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ORIGINAL PAPER

The behavior of key enzymes of xylose metabolism on the xylitol production by *Candida guilliermondii* grown in hemicellulosic hydrolysate

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Abstract A variety of raw materials have been used in fermentation process. This study shows the use of rice straw hemicellulosic hydrolysate, as the only source of nutrient, to produce high added-value products. In the present work, the activity of the enzymes xylose reductase (XR); xylitol dehydrogenase (XD); and glucose-6-phosphate dehydrogenase (G6PD) during cultivation of *Candida guilliermondii* on rice straw hemicellulosic hydrolysate was measured and correlated with xylitol production under different pH values (around 4.5 and 7.5) and initial xylose concentration (around 30 and 70 g l^{-1}). Independent of the pH value and xylose concentration evaluated, the title of XD remained constant. On the other hand, the volumetric activity of G6PD increased whereas the level of XR decreased when the initial xylose concentration was increased from 30 to 70 g l^{-1}. The highest values of xylitol productivity ($Q_P \approx 0.40$ g l^{-1}) and yield factor ($Y_{P/S} \approx 0.60$ g g^{-1}) were reached at highest G6PD/XR ratio and lowest XR/XD ratio. These results suggest that NADPH concentrations influence the formation of xylitol more than the activity ratios of the enzymes XR and XD. Thus, an optimal rate between G6PD and XR must be reached in order to optimize the xylitol production.

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Introduction

Generally, yeasts used in fermentation processes are able to consume hexoses as carbon source but not pentoses (e.g. xylose by *Saccharomyces cerevisiae*). Lignocellulosic materials represent the largest source of hexoses and pentoses with potential uses in chemical production [1], which have a considerable hemicellulosic fraction (18–34%) [2] composed mainly of D-xylose. This pentose and other sugars are easily obtained by acid hydrolysis of the hemicellulosic fraction [3]. Since D-xylose is the predominant carbon source formed, it is essential to resort to yeasts that also have the capacity to consume this pentose to obtain the full return from lignocellulosic materials [2]. The major interest compounds that can be produced from D-xylose by yeasts are ethanol and xylitol. According to variables of the fermentation process, some pentose-fermenting yeast produces mostly ethanol while others produce mainly xylitol as final product [4]. Besides, the knowledge of physiological and biochemical basis of the xylose metabolism can be useful to control and re-direct metabolite flow to enhance production of the desired compound [5]. The key enzymes for xylitol production in yeasts are xylose reductase (XR) and xylitol dehydrogenase (XD). Xylose reductase, using either NADH or NADPH, reduces D-xylose to xylitol, subsequently xylitol is oxidized to D-xylulose by a NAD-linked XD [4]. The metabolism will proceed through a phosphorylation step and the carbon enters into the pentose phosphate pathway (PPP) to yield

reducing power, mainly by the action of the enzyme glucose-6-phosphate dehydrogenase (G6PD), as well as metabolic intermediates that act as precursors for cell synthesis [6, 7].

Although there are studies focusing on the influence of process variables on D-xylose bioconversion to xylitol by different yeast strains, information on how these variables influence the activity of these important enzymes is limited. Therefore, this research investigated the influence of D-xylose concentration and initial pH of the medium on the activities of enzymes XR, XD and G6PD during xylitol production by *Candida guilliermondii,* which has been widely employed for this bioconversion [8].

Materials and methods

Hemicellulosic hydrolysate

The hemicellulosic hydrolysate was obtained from rice straw under optimized conditions of acid hydrolysis described by Roberto et al. [3]. The obtained hydrolysate (with about 19 g l^{-1} of xylose) was filtered and the liquid fraction (hemicellulosic hydrolysate) was treated with active charcoal for impurities removal. The treatment was based on the addition of 3.0 g charcoal per 100 g of hydrolysate at 45°C, 50 rpm for 30 min. Solids were removed by centrifugation at 1,100×*g* for 15 min and the hydrolysate was further concentrated under vacuum at 65°C to increase sixfold the xylose concentration (until about 110.0 g l^{-1} of xylose).The vacuum procedure was necessary to avoid sugar degradation.

