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Ethanol Fermentation of Various Pretreated and Hydrolyzed Substrates at Low Initial pH

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Abstract

Lignocellulosic materials represent an abundant feedstock for bioethanol production. Because of their complex structure pretreatment is necessary to make it accessible for enzymatic attack. Steam pretreatment with or without acid catalysts seems to be one of the most promising techniques, which has already been applied for large variety of lignocellulosics in order to improve enzymatic digestibility. During this process a range of toxic compounds (lignin and sugar degradation products) are formed which inhibit ethanol fermentation. In this study, the toxicity of hemicellulose hydrolysates obtained in the steam pretreatment of spruce, willow, and corn stover were investigated in ethanol fermentation tests using a yeast strain, which has been previously reported to have a resistance to inhibitory compounds generated during steam pretreatment. To overcome bacterial contamination, fermentations were carried out at low initial pH. The fermentability of hemicellulose hydrolysates of pretreated lignocellulosic substrates at low pH gave promising results with the economically profitable final 5 vol% ethanol concentration corresponding to 85% of theoretical. Adaptation experiments have shown that inhibitor tolerance of yeast strain can be improved by subsequent transfer of the yeast to inhibitory medium.

Index Entries: Inhibitors; lignocellulose; *Saccharomyces cerevisiae*; toxicity; yeast adaptation; bioethanol.

Introduction

Emission of greenhouse gases, especially carbon dioxide, has been steadily increasing since the industrial revolution. Apparently, the net carbon dioxide emission has been increasing exponentially during the last century mainly because of extensively growing energy demand. The transport sector, a key factor in economy as it facilitates movement of produced goods, accounts for more than 30% of the energy consumption in the European

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Community (EC). Carbon dioxide emission from transportation is expected to rise by 50% from 1990 for year 2010 to about 1113 million t for which road transportation can be held responsible with its 84% share to the total carbon dioxide generated *(1)*. At the present time transport sector is 98% oil dependent. Increasing the use of biofuels in road transportation is one of the key tools by which EC can reduce carbon dioxide emission to a great extent and at the same time also decrease the dependency on imported energy. Directive 2003/30/EC clearly sets the target shares of biofuels in transport sector for EC member states. The short-term target was set at 2% in 2005; however, at the end of the year it was clear that the European Union would not reach it. The long-term target is to increase the use of biofuels in energy consumption to 5.75% by 2010, but probably it could also fail to reach this goal.

Bioethanol (produced from biomass) has been long recognized as a possible alternative fuel. It can be mixed into both gasoline and diesel. As an alternative, ethyl tertiary butyl ether (ETBE) produced from bioethanol is also accepted in the EC; however, only 47% is of biological origin. For the production of bioethanol, large varieties of materials are available. However, as the share of raw material cost is calculated to be about 50% of total expenditures, the choice of feedstock and ethanol yields are two of the most important factors affecting the economy of fuel alcohol production. Lignocellulosics, the most abundant renewable resources on Earth, represent an enormous potential for large-scale bioethanol production. In spite of this, hydrolysis of polysaccharides in these materials is not an easy task to accomplish for which the complex and highly compact structure of lignocellulosics is responsible *(2,3)*. To be an attractive substrate for ethanol fermentation, pretreatment of lignocellulosic raw material is necessary to open up the structure and make it accessible for enzymatic attack. Despite the extensive research undertaken in the last decades on pretreatments (physical, chemical, enzymatic, or combinations of these methods), nowadays none of the available processes could be used as a general process, owing to the differences in composition of lignocellulosics; however, steam explosion is known as a highly efficient and economically feasible method *(4,5)*.

During pretreatment a range of toxic compounds are formed. Olsson et al. *(6)* divided inhibitors into different groups depending on the origin: acetic acid is released when the hemicellulose structure is degraded-furfural, 5-hydroxymethyl furfural (HMF) are produced because of sugar (pentose and hexose) degradation, whereas aromatic compounds originated from lignin degradation. Formic acid is formed when furfural and HMF are broken down and levulinic acid is formed by HMF degradation. Quantitative and qualitative composition of the inhibitors arising during pretreatment depends on the type of applied pretreatment and also on the origin of lignocellulosic material *(6)*. A detailed review on the generation of inhibitors, on the mechanisms of inhibition and on detoxification methods (biological, physical, and chemical) was reported by Palmqvist and Hahn-Hägerdal *(7,8)*.

