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Xylitol Production from Hardwood Hemicellulose Hydrolysates by *Pachysolen tannophilus*, *Debaryomyces hansenii*, and *Candida guilliermondii*

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Abstract

Three different yeasts, *Pachysolen tannophilus*, *Debaryomyces hansenii*, and *Candida guilliermondii*, were evaluated to ferment xylose solutions prepared from hardwood hemicellulose hydrolysates, among which *P. tannophilus* proved to be the most promising microorganism. However, the presence of both lignin-derived compounds (LDC) and acetic acid rendered a poor fermentation. To enhance the fermentation kinetics, different treatments to purify the hydrolysates were studied, including overliming, charcoal adsorption for LDC removal, and evaporation for acetic acid and furfural stripping. Under the best operating conditions assayed, 39.5 g/L of xylitol were achieved after 96 h of fermentation, which corresponds to a volumetric productivity of 0.41 g/L·h and a yield of product on consumed substrate of 0.63 g_p/g_s.

Index Entries: Xylitol; hemicellulose hydrolysate; *Debaryomyces hansenii*; *Candida guilliermondii*; *Pachysolen tannophilus*.

Introduction

Xylitol, a five-carbon polyol with sweetness comparable with that of sucrose, has found increasing use in the food industry thanks to several

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advantages. It has anticarcinogenic properties, is tolerated by diabetics, has been recommended for parenteral nutrition, does not cause acid formation, and has low viscosity and negative heat effect when dissolved in a solution *(1)*.

Currently, xylitol is manufactured by the chemical reduction of xylose present in lignocellulosic hydrolysates. Lignocellulosics are composed of cellulose (30–50%), hemicellulose (20–50%), and lignin (15–35%). These materials can be hydrolyzed to liberate sugars, but simultaneously some byproducts are formed. Thus, expensive separation and purification steps are necessary to remove these substances from xylose or xylitol *(2)*.

Xylitol can also be produced microbiologically from xylose solutions obtained by hydrolysis of lignocellulosic materials. In this way, hardwood hydrolysates have been employed by different investigators *(3–5)*. The biotechnological conversion of xylose solutions, which is a selective and promising process for xylitol production, can be carried out with fungi, bacteria, yeast, or purified enzymes from these microorganisms. The most studied xylitol producers are yeasts, with strains of the species *Candida guilliermondii*, *Pachysolen tannophilus*, and *Debaryomyces hansenii* among the best natural producers.

The fermentation of hydrolysates is hindered by inhibitors that can be present in the raw material or produced during chemical processing, such as furfural and hydroxymethylfurfural (generated by degradation of sugars); acetic acid (liberated from the acetyl groups of the raw materials); lignin degradation products and compounds derived from wood extractives (primarily composed of phenolic compounds); and inhibitors derived from the metals or minerals in wood, soil, or hydrolysis equipment *(6)*. To minimize their effects, several technologies have been employed, including adaptation of yeasts *(7–10)*, addition of reducing substances *(3,11–13)*, neutralization and overliming *(8,11,14–16)*, evaporation and steam stripping *(3,14,17)*, solvent extraction *(6,17,18)*, and charcoal adsorption *(5,6,12,13,19–23)*.

This present study deals with the evaluation of the three yeasts *D. hansenii*, *C. guilliermondii*, and *P. tannophilus* for xylitol production from hardwood lignocellulosic hydrolysates. To overcome the inhibitory effect of the foregoing substances present in the culture media, the hydrolysates were subjected to several detoxification methods. The combination of different treatments was necessary in order to carry out an effective fermentation of the xylose solutions into xylitol.

Materials and Methods

Preparation of Hemicellulose Hydrolysate

The hemicellulose hydrolysate, kindly supplied by Tennessee Valley Authority, was prepared from wood, primarily oak, impregnated under vacuum with 1% H_2SO_4 (w/w). After 1 h, chips were drained and exposed to about 12 atm steam for 4 min. The pressure was then released, and the residues were washed to extract the sugars. The sugar concentration was subsequently increased by washing successive batches of residue through

a countercurrent scheme. After removal of the residue, the remaining hydrolysate, consisting mainly of hemicellulosic sugars, was used for studies.

