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Fermentation Kinetics for Xylitol Production by a *Pichia stipitis* D-Xylulokinase Mutant Previously Grown in Spent Sulfite Liquor

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Abstract Spent sulfite pulping liquor (SSL) contains lignin, which is present as lignosulfonate, and hemicelluloses that are present as hydrolyzed carbohydrates. To reduce the biological oxygen demand of SSL associated with dissolved sugars, we studied the capacity of *Pichia stipitis* FPL-YS30 (*xyl3*Δ) to convert these sugars into useful products. FPL-YS30 produces a negligible amount of ethanol while converting xylose into xylitol. This work describes the xylose fermentation kinetics of yeast strain *P.stipitis* FPL-YS30. Yeast was grown in rich medium supplemented with different carbon sources: glucose, xylose, or ammonia-base SSL. The SSL and glucose-acclimatized cells showed similar maximum specific growth rates (0.146 h^{-1}). The highest xylose consumption at the beginning of the fermentation process occurred using cells precultivated in xylose, which showed relatively high specific activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49). However, the maximum specific rates of xylose consumption (0.19 g_{xylose}/g_{cel} h) and xylitol production (0.059 $g_{xylitol}/g_{cel}$ h) were obtained with cells acclimatized in

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glucose, in which the ratio between xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) was kept at higher level (0.82). In this case, xylitol production (31.6 g/l) was 19 and 8% higher than in SSL and xylose-acclimatized cells, respectively. Maximum glycerol (6.26 g/l) and arabitol (0.206 g/l) production were obtained using SSL and xylose-acclimatized cells, respectively. The medium composition used for the yeast precultivation directly reflected their xylose fermentation performance. The SSL could be used as a carbon source for cell production. However, the inoculum condition to obtain a high cell concentration in SSL needs to be optimized.

Keywords Xylitol · Yeast · Xylose · Ammonia spent sulfite liquor-SSL · Inoculum adaptation · Enzymes

Abbreviations

XR	xylose reductase
XDH	xylitol dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase
XK	xylulokinase
μ_x	maximum specific cell growth rate (h^{-1})
μ_s	maximum specific xylose consumption rate (g_{xylose}/g_{cell} h)
μ_p	maximum specific xylitol production rate ($g_{xylitol}/g_{cell}$ h)
$Y_{p/s}$	xylitol yield coefficient ($g_{xylitol}/g_{xylose}$)
$Y_{x/s}$	cell yield coefficient (g_{cell}/g_{xylose})
Q_p	xylitol volumetric productivity ($g_{xylitol}$/l h)

Introduction

In the sulfite-pulping process, about 50% of the wood (hemicellulose and lignin) is dissolved to produce cellulose for paper along with the effluent ("spent sulfite liquor," SSL). SSL is the only lignocellulosic hydrolysate available today in large quantities (about 90 billion liters annually worldwide) [1], and it is produced at a rate of 1 ton (dry basis) per ton of pulp [2]. Many investigators have studied the possibility of using microbes to convert SSL into usable products. Its sugar content ranges from 3 to 4%, depending on the source of wood being pulped [3]. Softwoods have been the traditional feedstock, and their hexose sugars constitute 74% of these hydrolysates. However, hardwood sulfite pulping is becoming more popular, and the pentose sugars, principally xylose, in hardwood SSL can be up to 50% [2].

Recently, the conversion of xylose into value-added chemicals, such as xylitol, ethanol, and lactic acid have made this process attractive to the fermentation industry [4]. In particular, bioconversion for xylitol production has been intensively studied during the last decade because xylitol can be used as a functional sweetener [5].

The xylose reductase (XR) catalyzes the first step of a fungal pathway that allows certain organisms to metabolize xylose, such as *Candida boidinii* [6], *Candida guilliermondii* [7], *Candida tropicalis* [8], *Candida parapsilosis* [9], and *Debaryomyces hansenii* [10]. After the reduction of xylose to xylitol by XR in a manner that can utilize nicotinamide adenine dinucleotide (reduced form; NADH) or nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), xylitol is re-oxidized to xylulose by xylitol dehydrogenase, which is often specific for nicotinamide adenine dinucleotide $(NAD)^+$ [11]. Xylulose is then phosphorylated. An efficient, pathway should recycle the cosubstrate such that there is no

net conversion of NADPH into NADH resulting from xylose metabolism. Perturbations in this ratio have been linked to cell stress and xylitol excretion [12]. *Pichia stipitis* alcohol dehydrogenase-disrupted [13], xylitol dehydrogenase-defective [14], and D-xylulokinase-disrupted [15] mutants also produce xylitol from xylose. In the D-xylulokinase mutant, a metabolic pathway via arabinitol and ribulose-5-phosphate that bypasses the xylulokinase step was proposed as an alternative pathway mediating xylose assimilation. This mutant was named *P. stipitis* FPL-YS30 (*xyl3*-Δ) [15]. This pathway is also involved in L-arabinose assimilation [15].

