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Co-fermentation of acetate and sugars facilitating microbial lipid production on acetate-rich biomass hydrolysates

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HIGHLIGHTS

- *Cryptococcus curvatus* co-utilizes acetate and sugars for lipid production.
- Acetate and sugar mixtures can give improved lipid production.
- Lipid content reached 60.8% on acetate-rich corn stover hydrolysates.
- Lipid samples had similar fatty acid compositional profiles to those of cocoa butter.

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ABSTRACT

The process of lignocellulosic biomass routinely produces a stream that contains sugars plus various amounts of acetic acid. As acetate is known to inhibit the culture of microorganisms including oleaginous yeasts, little attention has been paid to explore lipid production on mixtures of acetate and sugars. Here we demonstrated that the yeast *Cryptococcus curvatus* can effectively co-ferment acetate and sugars for lipid production. When mixtures of acetate and glucose were applied, *C. curvatus* consumed both substrates simultaneously. Similar phenomena were also observed for acetate and xylose mixtures, as well as acetate-rich corn stover hydrolysates. More interestingly, the replacement of sugar with equal amount of acetate as carbon source afforded higher lipid titre and lipid content. The lipid products had fatty acid compositional profiles similar to those of cocoa butter, suggesting their potential for high value-added fats and biodiesel production. This co-fermentation strategy should facilitate lipid production technology from lignocelluloses.

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1. Introduction

Microbial lipids from oleaginous yeasts have long been recognized as alternative resources for biodiesel production and substitutes for high value-added exotic fats such as cocoa butter equivalents (Papanikolaou and Aggelis, 2011a). However, the costs of microbial lipids remain prohibitively high for commercialization. To reduce the cost, considerable efforts have been devoted to exploring low cost substrates (Huang et al., 2013). Lignocellulosic biomass is one of the most abundant and sustainable resource in the biosphere. Cellulose, hemicelluloses and lignin are the three major components of lignocellulosic biomass. These components are tightly bound together by hydrogen bond and covalent bonds, causing biomass recalcitrance (Himmel et al., 2007). Monomeric sugars from lignocellulosic biomass include glucose, xylose, arabinose, galactose, and mannose, among others. Glucose and xylose, the two major ones, have been widely used as substrates for lipid production by oleaginous yeasts (Gong et al., 2013; Hu et al., 2011; Tsigie et al., 2011). Moreover, biomass hydrolysates have also been suggested as feedstock to produce lipids for sustainable biodiesel and oleochemical industries (Jin et al., 2015). Because hemicelluloses and lignin are acetylated to some extent, acetate up to 15 g/L was found in biomass hydrolysates (Wei et al., 2013). In addition, acetic acid-assisted pretreatment technologies have been designed to disrupt the rigid structure and enhance the accessibility of cellulose to hydrolytic enzymes, leading to acetate-rich hydrolysates ((Bondesson et al., 2014; Kim et al., 2015; Xu et al., 2010). While an artificial lignocellulosic hydrolysates containing

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low concentration of acetate (4 g/L) has been demonstrated for lipid production by the oleaginous fungus *Mortierella isabellina* (Ruan et al., 2015), hydrolysates with higher acetate concentrations have not been tested. Therefore, co-fermentation of acetate and sugars is critical to efficiently use biomass as raw materials for microbial lipid production.

Acetate has been known as a substrate for lipid production by several oleaginous species including *Cryptococcus curvatus* (Beligon et al., 2015; Chi et al., 2011; Christophe et al., 2012; Fei et al., 2011; Fontanille et al., 2012). A pH-stat culture of *C. curvatus* was established by feeding acetate as the sole carbon source, and cell mass and lipid content reached 168 g/L and 75.0%, respectively (Chi et al., 2011). However, when acetate co-existed with sugars, inhibitory effects were observed on oleaginous yeasts (Chen et al., 2009; Hu et al., 2009). The toxicity of acetate may be attributed to accumulation of intracellular anion since at acidic pH undissociated acetate can diffuse across the plasma membrane (Palmqvist and Hahn-Hagerdal, 2000).

In another aspect, lignocelluloses, such as agricultural residues and weeds, naturally contain high levels of nitrogenous components (Huang et al., 2011). The high nitrogen content sets a low carbon-to-nitrogen molar (C/N) ratio, which disfavors lipid accumulation because of inaccessible to a nitrogen-starvation environment (Ratledge and Wynn, 2002). To achieve high lipid content and lipid yield on sugars by oleaginous yeasts, the C/N ratio was routinely higher than 70 (Li et al., 2007). Interestingly, our recent work showed that lipid production under nitrogen-rich condition by *C. curvatus* was effective when acetate was used as the sole carbon source (Gong et al., 2015). In this study, acetate and sugars co-fermentation strategy was investigated to explore low value materials as substrates for lipid production by *C. curvatus*. It was found that acetate and sugars could be consumed simultaneously, and that lipid production was improved in the presence of acetate. This strategy provides a practical solution to use acetate-rich raw materials, which should facilitate designing more efficient microbial lipid bioprocess from lignocellulosic biomass.