Microorganism and inoculum cultivation

The yeast *C. guilliermondii* FTI 20037 (ATCC 201935) was maintained at 4°C on yeast–malt agar slant [9]. The inoculum was prepared by transferring the cells from the maintenance medium to 15 ml test tubes containing 5 ml sterile distilled water. Aliquots of 1 ml of this suspension were transferred to 500 ml Erlenmeyer flasks containing 200 ml of the hydrolysate-based medium. This medium was prepared by diluting the concentrated hydrolysate in sterile distilled water about four times (until about 30 g l^{-1} of xylose). The pH of the medium was adjusted to 6.2 with solid NaOH. This medium was not sterilized and the inoculum cultivation was made without nutrient supplementation. The flasks were incubated in a rotary shaker at 200 rpm, 30°C for 29 h. Subsequently, the cells were recovered by centrifugation (2,000×*g*, 20 min at 4°C), washed, and resuspended in the sterile distilled water. The final suspension obtained (about 15 g l^{-1}) was used to inoculate the fermentation medium.

Medium and fermentation conditions

To be used as fermentation medium, the concentrated hydrolysate was diluted with distilled water to obtain the initial xylose concentration of about 30 or 70 g l^{-1}. After that, the pH was adjusted to approximately pH 4.5 or 7.5 by addition of NaOH pellets, and the precipitate was removed by centrifugation (1,100×*g*, 20 min). No nutrient was added to the hydrolysate [10] and to avoid degradation of xylose during autoclavation, the hydrolysate-based medium was not sterilized. Fermentations were performed in 500 ml Erlenmeyer flasks containing 200 ml of medium. The flasks were incubated in a rotary shaker at 200 rpm and 30°C for 48 or 96 h with an initial cell concentration of 1.0 g l^{-1}.The fermentation runs were monitored through periodic sampling in order to determine cell growth, xylose consumption, enzymes level and medium pH. All assays were performed in duplicate.

Cell disruption

After each time of fermentation sampling, the cells were harvested by centrifugation (1,100×*g*, 20 min) washed with sterile distilled water and resuspended with 0.071 M buffer Tris–HCl buffer (pH 7.5) added with 10 mM β-mercaptoethanol, 2 mM aminocaproic acid and 0.2 mM EDTA. The final suspension (about 3 g l^{-1}) was mechanically disrupted in centrifuge tubes under vortex stirring using glass beads (Ø 0.5 mm) in a proportion of 3 ml of cell suspension to 3 ml of glass beads (1:1 v/v), at regular time of 5 min [11]. The disruption period was 1 min separated by 30 s interval in an ice bath. The samples were then centrifuged (6,725×*g*, 15 min, 5°C) and the supernatants were assayed for G6PD, XR and XD activities.

Enzymes assays

G6PD, XR and XD activities were determined through the oxidation or reduction of the coenzymes NADP+, NADPH and NAD+, respectively, by the change of absorbance at 340 nm at 30°C [12]. G6PD activity was determined in a reaction mixture (715 µl) containing 500 µl 70 mM Tris–HCl buffer (pH 7.5), 100 µl 35 mM $MgCl_2$, 5 µl 131 mM $NADP^+$, 10 µl 500 mM G6P and 100 µl crude enzyme extract. One unit of G6PD (U) was defined as the amount of the enzyme that catalyzes the reduction of 1 µmol of $NADP^+$ per minute. XR activity was determined in a reaction mixture (600 µl) containing 350 µl 70 mM Tris–HCl buffer (pH 7.2), 50 µl 1.2 mM NADPH, 50 µl 2 M xylose and 150 µl crude enzyme extract. One unit of XR (U) was defined as the amount of the enzyme that catalyzes the oxidation of 1 µmol of NADPH per minute. XD activity was determined in a reaction mixture (600 µl) containing 350 µl

70 mM buffer (pH 8.2), 50 µl 1.2 mM NAD^+, 50 µl 500 mM xylitol and 150 µl crude enzyme extract. One unit of XD (U) was defined as the amount of the enzyme that catalyzes the reduction of 1 µmol of NAD^+ per minute.