The xylose present in SSL is difficult to ferment using native xylose-fermenting yeast strains due to the presence of sugar degradation products, acetic acid, lignosulfonates, sulfate, and occasionally, ammonia [3]. These compounds can inhibit sugar fermentation. Keating et al. [16] studied the tolerance and adaptation of ethanologenic yeasts to lignocellulosic inhibitory compounds and observed that the strains were severely affected by hardwood SSL (HSSL). This was attributed to specific syringyl lignin-derived degradation products and synergistic interactions between inhibitors [16]. Helle et al. [3] observed that xylose fermentation by genetically modified *Saccharomyces cerevisiae* 259ST in spent sulfite liquor produced up to 130% more ethanol as compared to fermentations using nonxylose-fermenting yeast. Conditions optimized for fermentation of pretreated HSSL to ethanol using an adapted strain of *P. stipitis* increased from 6.7 to 20.2 g/l [17].

The composition of the medium used for cultivation of microorganisms is directly reflected in their physiological phenotype and their fermentation performance, which in turn affect the results of strain analyses and strain performance in industrial applications [18]. In the present work, we grew cells on SSL to determine the influence of ammonium sulfite spent liquor SSL on *P. stipitis* FPL-YS30 (*xyl3*-Δ) xylitol production. The present research used SSL derived from an ammonia sulfite pulping of southern pine.

Materials and Methods

Preparation of Ammonia SSL

Ammonia SSL from pine (evaporated to 50% solids), which was obtained from Rayonier Performance Fibers, LLC (Fernandia Beach, FL, USA), was diluted to 15% *w*/*w* with water and adjusted to pH 6.0 with sodium hydroxide. Suspended solids were removed by centrifugation at 5,000 rpm for 5 min, and the remaining liquid was autoclaved at 111°C for 15 min.

Yeast Strain and Inoculum Media

P. stipitis FPL-YS30 (*xyl3*-Δ) was maintained on yeast peptone dextrose (YPD) agar medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 18 g/l agar) and stored at 4°C then transferred to fresh plate to be used within 24 h of incubation at 30°C. Cells were grown in hydrophobic cotton-plugged 1,000-ml Erlenmeyer flasks containing 400 ml of either YPD (10 g/l yeast extract, 20 g/l peptone, and 30 g/l glucose), yeast peptone xylose (YPX; 10 g/l yeast extract, 20 g/l peptone, and 30 g/l xylose) or YPSSL (10 g/l yeast extract, 20 g/l peptone, and 30 g/l total sugar) in an orbital shaker at 30°C and 200 rpm. Following 24 h growth, cell cultures were harvested, centrifuged (3,000 rpm per 15 min at 21°C), and decanted to yield cell pellets. Pellets were then washed once with sterile deionized water and

subsequently adjusted to a calculated concentration of 30 g dry cell weight (DCW) per liter via standard curves relating 600 nm absorbance to DCW per liter concentration. An aliquot was transferred to fresh fermentation medium for an initial cell concentration of 0.2 g DCW/l.

Batch Fermentations

Fermentations were performed in hydrophobic cotton-plugged 125-ml Erlenmeyer flasks containing 50 ml YPX liquid medium (10 g/l yeast extract, 20 g/l peptone, 80 g/l autoclaved xylose) in an orbital shaker at 30°C and 200 rpm. Initially, the YPX media was inoculated with pure cultures from inocula grown in different media (YPD, YPX, and YPSSL) to achieve an initial cell concentration of 0.2 g DCW/l. Samples were aseptically withdrawn during fermentation in 12 h interval. All fermentation experiments were performed in duplicate.

