

Olive tree pruning as an agricultural residue for ethanol production. Fermentation of hydrolysates from dilute acid pretreatment

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Abstract

The use of agricultural residues for ethanol production constitutes one of the most promising alternatives from an environmental point of view for substituting fossil fuels in the transport sector. This work deals with the fermentability of hydrolysates obtained from olive tree pruning biomass and the influence of the pH of the culture medium. Hydrolysates of olive tree biomass were obtained by dilute acid pre-treatment of the raw material at 180 °C and 1% (w/v) sulfuric acid concentration. After pretreatment, solid residue and liquid were separated by filtration. The liquid fraction (hydrolysate) was then submitted to detoxification (overliming) before being used as fermentation medium. *Pichia stipitis* and *Pachysolen tannophilus* were compared as fermenting microorganisms. Different initial pH values were also tested. The best results in terms of ethanol yield were obtained by *P. tannophilus* with values as high as 0.44 g ethanol g⁻¹ sugar, and all liquids were fermented, to a different extent. *P. stipitis* could not ferment hydrolysates with initial pH below 6.5. It was also determined that ethanol production did not improve once glucose in the medium was totally converted, even if other sugars (xylose) were also consumed.

Additional key words: bioethanol; olive tree biomass; pentoses; *Pichia stipitis*; *Pachysolen tannophilus*.

Resumen

La poda de olivo como residuo agrícola para la producción de etanol. Fermentación de hidrolizados procedentes de pre-tratamiento con ácido sulfúrico diluido

El aprovechamiento de residuos agrícolas para la obtención de bioetanol es una de las alternativas más prometedoras desde el punto de vista medioambiental para la sustitución de los combustibles fósiles en el transporte. En este trabajo se estudia la fermentabilidad de hidrolizados procedentes de poda de olivo y se comprueba la influencia del pH del medio de cultivo. Se realizó un pre-tratamiento con ácido sulfúrico diluido (1% p/v) a 180 °C de biomasa procedente de poda de olivo. Tras el pre-tratamiento, el residuo sólido y la fracción líquida (hidrolizado) fueron separados por filtración. El hidrolizado fue sometido a una etapa de detoxificación (*overliming*) antes de ser empleado como medio de fermentación. Como microorganismos fermentativos se usaron las levaduras *Pichia stipitis* y *Pachysolen tannophilus*. También se evaluaron distintos valores de pH inicial de fermentación. Los mejores rendimientos de etanol se obtuvieron para *P. tannophilus* con valores de 0,44 g etanol g⁻¹ azúcar, y todos los líquidos fueron fermentados, en diferente extensión. *P. stipitis* no consiguió fermentar los hidrolizados con un pH inicial inferior a 6,5. Asimismo se detectó que la producción de etanol no mejoró una vez que la glucosa fue totalmente consumida, incluso cuando se consumieron otros azúcares como la xilosa.

Palabras clave adicionales: bioetanol; biomasa de olivo; pentosas; *Pichia stipitis*; *Pachysolen tannophilus*.

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Abbreviations used: C5 (pentoses); C6 (hexoses); CECT (Spanish Type Culture Collection); HMF (hydroxymethylfurfural).

Introduction

Because cellulose is the most abundant sugar polymer in lignocellulosic materials, glucose generation and its conversion is of key importance for bioethanol production. The pretreatment step of the raw material also generates a liquid fraction (hydrolysate) containing sugars from hemicellulose hydrolysis. Hemicellulosic sugars, especially xylose, can sum up to 45% of the total sugars in hardwoods and agricultural residues (Agbogbo *et al.*, 2008). However, relatively little attention has been paid to ethanol production from these sugars, due to a number of reasons. For example, in addition to sugars, hydrolysates contain sugar degradation products and lignin-derived products, some of which (*e.g.*, furfural, hydroxymethylfurfural [HMF], etc.) have been described as potentially inhibitors for yeast growth (Cara *et al.*, 2008; Pienkos & Zhang, 2009). Sometimes, a detoxification step must be included, for eliminating or reducing inhibitory compounds.

