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Xylitol Production from *Eucalyptus* Wood Hydrolysates in Low-Cost Fermentation Media

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Summary

Several aspects concerning the bioconversion of xylose-containing hydrolysates (obtained from *Eucalyptus* wood) into xylitol were assessed. *Debaryomyces hansenii* yeast strains were adapted to fermentation media (obtained either by prehydrolysis or autohydrolysis-posthydrolysis of wood) supplemented with low-cost nutrients. Media containing up to 80 g/L xylose were efficiently fermented when the hydrolysates were detoxified by charcoal adsorption and supplemented with corn steep liquor.

Key words: autohydrolysis, *Debaryomyces hansenii*, *Eucalyptus* wood, low-cost fermentation media, posthydrolysis, prehydrolysis, xylitol

Introduction

Xylitol (molecular weight, 152.15) is a polyalcohol of high added value used as sweetener with interesting dietary and technological properties (*1*,*2*). For example, xylitol does not undergo the Maillard reaction, it improves the colour and taste without affecting the shelf life of food products, it is suitable for parenteral and diabetics nutrition, it has anticariogenic properties and facilitates the remineralisation of caries lesions (*3*).

The microbiological production of xylitol is an alternative to the commercial chemical synthesis, but several aspects must be optimised in order to minimise the production costs, particularly those associated with detoxification and nutrient supplementation of the fermentation media. The optimal nitrogen source for fermenting a xylose solution depends on both the microorganism employed and the origin of the carbon source, since some agricultural residues already provide the nutritional requirements (*4*,*5*). On the other hand, organic sources are, in general, better than inorganic ones (*6*,*7*), although the successful use of inorganic sources has been reported (*8*,*9*). Fractions derived from the processing of substrates such as corn show promise as potential nitrogen sources.

In the production of xylose-containing fermentation media, lignocellulosics containing xylan (a polymer consisting of xylose units) as the major hemicellulose component are subjected to hydrolysis. *Eucalyptus* wood was selected in this work because of its comparative advantages (large availability, non-seasonal character and high xylan content).

Treatments of lignocellulosics with dilute mineral acids (prehydrolysis) allow an extensive solubilisation of hemicelluloses, whereas most of cellulose and lignin remain in solid phase. The drawbacks of this technology lie in the corrosion of common construction materials and in the costs derived from consumption of acid and neutralising agent as well as in handling the neutralisation sludges.

Soluble reaction byproducts generated in the prehydrolysis step (*e.g.* acid-soluble lignin, sugar-decomposition products or acetic acid derived from acetyl groups)

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may hinder the fermentation of hydrolysates. Inhibition has noticeable effects on growth, fermentation and microbial morphology (*10–13*). In order to overcome the inhibitory effect of these compounds, physico-chemical (*14–19*) or biological detoxification (*20,21*) have been proposed. Adaptation of microorganisms to hydrolysates is also a suitable strategy (*22,23*). Dealing with *Eucalyptus* wood acid hydrolysates, physical (*24,25*) and chemical (*16,18*) detoxification stages and the use of high inoculum concentrations (*15,24*) are possible options to improve the fermentation yield and productivity.

As the severity of the chemical treatment is closely related to the inhibitory potential of hydrolysates (*19,26,27*), mild operational conditions are desirable. Based on this idea, autohydrolysis treatments of xylan-containing raw materials (in which water is the only reagent) present several comparative benefits over prehydrolysis, including limited lignin solubilisation and decreased sugar decomposition. In this procedure, the hydronium ions from both water and *in situ* generated compounds (acetic, uronic and phenolic acids) are responsible for the catalytic depolymerisation of xylan. In autohydrolysis, corrosion is limited, no sludges are generated and low capital and operational costs are involved (*28*).

This work deals with the experimental assessment of biotechnological xylitol production from detoxified *Eucalyptus* wood hydrolysates supplemented with low-cost nutrients. In order to limit the severity of the conditions used in the hydrolysis of xylan, hydrolysates were obtained by sequential stages of autohydrolysis and posthydrolysis. Other factors affecting xylitol production (including microbial adaptation to the raw and detoxified hydrolysates, utilisation of increased substrate concentrations and type of *Debaryomyces* strain) are also considered.