Preparation of Cell Extract and Enzyme Assay

Cells were harvested by centrifugation (16,000×*g*) for 5 min at 4°C, immediately frozen in liquid nitrogen and stored at −80°C. For the enzymatic assays, the samples were thawed rapidly under running water and held on ice. These samples were washed once and suspended in buffer 0.1 M 3-(*N*-morpholino)propanesulfonic acid, pH 7.0 with the addition of 1 mM β-mercaptoethanol and 1 mM of a protease inhibitor cocktail (phenylmethylsulfonyl fluoride), and disrupted by vortexing with glass beads (Sigma G8772; 15 min; 0°C). Cell debris and glass beads from the cell extract were separated by centrifugation (16,000×*g*, 10 min 4°C. Enzymatic activity was measured with two different sample concentrations using a spectrophotometer (Molecular Devices spectra max plus) operating at 340 nm and 30°C. The XR (EC 1.1.1.21) activity was measured in a reaction mixture by standard assay [19] modified by using 100 mM Tris–HCl buffer, pH 7.2; 100 mM xylose; and 0.2 mM NADPH. The xylitol dehydrogenase (XDH; EC 1.1.1.9) activity was measured by standard assay [19] modified by using a reaction mixture containing 100 mM Tris–HCl buffer, pH 8.6; 0.4 mM NAD^+ ; 5 mM $MgCl_2$, and 100 mM xylitol. Glucose-6-phosphate dehydrogenase was assayed as previously described by [20]. The xylulokinase (EC 2.7.1.17) activity was measured by standard assay [19] modified by using a reaction mixture containing 50 mM Tris–HCl buffer, pH 8.6 plus 5 mM $MgCl_2$; 0.2 mM NADH; 1.5 phosphoenolpyruvate; 1.0 mM adenosine triphosphate; 4 U pyruvate kinase and 4 U lactate dehydrogenase. Specific activities are expressed as units per milligram of protein. Units are defined as micromoles of NADH or NADPH reduced or oxidized per minute.

Determination of Sugars and Extracellular Metabolites

Xylose, xylitol glycerol, arabitol, ethanol, and acetic acid were determined by high performance liquid chromatography using a refractive index detector (Hitachi High-Technologies model L-2490, Japan) and Bio Rad (Hercules, CA, USA) Aminex HPX-87H column (300×7.8 mm) at 55°C; 0.005 M H_2SO_4 as eluent, at a flow rate of 0.3 ml min^{-1} and injection volume of 20 μl. Samples of 50% SSL diluted to 15% *w*/*w* was analyzed using the same detector and a Bio-Rad Aminex HPX-87P column (300×7.8 mm) at 80°C; deionized water as eluent, at a flow rate of 0.6 ml min^{-1} and injection volume of 20 μl. Samples were appropriately diluted in deionized water, and then filtered through 0.45 μm polyvinylidene fluoride filters before injection (20 μl).

Determination of Biomass and Protein Concentration

Time-dependent offline sampling of 1 ml aliquots was performed aseptically during fermentations. Samples were mixed immediately before dilution in deionized water, and then subjected to duplicate absorbance determination in a spectrophotometer at 600 nm. Diluted cell-free medium was used to establish background readings and set zero absorbance levels. Values were averaged and corrected for dilution. Protein concentration in cell-free extracts was determined by Bradford method [21], using bovine serum albumin as a standard.

Determination of Total Phenolic Content

The phenolic content of 50% SSL diluted to 15% *w*/*w* samples was determined by Folin–Ciocalteu reagent [22]. Each sample (0.1 ml at proper dilution) was added to 4.2 ml of deionized water and 0.5 ml of Folin–Ciocalteu reagent (Sigma). After 1 min of mixing, 1 ml of an 80% solution of sodium carbonate and 4.2 ml of deionized water was added. The mixture was left for 2 h at room temperature in dark, and the absorbance at 760 nm was measured. The concentration of total phenolic content was determined by a comparison with the values obtained with a standard solution of vanillin (Sigma).

Results and Discussion

Xylose Fermentation and By-Products

The waste spent sulfite pulping liquor (SSL) had a high solid content, so dilution was necessary before fermentation. The 50% SSL after dilution to 15% *w*/*w* contained 2.04 g/l glucose; 4.69 g/l mannose; 0.94 g/l arabinose; 2.83 g/l xylose; 1.25 g/l galactose, and 11.2 g/l total phenolic compounds.

