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ORIGINAL PAPER

Evaluation of hexose and pentose in pre-cultivation of *Candida guilliermondii* on the key enzymes for xylitol production in sugarcane hemicellulosic hydrolysate

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Abstract The evaluation of hexose and pentose in pre-cultivation of *Candida guilliermondii* FTI 20037 yeast on xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes activities was performed during fermentation in sugarcane bagasse hemicellulosic hydrolysate. The xylitol production was evaluated by using cells previously growth in 30.0 gl^{-1} xylose, 30.0 gl^{-1} glucose and in both sugars mixture (30.0 gl^{-1} xylose and 2.0 gl^{-1} glucose). The vacuum evaporated hydrolysate (80 gl^{-1}) was detoxificated by ion exchange resin (A-860S; A500PS and C-150-Purolite®). The total phenolic compounds and acetic acid were 93.0 and 64.9%, respectively, removed by the resin hydrolysate treatment. All experiments were carried out in Erlenmeyer flasks at 200 rpm, 30°C. The maximum XR (0.618 Umg^{-1}_{Prot}) and XDH (0.783 Umg^{-1}_{Prot}) enzymes activities was obtained using inoculum previously growth in both sugars mixture. The highest cell concentration (10.6 gl^{-1}) was obtained with inoculum pre-cultivated in the glucose. However, the xylitol yield and xylitol volumetric productivity were favored using the xylose as carbon source. In this case, it was observed maximum xylose (81%) and acetic acid (100%) consumption. It is very important to point out that maximum enzymatic activities were obtained when the mixture of sugars was used as carbon source of inoculum, while the highest fermentative parameters were obtained when xylose was used.

Keywords Sugarcane bagasse · Hemicellulosic hydrolysate · Xylose reductase · Xylitol dehydrogenase · Xylitol

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Introduction

Xylitol, a five-carbon sugar alcohol ($C_5H_{12}O_5$), has a potential use in pharmaceutical, odontological and food industries (Hyvonen et al. 1982). Besides its anti cariogenetic proprieties (Mäkinen et al. 2001), xylitol can be used for diabetes treatments since its metabolism is independent of insulin, and other metabolic disorders such as deficiency in glucose-6-phosphate dehydrogenase enzyme. Xylitol compared to other sugars contributes for lower fat tissues formation, because of that it can be recommended to obese people (Manz et al. 1973; van Eyes et al. 1974). Also, xylitol could prevent otitis, osteoporosis and lung infection in patients with cystic fibrosis (Uhari et al. 1998; Matilla et al. 1998; Zabner et al. 2000). Yeasts are considered the best xylitol producers. In xylose-fermenting yeasts, the substrate is metabolized mostly via two-stage

