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Two consecutive step process for ethanol and microbial oil production from sweet sorghum juice

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ABSTRACT

The juice extracted from sweet sorghum stalks has been previously explored to produce ethanol and also to grow oleaginous yeasts and algae with the objective of producing microbial oil. In this paper we propose a different process route in order to produce ethanol and microbial oil in two consecutive fermentation steps. Ethanol is produced first followed by the growth of oleaginous yeast employing the residual carbon and nitrogen left from the first step. Two yeasts were compared for ethanol production. *Trichosporon oleaginosus* was cultivated for lipid production. The yeast selection for the first step was the most important factor for achieving a high ethanol yield and the effect of inorganic nitrogen addition was not significant. The remaining sugars consisted of a mixture of sucrose and fructose and no residual glucose was detected in any of the runs. In the second step *T. oleaginosus DSM 11815* grew in the pooled juices remaining from the first step for 168 h and produced biomass with 28% lipid content. Glucose showed the highest uptake rate, sucrose was utilized until low glucose values prevailed, and fructose was slowly metabolized and a substantial amount remained. Although the two step process has flexibility in choosing the proper microorganism for each step, it is necessary to look for a rapid fructose uptake strain for both fermentations.

1. Introduction

Sweet sorghum is a sugar rich crop that has been considered an alternative feedstock to sugarcane for first generation ethanol production in tropical countries, due to its efficient C4-photosynthesis, short production cycle, and nitrogen and water use efficiency, high tolerance to environmental stress and adaptability to marginal lands [1]. Sweet sorghum presents additional advantages: (a) it can be considered a multiproduct crop due to its high sugar productivity and its grain with adequate nutritional characteristics [2], (b) it can sustain a full year production cycle as sweet sorghum is capable in the tropics of ratoon crops [3], and c) it can be grown without the addition of chemical fertilizers [4].

A sweet sorghum disadvantage is a short harvest window, which can be overcome by employing cultivars with different plant cycles or using the same variety sowed at different dates, or both [1]. Moreover, the harvested stalk sugar content and proportions of individual sugars deteriorates rapidly at ambient temperatures with negative consequences for further processing. A practical suggestion is to extract and clarify the juice, then concentrate and store the syrup [5].

Fuel ethanol production from sugar or starch rich crops, byproducts or wastes has been investigated thoroughly; the research findings and the present industrial practice have been summarized in several recent reviews [6,7]. There are two mature technologies producing in an unprecedented scale ethanol as a biofuel, using corn starch as a raw material in the USA and cane juice and molasses in Brazil. In the former, glucose is the main carbon source, in the latter, a mixture of sucrose and reducing sugars. In Europe beet molasses, alone or in mixtures with cane molasses, are used as raw material for ethanol production; sucrose being the predominant sugar in beet molasses.

Other crops have also been considered; among those that excel are: (a) Jerusalem artichoke [8] which accumulates inulin as the major carbohydrate that can be hydrolyzed by inulinase to a mixture of fructose, glucose and fructo-oligosaccharides; (b) carob pods [9] sugars which are a mixture of sucrose, glucose and fructose; and (c) sweet sorghum which accumulates in the mature stem a mixture of sucrose, glucose and fructose [1–4].

Sweet sorghum has been studied extensively as a raw material for ethanol production [10,11]. However, there is presently no industrial ethanol production from this raw material. The extensive research published employing sorghum juice has been done

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in the laboratory or small pilot units in which juice extraction is done in small roller mills with very poor performance. In order to increase sugar extraction efficiency, the simultaneous extraction-fermentation technique employing sweet sorghum stem particles suspended in water has been employed in small scale [2–4]. Developments have taken place in solid substrate fermentation of milled sorghum stalks and continuous pilot units have been designed, built and operated [12,13]. New strategies have been proposed to transform simultaneously not only the soluble sugars but the carbohydrates produced by the hydrolysis of the lignocellulosic matrix [14,15]. Several researchers have investigated the very high gravity (VGH) ethanol fermentation from sweet sorghum syrups [16,17] in order to find the maximum sugar conversion into ethanol and the minimization of fermentation byproducts, by testing different ethanol tolerant yeasts, several nitrogen sources and adding other nutrients.

A general interest in producing microbial oils for further transformation into biodiesel has identified promising oleaginous algae, bacteria, fungi and yeast and has encouraged studies concerning their growth and lipid accumulation kinetics summarized in recent reviews [18,19]. Sweet sorghum juice and syrup have been used as carbon substrates for microbial oil production. Cui and Liang [20] employed the oleaginous yeast *Cryptococcus curvatus.* Gao et al. [21] grew heterotrophically the alga *Chlorella protothecoides*. In both cases the resulting microbial biomass had a high proportion of lipids. Oleaginous yeasts have been grown on sucrose, glucose and fructose containing raw materials. *Rhodotorula glutinis* and *Trichosporon fermentans* have been grown on cane molasses with acceptable growth and lipid accumulation [22,23]. *Rhosporidium toruloides*, *Rhodotorula mucilaginosa* and *Cryptococcus* sp. were grown successfully in hydrolyzed extracts of Jerusalem artichoke tubers, either by inulinase or by acids, producing cells with high lipid content [24–26].