Cell growth in yeast peptone (YP) medium with SSL as the carbon source (YPSSL) was only 80 or 94%, respectively, of cell growth in YPX and YPD medium (data not shown); however, precultivation in YPSSL resulted in the highest cell concentration (12.0 g/l; Fig. 1a) and cellular yield ($Y_{x/s}$; 0.198 g/g; Table 1) during fermentation of 80 g/l xylose. In this case, the maximum specific growth rate (μ_x; 0.143 h^{-1}) was similar to cells precultivated in YPD (0.148 h^{-1}). Jin et al. [23] previously reported the specific growth rates of *P. stipitis* FPL YS-30 and its parental strains (*P. stipitis* FPL-UC7) cultivated in YPX (40 g/l of xylose) as 0.06 and 0.28 h^{-1}, respectively (Table 1). The increase in xylose concentration to 80 g/l did not decrease the specific growth rate of the FPL-YS30. However, xylose consumption decreased 23% compared to Jin et al. [23].

Total xylose consumption was almost the same in all fermentations (Table 1), however, the specific xylose consumption rate (μ_s) varied. The highest (0.19 g_{xylose}/g_{cell} h) was obtained using cells precultivated in YPD. The next highest was with cells cultivated in YPSSL (0.141 g_{xylose}/g_{cel} h). The specific xylose assimilation rate by *P. stipitis* FPL YS-30 in YPX with 40 g/l of xylose was previously reported as 0.98 g_{xylose}/g_{cell} h [23], which was considerably higher than what we observed here in YPX with 80 g/l xylose.

The highest xylitol production that we observed (31.6 g/l; Fig. 2a) was obtained using cells precultivated in YPD. This inoculum condition also showed the highest specific xylitol production rate (μ_p; 0.059 $g_{xylitol}/g_{cell}$ h) and xylitol volumetric productivity (Q_p; 0.267 $g_{xylitol}$/l h; Table 1). However, the cells precultivated in YPX showed the highest

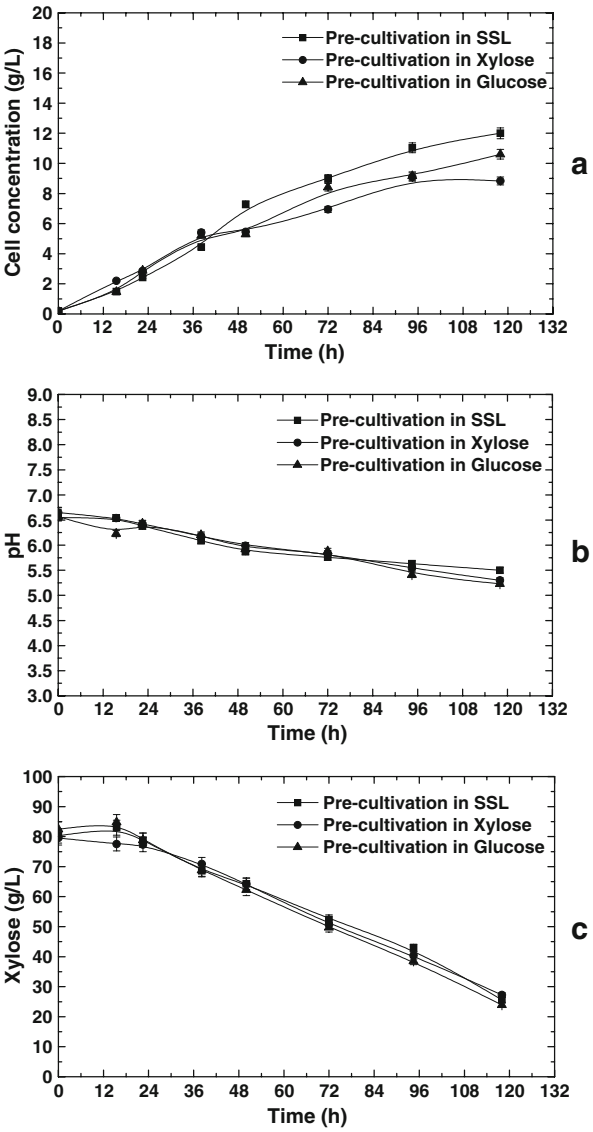

Fig. 1 Cell production (g/l; **a**), pH (**b**), and xylose consumption (g/l; **c**) during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in medium YPX

xylose conversion to xylitol ($Y_{p/s}$; 0.555 $g_{xylitol}/g_{xylose}$; Table 1) and a more stable specific xylitol production rate than cells precultivated in YPD. The highest xylitol concentration previously reported with YS30 was 13.2 g/l [23].