Preparation of Substrate

Hemicellulose hydrolysate contained 90.1 g/L of xylose, 5.0 g/L of glucose, 7.1 g/L of galactose, 5.2 g/L of arabinose, 6.9 g/L of mannose, 31.2 g/L of acetic acid, 1.2 g/L of furfural, and 2.9 g/L of hydroxymethylfurfural.

The hemicellulose hydrolysate, with a pH of 0.5 and a density of 1062 g/L, was neutralized by overliming, to eliminate inhibiting substances from the fermentation media. Overliming was performed by adding $Ca(OH)_2$ up to pH 10.0, filtering, and then adding sulfuric acid to pH 5.5. After treating with sodium sulfite (0.1% w/w), the precipitate was removed by filtration, and then the pH was readjusted to 5.5.

In selected experiments, previous to the overliming, a known volume of hydrolysate was evaporated to remove furfural and acetic acid, replacing any volume loss with heated distilled water.

Adsorption on Activated Charcoal

Powdered charcoal (Probus, Madrid, Spain) was mixed with neutralized hydrolysates for 1 h at the ratio 1:205 or 1:10 (g/g), as reported by Parajó et al. *(20)*. The liquors were recovered by filtration and treated again for an additional hour with the same amount of charcoal. The liquid phase was recovered by filtration and used for making culture media.

The charcoal was activated by boiling in distilled water for 3 h, filtering, and subsequently removing the excess water by evaporation at room temperature. The exhausted charcoal was regenerated following the same procedure.

Solvent Extraction

Solvent extraction of overlimed hydrolysate was evaluated by treating 50 mL of hydrolysate with 50 mL of diethyl ether in three successive steps for 6 h.

Microorganisms

Three different yeast strains were employed: *P. tannophilus* NRRL Y-2460, *D. hansenii* NRRL Y-7426 (kindly provided by the Northern Regional Research Laboratory, U.S.D.A., Peoria, IL), and *C. guilliermondii* NCR 5578 (kindly provided by L'Université Claude Bernard, Lyon 1, France).

Culture and Fermentation Media

The cells were grown in fermentation media containing: 10 g/L of pure xylose, 3 g/L of yeast extract, 3 g/L of malt extract, and 5 g/L of peptone. The microorganisms were maintained in agar slant tubes containing a medium formulated with the same components and concentrations as

the previous one plus 20 g of agar. Yeasts were adapted to hydrolysates by carrying out six successive batch cultures using the inocula obtained from the previous experiment. Fermentation media made from neutralized hydrolysates (with or without charcoal treatment or solvent extraction) were supplemented with 3 g/L of yeast extract, 3 g/L of malt extract, and 5 g/L of peptone, and sterilized in an autoclave. Incubation was conducted at 30°C under microaerophilic conditions in 100-mL Erlenmeyer flasks (containing 50 mL of culture media) placed in an orbital shaker at 200 rpm. The initial pH of media was adjusted to the desired value with H_2SO_4 or NaOH in the study of the optimization of the pH.

Analytical Methods

To obtain a semiquantitative estimation of the removal of phenolics, the 276 nm absorbance of hydrolysates was measured before and after charcoal adsorption *(20)*. At given fermentation times, samples from the fermentation media were taken, centrifuged, filtered through 0.45-μm membranes, and analyzed by high-performance liquid chromatography using two Shodex SH columns (mobile phase: H_2SO_4 0.01 *M*; flow rate: 0.7 mL/min; infrared and ultraviolet detection). This method allowed the determination of glucose, xylose, arabinose, acetic acid, ethanol, xylitol, and furfural. Biomass concentration was determined by dry weight filtering of known volumes of samples on 0.45-μm membrane filters.

