.1 and sterilized with autoclave sterilizer at 116 °C for 20 min. The algal seed liquid (the initial cell biomass was about 1.0 g/L) were inoculated into 250 mL flasks containing 100 mL of sterilized medium (1:100, v/v) and performed axenic culture (heterotrophic) at 28 °C and 160 rpm/min without light exposure.

2.4. Analytical methods

2.4.1. Biomass and residual reducing sugar measurement

The cultured cells were collected by centrifugation at 4000 rpm for 20 min, and the algal pellets were then washed thrice with distilled water. Finally, the algal cells were lyophilized by employing a freeze dryer, and the biomass was determined by the cell dry weight method. The residual RSC in the culture fluid was measured by the 3, 5-dinitrosalicylic acid (DNS) method.

2.4.2. Lipid content measurement

The distilled water and chloroform/methanol (v/v, 2:1) were added to the lyophilized algal powder. The mixtures were fiercely shaken for 20 min with hybrid oscillator and centrifuged (10,000 rpm) for 10 min. The chloroform phase was extracted from the bottom of the centrifugal tube. Finally, all the chloroform phases were collected together, evaporated and dried to constant weight under vacuum conditions. The total lipid was measured by cell dry weight method.

All biological assays were carried out in triplicate, and the mean values and standard deviation were calculated.

2.4.3. Proteomic analysis

The algal cells were collected and washed twice with ice phosphate buffered saline. Lysis buffer (7 M urea, 2% CHAPS, 2 M thiourea, and 20 mM Tris; pH 8.8) containing 1% protease inhibitors and nuclease mixture was added to lyse the algal cells. The lysate was then sonicated on ice with a one minute cool-down period between sonication cycles. After pelleting the insoluble material by centrifugation at 15,000*g* for 30 min, the supernatants