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Cultivation of oleaginous yeast using aqueous fractions derived from hydrothermal pretreatments of biomass

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HIGHLIGHTS

- Waste glycerol from hydrolysis of fats and oils was used for growing oleaginous yeast.
- Growth and lipid accumulation occurred at levels identical to commercial pure glycerol.
- Byproduct of hydrothermally processed *C. curvatus* was recycled for yeast growth.
- Recycling promoted higher biomass production without affecting lipid accumulation.

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ABSTRACT

This study addresses some of the current challenges in producing biofuels from yeast oils. Specifically, it valorizes byproduct waste streams of biomass processing technologies by integrating them as alternative carbon or nutrient sources in oleaginous yeast cultivation. Crude glycerol recovered from the thermal hydrolysis of various fats and oils was successfully used in culturing of the oleaginous yeast *Cryptococcus curvatus*, with growth and lipid accumulation occurring at levels identical to those achieved when commercially purchased glycerol was used. The aqueous byproduct stream from the hydrothermal processing of *C. curvatus* can also be recycled as a growth substrate for subsequent *C. curvatus* cultures. The addition of this stream promoted higher biomass production without affecting lipid accumulation and only moderately changing the fatty acid profile. Use of these recycling strategies reduces costs and environmental impact of current microbial biofuels production by providing accessible, non-expensive carbon sources and nutrients for oleaginous yeast cultivation.

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1. Introduction

Lipid-based renewable fuels are currently produced from a limited array of sources, particularly oilseeds. Providing additional low cost feedstocks to the industry is required to meet future global demands for a sustainable supply of biofuels (Balat, 2009). Lipids from oleaginous microorganisms, such as yeasts, are promising feedstocks for renewable fuels (Koutinas and Papanikolaou, 2011). However, it is necessary to develop cost-effective processing technologies to integrate yeast oils into biofuel production. Some of the current challenges to using oleaginous yeasts in fuel production include: (1) the identification of suitable and inexpensive carbon sources for yeast cultivation, (2) the creation of innovative conversion technologies, and (3) the development of methods to valorize byproducts generated during conversion (Koutinas and Papanikolaou, 2011).

An opportunity to address feedstock requirements for the cultivation of oleaginous yeast lies in the utilization of low-value carbon sources that do not have application as food. Some potential carbon sources include lignocellulosic raw materials or industrial waste streams (Koutinas et al., 2014). Fortunately, oleaginous yeasts can grow and produce lipids on a variety of substrates including sugar-containing byproduct streams and other organic-containing waste streams (Koutinas and Papanikolaou, 2011). The use of industrial waste streams as substrates for growing oleaginous yeast is economically attractive as such feedstocks are not likely to be expensive and would create value from waste.

An alternative methodology for the transformation of lipids and oils into biofuels has been recently proposed (Asomaning et al., 2014). This technology, which includes hydrothermal lipid hydrolysis followed by pyrolysis of fatty acids, has been applied not only to model vegetable oils, animal fats, and waste lipids (Asomaning

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et al., 2014), but also to oleaginous biomass feedstocks (Espinosa-Gonzalez et al., 2014). One byproduct of this technology is the glycerol contained in the recovered aqueous byproduct stream. According to projections, 4 billion gallons of glycerol will be produced by 2016, of which roughly 10% will be generated as a byproduct of biodiesel production (Quispe et al., 2013). Such supply levels are likely to surpass global glycerol consumption, which in 2008 was around 750 thousand tons (Quispe et al., 2013). Since there will likely be a significant excess of unutilized glycerol in future markets, it would be prudent to develop other processes to convert glycerol into value-added products. Glycerol can be used as a carbon source by oleaginous yeasts (Meesters et al., 1996); however, some of the additional compounds from biomass hydrolysis may serve as microbial nutrients or inhibitors. Thus, further investigation into the composition of hydrothermal byproduct aqueous streams and the ability of these streams to be recycled as substrates for yeast cultivation is required.

Several recycling methodologies have been previously implemented for industrial yeast processes, specifically for the production of enzymes, oils, and biofuels (Hsiao et al., 1994; Lam and Grootwassink, 1990; Yanagida and Matsumura, 2011). Although recycling was studied in these situations to reduce costs or to improve microbial performance, this strategy can also be applied to address environmental concerns. This includes the recycling of minerals, such as nitrogen and phosphorus, which are finite and represent a challenge for sustainable microbial biofuels production (Lardon et al., 2009).

In the present study, different aqueous waste streams were investigated as potential feedstocks for cultivation and lipid accumulation by the oleaginous yeast *Cryptococcus curvatus*. Model fats and oils as well as the oleaginous microorganism *C. curvatus* were subjected to thermal hydrolysis under optimized conditions for the Lipid-To-Hydrocarbon (LTH) process (Asomaning et al., 2014; Bressler, 2011). These hydrolysis conditions were chosen in hopes that the resulting fatty acids could be used directly in the second stage (pyrolysis) of the LTH process. Recycling of crude glycerol from the thermal hydrolysis of fats and oils as well as the aqueous byproduct stream from the thermal hydrolysis of *C. curvatus* were established as novel nutrient sources for yeast growth. In addition, it may be possible to use the two processing streams simultaneously to provide the required amount of glycerol and other nutrients for *C. curvatus* cultivation without further supplementation.

