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Fermentation of hardwood hemicellulose hydrolysate by *Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis*

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SUMMARY

Hardwood hemicellulose hydrolysate has been utilized as a substrate for ethanol production. Among the three different yeasts tested, the best performances have been obtained, in decreasing order, using *Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis*. Several pretreatments of this raw material have been studied to improve ethanol yields; in one such pretreatment a strain of *P. tannophilus* produced ethanol with a yield of 0.29 $g_{ethanol}/g_{sugars}$ (g_P/g_S); which is only 15% less than the values observed with synthetic media. Neither aeration nor acetone addition improved the fermentation of this substrate; in fact, only a marked stimulation of biomass growth has been observed at the expense of both ethanol and xylitol production.

INTRODUCTION

There is considerable literature regarding the biotransformation of lignocellulosics to ethanol, probably because of the huge amounts of this biomass available [22]. Lignocellulosics are composed of cellulose (30–50%), hemicellulose (20–50%) and lignin (15–35%). Lignocellulosics can be hydrolyzed to liberate sugars, with emphasis on large-scale production of ethanol by means of two different alternatives, acid or enzymatic hydrolysis. While only a limited margin for improvement remains to be made to acid hydrolysis, considerable and problematic enhancements are necessary before enzymatic hydrolysis becomes economically competitive [10].

Acid hydrolysis can be performed by employing strong acids at moderate temperature, or dilute sulphuric acid at high temperature. With the choice of strong acids at low temperature [3], the major problem is the waste of catalyst, whereas the use of dilute sulphuric acid at high temperature leads to the formation of numerous inhibiting compounds [18,19]. Various techniques [18] have been proposed to overcome inhibitor formation during acid hydrolysis and to increase yields.

The Tennessee Valley Authority (T.V.A.) scheme [4, 30], consisting of a two-stage hydrolysis of hardwood with dilute sulphuric acid at relatively high pressure and subsequent water extraction of sugars, generates first-stage hydrolysates of predominantly xylose and second-stage hydrolysates of predominantly glucose. While the second-stage hydrolysates usually can be biodegraded by the common hexose fermenting microorganisms, such as *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Zymomonas mobilis*, etc., biodegradation of first-stage hydrolysates, rich in pentoses, can be performed with microorganisms capable of using pentose directly (e.g. *Pachysolen tannophilus*, *Pichia stipitis*, *Candida shehatae*, *Clostridium thermosaccharolyticum*, etc.) [17]. An alternative to this procedure, consisting of the transformation of xylose to xylulose using xylulose-isomerase followed by fermentation with *S. cerevisiae* [9,16], is strongly affected by the high cost of the large amounts of enzyme required, and by the difference in optimal pH between the yeast (pH 5.0) and the enzyme (pH 7.0).

According to Strickland and Beck [30], warm treatment with $Ca(OH)_2$ at pH 5.5 with the addition of sulphite, or an overliming at pH 10.0, results in better yields in ethanol production from hemicellulose hydrolysates.

Hardwood hemicellulose fractions pretreated according to the T.V.A. scheme [30] have been fermented in this study by three different strains in order to select the most appropriate biocatalyst, and then subjected to further additional pretreatment steps devoted to the improvement of xylose fermentation. Furthermore, several attempts to reduce xylitol formation in favour of ethanol production resulted only in biomass growth stimulation.

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MATERIALS AND METHODS

Hemicellulose hydrolysate preparation. Hemicellulose hydrolysate, kindly supplied by T.V.A., was prepared from wood chips, primarily oak, impregnated under vacuum with 1% H_2SO_4 (w/w) [4,31]. 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 hydrolysate, consisting mainly of hemicellulosic sugars, has been used for studies.

Substrate preparation. Hemicellulose hydrolysate composition is reported in Table 1. Many earlier optimization studies [4,5,30] on the effects of different nutrients and pretreatments seem to suggest the following sequence as the best operation for eliminating inhibiting substances from the fermentation medium. A known volume of hydrolysate is heated to 100°C, held at that temperature for 10 min, replacing any volume loss with heated distilled water. The hydrolysate is then overlimed by adding $Ca(OH)_2$ up to pH 10.0, filtered, and then neutralized to pH 5.5 with sulphuric acid. After treating with sodium sulphite (0.1% w/w), the precipitate is removed by filtration, and then the pH is readjusted to pH 5.5. Nutrient additions consist of 0.2% urea (w/v), 0.2% yeast extract (w/v), and 0.05% KH_2PO_4 (w/v).