Analysis of substrate, xylitol and cell concentration

Xylose and xylitol concentrations were determined by high performance liquid chromatography (HPLC) using Waters equipment (Waters, Milford, MA, USA) with a refractive index (RI) detector (model 2414) and a HPX-87H column (Bio-Rad, Hercules, CA, USA). The operational conditions were: temperature of 45°C, 0.005 M sulfuric acid as eluent, flow of 0.6 ml min^{-1} and injection volume of 20 µl. To estimate cell concentration, a calibration curve of optical density at 600 nm per dry weight was prepared.

Results and discussion

Biomass and xylitol production

Published data have demonstrated that xylitol production by yeasts is influenced by experimental conditions such as substrate source (defined medium or hydrolysate) and its concentration, medium pH, temperature, inoculum level and aeration [13–15].

In this work, as expected, the xylitol production was dependent on the xylose concentration in fermentation medium since it has been shown to be critical for yeast growth and in absence of it, xylitol formation does not occur [4]. Regarding to another important factor, pH, it is well known that yeasts are generally cultivated at pH values between 4.0 and 6.0. If the medium pH was uncontrolled during fermentation process, pH would drop and therefore under such conditions the initial pH values have to be higher than under controlled conditions [4]. In this sense, the evaluated pH was 4.5 and 7.5 (Tables 1, 2). The exhaustion of xylose present in the medium occurred after 48 and 96 h with an initial xylose concentration of about 30 and 70 g l^{-1}, respectively. The total quantity of xylitol released in the medium by yeast *C. guilliermondii* varied from 16.5 to 40.1 g l^{-1}, depending on the initial xylose concentration as well as on the pH employed in the assays. The pH alteration in the assays using ≈30 g l^{-1} of xylose, from around 4.5 to 7.5, promoted an increase of biomass while xylitol production behavior did not change (Table 1). On the contrary, the increase in pH on the assays, which presented around 70 g l^{-1} of initial xylose concentration, had no marked effect on biomass concentration as noticed in the production of xylitol by the yeast cells (Table 2).

In the assays using the highest pH values (pH 7.5) there was a tendency for the pH to diminish slightly (around 0.8 units) during the experiments, being the pH value 6.7 at the

Table 1 Biomass, xylose and xylitol evolution in fermentation medium (initial xylose concentration around 30 g l^{-1} at pH 4.5 and 7.5)

Time (h)	pH 4.5				pH 7.5			
	X (g l^{-1})	Xylose (g l^{-1})	Xylitol (g l^{-1})	pH	*X* (g l^{-1})	Xylose (g l^{-1})	Xylitol (g l^{-1})	pH
0	0.7	33.4	–	4.6	0.7	33.8	–	7.6
12	2.4	24.7	2.9	4.7	2.7	25.7	2.5	6.6
24	2.6	16.1	8.4	5.0	3.0	17.0	7.3	6.6
36	2.9	8.5	13.5	4.9	3.8	8.7	13.5	6.7
48	3.4	1.5	16.5	4.8	4.1	1.5	16.7	6.7

The data points are the averages of duplicate experiments. The maximum variation between the duplicates was less than 10%. *X* biomass concentration (g l^{-1})

Table 2 Biomass, xylose and xylitol evolution in fermentation medium (initial xylose concentration around 70 g l^{-1} at pH 4.5 and 7.5)

Time (h)	pH 4.5				pH 7.5			
	X (g l^{-1})	Xylose (g l^{-1})	Xylitol (g l^{-1})	pH	*X* (g l^{-1})	Xylose (g l^{-1})	Xylitol (g l^{-1})	pH
0	1.2	66.1	–	4.6	1.5	71.9	–	7.4
24	2.3	60.4	3.9	4.5	3.6	51.5	6.9	6.3
48	3.8	42.9	12.9	4.7	5.3	35.1	20.1	6.4
60	3.8	31.9	20.0	4.8	4.8	24.3	26.1	6.5
96	4.9	3.8	38.5	4.8	5.1	1.8	40.1	6.7

The data points are the averages of duplicate experiments. The maximum variation between the duplicates was less than 10%. *X* biomass concentration (g l^{-1})

end of fermentation. The pH increase influenced the uptake of xylose and xylitol production in assays conducted with approximately 70 g l^{-1} (Table 2). In addition, the increase of xylitol accumulation observed in the higher levels of D-xylose could be a consequence of more severe oxygen-limited growth conditions as a result of higher cell densities rather than the effect of D-xylose concentration by itself as observed by Prior et al. [16]. The highest concentration of xylitol attained was 40.1 g l^{-1}, after 96 h of cultivation in the experiment conducted with 71.9 g l^{-1} of xylose, 1.5 g l^{-1} of inoculum and pH 7.4.