Materials and Methods

Raw material

Eucalyptus globulus wood chips obtained from a local pulp mill were milled to a particle size less than 1 mm, homogenised in a single lot, air-dried, stored and used for experimentation. The xylan content of samples was 17.4 % (oven-dry basis).

Prehydrolysis of wood

Eucalyptus wood milled samples were treated in autoclave at 130 °C with 3 % sulphuric acid during 1 hour using a liquid/wood mass ratio = 8 g/g. The liquid phase from the acid hydrolysis was neutralised with $CaCO_3$ to a final pH of 6.5, and the $CaSO_4$ precipitate was separated from the supernatant by filtration. Neutralised hydrolysates contained in 1 L on average 17.1 g xylose, 1.7 g glucose, 2.1 g arabinose, 5.2 g acetic acid and less than 0.5 g furfural.

Autohydrolysis-posthydrolysis of wood

Milled *Eucalyptus* wood samples were subjected to non-isothermal autohydrolysis for 40.9 min using water to solid ratio of 8 g/g to reach the maximum temperature of 195 °C. Under these conditions, 83 % of the xylan contained in the raw material is solubilised as xylo-oligosaccharides and xylose (*29*). Acid post-hydrolysis of autohydrolised liquors was carried out to split the xylo-oligosaccharides (which can not be fermented by the yeast) into xylose. The operational conditions (leading to maximal xylose concentrations with minimum sugar-dehydration products) were as follows (*29*): sulfuric acid concentration 1 % (mass), temperature 125 °C and reaction time 60 min. The liquid phase from posthydrolysis was neutralised with $CaCO_3$, and the $CaSO_4$ precipitate was separated from the supernatant by filtration. The liquors obtained after posthydrolysis contained 18.7 g/L xylose, 3.4 g/L glucose, 1 g/L arabinose, 4.1 g/L acetic acid, 0.1 g/L furfural and less than 0.08 g/L hydroxymethylfurfural.

Solvent extraction

Neutralised hydrolysates were detoxified by extraction with diethylether under reported operational conditions (*18*). Hydrolysates and organic solvent were mixed for 60 min at room temperature, and then the aqueous phase, which was concentrated under reduced pressure to remove traces of diethylether and used to prepare culture media, was separated.

Charcoal adsorption

Activated charcoal was prepared by treating powdered charcoal (Probus, Madrid, Spain) with hot water and dried at room temperature. Charcoal detoxification of hydrolysates was carried out using a hydrolysate to charcoal ratio of 10 g/g. Both phases were stirred at room temperature for one hour. The liquid phase was recovered by filtration and used for preparation of culture media.

Microorganisms

Two strains of the yeast *Debaryomyces hansenii* were used in experiments. *Debaryomyces hansenii* NRRL Y-7426 was kindly provided by the Northern Regional Research Laboratory (Peoria, Illinois) and *Debaryomyces hansenii* CCMI 942 was kindly provided by Dr. Gírio from INETI, Lisbon, Portugal.

Solid culture media

Freeze-dried cells were grown in a culture medium containing (*per* litre) 10 g commercial xylose, 3 g yeast extract, 3 g malt extract and 5 g peptone. Microorganisms were maintained in agar slant tubes containing a medium formulated with the same components and concentrations as the previous one plus 20 g/L agar. The adapted yeasts were maintained in agar slants made from hydrolysates (diluted when necessary).

Fermentation media and conditions

Bioconversion of media made from hydrolysates was performed at 30 °C in 100 mL Erlenmeyer flasks containing the desired amount of culture media placed in an orbital shaker at 200 or 300 rpm. The initial pH of the media was set at 5.5–6.5 and the initial biomass concentration was in the range of 0.1–30 g/L. Additional

experiments were carried out in a Braun Biostat B fermentor with pH, temperature and dissolved oxygen control.

