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Simultaneous Saccharification and Fermentation of Steam-Pretreated Spruce to Ethanol

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Abstract

Ethanol production was studied in simultaneous saccharification and fermentation (SSF) of steam-pretreated spruce at 42°C, using a thermotolerant yeast. Three yeast strains of *Kluyveromyces marxianus* were compared in test fermentations. SSF experiments were performed with the best of these on 5% (w/w) of substrate, at a cellulase loading of 37 filter paper units/g of cellulose, and a β-glucosidase loading of 38 IU/g of cellulose. The detoxification of the substrate and the lack of pH control in the experiments increased the final ethanol concentration. The final ethanol yield was 15% lower compared to SSF with *Saccharomyces cerevisiae* at 37°C, owing to the cessation of ethanol fermentation after the first 10 h.

Index Entries: Simultaneous saccharification and fermentation; *Kluyveromyces marxianus*; ethanol fermentation; steam-pretreated spruce.

Introduction

Cellulose, the main component of all higher plants, is a renewable carbon and energy source, which is produced in large amounts worldwide. The cellulose content of lignocellulosic materials can be hydrolyzed to glucose by cellulolytic enzymes, and the released fermentable sugars can be converted to ethanol by yeasts. The ethanol produced from this biomass can be used as a transportation fuel. The use of ethanol as a biofuel has environmental benefits and could help relieve future energy scarcity *(1)*. Simultaneous saccharification and fermentation (SSF) is the most promising process for the production of ethanol from lignocellulosics. In SSF, enzymatic hydrolysis of cellulose to glucose and fermentation of sugars to ethanol are

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carried out simultaneously, in the same vessel. The rate of hydrolysis is increased by reduced glucose inhibition of the cellulases, in comparison to separate hydrolysis and fermentation, which results in higher productivity or reduced enzyme consumption for the same productivity *(2,3)*. Another advantage of SSF is a lower capital cost owing to the reduced number of vessels needed *(4)*.

One disadvantage of SSF is the difference in optimum conditions (pH and temperature) for hydrolysis and fermentation. The optimal pH is 4.8 for the cellulase enzyme system and between 4.0 and 5.0 for the yeast cell. Accordingly, if the pH is maintained between 4.5 and 5.0 during the SSF, it is convenient for both the enzyme and the yeast. The optimal temperature is below 35°C for yeasts normally used for producing ethanol. Temperatures above 40°C lower the viability of most yeast cultures. The rate of hydrolysis is highest at temperatures in the 40–50°C range, since the cellulase enzyme system acts optimally at about 50°C. At lower temperatures, the rate of saccharification decreases considerably. For example, at 30°C the enzyme system is working at approx 50% of its maximum activity *(5)*.

The temperature in SSF experiments using *Saccharomyces cerevisiae* as a fermenting yeast is usually 37°C *(6–9)*. However, at this temperature, the degree of saccharification, which is the rate-limiting step in a major part of SSF *(10)*, does not reach the maximum. One possible way to overcome this problem is to use such a yeast for fermentation that can grow and ferment at higher temperatures. In addition, the yeast must also tolerate inhibitors in the hydrolysate and have a high tolerance for ethanol.

In several studies *(11–13)*, *Saccharomyces*, *Candida*, and *Kluyveromyces* strains have been screened for their ability to grow and ferment glucose at temperatures above 40°C. In these studies the investigators have shown *Kluyveromyces* strains to be the most thermo and ethanol tolerant, capable of producing ethanol from glucose at the temperature interval of 40–50°C.

Szczodrak and Targonski *(11)* have tested the fermentative properties of yeast strains with respect to the hydrolysis products of hemicellulose and cellulose. The selected strains, belonging to genera *Kluyveromyces*, *Saccharomyces*, and *Fabospora* were able to ferment glucose, galactose, and mannose at 40, 43, and 46°C, respectively.

Accordingly, *Kluyveromyces marxianus* has been the most used thermotolerant strain for SSF *(12,14–18)*. When ethanol production was performed with purified cellulosic substrate and glucose as carbon sources, it yielded high ethanol concentrations, before the sensitivity of yeast to ethanol appeared at the elevated temperatures *(12,14)*.