Evaluation of key enzymes of xylose metabolism in *C. guilliermondii* during xylitol production

In order to obtain a supplementary evaluation of the effect of initial xylose concentration and medium pH on xylose conversion to xylitol by *C. guilliermondii*, the behavior of three important enzymes involved in the xylose metabolism, XR, XD and G6PD, were analyzed. In scientific literature, there is no sufficient information regarding to the influence of xylose concentration connected to medium pH over the enzymes of xylose metabolism, although some efforts have been made in last years in order to elucidate the importance of other variables over this metabolism, for example: the effect of oxygen transfer rates in *Debaryomyces hansenii* [7]; the induction of aldose reductase and XD activities from *C. guilliermondii* on mixed sugars [5], the effect of nitrogen sources on the levels of aldose reductase and XD activities in *C. tenuis* [17] and the effect of acetic acid present in bagasse hydrolysate on the activity of XR and XD in *C. guilliermondii* [18]. However, none of those researchers mentioned about the behavior of another important enzyme, G6PD, belonging to the PPP, the main source of NADPH, the XR cofactor. It has been shown that xylose metabolism in yeast yields different carbon containing products such ethanol and xylitol [2, 4, 9]. In this manner, xylitol yield is dependent upon the regulation of carbon flow through available metabolic routes. The evaluation of XR, XD and G6PD behaviors during the bioconversion of xylose to xylitol are presented in Figs. 1 and 2.

In Figs. 1 and 2, it can be noticed that G6PD has a high volumetric activity in the beginning of the fermentation, around 130 U l^{-1}. This behavior must be due to the fact that G6PD is a constitutive enzyme of yeast cell [19]. Though, in the assays performed with ≈30 g l^{-1} of xylose, G6PD decreased from 132 to 93 U l^{-1} in the first 12 h owing to the low pH (Fig. 1a); differently, at pH 7.5, G6PD remained almost constant (Fig. 1b). As can be seen in Fig. 2a, by increasing xylose concentration from 30 to 70 g l^{-1}, the pH change did not affect the volumetric activity of G6PD during the xylitol production. Probably, the studied xylose concentration has no direct effect on the level of G6PD in a different way of medium pH and the combination of these parameters.