2. Methods

2.1. Reagents, strain and media

Cellulase was purchased from Imperial Jade Bio-Technology Co., Ltd. (Yinchuan, China) and had a filter paper activity of 49.0 FPU/mL. β-Glucosidase and xylanase were purchased from Sigma and Imperial Jade Bio-Technology Co., Ltd., respectively, and used as described (Gong et al., 2014). Yeast extract (containing 3% [wt/wt] ammonium-N and 9.0% [wt/wt] total nitrogen) and peptone (animal-tissue based containing 3% [wt/wt] ammonium-N and 14.5% [wt/wt] total nitrogen) were obtained from Aoboxing Biotech. Co. Ltd. (Beijing, China). Other reagents used were analytical grade and purchased from local company.

C. curvatus ATCC 20509 was purchased from the American Type Culture Collection (ATCC). Cells were propagated on yeast peptone dextrose (YPD) agar slants (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L, agar 15 g/L, pH 6.0). Yeast inocula were prepared from YPD liquid medium (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L, pH 6.0).

Sugars fermentation media contained (g/L): $(NH_4)_2SO_4$ 2.0, yeast extract 1.0, KH_2PO_4 2.7, $Na_2HPO_4{\cdot}12H_2O$ 2.4, $MgSO_4{\cdot}7H_2O$ 0.5, EDTA 0.1, trace element solution 1% (v/v), and single or mixed sugars at various concentrations as the carbon source. Initial pH was adjusted to 5.5.

Sugar/acetate mixture media contained (g/L): $(NH_4)_2SO_4$ 2.0, yeast extract 1.0, KH_2PO_4 2.7, $Na_2HPO_4{\cdot}12H_2O$ 2.4, $MgSO_4{\cdot}7H_2O$ 0.5, EDTA 0.1, trace element solution 1% (v/v), and sugars/acetate mixtures at various concentrations and mass ratios as the carbon source. Initial pH was adjusted to 7.0.

The composition of the trace element solution contained (g/L): $CaCl_2{\cdot}2H_2O$ 4.0, $FeSO_4{\cdot}7H_2O$ 0.55, citric acid·H_2O 0.52, $ZnSO_4{\cdot}7H_2O$ 0.10, $MnSO_4{\cdot}H_2O$ 0.076 and 100 μL of 18 M H_2SO_4 (Meesters et al., 1996). All media were sterilized by autoclaving at 121 °C for 20 min before use.

2.2. Pretreatment of corn stover with mixed acids

Corn stover harvested from countryside of Henan Province, China, was milled and passed through 1 mm size screen. The milled materials were washed to remove the field dirt, dried at 105 °C to a constant weight. It was pretreated with mixed acids of 1% (w/v) sulfuric acid and 2% (w/v) acetic acid at 120 °C for 60 min with a solid–liquid ratio of 1:10 (w/v).

2.3. Lipid production on mixtures of acetate and sugar

All pre-cultures were made in YPD medium at 30 °C, 200 rpm for 24 h unless otherwise specified. Lipid fermentations were initiated upon 45 mL of the culture medium inoculated with 5 mL of pre-cultures in 250-mL unbaffled conical flasks. The cultures were held at 30 °C, 200 rpm. The pH was periodically adjusted to the initial values using 4 M NaOH or 4 M HCl in 12 h time intervals. All experiments were done in triplicates and data were presented as mean value ± standard deviation.

2.4. Lipid production on corn stover hydrolysates

The pretreated sample (Section 2.2) was adjusted to pH 4.8 and loaded at 8% (w/v) solid loading and hydrolyzed at 50 °C for 48 h in the presence of 15 FPU cellulase, 30 CBU β-glucosidase and 5 mg xylanase per gram corn stover. The hydrolysates were boiled for 10 min and filtered to remove residual solids and precipitated proteins. The pH of the hydrolysates was adjusted to 7.0 before sterilization.

Cultures were initiated upon 45 mL of the hydrolysates inoculated with 5 mL of pre-cultures in 250-mL unbaffled conical flasks. The cultures were held at 30 °C, 200 rpm for 96 h. Cultivation pH was adjusted to 7.0 in 12 h time intervals. Experiments were done in triplicates.

2.5. Analytical method

Glucose was determined using an SBA-40E glucose analyzer (Shandong Academy of Sciences, Jinan, China). Xylose, when used as the sole sugar derived carbon source, was quantified according to the dinitrosalicylate (DNS) method (Miller, 1959). Because of its complexity, xylose was measured by K-XYLOSE assay kit from Megazyme when it co-existed with other sugars. Acetate was measured by K-ACETAF assay kit from Megazyme (Gong et al., 2015). Nitrogen was determined according to the Kjeldahl method (Morgan et al., 1957).

Cell mass was harvested by centrifugation and washed twice with distilled water and determined gravimetrically after drying the wet cells at 105 °C for 24 h. Non-lipid cell mass was calculated after subtraction of lipids extracted from cell mass.

Lipid extraction was performed according to a published procedure (Gong et al., 2012). Lipid content was expressed as gram lipid per 100 g lipid-containing dry cell weight. Lipid yield was calculated as gram lipid per gram carbon sources (containing glucose, xylose and acetate) consumed.

The fatty acid compositional profiles of lipid samples were determined by gas chromatography (GC) after transmethylation according to a published procedure with minor modifications

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