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ORIGINAL PAPER

Xylitol production from DEO hydrolysate of corn stover by *Pichia stipitis* YS-30

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Abstract Corn stover that had been treated with vapor-phase diethyl oxalate released a mixture of mono- and oligosaccharides consisting mainly of xylose and glucose. Following overliming and neutralization, a D-xylulokinase mutant of *Pichia stipitis,* FPL-YS30 (*xyl3*-Δ1), converted the stover hydrolysate into xylitol. This research examined the effects of phosphoric or gluconic acids used for neutralization and urea or ammonium sulfate used as nitrogen sources. Phosphoric acid improved color and removal of phenolic compounds. D-Gluconic acid enhanced cell growth. Ammonium sulfate increased cell yield and maximum specific cell growth rate independently of the acid used for neutralization. The highest xylitol yield (0.61 $g_{xylitol}/g_{xylose}$) and volumetric productivity (0.18 $g_{xylitol}/g_{xylose}$ l) were obtained in hydrolysate neutralized with phosphoric acid. However, when urea was the nitrogen source the cell yield was less than half of that obtained with ammonium sulfate.

Keywords Xylitol · *Pichia stipitis* FPL-YS30 · Corn stover hemicellulosic hydrolysate · Nitrogen source · Vapor-phase diethyl oxalate pretreatment · Overliming neutralization

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Introduction

Corn stover, the largest agricultural biomass resource in the USA, constitutes more than one-third of the total solid residues produced, including municipal solid waste [8]. Harvesting only 30–60% of the of 220 million tons available each year would yield 80–120 million tons of residue from this source alone [22]. Its composition is about 70% cellulose and hemicellulose and 15–20% lignin [22]. The hemicellulose components can be converted to monomeric and oligomeric sugars by various hydrolysis processes, to form a xylose-enriched liquid hydrolysate fraction [17].

Kenealy et al. [14] recently developed a process to treat wood chips of pine, spruce, aspen, and maple at 135–140°C with vapor-phase diethyl oxalate (DEO). Under the conditions used, DEO enters the lumen of the cell where water de-esterifies it to ethanol and oxalic acid (OA). Oxalic acid hydrolyzes the hemicellulose, and the sugars are then washed from the chips, concentrated, and fermented. The treated cellulose can be used for paper fiber or hydrolyzed to glucose. The amount and identity of carbohydrates released from the chips depend on the wood species [14]. Increasing the amount of DEO, reaction time, or temperature results in greater carbohydrate release. Mild treatment of wood chips with DEO can release carbohydrates suitable for fermentation and leave the cellulose largely intact. With more severe treatments, several by-products are formed or released in the hydrolysis process. The most important are furans (furfural and 5-hydroxymethylfurfural), carboxylic acids (i.e., levulinic, formic, and acetic acids), and phenolics (e.g., phenol, vanillin, vanillic acid, coumaric acid,

syringaldehyde, syringic acid, and 4-hydroxybenzaldehyde) [3, 26].

Various approaches are being considered to remove or to minimize fermentation inhibitors. Post-hydrolysis treatments include alkali or acid, activated charcoal, resin, solvent extraction, and vacuum evaporation [1, 6, 28]. Recently, the conversion of xylose into value-added chemicals, such as xylitol, ethanol, and lactic acid, has been an attractive goal for the fermentation industry [20]. The most important method utilized in the synthesis of xylitol involves the chemical reduction of xylose, which in turn is obtained by the acid hydrolysis of xylan present in the hemicellulose of birchwood, beechwood, or the structural plant tissues such as corn stalks, wheat straw, cotton seed, peanut hulls, sugarcane bagasse, wood pulp, and flax straw [20]. In particular, the bioconversion process for xylitol production has been intensively studied during the last decade because xylitol can be used as a functional sweetener [21].

Many yeasts will convert xylose to xylitol. *Pichia stipitis* normally has a very low xylitol yield, but it increases following some genetic modifications. Xylose reductase (XR) catalyzes the first step of a fungal pathway that allows certain organisms, such as *Candida boidinii* [31], *Candida guilliermondii* [27], *Candida tropicalis* [16], *Candida parapsilosis* [24], and *Debaryomyces hansenii* [4], to metabolize xylose. After XR reduces xylose to xylitol using NADH or NADPH, xylitol is re-oxidized to xylulose by an NAD^+-coupled xylitol dehydrogenase [19]. Xylulose is then phosphorylated by D-xylulokinase.