When using a lignocellulosic material such as wood as a substrate, pretreatment is required so that the cellulose will be more available to the enzymatic attack *(19)*. Steamexplosion is a widely utilized method for the pretreatment of lignocellulosics *(20,21)*. However, a major problem associated with steam pretreatment is the formation of undesirable degradation products (such as furfural, phenolic compounds, 5-hydroxymethyl furfural [HMF], and acetic acid), which have well-known toxic effects on the growth and fermentability of yeast *(22,23)*.

The negative effects of these degradation products can be alleviated by the detoxification of the hydrolysate of pretreated lignocellulosics. Overliming is one of the most widely used *(24)* and least costly *(25)* detoxification methods. The increase in ethanol yield, owing to increased fermentability of yeast, with the use of overliming to detoxify the hydrolysate of pretreated wood was demonstrated *(26,27)* when *S. cerevisia* was used for fermentation.

In the present study, three thermotolerant yeast strains belonging to the *K. marxianus* group were screened for their ferment ability at higher temperatures. SSF experiments on steam-pretreated spruce (SPS) were carried out at 42°C using the previously selected yeast *K. marxinus* Y.00243, which showed less sensitivity to inhibitors with the highest ethanol productivity. In some experiments, the hydrolysate was detoxified by overliming to increase the ethanol yield.

Materials and Methods

Raw Material

Chips of spruce, *Picea abies*, free from bark, were obtained from a sawmill (Harry Nilsson, Hästveda, Sweden). The wood chips were further chipped and sieved, and fractions between 2.2 and 10 mm were used. The dry matter (DM) content was 47.0%. Table 1 gives the composition of the raw material, analyzed according to Hägglund *(28)*.

Pretreatment

Prior to pretreatment, the chips were impregnated with SO_2, 2.5% (w/w) (based on the moisture in wood) for 20 min. The chips were pretreated at 215°C for 5 min, as described in detail previously *(29)*. The pretreated material was stored at 4°C.

Substrate

After pretreatment, the total and insoluble DM contents were 18.6 and 13.0%, respectively. The yield of fibrous material was 64% (based on original dry wood). The lignin and glucan contents of the solid material were 39 and 51.5%, respectively. The concentrations of sugars in the liquid after pretreatment were 13.3 g/L of glucose, 3 g/L of arabinose, and 25 g/L of xylose + galactose + mannose. The concentrations of furfural, HMF, and acetic acid were 0.8, 1.7, and 4 g/L, respectively.

Enzymes

Two commercial enzymes from NOVO Industries, Denmark, were employed: Celluclast 2L, a cellulase enzyme system from *Trichoderma reesei* with an activity of 80 filter paper units (FPU)/mL *(30)* and β-glucosidase activity of 21 IU/mL *(31)*; and Novozym 188, a cellobiase from *Aspergillus niger* with a β-glucosidase activity of 490 IU/mL.

Table 1
Composition of Raw Material and Pretreated Material

Component	Raw material (% of DM)
Glucan	43.3
Xylan	4.5
Galactan	2.1
Arabinan	0.8
Mannan	12.7
Lignin	28.1

Microorganisms

Three yeast strains, kindly provided by the National Collection of Agricultural and Industrial Microorganisms, University of Horticulture and Food Industry (Budapest, Hungary), belonging to the genera *K. marxianus* (*K. marxianus* Y.00243, *K. marxianus* Y.00242, and *K. marxianus* Y.01070), were investigated. Commercial baker's yeast, *S. cerevisiae*, was used as a control (Kron Jäst from Jästbolaget, Sweden).