Analytical methods

Xylose, ethanol, xylitol, glucose, arabinose and acetic acid were analysed by HPLC using an interaction ion column (mobile phase, H_2SO_4 0.01 M; flow rate 0.4 mL/min; IR and UV detection), after centrifugation and filtration of samples through 0.45 μm membranes. Dry cell weight was determined after successive steps of centrifugation, washing and oven-drying.

Results and Discussion

Adaptation of the yeasts to progressively lower supplementation

Corn Steep Liquor (CSL) and ammonium sulfate (AS) were used as nitrogen sources. Both products show economic advantages as compared to the typical supplementation, yeast extract (YE) – malt extract (ME) – peptone (P), usually employed in xylitol fermentation, which was also employed in this work as a reference. Combinations of CSL or AS with yeast extract and/or malt extract and/or peptone were also considered (see below). The strain *Debaryomyces hansenii* CCMI 942 was adapted to progressively reduced concentrations of the selected nitrogen sources by performing a series of successive fermentations. After each fermentation run, the adapted yeasts were grown in agar plates formulated with the same type and concentration of nitrogen source employed in the corresponding fermentation, stored at 4 °C and used to inoculate the next medium. The microorganisms were considered to be adapted to a given medium when successive fermentations did not result in shorter *lag* periods and/or fermentation times.

Table 1 summarises the composition of the fermentation media before and after fermentation as well as the main fermentation parametres achieved with fully adapted microorganisms. Media containing 5 g/L of CSL or AS led to remarkable product yields ($Y_{P/S}$). When the concentration of CSL was reduced from 5 to 3 g/L, little variation in volumetric productivity (Q_p) was observed. At the same concentration of 3 g/L, better results were obtained in media containing CSL than in media made with AS. Because of this, the rest of experiments were carried out with media containing 3 g/L of CSL. Considering that all the assays were started with a loopful of cells, the data of Table 1 show a significant production of biomass, a situation similar to that reported for the same yeast strain with mixtures of sugars in batch cultures (*30*) and in continuous operation (*31*).

Comparison of prehydrolysis and autohydrolysis-posthydrolysis

The bioconversion of prehydrolysates with the strain *D. hansenii* CCMI 942 was characterised by the presence of *lag* phase and prolonged fermentation times. In order to improve both aspects, detoxification steps were implemented, and hydrolysates were obtained by a different method (autohydrolysis-posthydrolysis instead of prehydrolysis). Fermentation media made with this procedure were neutralised to pH = 5.5, inoculated with a loopful of adapted yeasts and fermented in Erlenmeyer flasks placed in an orbital shaker (200 rpm) at 30 °C, using a ratio (volume of medium):(volume of flask) = 1/5. The media made from autohydrolysis-posthydrolysis had an advantage over media made from prehydrolysis, with 25 % increase in xylitol production after 100 h of fermentation. However, other aspects concerning fermentation (such as the presence of *lag* phase, slow fermentation kinetics and excess of biomass production) were still not favourable (data not shown).

In previous studies, the operational conditions for the fermentation of acid hydrolysates from *Eucalyptus* wood with *D. hansenii* NRRL Y-7426 were optimised (*16,25*). Due to the high biomass production observed

Table 1. Data obtained in fermentations with *Debaryomyces hansenii* CCMI 942 cells adapted to different media