An efficient, high-flux pathway should recycle the cofactor so that there is no net conversion of NADPH into NADH resulting from xylose metabolism. Perturbations in this ratio have been linked to cellular stress and xylitol excretion [25]. *P. stipitis* mutants defective in alcohol dehydrogenase (ADH) [5], xylitol dehydrogenase [15], or D-xylulokinase each produce xylitol from xylose [11]. In the *P. stipitis* D-xylulokinase mutant, FPL-YS30 (*xyl3*-Δ1), an alternative metabolic pathway, perhaps via arabinitol and ribulose-5-phosphate, bypasses the xylulokinase step during xylose assimilation. This pathway is also involved in L-arabinose assimilation [11].

The fermentability of a hydrolysate can be affected by the acid used for neutralization. Phosphoric acid is much more effective than sulfuric, but also considerably more expensive. D-Gluconic acid is a non-toxic, easily biodegradable [7] hydroxycarboxylic chelating sugar acid that can be used as a sequestering agent for heavy metals in alkaline cleansing solutions and as a scale inhibitor [7]. Kim et al. [15] selected D-gluconic acid as a co-substrate for high-yield production of xylitol from xylose using the xylitol dehydrogenase defective mutant PXM-4 of *P. stipitis* CBS 5776 because gluconic acid neither blocked xylose transport nor repressed xylose reductase expression. In the present studies, we examined its efficacy as a neutralizing agent following $Ca(OH)_2$ overliming.

Materials and methods

Pretreatment of corn stover

The vapor-phase diethyl oxalate (DEO) pretreatment of corn stover was developed by Kenealy et al. [14]. Prior to treatment, corn stover was soaked in water for 10 min then drained. DEO (100 ml/0.75 kg stover) was added to a semi-pilot reactor, the configuration of which was described previously [13]. Following hydrolysis at 156°C for 30 min, the sugars were extracted with water, concentrated by reverse osmosis, and refrigerated at 3°C until use.

Treatment of hemicellulosic hydrolysate

Powdered $Ca(OH)_2$ (commercial grade) was slowly added to the hydrolysate at room temperature with constant mixing, thereby increasing the pH from 1.12 to 11.99. The pH was then reduced to 5.6 with 85% H_3PO_4 (Fisher Scientific) or 45–50 wt% D-gluconic acid (Sigma–Aldrich). After each pH change, precipitates from the overliming or acidification were removed by centrifugation at 16,279 *g*. Fermentation experiments were conducted in overlimed hydrolysate with added phosphoric (HP) or gluconic (HG) acids. Before fermentation, hydrolysates (HP and HG) were sterilized though 0.2 μm cellulose nitrate membranes (Nalgene).

Yeast strain and inoculum medium

Pichia stipitis (*xyl3*-Δ1) FPL-YS30 (=NRRL Y-30785) was maintained on malt agar medium (Difco Laboratories) and stored at 4°C. Cells were cultivated on fresh malt agar plates to be used within 24 h of incubation at 30°C. Cells were grown in 1,000-ml Erlenmeyer flasks containing 400 ml of YPX (10 g/l yeast extract, 20 g/l peptone, and 30 g/l xylose) in an orbital shaker (200 rpm) at 30°C. After 24 h, cell cultures were harvested, centrifuged (4,068 *g* per 15 min at 21°C), and decanted to yield cell pellets, which were washed once with sterile deionized water, and adjusted to a calculated concentration of 30 g dry cell weight (g_{DCW})/l using standard curves that related 600 nm absorbance to g_{DCW}/l. An aliquot was transferred to fresh fermentation medium for an initial cell concentration of 1.0 g/l.

Batch fermentations

Fermentations were performed in 125-ml Erlenmeyer flasks containing 50 ml corn stover hemicellulosic

hydrolysate treated as above. Both hydrolysates were supplemented with 5 g/l yeast extract supplemented with 5 g/l urea or 5 g/l $(NH_4)_2SO_4$ as a nitrogen source. The experiments were conducted in an orbital shaker at 30°C and 200 rpm. The hydrolysate media were inoculated with pure cultures grown previously in YPX media to achieve an initial cell concentration of 1.0 g_{DCW}/l. Samples were aseptically withdrawn during fermentation. All fermentation experiments were performed in duplicate.

