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Solvent extraction of hemicellulosic wood hydrolysates: a procedure useful for obtaining both detoxified fermentation media and polyphenols with antioxidant activity

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Abstract

Eucalyptus wood hydrolysates were extracted with organic solvents (ethyl acetate and diethyl ether) to remove part of the phenolics derived from lignin. In order to obtain increased phenolic removal, the effects of the major experimental variables affecting extraction (type of solvent, hydrolysate to solvent volume ratio, temperature, pH and coupling of extraction stages) were explored. Under the best operational conditions, up to 84% of the initial lignin-derived compounds were extracted. The phenolic compounds extracted by solvents showed antioxidant activity. Under the best conditions, the antioxidant activity coefficient was 64% of the value found with a synthetic antioxidant (BHT). Extracted hydrolysates led to xylose solutions, allowing an enhanced fermentative production of xylitol with the yeast *Debaryomyces hansenii*.

Keywords: Antioxidants; *Debaryomyces hansenii*; *Eucalyptus* wood; Hemicellulose hydrolysates; Xylose; Xylitol

1. Introduction

Hemicellulosic acid hydrolysates of lignocellulosic materials contain inhibitors of microbial growth and fermentation that can hinder or even prevent fermentation (Watson, Prior & Lategan, 1984). When hardwoods are used as raw materials, xylose is the main reaction product, but the concentration of compounds derived from the acid-soluble lignin fraction of woods is also important (Wayman, Seagrave & Parekh, 1987).

A variety of procedures have been employed for removing inhibitory compounds from hydrolysates and/or for limiting their inhibitory action on the fermentation. For example, physico–chemical procedures, including adsorption in activated charcoal (Gong, Chen & Chen 1993; Parajó, Domínguez & Domínguez, 1996; Tran & Chambers, 1985), neutralization and overliming (Perego, Converti, Zilli & Del Borghi, 1994; Roberto, Lacis, Barbosa & Mancilha, 1991) and extraction with organic solvents (Frazer & McCaskey 1989; Parajó et al. Domínguez et al., 1997a) have been used for this purpose. Microorganism adaptation (Amartey & Jeffries 1996; Chen & Gong, 1985; Parajó, Domínguez et al., 1995) and biological detoxification (Palmqvist, Hahn-Hägerdal, Szengyel, Zachhi & Réczey, 1997; Schneider, 1996) have also been employed for overcoming the presence of inhibitors.

Among the above detoxification strategies, solvent extraction shows several interesting features, including: (i) good fermentation performance achieved using solvent-detoxified media, particularly when the bioconversion of hydrolysates is directed towards xylitol (Parajó, Domínguez et al., 1997a), (ii) easy recovery of solvents, owing to their low boiling point, an aspect that favours the overall economic features of the process, and (iii) easy recovery of solubilized lignin fractions, which might be used as a cheap, renewable source of food antioxidants (see below). Fig. 1 shows the general idea of the proposed process.

This last idea should be evaluated in the context of the worldwide trend to avoid or minimize the use of synthetic food additives, which is leading to a great interest in the search for new antioxidants. Lipid oxidation is a major cause of food deterioration, not only by producing rancid odours and flavours but also by decreasing both nutritional quality and safety by the formation of secondary products during processing.

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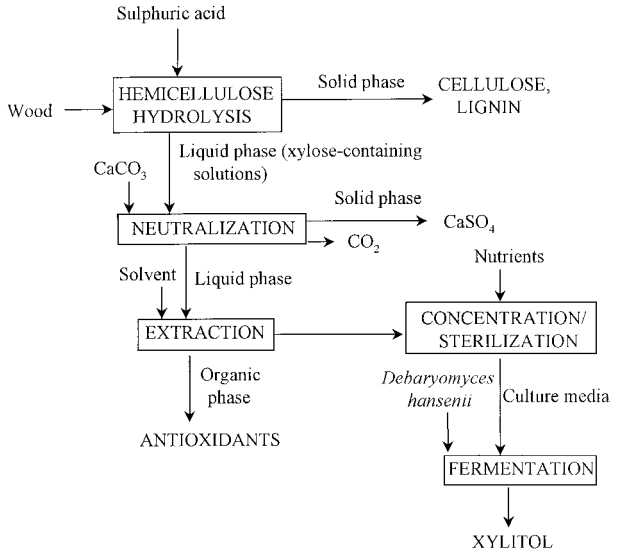

Fig. 1. General idea of the studied process.

