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Effect of Lignocellulosic Degradation Compounds from Steam Explosion Pretreatment on Ethanol Fermentation by Thermotolerant Yeast *Kluyveromyces marxianus*

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Abstract

The filtrate from steam-pretreated poplar was analyzed to identify degradation compounds. The effect of selected compounds on growth and ethanolic fermentation of the thermotolerant yeast strain *Kluyveromyces marxianus* CECT 10875 was tested. Several fermentations on glucose medium, containing individual inhibitory compounds found in the hydrolysate, were carried out. The degree of inhibition on yeast strain growth and ethanolic fermentation was determined. At concentrations found in the prehy-drolysate, none of the individual compounds significantly affected the fermentation. For all tested compounds, growth was inhibited to a lesser extent than ethanol production. Lower concentrations of catechol (0.96 g/L) and 4-hydroxybenzaldehyde (1.02 g/L) were required to produce the 50% reduction in cell mass in comparison to other tested compounds.

Index Entries: Ethanol production; *Kluyveromyces marxianus*; poplar biomass; inhibitors; fermentation.

Introduction

Conversion of lignocellulosic biomass into ethanol as an alternative to conventional petroleum transportation fuels is currently under extensive investigation *(1,2)*. The simultaneous saccharification and fermentation (SSF) process has been suggested as one of the most promising systems because the continuous removal of the sugars by the microorganism reduces the end-

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product inhibition of the enzyme complex. Over the past 10 years, our laboratory has selected a thermotolerant strain of *Kluyveromyces marxianus* capable of ethanol fermentation of glucose from cellulose in an SSF process at 42°C with a good ethanol yield *(3)*.

Steam explosion has been proposed as an efficient pretreatment of lignocellulosic materials and has the advantage of being able to be developed on a commercial scale *(4,5)*. During steam explosion pretreatment, some degradation products are formed that may be potential inhibitors during fermentation of the sugar fraction. After pretreatment, these inhibitors are in the aqueous portion of the pretreatment hydrolysate slurry. In an environmentally sustainable lignocellulose-to-ethanol process, this aqueous fraction should be used as fermentation broth to minimize fresh water requirements and decrease the amount of water waste produced.

Several researchers have investigated the nature of the inhibitors present in dilute-acid hydrolysates and steam explosion–pretreated biomass *(6–9)*. Nevertheless, not only does the concentration of the final inhibiting compounds vary greatly with the pretreatment conditions and the raw material used, but also their effects depend on the nature of the microorganism, as well as pH and temperature conditions of the fermentation broth.

In the present study, the filtrate from steam-exploded poplar was analyzed to identify degradation compounds. The effect of selected compounds on ethanol fermentation of the thermotolerant yeast strain *K. marxianus* CECT 10875 was tested.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO), except for 4-hydroxy-3,5-dimethylbenzyl alcohol (syringyl alcohol), which was obtained from Lancaster Synthesis (Morecambe, England).

Preparation of Poplar Hemicellulose Hydrolysate

One hundred grams of poplar biomass was subjected to pretreatment in a steam explosion pilot unit at 210°C and 4 min, operated by batches and equipped with a 2-L reaction vessel. The plant description and working methodology were described previously *(10)*. The pretreated material was suction filtered, and the filtrate was collected (approx 1 L) and analyzed.

Microorganism and Fermentation Conditions

K. marxianus CECT 10875, a thermotolerant strain obtained in our laboratory, was used in fermentation experiments. Active cultures for inoculation were prepared by growing the organism overnight on a rotary shaker at 150 rpm and 42°C in a growth medium (initial pH 5.5) containing: 5 g/L of yeast extract, 2 g/L of NH_4Cl, 1 g/L of KH_2PO_4, 3 g/L of $MgSO_{4}7H_2O$, and 30 g/L of glucose.

Experiments were carried out in 100-mL Erlenmeyer flasks each containing 25 mL of the growth medium under nonsterile conditions and agitated at 150 rpm. Flasks were inoculated at 4% (v/v).

To study the effect of the concentration of toxic substances on growth and ethanol production, different amounts of individual inhibitory compounds were added to the fermentation medium. A culture without toxic compounds was used as control. After 24 h of fermentation, the flasks were checked for cell growth and ethanol production.

In experiments with organic acids supplementation, the broth was adjusted to pH 5.5 with 2 *N* NaOH. In experiments studying the assimilation profile of aldehydes and glucose fermentation, samples were taken periodically. All experiments were carried out in triplicate.