Determination of sugars and extracellular metabolites

Xylose, xylitol glycerol, ethanol, D-gluconic acid, and acetic acid were determined using high-performance liquid chromatography using a refractive index detector (Hitachi High-Technologies Corporation model L-2490, Japan) and Bio Rad (Hercules, CA) Aminex HPX-87H column (300 × 7.8 mm) at 55°C using 0.005 M H_2SO_4 as eluant, at a flow rate of 0.3 ml/min and an injection volume of 20 µl. Samples were appropriately diluted in deionized water, and then filtered through PrepSEP C18 (Fisher Scientific) filters prior to injection (20 µl).

Determination of biomass

Aseptic samples (1 ml), taken with time, were mixed immediately prior to dilution in deionized water, and then subjected to duplicate absorbance determinations 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.

Determination of total phenolic content

The phenolic content of hydrolysate samples was determined using Folin–Ciocalteu reagent [30]. 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–Aldrich). After 1 min of mixing, 1 ml of an 80% solution of sodium carbonate and 4.2 ml of deionized water were added. The mixture was left for 2 h at room temperature in the 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–Aldrich).

Determination of color

The International Commission for Uniform Methods of Sugar Analysis (ICUMSA) color index was determined according to methodology developed by Napoles et al. [23]. Absorbance readings at 420 nm and degree Brix (% soluble solids) values of the hydrolysates (HG and HP) were determined in order to calculate this parameter.

Determination of specific rates

Specific rates of growth (μ), xylose consumption (μ_s), and xylitol production (μ_p) during the experiments were calculated according to the following equation:

$$\mu = \frac{1}{x}\frac{dx}{dt} \quad \mu_s = \frac{1}{x}\frac{ds}{dt} \quad \mu_p = \frac{1}{x}\frac{dp}{dt}$$

where μ = specific growth rate (h^{-1}), x = biomass concentration (g_{DCW}/l), μ_s = specific xylose consumption rate ($g_{xylose}/g_{cell}.h$), s = xylose concentration (g_{DCW}/l), μ_p = specific xylitol production rate ($g_{xylitol}/g_{cell}.h$), and p = xylitol concentration (g_{DCW}/l). The derivatives, dx/dt, ds/dt, and dp/dt, were calculated using a geometric method [18].

Results

Addition of D-gluconic and phosphoric acids to corn stover hemicellulosic hydrolysate

The corn stover hemicellulosic hydrolysate had the following partial composition before overliming and neutralization: 2.39 g/l glucose; 21.50 g/l xylose; 5.12 g/l arabinose; 1.6 g/l acetic acid; and 5.20 g/l total phenolic compounds. During the overliming using $Ca(OH)_2$ the xylose loss was ≈5%, glucose and arabinose removal was less than 5%; acetic acid was not removed. The DEO pretreatment generates oxalate, which precipitated under acidic conditions (pH 1.12) when the hydrolysate was stored at 4°C. It could be partially removed by centrifugation, and the remaining oxalate reacted with $Ca(OH)_2$ to form calcium oxalate. Given the low solubility of calcium oxalate, the bulk of the residual oxalic acid was precipitated in this step. The amount of D-gluconic acid required to decrease the hydrolysate pH to 5.6 was almost one-third of the amount of lime (80.7 g/l) used during the overliming process. The amount of phosphoric acid used to reach the same pH was 2.3 g/l, almost one-eleventh of the amount of D-gluconic acid. However, a significant precipitate was observed in the hydrolysate with phosphoric acid, which could be attributed to the formation of calcium phosphate. Phosphoric acid removed 13% more color and 23% more phenolic compounds from the hydrolysate than gluconic acid did.

Fermentation kinetics for xylitol production: the role of cell growth

Gluconic acid gave 60% more cell growth than phosphoric acid, even though more phenolic compounds were present.