In order to maintain food quality, chemical antioxidants are widely used because of their effectiveness and low cost but important aspects, such as their toxicity and safety, have been questioned. An important number of natural extracts (oat groats and hulls, mung bean hulls, peanut hulls) were tested to prevent the oxidation of different oils (Duh & Yen, 1997; Duh, Yen, Du & Yen, 1997; Xing & White, 1997). Phenolics extracted from different kinds of wood reduced the harshness of young brandy and improved its taste (Venkataramu, Patel & Subba, 1983). Extracts of ground black pepper have also been employed to reduce lipid oxidation of cooked ground pork (Tipsrisukond, Fernando & Clarke, 1998). On the other hand, natural food antioxidants show beneficial properties, including: (i) anticarcinogenic activity, (ii) ability for inhibiting oxidation reactions that could play a role in heart and in neurodegenerative diseases, as well as aging processes (Aruoma et al., 1997; Hollman, Hertog & Katan, 1996), (iii) it has been suggested that some phenolic compounds might function as natural biological response modifiers, affecting the autoimmune system (Nakagami, Nanaumi-Tamura, Toyomura, Nakamura & Shigehisa, 1995). Finally, it must be cited that a prooxidant effect of phenolics has also been reported (Yen, Chen, & Peng, 1997).

Compounds with strong antioxidant properties have been identified in plants, and special attention has been devoted to the extraction of this type of compound from residual sources, such as peanut hulls (Duh & Yen 1995; 1997; Watanabe, Ohshita & Tsushida, 1997), buckwheat hulls (Watanabe et al., 1997), oat groats and hulls (Xing & White, 1997), red grape pomace peels (Larrauri, Rupérez & Saura-Calixto, 1997) or grape seeds (Saito, Hosoyama, Ariga, Kataota & Yamaji, 1998), potato peel waste (Rodríguez de Sotillo, Hadley & Holm, 1994), olive rape (Sheabar & Neeman, 1988), barks (Marinova, Yanishlieva & Kostova, 1994) and corn bran hemicellulose fragments after acid hydrolysis (Ohta, Yamaski, Egashira & Sanada, 1994).

Several solvents have been used for antioxidant extraction, the activity of these compounds being closely dependent on the solvent used (Marinova & Yanishlieva, 1997; Tiito, 1985). For example, ethers and ketones are among the most employed solvents for removing phenolics from water, whereas ethyl acetate and diethyl ether have been used for extracting low molecular weight phenolics from oak wood (Fernández de Simón, Cadahía, Conde & Garcia-Vallejo, 1996). Strong antioxidant activity has also been reported for polyphenols extracted with ethyl acetate from natural materials (Marinova & Yanishlieva, 1997; Tsuda, Ohshima, Kawakishi & Osawa, 1994). The amount of phenolics extracted was also affected by temperature and other operational factors (Tiito, 1985).

The main purpose of this work was to select the optimum operational conditions for the solvent extraction of *Eucalyptus* wood hemicellulosic hydrolysates. After extraction of hydrolysates with diethyl ether or ethyl acetate, the antioxidant activity of phenolics present in the organic phase was measured. Further experimentation confirmed the suitability of extracted hydrolysates for making culture media easily fermentable with *Debaryomyces hansenii* for xylitol production.

2. Materials and methods

2.1. *Acid hydrolysis of* Eucalyptus *wood*

Eucalyptus globulus wood chips were subjected to acid hydrolysis under conditions previously reported (Parajó, Vázquez, Alonso, Santos & Domínguez, 1994). The hydrolysates contained 18 g xylose $litre^{-1}$, 3.6 g glucose $litre^{-1}$, 2.2 g acetic acid $litre^{-1}$, 0.6 g arabinose $litre^{-1}$ and less than 0.5 g furfural $litre^{-1}$.