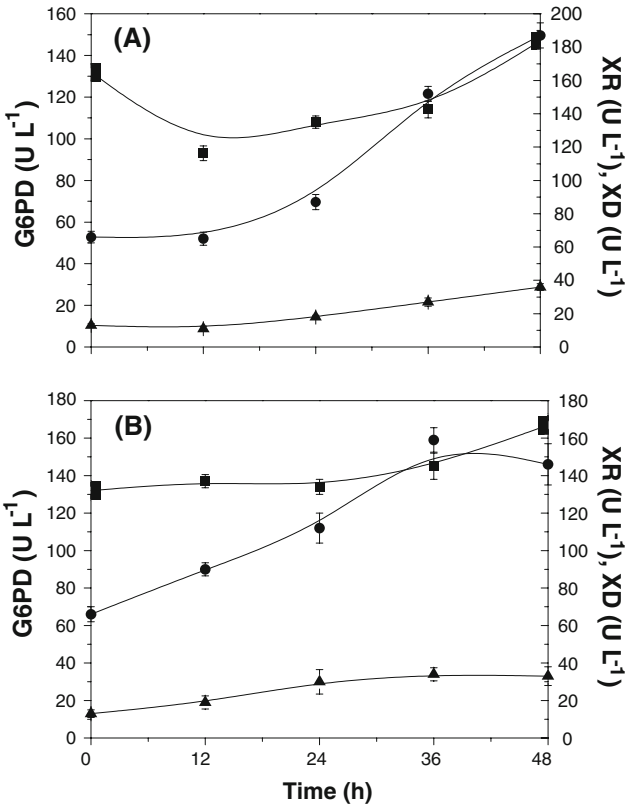

Fig. 1 **a** Activities of G6PD (*filled square*), XR (*filled circle*) and XD (*filled triangle*) as a function of time in a medium containing ≈30 g $l^{-1}$of xylose, 1 g l^{-1} of inoculum and at pH 4.5; **b** activities of G6PD (*filled square*), XR (*filled circle*) and XD (*filled triangle*) as a function of time in a medium containing ≈30 g $l^{-1}$of xylose, 1 g l^{-1} of inoculum and at pH 7.5. *Error bar* indicates the standard deviation of the measured values with 95% confidence intervals

These results are very important for xylitol production as, recently, Jeppsson et al. [20] showed that NADPH-dependent xylose reduction promoted the most of xylitol production in recombinant *Saccharomyces cerevisiae*. These authors proved that by blocking the oxidative PPP the xylitol yield decreases and xylose consumption rate is reduced promoting a higher ethanol yield. These authors suggested that the low xylitol yield is directly linked to the depletion of NADPH in strains with defective PPP. In other study, Jeppsson et al. [21] observed that recombinant strains with low G6PD activity grow slower in a lignocellulose hydrolysate than the strain with wild-type G6PD activity, which denoted that the availability of intracellular NADPH is correlated with tolerance towards lignocellulose-derived inhibitors, such as furan derivatives (5-hidroximethylfurfural). Besides, this cofactor is also needed for biomass formation. Consequently, G6PD is an important enzyme for the bioconversion of xylose to xylitol, since this

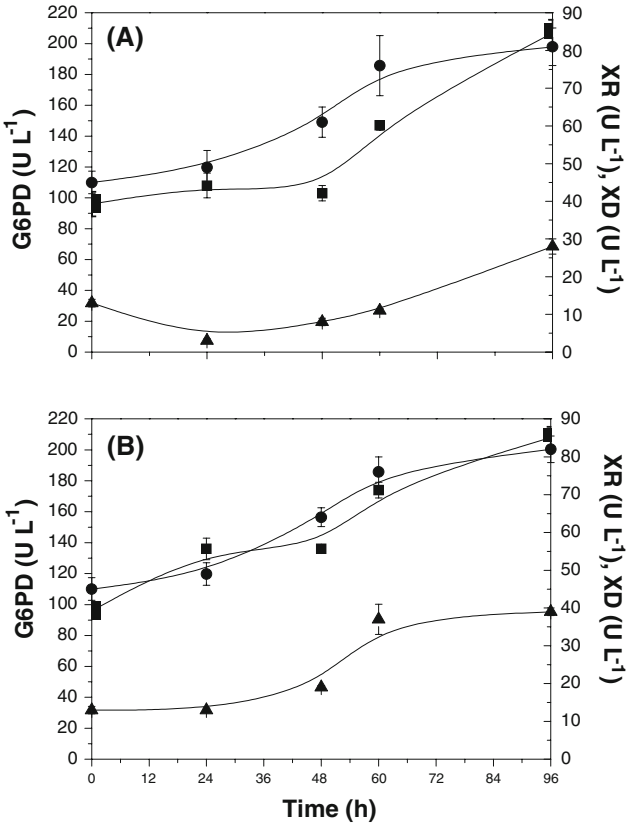

Fig. 2 **a** Activities of G6PD (*filled square*), XR (*filled circle*) and XD (*filled triangle*) as a function of time in a medium containing ≈70 g $l^{-1}$of xylose, 1 g l^{-1} of inoculum and at pH 4.5; **b** activities of G6PD (*filled square*), XR (*filled circle*) and XD (*filled triangle*) as a function of time in a medium containing ≈70 g $l^{-1}$of xylose, 1 g l^{-1} of inoculum and at pH 7.5. *Error bar* indicates the standard deviation of the measured values with 95% confidence intervals

enzyme is the first enzyme of the PPP and yield cofactors NADPH for the xylose reduction [10].