Analytical Procedures

Vanillin, vanillyl alcohol, syringaldehyde, syringyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, catechol, guaiacol, 4-hydroxybenzoic acid, syringic acid, and vanillic acid analyzes were performed on a high-performance liquid chromatography (HPLC) system. An 1100 Hewlett Packard liquid chromatograph with an Aminex HPX-87H stainless steel (300 × 7.6 mm) column (Bio-Rad, Hercules, CA) and a 1040A Photodiode-Array detector was used. The mobile phase was 82% 5 m*M* H_2SO_4 and 18% acetonitrile at a flow rate of 0.3 mL/min and temperature of 55°C. For 5-hydroxymethylfurfural (HMF), furfural, and furfuryl alcohol analysis, a reversed-phase (250 × 4 mm) Lichrosorb RP18 column was employed. The mobile phase was a mixture of buffer solution of 1.25 g/L of monobasic sodium phosphate and 1.25 g/L of dibasic sodium phosphate, and methanol in a proportion of 90–10%. A 0.6 mL/min flow rate and 50°C temperature were used.

Acetic, levulinic, and formic acid analysis was carried out with an HPLC system with a refractive index detector. Separation was performed with an Aminex HPX-87H column (Bio-Rad). The mobile phase was 5 m*M* H_2SO_4, at a flow rate of 0.6 mL/min and temperature of 65°C.

Ethanol was measured by gas chromatography using a Hewlett Packard 5890 Series II apparatus with a flame ionization detector and a columm of Carbowax 20M (2 m × 32 mm) at 85°C. Injector and detector temperatures were 150 and 190°C, respectively.

Cell mass was determined by optical density measurements at 600 nm using a Jasco V-530 UV/VIS spectrophotometer, and gravimetrically by dry weight as follows: culture liquid (5 mL) was filtered (0.45-µm HA; Millipore) and the filter washed with water and dried to constant weight in a microwave oven for 15 min. Measurements of cell growth were done in triplicate.

Results

Identification of Degradation Compounds in Hydrolysate

The hydrolysate from steam explosion pretreatment of poplar at 210°C and 4 min residence time was analyzed. Table 1 shows the quantitative determination of degradation compounds identified. Results are expressed as milligrams of the compound per liter of filtrate.

Table 1
Degradation Products Composition of Liquid Fraction Obtained After Steam Explosion Pretreatment of Poplar

Compound	Concentration (mg/L)[a]
Acetic acid	2100
Formic acid	430
Levulinic acid	NQ
Furfural	490
5-HMF	80
4-Hydroxybenzaldehyde	NQ
4-Hydroxybenzoic acid	100
Catechol	30
Guaiacol	NQ
Syringaldehyde	50
Syringic acid	NQ
Vanillin	14
Vanillic acid	NQ

[a]NQ, not quantified.

Acetic acid (2100 mg/L) and furfural (490 mg/L), from hemicellulose degradation of hardwood poplar, were the main compounds present in the hydrolysate. 4-Hydroxybenzoic acid (100 mg/L) and syringaldehyde (50 mg/L) constituted a large fraction of the lignin-derived compounds in the hydrolysate.

Effects of Degradation Compounds on Growth and Fermentation

The inhibitory effect of various concentrations of toxic compounds on growth and ethanol fermentation of *K. marxianus* CECT 10875 was investigated. Cultures of yeast strain were grown in a glucose-containing medium and supplemented with varying initial concentrations of degradation compounds identified in the hydrolysate. Dose-response curves for ethanol production and growth at 24 h were determined for all compounds. Results of growth and ethanol concentration were expressed as percentage of the control (one hundred percent of the control growth and ethanol production is equivalent to 3.4 ± 0.1 g/L and 12.3 ± 0.5 g/L, respectively). Results for acetic acid, furfural, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and catechol are shown in Fig. 1. Experiments using formic and levulinic acids showed results similar to acetic acid assays, results obtained using 5-HMF were similar to 5-furfural, and those using syringaldehyde were similar to 4-hydroxybenzaldehyde (data not presented).

