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Technical note

Bioethanol production from hydrolyzed sugarcane bagasse supplemented with molasses “B” in a mixed yeast culture

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ABSTRACT

Lignocellulosic biomass is an abundant renewable source of xylose and glucose, which can be converted into ethanol using microorganisms. The aim of this study was to use sugarcane bagasse hydrolyzate (HBC) supplemented with sugarcane molasses “B”, one of the liquors resulting from the crystallization step in cane sugar production process, in a mixed yeast culture (*Saccharomyces cerevisiae* ITV-01 and *Scheffersomyces* (formerly *Pichia) stipitis* NRRL Y-7124) in order to increase ethanol production and the complete utilization of hydrolyzate sugars. The culture of mixed yeast in a medium with 50% HBC supplemented with 8% v/v intermediate molasses “B” after 42 h fermentation exhibited a significant increase in ethanol production (53.80 gL^{-1}), yield (0.45 gg^{-1}) and productivity (1.07 gL^{-1} h^{-1}). The novel strategy of using 8% v/v sugar cane molasses “B”, promotes complete sugar consumption and a 74% increase in ethanol fermentation by the mixed culture of *S. cerevisiae* ITV-01 and *Scheffersomyces stipitis* NRRL Y-7124.

1. Introduction

The bioethanol industry has developed rapidly in recent years to cope with the depletion of fossil fuel. Materials unsuitable for human consumption e.g. lignocellulose [1] are considered ideal substrates for bio-ethanol production. Lignocellulosic biomass represents a potential source of carbohydrates (cellulose and hemicellulose) for microbial fermentation into ethanol and other chemicals [2]. Dilute sulfuric acid has been widely used to pretreat sugar cane bagasse and this results in the hemicellulose fraction being released as a pentoses xylose and arabinose [3]. Moreover, soluble side products (furfural, 5-hydroxymethyl furfural, acetate, phenolics and others) formed during dilute acid hydrolysis inhibit microbial growth and retard fermentation [2,4]. The optimization of fermentation efficiency, with the aim of industrial scale production of ethanol from lignocellulosic biomass, has been evaluated. *Saccharomyces cerevisiae*, with its high ethanol tolerance and high yields and rates of fermentation, is unable to ferment xylose, the second most abundant sugar in nature, which limits its use in biofuel production [5,6]. Some yeast such as *Scheffersomyces* (formerly *Pichia) stipitis* and *Candida shehatae* can ferment xylose and other important hexoses with relatively high yields and rates, but they have low ethanol tolerance and ethanol concentrations above 30 to 35 gL^{-1} inhibit their reactions [6,7]. Previously, the culture medium optimization of nitrogen source and mineral increased the ethanol titer obtained using *Scheffersomyces stipitis* was 61 gL^{-1} [8]. So, the presence of mineral and amino acids is key to improve ethanol production and tolerance in *S. stipitis*. The culture medium enrichment could result in increasing its price [8], thus the use of new feedstocks should be study in order to improve the performance of *S. stipitis* in a cheap culture media.

Since there is no wild type microorganism that could efficiently accomplish this process, the utilization of two microorganisms has been evaluated [1,9–11]. The main difficulty of using two microorganisms for the co-fermentation of these two sugars (glucose and xylose) is the inability to provide optimal environmental conditions for two strains simultaneously [1]. A majority of previous studies on strain mixed cultures reported that while glucose fermentation in

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the sugar mixture proceeded efficiently with a traditional glucose fermenting strain, xylose fermentation was often slow and inefficient due to the conflicting oxygen requirements between the two strains and/or catabolite repression on xylose assimilation caused by glucose [11].

During sugar production from sugarcane (*Saccharum officinarum*), three vacuum evaporators-crystallizers are employed. The fluid feed to the first one is called "cooked mass A", and the product is "Molasses A". This are feed to the second one, and the product is "Molasses B", and they are feed to the third one and the final product is called "Molasses C" also know as sugarcane molasses or final molasses. So, Molasses B are an intermediary from sugarcane production 15% richer in sugar content than the final molasses [12]. Molasses "B" contains the following: 56% w/w fermentable sugars, 18% dried matter (non sugars), 17% water, 7% ash and 2% nitrogen compounds. In addition, molasses "B" also contains vitamins and minerals (biotin >0.36; choline >745; pantotenic acid >21; riboflavin >1.8; and thiamine >0.9 mg kg^{-1}) and it has exhibited a buffering capability maintaining pH fermentation values between 5.3 and 5.6 [13]. This substrate was previously used to produce ethanol using *S. cerevisiae* ITV-01 [14]. Therefore, the objective of this study was to determine the effect on growth and ethanol production of adding sugarcane molasses "B" to a medium with sugarcane bagasse hydrolyzate by separate microbial communities of *S. cerevisiae* ITV-01 and *S. stipitis* NRRL Y-7124, and also these communities in co-culture. Fermentation kinetics and yield parameters were obtained using established models to aid in the interpretation of the data.