Further, a minimum xylose concentration has been shown to be critical for yeast growth and xylitol formation. It is a consequence of xylose requirement for XR and XD induction in yeasts [4]. In the present study, XR showed low activity in the beginning of cultivation, which increased during the course of the experiment. The volumetric activity of XR was higher in the cultivations conducted with an initial xylose concentration of 30 g l^{-1} when compared to 70 g l^{-1} (Figs. 1, 2). In assays where 30 g l^{-1} of initial xylose concentration was employed, XR volumetric activity varied from 66 to 187 U l^{-1} at pH 4.5, and from 66 to 146 U l^{-1} at pH 7.5 (Fig. 1a, b). In other words, in the experiments using ≈30 g l^{-1} of initial xylose concentration an enhancement of XR volumetric activity of around 100 U l^{-1} was noticed from the beginning until the end of fermentation, while an increase of 36.5 U l^{-1} in the assays carried with ≈70 g l^{-1} of xylose concentration could be observed. In fact, due to the mixture of sugars obtained in the hemicellulosic hydrolysate, the enhancement on xylose concentration promotes an increase in glucose available in fermentation medium. The presence of intracellular glucose may reduce the differential rate of XR synthesis induced by xylose, a phenomenon called partial or weaker carbon catabolite repression according to Sugai and Delgenes [22]. Furthermore, in the latter effect, glucose does not disable the entry of xylose, but it may compete for the transport system in *Candida guilliermondii*, as noticed in *Pichia stipitis* and *Candida shehatae*. Since glucose and xylose are thought to be transported by the same family of hexoses transporters, in a mixture, glucose and xylose will have to compete for these transporters [22].

Candida guilliermondii can be used efficiently in the bioconversion of xylose to xylitol due to the presence of NADPH-dependent XR and the NAD-dependent XD. Richard et al. [23] demonstrated that the gene *YLR070c* of *Saccharomyces cerevisiae* encodes a NAD-dependent XD implying that this yeast can naturally produce the enzyme when are grown in presence of xylose but not during the growth on glucose, aiming the production of ethanol in mixed glucose–xylose medium as the hemicellulosic hydrolysate. According to these authors, the xylose concentration of the culture medium affected the XD in *S. cerevisiae* denoting an improvement in XD activity with the increase in the initial xylose concentration. Probably, this may reflect on the regulation of the expression level of XD likewise in cells of *C. guilliermondii*. Figures 1 and 2 also present the behavior of the enzyme xylitol dehyrogenase during xylitol formation on studied medium conditions. On the contrary to G6PD and XR, the enzyme XD showed low activity. The XD volumetric activity variation was 21 U l^{-1}, approximately. Despite the low volumetric activity, NAD-dependent XD enzyme is extremely important since the excess NADH can be shuttled into xylitol formation during oxygen-limited fermentation [24], by yeasts such as *C. guilliermondii*, after reduction of xylose in the reaction with the enzyme XR exclusively NADPH-dependent [25]. On the contrary, in studies of alcohol production by recombinant *Saccharomyces cerevisiae* the presence of a NADPH/NADH-dependent XR was noticed. In this process, the excess of NADH must be removed with an acceptor of electrons, such as oxygen, or the xylitol formation will occur at the expense of the ethanol yield on xylose due to the presence of dual cofactors (NADPH/NADH) [24, 26–28].

Table 3 presents an assays approach on samples withdrawn after 48 or 96 h of fermentation, depending on the medium conditions. It can be observed that xylitol concentration varied from 16.5 to 40.1 g l^{-1} when the initial xylose concentration was increased from ≈30 to 70 g l^{-1}. The product yields, $Y_{P/S}$, and volumetric productivity, $Q_{P,}$ (0.52 g g^{-1} and 0.34 g l^{-1} h^{-1}) were equal in the assays

Table 3 Residual xylose concentration, product formation, xylitol yield ($Y_{P/S}$) and productivity (Q_P) and level of enzymes G6PD, XR and XD at different medium conditions