In all cultures, growth inhibition was more intensive than ethanol production. Dose-response curves for growth in cultures supplemented with 4-hydroxybenzaldehyde and furfural exhibited a sigmoidal pattern, while catechol followed almost a linear pattern. No total inhibition of both ethanol

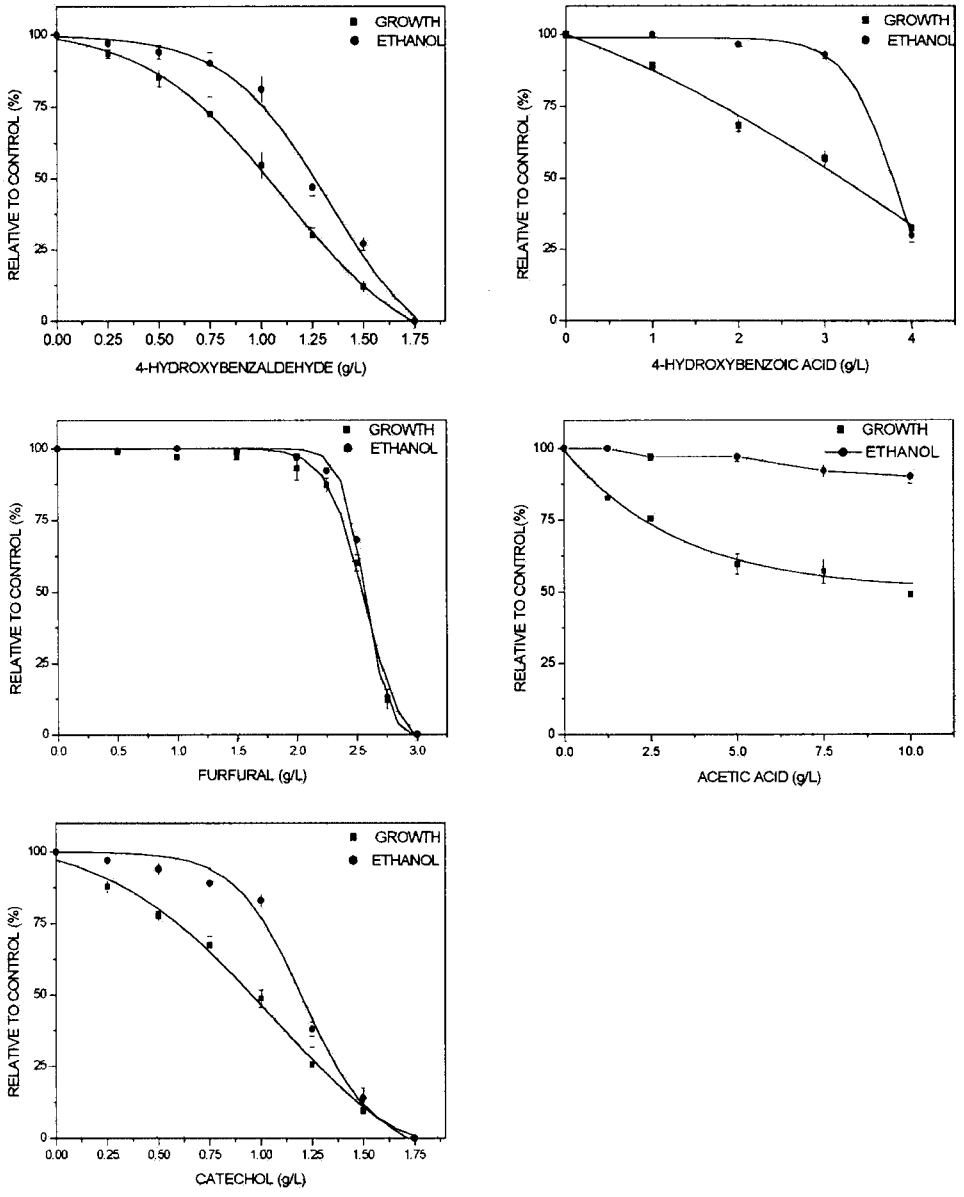


Fig. 1. Effect of degradation compounds produced in steam explosion pretreatment of poplar biomass on growth and ethanol production of *K. marxianus* CECT 10875 after 24 h. SDs are represented by error bars. One hundred percent control equivalent to growth = 3.45 g/L, and ethanol production = 12.3 g/L.

production and growth was observed at the highest 4-hydroxybenzoic acid concentration (4 g/L) assayed.