Biomass growth and ethanol production rate is hampered in the presence of weak acids (acetic and lactic acid) *(9–11)*. The inhibitory effect of these acids depend heavily on pH *(8,9)*, which can be reduced by maintaining higher fermentation pH. The tendency is to increase pH to values higher than 5.0 or even 5.5 *(12–17)* to demonstrate fermentability of pretreated raw materials; however, the optimum fermentation pH is in the range of 4.0–5.0 *(18)*. Above pH 5.0 bacteria can grow much faster than yeasts and on industrial scale uncontrollable fermentations will be observed in a nonsterile continuous process lay out. The principal bacterial contaminants in a distillery are those that form lactic acid. Some effects of contamination on flavor are known, but normally do not cause serious problems *(19)*. Although the production of fuel alcohol is not concerned with the taste of the product, any lactic acid formed subtracts from the yield of alcohol, furthermore, inhibit yeast growth and metabolism *(20)*. The production of lactic acid and other contaminants should therefore be avoided as much as possible. The development of these microorganisms is severely repressed at pH values under 5.0. This article presents a study on inhibitory effects of hemicellulose hydrolysates (HH) obtained after steam pretreatment of spruce, willow, and corn stover on ethanol fermentations at low pH (pH < 5.0) using an inhibitor resistant *Saccharomyces cerevisiae* strain, and a strategy to adopt the microorganism to inhibitors present in the hydrolysates.

Materials and Methods

Microorganisms and Culture Media

S. cerevisiae ATTC 26602, obtained from the American Type Culture Collection (ATCC), was used throughout this study. The yeast strain was maintained at –85°C in the mixture of 50 vol% glycerol and yeast, peptone, glucose (YPD) solution, which contained per liter demineralized water: 20.0 g of bacto peptone, 10.0 g of yeast extract, and 10.0 g of glucose. The pH was adjusted with 0.1 *M* KOH to 6.5 and sterilized for 15 min at 125°C. To control the procedure of freezing, yeast strain was plated on YPD agar contained per liter demineralized water: 20.0 g of bacto peptone, 10.0 g of yeast extract, 10.0 g of glucose, and 15.0 g of bacto agar. The pH was adjusted with 0.1 *M* KOH to 6.5 and sterilized for 15 min at 125°C.

Yeast Cultivation

Starter culture of *S. cerevisiae* ATCC 26602 was grown in 1000-mL cap flasks containing 500 mL of culture medium. The medium for growth of yeasts contained per 0.5 L demineralized water: 114.0 g of Nedalco standard beet molasses (45–50% sugar content) and 1.2 g of $(NH_4)_2PO_4$. The pH was adjusted to 4.8 with 25% H_2SO_4, the medium was sterilized by autoclaving at 110°C for 30 min at 0.5 bar and was inoculated with freezed stock solution (1.5 mL) of yeast. After 1 d of incubation at 32°C cultures

were centrifuged at 1750*g* for 10 min (Rotanta 46, Hettich Zentrifugen, Germany), washed with demi water and harvested in demi water.

Substrates

Spruce, willow, and corn stover containing about 45% cellulose based on dry matter were obtained from Sweden. Pretreatment of raw materials were carried out at Lund University (Sweden). Pretreatment of spruce, willow, and corn stover were carried out at 215°C for 5 min, 205°C for 4 min, and at 190°C for 5 min, respectively, with SO_2 impregnation. Pretreatment of willow was also carried out without SO_2 impregnation at 210°C for 14 min. Complete analysis of pretreated materials was carried out at the Budapest University of Technology and Economics (Hungary) (Table 1) according to the National Renewable Energy Laboratory Analytical Procedures. The liquid part of pretreated material (HH) was separated by centrifugation at 2625*g* for 10 min. Other batches of pretreated corn stover, originating from Italian National Agency for New Technologies, Energy and Environment, Italy (ENEA), pretreated at 190°C for 5 min and 210°C for 5 min, were also tested for ethanol fermentation.