Cultivation of Yeasts

The yeasts were incubated at 26°C on agar slant containing 1% (w/w) glucose, 1% (w/w) yeast extract, 1% (w/w) peptone, and 2% (w/w) agar. The slants incubated for 3 d and were washed with sterile distilled water. The yeast suspension was used for the inoculation of the growth medium with about 0.5 g/L of initial cell concentration (based on oven dry matter [ODM]). The yeasts were grown under sterile conditions at 30°C in 1-L, baffled Erlenmeyer flasks with 150 mL of medium, and were stirred on a rotary shaker at 64*g*. The medium for the production of yeast cells consisted of 2.5 g/L of yeast extract, 5.0 g/L of peptone, 1 g/L of KH_2PO_4, 0.3 g/L of $MgSO_4 \cdot 7H_2O$, 2.0 g/L of NH_4Cl, and 50.0 g/L of glucose *(12)*. The glucose was consumed after 10 h of cultivation (paper test; Clinistix, Bayer), and the final cell mass was 7 to 8 g of ODM/L. Before the inoculation of the SSF reactor, the yeast culture was centrifuged for 10 min at 15,000*g* (Beckman Avanti TM J-251 High Performance Centrifuge, Beckman, Palo Alto, CA), and was either stored at 4°C for a night (1-d culture) or used immediately after the centrifugation and determination of dry weight (fresh culture).

SSF Experiments

SSF experiments were run for 96 h, in 1-L fermenters (Belach AB, Stockholm, Sweden) with 750 g of SSF medium. The whole slurry from the pretreatment stage, diluted to 5% (w/w) dry wt of solid material, was used as substrate. In some experiments, the hydrolysate was detoxified with

$Ca(OH)_2$ by adjusting the pH to 10.0. After 1 h of mixing, the solution was filtered through a 0.45-μm cellulose filter. After filtration, the pH of the filtrate was readjusted to 5.5 with H_2SO_4 *(27)*.

The amount of Celluclast 2L added was 24% (w/w) insoluble DM corresponding to the cellulase activity of 37 FPU/g of cellulose. The amount of Novozym 188 added was 4% (w/w) insoluble DM corresponding to the β-glucosidase activity of 38 IU/g of cellulose. The composition of nutrients was 1.0 g/L of yeast extract, 0.5 g/L of $(NH_4)_2HPO_4$, 0.025 g/L of $MgSO_4 \cdot 7H_2O$. The SSF cultures were inoculated to a concentration of 5 g of DM/L.

The experiments were carried out at 42°C with and without pH control. The initial pH was adjusted to 5.0 with $Ca(OH)_2$ in all experiments. In the case of pH control, the pH was maintained at 4.8–5.0, by the addition of 10% (w/w) NaOH and 10% (w/w) H_2SO_4.

Fermentation Tests

Three *K. marxianus* yeast strains were tested in fermentation experiments, using *S. cerevisiae* as a control. The test fermentations were performed in 25-mL glass flasks as previously described *(32)*. The conditions (i.e., concentrations of nutrients and inoculum, temperature, and initial pH) were the same as in the SSF experiments. The fermentations were run with filtrate and with detoxified filtrate, using the hydrolysate diluted to the same concentration as that used in the SSF experiments with 5% DM. The broth was supplemented with glucose to a total concentration of fermentable sugar (mannose, xylose, galactose, and glucose) of 30 g/L. The fermentation tests were run for 96 h. Samples were drawn every hour for the first 8 h, and seven more times between 8 and 96 h of fermentation.

Analysis

The samples from SSF were centrifuged for 5 min at 6100*g*, and the supernatant was filtered (MFS-13: CA membrane, 0.2-μm pore size, MFS Inc.), diluted 10 times with Millipore water, and analyzed using high-performance liquid chromatography (Shimadzu LC-10A, Shimadzu, Kyoto, Japan) equipped with an RI detector. The samples from the fermentation tests were filtered and diluted before the analysis. Ethanol, glucose, mannose, xylose, galactose, arabinose, lactic acid, glycerol, acetic acid, HMF, and furfural were analyzed on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65°C. This column cannot separate xylose and galactose from mannose; therefore, the change of the collective peak area (man/xyl/gal) had to be used to estimate the consumption of these sugars. The mobile phase was 5 m*M* H_2SO_4 at a flow rate of 0.5 mL/min.

The yeast cell concentration was determined by measuring the DM content. A 5-mL sample taken at the end of the yeast cultivation was centrifuged at 6100*g* for 10 min, washed twice with distilled water, and dried in an oven at 105°C to constant mass. After about 1 d, the dried sample was cooled to room temperature in a desiccator and weighed *(33)*.