Ethanol production was not affected by the presence of acetic acid at the concentrations tested. At the highest acetic acid concentration (10 g/L), growth was 53% of the control (one hundred percent of the control growth

Table 2
Concentration of Toxic Compounds Required to Inhibit 50% of Growth and Ethanol Production of *K. marxianus* CECT 10875 at 24 h

	Inhibitory concentration (g/L)	
	Growth	Ethanol
Furfural	2.53	2.60
5-HMF	4.01	4.20
Vanillin	2.55	2.67
Syringaldehyde	2.86	3.50
4-Hydroxybenzaldehyde	1.02	1.24
Catechol	0.96	1.19
Guaiacol	1.43	1.75
4-Hydroxybenzoic acid	3.10	3.86

is equivalent to 3.4 ± 0.1 g/L). Dose-response curves for ethanol production followed a sigmoidal pattern for all compounds except acetic acid (Fig. 1) and formic and levulinic acids (data not shown).

To facilitate comparison of toxicity for the compounds assayed, the concentrations of the test agents that caused 50% inhibition of growth and ethanol production are given in Table 2. Catechol and 4-hydroxybenzaldehyde showed the highest inhibitory effect. An initial concentration of these compounds close to 1 g/L caused 50% inhibition of both growth and ethanol production processes. Furfural was more toxic than 5-HMF, and a twofold higher concentration of HMF was necessary to reach the 50% level of inhibition.

Effect of Initial pH on Toxicity of Organic Acids

Considering that the initial pH of the fermentation medium has a marked effect on toxicity of acids, the influence of the different acids found in the hydrolysates on ethanol production was examined, individually, at two different pHs (Fig. 2). The toxic effect of all acids tested, at a concentration of 5 g/L, decreased with increasing pH. At pH 5.5, the addition of 5 g/L of organic acids had almost no effect on ethanol production. However, at pH 4.0, the presence of 5 g/L of levulinic, formic, or vanillic acid, individually, blocked ethanol production (Fig. 2). Less inhibition was observed for 4-hydroxybenzoic and syringic acid (30 and 75% of the control, respectively).

Assimilation Profiles of Aldehydes

Analysis of culture media extracts supplemented with inhibitors over the course of fermentations by *K. marxianus* CECT 10875 showed that acids and alcohols were not metabolized by the yeast, and that their concentration remained constant during fermentation (data not shown).

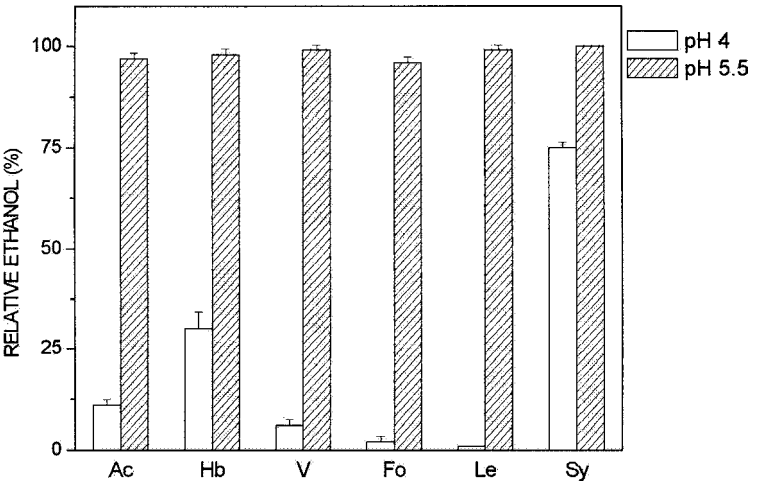


Fig. 2. Effect of organic acids (5 g/L concentration) on ethanol production by *K. marxianus* CECT 10875 at different initial pHs. Ac, acetic acid; Hb, 4-hydroxybenzoic acid; V, vanillic acid; Fo, formic acid; Le, levulinic acid; Sy, syringic acid.

In the case of furfural, 5-HMF and aromatic aldehydes, the microorganism had the ability to assimilate these compounds. In Fig. 3 the assimilation profiles for several aldehydes are shown at two different initial concentrations.

At a concentration of 1 g/L (Fig. 3A), furfural was completely metabolized in the first 4 h of fermentation. 5-HMF, vanillin, and syringaldehyde were also metabolized by the yeast strain, but their assimilation rates were slower. The concentration of these compounds decreased during fermentation and they became undetectable after 8 h of fermentation. The assimilation of 4-hydroxybenzaldehyde was significantly slower; thus after 8 h of fermentation, only 0.1 g/L of this compound had been assimilated. After 24 h, however, no 4-hydroxybenzaldehyde remained in the medium.