γ (supplementation)/(g/L)					γ (initial concentration)/(g/L)					γ (final concentration)/(g/L)					
YE	ME	P	CSL	AS	Xylose	Glucose	Arabinose	Acetic acid	Ferment. time / h	Biomass	Xylitol	Ethanol	Consumed xylose / %	$\frac{Y_{P/S}}{g/g}$	$\frac{Q_P}{g \cdot L^{-1} \cdot h^{-1}}$
3	3	5	0	0	27.90	3.08	4.28	1.94	216	6.07	13.01	0.36	86.52	0.54	0.06
3	0	4	1	0	33.38	4.11	9.38	2.80	72	2.51	15.73	0.84	81.25	0.58	0.22
3	0	3	2	0	16.99	1.78	7.99	3.20	72	5.09	5.93	0.17	64.27	0.54	0.08
2	0	3	2	0	14.19	0.00	6.18	3.34	48	4.54	6.76	0.09	50.18	0.95	0.14
1	0	2	5	0	25.38	4.18	0.09	0.03	192	5.18	10.03	1.75	81.64	0.48	0.05
0	0	2	5	0	25.38	4.18	0.09	0.03	192	6.26	12.97	1.05	80.06	0.64	0.07
0	0	0	5	0	20.41	2.61	9.13	1.94	216	5.87	15.70	0.07	87.56	0.88	0.07
0	0	0	3	0	28.54	4.46	6.36	2.31	192	6.01	15.83	1.02	82.34	0.67	0.08
0	0	0	1	0	28.83	7.87	1.87	2.58	96	4.65	2.86	0.00	38.04	0.26	0.03
1	0	2	0	5	22.60	3.38	0.10	0.02	192	6.45	11.50	0.95	79.87	0.64	0.06
0	0	0	0	5	25.88	0.00	6.51	2.64	216	5.81	14.93	0.03	89.99	0.64	0.07
0	0	0	0	3	29.07	3.65	6.20	2.20	192	6.58	9.09	0.00	63.91	0.49	0.05
0	0	0	0	1	24.59	0.91	7.63	1.34	120	6.23	2.12	0.00	12.60	0.68	0.02

with the CCMI 942 strain, the operational conditions for this yeast (especially the oxygenation of cultures in Erlenmeyer flasks) were also optimised (data not shown). For this set of fermentations, which were carried out at 30 °C, the best conditions were pH=5.5, 300 rpm and a medium to flask volume ratio of 0.5. In further experiments, each strain was used at their respective optimal conditions (for *D. hansenii* NRRL Y-7426: 200 rpm, volume ratio 0.2; for *D. hansenii* CCMI-942:300 rpm, volume ratio 0.5).

Effect of aeration on cell adaptation

The beneficial effect obtained by recycling the biomass in a sequence of repeated batch cultures has been confirmed both in experiments carried out in Erlenmeyer flasks (*32*) and in assays performed in a stirred fermentor with oxygen supply (*25*). In the fermentation of hydrolysates, the aeration rate can affect the yeast adaptation to the medium by overcoming the inhibitory effect of some compounds (*4,33,34*). As the stirring rate has an effect on the oxygen transfer coefficient approximately twice stronger than the aeration rate, new fermentation assays were carried out in a commercial bioreactor with stirring control in order to speed up the adaptation of yeasts. Fig. 1 shows data corresponding to a sequence of trials carried out in the stirred fermentor at 700 rpm (Figs. 1.a, 1.b and 1.c). In the experiments presented in Figs. 1.a to 1.c, a high stirring rate (leading to high oxygenation transfer rate) was selected to overcome inhibitory effects. Under the conditions in Fig. 1.a, the *lag* phase lasted for almost 100 h and then the sugars were utilised successively, as it was described for both synthetic mixtures (*30,31,35*) and hydrolysates (*36*). Xylose was metabolised only after glucose depletion, leading to biomass and ethanol as major fermentation products. In the second stage (Fig. 1.b) no *lag* phase was observed, and glucose was consumed faster than in the first culture. Finally, the overall fermentation time was considerably reduced in the third fermentation (Fig. 1.c), and the xylitol production increased significantly. Operation under high aeration conditions results in a remarkable decrease in the number of successive fermentations needed to achieve the full adaptation of cells to the corresponding media.