oxidative-reductive pathway consisting in reduction of xylose to xylitol by an NAD(P)H dependent xylose reductase (EC 1.1.1.21, XR) and oxidation of xylitol to xylulose by an NAD^+ dependent xylitol dehydrogenase (EC 1.1.1.9, XDH) yielding D-xylulose. Xylulose is then phosphorylated by xylulose kinase to D-xylulose-5-phosphate, which can be catabolized by pentose phosphate, Embden-Meyerhof-Parmas, or by phosphoketolase pathways. An imbalance of the NAD^+:-NADH redox system in D-xylose metabolism of yeasts is avoid by reducing xylose to xylitol with NADH. The oxygen availability greatly influences the cofactors requirements of these enzymes. Anaerobic or oxygen-limited conditions cause a redox imbalance which interferes with the xylitol and the by-products production of this metabolism (ethanol and glycerol) (Felipe 2004). The activities of XR and XDH are believed to be influence by other carbohydrates (Sugai and Delgenes 1995; Lee et al. 1996; Kastner et al. 2001). Because a significant fraction of xylose is assimilated as a consequence, a high XR:XDH ratio was hypothesized as a desirable feature to optimize xylitol productivity (Alves et al. 2002). *Candida guilliermondii* yeast, one of the best xylitol producers, has NADPH-dependent xylose reductase and NAD^+-dependent xylitol dehydrogenase. These enzymes are both induced by xylose (Sene et al. 2000; Felipe 2004). For xylitol biotechnological production, the semi-synthetic fermentation medium could be replaced by hemicellulosic hydrolysates obtained from lignocellulosic materials as sugarcane bagasse, corn stover, corncob, rice straw, etc. These hydrolysates contain a complex mixture of sugars including pentoses (xylose and arabinose) and hexoses (glucose and mannose). Also, they can contain inhibitory compounds formed or released during the hydrolysis of the lignocellulosic materials, which could affect negatively the bioconversion of xylose-to-xylitol. Therefore, removing inhibitors (organic acids, sugar degradation compounds and lignin derivatives) from hydrolysate before its fermentation improve the bioconversion rates and yields (Felipe 2004). For hydrolysate detoxification, a number of methods can be used as pH adjustment (Martinez et al. 2001; Chandel et al. 2007), adsorption into ion exchange resins (Villarreal et al. 2006; Canilha et al. 2010) and adsorption into active charcoal (Marton et al. 2006; Canilha et al. 2010). The later one presents a high efficiency associated to a low-cost (Marton et al. 2006; Sreenivas Rao et al. 2006). Besides hydrolysates destoxification, cell recycle could be used to improve the xylose bioconversion rate (Sene et al. 1998). According to Silva and Felipe (2006) inoculum previously growth in glucose induced the enzymes expression necessary for xylose metabolism, as xylose reductase and xylitol dehydrogenase. According to Walther et al. (2001) the xylose-to-xylitol bioconversion is influenced by the type of sugar and sugars ratio in the fermentation media. Also, these authors observed improvement in fermentation of hemicellulosic hydrolysate by using an adequate pre-cultivation medium to induce the rate of xylose to xylitol bioconversion by the yeast *Candida tropicalis*. In this context, the aim of this work was to evaluate the presence of hexose and pentose in pre-cultivation of *Candida guilliermondii* on the key enzymes for improvement of xylitol production in sugarcane bagasse hemicellulosic hydrolysate.

Materials and methods

Microorganism and cells pre-cultivation media

The yeast *Candida guilliermondii* FTI 20037 was maintained at 4°C on malt-extract agar slants. The cells pre-cultivation media containing 30.0 g l^{-1} xylose or 30.0 g l^{-1} glucose or both sugars mixture (2.0 g l^{-1} glucose and 30.0 g l^{-1} xylose) was added with the following nutrients: 20.0 g l^{-1} rice bran extract, 2.0 g l^{-1} $(NH_4)_2SO_4$ and 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$. The inoculum was carried out in Erlenmeyer flasks (125 ml) containing 50 ml of inoculated pre-cultivation medium on a rotary shaker (200 rpm) at 30°C for 24 h. Afterwards, the cells were separated by centrifugation (2000×*g*; 20 min), rinsed twice with distilled water and the cell pellet was ressuspended in an adequate volume of distilled water and used as an inoculum.

Obtainment, vacuum evaporation and resin treatment of the sugarcane bagasse hemicellulosic hydrolysate

The hydrolysis of the sugarcane bagasse was carried out using diluted H_2SO_4 (100 mg of H_2SO_4 per gram of dry matter) and a solid/liquid ratio of 1.75:10 at 150°C for 30 min in a 100 l steel reactor. The sugarcane bagasse hemicellulosic hydrolysate, which