At an initial aldehyde concentration of 2 g/L (Fig. 3B), furfural and 5-HMF were completely assimilated after 8 h incubation, and vanillin and syringaldehyde were assimilated after 16 h. *K. marxianus* CECT 10875 only assimilated 30 % of the 4-hydroxybenzaldehyde after 24 h of incubation.

Effect of Aldehydes on Glucose Fermentation

Fermentation profiles in the presence of 2 g/L of furfural, vanillin, and syringaldehyde, and 1 g/L of 4-hydroxybenzaldehyde are presented in Fig. 4. The initial aldehyde concentration was selected according to the maximum concentration that allows the yeast to reach a final ethanol concentration similar to control (12.3 g/L).

As can be seen in Fig. 4A, furfural was completely reduced to furfuryl alcohol in the first 8 h of fermentation. No ethanol production was observed when furfural was present in the medium. A final ethanol concentration of 12.1 g/L was reached after 20 h of fermentation.

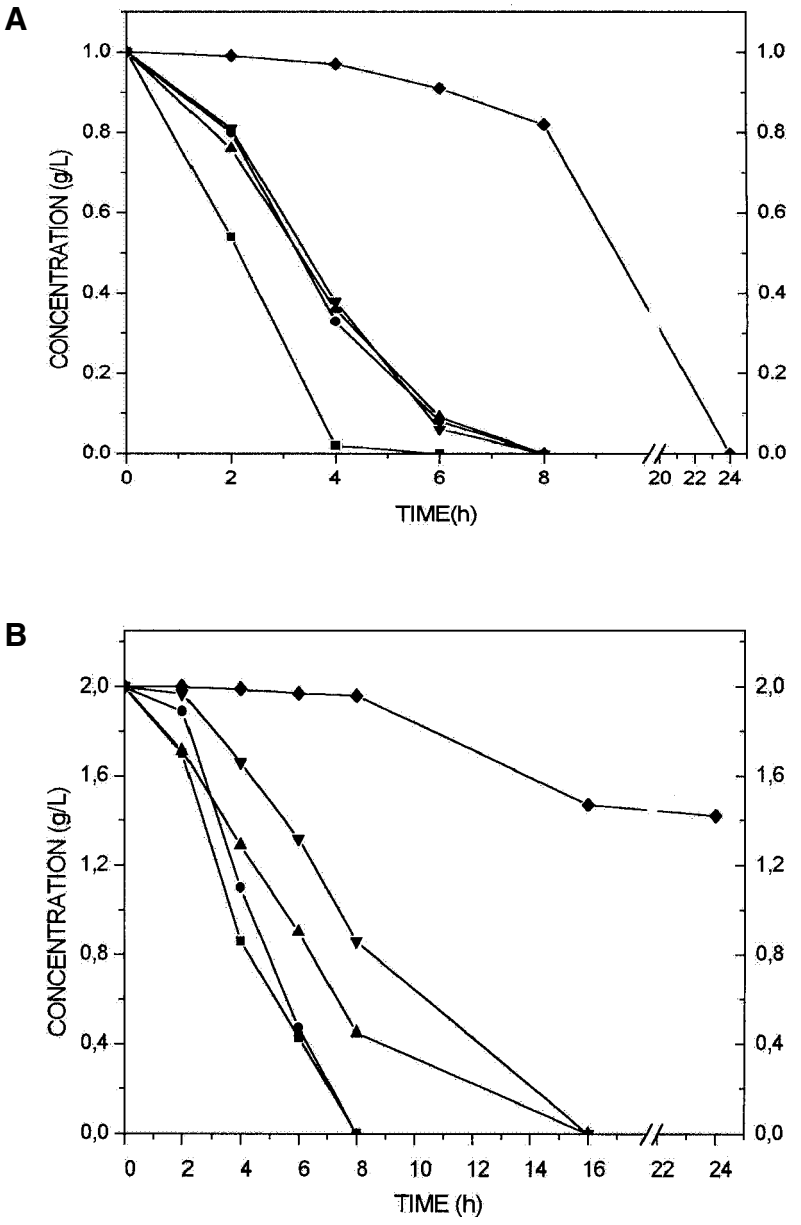
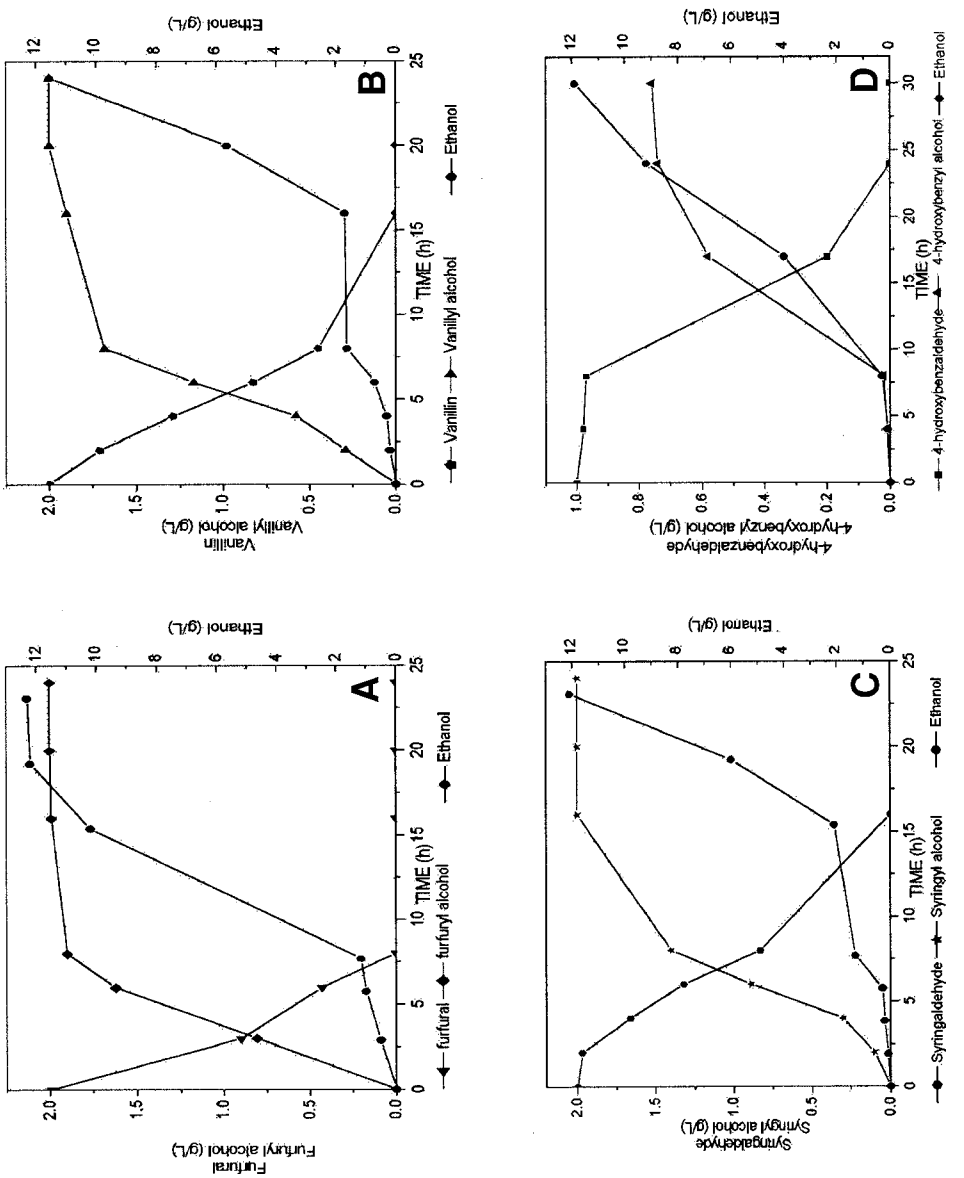


Fig. 3. Assimilation profiles for *K. marxianus* CECT 10875 of **(A)** 1 g/L and **(B)** 2 g/L of different aldehydes obtained in hydrolysate after steam explosion pretreatment of poplar. (■) Furfural; (●) 5-Hydroxymethylfurfural; (▲) Vanillin; (▼) Syringaldehyde; (◆) 4-Hydroxybenzaldehyde.