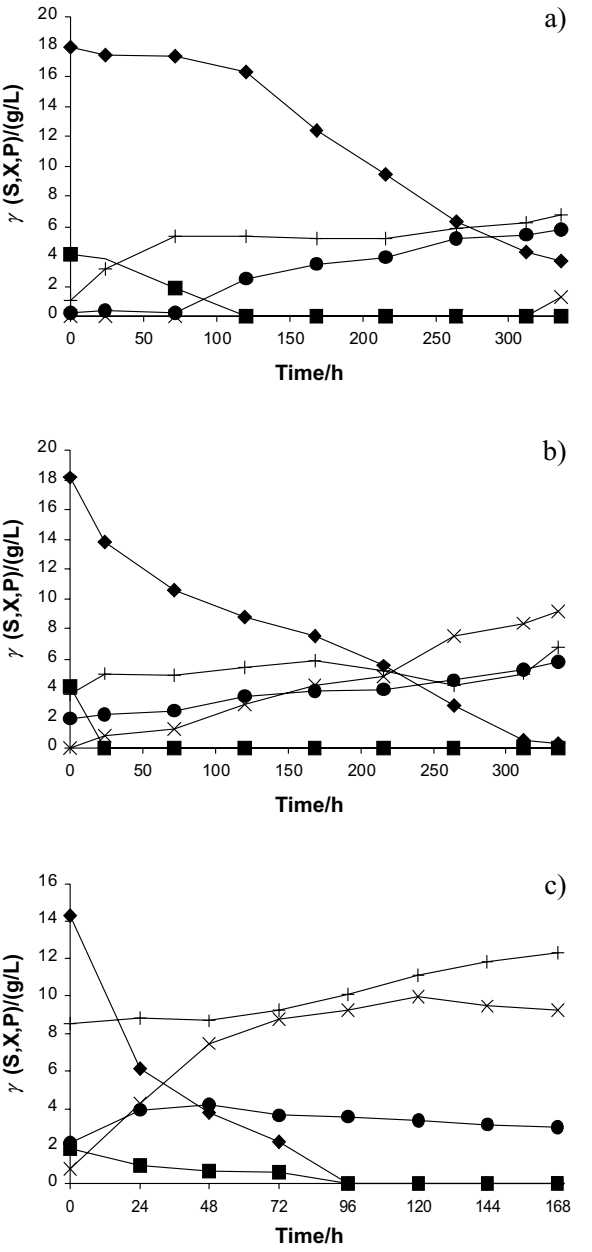

Fig. 1. Adaptation of *Debaryomyces hansenii* CCMI 942 to acid hydrolysates of *Eucalyptus globulus* wood in a Bran Biostat B fermentor at 700 rpm. a) experiment with non-adapted cells; b) first adaptation run; c) second adaptation run. Symbols: xylose (♦); xylitol (x); biomass (+); ethanol (•); glucose (■).

Experiments with concentrated hydrolysates

As the comparatively low xylose concentration of hydrolysates was one of the factors limiting the xylitol production, the hydrolysates were concentrated under reduced pressure to reach xylose concentrations in the range of 40–50 g/L. According to reported data (*37*), fermentation of media made with pure xylose is not significantly affected when the substrate concentration is below 200 g/L, but it must be taken into account that the increase in xylose concentration in media made from hydrolysates is associated with a proportional increase in the concentration of other non-volatile substances with inhibitory potential.

In order to adapt the yeasts to the concentrated hydrolysates, the following strategy was followed: *D. hansenii* CCMI 942 cells adapted to raw, unconcentrated hydrolysates were grown in this type of media and, once proliferated, the cells were used to prepare dense inocula (*24,38*). The inocula were then used to seed the progressively concentrated media, and consecutive fermentations were carried out in order to achieve a complete adaptation.

Although limited use of detoxification stages is desirable to minimise the operational costs, adaptation of yeasts to hydrolysates was not enough to overcome the inhibitory effects, and detoxification by charcoal adsorption or diethyleter extraction was applied. In some cases, solvent extraction was not applicable to hydrolysates due to the poor manageability of the system and difficulties in the separation of phases.