This was independent of the nitrogen source used during fermentation (Fig. 1). Ammonium sulfate improved the cell yield ($Y_{x/s}$), independently of which acid was used for neutralization. Ammonium sulfate increased cell growth by 3.6 and 27.6% and the maximum specific growth rate by 82.3 and 96.8% using gluconic or phosphoric acid, respectively. The final pH decreased by 0.3 units with $(NH_4)_2SO_4$ and increased by 1.6 units when urea was used. These changes were independent of gluconic or phosphoric acid addition. The improved cell growth (Fig. 1) in phosphoric/$(NH_4)_2SO_4$ could be related to the medium pH. However, with gluconic/$(NH_4)_2SO_4$ additions and gluconic/urea additions the cell growth rates (data not shown) were similar, despite the pH variation (Fig. 1).

In all experiments, the small amount of acetic acid present ($\sim$1.6 g/l) was totally consumed concurrently with xylose, independently of which acid or nitrogen source was used in the hydrolysate (Fig. 2b). We did not observe significant differences in the consumption of acetic acid during the experiments (Fig. 2b). Also, we did not observe any inhibition that could be attributed to the presence of oxalate over the pH range used for fermentation.

D-Gluconic acid metabolism generates NADPH, which is essential for xylose consumption by yeasts. However, *P. stipitis* FPL-YS30 did not consume the gluconic acid added to the hydrolysate (26 g/l), perhaps due to inhibition by other sugars present in the hydrolysate. The gluconic/$(NH_4)_2SO_4$ treatment showed the highest cell yield (0.40 g_{cell}/g_{xylose}) along with the lowest xylitol yield (0.44 $g_{xylitol}/g_{xylose}$) and lowest xylitol volumetric productivity (0.12 $g_{xylitol}/g_{xylose}$ l). Using urea instead of $(NH_4)_2SO_4$ increased xylose consumption and xylitol production rates (Figs. 3a, 4a) by 25 and 34%, respectively. Also, urea increased xylose consumption by 47–50% in the hydrolysates in which gluconic or phosphoric acid were added (Fig. 3b). Similar high values for xylitol yield (0.61 $g_{xylitol}/g_{xylose}$) and xylitol volumetric productivity (0.18 $g_{xylitol}/g_{xylose}$ l) were obtained with phosphoric/urea and phosphoric/$(NH_4)_2SO_4$ treatment. However, the cell yield with phosphoric/urea was 56.8% less. Phosphoric/urea also favored xylitol production (Fig. 4b). Under this condition we observed the highest rates for xylose consumption (0.23 $g_{xylose}/g_{cell}.h$), and xylitol production (0.15 $g_{xylitol}/g_{cell}.h$) (Figs. 3a, 4a), and the highest arabinose consumption (Fig. 2a). However, xylitol production did not show a further clear difference in phosphate-neutralized hydrolysate using urea and ammonium sulfate as nitrate source (Fig. 4b). When using gluconic acid almost 50% improvement in xylitol production was found in hydrolysate adding urea rather than ammonium sulfate (Fig. 4b). In general, the *P. stipitis* FPL YS30 produced an insignificant amount of ethanol (less than 0.1 g/l), which was consumed during the fermentation. Glycerol was not detected under the conditions used.

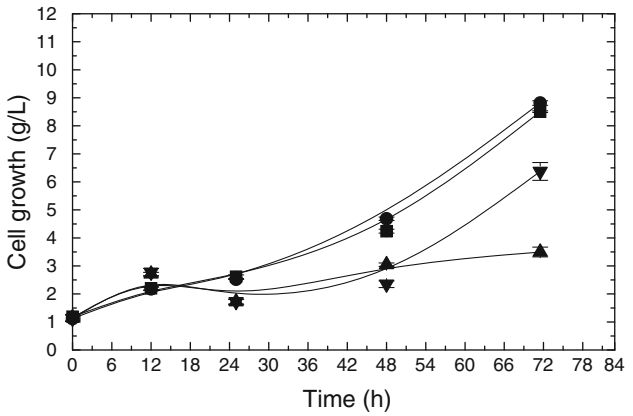

Fig. 1 Cell production during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in corn stover hemicellulosic hydrolysate with D-gluconic acid neutralization and urea as a nitrogen source (*filled squares*), gluconic acid with $(NH_4)_2SO_4$ (*filled circles*), phosphoric acid with urea (*filled triangles*), or phosphoric acid with $(NH_4)_2SO_4$ (*filled inverted triangles*)