was separated from the solid residue by vacuum filtration using qualitative paper, was vacuum evaporated at 70°C to increase fivefold its original xylose concentration. The vacuum evaporated sugarcane bagasse hemicellulosic hydrolysate was treated by the following sequence of ion-exchange resins (Purolite®):A-860S (macroporous strong base anion exchanger), A-500PS (macroporous type I strong base anion exchanger) and C-150 (macroporous strong acid cation exchanger). The procedure during ion-exchange resins (IER) treatment involved the following stage: (1) Regeneration of the anion-exchangers and cation-exchanger resins by using 10% NaOH and 5% HCl solutions, respectively; (2) Washing resins by using deionized water; (3) Treatment of the sugarcane hemicellulosic hydrolysate by maintaining it in contact with the resins at the ratio of 1:2 v/v, respectively; and (4) Washing resins by using deionized water. The resins treatments were carried out in Erlenmeyer flasks in a rotary shaker at 200 rpm and 30°C for 1 h. After each stage, the resins were recovered by filtration.

Medium and fermentation conditions

The treated sugarcane bagasse hemicellulosic hydrolysate was autoclaved at 111°C, under 0.5 atm, and it was used as fermentation medium. The fermentation medium was supplemented with the same nutrients used in the inoculum medium preparation, but without glucose and xylose addition. The batch process was carried out in Erlenmeyer flasks (125 ml) containing 50 ml of the fermentation medium (pH 5.5), on a rotary shaker at 200 rpm, 30°C for 120 h. The initial cell concentration on all experiments was 1 g l^{-1}. The fermentation experiments were performed in triplicate.

Analytical methods

For cell disruption and enzyme assays, the cells from fermentation samples (0, 24, 48, 72, 96, 120 h) were harvested by centrifugation (2000×*g*, 15 min) washed with sterile distilled water, resuspended with 0.1 M potassion phosfate buffer (pH 7.2) and stored in a freezer (−4°C). The cell suspension (12 g l^{-1}) was thawed, and mechanically disrupted by glass beads (0.5 mm of diameter) in centrifuge tubes under vortex agitation. The proportion of cell suspension to glass beads was 1:1 v/v. According to Gurpilhares et al. (2009), the disruption period of 5 min was intercalated minute-by-minute in ice bath for 30 s. After cell disruption, the samples were centrifuged (6700×*g*, 10 min, 4°C) and the supernatants were assayed for XR and XDH activities. Enzymes activities were determined by spectrophotometer at 340 nm, at room temperature as described by Alexander (1985). One XR or XDH units (U) was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADPH min^{-1} or 1 μmol of NAD^+ min^{-1}, respectively. Specific activities were expressed as U mg_{Prot}^{-1} as determined by the dye-binding technique using Coomassie Blue Reagent (Bradford 1976). Each enzymatic activity determination was done in triplicate, with the coefficient of variation being not higher than 5%.

The concentrations of xylose, glucose, arabinose, acetic acid, xylitol, glycerol, and ethanol were determined by high-performance liquid chromatography (Shimadzu LC-10AD, Kyoto, Japan) using a refractive index detector and Bio Rad (Hercules, CA) Aminex HPX-87H column at 45°C, 0.01 N H_2SO_4 as eluent, at flow rate of 0.6 ml min^{-1} (Rodrigues et al. 2006). The 5-hydroxymethylfurfural, furfural and phenolic compounds concentrations were determinated as described by Villarreal et al. (2006).

Results and discussion

Sugarcane bagasse hemicellulosic hidrolysate composition

Table 1 shows the composition of the original (H1), vacuum evaporated (H2) and resin treated (H3) sugarcane bagasse hemicellulosic hydrolysates.

The sugars concentration in the original sugarcane hemicellulosic hydrolysate increased proportionally to the concentration factor used during the vacuum evaporation process. The resin treatment did not remove significantly xylose from the hydrolysate. However, it was observed removal of toxic compounds after resin hydrolysate treatment (Table 1). The removal of toxic compounds from the hydrolysate could improve xylitol fermentation by yeast fermentation (Alves et al. 1998; Rodrigues et al. 2001; Canilha et al. 2010). Similar original sugarcane hydrolysate composition was reported by Pessoa Júnior et al. (1997) and Marton et al. (2006).

