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DETOXIFICATION OF HEMICELLULOSE ACID-HYDROLYZATES FOR XYLITOL PRODUCTION BY YEASTS

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Abstract. *Candida guilliermondii, Debaryomyces hansenii and Pachysolen tannophilus were evaluated to ferment xylose solutions prepared from hardwood hemicellulose hydrolyzates. Pachysolen tannophilus proved to be the most promising microorganism but the presence of both lignin derived compounds (LDC) and acetic acid rendered a poor fermentation. In order to enhance the fermentation kinetics, different treatments to purify the hydrolyzates were studied, including overliming, charcoal adsorption for the lignin derived compounds removal, and evaporation for acetic acid and furfural stripping. Under the best operating conditions assayed, 39.5 g_P/L of xylitol were achieved after 96 hours of fermentation, which correspond to a volumetric productivity of 0.41 $g_P/L \cdot h$ and a yield of product on consumed substrate of 0.63 g_P/g_S.*

Keywords: *Xylitol; Hemicellulose hydrolyzate; Debaryomyces hansenii; Candida guilliermondii; Pachysolen tannophilus.*

1. INTRODUCTION

Xylitol, a five carbon polyol with sweetness comparable to that of sucrose, has found increasing use in the food industry thanks to a certain number of advantages. In fact, it has anticariogenic 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 (Pepper and Olinger, 1988).

Currently, xylitol is manufactured by the chemical reduction of xylose present in lignocellulosic hydrolyzates. 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 (Hyvönen *et al.* 1982).

Xylitol can also be produced microbiologically from xylose solutions obtained by hydrolysis of lignocellulosic materials. In this way, hardwood hydrolyzates have been employed by different authors (Domínguez et al. 1996, Heikkilä et al. 1991, Perego et al. 1990). 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 the yeasts *Candida guilliermondii*, *Pachysolen tannophilus*, and *Debaryomyces hansenii* which are the best natural producers.

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The fermentation of hydrolyzates 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 (Parajó et al. 1997a). In order to minimize their effects, several technologies have been employed, including adaptation of yeasts (Amartey and Jeffries, 1996, Chen and Gong, 1985, Parajó et al. 1995, Roberto et al. 1991a), addition of reducing substances (Leonard and Hajny, 1945, Perego et al. 1990, Tran and Chambers, 1985, 1986), neutralization and overliming (Du Preez, 1994, Leonard and Hajny, 1945, Roberto et al. 1991a, b, 1994), evaporation and steam stripping (Delgenes et al. 1996, Perego et al. 1990, Roberto et al. 1991b, Wilson et al. 1989), solvent extraction (Parajó et al. 1997a, Wilson et al. 1989), and charcoal adsorption (Domínguez et al. 1996, 1999, Parajó et al. 1996a-c, 1997a, b, Tran and Chambers, 1985, 1986).

This work deals with the evaluation of the three yeasts *Debaryomyces hansenii, Candida guilliermondii* and *Pachysolen tannophilus* for the xylitol production from hardwood lignocellulosic hydrolyzates. In order to overcome the inhibitory effect of the foregoing substances present in the culture media, the hydrolyzates were undergone 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.

2. MATERIALS AND METHODS

2.1 Hemicellulose hydrolyzate preparation

The hemicellulose hydrolyzate, kindly supplied by Tennessee Valley Authority (TVA), 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 the residue removal, the remaining hydrolyzate, consisting mainly of hemicellulosic sugars, was used for studies.

2.2 Substrate preparation

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

The hemicellulose hydrolyzate, with pH 0.5 and density of 1062 g/L, was neutralized by overliming, in order 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 sulphuric acid to pH 5.5. After treating with sodium sulphite (0.1 % w/w), at room temperature, the precipitate was removed by filtration, and then the pH readjusted to pH 5.5. According to literature (Tran and Chambers, 1986), the improvements obtained with sulfitation could be related with the structural alterations induced in phenolic inhibitors

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

2.3 Adsorption on activated charcoal

Powdered charcoal (Probus, Madrid, Spain) was mixed with neutralized hydrolyzates during one hour in the ratio 1/205 or 1/10 g/g, as reported by Parajó *et al.* (1996b). The liquors were recovered by filtration and treated again during one 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.

