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Effects of selected ionic liquids on lipid production by the oleaginous yeast *Rhodosporidium toruloides*

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HIGHLIGHTS

- Oleaginous yeast *R. toruloides* tolerated ionic liquids used for biomass pretreatment.
- Assimilation of the anion of [Emim][OAc] led to a rapid alkaline-pH shift.
- Ionic liquids tended to increase saturated fatty acids contents of lipid samples.

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ABSTRACT

Lignocellulosic biomass pretreatment with ionic liquids (ILs) has been emerged as a new technology, but the effects of residual ILs on the downstream biotransformation remain largely unknown. Here, three typical ILs were tested for their effects on lipid production by the oleaginous yeast *Rhodosporidium toruloides* AS 2.1389. When cultures were maintained at pH 6.0 in the presence of 30 mM ILs, [Emim]Cl, [Emim][DEP], or [Emim][OAc], minor inhibition effects were observed. When cultures were performed in the presence of 60 mM ILs or without pH control, inhibition was largely dependent on ILs. Detailed analysis indicated that the anion of [Emim][OAc] was assimilated, leading to a rapid alkaline-pH shift and enhanced inhibition on cell growth and lipid production. Our results demonstrated that *R. toruloides* is a robust lipid producer tolerating ILs at low concentrations, and that care should be taken in bioprocess control and data analysis when ILs are involved.

1. Introduction

Increasing energy demand, together with rapid depletion of fossil fuels, necessitated searching for renewable fuels (Padmanabhan et al., 2011). Lignocellulosic biomass has received much attention as a potential feedstock for biofuel production (Lynd et al., 2008). However, lignocellulose is a highly ordered and rigid polymer, and the crystalline structure limits biodegradation by cellulolytic enzymes (Nakashima et al., 2011). To ensure the enzymatic hydrolysis process practical, lignocellulose is typically pretreated by using various procedures (Ouellet et al., 2011). The most widely used method is dilute acid pretreatment, which can depolymerize hemicellulose, remove some lignin, and make the cellulose more amenable to enzymatic hydrolysis (Wyman et al., 2005). However, this method is known to rust equipment and produce inhibitors detrimental to downstream microbial transformations (Palmqvist and Hahn-Hagerdal, 2000a; Palmqvist and Hahn-Hagerdal, 2000b; Pienkos and Zhang, 2009). Hence, alternative pretreatment methods have been emerging. Recently, pretreatment with ionic liquids (ILs) has been received intensive study (Klein-Marcuschamer et al., 2011), and more biocompatible ILs have been developed. Regardless of detailed procedures (Li et al., 2010; Ninomiya et al., 2012; Wang et al., 2011), however, ILs are carried over and present at various concentrations in the stream used for fermentation (Zhao, et al., 2009). This may cause unacceptable effects. For example, the presence of [Emim][OAc] at 33–52 mM in the corn stover and switchgrass hydrolysates showed significant inhibition on cell growth and ethanol production by *Saccharomyces cerevisiae* (Ouellet et al., 2011). Hexyl- and octyl-imidazolium and pyridinium bromides at concentrations as low as 1000 ppm had significant antimicrobial activity to *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *S. cerevisiae*

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(Docherty and Kulpa, 2005). Yet, *S. cerevisiae* MT8-1/cocdBEC1 could achieve *in situ* saccharification and fermentation in the presence of 200 mM [Emim][DEP], a more biocompatible IL (Nakashima et al., 2011). Therefore, the effects of residual ILs on microbial production of bio-based products warrant extensive assessment.

Some oleaginous species are exceptionally robust in terms of converting carbohydrates into lipids such as triacylglycerols (Papanikolaou and Aggelis, 2011). Microbial lipid has been considered as an alternative feedstock for biodiesel production (Liu and Zhao, 2007; Meng et al., 2009). *Rhodosporidium toruloides* is a non-pathogenic basidiomycetous fungus that can accumulate lipids over 70% of its dry cell weight (DCW) (Ratledge and Wynn, 2002; Li et al., 2007). More importantly, *R. toruloides* had excellent tolerance towards inhibitory compounds found in biomass hydrolysates (Hu et al., 2009; Zhao et al., 2012). In this study, *R. toruloides* was cultivated under various conditions in the presence of three imidazolium ILs, [Emim]Cl, [Emim][OAc] and [Emim][DEP], that have been explored for biomass pretreatment. It was found that *R. toruloides* was resistant to these ILs at up to 30 mM when culture pH was maintained. Our data indicated that the anion of [Emim][OAc] was assimilated, leading to rapid pH shift to over pH 8.0 and strong inhibition to cell growth. Our results provided valuable information for the incorporation of ILs-based biomass pretreatment into biorefinery, particularly in terms of microbial lipid technology.

2. Methods

2.1. Strain, chemicals, medium and culture conditions

Yeast strain *R. toruloides* AS 2.1389 was obtained from the China General Microbiological Culture Collection Centre. Yeast extract (containing 1.4% ammonium-N and 8.7% total nitrogen) and peptone (containing 0.73% ammonium-N and 15.3% total nitrogen) were purchased from Aoboxing Biotech (Beijing, China). YNB was obtained from Jingkehongda Biotech (Beijing China). 1-Ethyl-3-methylimidazolium chloride ([Emim]Cl > 99%), 1-ethyl-3-methylimidazolium acetate ([Emim][OAc] > 98.5%) and 1-ethyl-3-methylimidazolium diethyl phosphate ([Emim][DEP] > 99%) were supplied by Lanzhou Greenchem ILs (Lanzhou, China). All other chemicals were reagent grade.