Microorganisms. All three yeasts tested, *Pachysolen tannophilus* (NRRL Y 2460), *Candida shehatae* (ATCC 22484), and *Pichia stipitis* (CBS 5773) were maintained on agar slants containing 0.1% (w/v) yeast extract and 50.0% (v/v) hemicellulose hydrolysate. The cells were grown aerobically at 32°C for 72 h on a medium containing 50.0 g/l D-xylose, 5.0 g/l KH_2PO_4, 2.0 g/l $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4 \cdot 7\ H_2O$, using loosely capped 250 ml Erlenmeyer flasks on an orbital shaker set at 100 rpm. After separation from the growth media by centrifugation, the cells were washed twice and resuspended in sterile water to give a yeast density of 1.0 g/l (dry weight) before the addition to the hydrolysate.

TABLE 1

Average composition of hardwood hemicellulose hydrolysate

Component	Concentration (g/l)
Xylose	43.5
Glucose	9.0
Galactose	3.3
Arabinose	2.9
Mannose	3.4
Acetic acid	10.9
Furfural	0.3
Hydroxymethylfurfural	0.9
Minerals:	
Cr	0.002
Ni	0.003
Fe	0.038
Mn	0.014
Density	1024

Fermentation conditions. A 5.0-liter Gallenkamp FBL-195 chemostat with working volume of 3.0 l, stirred at 300 rpm, was employed for the batch fermentations. Microaerophilic conditions were maintained by covering the gas output of the fermentor with sterile cotton caps. Aerobic and anaerobic conditions were assured by flushing alternatively nitrogen at a rate of 0.025 *Va*/*Vb* min^{-1} (volume added/volume broth min^{-1}) or air at the selected rate value in the range between 0.025 and 0.180 *Va*/*Vb* min^{-1}. The pH of the fermentation broth was automatically regulated, to an accuracy of 0.1 pH units, by a pH control module FBL-725, provided with a peristaltic pump which injected a fine stream of 1 N NaOH solution. The temperature was kept at a constant value of 32 ± 0.5°C by a temperature control module FBL-360. The fermentor and the media were sterilized by autoclaving at 120°C for 20 min, unless specified otherwise.

Analytical measurements. The initial sugar composition of the hydrolysates, as well as the sugar consumption during the fermentations, were determined by HPLC (Waters ALC 201 with RI detector). A Biorad HPX-42 column was used with 70 : 30 acetonitrile-water as the mobile phase at a flow rate of 0.8 ml/min.

Ethanol formation was followed, during the batch runs, by a gas chromatograph (Fractovap model C Type ATC/t Carlo Erba Milano), with a column packed with Cromosorb W coated with Carbowax 1500. The column was kept at 187 mA. Helium at 1.5 atm. was used as a carrier gas. The gas chromatograph was calibrated several times during each run by means of standard ethanol-water solutions.

The cell concentration was determined by filtering a known volume of culture broth on 0.45-μm autoclavable filters. The filters were dried at 105°C until no weight change between consecutive measurements was observed. In the presence of impurities in the broth, it was necessary to subtract, from the total weight, the fraction imputable to the precipitate, which had been previously determined with the same method before inoculating. In order to obtain the same starting biomass concentration for all the runs (1 g/l dry weight), a turbidimetric assay

described in detail in a previous work [11] has been utilized, using a Bausch and Lomb colorimeter at 595 nm wavelength.

The metal contents in hemicellulose hydrolysate were determined by atomic absorption spectrophotometry [12].

Furfural and hydroxymethylfurfural concentrations were measured using a gas chromatograph containing a Porepack Q column. Measures were carried out using nitrogen as a carrier gas and *n*-propanol as the internal standard, at oven and injection temperatures of 230 and 275°C, respectively.

RESULTS AND DISCUSSION

Choice of the best microorganism for ethanol fermentation

Several bacteria, fungi and yeasts are able to produce ethanol from pentoses, but yeasts appear to be the most suitable to ferment xylose, with particular regard to *P. tannophilus* [2,27,29,32], *C. shehatae* [14] and *P. stipitis* [32]. From those reports, *P. tannophilus* appears to be, in aerated cultures, the best microorganism for the fermentation of pure xylose solutions in terms of ethanol yields, whereas *C. shehatae* shows the highest fermentation rates. On the other hand, the specific productivity is nearly the same under anaerobic conditions. Up to now, however, hardwood hemicellulose hydrolysate has been successfully fermented only by *P. tannophilus* [4,23,24,30].