In general, cells precultivated in YPSSL and YPX showed similar and more stable values for specific xylose consumption and xylitol production rates than cells from YPD medium (data not shown). However, fermentation using cells precultivated in YPSSL showed lower values for specific xylose and xylitol production rates than cells precultivated in YPX.

Table 1 Parameter kinetics for xylitol production by a *P. stipitis* D-xylulokinase mutant previously grown in YP-SSL, YPX, and YPD media.

Parameters	Inoculum media		
	YP-SSL	YP-X	YP-D
Xylose consumption (%)	68.0±1.41	65.8±1.13	71.0±1.41
μ_x (h^{-1})	0.143±0.004	0.093±0.006	0.148±0.011
μ_s (g_{xylose}/g_{cell} h)	0.141±0.001	0.107±0.011	0.190±0.014
μ_p ($g_{xylitol}/g_{cell}$ h)	0.046±0.006	0.047±0.009	0.059±0.006
$Y_{p/s}$ (g/g)	0.469±0.004	0.555±0.078	0.539±0.013
$Y_{x/s}$ (g/g)	0.198±0.003	0.165±0.009	0.178±0.004
Q_p ($g_{xylitol}$/l h)	0.217±0.003	0.246±0.01	0.267±0.01

We did not observe any ethanol or acetic acid production. However, glycerol and a negligible amount of arabitol were formed (Fig. 2b, c). Cells precultivated in YPSSL showed the highest glycerol production (6.26 g/l; Fig. 2c) and the highest cellular growth (12.0 g/l; Fig. 1a). The inverse was observed using cells precultivated in YPX, which showed the lowest value for glycerol production (3.79 g/l; Fig. 2c) and cellular growth (8.8 g/l; Fig. 1a). Cells cultivated on YPSSL showed a strong tendency to produce more glycerol than cells precultivated in YPX and YPD (Fig. 2b, c).

Glycerol production could be related to cellular growth. Glycerol offsets redox imbalances that occur during yeast growth, and glycerol yields decrease as yeast growth decreases [24]. Differences in glycerol yields from cells cultivated on different media (Fig. 2b, c) indicates that the cell redox metabolism may differ. A lower glycerol yield on cells precultivated in YPX could be attributed to higher respiratory activity during growth in xylose, leading to a reduced need for glycerol production for maintenance of redox balance. The YP medium had an inherent buffering capacity (Fig. 1b) that maintained the pH around 6.0 (Fig. 1b). Glycerol yields are increased in fermentations near pH 7 [25]. However, the increased glycerol production (29%) in cell precultivated in YPSSL reduced the xylitol production (19%) compared with cells precultivated in YPD.

Cells precultivated in YPX showed the highest arabitol production (0.21 g/l; Fig. 2b). Jin et al. [15] previously proposed an alternative pathway for xylose assimilation in the D-xylulokinase mutant, *P. stipitis* FPL-YS30 (*xyl3-Δ*). This pathway (via arabinitol and ribulose-5-phosphate) is also involved in L-arabinose assimilation. Many yeast and fungi can aerobically assimilate L-arabinose, but most are enable to ferment it to ethanol or they exhibit only very low ethanol production rates and yields [29]. The rarity of ethanolic arabinose fermentation may be due to a redox imbalance in the fungal arabinose pathway as a result of the use of NADPH for the reductive reactions and the production of NADH in the oxidation reactions [29]. *P. stipitis* FPL-YS30 (*xyl3-Δ*) produces xylitol mainly because of insufficient glycolytic capacity of the alternative pathway rather than because of a redox imbalance, indicating that the oxidative phase of the pentose phosphate pathway, which produces NADPH for xylitol accumulation, is sufficient even without activity coded for by *XYL3* [15].

According to Shi et al. [26], there is a fundamental difference between the respiratory machinery supporting xylose and glucose metabolism using *P. stipitis* wild-type CBS 6054. However, addition of electron acceptors such as ketones and aldehydes in the medium [27] reduces xylitol formation. Such compounds are present also in lignocellulose hydrolysates [28] and most likely also in YP medium [29].