Concerning sugars, both C5 (pentoses: xylose, arabinose, mannose) and C6 (hexoses: glucose, galactose) are present in hydrolysates, making fermentation more difficult than from a single sugar. Yeasts able to ferment both glucose and xylose must be used. *Pichia stipitis* has been recognised as the most promising naturally occurring C5 fermenting microorganism (Agbogbo & Coward-Kelly, 2008); the fermentation of hydrolysates from olive tree cuttings has been reported (Díaz *et al.*, 2009). *Pachysolen tannophilus* is also well known pentose-fermenting yeast. The fermentation of olive tree biomass hydrolysates, obtained by sulphuric and phosphoric acid pre-treatment under atmospheric pressure (90 °C), has been reported with *P. tannophilus* (Romero *et al.*, 2007a,b).

Olive tree biomass is one of the main agricultural residues especially in Mediterranean countries. A special feature of olive tree biomass is its high extractive content, up to 30% including 8% of free glucose. This fact makes that liquid fractions issued from pre-treatment have unusually high glucose content, together with other hemicellulosic sugars. The conversion into ethanol of these sugars is important from an economic point of view, contributing to the feasibility of the whole process.

This work deals with the fermentation of olive tree biomass hydrolysates by *P. stipitis* and *P. tannophilus*. The main objective is to assess the fermentability of hydrolysates by yeasts, with especial focus on pentose uptake, as well as the influence of pH. The fermentation performance is compared based on substrate consumption and ethanol production.

Material and methods

Hydrolysates from olive tree biomass

The hydrolysates used in this study were obtained from olive tree biomass submitted to dilute sulfuric acid (1% w/w) pretreatment at 180 °C for 10 min in a laboratory scale stirred Parr reactor. The liquid fraction issued from pretreatment (hydrolysate) was used as carbon source for fermentation in this study. This hydrolysate was chosen because it presented high sugar recovery and, at the same time, the corresponding pretreated solids were efficiently transformed into glucose by enzymatic hydrolysis in a previous work (Cara *et al.*, 2008).

Microorganisms and fermentation

Fermentation experiments were performed by *P. tannophilus* obtained from the Spanish Culture Type Collection (CECT No. 12920), and *P. stipitis* (CECT No. 1922). The inoculum was obtained from a liquid culture in a medium containing (g L⁻¹) yeast extract, 5; NH₄Cl, 2; KH₂PO₄, 1; MgSO₄·7H₂O, 0.3; and xylose or glucose, 30. The inoculum culture was maintained on a rotary shaker at 30 °C, 150 rpm for 36 h and the amount of inoculum was calculated for 1 g L⁻¹ yeast initial concentration.

Fermentations were performed in 250 mL Erlenmeyer flasks with 100 mL fermentation medium on rotary shaker (Certomat-R, B-Braun, Germany) at 150 rpm. Temperature was constant at 30 °C. Experiments were performed in triplicate and average results are shown.

Hydrolysate conditioning

Hydrolysates were used as carbon source for fermentation experiments in two ways: first, they were used without further conditioning, except pH adjust to 4.5, 5.5 or 6.5. The desired pH was adjusted by adding drops of NaOH solutions (5 N and 1 N) at room temperature and under agitation. The hydrolysate that was just conditioned to pH 4.5 showed no cellular growth, and neither sugar consumption nor product formation were detected (data not shown). Another set of hydrolysates were submitted to overliming conditioning. Overliming was performed by adding calcium hydrox-

ide to increase hydrolysate pH from its initial value (1.5) to pH 10, maintaining it on stirred bath at 50 °C for 30 min; then, sulfuric acid was added to pH 4.5, 5.5 or 6.5 and the precipitate was removed by centrifugation (Purwadi *et al.*, 2004).

Analytical methods

The cellular growth was determined by measuring the fermentation broth UV-spectrophotometric absorbance at 620 nm and correlated to a dry weight calibration line. Analytical determinations were performed by HPLC in a Varian Prostar liquid chromatograph, equipped with refractive index detector. A Transgenomic CHO-682 column was used to quantify sugars with ultrapure water as the mobile phase, at a flow rate of 0.4 mL min⁻¹ and 80 °C temperature. Bioproducts (ethanol and xylitol) and other compounds as acetic acid, formic acid, furfural and HMF were measured with an Aminex HPX-87H column; operation conditions included 5 mM sulfuric acid solution as mobile phase, at a flow rate of 0.5 mL min⁻¹ and 65 °C.