Results and Discussion

Detoxification of Hydrolysate by Overliming and Adsorption on Activated Charcoal

Preliminary experiments were focused on the fermentation of raw hydrolysates neutralized by overliming. The hydrolysates were diluted up to one-half of the starting concentration in order to diminish the inhibitory effect provoked by the presence of inhibitory compounds. The overliming is known to ensure several beneficial effects, including partial removal of acids (acetic and tannic acids) and phenolic compounds *(14)*, precipitation of heavy metal ions *(24)*, as well as conversion of furfural into furfuryl acid *(3,12,24)*. As can be seen in Fig. 1, although the overliming was able to remove 61.15% of the lignin-derived compounds (LDC), no yeast was able to ferment this medium unless it was submitted to additional treatments. Xylose was barely consumed and negligible amounts of xylitol were detected, reaching a volumetric productivity of only 0.02 g_p/L·h (*see* Table 1).

To overcome the inhibitory effect of the LDC, the hydrolysates were treated with activated charcoal as described Parajó et al. *(21)*. Figure 1 shows that the higher the activated charcoal, the higher the LDC removal. The activated charcoal:hydrolysate ratio of 1:10 (g/g), which allowed the elimination of 95.40% of the LDC, was considered optimal. In fact, increasing the charcoal/hydrolysate ratio to 1:5 (g/g) did not represent a significant

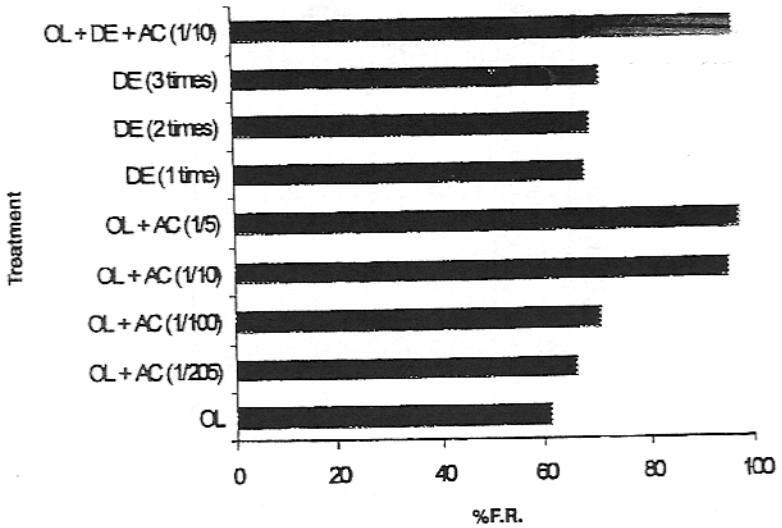

Fig. 1. Percentage of LDC removed from the hydrolysates after different treatments. OL, overliming; OL + AC (1/205), overliming followed by adsorption with activated charcoal in the charcoal:hydrolysate ratio 1:205 (g/g); OL + AC (1/100), overliming and charcoal:hydrolysate ratio 1:100 (g/g); OL + AC (1/10), overliming and charcoal: hydrolysate ratio 1:10 (g/g); OL + AC (1/5), overliming and charcoal:hydrolysate ratio 1:5 (g/g); DE (1 time), one extraction of overlimed hydrolysate with diethyl ether; DE (2 times), two successive extractions of overlimed hydrolysate with diethyl ether; DE (3 times), three successive extractions of overlimed hydrolysate with diethyl ether; OL + DE + AC (1/10), one extraction of overlimed hydrolysate with diethyl ether follow by adsorption with activated charcoal in the charcoal:hydrolysate ratio 1:10 (g/g).

improvement in the elimination of LDC removed, but increased the cost of treatment significantly.

Because an activated charcoal:hydrolysate ratio of 1:10 (g/g) is surely too expensive for practical application, the possibility of charcoal regeneration and reuse has been considered. The effect of six successive treatments with activated charcoal on LDC content of detoxified hydrolysate was evaluated. A progressive decrease in the adsorption efficiency was found. While a negligible loss of adsorption capacity was evident after the third treatment (reduction of LDC removal from 95.4 to 92.6%), the last three regeneration steps were responsible for a more marked effect (reduction up to 81.6%). These results suggest that the activated charcoal can certainly be reused three times without significant activity loss. However, an economic evaluation appears to be necessary to establish the optimal number of regeneration steps consistent with the detoxification requirements of the fermentation process.