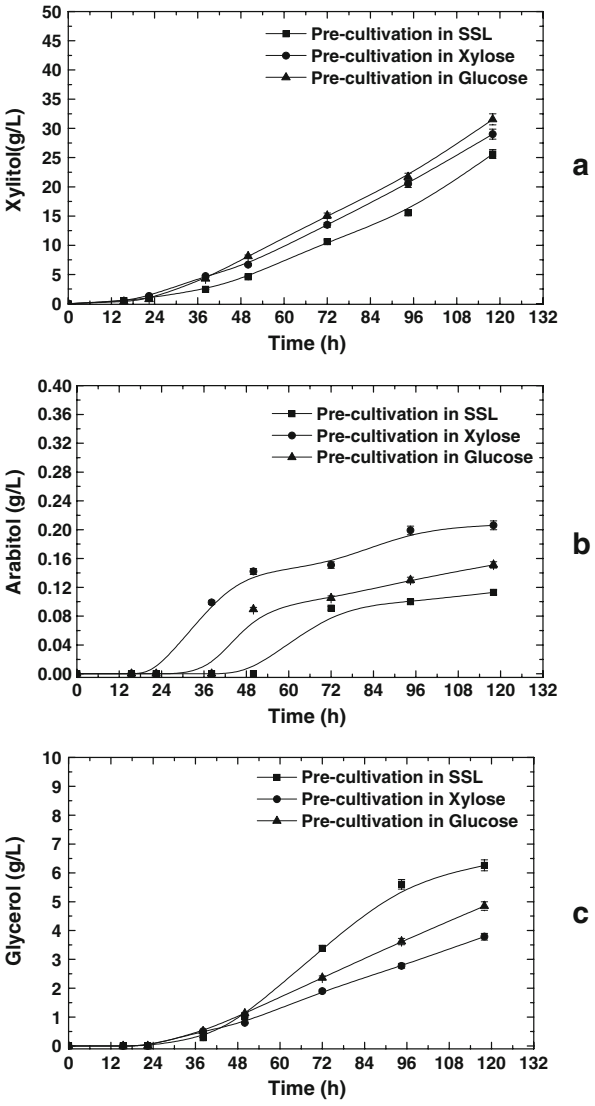

Fig. 2 Xylitol (**a**), arabitol (**b**), and glycerol (**c**) production during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in medium YPX

Enzyme Activities

Precultivation in YPX provided the highest maximum specific xylose reductase activity (0.349 U/mg_{prot}) after 38 h of fermentation (Fig. 3a). However, cells precultivated in YPSSL and YPD showed similar values for maximum specific XR activity (approximately 0.25 U/mg_{prot}) at 38 and 95 h, respectively. Initially, the XR activity in cells precultivated in YPD was not detectable, but after 23 h it was ≈0.22 U/mg_{prot}. This was also observed for cells precultivated in YPX. However, for cells precultivated in YPSSL XR, specific activity decreased approximately 44% after 38 h. Xylose consumption (Fig. 1c) and xylitol