Instead of employing sweet sorghum juice as raw material in different processes to produce either ethanol or biodiesel, we are proposing a different alternative route in order to obtain ethanol and microbial oil as main products in two consecutive fermentation steps. The first step consists of ethanol production and the second step the growth of oleaginous yeast employing the residual carbon and nitrogen left from the first step. In between the steps, *Saccharomyces cerevisae* is separated by centrifugation and ethanol by distillation. At the end of the production line the oleaginous yeast biomass oil can be converted to fatty acids ethyl esters or FAEE employing part of the ethanol produced in the first step. In this paper we present experimental results of the two fermentation steps with the main objective of providing a proof of concept.

2. Material and methods

2.1. Sweet sorghum juice

Five stalks from four sweet sorghum varieties, Della, M81-E, Sugar Drip, Top 76-6 and Umbrella were brought from our experimental agricultural station stripped of adhering leaves, leaf sheaths, and top and were kept at −10 °C until processed. The thawed stalks were pressed employing a stainless steel pilot three roll crushing mill (*Vencedora Maqtron Model 721*) with a 2-HP motor. Juice Brix was measured with a digital refractometer (*Model 300034 Sper Scientific Ltd*) and a juice sample was used for sugar analysis as described below. The juice mixture soluble solids were 17.4 Brix. Three different concentrations were prepared diluting with distilled water, 17, 15 and 13 Brix. Their total sugar content was 15.3, 13.5 and 11.7 wt%, respectively of which 69% was sucrose, 19% was glucose and 12% fructose. Kjeldahl nitrogen was 0.42 g/L and pH 5.2. Starch, mannitol, phosphorus and organic acids contents were not quantified.

2.2. Microorganisms and inoculum preparation

The ethanol production first step was done employing two *Saccharomyces cerevisae* yeasts separately as explained below, CBS 400 and CBS 459 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). In the second step, the oleaginous yeast *Trichosporon oleaginosus DSM 11815* (Leibnitz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was cultivated for lipid production.

The inoculum for all yeasts was prepared as follows: a pure culture sample was grown in a 30 g/L Sabouraud broth (Merck, 2% glucose, 0.5% animal peptone, and 0.5% casein peptone) plus 1% additional sucrose. One hundred and twenty-five milliliters of broth was added into a 250 mL flask, sterilized at 121 °C for 20 min, cooled, inoculated, and agitated at 250 rpm at 30 °C for 48 h (Incubator Shaker Lab Companion Model SI-600). The suspension was centrifuged at 1600*g* for 5 min at 10 °C (Eppendorf Table-top Refrigerated Centrifuge Model 5804R). The solid pellet was suspended in deionized water and the optical density adjusted approximately to a value of 1.8.

2.3. Experimental strategy

A two level factorial design for three factors was used for the ethanol production step (Design Expert 9.0.5). The factors were: (a) the yeast culture, (b) sweet sorghum juice initial Brix and (c) the quantity of ammonium phosphate added. Note that the first factor is categorical and the other two are numerical. The design consisted in 14 runs including six central point repetitions. Center points were duplicated at both the low and high value of the categorical factor. We decided to include the yeast strain as an experimental factor in order to compare its effect to those effects from the other two design variables. Previous research on industrial ethanol fermentations from sugarcane byproducts under normal and high gravity conditions have shown the importance of the yeast strain on the fermentation outcome [27].

For each run, 100 mL of the prepared sweet sorghum juice were placed in 250 mL Erlenmeyer flasks and 10 mL of the yeast inoculum were added. The flasks were kept for 48 h at 30 °C under static conditions. The yeast was separated by centrifugation at 2000*g* for 5 min at 10 °C (Sorvall RT7 refrigerated centrifuge) and a liquid sample was sent for sugar and ethanol analysis as explained below. All remaining liquids were then pooled and ethanol was separated by distillation. The hot residual liquid was placed in a 1 Liter bioreactor (BioStat® A Plus-Sartorius Stedim), cooled, had a pH of 4.8 and inoculated with the oleaginous yeast. Temperature was set at 30 °C, an air flow of 1.2 vvm, a controlled pH of 4.80, 350 rpm and a working liquid volume of approximately 750 mL. It was operated for 168 h and samples were taken periodically. Yeast dry weight and total sugars were determined as explained below. Yeast biomass was recuperated by centrifugation at 1600*g* for 5 min at 10 °C (Eppendorf Table-top Refrigerated Centrifuge Model 5804R). It was kept frozen for two days at −10 °C. It was thawed, placed on a tray and dried with air at 65 °C. Moisture and oil content were determined as explained below.

2.4. Analysis

Sugars in the filtrate were determined with an Agilent 1100 high pressure liquid chromatograph, an Agilent 1200 refractive index detector, a Zorbax NH2, 25 cm long, 4.6 mm internal diameter column, employing acetonitrile in water (70–30), as the solvent phase. Ethanol was quantified employing an Agilent 6890 N gas chro-