In order to select the best yeast for hemicellulose hydrolysate fermentation, three good ethanol producing strains of *P. tannophilus*, *C. shehatae* and *P. stipitis* were tested under microaerophilic conditions.

As the results presented in Fig. 1 show, *P. tannophilus* fermented hemicellulose hydrolysate pretreated according to the T.V.A. procedure with an ethanol yield of 0.25 g_P/g_S. Xylose and glucose were utilized simultaneously by *P. tannophilus* from the beginning of the fermentation, but xylose disappeared more slowly than glucose, before the latter sugar was completely consumed, which gives a partial confirmation of the observations of Beck and Strickland [4,30,31]. The novelty for this substrate is, on the contrary, the simultaneous ethanol and xylose consumptions after the product reaches its maximum concentration, with ethanol disappearing at a faster rate than xylose. In fact, this effect also was observed for the first time by the above authors but only during the fermentation of second-stage hydrolysates; on that occasion, perhaps, an insufficient fermentation time passed to observe it also in the hemicellulosic fraction. Moreover, hemicellulose hydrolysates containing higher glucose contents showed slightly lower yields in a previous work [24], which suggests that xylose consumption [4] is stimulated by glucose only when cellulose hydrolysate fractions are added to the medium.

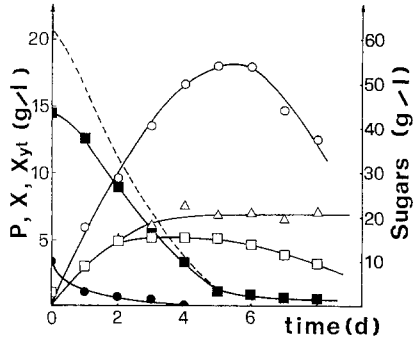

Fig. 1. Batch fermentation of hardwood hemicellulose hydrolysate by *P. tannophilus*. (○) P = ethanol; (△) Xyt = xylitol; (□) X = biomass; (-------) total sugars; (■) xylose; (●) glucose.

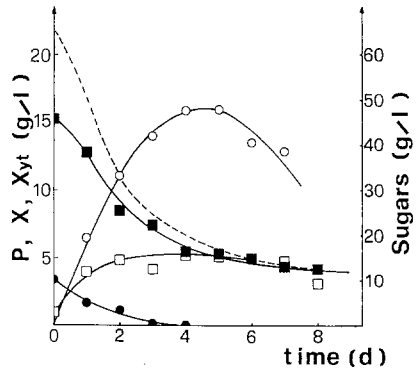

Fig. 2. Batch fermentation of hardwood hemicellulose hydrolysate by *C. shehatae*. (○) P = ethanol; (□) X = biomass; (-------) total sugars; (■) xylose; (●) glucose.

Hardwood seems to be, on the whole, a better raw material for ethanol production than wheat straw, whose hemicellulose hydrolysate with comparable sugar concentration (43 g/l) yielded only 0.23 g_P/g_S and 10 g/l ethanol. An interesting improvement of these yields was obtained by Fanta et al. (0.29 g_P/g_S) by carrying out the hydrolysis with trifluoroacetic acid in order to avoid the formation of toxic by-products [15].

The figure also shows a considerable production of xylitol, a by-product of alcohol fermentation which, for its dietetic and clinical properties, is gaining increasing interest and may be assumed also as a sweetener for diabetics [20]. Since, however, the aim of this study is the optimization of ethanol production, further batch fermentations, whose results are presented later on, have been carried out making an attempt to inhibit xylitol formation and stimulate, as a consequence, ethanol synthesis.

C. shehatae has shown, under the same conditions, a lower ethanol yield (0.21 g_P/g_S), but a higher fermentation rate. Fig. 2, where the fermentation parameters have been plotted versus the fermentation time, shows the same

ethanol metabolism previously evidenced for *P. tannophilus*, an incomplete consumption of sugars even after 8 days, but no formation of xylitol.