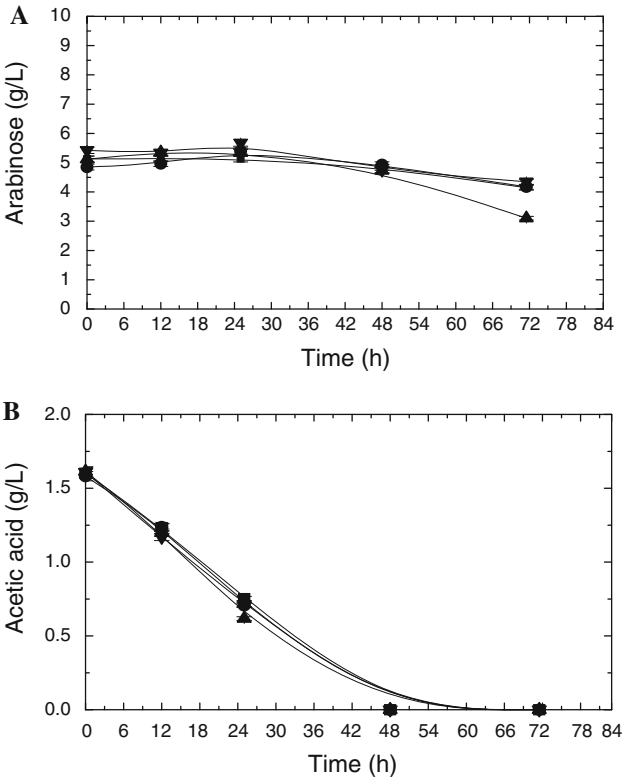

Fig. 2 Arabinose (**a**) and acetic acid (**b**) consumption during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in corn stover hemicellulosic hydrolysate with D-gluconic acid neutralization and urea as a nitrogen source (*filled squares*), gluconic acid with $(NH_4)_2SO_4$ (*filled circles*), phosphoric acid with urea (*filled triangles*), or phosphoric acid with $(NH_4)_2SO_4$ (*filled inverted triangles*)

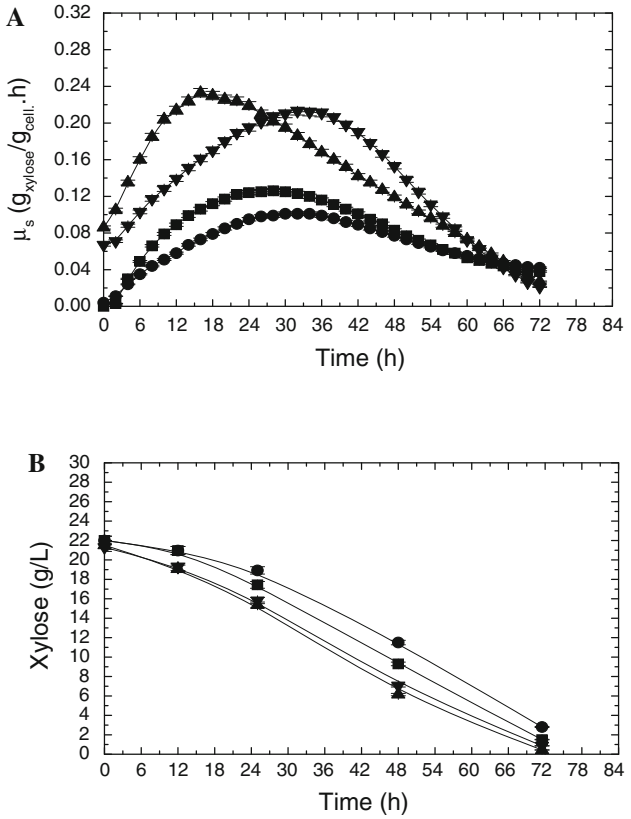

Fig. 3 Xylose consumption rate (**a**) and xylose consumption (**b**) during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in corn stover hemicellulosic hydrolysate with D-gluconic acid neutralization and urea as a nitrogen source (*filled squares*), gluconic acid with $(NH_4)_2SO_4$ (*filled circles*), phosphoric acid with urea (*filled triangles*), or phosphoric acid with $(NH_4)_2SO_4$ (*filled inverted triangles*)