Results and discussion

Hydrolysate composition

Hydrolysate composition, as issued from pretreatment and after overliming treatment, is shown in Table 1. Sugar concentration was approximately 50 g L⁻¹ while

Table 1. Composition (g L⁻¹) of the initial, untreated hydrolysate obtained after 1% (w/w) sulfuric acid pre-treatment of olive tree biomass at 180 °C for 10 min and after overliming detoxification treatment. Mean values and standard deviations from three determinations

Composition	Untreated	Overlimed
Glucose	19.42 ± 0.50	20.67 ± 0.53
Xylose	15.05 ± 0.27	15.95 ± 0.59
Galactose	5.33 ± 0.41	6.47 ± 0.61
Arabinose	6.30 ± 0.69	6.68 ± 0.67
Mannose	4.89 ± 0.61	4.70 ± 0.90
Acetic acid	5.43 ± 0.39	6.20 ± 0.09
Formic acid	1.16 ± 0.04	1.38 ± 0.05
Furfural	1.17 ± 0.36	0.70 ± 0.04
Hydroxymethylfurfural	0.80 ± 0.09	0.51 ± 0.05

other non sugar components, *e.g.* acetic acid, formic acid, furfural and HMF globally summed up to 8.5 g L⁻¹. Only the concentrations of furfural and HMF were lower in overlimed hydrolysate as compared to the untreated one, while the rest of the components slightly increased their concentration, probably due to some water evaporation during the detoxification process.

Microbial growth and substrate consumption

Growth curves showed a longer lag phase of growth by *P. tannophilus* when using the hydrolysate directly, in comparison with the cultures in which overliming treatment was done. It is likely that, in that period of time, the enzyme system of the yeast is adapting to the new environment, before cellular growth reached its exponential rate. In contrast, *P. tannophilus* fermentation experiments using pure glucose and xylose exhibited a much reduced lag phase, lower than 2 h (Sánchez *et al.*, 1999).

Concerning substrate consumption, glucose was completely consumed by *P. tannophilus*, while *P. stipitis* was only able to use up glucose contained in the pH 6.5 hydrolysate, as depicted in Fig. 1a. Glucose from untreated hydrolysates at 5.5 initial pH was consumed by *P. tannophilus*.

Concerning time evolution of xylose concentration, Fig. 1b shows that, as known, both *P. tannophilus* and *P. stipitis* consumed this pentose. Nevertheless, under the assayed conditions, xylose uptake by *P. stipitis* was only detected in the 6.5 initial pH experiment, and at a higher rate than that exhibited by *P. tannophilus* which, in turn, consumed xylose from hydrolysates at any condition.

Time evolution of other medium components

The time evolution of acetic acid, formic acid, furfural and HMF was followed throughout the fermentation experiments because all of them have been described as potential inhibitors of yeast growth (Pienkos & Zhang, 2009). As an example, Fig. 1c shows the time evolution for acetic acid concentration, the main compound in hydrolysates besides glucose and xylose. Acetic acid was also consumed by *P. tannophilus* during fermentation, although 1 g L⁻¹ concentration remained after 150 h fermentation time, no matter the treatment of the hydrolysate. Acetic acid has been de-