As regards *P. stipitis*, since Bruinenberg et al. [7] have found for synthetic substrates that xylose reductase in this yeast is subjected to the regeneration of both, NADPH and NADH, thus showing better ethanol yields with respect to *P. tannophilus*, we have researched the possibility that the same effect could occur also for hardwood hemicellulose hydrolysate. This yeast showed itself able to ferment with excellent yields (0.34 g_P/g_S) the synthetic substrate in about 10 days (Fig. 3) under microaerophilic conditions, with a concomitant production of xylitol of 0.15 $g_{xylitol}/g_{sugars}$ and a rapid decrease of ethanol after the achievement of its maximum value. On the other hand, such unacceptably low ethanol yields have been obtained for hardwood hemicellulose hydrolysate (Fig. 3), as to advise one against the utilization of *P. stipitis* for fermenting this substrate, unless specific pretreatment operations of hemicellulose hydrolysate are able to improve its performance.

Identification of the best pretreatment procedure

The above described T.V.A. pretreatment procedure was able to reduce the negative effects on the fermentation provoked by inhibiting substances present in hemicellulose hydrolysate. The formation of these substances occurs during the lignocellulose acid-catalyzed fractioning: pentoses and hexoses are partially dehydrated [18] and converted to furfural, hydroxymethylfurfural, their precursors and phenolic compounds [8], that are included in hemicellulose and are toxic for many microorganisms. Volatile compounds, such as furfural and phenols, are stripped by boiling, while overliming with $Ca(OH)_2$ removes other acid components (acetic and tannic acids). Furthermore, the pH increase up to 10.0, due to overliming, results in precipitation of heavy metals,

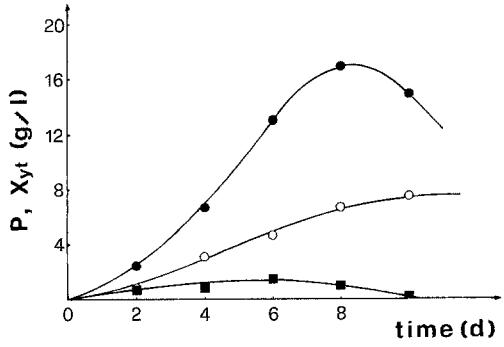

Fig. 3. Batch fermentations by *P. stipitis*. (●) P = ethanol and (○) Xyt = xylitol from hardwood hemicellulose hydrolysate; (■) P = ethanol from synthetic xylose substrate (Initial xylose concentration = 50 g/l).

TABLE 2

Tested combinations of various pretreatment operations

	Filtration after overliming		Sterilization		Boiling after Na_2SO_3 addition	
	warm	cold	wet	filter	yes	no
A	×			×		×
B	×		×			×
C	×		×	×		×
D		×		×		×
E		×	×			×
F		×	×	×		×
G	×			×	×	
H	×		×		×	
I	×		×	×	×	

mainly Fe and Mn, that are the most abundant, as the values of Table 1 show. Contemporaneously, furfural is transformed into furfuryl acid, which condenses with other components of hydrolysate [31]. In spite of this treatment, however, xylose is not completely consumed by *P. tannophilus* [4], thus yielding ethanol levels lower than those found in literature for synthetic substrates.

The fermentation efficiency has been improved in this work making a few additions and/or small variations in the original T.V.A. procedure, such as: (a) overliming has been followed by warm-filtration; (b) boiling after Na_2SO_3 addition has been eliminated; (c) the procedure included a final step of filtration through 0.45 μm filters.

Table 2 lists the tested combinations of the above variations; each of them is marked by a different letter, in order to make its reference in the text easier.

Table 3, on the other hand, shows the main batch results obtained with *P. tannophilus* under microaerophilic conditions, using hemicellulose hydrolysate prepared through the various pretreatment operations described above. The best results have been obtained with the substrate warm-filtered after overliming and finally filtered through 0.45 μm filters (combination A), with ethanol concentration and ethanol yield increasing up to 18 g/l and 0.29 g_P/g_S, respectively. This is a very encouraging result, if compared with the ethanol yields (0.34 g_P/g_S) observed by Slininger et al. [29] and Maleszka et al. [21] for synthetic substrates with a comparable starting sugar concentration. Furthermore, this ethanol yield is higher than the ones obtained with 0.2 μm filters by Strickland and Beck (up to 0.25 g_P/g_S) [30,31], though they were able to produce a good 19 g/l in seven days.