Table 1 Sugarcane bagasse hemicellulosic hydrolysates compositions

Composition (g l^{-1})	H1	H2	H3
D-glucose	1.82	8.89	4.88
D-xylose	15.73	77.26	74.99
L-arabinose	1.45	6.52	4.36
Acetic acid	2.31	5.28	1.85
Total phenolic compounds	5.48	12.74	0.96
Furfural	0.19	0.08	0.03
Hydroxymetylfurfural	0.02	0.07	0.02

H1 original sugarcane bagasse hemicellulosic hydrolysate, *H2* vacumm evaporated sugarcane bagasse hemicellulosic hydrolysate, *H3* resin treated sugarcane bagasse hemicellulosic hydrolysate

Candida guilliermondii pre-cultivation in hexose and pentose: effect on the key enzymes for xylitol production in sugarcane hemicellulosic hydrolysate

The maximum XR (0.618 U mg^{-1}_{Prot}) and XDH (0.783 U mg^{-1}_{Prot}) activities (Fig. 1) were observed using inoculum previously grown in a mixture of glucose and xylose. However, the xylose consumption was not improved by this inoculum, which was lower 12.5 and 21.7% than inoculum cultivated only in glucose or xylose as a carbon source, respectively. This fact could be observed by analyzing the angular coefficients formed by linear xylose consumption (Fig. 2). Silva et al. (2005) using the same yeast, but a different sugarcane hemicellulosic hydrolysate treatment observed an improvement in xylose consumption by using an inoculum previously growth in a mixture of glucose and xylose. This behavior could be explained by the multiple uptake systems for D-xylose assimilation, for example according to Lucas and van Uden (1986) when *C. shehatae* was grown on either D-glucose or D-xylose it produces a facilitated diffusion system which can transport D-glucose with high affinity/low capacity, while D-xylose transportation is low affinity/high capacity.

In general, the consumption of sugars by the yeast *Candida guilliermondii* showed total glucose consumption (24 h) followed by xylose and arabinose, independently of inoculum used during the fermentation. However, only part of the arabinose was consumed (data not shown). A similar behaviour of *C. guilliermondii* yeast in sugarcane bagasse hydrolysate was reported by Silva et al. (2005) and Sarrouh et al. (2009). Also, it was observed total acetic acid consumption independently of carbon source used during the inoculum growth (data not shown). In all experiments, the medium pH increased. This fact could be associated to acetic acid consumption by *C. guilliermondii* in the hydrolysate as noted by Morita et al. (2000) using the same yeast in sugarcane bagasse hemicellulosic hydrolysate. Besides xylose consumption, inoculum cultivated in glucose favored the cellular growth during fermentation (Fig. 3a). In this case, it was obtained the highest cell biomass (10.6 g l^{-1}), which was 7.5 and 20.0% higher than inoculum previously grown in xylose and in a mixture of glucose and xylose, respectively. This behavior was not verified by Canilha et al. (2008) using the same yeast pre-grown in a medium