2.4 Solvent extraction

Solvent extraction of the overlimed hydrolyzate was evaluated by treating 50 mL of hydrolyzate with 50 mL of diethyl ether in three successive steps during 6 hours (Parajó *et al.* 1997a).

2.5 Microorganisms

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

2.6 Culture and fermentation media

The cells were grown in fermentation media containing per liter: 10 g pure xylose, 3 g yeast extract, 3 g malt extract, and 5 g 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 hydrolyzates by carring out six successive batch cultures using the inocula obtained from the previous experiment. Fermentation media made from neutralized hydrolyzates (with or without charcoal treatment or solvent extraction) were supplemented with 3 g yeast extract/L, 3 g malt extract/L and 5 g peptone/L, and sterilized in autoclave. Incubation was conducted at 30°C under microaerophilic conditions in 100 mL Erlenmeyers flasks (containing 50 mL of culture media) placed in an orbital shaker at 200 rpm.

2.7 Analytical methods

In order to obtain a semiquantitative estimation of the removal of phenolics, the 276 nm absorbance of hydrolyzates was measured before and after charcoal adsorption (Parajó et al. 1996b). At a given fermentation time, samples from the fermentation media were taken, centrifuged, filtered through 0.45 μm membranes and analyzed by HPLC using two Shodex SH columns (mobile phase, H_2SO_4 0.01 M; flow rate, 0.7 mL/min; IR and UV detection). This method allowed the determination of glucose, xylose, arabinose, acetic acid, ethanol, xylitol, and furfural. Biomass concentration was determined by dry weight of filtered known volumes of samples on 0.45 μm membrane filters.

3. RESULTS AND DISCUSSION

3.1 Hydrolyzate detoxification by overliming and adsorption on activated charcoal

Preliminary experiments were focused on the fermentation of raw hydrolyzates neutralized by overliming. The hydrolyzates 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 benefic effects, including a partial removal of acids (acetic and tannic acids) and phenolic compounds (Roberto et al. 1991b), precipitation of heavy metal ions (Strickland and Beck 1984) as well as conversion of furfural into furfuryl acid (Perego et al. 1990, Strickland and Beck, 1984, Tran and Chambers, 1985). As it can be seen in Figure 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 hardly consumed and negligible amounts of xylitol were detected, reaching a volumetric productivity of only 0.02 $g_P/L \cdot h$ (see Table 1).