Fig. 4. *(opposite page)* Conversion of aldehydes and ethanol production during glucose fermentation by *K. marxianus* CECT 10875. **(A)** Furfural, **(B)** Vanillin, **(C)** Syringaldehyde, and **(D)** 4-Hydroxybenzaldehyde.


A
B
C
D
Furfural
Furfuryl alcohol (g/L)
Ethanol (g/L)
TIME (h)
furfural
furfuryl alcohol
Ethanol
Vanillin
Vanillyl alcohol (g/L)
Vanillin
Vanillyl alcohol
Ethanol
Syringaldehyde
Syringyl alcohol (g/L)
Syringaldehyde
Syringyl alcohol
Ethanol
4-hydroxybenzaldehyde
4-hydroxybenzyl alcohol (g/L)
4-hydroxybenzaldehyde
4-hydroxybenzyl alcohol
Ethanol

Ethanol production in the presence of 2 g/L of vanillin (Fig. 4B) and syringaldehyde (Fig. 4C) followed a similar pattern. These compounds were metabolized completely by the microorganism after 16 h of fermentation. As observed with furfural, the reduction of toxic aldehydes to their corresponding alcohols was necessary for ethanol production to proceed. Final ethanol concentrations close to 12 g/L were obtained in both experiments after 24 h of fermentation.

The results of Fig. 4D show that *K. marxianus* had the ability to reduce 1 g/L of 4-hydroxybenzaldehyde after a lag period of 8 h. The reduction rate was significantly slower in comparison to the other aldehydes tested, so the microorganism needed an incubation period of 24 h to metabolize all the 4-hydroxybenzaldehyde. At this time only 0.76 g/L of 4-hydroxybenzyl alcohol was obtained. A final ethanol concentration of 12 g/L was achieved after 30 h of fermentation.

Discussion

Degradation Compounds in Hydrolysate

All compounds found in the liquid fraction obtained from steam explosion pretreatment of poplar biomass have been previously identified in other hardwood hydrolysates *(6–9)*. Acetic acid from hydrolysis of hemicellulose and furfural from degradation of xylose were obtained as a consequence of the high xylan content in hardwood. Vanillin, which is formed by degradation of guaiacylpropane units of lignin and syringaldehyde, which are formed in turn by the degradation of syringyl propane units, has been reported in hydrolysates from other hardwoods such as poplar *(6,7)* and red oak *(8)*. 4-Hydroxybenzoic acid constitutes a large fraction of lignin-derived compounds in hydrolysates from the hardwoods poplar *(7)* and willow *(9)*.

Effect of Degradation Compounds on Growth and Fermentation

The effect of toxic compounds generated in the pretreatment of lignocellulosic biomass has been examined in different microorganisms such as prokaryotes *(11–15)*, xylose-fermenting yeasts *(16,17)*, and *Saccharomyces cerevisiae (17–23)*. However, no studies of the inhibitory effect on growth and ethanol production of these compounds have been reported for the thermotolerant yeast *K. marxianus*.

To interpret the results in the literature, it should be kept in mind that the solubility of these compounds is limited. Thus, when a high concentration of a compound is tested, it is possible that, in fact, the microorganism is exposed to a lower concentration *(24)*.

At the concentrations tested, growth inhibition was observed for all compounds examined. Cell growth was generally more influenced than ethanol production for all conditions assayed.

Results from dose-response curves of the different compounds tested showed that catechol and 4-hydroxybenzoic acid inhibit the growth of the

yeast strain in a near linear pattern, indicating that the inhibition of growth is closely related to the initial concentration of these molecules. However, aldehydes exhibited a sigmoidal inhibition with a shoulder at low concentrations. Bacteria and yeasts have been shown to metabolize furans *(20–22)*, and aromatic aldehydes *(15,17–19)*, although enzymes involved in the metabolic pathways remain unknown in most cases. In our study the assimilation of furfural, HMF, vanillin, syringaldehyde, and 4-hydroxybenzaldehyde by *K. marxianus* was demonstrated. The strain exhibited higher assimilation rates for all aldehydes compared to what has been reported for other glucose-fermenting microorganisms such as *Klebsiella pneumoniae (15)*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis (17)*. According to Delgenes et al. *(17)*, *S. cerevisiae* exhibited a lag assimilation period of 24 and 30 h at an initial concentration of 2 g/L of furfural and vanillin, respectively. *K marxianus*, however, required only 8 and 16 h, respectively, to completely assimilate these compounds.

