Biochemical Engineering Journal 93 (2015) 229–234

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular Article

Sweet sorghum syrup as a renewable material for microbial lipid production

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ARTICLE INFO

Article history:
Received 8 March 2014
Received in revised form 18 June 2014
Accepted 29 September 2014
Available online 14 October 2014

Keywords:
Sorghum syrup
Cryptococcus curvatus
Microbial lipids
Fed-batch
Microwave

ABSTRACT

Cryptococcus curvatus, an oleaginous yeast strain was observed to grow on sweet sorghum syrup derived from sorghum juice. In a batch cultivation mode, within the tested range of total sugar concentrations from 11.7 to 34.0 g/L, no substrate inhibition to cell growth was detected. *C. curvatus* utilized glucose, fructose, and sucrose sequentially. In a fed-batch process, however, this yeast consumed the three sugars more rapidly and simultaneously. Within three days, a biomass density of 23.6 g/L was obtained. The overall lipid productivity was 4.0 g/L-day. Through use of microwave-assisted lipid extraction, 78.1% of available lipid was extracted out of *C. curvatus* cells in 4 min using methanol as the only solvent. This study demonstrated that fermentation of *C. curvatus* using sorghum syrup will result in high lipid yield.
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1. Introduction

Sweet sorghum, a C4 crop, has several features that make it a desirable plant for serving as a biomass feedstock. First, it has a high carbon assimilation capacity of 50 g/m^2-day [1]. Second, it can reach maturity in 3–5 months [1]. Third, it has greater water use efficiency than corn, sugar beets, or fodder beets. Water usage for sweet sorghum is 8000 m^3/ha due to extensive root systems and short growth period. For sugarcane which requires 12–13 months to mature, a volume of 36,000 m^3 water per ha is needed [1]. Fourth, sweet sorghum requires only 36% of the nitrogen fertilizer needed for corn [2]. Fifth, in spite of a native to tropics, sweet sorghum has well adapted to temperate climate [3]. Sixth, stalk of sweet sorghum contains high levels of extractable sugars ranging from 11% to 24% depending on sorghum variety, location, and other growth conditions [4].

Juice extracted from stalks is composed of glucose, fructose, and sucrose [5]. Traditionally, this juice has been used for making white sugar [3]. Nowadays, due to energy crisis, sweet sorghum juice has been examined for producing various biofuels through microbial fermentation, such as: ethanol [5–10], hydrogen [11], and microbial lipids [4,12,13].

Production of microbial lipids or single cell oils represent an interesting area for research and development as these lipids resembling vegetable oils can be converted to biodiesel or other liquid transportation biofuels [14]. However, the three oleaginous microbes that have been tested so far, *Chlorella protothecoides* [12], *Schizochytrium limacinum* SR21 [4] and *Mortierella isabellina* [13] do not utilize sucrose, the main sugar in sorghum juice.

In addition to this issue of sucrose utilization, there is another concern for using sorghum juice for producing biofuels at industrial scales. Unlike sorghum stalks after juice expression that can be stored in the field for 4–5 months as silage without quality comprise in northern atmosphere [3], sorghum juice has a poor storability and requires immediate processing [3,5,15]. Generally, freshly squeezed juice spoils within 5–12 h at ambient temperatures due to the presence of various microorganisms in the juice (10^8 CFU/ml), such as *Leuconostoc mesenteroides*, yeasts, and nonfecal coliform bacteria. Therefore, if sweet sorghum is to be used as a raw material for fuels or chemicals, this deterioration must be prevented. More importantly, if sweet sorghum is to reach its potential as a biomass crop, the storage time after harvesting must be at least 6 months. Any short processing time will increase capital costs as oversized equipment will be needed [16].

One way for long-term storage is to store the syrup obtained after evaporating the juice. By doing so, the concentrated syrup will be stable and can be easily transported and used as a year-around supply [17]. In addition, this juice-to-syrup pathway has been practiced by sorghum growers for years with equipment and experience available [18].

To solve the problem of sucrose utilization as identified above, we aim to investigate an oleaginous yeast strain *Cryptococcus*

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http://dx.doi.org/10.1016/j.bej.2014.09.013
1369-703X/Published by Elsevier B.V.

curvatus. Research in our lab and others has shown that this yeast can accumulate lipids when grown on glycerol, cheese whey and hydrolysates of sorghum bagasse [19–22]. Compared with other oleaginous yeasts, this strain produced the highest lipid productivity of 2.48 g/L-day on biomass hydrolysate only [23]. Thus, in this study, we aimed to evaluate growth and lipid productivity of this yeast strain on sorghum syrup at batch and fed-batch fermentation modes.

Besides evaluating lipid productivity, we also sought to develop a fast and effective method for extracting microbial lipids. Unlike pressing oil out of seeds, extracting lipids out of microbial cells involves two steps: cell wall disruption and lipid extraction. To disintegrate microbial cells, various approaches, such as: ultrasonic, bead beating, French press, and microwave irradiation have been investigated. Among these methods, microwave irradiation has been reported to be the most efficient since heat and pressure generated rapidly within the biological system can disrupt the cell wall structures and force compounds out of biological matrix, producing good quality extracts in a very short time [24]. Following cell disruption, most studies employed the conventional two-solvent (chloroform and methanol) system to extract lipids. Some protocols avoid the use of organic solvents by adopting supercritical fluid. But from the perspective of producing microbial lipids for cheap biofuel production, lipid extraction conducted under supercritical conditions is just too expensive to be practical. Thus, for this study, we evaluated the efficiency of lipid extraction from *C. curvatus* cells through microwave-assisted extraction (MAE).