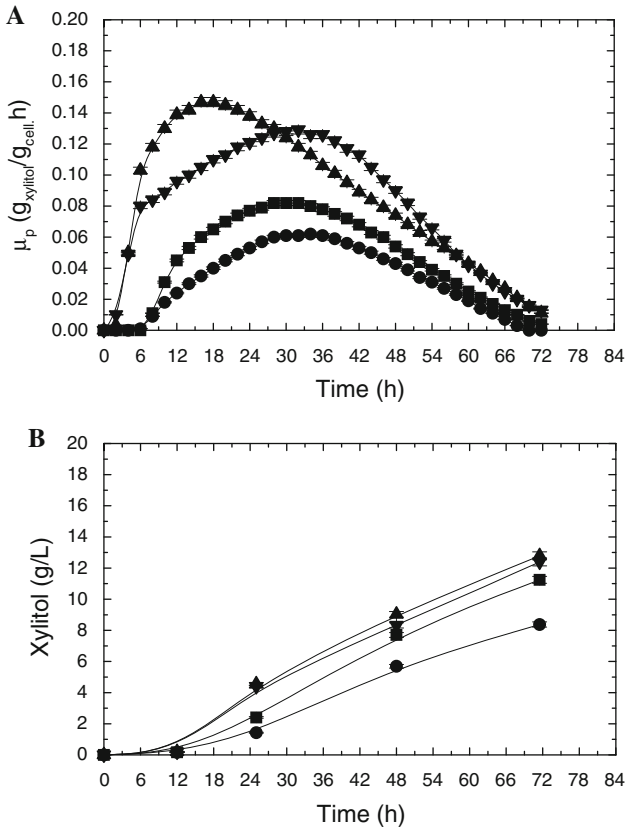

Fig. 4 Xylitol production rate (**a**) and xylitol production (**b**) during xylose bioconversion to xylitol by *P. stipitis* D-xylulokinase mutant in corn stover hemicellulosic hydrolysate with D-gluconic acid neutralization and urea as a nitrogen source (*filled squares*), gluconic acid with $(NH_4)_2SO_4$ (*filled circles*), phosphoric acid with urea (*filled triangles*), or phosphoric acid with $(NH_4)_2SO_4$ (*filled inverted triangles*)

Discussion

The DEO pretreated corn stover hemicellulosic hydrolysate had little acetic acid and total phenolics. Phosphoric acid neutralization further reduced phenolic compounds. This did not improve cell growth, but it did increase xylitol production—independently of the nitrogen source used during fermentation. In general, the lowest specific cell growth rate associated with the highest specific xylose consumption rate coincided with the highest conversion of xylose to xylitol and the highest xylitol volumetric productivity. The improvement observed in cell growth (Fig. 1) with phosphoric/$(NH_4)_2SO_4$ addition could be related to the medium pH. The additional role of urea in preventing rapid acidification during fermentation could negatively influence *P. stipitis* YS-30 growth in the hemicellulosic hydrolysate.

Acetic acid is a strong inhibitor of fermentative activity at acidic pH. We did not observe significant differences in the consumption of acetic acid during the experiments (Fig. 2b). Moreover, we did not observe any inhibition that could be attributed to the presence of oxalate over the pH range used for fermentation. Kim et al. [15] found that gluconic acid reduced the rates of xylitol production and cell growth by decreasing medium pH, and that the optimal concentration of gluconic acid was 20 g/l with approximately 100% xylitol conversion yield.

D-Gluconic acid metabolism generates the cofactor NADPH, which is essential to xylose consumption by yeasts. However, *P. stipitis* FPL-YS30 did not consume the gluconic acid added to the hydrolysate (26 g/l). This could be attributed to inhibition of assimilation by the presence of sugars in the hydrolysate. The hydrolysate is a complex medium, and formation of salts could occur very easily by addition of phosphoric acid. In this case, the removal of some essential micronutrient or mineral could reduce the cell growth rate. Addition of high amounts of sugar or salt to a growing culture of yeast stops growth [2]. This rapid impact has been explained by cell dehydration, through a purely osmotic process. Guebel and Nudel [9] obtained

maximum cell growth rates at low Ca^{2+} (0.34 mM) concentrations and low growth at high Ca^{2+} (1 mM) concentrations.