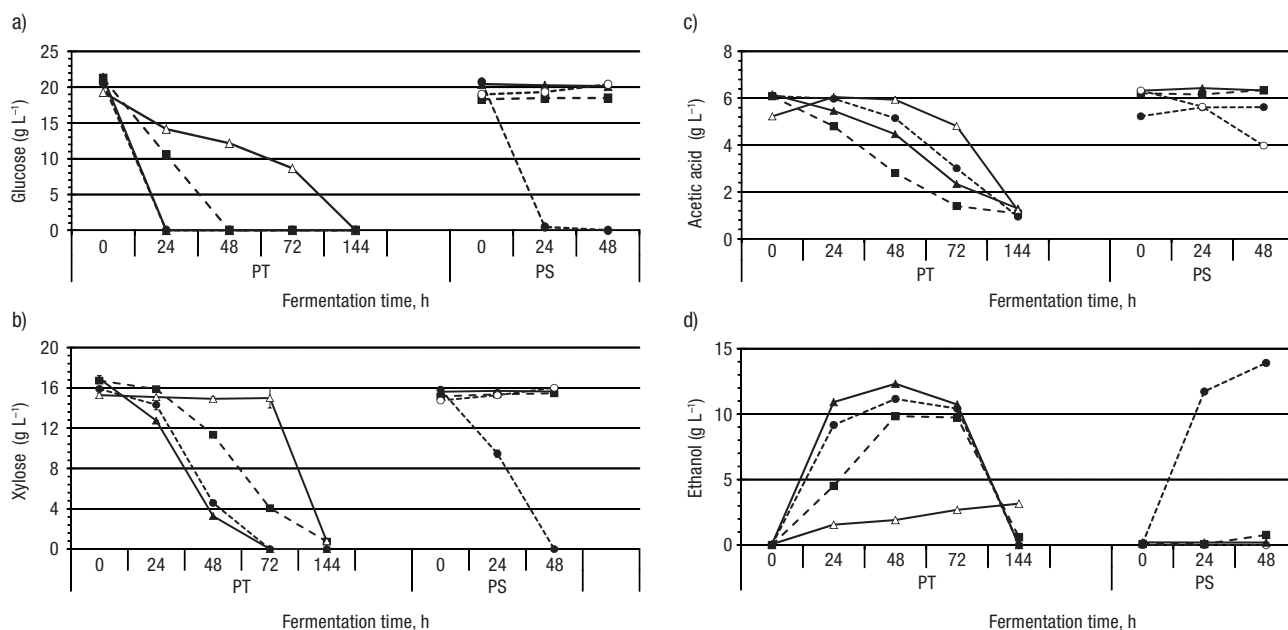


Figure 1. Time evolution of glucose (a), xylose (b), acetic acid (c) and ethanol production (d) at different initial pH and fermentation conditions (4.5 overlimed ■; 5.5 overlimed ▲; 5.5 untreated △; 6.5 overlimed ●; 6.5 untreated ○) for *Pachysolen tannophilus* (PT) and *Pichia stipitis* (PS).

scribed as exerting different influence on fermentation depending on initial pH. For example neither production of xylitol nor acetic acid consumption was detected at pH 5.0, but occurred at initial pH of 5.5 and 6.5 (Parajó *et al.*, 1998), when using media made from *Eucalyptus* wood hydrolysates with 10.1-10.5 g acetic acid L⁻¹. In this work, *P. stipitis* did not consume acetic acid except partially in the 6.5 initial pH hydrolysate.

Concerning furfural and HMF, both compounds were rapidly and completely consumed at any hydrolysate conditions; only in fermentations performed by *P. stipitis* these compounds were not entirely used, except for the hydrolysate with initial pH 6.5. The time evolution of furfural during the fermentations is shown in Table 2. This result agrees with that reported on *P. stipitis* fermentation grown on similar olive tree hydrolysates (Díaz *et al.*, 2009), although in this case the initial pH of the hydrolysates was 4.5 and it was required dilution for cellular growth to be detected.

Ethanol production

Ethanol was the main fermentation product. The time evolution of the ethanol concentration is shown

in Fig. 1d and the highest concentrations attained at each experiment are presented in Table 3. *P. tannophilus* efficiently converted sugars into ethanol. Untreated hydrolysate produced the lowest ethanol production throughout fermentation; a maximum ethanol concentration of 3.2 g L⁻¹ was achieved after 144 h fermentation. In contrast, ethanol concentrations ranging from 9.8 to 12.3 g L⁻¹ were obtained from overlimed hydrolysates depending on hydrolysate conditions. It seems clear that fermentation resulted inhibited in the hydrolysate that was not overlimed. Concerning overlimed hydrolysates, those of initial pH 5.5 and 6.5 gave the best results in terms of ethanol concentration. Fig. 1d shows that ethanol reached its maximum concentration from overlimed hydrolysates after 48 h fermentation time; beyond this time, ethanol itself was also used as substrate and it was virtually consumed at the end of the fermentation experiment (144 h). This finding was also reported for pure xylose fermentation at various initial sugar concentrations. In all cases, ethanol concentration began to decline when there was still 20% of the initial xylose present in the fermentation broth (Zhao *et al.*, 2010). Nevertheless, overlimed hydrolysates at pH 5.5 and 6.5 produced more than 80% of the maximum ethanol within the first 24 h of fermentation. On the contrary, no consumption of the ethanol pro-

Table 2. Time evolution (h) of furfural (g L⁻¹) at different initial pH and fermentation conditions

