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Hydrothermal treatment of oleaginous yeast for the recovery of free fatty acids for use in advanced biofuel production

Isabel Espinosa-Gonzalez, Archana Parashar, David C. Bressler*

Biorefining Conversions and Fermentation Laboratory, Department of Agricultural, Food and Nutritional Science, 4-10 Agriculture/Forestry Centre, University of Alberta, Edmonton, AB, Canada T6G 2P5

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ABSTRACT

Microbial oils hold great potential as a suitable feedstock for the renewable production of biofuels. Specifically, the use of oleaginous yeasts offers several advantages related to cultivation and quality of lipid products. However, one of the major bottlenecks for large-scale production of yeast oils is found in the lipid extraction process. This work investigated the hydrothermal treatment of oleaginous yeast for hydrolysis and lipid extraction resulting in fatty acids used for biofuel production.

The oleaginous yeast, *Cryptococcus curvatus*, was grown in 5 L bioreactors and the biomass slurry with 53 ± 4% lipid content (dry weight basis) was treated at 280 °C for 1 h with an initial pressure of 500 psi in batch stainless steel reactors. The hydrolysis product was separated and each of the resulting streams was further characterized. The hexane soluble fraction contained fatty acids from the hydrolysis of yeast triacylglycerides, and was low in nitrogen and minerals and could be directly integrated as feedstock into pyrolysis processing to produce biofuels.

The proposed hydrothermal treatment addresses some current technological bottlenecks associated with traditional methodologies such as dewatering, oil extraction and co-product utilization. It also enhances the feasibility of using microbial biomass for production of renewable fuels and chemicals.

1. Introduction

The renewable production of hydrocarbons for biofuels has mainly been explored utilizing edible vegetable oils (Gui et al., 2008), which has raised issues related to land usage, competition for food and uncertainties for climate changes. Although other sources (such as animal or inedible oils) have been explored, the lack of consistency and supply has resulted in an urgent need to find other more reliable and cost-effective alternatives. Microbial oils are potential suitable feedstocks because they are able to overcome several major challenges associated with alternatives (Galafassi et al., 2012; Meng et al., 2009). Of the known microbial sources, oleaginous yeasts are very promising since they accumulate lipids in more than 20% of their cellular dry weight, have short duplication times (<1 h), offer easy scale-up, and alter their fatty acid composition (Ageitos et al., 2011). *Cryptococcus curvatus* is an excellent candidate for biodiesel production due to its high lipid content and suitability for high cell density cultivation in fed-batch, as well as its ability to utilize alternative carbon sources for growth (Meesters et al., 1996; Thiru et al., 2011; Zhang et al., 2011).

Biodiesel production has been generally based on transesterification reactions of triglycerides with methanol and a catalyst to yield fatty acid methyl esters along with glycerol (Fjerbaek et al., 2009; Parawira, 2009). The utilization of alternative feedstocks for biodiesel production—especially oleaginous yeast—would require modifications to the traditional process such as the use of novel catalysis to avoid undesired and prejudicial saponification reactions (Azocar et al., 2010). Another major challenge will be the lipid extraction process due to the complex treatments demanded by yeast. Lipids in yeast are intracellular requiring breakage of the thick yeast cell wall; this process is commonly achieved by using acid, base, enzymes, or by applying physical and mechanical pressure such as glass shear, osmotic shock, pressing or sonication (Jacob, 1992). These downstream processes for lipid recovery are major bottlenecks especially for large-scale production (Cooney et al., 2009). Furthermore, some lipid extraction methods demand the use of dry cells—requiring extensive dewatering—which is cost-intensive for large-scale production of microbial lipids (Cescut et al., 2011).

* Corresponding author. Tel.: +1 780 492 498; fax: +1 780 492 4265.
E-mail addresses: david.bressler@ualberta.ca, dbressle@ualberta.ca (D.C. Bressler).

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In recent years, several yeast lipid extraction technologies have been proposed; these involve the use of solvents in near-critical or supercritical conditions or the use of pressurized liquids of different solvent mixtures (Catchpole et al., 2010; Cescut et al., 2011; Hegel et al., 2011; Milanesio et al., 2013). Another technique (direct extraction from fermentation broths) has been also studied by using homogenizers (high pressure), microwaves (followed by enzymes), and solvents (Jin et al., 2012; Thiru et al., 2011). However, only some of these approaches have been scaled-up, and their feasibility and cost-effectiveness are justified only when value-added specialty lipids or metabolites are produced.

The goal of this work was to investigate the potential application of the pre-processing step of a patented thermal lipid-to-hydrocarbon (LTH) technology for conversion of oil into fuels and chemicals (Bressler, 2011). This pre-processing involves the hydrolysis of oils and fats. Hydrothermal treatment of oleaginous yeast biomass was evaluated as an alternative bioprocessing strategy for hydrolysis and lipid extraction resulting in fatty acids used for biofuel production. Further, each byproduct stream was characterized to assess its suitability for biofuel production and other potential uses.