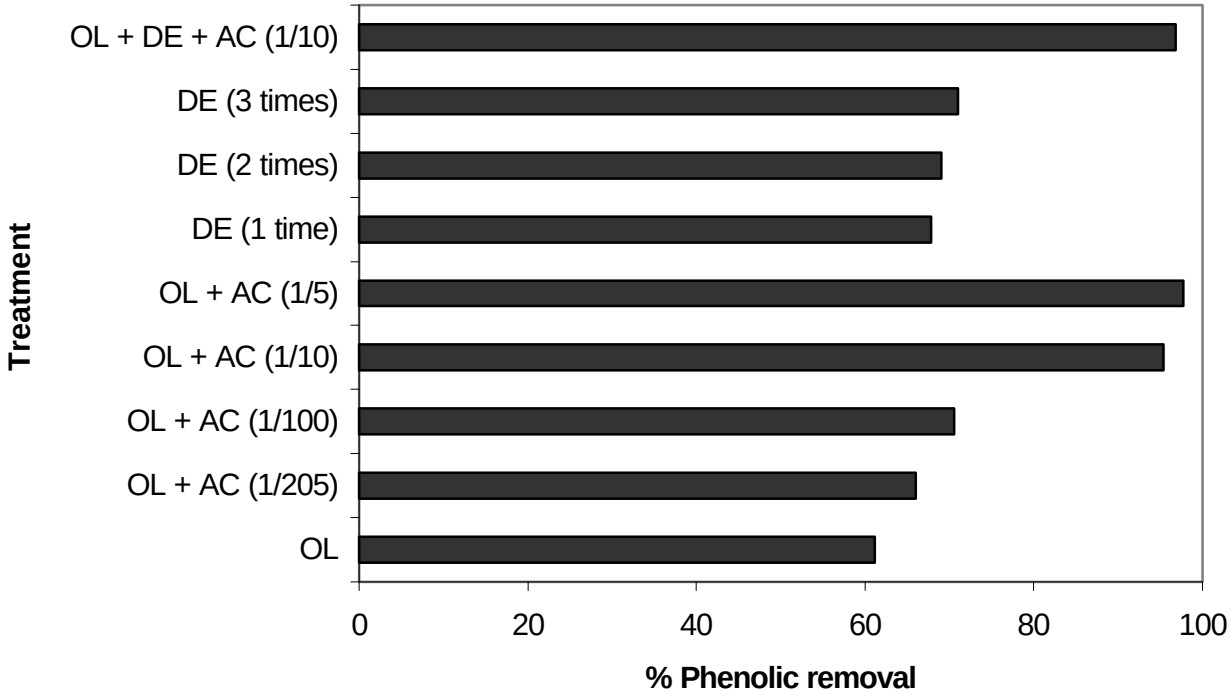

Fig. 1. Percent of lignin derived compounds removed from the hydrolyzates after different treatments: OL = overliming; OL + AC = overliming followed by adsorption with activated charcoal in different charcoal/hydrolyzate ratios; DE (n times) = n extractions of overlimed hydrolyzate with diethyl ether.

To overcome the inhibitory effect of the lignin-derived compounds, the hydrolyzates were treated with activated charcoal as described by Parajó *et al*. (1996c). Figure 1 shows that the higher activated charcoal the higher LDC removal. The activated charcoal/hydrolyzate ratio 1/10 g/g, which allowed elimination of 95.40 % of the LDC, was considered optimal. In fact, increasing the charcoal/hydrolyzate ratio up to 1/5 g/g did not represent a significant improvement in the elimination of LDC, while increasing the treatment cost significantly.

Since an activated charcoal/hydrolyzate ratio of 1/10 g/g is surely too expensive for practical application, the possibility of charcoal regeneration and re-use has been considered. The effect of 6 successive treatments with activated charcoal on LDC is shown in Table 2, which demonstrates a progressive decrease of the adsorption efficiency. While a negligible loss of adsorption capacity is evident after the third treatment (reduction of LDC removal from 95.4 to 92.6%), the last three regeneration steps were responsible for a lower effect (reduction up to 81.6%). These results suggest that the activated charcoal can certainly be re-used for three times without significant activity loss, while an economic evaluation appears to be necessary to establish the optimal number of regeneration steps consistent with the detoxification requirements of the fermentation process.

Table 1. Fermentation parameters for the different yeast employed using raw or charcoal treated hydrolyzates

Yeast	Charcoal/ Hydrolyzate 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/Lh)	$Y_{P/S}$ (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	0.30
	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	0.46
	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

3.2 Selection of the best microorganism for xylitol production

Several yeasts are reported to produce xylitol from different lignocellulosic hydrolyzates with particular regard to *Candida guilliermondii, Pachysolen tannophilus*, and *Debaryomyces hansenii*. *Candida guilliermondii* was used with sugar cane bagasse (Felipe et al. 1993, 1996a, 1997, Pfeifer et al. 1996, Roberto et al. 1995a), rice straw hydrolyzates (Roberto et al. 1994, 1995a, 1995b, 1996a, 1996b), and *Eucalyptus* wood hydrolyzates (Felipe et al. 1996b). *Pachysolen tannophilus* was utilized with sugar cane bagasse (Watson et al. 1984), sulphite liquors (Lindén and Hahn-Hägerdal, 1989), and hardwood hydrolyzates (Perego et al. 1994). Finally, *Debaryomyces hansenii* was reported to produce xylitol from hardwood hydrolyzates (Heikkilä et al. 1991) and *Eucalyptus* wood hydrolyzates (Parajó et al. 1995, 1996 a-c, 1997a, b, 1999, Domínguez et al. 1999).

To make a comparison on the relative ability of the selected microorganisms in producing xilitol, batch fermentations have been carried out on detoxified hydrolyzates subsequently treated with activated charcoal. The results obtained using two different charcoal/hydrolyzate ratios (1/205 and 1/10 g/g) are shown in Table 1. Both *Candida guilliermondii* and *Debaryomyces hansenii* consumed the xylose slowly in spite of the high initial cell concentration (30-43 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/hydrolyzate ratio from 1/205 to 1/10 g/g hardly improved the fermentation parameters.