Selection of the Best Microorganism for Xylitol Production

Several yeasts are reported to produce xylitol from different lignocellulosic hydrolysates with particular regard to *C. guilliermondii*, *P. tannophilus*, and *D. hansenii*. *C. guilliermondii* was used with sugar cane bagasse

Table 1
Fermentation Parameters for Different Yeasts Employed Using Raw or Charcoal-Treated Hydrolysates

Yeast	Charcoal:hydrolysate ratio (g/g)	S_0 (g_S/L)	X_0 (g_X/L)	Time (h)	S (g_S/L)	P (g_P/L)	Q_P (g_P/L·h)	$Y_{P/S}$ (g_P/g_S)
Candida guilliermondii	Raw	57.4	40.2	163.8	50.6	3.1	0.02	0.46
	1/205	64.6	37.3	115.3	7.8	14.1	0.12	0.25
	1/10	53.0	43.1	115.3	3.2	15.5	0.13	0.31
Debaryomyces hansenii	Raw	51.4	31.9	163.8	46.3	2.8	0.02	0.55
	1/205	53.9	30.6	115.3	9.5	15.5	0.13	0.35
	1/10	52.8	32.6	115.3	2.5	16.3	0.14	0.33
Pachysolen tannophilus	Raw	53.6	24.4	158.3	45.7	3.1	0.02	0.39
	1/205	59.5	31.7	115.3	12.0	12.5	0.11	0.26
	1/10	44.3	31.6	115.3	3.5	19.9	0.17	0.49

(25–29), rice straw hydrolysates (15,26,30–32), and *Eucalyptus* wood hydrolysates (33). *P. tannophilus* was utilized with sugar cane bagasse (34), sulfite liquors (35), and hardwood hydrolysates (36). Finally, *D. hansenii* was reported to produce xylitol from hardwood hydrolysates (4) and *Eucalyptus* wood hydrolysates (6,9,19–23).

To compare the relative ability of the selected microorganisms in producing xylitol, batch fermentations were carried out on detoxified hydrolysates subsequently treated with activated charcoal. Table 1 presents the results obtained using two different charcoal:hydrolysate ratios (1:205 and 1:10 [g/g]). Both *C. guilliermondii* and *D. hansenii* consumed the xylose slowly in spite of the high initial cell concentration (30 and 43 g/L, respectively), leading to relatively low concentrations of xylitol (14.1–16.3 g/L) and volumetric productivities (0.12–0.13 g/L·h). In both cases, increasing the charcoal:hydrolysate ratio from 1:205 to 1:10 (g/g) barely improved the fermentation parameters.

By contrast, using the yeast *P. tannophilus*, the adsorption with charcoal represented a strong increment in the xylitol concentration, which increased from 3.1 g/L with the raw hydrolysate to 12.5 g/L with the charcoal:hydrolysate ratio 1:205, and 19.9 g/L with the ratio 1:10 (g/g). These results suggest that, in spite of the low kinetic parameters achieved, *P. tannophilus* is the best yeast to ferment this hydrolysate. Thus, it was selected to perform the following experiments. The different behavior observed is owing to the concentration of a given inhibitor hindering the bioconversion of hydrolysates, which depends on the type of microorganism, the type of bioconversion assayed, and the operating conditions (9,37).

Extraction of LDC with Diethyl Ether

Finally, to confirm that the low kinetic parameters achieved were not owing exclusively to the LDC, another technique of elimination of these substances was studied. Parajó et al. (6) considered the detoxification of *Eucalyptus* wood hydrolysates using extraction with organic solvents, which removed some volatile compounds and found diethyl ether as the best compound assayed. However, in our case, this organic solvent showed a lower percentage of phenolic compounds removed (*see* Fig. 1), thus increasing the fermentation time with respect to charcoal treatment (from 120 h with charcoal treatment to 206.5 h with diethyl ether extraction) and decreasing considerably the final xylitol concentration (from 19.2 g/L with charcoal treatment to 11.8 g/L with diethyl ether extraction).