Overliming provides an excess of calcium in the hydrolysate. By using D-gluconic acid instead of phosphoric acid, the cell growth was favored (60%), even with a higher concentration of phenolic compounds. This might result from a reduction of toxic metals present in the hydrolysate. D-Gluconic acid could reduce the concentration of free metal ions by converting them to soluble chelates. In the presence of D-gluconate, calcium, iron, copper, aluminum, and other heavy metals are firmly chelated in alkaline solution and masked in such a way that their interferences are eliminated [29]. Aqueous solutions of D-gluconic acid added to a soil polluted with heavy metals (Cd, Cr, Cu, Ni, Pb, Zn) showed that their extractability was low under near-neutral and slightly basic pH conditions and that it increased drastically between pH 12 and 13. Applying D-gluconic acid at high pH (11.99) in the hydrolysate could play a role in controlling metal ion concentrations by formation of very stable coordination compounds [7].

Pichia stipitis FPL-YS30 cultivated in YPX (40 g/l of xylose) showed severely hindered xylose assimilation (0.98 $g_{xylose}/g_{cell}.h$ compared with 4.2 $g_{xylose}/g_{cell}.h$ for its parental strain FPL-UC7) [12]. However, comparing our results with those of Jin et al. [12], the *P. stipitis* FPL-YS30 cultivated in the hydrolysate (21 g/l of xylose) decreased the xylose consumption rate and xylitol volumetric productivity 76 and 18%, respectively, even while the xylitol yield increased 124%. The highest arabinose consumption by *P. stipitis* FPL-YS30 (39.42%), which also had the highest xylose consumption (98%), was obtained using phosphoric/urea addition (Fig. 2a). Rodrigues et al. [27] reported that *Candida guilliermondii* FTI 20037 consumed 13% of the arabinose from sugarcane hemicellulosic hydrolysate only after 98% of the xylose had been taken up. However, arabinose consumption was better when the inoculum was previously grown in hydrolysate and under those conditions, both xylose and arabinose were consumed simultaneously by the cells.

P. stipitis FPL-YS30 is a *xyl3* mutant [11]. It therefore must use some other pathway for xylulose phosphorylation. Jin et al. [11] hypothesized that YS30 xylose metabolism proceeds via arabitol and ribulose-5-phosphate, bypassing the D-xylulokinase step. This pathway is also involved in L-arabinose assimilation in *P. stipitis*. Relatively little is known about L-arabinose uptake by yeasts [10], so *P. stipitis* FPL-YS30 could be useful for this study. Further improvements to increase the xylose consumption rates by *P. stipitis* FPL-YS30 are needed.

Conclusions

The diethyl oxalate pretreated corn stover hemicellulosic hydrolysate generated a fermentable hemicellulosic hydrolysate with low concentrations of acetic acid and phenolic compounds. Phosphoric acid neutralization removed more color and phenolic compounds from the hydrolysate than gluconic acid did. However, D-gluconic acid neutralization enhanced cell growth. Ammonium sulfate increased cell yield and the maximum specific cell growth rate independently of which acid was used for neutralization. The highest xylitol yield (0.61 $g_{xylitol}/g_{xylose}$) and xylitol volumetric productivity (0.18 $g_{xylitol}/g_{xylose}$ l) were obtained similarly in hydrolysate with added phosphoric acid/urea and phosphoric/$(NH_4)_2SO_4$. However, with phosphoric/urea addition the cell yield was less than half of that obtained with phosphoric/$(NH_4)_2SO_4$. In general, the lowest specific cell growth rate associated with the highest specific xylose consumption rate coincided with the highest conversion of xylose to xylitol and the highest xylitol volumetric productivity. *P. stipitis* FPL-YS30 produced insignificant amounts of ethanol (less than 0.1 g/l), which was consumed during the fermentation and glycerol formation was not detected under the conditions used. Further improvements to increase the xylose consumption rates by *P. stipitis* FPL-YS30 are needed.

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