Table 2. Effect of the degree of concentration of hydrolysates on the fermentability of raw and detoxified autohydrolysis-posthydrolysis media supplemented with γ (CSL)=3 g/L. Results of fermentations performed with the strain *Debaryomyces hansenii* CCMI 942

Concentration ratio (Final/initial volume ratio)	1:1		1:2		1:3		1:4		
Detoxification	Raw	Charcoal	Raw	Charcoal	Raw	Charcoal	Raw	Charcoal	Diethylether
γ (xylose$_0$) / (g/L)	17.82	11.16	39.23	24.59	59.72	48.34	66.82	59.32	67.62
γ (glucose$_0$) / (g/L)	1.79	1.02	3.95	2.42	7.38	6.66	8.80	8.34	13.99
γ (arabinose$_0$) / (g/L)	0.31	0.18	1.18	0.70	1.99	2.19	2.71	2.36	3.27
Fermentation time / h	24	24	72	48	48	48	48	48	72
Conc. xylose / %	91.95	98.42	91.91	97.99	12.54	6.74	10.93	6.18	0.00
γ (xylitol) / (g/L)	3.73	0.65	6.73	5.69	0.69	1.22	0.49	0.79	0.00
γ (biomass) / (g/L)	2.89	2.18	1.76	2.32	0.00	1.35	0.90	0.45	0.22
γ (ethanol) / (g/L)	2.19	1.29	2.42	4.07	0.00	2.70	0.00	0.00	0.00
Q_S / $(g \cdot L^{-1} \cdot h^{-1})$	0.68	0.46	0.50	0.50	0.16	0.07	0.15	0.08	–
Q_P / $(g \cdot L^{-1} \cdot h^{-1})$	0.15	0.03	0.09	0.12	0.01	0.02	0.01	0.02	–
ζ ($Y_{P/S}$) / (g/g)	0.22	0.06	0.19	0.24	0.09	0.37	0.07	0.21	–
Q_X / $(g \cdot L^{-1} \cdot h^{-1})$	0.12	0.09	0.02	0.05	0.00	0.03	0.02	0.01	0.003
ζ ($Y_{X/S}$) / (g/g)	0.17	0.20	0.04	0.10	0.00	0.41	0.12	0.13	–

The fermentation process was performed under the operational conditions previously selected: pH=5.5, (volume of medium):(volume of flask) ratio =1/2, 30 °C, operation in Erlenmeyer flasks shaken at 300 rpm.

The initial substrate concentration was a key factor affecting both productivity and yield. Table 2 summarises the results obtained in fermentations of unconcentrated liquors compared to those concentrated in volume ratios 1:2, 1:3 and 1:4. Using unconcentrated media, the xylose loss caused by charcoal adsorption resulted in decreased xylitol production compared to the non-detoxified medium. Charcoal detoxification of concentrated hydroysates resulted in 25–400 % increase in product yield compared to unconcentrated hydrolysates. In literature, increased product yields have been reported for detoxified hydrolysates (up to 50 % increase compared to raw hydrolysates) in assays with *D. hansenii* NRRL-Y 7426 (*39*).

Comparative performance of the two Debaryomyces hansenii strains

The ability of two adapted strains of *Debaryomyces hansenii* (NRRL Y-7426 and CCMI 942) for xylitol production from hydrolysates made by autohydrolysis-posthydrolysis was compared in media supplemented with yeast extract, malt extract and peptone. For this purpose, the assays were performed in Erlenmeyer flasks under the conditions previously selected. The results presented in Table 3 show that the NRRL strain was superior to the CCMI strain.

Fermentation of concentrated, charcoal-detoxified hydrolysates

Fig. 2 shows the results obtained in the fermentation with the strain *D. hansenii* NRRL-Y7426 of media detoxified with charcoal and concentrated to reach the desired initial xylose concentrations. Figs. 2a to 2c correspond to the experiments carried out with media supplemented with yeast extract, malt extract and peptone, whereas the media presented in Figs. 2d to 2f were supplemented only with 3 g/L of CSL. The conditions used in experiments were the same as previously specified. The NRRL strain performed slightly better in the CSL-supplemented medium than in the YMP medium containing 35 g/L of initial xylose or less (Figs. 2a, 2b, 2d and 2e), particularly in terms of product yield. For media containing more than 45 g/L of initial xylose (Figs. 2c and 2f), the most remarkable finding was the prolonged fermentation time necessary to deplete the substrate in the CSL-supplemented medium.