Experiment	<i>Pachysolen tannophilus</i>			<i>Pichia stipitis</i>		
	0	24	48	0	24	48
4.5overlimed	0.71 ± 0.02	0	0	0.54 ± 0.03	0.48 ± 0.05	0.46 ± 0.05
5.5untreated	0.94 ± 0.08	0	0	–	–	–
5.5overlimed	0.70 ± 0.09	0	0	0.73 ± 0.04	0.19 ± 0.02	0.20 ± 0.05
6.5untreated	–	0	0	0.67 ± 0.06	0	0
6.5overlimed	0.72 ± 0.08	0	0	1.00 ± 0.08	0.54 ± 0.06	0.44 ± 0.04

Untreated: hydrolysates used without further conditioning, except pH adjust to 4.5, 5.5 or 6.5. Overlimed: hydrolysate conditioned by adding calcium hydroxide to pH 10, maintaining it stirred at 50°C for 30 min; then, adding sulfuric to pH 4.5, 5.5 or 6.5 and further centrifugation.

duced from untreated hydrolysate was detected; moreover, ethanol production increased continuously with fermentation time, although lower concentrations were attained, as explained above.

P. stipitis was not able to produce ethanol, except from the overlimed hydrolysate with 6.5 initial pH, which reached the maximum ethanol concentration of all the performed experiments.

Ethanol yields were calculated as ethanol formed divided by substrate consumed (glucose and xylose) and ethanol productivity as ethanol formed divided by the elapsed fermentation time. Results are shown in Table 2, based on ethanol production after 24 h fermentation time. Untreated hydrolysate produced a relatively high ethanol yield of 0.29 g g⁻¹, but it must be taken into account that, at that time, sugar consumption was still limited. A maximum ethanol yield of 0.43 g g⁻¹ was obtained from overlimed hydrolysate at 5.5 initial pH, and the ethanol productivity was also the highest one.

Xylitol production

The highest values of xylitol concentrations corresponding to each experiment are summarized in Table 2. When *P. tannophilus* was used, the untreated hydrolysate produced as much xylitol as ethanol, while overlimed hydrolysates produced different concentrations of xylitol as coproduct, but always at much lower concentrations than those of ethanol. Contrarily to ethanol production, xylitol concentration increased with fermentation time in all cases.

As a result of the performed experiments, it can be concluded that the fermentation of hydrolysates produced from olive tree pruning by *Pachysolen tannophilus* and *Pichia stipitis* is clearly influenced by the pH value of the culture medium. *P. tannophilus* is able to ferment the sugars at the different pH assayed (4.5-6.5), the best ethanol results being attained at pH 5.5. On the other hand, no ethanol was produced when *P. stipitis* was used at pH values below 6.5. Nev-

Table 3. Maximum product in ethanol concentration (g L⁻¹), xylitol concentration (g L⁻¹), maximum ethanol yield (g ethanol/g consumed sugar) attained at the fermentation experiments

Experiment	<i>Pachysolen tannophilus</i>			<i>Pichia stipitis</i>		
	Ethanol	Xylitol	Ethanol yield	Ethanol	Xylitol	Ethanol yield
4.5overlimed	9.84 (48)	1.70 (144)	0.39 (24)	0	0	0
5.5untreated	3.16 (144)	3.38 (144)	0.29 (24)	–	–	–
5.5overlimed	12.31 (48)	2.51 (144)	0.43 (24)	0	0	0
6.5untreated	–	–	–	0	0	0
6.5overlimed	11.16 (48)	3.65 (144)	0.41 (24)	13.90 (48)	0	0.40 (24)

Data in parentheses stand for the corresponding fermentation time. Untreated: hydrolysates used without further conditioning, except pH adjust to 4.5, 5.5 or 6.5. Overlimed: hydrolysate conditioned by adding calcium hydroxide to pH 10, maintaining it stirred at 50°C for 30 min; then, adding sulfuric to pH 4.5, 5.5 or 6.5 and further centrifugation.

ertheless, when pH 6.5 experiments were compared, sugar consumption was faster and ethanol concentration was a little higher when *P. stipitis* was used as a fermentative microorganism, compared to *P. tannophilus*. Finally, it has been demonstrated that conditioning by overliming clearly improves fermentability of olive tree pruning hydrolysates.

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