2.2. *Solvent extraction of hydrolysates*

Neutralized hydrolysates were extracted, in duplicate, with diethyl ether or ethyl acetate under a variety of operational conditions (see below). Hydrolysates and the selected solvent were contacted in 250 ml baffled Erlenmeyer flasks with orbital shaking (300 rpm) at constant temperature (in the range 10–40°C). pH of hydrolysates was readjusted to 3 or 6.5 (with $CaCO_3$) or to alkaline values (with $CaOH_2$). Organic phases were vacuum-evaporated at temperatures under 40°C, and the residual water was removed by freeze-drying. The resulting solids were used to calculate the extract yields and to measure their antioxidant activity (see below). Aqueous phases from extraction were used to determine residual

lignin (by UV absorbance readings, see below) and to prepare culture media, with or without a previous concentration step.

2.3. Measurement of antioxidant activity of extracts

Powdered extracts were redissolved in ethanol, and assayed for antioxidant activity by the spectrophotometric method of Marco (1968) as modified by Miller (1971), based on the ability of the different extracts to hinder the oxidative degradation of β-carotene in an emulsion containing linoleic acid. Antioxidant activity was measured from absorbance readings in terms of the antioxidant activity coefficient AAC, defined as:

$$AAC = \frac{\begin{pmatrix}\text{Absorbace of extracts}_{120\,h} - \\ \text{Absorbance of control}_{120\,h}\end{pmatrix}}{\begin{pmatrix}\text{Absorbace of control}_{0\,h} - \\ \text{Absorbance of control}_{120\,h}\end{pmatrix}} \times 1000$$

which provides an estimate of the relative degree of oxidation in the presence of extracts with respect to the degree of oxidation in the presence of a control sample containing ethanol. In order to provide comparative data, additional experimentation was carried out with butylated hydroxytoluene (BHT), a model antioxidant. All determinations were perfomed in duplicate.

2.4. Fermentation of hydrolysates

Culture media made from raw or solvent-extracted hydrolysates were supplemented with yeast extract and peptone (Parajó et al., 1995), autoclaved and used for xylitol production with the yeast *Debaryomyces hansenii* NRRL Y-7426. Batch fermentations were carried out in triplicate at 30°C and initial pH = 6.5 in Erlenmeyer flasks with orbital shaking (200 rpm) under microaerobiosis.

2.5. Analytical methods

Xylose, glucose, xylitol, acetic acid and furfural were analysed by HPLC, using an Interaction Ion column (mobile phase, 0.005 M H_2SO_4; flow rate, 0.4 ml/min; RI and UV detection). Soluble lignin was estimated by UV spectrophotometry at 279 nm (Browning, 1967; Maekawa, Ichizawa & Koshijima, 1989; Tran & Chambers, 1985).

3. Results and discussion

3.1. Kinetics of solvent extraction

A preliminary set of experiments was performed in order to establish the inter-relationship between contact time and percent of phenolics extraction (that was considered to be equal to the percent of absorbance decrease). The experimental results obtained in assays with diethyl ether at several hydrolysate/extracting solvent volume ratios (H/E) under fixed operational conditions (pH = 6.5, temperature = 30°C) are shown in Fig. 2. In all the cases considered, equilibria were reached in a few minutes, independently of the volume ratio of phases utilized. As similar results were achieved with ethyl acetate (data not shown), contact times of 30 min were considered enough to reach equalibrium and selected for further experimentation.

3.2. Extraction equilibrium

In order to obtain information on the maximum percent of lignin extractable in a single step under defined experimental conditions, a set of equilibrium extractions was carried out at 25 or 30°C using solvent:hydrolysate volume ratios in the range 1:1 to 1:9. Fig. 3 shows some experimental results achieved for both solvents. Little effects were observed in ethyl acetate extractions when hydrolysate:solvent volume ratio was increased over 1:3, the maximum percent of extractable lignin being about 84%. On the other hand, lignin extraction steadily increased in experiments with diethyl ether when the solvent:hydrolysate volume ratio varied from 1:1 to 1:7.

The absorbance of neutralized hydrolysates decreased slightly (by about 4%) after prolonged storage at 4°C. This fact is in agreement with the findings of Friedman (1997), who detected time-and light-dependent changes of polyphenolic compounds by UV measurements.