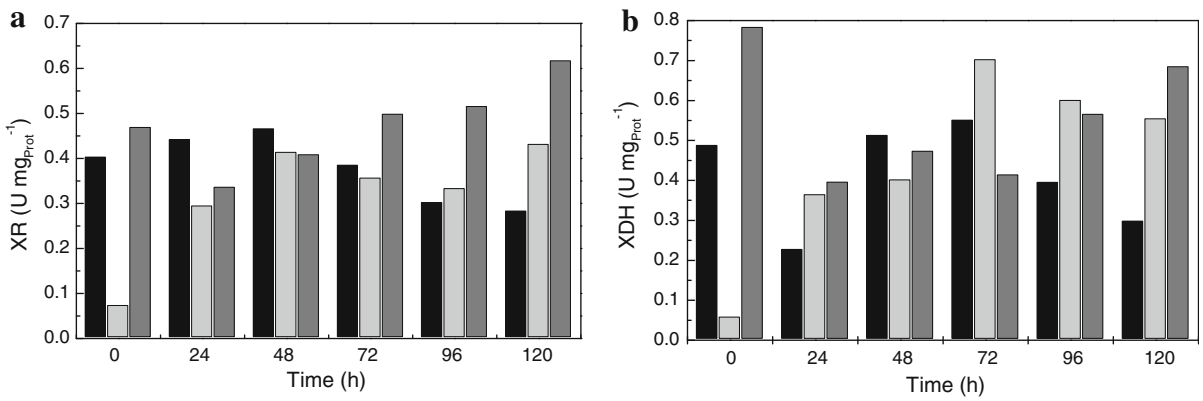

Fig. 1 XR (**a**) and XDH (**b**) enzymes activities during sugarcane bagasse hemicellulosic hydrolysate fermentation using *C. guilliermondii* previously growth on: xylose (*black bar*) or glucose (*light grey bar*) or both sugars (*dark grey bar*)

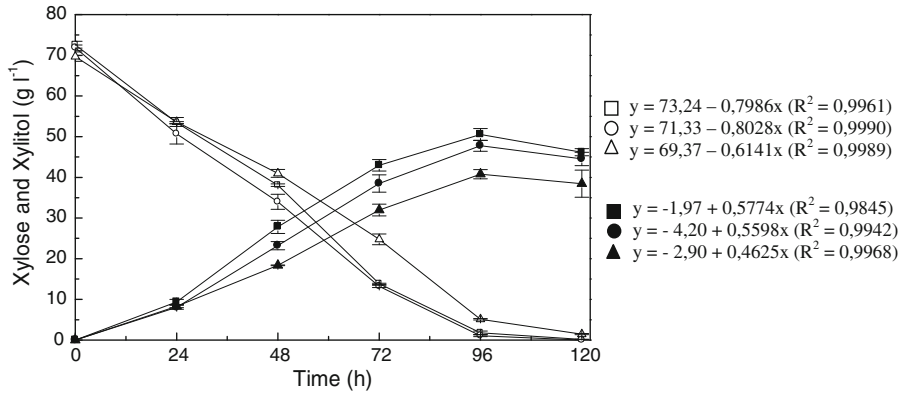

Fig. 2 Xylose consumption (*white symbols*) and xylitol formation (*black symbols*) during sugarcane bagasse hemicellulosic hydrolysate fermentation using *C. guilliermondii* previously growth on: xylose (*square*) or glucose (*circle*) or both sugars (*triangle*)

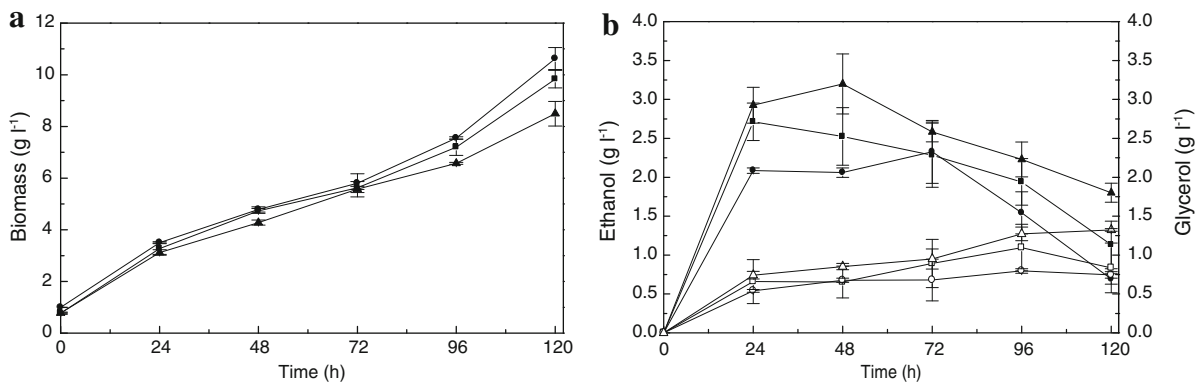

Fig. 3 Biomass (**a**); Ethanol (*black symbols*) and glycerol (*white symbols*) (**b**) formed during sugarcane bagasse hemicellulosic hydrolysate fermentation using *C. guilliermondii* previously growth on: xylose (*square*); glucose (*circle*) or both sugars (*triangle*)

elaborated with xylose + glucose or just glucose and cultivated in wheat straw hemicellulosic hydrolysate detoxified with 2.5% active charcoal once these authors found a higher biomass when a mixture of sugars was employed.