2. Materials and methods

2.1. Sweet sorghum syrup

Fresh sweet sorghum crops (KN Morris) grown at Heil's Farm at Cobden, Illinois, USA, were harvested in early October of 2011. After the heads were cut and leaves removed, the stalks were stored in shade for seven days before they were squeezed by a mill to obtain fresh juice which was then cooked to attain syrup. Bottled syrup used in this study has been stored at room temperature. Before use, the dense syrup was diluted by distilled and deionized water (DDW) 25 times (weight basis) and quantified by HPLC. Syrup used in fermentation studies was autoclaved at 121 °C for 30 min. To understand effect of sterilization on syrup sugar composition, diluted syrup with a dilution factor of 5 was autoclaved for five times consecutively. After each autoclave, samples were withdrawn and further diluted by DDW for HPLC analysis.

2.2. Microorganism and inoculum preparation

Inoculum of *C. curvatus* (ATCC 20509) was grown in a liquid medium containing 2% peptone, 1% yeast extract, and 2% of glucose at room temperature on a rotary shaker set at 150 rpm. A minimal medium that was used in this study contained (per liter): 2.7 g KH_2PO_4; 0.95 g Na_2HPO_4; 0.2 g $MgSO_4{\cdot}7H_2O$; 0.1 g yeast extract; and 0.1 g EDTA. After the pH was adjusted to 5.5 by adding concentrated HCl, the medium was supplemented with a 100× spores stock solution consisting of (per liter): 4 g $CaCl_2{\cdot}2H_2O$; 0.55 g $FeSO_4{\cdot}7H_2O$; 0.52 g citric acid; 0.10 g $ZnSO_4{\cdot}7H_2O$; 0.076 g $MnSO_4{\cdot}H_2O$; and 100 μL 18 M H_2SO_4 [25]. The medium was autoclaved at 121° C for 30 min before use. All chemicals for making medium and those used in the following procedures were purchased from either Fisher Scientific (Pittsburgh, PA, USA) or Sigma Aldrich (St. Louis, MO, USA) with the highest grade possible.

2.3. Batch stage fermentation with sweet sorghum syrup

The sticky sorghum syrup was diluted by DDW and quantified by HPLC as described below. Diluted syrup that was autoclaved was then added to different flasks at different volumes to achieve different sugar concentrations. Total sugar concentrations ranging from 12 to 34 g/L were tested. Size of inoculum was always 10% of the final total volume. All cultures were grown in 250-mL Erlenmeyer flasks with a final volume of 100 mL on a rotary shaker set at 150 rpm at room temperature. At different time points, 10 mL samples were withdrawn under sterile conditions and analyzed as illustrated in the following. The same experiment was conducted twice to evaluate the reproducibility of the obtained results.

2.4. Fed-batch fermentation with sweet sorghum syrup

Fed-batch experiments were conducted adopting a procedure that was used previously [19]. Briefly, after minimal medium was filled in and pH and DO probes were connected and calibrated, the 2-L fermentor with a bladed stirrer (New Brunswick, Edison, NJ, USA) was autoclaved at 121 °C for 30 min. Diluted and sterile sorghum syrup was then supplemented together with the yeast inoculum. During fermentation, pH was maintained at 5.5 by automatic pumping of NaOH. Temperature was controlled at 28 °C. Incoming air was filtered through 0.2 μm filter. DO concentration was kept at 50% of saturation by controlling air flow rate. The stirring speed was set at 720 rpm. The whole culture volume was maintained at approximately 1.5 L. To prevent excess foam formation, silicone antifoam emulsion (J.T. Baker, Phillipsburg, NJ, USA) was added. At 48 and 60 h, certain volume of sorghum syrup was added to the fermentor to provide enough carbon source to sustain cell growth. On a daily basis, a 50 mL sample was taken from the fermentor and analyzed for biomass dry weight (DW), sugar concentrations, and other measurements as described below. The culture was terminated after three days.

2.5. Analysis

Samples harvested at different time points were centrifuged first at 5000 × *g* for 5 min. The supernatant was filtered through 0.2 μm nylon filter for HPLC determination of sugar concentrations. The pellets were then washed with DDW twice and freeze-dried overnight to obtain biomass DW. The dried biomass was also used for lipid content analysis.

Sugar concentrations were determined by HPLC (Shimadzu Scientific Instrument, Inc., Columbia, MD, USA) with a refractive index detector. A Supelcosil LC-NH_2 column (5 μm, 25 cm × 4.6 mm) was used with 75% acetonitrile in DDW as the mobile phase. The flow rate was 1 mL/min. The injection volume was 20 μL. Concentrations of glucose, fructose, and sucrose were calculated based on calibration curves built for these three sugars using standard chemicals.

Cellular lipid content was determined following a procedure developed in our laboratory [26]. In short, 0.5 g dried cell pellet was transferred to a 7-mL chamber of a bead-beater (Bio-Spec Products, Bartlesville, OK, USA). This chamber was filled with 0.5 mm zirconium beads to approximately 5 mL. Methanol was then added to fill the rest of the chamber. After cells were disrupted by bead-beating for 2 min, the entire content was transferred to a 50-mL plastic centrifuge tube. The chamber was washed twice using methanol (total 10 mL) to collect the yeast residue. Chloroform was then added to the tube to make the chloroform/methanol ratio of 2:1 (v/v). The tube was vortexed for 5 min and was allowed to stand for 24 h. After that, the liquid layer was transferred to another tube and centrifuged at 5000 × *g* for 5 min to remove the zirconium beads and yeast residuals. The collected supernatant was then transferred