The scarce influence of the extraction with organic solvents was shown because the results attained combining both treatments (extraction and adsorption) were even worse (17.8 g/L of xylitol) than those obtained detoxifying exclusively with activated charcoal.

Elimination of Acetic Acid by Evaporation

Once it was shown that the LDC were not the only inhibitors present in the hydrolysates able to affect the yeast fermentation, we focused our

research on acetic acid removal. The acetic acid inhibitory action depends on the concentration of the undissociated form, which is a function of both concentration and pH. The sensitivity of yeasts to acetic acid depends on the microorganism considered. For the yeast *P. tannophilus*, Watson et al. *(34)* reported that acetic acid concentrations higher than 1.45 g/L inhibit its growth completely.

Evaporation can remove acetic acid, furfural, and some other volatile compounds *(3,14,17)*, allowing a faster fermentation *(38)*. To remove acetic acid from the fermentation broth, the hydrolysate was boiled for 3 h and samples were taken every 20 min. A boiling time of 160 min was found to be sufficient to decrease the acetic acid concentration from 31.2 to 1.0 g/L, which is below the inhibition threshold determined by Watson et al. *(34)* for this yeast. In addition, the furfural concentration decreased from 1.2 to <0.5 g/L.

Hydrolysates adequately stripped of acetic acid according to the preceding procedure were then used at different starting pH values in order to select the optimal value of this parameter as well as to investigate its effect on the kinetics of xylitol production by *P. tannophilus* under microaerophilic conditions. The results in Fig. 2 show three similar bell-shaped behaviors for all the kinetic parameters considered in this study, namely, the volumetric productivity (Q_p), the maximum specific productivity (v_m), and the yield of product on consumed substrate ($Y_{P/S}$). As a whole, these results indicate 6.0 < pH < 7.5 as the optimal pH range for xylitol production. This range is substantially lower than the optimal value (8.0) reported in the literature for *P. tannophilus* growth *(39)*, and much higher (5.5–6.0) than that observed by Beck and Strickland *(40)* for alcohol fermentation by the same yeast. This result confirms the hypothesis of a previous study in which the progressive adaptation of this microorganism was recognized as the major factor controlling the orientation of its metabolism toward xylitol or ethanol production *(41)*.

These promising results obtained using well-detoxified hydrolysates with relatively low starting xylose concentration suggested that the preceding detoxification treatments on the fermentability of the raw concentrated hydrolysate (without preliminary dilution and stripped of acetic acid) should be tested. Thus, batch fermentations were conducted with or without adsorption on charcoal to evaluate independently the inhibition of the acetic acid and the acetic acid and LDC together (Fig. 3). In the latter case (without charcoal adsorption), in spite of the absence of acetic acid, the LDC clearly hindered the bioconversion. This happened in a relatively less marked way when charcoal-treated hydrolysates at a ratio of 1:205 (g/g) were used. By contrast, using a charcoal:hydrolysate ratio of 1:10 (g/g), the fermentation was carried out efficiently, reaching 39.5 g_P/L of xylitol from 89 g_S/L of xylose after 96 h of fermentation, which corresponds to a volumetric productivity of 0.41 g_P/L·h and a product yield of 0.63 g_P/g_S. This result is indeed very promising considering the high xylose concentration of these hydrolysates.