The superiority of ethyl acetate over diethyl ether was also confirmed for freshly prepared hydrolysates, the percentages of phenolics removal being 88.1 and 79.9%,

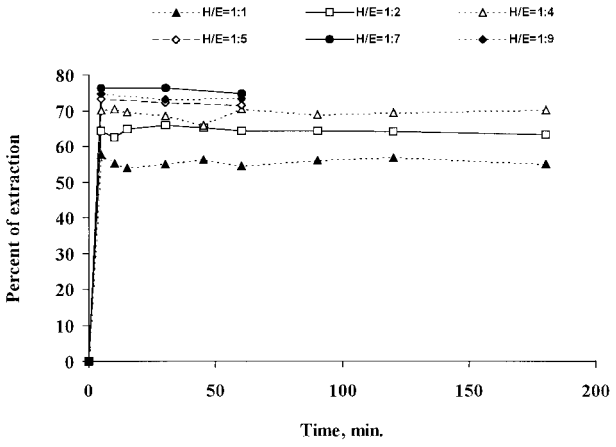

Fig. 2. Dependence of the percent of lignin-derived compounds extracted from neutralized hydrolysates on the contact time in experiments performed at selected solvent/hydrolysate volume ratios (H/E). Operational conditions: solvent = diethyl ether; PH = 6.5, temperature = 30°C.

respectively. Therefore, for further experiments, ethyl acetate was used.

Operating with ethyl acetate at H/E = 5 during 30 min, the percent of phenolics extraction was not affected by temperature in the range explored (10–40°C), the experimental values being in the range 83–84.

3.3. Coupling of extraction stages

As a way for improving the removal of phenolic compounds, the benefits derived from performing sequential extraction stages, of neutralized hydrolysates, with ethyl acetate, were studied. Operating at H/E = 1:1, a single extraction stage removed 75.4% of phenolics, in comparison with 84.1% extraction when a second extraction stage was performed. This last value was near the results obtained in experiments with a single extraction stage and H/E in the range 1:2 to 1:9, in which the percentage of phenolics removal were in the range 80.5–83.5%.

3.4. Effect of pH on single and two-stage extraction

Extraction of hydrolysates was performed at acidic, neutral and alkaline conditions (pH 3, 6.5 and 10) in order to establish the effects of this variable on the removal of phenolic compounds. Operating at an H/E = 1:3, the highest percent of phenolics removal (81.9%) was achieved at pH = 3, in comparison with 79.5% at pH = 6.5 and 66.4% at pH = 10. Similar behaviour was observed when coupling two extraction stages performed, with H/E = 1:1, pH = 3 being the most favourable one (86.1% removal of phenolics), in comparison with 81.9% phenolics removal reached at pH = 6.5 and 68.9% phenolics removed at pH = 10. This experimental trend was similar to that reported by Sheabar and Neeman (1988), who found improved extraction yeilds of polyphenolics from rape of olives with ethyl acetate and with isobutyl alcohol operating at pH = 4.

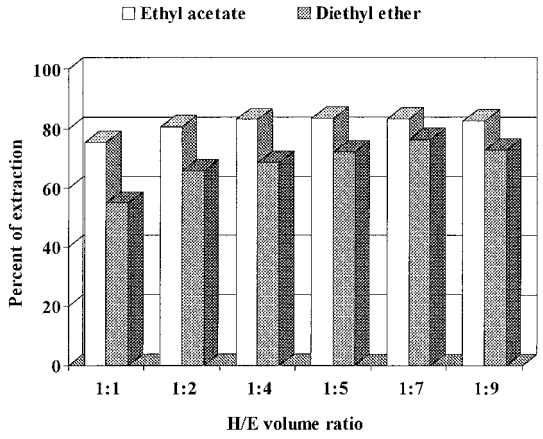

Fig. 3. Effect of the hydrolysate/solvent volume ratio (H/E) on the percentage of lignin-derived compounds extracted. Operational conditions: pH = 6.5, temperature = 30°C; contact time, 30 min.