2. Material and methods

2.1. Materials

Sugarcane bagasse and molasses "B" were obtained from "El Modelo" factory in January, 2012. The reagents employed were: $(NH_4)_2SO_4$, J.T. Baker; $MgSO_4 \cdot 7H_2O$, J.T. Baker; KH_2PO_4, J.T. Baker; NaCl, J.T. Baker; Glucose, Golden Bell Reactivos; Fructose, J.T. Baker; Xylose, Golden Bell Reactivos; Xylitol, Sigma–Aldrich; Yeast extract, Bioxon (Becton Dickinson); Agar, Bioxon (Becton Dickinson); H_2SO_4 96% J.T. Baker; Glycerol, J.T. Baker; Acetic acid, J.T. Baker; Furfural, J.T. Baker; and Ethanol anhydrous, J.T. Baker.

2.2. Preparation of hemicellulose hydrolyzate fraction

Sugarcane bagasse hydrolyzate was obtained after sulfuric acid pretreatment under the following conditions: 121 °C, 40 min, 2% (v/v) sulfuric acid concentration, 1:6.25 (w/v) ratio. The pH was increased to 5.0 with NaOH and the sediment formed was separated by centrifugation (Refrigerated Superspeed Centrifuge RC-SB, Du Pont Instruments, Cincinnati OH 45237, EUA) at 25 °C and 142 × g then enriched with the following (gL^{-1}): $(NH_4)_2SO_4$, 3.0; $MgSO_4$ 7; H_2O, 1.0; KH_2PO_4, 5.0 and yeast extract, 2.0.

2.3. Yeasts strains

S. stipitis NRRL Y-7124 was supplied by the American Type Culture Collection and *S. cerevisiae* ITV-01 is a wild type yeast previously isolated from sugarcane molasses from the region of Cardel, Veracruz, Mexico [15].

2.4. Culture media

The yeasts were stored at 4 °C using a culture medium with the following composition (gL^{-1}): glucose, 20; yeast extract, 20; and agar, 20. Preculture medium for *S. cerevisiae* ITV-01 and *S. stipitis* NRRL Y-7124 were 20 gL^{-1} glucose and 20 gL^{-1} xylose respectively, complemented with (gL^{-1}) yeast extract, 2.0; KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 3.0 and $MgSO_4 \cdot 7H_2O$, 1.0 and then sterilized for 15 min at 121 °C. The fermentation medium was prepared by diluting the acidic hydrolyzate to a concentration of 25, 50, 75 and 100%, supplemented with the same nutrients as the preculture medium and also with 8% v/v molasses "B". For the adaptation procedure, the medium was prepared by dilution of hydrolyzate to a concentration of 50%, supplemented with the same nutrients as preculture medium and 8% v/v molasses "B".

2.5. Preculture conditions

The preculture for each yeast was made in a 250 mL Erlenmeyer flask with 100 mL synthetic medium and stirred at 150 rpm. After inoculation, each Erlenmeyer flask was incubated at 30 °C for 12 h. The inoculum size was 6×10^6 viable cell mL^{-1}. After that the adaptation procedure was made twice in a 250 mL Erlenmeyer flask with 150 mL liquid hemicellulosic hydrolyzate and stirred at 150 rpm, 30 °C for three days. The cells were harvested in the exponential phase by centrifugation at 5000 rpm for 20 min (Refrigerated Superspeed Centrifuge RC-SB, Du Pont Instruments, Cincinnati OH 45237, EUA), washed once with 0.9% (w/v) NaCl.

2.6. Culture conditions

The performance of the adapted strain was evaluated in bagasse hydrolyzate fermentations. Both independent and co-culture fermentations were performed in 250 mL Erlenmeyer flasks with 150 mL hydrolyzate medium; this was, respectively, for 80 h and 40 h by *S. stipitis* NRRL Y-7124 and *S. cerevisiae* ITV-01 separately, and for 60–80 h in co-culture. Agitation was fixed at 150 rpm (New Brunswick Scientific classic series C24KC Refrigerated Incubator, Shaker Edison NJ, USA) and 30 °C. All experiments were carried out in duplicate.

2.7. Analytical techniques

The culture medium was centrifuged for 10 min at 10000 rpm using an Eppendorf Centrifuge 5424 (Germany). The supernatant was stored at −20 °C until analysis. Glucose, fructose, xylose, xylitol, glycerol, acetic acid, furfural and ethanol were measured by high performance liquid chromatography (Waters 600, TSP Spectra System, Waters, Milford, MA, USA) using a Shodex sugar SH1011 column (Shodex Group, NY 10017, USA). The temperature was 50 °C, mobile phase 5 mmol L^{-1}sulfuric acid, 0.6 mL min^{-1} flow rate and an index refraction detector (Waters 2414, TSP Refractor Monitor V, Waters) was used.

2.8. Yields and productivities calculation

The ethanol and xylitol yields ($Y_{et/s}$ and $Y_{xyl/s}$, respectively) were calculated based upon the ratio of maximum product titer and beginning sum of glucose, fructose and xylose concentrations of the media. Ethanol productivity (Q_p) was calculated by ratio of ethanol production and fermentation time. The values reported in Tables are the average of two independent experiments.

3. Results and discussion

3.1. Fermentation on hemicellulosic hydrolyzate

Sugarcane bagasse was degraded to simple sugars by acidic hydrolysis using sulfuric acid. The composition of the acidic hydrolyzate obtained was 21 g xylose L^{-1}, 2.7 g glucose L^{-1}, 4.5 g