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High yields of fatty acid and neutral lipid production from cassava bagasse hydrolysate (CBH) by heterotrophic *Chlorella protothecoides*

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HIGHLIGHTS

- High yield of reducing sugar was produced by cellulase hydrolysis of cassava bagasse.
- Cassava bagasse hydrolysate (CBH) was a superior carbon source instead of glucose.
- Fatty acid and neutral lipid production from CBH were achieved by *C. protothecoides*.
- The byproducts in concentrated CBH had a limited inhibition effect on fermentation.
- Intercellular fatty acids were suitable to prepare high-quality biodiesel.

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ABSTRACT

The fermentation process for high yields of fatty acid and neutral lipid production from cassava bagasse hydrolysate (CBH) was developed by heterotrophic *Chlorella protothecoides*. An efficient single-step enzymatic hydrolysis of cassava bagasse (CB) by cellulase was firstly developed to produce >30 g/L of reducing sugars. The concentrated CBH was subsequently applied in a batch culture, producing 7.9 g/L of dry biomass with yield of 0.44 g/g reducing sugar and 34.3 wt% of fatty acids and 48.6 wt% of neutral lipids. Furthermore, fed-batch fermentation using CBH achieved higher yields of fatty acids (41.0 wt% and a titer of 5.83 g/L) and neutral lipids (58.4 wt% and yield of 0.22 g/g reducing sugar). Additionally, the fatty acid profile analysis showed that the intercellular lipid was suitable to prepare high-quality biodiesel. This study demonstrated the feasibility of using CBH as low-cost feedstock to produce crude algal oil for sustainable biodiesel production.

1. Introduction

With the increasing concern about the shortage of fossil fuel and the rising oil price, it has become increasingly attractive to develop alternative renewable fuels including bio-ethanol and biodiesel. Bio-ethanol, produced from various renewable feedstocks such as sugarcane, corn and cassava, have already been put into industrial production in the world recently (Baeyens et al., 2015; Kang et al., 2014). In contrast to bio-ethanol, biodiesel is considered as another promising alternative fuel to partly replace fossil-derived fuels with the great potential of reducing the greenhouse gas emissions significantly. Biodiesel is a clean and renewable biofuel. It has a good performance in CI (compression ignition) engines and has environmental benefit as CO_2 neutral. The demands for biodiesel are increasing progressively due to the common worldwide problems of energy shortage and the concerns toward climate change caused by excessive CO_2 emissions. Biodiesel can be synthesized from triglyceride (neutral lipid) or free fatty acids with short chain alcohol by a transesterification process. However such biodiesel feedstock are too viscous for direct use in modern diesel engines (Rawat et al., 2013). The high content of stable, long chain, unbranched fatty acids in the crude oil, ranging from C16 to C20, is important for high-quality biodiesel production. Biodiesel has been commercially produced from various renewable feedstocks, such as vegetable oils (i.e., rapeseed oil, soybean oil, palm oil and waste cooking oil), animal fats, microbial lipid (Chisti, 2007). The production of biodiesel from algal feedstocks was also under research currently. Although the

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modern economy of biodiesel production is very poor, this is valid for all biofuels production. The high costs of raw materials, competition with food, and costs of processing hamper the production of biodiesel.

Microalgae are one of the most efficient producers of crude oil for biodiesel and can produce lipids in large amounts with faster growth-rates than terrestrial crops, which make algal oil be one of the most potential feedstocks of renewable biodiesel, capable of meeting the global demand for transport fuels (Demirbas, 2011). Additionally, starch and cellulose in the microalge biomass could be used as a fermentation feedstock for bio-ethanol production, which makes microalgae the most promising strain for biodiesel and bio-ethanol production (Baeyens et al., 2015). As the most promising producer of algal oil, the green microalgae *Chlorella* has been applied in lipid production by fermentation using various carbon substrates, such as glucose, xylose, and polysaccharides derived from biomass feedstock (Chisti, 2007). Recently, the heterotrophic culture of *Chlorella protothecoides* under nitrogen starvation has been developed to produce lipid for clean type of non-fossil biodiesel (Gao et al., 2010; Miao and Wu, 2004). High oil content has been achieved by *C. protothecoides* using glucose or glycine as substrate in previous studies (Chen and Walker, 2012; Miao and Wu, 2004), but the high cost of carbon source (about 80% of medium cost) has limited the large-scale fermentation of algal oil production. It was reported that sugar cane, corn and wheat were the main feedstocks for bio-ethanol in Brazil and US, while in developing countries, it's necessary to utilize the non-food feedstocks such as cassava to replace the food-related raw materials for biofuel production (Baeyens et al., 2015). Therefore, a cost-effective lipid fermentation process using alternative non-food feedstock, such as cassava starch, cassava bagasse and waste molasses, has become more attractive in biodiesel production by fermentation from *Chlorella* (Wei et al., 2009; Yan et al., 2011).