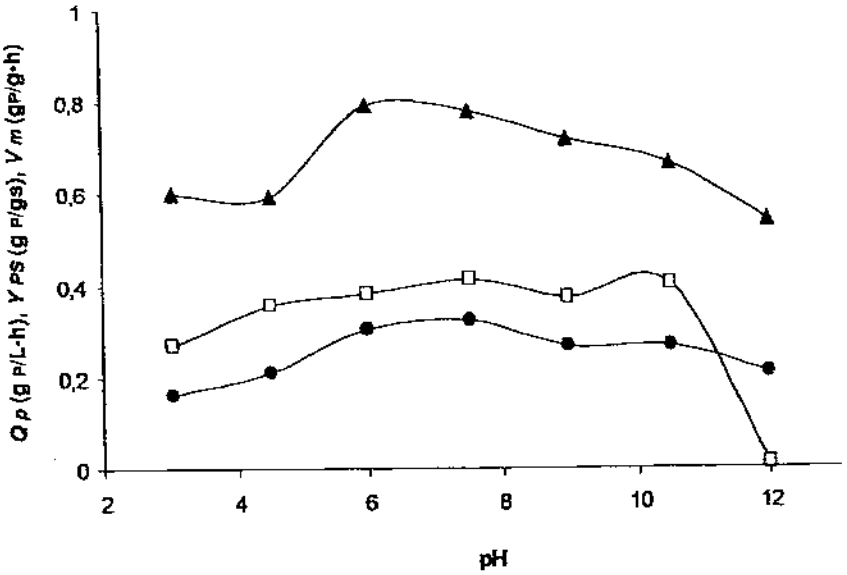

Fig. 2. Kinetic results of batch fermentations of hemicellulose hydrolysates carried out at variable pH with *P. tannophilus*. (●) Volumentric productivity, Q_p (g_p/L-h); (□), maximum specific productivity, v_m ($g_p/g_x h$); (▲), yield of product on consumed substrate, $Y_{p/s}$ (g_p/g_s).

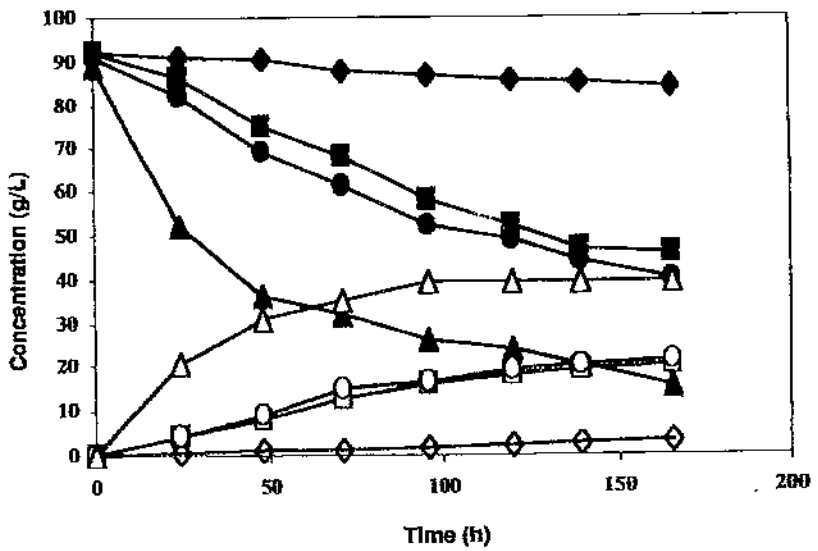

Fig. 3. Time course of xylose (solid symbols) and xylitol (open symbols) concentrations in batch xylitol fermentations by *P. tannophilus* from differently detoxified hydrolysates. (◆), Overliming without dilution nor acetic acid stripping. Treatment in addition to overliming and acetic acid stripping: (□) none; (○) activated charcoal adsorption at the ratio 1:205 (g/g); (△) activated charcoal adsorption at the ratio 1:10 (g/g).

Conclusion

From this investigation it can be concluded that, among the tested yeasts, *P. tannophilus* is the best xylitol producer using these hydrolysates, providing an efficient progressive adaptation of this microorganism. To minimize the inhibitory effects that certain substances present in the culture media have on the fermentation, it was necessary to submit the hydrolysates to several detoxification treatments. The overliming and the activated charcoal adsorption removed most of the LDC, and the evaporation was able to reduce the acetic acid concentration below the inhibition threshold. The combination of all these treatments allowed an efficient fermentation of the xylose solutions into xylitol.

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