2. Materials and methods

2.1. Materials

Yeast *C. curvatus* (ATCC 96219) was directly obtained from the American Type Culture Collection Centre (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 (transferred to fresh media every 4 weeks). Starter cultures in YEPD broth (inoculated with a colony from short term stocks) were grown for 24 h. All chemicals used for media preparation were purchased from Sigma–Aldrich (St. Louis, MO). Sulfuric acid and hexane (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ) and nitrogen (99.998%) was obtained from Praxair (Mississauga, ON). All chemicals were used as received.

2.2. Yeast culture in batch mode

C. curvatus was cultured in 250 mL Erlenmeyer flasks with 100 mL base mineral media (Hassan et al., 1993). Glucose was used as the main carbon source added to a final concentration of 30 g/L (1 C mol/L). Yeast extract was used as the main nitrogen (N) source added to a final concentration of 1.2 g/L (9.2 E^{-3} mol/L) for establishing a final carbon to nitrogen C to N ratio of 100:1. Flasks were inoculated with 5% (v/v) starter culture grown to exponential phase (24 h). Cultures were grown at 30 °C, 200 rpm with intermediate sampling intervals for a total of 72 h. pH of the cultures was initially adjusted to 5.4 with 2 M KOH. Each experimental unit was monitored for contamination by microscopic examination. All experiments were done in triplicate.

2.3. Yeast culture in fed-batch mode

2.3.1. Small scale

C. curvatus was grown in fed-batch mode in 500 mL flasks with 200 mL mineral media (as described above for batch cultures). Flasks were inoculated with 5% (v/v) starter culture grown to exponential phase (24 h). 8.5 mL of a concentrated glucose solution (200 g/L glucose) and 0.50 mL of a concentrated yeast extract solution (140 g/L) were added to the fermentation as needed to maintain glucose concentration around 10 g/L. The experiment was conducted in triplicate.

2.3.2. Large scale

C. curvatus was grown in fed-batch mode in 5 L Infors-HT bioreactors (Bottmingen, Switzerland). Media used for growth was the same as described above for batch cultures. Flasks were inoculated with 5% (v/v) starter culture in exponential growth phase (24 h). Concentrated glucose and yeast extract solution (750 g/L glucose, 30 g/L yeast extract) was added to the fermentation as needed to maintain glucose concentration below 60 g/L. Aeration rate and stirrer speed were varied between 1–2 vvm and 100–200 rpm, respectively, to keep air saturation over 30% (Zhang et al., 2011). When necessary, pure O_2 was mixed with air for the same purpose. During kinetics, glucose concentration was monitored by the glucose oxidase peroxidase (GOPOD) enzymatic procedure (Megazyme, kit), growth was measured by OD_{600nm} using a standard curve for dry biomass, and lipid accumulation was determined gravimetrically following the extraction method described by De la Hoz (2012). At harvest, yeast biomass was concentrated by centrifugation (5000 × *g* for 10 min) in an Avanti, Beckman Coulter centrifuge (Brea, CA) and the slurry was stored in a fridge (2 °C) until further use. Fermentations were done in triplicate. Yeast slurries were pooled and homogenized before hydrolysis reactions. A sample of final slurry was stored in a freezer (−20° C) before freeze drying (for lipid and amino acid analysis).

2.4. Hydrolysis

Hydrolysis reactions were conducted in either small or large scale reactors (Maher et al., 2008). For small scale, 15 mL stainless steel batch reactors heated in a Techne model SBS-4 fluidized sand bath with a Techne TC-8D temperature controller (Burlington, NJ) were used and a total of five reactions were done to evaluate product distribution. For large scale, a 5.5 L batch stainless steel reactor (Parr Series 4582, Parr Instrument Co., Moline, IL) was used. In both cases, hydrolysis reactions were conducted at 280 °C for 1 h with an initial pressure of 500 psi. The reactors were loaded with 10 g or 2.4 kg yeast slurry, (for 15 mL and 5.5 L reactor, respectively) purged three times at 500 psi and pressurized to the desired initial pressure with nitrogen. Hydrolysis time was considered when the set temperature was reached, and the reaction was stopped by quenching in a water bath for the small reactor and by using an external cooling unit for the 5.5 L reactor.

2.5. Post-hydrolysis treatment

2.5.1. Large scale reactor

Liquid and solid product samples were collected using plastic containers. A Büchner funnel with a glass fibre Whatman GF/A filter (Whatman, Maidstone, Kent) was used for initial separation of the aqueous fraction and paste. Paste was then successively washed with de-ionized water and hexane, and the filtrates were collected in separate containers. Hexane solubles were recovered by evaporating hexane in a rotovapor (Büchi, Flawil, Switzerland), and were further dried in a convection oven for 2 h at 105 °C to determine their mass gravimetrically. The insoluble paste retained on the filter was dried in a convection oven at 105 °C to a constant weight and this fraction was considered as insoluble solids.

2.5.2. Small scale reactor

The mass of gas was determined by measuring the weight of the reactor before and after venting (Maher et al., 2008). The reaction product was collected in pre-weighed 50 mL plastic conical centrifuge tubes (Fisher Scientific, Fairlawn, NJ), subsequently frozen at −80 °C and freeze dried for 48 h. The weight of the freeze dried material was used to calculate the mass of the aqueous and soluble products by difference.