3.5. Antioxidant activity of extracts

Fig. 4 shows the time course of the absorbance of an emulsion containing β-carotene, Tween 40, linoleic acid and phenolic extracts from hydroysates in typical experiments made to measure the antioxidant activity of this latter type of compound (Miller, 1971). For this part of the experimental work, extracts obtained from hydrolysates at pH 3, 6.5 or 10 were considered, as well as two values of the hydrolysate/solvent volume ratio (H/E of 1:1 and 1:3). Table 1 shows the values of the antioxidant activity coefficients AAC of ethyl acetate extracts of *Eucalyptus* wood hydrolysates calculated from these experimental data. A comparative analysis of both the experimental data shown in Fig. 4 and the

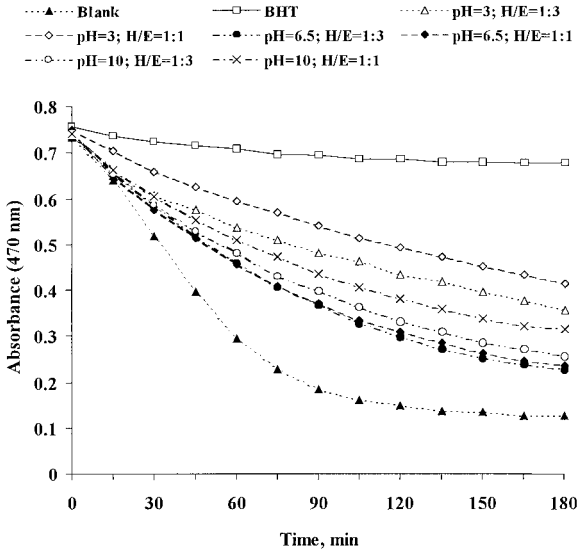

Fig. 4. Bleaching of β-carotene in the presence of linoleic acid, Tween 40 and ethyl acetate extracts of *Eucalyptus* wood acid hydrolysates.

Table 1
Values of the antioxidant activity coefficient (AAC) determined for ethyl acetate extracts of *Eucalyptus* wood acid hydrolysates and for a model compound (BHT)[a]

Sample	pH	Solvent/hydrolysates volume ratio (H/E)	AAC
Extracts from hydrolysates	3	1:3	485 ± 7.3
Extracts from hydrolysates	3	1:1	588 ± 8.5
Extracts from hydrolysates	6.5	1:3	255 ± 6.0
Extracts from hydrolysates	6.5	1:1	272 ± 4.8
Extracts from hydrolysates	10	1:3	313 ± 3.6
Extracts from hydrolysates	10	1:1	397 ± 10.9
BHT	–	–	916 ± 6.0

[a] Concentration used in tests: 400 mg litre^{-1}.

values of AAC shown in Table 1 shows that the pH of hydrolysates was the most influential variable on the antioxidant activity of extracts. The highest AAC (588) was obtained operating with H/E = 1:1 and pH = 33. Little effects were associated with the variation of the hydrolysate/solvent volume ratio H/E, the best behaviour being observed for H/E = 1:1, a fact suggesting that compounds extracted at this volume ratio have different chemical natures from the additional fraction extracted using H/E = 1:3.

The antioxidant ability of extracts is difficult to compare with literature data owing to the variety of methods employed in extraction and analytical procedures. In general terms, polyphenolic extracts from plants show lower antioxidant activity than compounds like butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) (Duh & Yen, 1997; Kim, Kim, Kim, Oh & Yung, 1994; Zygadlo, Lamarque, Maestri & Grosso, 1995). von Gadow, Joubert, and Hansmann (1997) used the β-carotene bleaching method for comparing the antioxidant activity of several polyphenolic compounds,