2. Methods

2.1. Materials

Yeast *C. curvatus* (ATCC 96219) was obtained from the American Type Culture Collection (Manassas, VA). For long term storage, glycerol stocks were kept at −20 °C. Yeast Extract Peptone Dextrose (YEPD) agar was used for short term agar plate stocks, which were transferred to fresh media every 4 weeks. Colonies from these plate stocks were used to inoculate small amounts of YEPD broth that were grown for 24 h at 30 °C to produce starter cultures. Base mineral media used for yeast cultivation (1×) contained ammonium chloride (99%; 0.645 g/L), potassium phosphate monobasic (99%; 7.0 g/L), sodium phosphate dibasic dodecahydrate (99%; 2.0 g/L), magnesium sulfate heptahydrate (99%; 1.5 g/L), calcium chloride dihydrate (99%; 0.1 g/L), iron(III) chloride hexahydrate (98%; 24 mg/L), zinc sulfate heptahydrate (99%; 5 mg/L), manganese(II) sulfate monohydrate (98–101%; 2 mg/L), copper(II) sulfate (99%; 7 mg/L) and thiamine hydrochloride (99%; 40 μg/L) (Hassan et al., 1993). All chemicals in the base mineral media were obtained from Sigma–Aldrich (St. Louis, MO).

Sunflower, soybean, canola, camelina, and peanut oils were purchased from a local retail store. Beef and poultry tallow were obtained directly from rendering industries. All fats and oils were used "as is" with no additional pretreatment. Potassium hydroxide (85–90%), phthaldialdehyde (99%), amino acids in solution (2.5 μm/mL), β-amino-N-butyric acid (97%), yeast extract (11% nitrogen), and hydrochloric acid (37%) were obtained from Sigma–Aldrich (St. Louis, MO). Sodium meta-bisulfite (97%), hexane (HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade), dichloromethane (HPLC grade), and sulfuric acid (2 N), were obtained from Fisher Scientific (Fairlawn, NJ). Nitrogen (99.998%) was obtained from Praxair (Mississauga, ON).

2.2. Methods

2.2.1. Yeast culture in glycerol

C. curvatus was cultured in 250 mL Erlenmeyer flasks with 100 mL of base mineral media (Hassan et al., 1993). Crude glycerol recovered from hydrothermally processed byproduct streams or commercially purchased glycerol was used as the main carbon source at a final concentration of 20 g/L (0.65 C mol/L). To promote lipid accumulation, a low concentration of nitrogen (yeast extract at 0.8 g/L; 6.0 mmol/L) was used in the growth media to establish a carbon to nitrogen ratio of 100:1 (Espinosa-Gonzalez et al., 2014). The pH of the media was initially adjusted to 5.4 with 2 M KOH. Flasks were inoculated with a starter culture (5% v/v) in exponential growth phase (24 h). Cultures were grown at 30 °C with agitation (200 rpm) for a total of 168 h. Small samples were taken at the beginning and end of the experiment to monitor growth. All experiments were done in triplicate and assessed for contamination microscopically.

2.2.2. Hydrothermal processing

2.2.2.1. Hydrolysis of fats and oils. Hydrolysis of fats and oils was conducted in a 5.5 L batch stainless steel reactor (Parr Series 4582, Parr Instrument Co., Moline, IL). The reactors were loaded with 2 kg of fats or oils, and 2 kg of water, which was standardized to a water ratio of 1:1 (by mass) to balance hydrolysis with product recovery and separation. Reactors were purged three times at 500 psi, and then pressurized to 500 psi with nitrogen. Hydrolysis time started when the temperature reached 280 °C. The reaction was stopped after 1 h using an external glycol cooling unit. After cooling, organic and aqueous phases were separated using a separatory funnel. Aqueous fractions were stored at 2 °C until needed.

2.2.2.2. Hydrolysis of yeast biomass. The hydrolysis of lipid-containing yeast biomass was conducted under the same conditions as described in 2.2.2.1. Separation of the aqueous stream from the hydrolysis product was done using a Büchner funnel and a Whatman® glass microfiber filter (GF/A grade; Whatman, Maidstone, Kent). The recovered filtrate was the yeast hydrolysate (Espinosa-Gonzalez et al., 2014).

2.2.3. Liquid–liquid organic extractions

Liquid–liquid extraction of the organic fraction of the yeast hydrolysate was conducted as reported by Pham et al. (2013). Dichloromethane (DCM) was the organic solvent used in sequential extractions of the yeast hydrolysate. The pH of the aqueous fraction was adjusted to 12 with 5 M KOH prior to extraction with DCM, and then to 5 with 6 M HCl followed by another DCM extraction. Organic extracts were pooled, concentrated and re-suspended in distilled water to 10X and 1X (v/v) concentrations, with respect to the volume of the original aqueous fraction.

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