Response	Medium conditions			
	1[a]	2[a]	3[b]	4[b]
Xylose (g l^{-1})	1.5	1.5	3.8	1.8
Xylitol (g l^{-1})	16.5	16.7	38.5	40.1
$Y_{P/S}$ (g g^{-1})	0.52	0.52	0.62	0.57
Q_P (g $l\,h^{-1}$)	0.34	0.34	0.40	0.42
G6PD (U l^{-1})	147	167	207	208
XR (U l^{-1})	187	146	81	82
XD (U l^{-1})	36	33	28	39
XR/XD	5.2	4.4	2.9	2.1
G6PD/XR	0.78	1.1	2.5	2.5
Final medium pH	4.8	6.7	4.8	6.7

1 Initial conditions: pH 4.6 and 33.4 g l^{-1} of xylose; *2* initial conditions: pH 7.6 and 33.8 g l^{-1} of xylose; *3* initial conditions: pH 4.6 and 66.1 g l^{-1} of xylose and *4* initial conditions: pH 7.4 and 71.9 g l^{-1} of xylose

$Y_{P/S}$, xylitol yield factor for xylose consumed; Q_P, xylitol volumetric productivity

[a] 48 h of fermentation

[b] 96 h of fermentation

performed with 30 g l^{-1} of initial substrate concentration and about 0.60 g g^{-1} and 0.40 g l^{-1} h^{-1}, respectively, in the assays which were employed 70 g l^{-1} of xylose, for both pH values evaluated. In the present studies, the XR/XD ratios were higher than 1.0 denoting high values of XR activities compared to XD activities. The highest volumetric xylitol production, 0.42 g l^{-1} h^{-1}, occurred with the lowest rate XR/XD, 2.1. This behavior is similar to that observed by Alexander et al. [29] during cultivations of a xylose-grown *C. shehatae* in chemostat with different aeration levels which XD activity was always lower than XR activity. Many authors' observations lead to a conclusion that at low XD activity and high XR activity there is a tendency to an accumulation of xylitol in the medium will succeed. According to authors cited by Jeppsson et al. [20], strains with a low XR/XD ratio form less xylitol than strains with a high ratio. Nevertheless, in the xylose-fermenting *Pichia stipitis*, the XR/XD ratio was much higher than in a recombinant *Saccharomyces cerevisiae* and, on contrary, *P. stipitis* produced less xylitol. The authors concluded that cofactor concentrations may influence the formation of xylitol more than the activity ratios of the enzymes XR and XD do. On the other hand, none mentioned the ratio of G6PD/XR, which could elucidate the results. In the present work, the highest values of Q_P and $Y_{P/S}$ were reached at high values of G6PD/XR, 2.5. This means that the levels of NADPH are higher than $NADP^+$ leading to a xylitol accumulation in the fermentation medium. In addition, as it can be seen in Table 3, at the highest XR/XD ratio, 5.2, a lower Q_P was reached (0.34 g l^{-1} h^{-1}). Jeppsson et al. [26] demonstrated that a recombinant strain of *S. cerevisiae* TMB 3260, in which *ZWF1* gene encoding G6PD had been deleted and XR activity was overexpressed, had a XR/XD ratio of 2.0 and besides the enhanced xylose consumption rate could be observed, a decrease in xylitol yield occurred when compared to the control strain TMB3001. A possible explanation could be that higher XR activity may result in an enhanced turnover of xylose to xylitol pushing the kinetically unfavorable XD reaction and resulting in higher specific xylose consumption and a lower xylitol yield.

The present work showed that even if the volumetric activity of XR is higher, an optimal rate between this enzyme and G6PD must be reach in order to optimize the xylitol production.

Conclusions

The efficiency of xylose conversion to final products of xylose metabolism is strictly connected to the flux of this carbon source through the available pathways. The knowledge of the key enzymes of xylose metabolism behavior on xylitol production could also be an attractive aim for the attainment of different high added-value products, such as G6PD, XR and XD in biotechnological process using hydrolysates from agricultural residues as a low cost raw material. In addition, the present work proved that it would be possible to join the production of xylitol with these enzymes and make a process completely feasible. Besides, the study of G6PD, XR and XD in wild yeast as *Candida guilliermondii* is an important tool to yeasts metabolic engineering towards xylose utilization to fulfill the requirements of biotechnological industry.

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