Results

Screening K. marxianus *Strains for Fermentation of Hydrolysate*

In the test fermentations, the ethanol production was investigated on SPS supernatants under anaerobic conditions. Table 2 presents the results. *K. marxianus* Y.00243 consumed almost all the glucose in the filtrate within 8 h and produced 6.3 g/L of ethanol. The glucose consumption was faster on detoxified filtrate, with the same amount of glucose consumption reached as on filtrate after 6 h, and a 7% higher ethanol yield after 8 h.

K. marxianus Y.01070 consumed the glucose slowly on the filtrate. The maximum ethanol concentration of 4.9 g/L was reached in 6 h, but 4.6 g/L of glucose and a high amount of man/xyl/gal (65% of initial amount) were still available. When the filtrate was only detoxified 0.5 g/L of glucose remained after 3 h, resulting in an ethanol concentration of 7.5 g/L after 24 h. The ethanol yield was 6% higher than that reached with *S. cerevisiae* on filtrate.

K. marxianus Y.00242 proved to be quite sensitive to inhibitors in the filtrate. The concentration of glucose remained at 12.6 g/L after 96 h of fermentation. Ethanol production was low (3.7 g/L after 3 h), and did not change in the rest of the 96-h observation period. This strain gave a relatively high ethanol concentration (7.4 g/L on detoxified filtrate) after 24 h (50% higher than that obtained on filtrate).

A decrease in the amount of other sugars (man/xyl/gal) was observed simultaneously with glucose consumption for all thermostable yeasts. After this decrease, the remaining amount of these sugars was much higher on filtrate, 65 and 74% of the initial amount for *K. marxianus* Y.01070 and *K. marxianus* Y.00242, respectively, than on detoxified filtrate, in which 44 and 35% of the initial amount was detected, respectively. *K. marxianus* Y.00243 was an exception, because the amount of the remaining sugars (man/xyl/gal), 34% of the initial amount, was the same in both mediums.

The inhibitor sensitivity of yeasts can be seen: the glucose was almost completely consumed on detoxified filtrate, and a higher ethanol yield was obtained than with fermentation on filtrate with *K. marxianus* Y.00243 and *K. marxianus* Y.01070.

K. marxianus Y.00243 was chosen for SSF experiments, although the ethanol yield reached was only 49% of theoretical, whereas *K. marxianus* Y.01070 gave a 6% higher theoretical yield, but the high amount of other sugars remained in the filtrate. *K. marxianus* Y.00243 reached the maximum ethanol yield in a much shorter time than *K. marxianus* Y.01070. *K. marxianus* Y.00242 gave a high ethanol yield, but a high concentration of glucose and the amount of other sugars remained in the filtrate. Although the ethanol yield reached with *S. cerevisiae* was the same as that obtained with *K. marxianus* Y.00243, it required 43% longer time to reach its maximum yield.

Table 2
Results of Fermentation by Different Yeast Strains
at 42°C in Medium Containing 30 g/L Fermentable Sugars (filtrate, detoxified filtrate)

Yeast strain	Substrate	Time (h)[a]	Initial glucose (g/L)	Residual glucose (g/L)	Remaining xyl/gal/man (%)	Maximum ethanol (g/L)	Ethanol yield (g/100 g)[b]
K. marxianus Y.00243	Filtrate	8	18.1	0.8	34	6.3	25.0
K. marxianus Y.00243	Detoxified filtrate	8	16.6	0.7	34	6.7	27.2
K. marxianus Y.01070	Filtrate	6	19.3	4.6	65	4.9	26.6
K. marxianus Y.01070	Detoxified filtrate	24	17.0	0.5	44	7.5	31.5
K. marxianus Y.00242	Filtrate	3	20.3	12.6	74	3.7	36.0
K. marxianus Y.00242	Detoxified filtrate	24	16.5	0.5	35	7.4	30.0
S. cerevisiae	Filtrate	14	15.3	0.0	44	5.9	25.0

[a]Time when maximum ethanol concentration was measured.
[b]Ethanol yields expressed as g/100 g were calculated from the glucose and other sugar consumption.