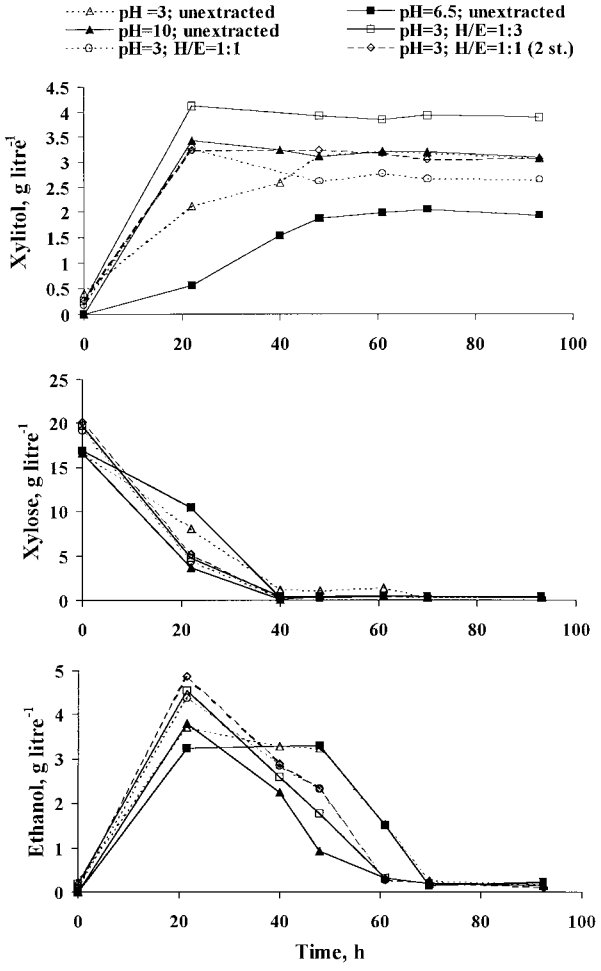

Fig. 5. Fermentation of hydrolysates extracted with ethyl acetate at different pH values and hydrolysate/solvent volume ratios (H/E). Values are the averages of three independent experiments. Standard deviations were in the range of 3–10% of the mean.

and found increasing antioxidant activities for *p*-hydroxybenzoic acid, syringic acid, ferulic acid and vanillic acid. The AAC of these compounds were 33–50% lower than the one calculated for BHT. Since these organic acids are model compounds for hardwood lignins, a similar behaviour could be expected for the extracts from *Eucalyptus* wood hydrolysates. The results shown in Table 1 confirm the interrelationship between the antioxidant activity of extracts and lignin-model compounds.

3.6. Fermentation of extracted hydrolysates

In order to obtain data on the ability of solvent extraction as a detoxification method for improving the fermentability of hydrolysates for xylitol production, a set of shaker fermentation experiments was carried out using initial substrate concentrations in the range 15–20 g litre^{-1} with a limited initial cell concentration (0.1 g litre^{-1}) in this work. Hydrolysates at pH 3, 6.5 or 10 were extracted with ethyl acetate in single stage at hydrolysate/solvent volume ratios of 1:1 and 1:3, and in a two-stage extraction using a hydrolysate/solvent volume ratio of 1:1. Both unextracted hydrolysates (at pH 3, 6.5 and 10) and the extracted hydrolysates specified above were kept at the desired pH, supplemented with nutrients and used as fermentation media. The experimental results obtained are shown in Figs. 5 and 6. As expected, unextracted hydrolysates at pH 6.5 and 3 led to slow xylose consumption and xylitol production. Better results were obtained with unextracted extracts overlimed to pH = 10, confirming the validity of this method for improving fermentation. Overliming has been reported as an efficient method for removing inhibitors from fermentation media (Parajó, Domínguez et al., 1997b). Extracted hydrolysates showed an enhanced susceptibility towards fermentation in relation to unextracted hydrolysates at pH 6.5 and 3, the best results corresponding to hydrolysates at pH = 3 extracted with a hydrolysate/solvent volume ratio of 1:3. It is notable that pH = 3 was the one leading to the highest percent of polyphenol removal, so a qualitative agreement between degree of extraction and xylitol productivity exists. On the other hand, sequential extraction stages did not improve fermentability, suggesting that a single extraction was enough to remove the compounds with a chemical nature appropriate for inhibitors. This finding is in agreement with the general behaviour observed for the antioxidant activity coefficients of extracts. On the basis of the above ideas, the operational conditions defined by pH = 3 and hydrolysate/solvent volume ratio = 1:3 were selected for further experimentation with concentrated hydrolysates.