YEPD liquid medium contained (g/L): glucose 20, yeast extract 10, and peptone 10. Domestication medium (g/L): glucose 1, YNB 1.7, $(NH_4)_2SO_4$ 5. Assimilation medium contained (g/L): YNB 1.7, $(NH_4)_2SO_4$ 5. The lipid production medium contained (g/L): glucose 60, $(NH_4)_2SO_4$ 0.1, yeast extract 0.75, KH_2PO_4 0.4, and $MgSO_4{\cdot}7H_2O$ 1.5. After the medium was sterilized at 121 °C for 18 min, it was supplemented with 1 vol.% trace element solution contained (g/L): $CaCl_2{\cdot}2H_2O$ 4.0, $FeSO_4{\cdot}7H_2O$ 0.55, citric acid$\cdot H_2O$ 0.52, $ZnSO_4{\cdot}7H_2O$ 0.10, $MnSO_4{\cdot}H_2O$ 0.076 and 18 M H_2SO_4 100 uL (Wu et al., 2011). ILs were supplemented to the above medium at appropriate concentrations after the medium was sterilized.

All cultures were incubated at 30 °C with a rotary rate of 200 rpm. Experiments were done in triplicate and data were presented as means ± standard error of mean of triplicate experiments.

2.2. Cell growth in the presence of ILs

ILs were added to final concentrations at 30 or 60 mM to sterilized YEPD liquid medium. The starter culture of *R. toruloides* was diluted to a final optical density at 600 nm (OD_{600}) of 0.1 into 50 mL of the IL-supplemented medium in 250 mL shake flask, and cells were grown at 30 °C, 200 rpm. Samples were collected in three-hour intervals. Cell densities were estimated by measuring OD_{600} values upon appropriate dilution.

To determine whether *R. toruloides* can use ILs as the sole carbon source, cells were pre-cultured in YEPD liquid medium for 24 h, collected by centrifugation at 6200*g*, washed with sterile water for three times, and then incubated in the domestication medium for 24 h to consume the internal carbon sources. About 1 mL of the above culture was transferred into the assimilation medium to a final OD_{600} of 0.04. Small samples were withdrawn periodically to monitor the cell density and the concentrations of $[Emim]^+$ and $[OAc]^-$.

2.3. Lipid production in the presence of ILs

Precultures were made by inoculating for 24 h in 50 mL YEPD medium in 250 mL shake flasks. Exactly 5 mL of the preculture was transferred into 45 mL of the lipid production medium supplemented with appropriate amount of ILs, and the cultures were performed for 100 h. Small samples were withdrawn to monitor culture pH and $[OAc]^-$ concentration. Cells were collected by centrifugation and washed with distilled water for biomass and lipid analysis.

To stabilize the culture pH in the lipid production experiments, the medium was made with 0.1 M phosphate buffer, pH 6.0 or 8.0, in lieu of water. During the culture process, culture pH was monitored and adjusted to the specified value with 4.0 M NaOH or 4.0 M HCl when necessary.

2.4. Analytical methods

The optical density of the culture was determined at 600 nm with an appropriate dilution with a JASCO V-530 UV–visible spectrophotometer (JASCO, Japan). The pH of the culture was monitored with a pH/Ion 510 acidometer (Eutech Instruments, Singapore). Residual glucose was analyzed by using a SBA-50B glucose analyzer (Shandong Academy of Sciences, Jinan, China). Cells in 30 mL culture broth were harvested by centrifugation, washed twice with distilled water, and then dried at 105 °C for 24 h to obtain DCW. Cellular lipid was extracted with chloroform–methanol (Li et al., 2007). Lipid content was calculated as g lipid per g DCW. Lipid coefficient was calculated as g lipid produced per g glucose utilized and then multiplied by 100%. The fatty acid composition of the lipid were determined using a 7890F gas chromatography (GC) instrument (Techcomp Scientific Instrument Co. Ltd., Shanghai, China) according to the published procedure (Li et al., 2007).

Concentrations of $[Emim]^+$ and $[OAc]^-$ were determined by ion chromatography (IC) on the Dionex ICS2500 system (Dionex, USA) at 30 °C with a flow rate at 1 mL per minute. $[Emim]^+$ was separated using an analytical column IonPac CS12A 4 × 250 mm and the guard column IonPac CG12A 4 × 50 mm by isocratic elution with a mobile phase consisting of 13 mM methanesulfonic acid and 15% acetonitrile. $[OAc]^-$ was separated using an analytical column IonPac AS11-HC 4 × 250 mm and the guard column IonPac AS11-GC 4 × 50 mm by isocratic elution. The mobile phases for the analysis of $[OAc]^-$ in the assimilation medium and the lipid production medium were 18 mM NaOH and 2 mM NaOH, respectively.

3. Results and discussion

3.1. R. toruloides growth profile in the presence of ILs

A number of ILs have been tested for biomass pretreatment. Specifically, [Emim]Cl is the most commonly used, and [Emim][OAc] is much more effective for the pretreatment of lignocelluloses (Zavrel et al., 2009). [Emim][DEP] has good biological compatibility and good solubility for biomass (Su et al., 2012). When [Emim][OAc]