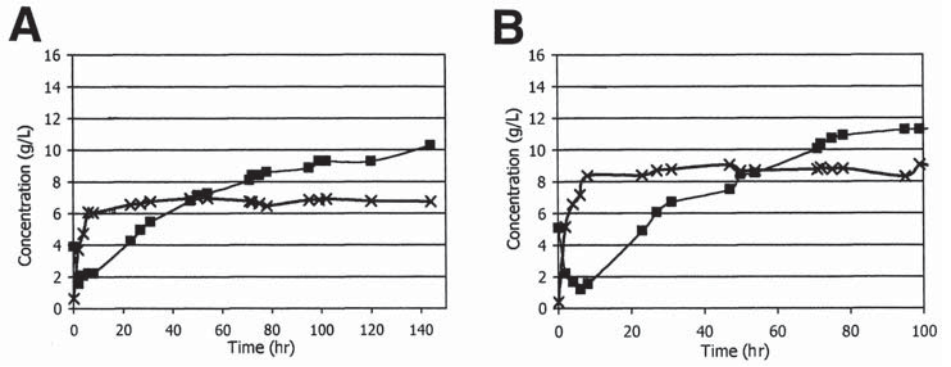

Fig. 1. Glucose (■) and ethanol (×) concentration vs time for SSF using 1-d culture of *K. marxianus* Y.00243 with pH control (4.8–5.0) **(A)** on SPS and **(B)** detoxified SPS.

SSF Experiments

The SSF experiments were run with the selected thermotolerant yeast *K. marxianus* Y.00243. Using a 1-d-old yeast culture for inoculation, the centrifuged yeast was suspended in the nutrient solution of the SSF, stirred at room temperature for half an hour, and then added to the SSF reactor.

Figure 1 presents the results of the SSF experiment run in SPS and in detoxified SPS with *K. marxianus* Y.00243. When SPS was used as a substrate at an initial pH of 5.4 (Fig. 1A), the glucose was initially consumed quickly until 2 h into the fermentation, when an increase in glucose concentration was observed, as the result of enzymatic hydrolysis and a decrease of yeast fermentability, reaching 10.3 g/L after 144 h. The maximum ethanol concentration obtained was 7 g/L after 50 h. Man/xyl/gal were consumed in the beginning in the same way as glucose, and after 8 h 43% of the initial amount of the sugars remained. For the detoxified substrate (Fig. 1B), with an initial pH of 4.8, the consumption of glucose and man/xyl/gal was fast, as the initial amounts decreased to 40% after 6 h. The concentration of glucose reached 11.5 g/L during 120 h. The ethanol concentration increased rapidly in the beginning and slowly from the eighth to the forty-seventh hours. The ethanol concentration was 9.1 g/L after 47 h. On detoxified substrate, 10.5 g/100 g of DM maximal ethanol yield was reached after 47 h, whereas without detoxification, 7.7 g/100 g of DM was obtained.

In an attempt to improve ethanol yield, a fresh yeast culture was added immediately to the SSF after centrifugation. Figure 2 presents the results. The glucose was consumed slightly for 2 h, and then increased to a concentration of 12 g/L after 96 h, indicating the continuation of cellulolytic activity, while the alcoholic fermentation slowed down. After a slight decrease, the amount of the man/xyl/gal remained unchanged at a high level, 69% of the initial amount, during the fermentation. The maximum in ethanol concentration (4.9 g/L) was obtained after 8 h. Using fresh yeast the obtained ethanol yield was 5.5 g/100 g of DM after 8 h, whereas the 1-d culture gave 6.6 g/100 g of DM after 8 h, with a maximum yield of 7.7 g/100 g of DM after 47 h.

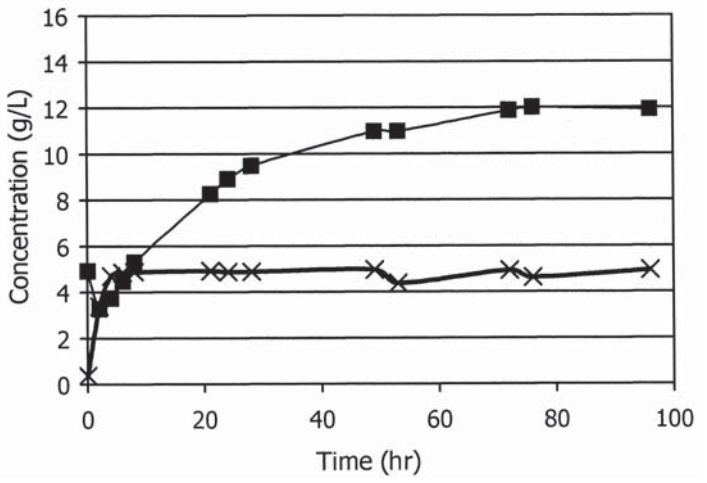

Fig. 2. Glucose (■) and ethanol (×) concentration vs time for SSF using fresh culture of *K. marxianus* Y.00243 with pH control (4.8–5.0) on SPS.