Fig. 7 shows the time course of a fermentation experiment carried out with vacuum-concentrated hydrolysates detoxified with ethyl acetate under the conditions specified above. Using a reduced initial cell concentration (0.61 g litre^{-1}), the maximum xylitol

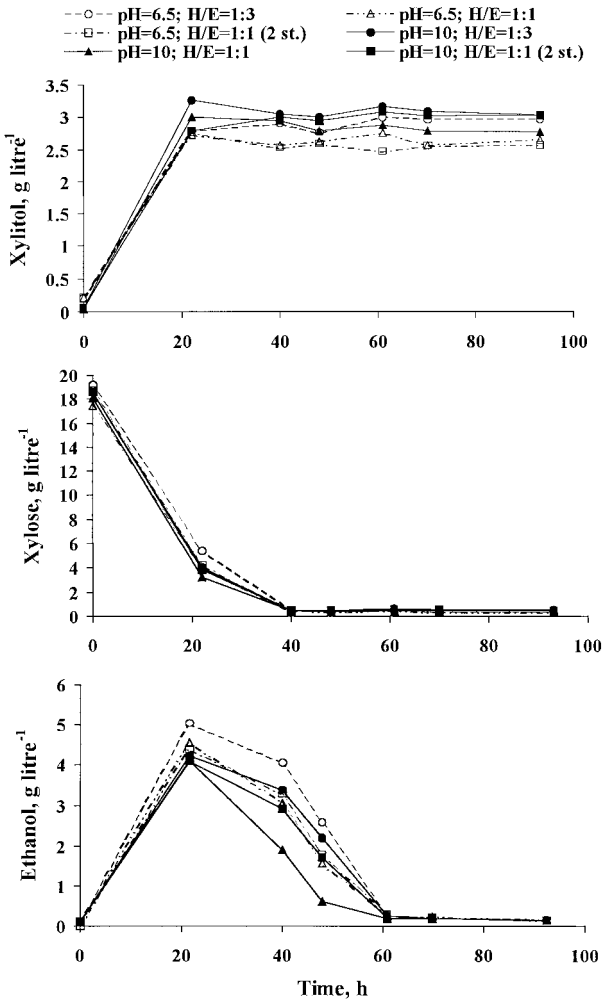

Fig. 6. Fermentation of hydrolysates extracted with ethyl acetate at different pH values and hydrolysate/solvent volume ratios (H/E). Values are the averages of three independent experiments. Standard deviations were in the range of 3–10% of the mean.

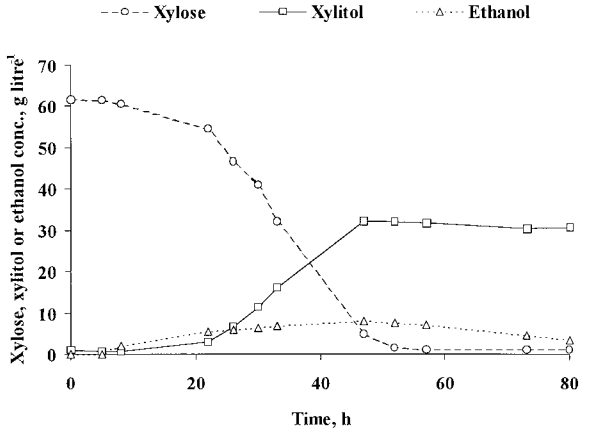

Fig. 7. Fermentation of concentrated hydrolysates (final volume:initial volume ratio = 1:3) after extraction with ethyl acetate. Operational conditions used in extractions: pH = 3, hydrolysate/solvent volume ratio = 1:3. Initial cell concentration in fermentation = 0.61 g litre^{-1}. Values are the averages of three independent experiments. Standard deviations were in the range of 4–8% of the mean.

concentration (32.2 g litre^{-1} was achieved after 47 h of fermentation. The volumetric xylitol productivity (0.66 g litre^{-1} h^{-1}) and the product yield [0.511 g xylitol (g xylose consumed)$^{-1}$] were satisfactory, considering the low initial cell concentration. Ethanol (maximum concentration, 8.1 g litre^{-1} after 47 h) was a valuable fermentation byproduct.

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