Table 2. Percent of lignin derived compounds removed from the hydrolyzates after six successive steps of adsorption on activated charcoal, at the ratio 1/10 g/g

Nº of treatments	% Phenolic removal
1	95.40
2	92.81
3	92.57
4	86.57
5	85.97
6	81.57

On the contrary, using the yeast *Pachysolen tannophilus*, the adsorption with charcoal represented a strong increment in the xylitol concentration, which increased from 3.1 g/L with the raw hydrolyzate to 12.5 g/L with the charcoal/hydrolyzate 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, *Pachysolen tannophilus* is the best yeast to ferment this hydrolyzates, thus being selected to perform the following experiments. The different behavior observed is due to the concentration of a given inhibitor hindering the bioconversion of hydrolyzates, which depends on the type of microorganism, the type of bioconversion assayed, and the operating conditions (Maiorella et al, 1983, Parajó et al. 1995).

3.3 Extraction of LDC with diethyl ether

Finally, in order to confirm that the low kinetic parameters achieved were not due exclusively to the LDC, another technique of elimination of these substances was studied. Parajó *et al*. (1997a) considered the detoxification of *Eucalyptus* wood hydrolyzates 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 Figure 1), thus increasing the fermentation time and decreasing considerably the final xylitol concentration with respect to charcoal treatment (Table 3).

Table 3. Fermentation parameters for the yeast *P. tannophilus* using hardwood hemicellulose hydrolyzates subjected to different detoxification treatments

Treatment	S_0 (g_S/L)	X_0 (g_X/L)	Time (h)	S (g_S/L)	P (g_P/L)	Q_P (g_P/Lh)	$Y_{P/S}$ (g_P/g_S)
Adsorption with charcoal (1/10 g/g)	46.2	25.4	120	4.1	19.2	0.16	0.46
Extraction with diethyl ether	64.8	26.2	206.5	23.0	11.8	0.06	0.28
Extraction with diethyl ether + Adsorption with charcoal (1/10 g/g)	46.0	24.6	206.5	11.6	17.8	0.09	0.52

The low efficiency of the extraction with organic solvents was shown by the lower result obtained combining both treatments, extraction and adsorption, (17.8 g/L of xylitol) when comparing to that obtained detoxifying exclusively with activated charcoal (19.2 g/L of xylitol).

3.4 Acetic acid elimination by evaporation

Since it was shown that the LDC were not the only inhibitors present in the hydrolyzates able to affect the yeast fermentation, our research was focused on the 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 *Pachysolen tannophilus* Watson *et al*. (1984) reported that acetic acid concentrations higher than 1.45 g/L inhibit completely its growth.

Evaporation can remove acetic acid, furfural, and some other volatile compounds (Perego et al, 1990, Roberto et al. 1991b, Wilson et al. 1989), allowing a faster fermentation (Parekh et al. 1987). In order to remove acetic acid from the fermentation broth, the hydrolyzate was boiled for 3 hours and samples were taken every 20 min. As it can be seen in Figure 2, a boiling time of 160 minutes was enough to decrease the acetic acid concentration from 31.2 to 1.0 g/L, which is below the inhibition threshold mentioned by Watson *et al*. (1984) for this yeast. In addition, the furfural concentration decreased from 1.2 g/L to less than 0.5 g/L.