The explanation of these results is provided by some observations made during the experimentation and by the

TABLE 3

Batch fermentations of hardwood hemicellulose hydrolysate by *Pachysolen tannophilus*, under microaerophilic conditions

	Initial sugars (g/l)	Ethanol (g/l)	Time (days)	Xylitol (g/l)	Ethanol yield (g_p/g_S)	% of theoretical yield
A	61.6	18.0	5	7.5	0.29	57.3
B	63.0	13.1	4	6.5	0.21	40.7
C	66.7	12.1	3	7.5	0.18	35.6
D	62.3	15.3	6	9.0	0.25	48.1
E	58.0	11.1	5	11.0	0.19	37.5
F	58.1	9.6	5	8.0	0.16	32.4
G	63.8	11.6	3	7.5	0.18	35.6
H	62.7	11.4	5	7.0	0.18	35.6
I	63.7	11.6	5	7.0	0.18	35.7

Values refer to the maximum ethanol concentration.

comparison among the results obtained with the above nine combinations, as described below.

Warm filtration after overliming (A–C of Table 2) is more effective than cold filtration (D–F) in removing inhibiting substances from the broth, thus yielding higher ethanol concentrations. This finding can be explained with the roles of overliming and heat in reducing the concentration of volatile compounds, such as furfural, a potential inhibitor of ethanol production formed during hemicellulose hydrolysis [30]. Toxic substances could precipitate together with the crystals in formation of $CaSO_4$, whose solubility decreases with increasing temperature [19], thus assuring a more complete removal of them. The increase of xylitol formation resulting from cold filtration (D–F) could be ascribed to the insufficient solubilization and the consequent partial removal of not well identified hydrogen acceptors; the resulting reducing environment would support the transformation of xylose to xylitol, following the mechanism explained in more detail in the next section.

Analyses of the broth composition have demonstrated that wet sterilization (B, E, H) provokes the combination and the consequent precipitation of some nutrients or positive modulators, thus yielding a worsening of the fermentation, although the starting production rate appears to remain unvaried. This effect disappears in run H because boiling after Na_2SO_3 addition removes them in a previous step.

The above phenomenon becomes more marked in C and F where the precipitate formed during wet sterilization is completely removed by micro-filtration. For the boiling operation after Na_2SO_3 addition (I), the same considerations can be formulated.

A possible interpretation of the negative effect of boiling after Na_2SO_3 addition has been proposed above. A further cause could be the reaction of sulphite with the aldehydic groups of sugars, that partially prevents their degradation, and in a partial caramelization during this heating. This last supposition is suggested by the observation that the subsequent sterilization in runs H and I does not significantly modify the fermentation yields with respect to G.

Selection of the optimal fermentation conditions

The final phase of this study has been devoted to the selection of the best fermentation conditions, especially in terms of oxygen requirements.

Several authors have reported on the possibility of a few microbial strains producing ethanol better from pentoses in semiaerobic than in anaerobic conditions [22]. Bruinenberg et al. [7] suggested that the oxygen involvement in xylose fermentation may be due to the cofactor specificity of the first two enzymes in the xylose metabolic pathway.

In the absence of oxygen, ethanol production by *P. tannophilus* seems to be prevented or inhibited by a shortage of NAD^+ [6], the cofactor of xylitol dehydrogenase, that oxidizes xylitol to xylulose, a more easily fermentable sugar. On the contrary, the reducing environment could support the synthesis of NADPH, the cofactor of xylose reductase, that reduces xylose to xylitol [1].

In order to increase ethanol yields, it is necessary to activate electron transport in the respiratory chain, using either aerobic conditions or providing the culture broth with hydrogen acceptors. To this purpose, good results have been obtained by other workers with synthetic substrates, using as oxidizing compounds air oxygen or acetoin for *Brettanomyces* and other yeasts [25,26], acetoin for *C. utilis* [6], air oxygen [28,29] and acetone [2] for *P. tannophilus*.

TABLE 4

Effects of aeration and acetone addition on the fermentation of hemicellulose hydrolysate by *P. tannophilus*

	A[a]	M[b]	Acetone		Aeration (Va/Vb min^{-1})			
			50 mM	100 mM	0.025	0.050	0.090	0.180
Initial sugars (g/l)	58.4	61.6	55.9	51.1	54.9	70.1	54.5	58.4
Ethanol (g/l)	9.0	18.0	11.6	3.7	11.4	6.3	4.4	3.7
Time (days)	14	5	5	4	6	4	3	3
Ethanol yield (g_P/g_S)	0.15	0.29	0.21	0.07	0.21	0.09	0.08	0.06
% of theoretical yield	30.2	57.3	40.7	14.2	40.7	17.6	15.8	12.4
Xylitol (g/l)	8.5	7.5	2.6	2.7	3.0	2.3	2.2	1.5
Biomass (g/l)	1.2	5.2	6.5	6.0	9.0	10.5	11.5	13.9

Values refer to the maximum ethanol concentration.