Cassava (*Manihot esculenta* Cranz), an important starch source for food and sugar refinery industry, is widely planted in southern China, Thailand, Latin America and Africa, while the annual yield of cassava in southern China was ca. 9,110,000 tonnes which are mainly produced for cassava starch (Jansson et al., 2009; Okudoh et al., 2014). Cassava bagasse is the most abundant byproduct of cassava, approximately 90% of wet weight (Gaewchingduang and Pengthemkeerati, 2010). CB contains high residue content (40–64%) of starch, cellulose, hemicellulose and lignin in fiber (15–50%) (Martin et al., 2007; Pandey et al., 2000). The conversion technology of cassava starch to bio-ethanol is well studied, while the conversion of lignocellulose-to-bioethanol or biodiesel at present were still at a plot and demonstration stage due to high treatment cost and the inhibitors in the hydrolysate. Certain amounts of CB are currently utilized for the production of aroma compounds, mushrooms, animal feed, organic acid, biochemicals, and Kraft paper pulp (Martin et al., 2007; Pandey et al., 2000). The biodiesel production using cassava bagasse is feasible to meet the purpose of lowering the substrate costs of the cultivation and reducing the environmental pollution caused by bagasse disposal. Although a high portion of CB has been used as an alternative substrate to produce bio-ethanol and biobutanol (Lu et al., 2012; Nuwamanya et al., 2012; Pandey et al., 2000), the production of fatty acid and neutral lipid for biodiesel from cassava bagasse is still under research which certainly needs additional research and then can be considered for industrial application.

The objective of this study was to develop an integrated lipid fermentation process from low-cost cassava bagasse hydrolysate (CBH). The CB hydrolysis was investigated to produce high titer of reducing sugars. Both batch and fed-batch fermentations by *C. protothecoides* were studied to assess the feasibility of fatty acid and neutral lipid production using CBH as carbon source. The effect of CBH on cell growth, yields of fatty acid and neutral lipid, and fatty acid composition was studied and discussed.

2. Methods

2.1. Hydrolysis of cassava bagasse

The fresh cassava bagasse was obtained from Dongguan Dongmei Starch Company (Guangdong, China), sun-dried to less than 5% moisture, and stored at room temperature until use. The dried CB was milled to superfine powder using a superfine grinder (WZJ6-BFM6 Model, Jinan Billionpowder Tech. & Eng., Shandong, China) for 50 min. The particle size was about 50–100 μm with high specific surface area, conducible for acid and enzymatic hydrolysis. The hydrolysis process of superfine powder was developed by comparing multiple operations, including acid treatment, gelatinization, amylase hydrolysis and cellulase hydrolysis. Single-step and multiple-step hydrolysis procedures were investigated to obtain high yields of reducing sugars with low cost.

2.1.1. Acid hydrolysis

The acid hydrolysis of CB powder was carried out in a reagent bottle with a cap. Each bottle, containing 10 g of CB powders (10%, w/v) in 90 mL of 0.1 N HCl, was autoclaved at 121 °C for 15 min. The undigested CB was removed by centrifugation at 7000 r/m for 10 min, and the supernatant was filtered to collect hydrolysate containing fermentable sugars.

2.1.2. Enzymatic hydrolysis using amylase

The 10% (w/v) CB powder was firstly gelatinized by mixing in Na_2HPO_4–citrate acid buffer (pH 5.6) at 60 °C for 30 min. Next, $CaCl_2$ (0.01 M as final concentration) and α-amylase (24 mg/g CB, Beijing Aoboxing Biotechnology Co., Ltd.) were added into the mixture for liquefaction at 55 °C and pH 5.6 for 30 min, followed by 5-min boiling to deactivate α-amylase. Finally, 65.5 mg/g of glucoamylase (Beijing Aoboxing Biotechnology Co., Ltd.) was added for saccharification at 75 °C and pH 4.0 for 4 h, and boiled for 5 min to deactivate gulcoamylase. These hydrolysis reactions were performed in a carousel 12 place reaction station (12PRS, Radleys Discovery Technologies, Essex, UK) following the procedure developed by Wei et al. (2009). The residue of CB was removed by centrifugation, and the supernatant was filtered as cassava bagasse hydrolysate (CBH).

2.1.3. Enzymatic hydrolysis using cellulase

The commercial cellulase Accellerase 1500 (A1500, CMCase: 2274 U/g, β-glucosidase: 553 pNPG U/g, xylanase: 982 U/g protein, pH 4.8) was a gift from Genencor Bio-Products Co. (NY, USA). The dosage of A1500 at 0.2 mL/g CB or 0.05 mL/g CB was used for hydrolysis at 50 °C and pH 4.8 for 48 h in the carousel 12PRS. Direct hydrolysis by cellulase, multi-step enzymatic hydrolysis with acid pretreatment or gelatinization and amylase were carried out and compared. Centrifugation and filtration were applied to collect clear CBH liquid.

2.2. Scale-up of cassava bagasse hydrolysis

To evaluate the scalability of a direct single-step hydrolysis of CB using cellulase, low dosage of A1500 (0.05 mL/g CB) was added to CB powder suspension without pretreatment at 50 °C and pH 4.8 for 36 h. The 5-L suspension (10%, w/v) in 7-L bioreactor (BTF-A5L, Biotop, Taiwan, China) and 50-L suspension (10%, w/v) in 100-L stirred tank bioreactor (PG-100, Meili Light Chemical Machinary Factory, Shaanxi, China) were performed with agitation at 140 r/m and 150 r/m, respectively. To study the kinetics of