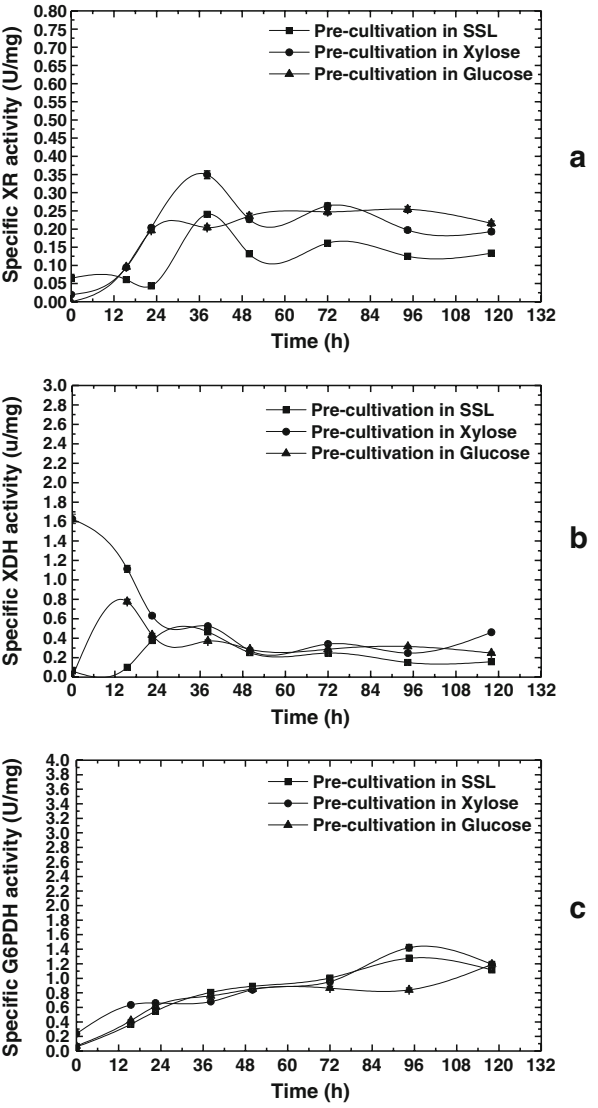

Fig. 3 Specific enzyme activities of XR (**a**), XDH (**b**), and G6PDH (**c**) during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in medium YPX

production decreased concomitantly (Fig. 2a) with enzymatic activity. The cells precultivated in YPD showed stability in XDH values around 0.3 U/mg_{prot} after 23 h of fermentation (Fig. 3b). In the other cases, there was an observed decrease in XDH maximum specific activity (Fig. 3b). The maximum specific xylitol dehydrogenase activity (1.11 U/mg_{prot}) was found using cells precultivated in YPX, followed by cells precultivated in YPD (0.778 U/mg_{prot}), in both cases after 16 h of fermentation. However, xylitol production has been partially ascribed to the difference in cofactor usage in the NAD(P)H-dependent XR and the NAD^+-dependent XDH reactions [7]. The highest xylitol production was obtained using cells precultivated in YPD, where this ratio XR/XDH was kept at higher

level (0.82). This behavior may have favored the specific xylitol production rate for cells precultivated in YPD.

A higher ratio of XR activity to XDH activity is essential for xylitol accumulation [12]. After the reduction of xylose to xylitol by XR in a manner that can utilize NADH or NADPH, xylitol is re-oxidized to xylulose by xylitol dehydrogenase, which is often specific for NAD^+ [11]. Xylulose can then be phosphorylated and enter general metabolic pathways. An efficient, high-flux pathway should recycle the cosubstrate such that there is no net conversion of NADPH into NADH resulting from xylose metabolism. In the D-xylulokinase mutant, *P. stipitis* FPL-YS30 (*xyl3-1*) [15], a metabolic pathway via arabinitol and ribulose-5-phosphate that bypasses the xylulokinase step, was proposed as an alternative pathway mediating xylose assimilation. This pathway is also involved in L-arabinose assimilation [15]. During the fermentations, the glucose 6-phosphate dehydrogenase (G6PDH) activity increased and practically reached a plateau of approximately 1.2 U/mg_{prot} (Fig. 3c). However, cells precultivated in YPX presented the highest G6PDH specific activity, 0.24 and 1.4 U/mg_{prot} at initial and 95 h of fermentation, respectively. The G6PDH enzyme regenerates NADPH, which is necessary for the consumption of xylose. The highest xylose consumption at the beginning of the fermentation process occurred using cells precultivated in YPX, which showed relatively high specific activity of G6PDH.

Conclusions

The medium composition used for *P. stipitis* FPL-YS30 inoculum development directly affected subsequent xylose fermentations. SSL could be used for cell production, but the growth conditions are not ideal. The capacities of cells cultivated in different media (YPSSL, YPX, and YPD) to procduce xylitol were equal, and thus the yields of xylitol were similar. However, differences in glycerol and arabitol yields showed that these may differ in subtle ways. We did not observe ethanol or acetic acid production. Cells exposed to a stressful environmental (YPSSL) showed high tendency to produce more glycerol than cells precultivated in YPX and YPD. The inverse was observed using cells precultivated in YPX, which showed the lowest value for glycerol production and cellular growth. However, cells precultivated in YPX showed the highest arabitol production. A portion of the reducing power is consumed in glycerol and arabitol formation that could improve xylitol production. Glycerol production in particular lowers the yield of xylitol. The highest xylitol production was obtained using cells precultivated in YPD, where the ratio XR/XDH was kept at higher level (0.82).The highest xylose consumption at the beginning of the fermentation process occurred using cells precultivated in YPX, which showed relatively high specific activity of G6PDH.

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