[a] Anaerobic conditions.

[b] Microaerophilic conditions.

As the results of Table 3 show, the hemicellulose hydrolysate fermentation by *P. tannophilus* also leads to the formation of considerable amounts of xylitol, never detected up to now for this substrate. In accordance with these suggestions, batch fermentations of hemicellulose hydrolysate, prepared according to the most effective pretreatment operation (A), have been carried out using, alternatively, two different starting acetone concentrations (50 and 100 mM) or 4 air flows per reactor volume (namely 0.025, 0.050, 0.090, and 0.180 Va/Vb min^{-1}); the ranges of acetone concentration and air flow rate tested in this work have been chosen among the values reported in literature for synthetic substrates.

The main results of these tests are compared in Table 4 with those obtained under anaerobic and microaerophilic conditions. In the absence of oxygen in the broth, an ethanol yield of only 0.18 g_P/g_S and a simultaneous xylitol production of 8.5 g/l, obtained after about 15 days, are responsible for nearly the same percentage of xylose consumption. The production of biomass has been strongly affected by the absence of oxygen, reaching not more than 1.5 g/l dry weight. On the other hand, in comparison with microaerophilic tests, both additions of air and acetone actually repress xylitol formation but stimulate, at the same time, biomass growth rather than ethanol production, with particular concern to air addition. This finding gives a partial confirmation of the results obtained with air oxygen by Slininger et al. [29] for synthetic xylose substrate. In particular, the Pasteur effect observed even at the lowest air flow rate (0.025 Va/Vb min^{-1}) seems to point out that the oxygen requirements for NADH regeneration in *P. tannophilus* could be less in hardwood hemicellulose hydrolysate than the lowest value found with synthetic medium. From these considerations, it can

TABLE 5

Effects of aeration and acetone addition on the fermentation of hemicellulose hydrolysate by *C. shehatae*

	A[a]	M[b]	Acetone	Aeration (Va/Vb min^{-1})		
			50 mM	0.025	0.050	0.090
Initial sugars (g/l)	59.1	65.4	65.9	66.5	64.2	67.2
Ethanol (g/l)	1.0	16.0	14.8	9.6	3.3	2.4
Time (days)	8	5	6	4	5	3
Ethanol yield (g_P/g_S)	0.02	0.24	0.22	0.14	0.05	0.04
% of theoretical yield	3.3	47.7	44.0	28.3	10.1	7.0
Xylitol (g/l)	0	0	0	0	0	0
Biomass (g/l)	1.4	5.2	5.5	6.2	9.0	11.0

Values refer to the maximum ethanol concentration.

[a] Anaerobic conditions.

[b] Microaerophilic conditions.

be deduced that results obtained on the yeast metabolism using substrate of either synthetic or other nature are not directly transferable to hemicellulose hydrolysates.

Since the optimal fermentation conditions for other yeasts might not be the same as *P. tannophilus*, acetone and aeration effects on ethanol yields by *C. shehatae* have been also studied, notwithstanding the absolute absence of xylitol formation.

The experimental results obtained with *C. shehatae*, listed in Table 5, show a metabolism strongly reduced under anaerobic conditions with no ethanol production, confirming the results obtained by other authors with synthetic xylose medium [13]. Furthermore, a negative effect of both acetone and oxygen on ethanol concentration, as well as a corresponding increase in biomass production, proportional to the air flow have been detected, but these effects are very less marked than those observed for *P. tannophilus*. These observations on the whole suggest the xylitol pathway for *C. shehatae* could be completely different.

Lastly, none of the conditions tested in this study has been able to enhance the insufficient performance of *P. stipitis*, thus proving this strain to be absolutely unsuitable for hardwood hemicellulose hydrolysate fermentation.

The results of this study are slightly surprising because *P. stipitis* and *C. shehatae* are generally acknowledged to be better fermentors of xylan than *P. tannophilus*. The point is well made, however, that hydrolysates differ in many ways from pure sugars and synthetic substrates. It is possible that the acetic acid is playing an inhibitory role.

In conclusion, it is not clear why *P. stipitis* (which does not differ in many respects from *C. shehatae*) has performed so poorly although adequate parallel controls have been performed with all three organisms under similar conditions. The object of further studies will be determining the reason of this anomalous behaviour, which in our opinion should be explored in the possible specific sensitivity of this strain on substances contained in hemicellulose hydrolysate.

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