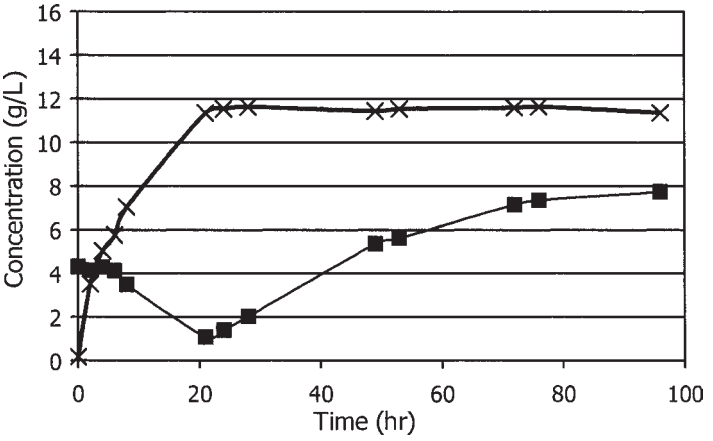

Fig. 3. Glucose (■) and ethanol (×) concentration vs time for SSF using fresh culture of *K. marxianus* Y.00243 without pH regulation on SPS.

K. marxianus Y.00243 seems to be sensitive to changes in pH, on the basis of previous observations (data not shown); therefore, pH was measured during the SSF experiments. The experiments with SPS using a fresh yeast culture for inoculation were performed without pH control, with an initial pH of 5.4 (Fig. 3). The sugar consumption stopped after 21 h, reaching a low value, 32% of the initial amount of xyl/gal/man, and a glucose concentration of 1.1 g/L. The concentration of glucose increased to 7.7 g/L at 96 h, as the result of hydrolysis, and ethanol fermentation was not observed. The ethanol concentration reached a maximum (11.6 g/L) after 28 h.

The ethanol yield reached 14 g/100 g of DM after 23 h with fresh yeast culture without pH regulation. The pH was between 5.2 and 5.3 up to 21 h and remained at 5.1 between 21 and 96 h.

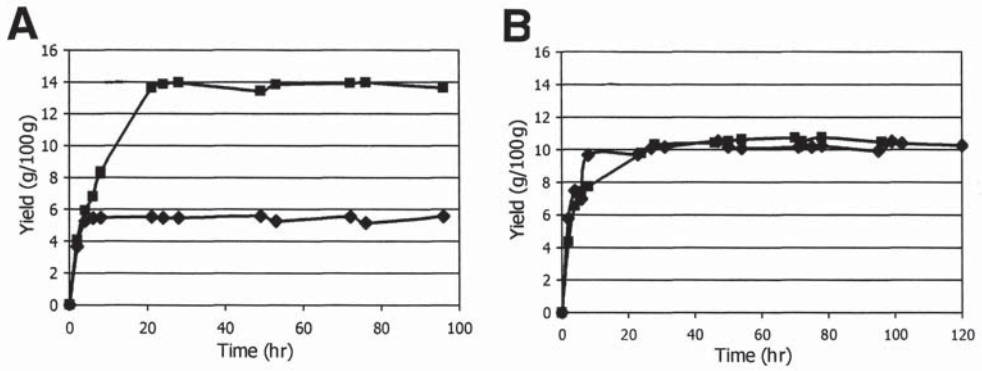

Fig. 4. Obtained ethanol yields in SSF using *K. marxianus* Y.00243 (◆) with pH regulation and (■) without pH regulation **(A)** on SPS with fresh yeast culture and **(B)** on detoxified SPS with 1-d yeast culture.