Some works related that xylose induces XR and XDH activities and glucose can repress this induction (Sugai and Delgenes 1995; Lee et al. 1996). According to Rosa et al. (1998) cultivation of *C. guilliermondii* in synthetic medium, showed that XR and XDH activities were sensitive to glucose when the ratio between glucose and xylose was above 10% in the medium. The highest xylitol production (50.5 g l^{-1}) was obtainned by using inoculum previously grown in xylose (Fig. 2), followed by using inoculum previouly grown only in glucose (47.7 g l^{-1}) and in both sugars (40.8 g l^{-1}) in 96 h of fermentation. Similar to this work, Kastner et al. (2001) during synthetic medium fermentation showed a enhancement on xylose consumption and xylitol production by using *C. tropicalis* previously grown in xylose instead of glucose. In the other hand Canilha et al. (2008) during the bioconversion of xylose-to-xylitol in wheat straw hemicellulosic hydrolysate by *C. guillliermondii* which was pregrown in a medium containing glucose (37 g l^{-1}) as the sole carbon source verified that the bioconversion parameters were slightly better than those observed when both glucose (7 g l^{-1}) and xylose (30 g l^{-1}) were used as carbon sources in the medium used to grow the inoculum. According to these authors, the maximum xylitol production (30.5 g l^{-1}) was achieved after 72 h of fermentation, resulting in productivity of 0.42 g l^{-1} h^{-1} and bioconversion yield of 0.59 g g^{-1}. Such findings suggest that the cheaper sugar glucose can be used as the sole carbon source to grow the *C. guilliermondii* FTI 20037 cells for a subsequent xylose-to-xylitol bioconversion in wheat straw hemicellulosic hydrolysate.

Similar to that observed in xylitol production in this work, the highest xylitol yield ($Y_{P/S}$ = 0.81 g g^{-1}) and

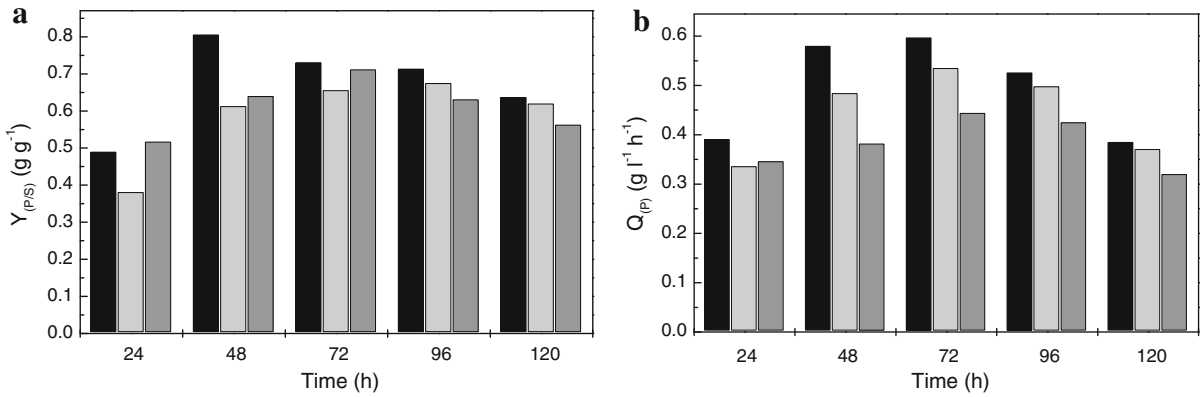

Fig. 4 Xylitol yield (**a**) and xylitol volumetric productivity (**b**) obtained during sugarcane bagasse hemicellulosic hydrolysate fermentation by using *C. guilliermondii* previously grown on: xylose (*black bar*) or glucose (*light grey bar*) or both sugars (*dark grey bar*)