Table 3. Results achieved with two *Debaryomyces hansenii* strains in the fermentation media made by autohydrolysis-posthydrolysis. Operational conditions: for the yeast *D. hansenii* NRRL-Y7426, medium to Erlenmeyer flask volume ratio = 1/5, 200 rpm, pH= 6.5, 30 °C; for the yeast *D. hansenii* CCMI 942, medium to flask volume ratio = 1/2, 300 rpm, pH=5.5, 30 °C

	Debaryomyces hansenii	
	NRRL Y-7426	CCMI 942
γ (initial xylose) / (g/L)	15.4	14.79
γ (initial glucose) / (g/L)	3.04	3.19
Time / h	96	168
Consumed xylose / %	93.8	93.37
γ (xylitol) / (g/L)	3.23	1.37
γ (biomass) / (g/L)	7.86	11.8
Q_P / $(g/L \cdot h^{-1})$	0.033	0.008
ζ ($Y_{P/S}$) / (g/g)	0.22	0.09
Q_S / $(g/L \cdot h^{-1})$	0.08	0.07
ζ ($Y_{X/S}$) / (g/g)	0.54	0.85

On the basis of the results above, additional experiments were carried out to assess the maximum concentration degree of hydrolyzates leading to media suitable

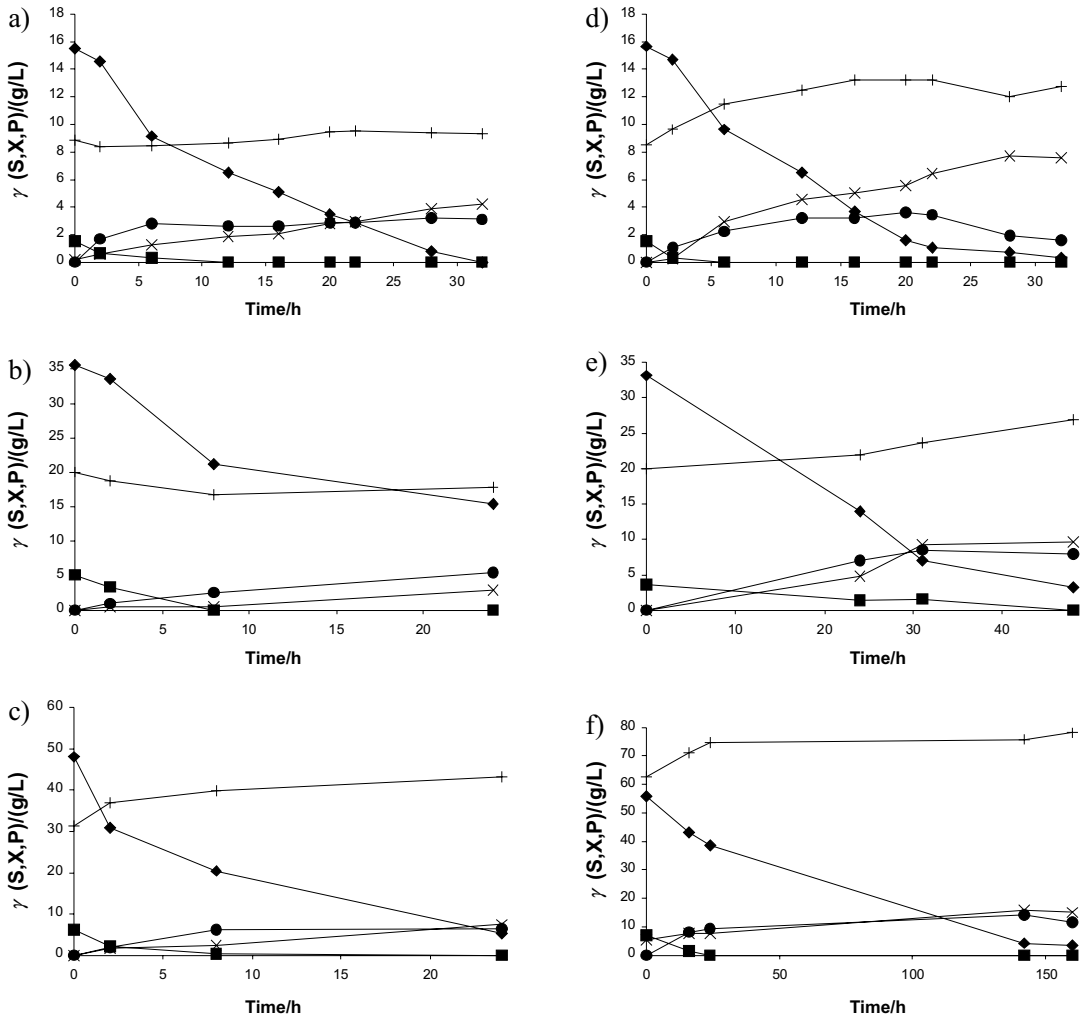

Fig. 2. Bioconversion with *D. hansenii* NRRL Y-7426 of media obtained by autohydrolysis-posthydrolysis, detoxified by charcoal adsorption and concentrated in various initial xylose concentrations. a), b), and c): experiments in media supplemented with yeast extract, malt extract and peptone (YMP medium). d), e) and f): media supplemented with corn steep liquor (CSL). Symbols: xylose (♦); xylitol (x); biomass (+); ethanol (•); glucose (■).

for fermentation purposes using corn steep liquor as the unique supplementation source. The best results (see Fig. 3) were obtained when the original hydrolysates were concentrated to reach an initial xylose concentration of 80 g/L and the medium was supplemented with CSL concentration of 3 g/L. Under these conditions, the maximum xylitol concentration (40 g/L) was achieved in 100 h of fermentation, with a volumetric productivity of 0.4 g/L·h and a product yield of 0.57 g/g.