The reduction of furfural to furfuryl alcohol at the beginning of fermentation has been observed by other investigators *(21,24)*, and is generally believed to be catalyzed by NADH-dependent alcohol dehydrogenase *(16)*. Furfural is a strong inhibitor of ethanol fermentation of *K. marxianus*. A total conversion of furfural to the corresponding alcohol is needed to start ethanol production. The conversion of furfural to furfuryl alcohol and furonic acid has been reported for a number of yeasts such as *S. cerevisiae (20–22)*, *Pichia (16)*, *Turolopsis*, and *Rhodotorula (25)*. We have found furfuryl alcohol as the only metabolite from furfural assimilation.

Likewise, vanillin and syringaldehyde were metabolized by *K. marxianus* in the fermentation process and totally converted into their corresponding alcohols. In experiments with 4-hydroxybenzaldehyde, however, although all the aldehyde was assimilated by the tested strain, it was only partially transformed to 4-hydroxybenzyl alcohol (76%), and an unidentified compound was detected by HPLC analysis.

4-Hydroxybenzaldehyde was the most toxic aromatic aldehyde for *K. marxianus*. At low concentration (1 g/L), the yeast exhibited a lag period of 8 h, but after prolonged incubation (24 h), this compound was completely reduced. However, at a higher concentration (2 g/L), only 25% of the initial 4-hydroxybenzaldehyde was metabolized after 24 h of incubation.

The conversion of vanillin *(15,19)* and syringaldehyde *(15)* to their corresponding alcohols by microorganisms has been previously observed. By contrast, Nishikawa et al. *(15)* found that microbial metabolism of vanillin and syringaldehyde led to the production of other compounds. In the metabolism of vanillin, besides vanillyl alcohol, veratrole and several unidentified self-condensation products were detected. Analogously, in the metabolism of syringaldehyde a multitude of minor products, none dominating, was observed.

Several potential mechanisms for the toxicity of aldehydes have been suggested *(11)*, including damage from chemical reactivity, direct inhibition of glycolysis and fermentation, and plasma membrane damage.

Zaldivar et al. *(11)* suggested that the toxic effects on growth and fermentation owing to aromatic aldehydes and HMF appear similar. Previous studies *(26)* have suggested that the mechanisms for inhibition by these compounds were cell damage and direct interference with biologic membranes, which affect their ability to serve as selective barriers and enzyme matrices. Furfural, however, appears to have a direct effect on either the glycolytic or fermentative enzymes *(11)*.

It is worthwhile to mention that *K. marxianus* CECT 10875, a strain selected for resistance to temperature, also exhibits higher resistance to aromatic compounds and HMF, but not to furfural, in comparison with other microorganisms *(11)*. Since there is evidence in the literature that temperature can change fatty acid composition, the composition of plasma membrane of the thermotolerant *K. marxianus* strain could be the basis for its resistance to aromatic aldehydes and HMF.

The decrease in biomass formation when aliphatic acids were added to the fermentation medium has been previously reported in *S. cerevisiae (27)*. Concentrations of acid that inhibited growth by 50% caused only modest inhibition of ethanol production. However, we did not find increased ethanol yield with the addition of a low concentration of acetic acid to the fermentation medium. Differences in pH have a pronounced effect on the toxicity of acids but not aldehydes or alcohols. The increase in organic acid toxicity at reduced pH is consistent with the mechanism observed for other microorganisms: uncoupling and intercellular ion accumulation *(28)*.

Conclusion

Growth and alcoholic fermentation of glucose by *K. marxianus* CECT 10875 was significantly affected by the presence of biomass decomposition products. The results showed that growth is more affected than ethanol production. The degree of inhibition caused by the toxic compounds greatly depended on the nature and concentration of inhibitor. At inhibitor concentrations found in the hydrolysate of steam-exploded poplar biomass, no single inhibitor completely inhibited growth or fermentation.

4-Hydroxybenzaldehyde and catechol were the most powerful inhibitors of growth and ethanol production. Many of the aldehydes were metabolized by the microorganism to their corresponding alcohols, which resulted in the removal of the toxic compounds and partial recovery of both growth and ethanol production.

K. marxianus CECT 10875, a strain selected for resistance to temperature, exhibited resistance to aromatic compounds and HMF. The yeast strain showed higher aldehyde assimilation rates in comparison with other fermentation microorganisms.

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