xylitol volumetric productivity ($Q_P = 0.60$ g l^{-1} h^{-1}) was observed by using inoculum previously grown only in xylose after 48 h and 72 h of fermentation, respectively (Fig. 4). This results corresponded an increase of 25.6 and 20.6% in xylitol volumetric productivity and xylitol yield, respectively related to inoculum previouly grown in both sugars. The xylitol was assimilated as a carbon source by the yeast (Fig. 2) after total xylose consumption (96 h), arabinose and acetic acid were partially consumed (data not shown). Same results were found by others authors during *C. guilliermondii* cultivation in synthetic medium (Felipe et al. 1995) and in sugarcane bagasse hydrolysate (Silva et al. 2007). Pfeifer et al. (1996) observed the xylitol formation by yeast *C. guilliermondii* was greatly affected by the composition of different types of medium culture. However, the highest volumetric productivity (0.53 g l^{-1}h^{-1}) was found when the yeast grown in medium containing only xylose, similar to our study. According to these authors, when only xylose was used for cell growth, the xylitol was used for regeneration of NADPH, which was used in the first step of xylose metabolism. It is very important to point out that maximum enzymatic activities and maximum xylitol production were obtained at different conditions of inoculum preparation. The same results were found during the cultivation of *C. guilliermondii* in sugarcane bagasse hydrolysate by Silva and Felipe (2006). Also, we observed the ethanol and glycerol formation as by-products of xylose-to-xylitol bioconversion by *C. guilliermondii* (Fig. 3b). Similar results were found by Arruda and Felipe (2009) using the same yeast cultivated in semi-synthetic medium. The highest ethanol and glycerol production were obtained in the beginning of the fermentations, independent of the inoculum used during the fermentation. As observed by enzymes activities, the highest glycerol (1.32 g l^{-1}) and ethanol (3.20 g l^{-1}) production were favored by using inoculum previously grown in both sugars followed by inoculum previously grown in xylose or in glucose. According to Silva et al. (2005) the highest glycerol formation were observed when *C. guilliermondii* was previously grown in both sugars and xylose, respectively using sugarcane bagasse hydrolysate as a fermentation medium.

Conclusions

The evaluation of hexose and pentose in pre-cultivation of *C. guilliermondii* FTI 20037 yeast on xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes activities was performed during fermentation in sugarcane bagasse hemicellulosic hydrolysate. The resin treatment of the sugarcane hemicellulosic hydrolysate removed phenolic compounds (93%) and acetic acid (64.9%) in the medium. The inoculum previously growth in mixture of glucose and xylose improved XR (0.618 U mg^{-1}_{Prot}) and XDH (0.783 U mg^{-1}_{Prot}) enzymes activities together with glycerol (1.32 g l^{-1}) and ethanol (3.20 g l^{-1}) formation during the fermentation. Inoculum pre-cultivated in the glucose favored the highest cell concentration (10.6 g l^{-1}), while the highest xylose consumption

was obtained in inoculum pre-cultivated in xylose. The maximum xylitol production (50.5 g l^{-1}) was obtained by using inoculum previously grown in xylose followed by using inoculum previouly grown only in glucose (47.7 g l^{-1}) and in mixture of glucose and xylose (40.8 g l^{-1}) Also, the highest xylitol yield ($Y_{P/S} = 0.81$ g g^{-1}) and xylitol volumetric productivity ($Q_P = 0.60$ g l^{-1} h^{-1}) were obtainned by using inoculum previously grown only in xylose. It is very important to point out that maximum enzymatic activities and maximum xylitol production were obtained at different conditions of inoculum preparation.

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