In SSF experiments regulating pH, the pH was maintained at 4.98. Ethanol yield with pH regulation was only 39% of that reached with fresh yeast without pH regulation using SPS as substrate (Fig. 4A). No difference in ethanol yield was observed, with and without pH regulation, for the detoxified substrate using a 1-d yeast culture (Fig. 4B). The obtained yield was 10 g/100 g of DM in the two cases, after 31 and 28 h, respectively.

Discussion

K. marxianus is the most frequently used thermotolerant strain in SSF performed at temperatures above 40°C *(14–18)*. In the present study, fermentation tests were conducted to investigate the sensitivity of three thermotolerant *K. marxianus* strains to the inhibitors (furfural, HMF, phenolic compounds, and acetic acid) present in the hydrolysate of SPS.

Detoxification by overliming resulted in an 8 and a 15% increase in ethanol yield for *K. marxianus* Y.00243 and *K. marxianus* Y.01070, respectively, and utilization of a higher amount of sugars in the test fermentations. These results can be explained by the fact that the amounts of inhibitory compounds (furfural, HMF, and phenolic compounds) are reduced by overliming *(27)*.

Larsson et al. *(27)* have reported a reduction of about 20% in the amount of furfural, HMF, and phenolic compounds owing to detoxification by overliming and, consequently, an increase of 27% in ethanol yield in the fermentation of the hydrolysate of SPS with *S. cerevisiae*.

The favorable effect of detoxification was observed in SSF experiments as well, resulting in a 27% increase in ethanol yield. The effect of pH proved to be significant on ethanol yield when the substrate was not detoxified (Fig. 4). Maintaining the pH above 5.0 during SSF was favorable for the ethanol yield. The pH strongly influences the inhibitory effect of acetic acid *(34)*. The negative effect of acetic acid can be compensated by increasing the pH *(24)*.

Table 3
Ethanol Yields in SSF on SPS and Detoxified SPS
Using *K. marxianus Y.00243* at 42°C and *S. cerevisiae* as Control at 37°C

Yeast strain	Inoculum	Substrate	PH control[b]	EtOH yield (g/100 g of DM)/ (% of theoretical)[a]
K. marxianus	Fresh	SPS	+	5.5/17
K. marxianus	1-d	SPS	+	7.7/24
K. marxianus	1-d	Detoxified SPS	+	10.5/33
K. marxianus	Fresh	SPS	–	13.9/44
K. marxianus	1-d	Detoxified SPS	–	10.3/33
S. cerevisiae	Dry	SPS	+	16.4/52

[a]Based on original dry wood.
[b]+, Adjusted pH; –, nonadjusted pH.

Olsoon and Hahn-Hägerdal *(24)* have reported a 50% inhibition of fermentability of *S. cerevisiae* at an acetic acid concentration of 1.4 g/L when the pH was 4.5. At a pH of 5.5, the same inhibition (50%) was observed when the concentration of acetic acid was 4.2 g/L *(24)*. In the present SSF experiments, the acetic acid concentrations were between 1.7 and 1.9 g/L.

Therefore, the favorable effect of higher pH on ethanol yield is probably owing to the reduced toxic effect of acetic acid. On the basis of the results, a fresh culture of yeast is more sensitive to inhibitors present in the substrate than a 1-d-old culture (Fig. 3).

The highest ethanol yield was 14 g/100 g of dry substrate obtained after 23 h (Table 3). Ballesteros et al. *(12)* reported an ethanol yield of 0.50 g/g of cellulose in 78 h in SSF experiments running on Solka Floc 200 (FS&D, Urbana, IL), at 42°C with *K. marxianus* strains. This ethanol yield was 36% higher than that obtained in the present study. However, in the present SSF experiments, the concentration of glucose increased continuously after 10 h, indicating continued cellulolytic activity, and the sugar was not fermented by yeast, which resulted in a rather poor ethanol yield. The cessation of ethanol production indicated the cessation of yeast fermentability, which could be caused by the inhibitory compounds of hydrolysate. The favorable effect of detoxification and the higher pH confirmed this assumption. These results pointed out the high sensitivity of this thermotolerant strain to inhibitors present in hydrolysate.

In the future, the adaptation of strains to the hydrolysate and the use of more efficient detoxification methods should be investigated.

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