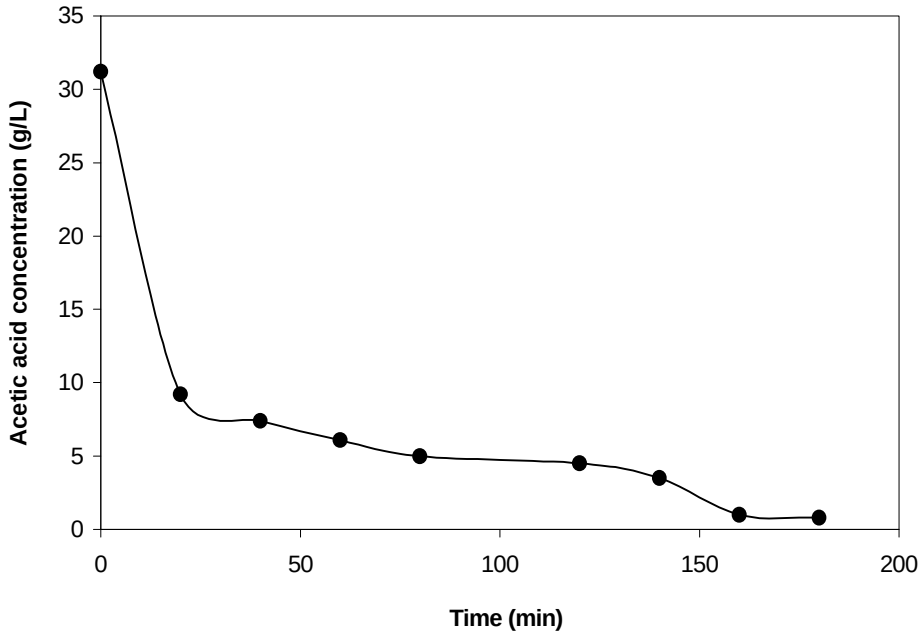

Fig. 2. Acetic acid concentration at different times during the stripping of hydrolyzates.

These promising results obtained using well-detoxified hydrolyzates with relatively low starting xylose concentration suggested to test the above detoxification treatments on the fermentability of the raw concentrated hydrolyzate (without preliminary dilution and stripping of acetic acid). Thus, batch fermentations were carried out without or with adsorption on charcoal to evaluate independently the inhibition of the acetic acid and the acetic acid and LDC together (Figure 3). In the former case (without charcoal adsorption), in spite of the absence of acetic acid, the lignin derived compounds clearly hindered the bioconversion. This happened in a relatively less marked way when charcoal-treated hydrolyzates in the ratio 1/205 g/g were used. On the contrary, using a charcoal/hydrolyzate ratio 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 hours of fermentation, which corresponds to a volumetric productivity of 0.41 g_P/Lh

and a product yield of 0.63 g_P/g_S. This result is indeed very promising also considering the high xylose concentration of these hydrolyzates.

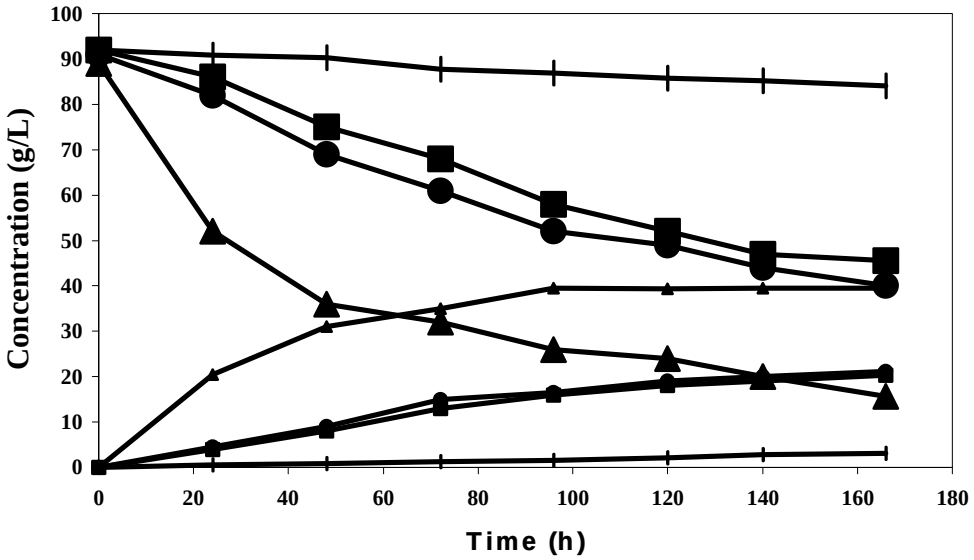

Fig. 3. Time course of xylose (higher symbols) and xylitol (smaller symbols) concentrations in batch xylitol fermentations by *P. tannophilus* from differently detoxified hydrolyzates. Overliming without dilution nor acetic acid stripping (→). Additional treatment besides overliming and acetic acid stripping: ( ) none; ( ) activated charcoal adsorption in the ratio 1/205 g/g; ( ) activated charcoal adsorption in the ratio 1/10 g/g.

4. CONCLUSIONS

Among the tested yeasts in this investigation, *Pachysolen tannophilus* is the best xylitol producer using these hydrolyzates. It was necessary to submit the hydrolyzates to several detoxification treatments in order to minimize the inhibitory effects that certain substances present in the culture media have on the fermentation,. The overliming and the activated charcoal adsorption removed most of the lignin derived compounds, while 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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