Conclusions

Fermentation of media made by autohydrolysis-posthydrolysis of *Eucalyptus* wood has been assayed for xylitol production. Among the approaches considered in this work, the best results were achieved when hydrolysates concentrated under reduced pressure, containing 80 g/L of xylose, were detoxified with charcoal, supplemented with 3 g/L of CSL and fermented with the strain *D. hansenii* NRRL Y-7426. In this case, volumetric xylitol productivity of 0.4 g/L·h was achieved at a product yield of 0.57 g/g.

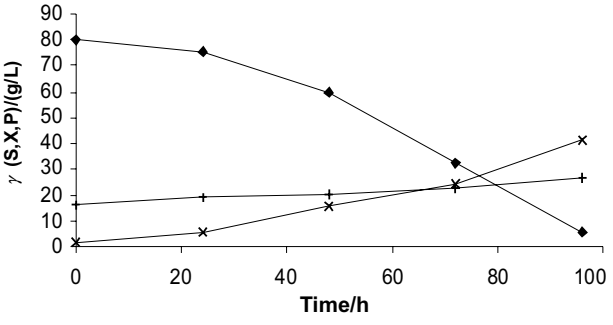

Fig. 3. Fermentation with *Debaryomyces hansenii* NRRL Y-7426 of media made from autohydrolysis-posthydrolysis, detoxified with charcoal and concentrated to an initial xylose concentration of 80 g/L. Symbols: xylose (♦); xylitol (x); biomass (+).

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Proizvodnja ksilitola iz hidrolizata eukaliptusova drva u jeftinim fermentacijskim podlogama

Sažetak

Ispitano je nekoliko mogućnosti biokonverzije ksiloze iz hidrolizata, dobivenih iz eukaliptusova drva, u ksilitol. Sojevi *Debaryomyces hansenii* adaptirani su na fermentacijske podoge (dobivene predhidrolizom ili autohidrolizom-posthidrolizom drva) uz jeftine hranjive dodatke. Fermentacija je bila uspješna u podlozi s 80 g/L ksiloze ako su hidrolizati bili detoksificirani adsorpcijom na aktivnom ugljenu uz dodatak kukuruznog ekstrakta (CSL).