Fermentation Assays

Batch fermentations were carried out in stirred flasks with online measuring CO_2 production. Experiments were performed in 0.5- or 0.25-L capped flasks containing 100 or 50 mL of fermentation broth agitated at 300 rpm by magnetic stir bars and incubated in water bath at 32°C until the end of the fermentation (1 d). The fermentation medium contained per liter: liquid phase of pretreated material (HH) at different vol%, 16 mL of mineral solution, 1 mL of trace element solution, 1 mL of vitamin solution, and 1 mL of 30% (w/w) $FeSO_4 \cdot 7H_2O$ *(21)*. The mineral solution contained per liter *(12)*: 250.0 g of $(NH_4)_2SO_4$, 125.0 g of KH_2PO_4, 31.25 g of $MgSO_4$; the trace element solution contained per liter: 4.5 g $ZnSO_4 \cdot 7H_2O$, 1.0 g $MnCl_4 \cdot 4H_2O$, 0.3 g $CuSO_4 \cdot 5H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 4.5 g $CaCl_2 \cdot 2H_2O$, 0.4 g $Na_2MoO_4 \cdot 2H_2O$, 1.0 g H_3BO_3, and 0.1 g KI; the vitamin solution contained per liter: 0.05 g of biotin, 1.0 g calcium-pantothenate, 1.0 g nicotinic, 25.0 g inositol, 1.0 g thiamin-HCl, 1.0 g pyridoxin-HCl, and 0.2 g *p*-aminobenzoic acid. Glucose was added to the medium according to the cellulose content of the pretreated material (Table 1). The additional glucose was calculated as:

$$\text{Glucose (g)} = \frac{\begin{array}{c}[(\text{cellulose content}(\%) \cdot (\text{amount of HH(L)} \cdot \text{density of HH(kg / L)} / 0.9)] + \\ [(\text{cellobiose content}(\%) \cdot (\text{amount of HH(L)} \cdot \text{density of HH (kg / L)} / 0.95)]\end{array}}{100 \cdot \text{working volume (mL)}}$$

Harvested yeast was added to correspond to 1.5 g dry weight per liter. Dry matter content of harvested yeast was determined as described in Analytical Procedures section. The pH of the broth was adjusted initially to

Table 1
Composition of Pretreated Materials by Steam Explosion

	Spruce	Willow ($-SO_2$)[a]	Willow ($+SO_2$)[a]	Corn stover	Corn stover[b]
Pretreated material (%)					
Dry matter	23.76	18.61	24.70	10.03	35.01
Cellulose[c]	8.95	9.05	10.01	4.12	~9.62
Lignin[c]	7.40	5.08	5.38	1.50	~8.4
Ash[c]	0.01	0.10	0.09	0.29	~3.4
Liquid part: HH[d] (%)					
Cellulose	0.23	0.03	0.11	0.01	nd
Glucose	3.07	0.14	0.79	0.23	nd
Mannose	2.73	–	–	–	nd
Xylose	–	0.30	2.12	1.16	nd
Arabinose	0.07	0.01	0.05	0.12	nd
Formic acid	0.18	0.11	0.12	0.06	nd
Acetic acid	0.52	0.67	0.70	0.14	nd
Levulinic acid	0.13	–	–	–	nd
HMF[e]	0.38	0.10	0.10	–	nd
Furfural	0.25	0.22	0.15	0.01	nd

nd, not determined.
[a]Willow impregnated with (+) or without (–) SO_2.
[b]Pretreated at ENEA (Italy).
[c]Based on dry matter content.
[d]Based on pretreated material (mass/volume).

4.0 with 10% NaOH. Flasks were sampled at the end of fermentation, and analyzed for concentrations of biomass, sugars, metabolites, and ethanol by high-performance liquid chromatography (HPLC) at the following described conditions. Calculations were based on data obtained at the point from which no additional CO_2 production was achieved.

Analytical Procedures

- Changes in biomass concentration throughout the fermentation process were measured by optical density measurement at 700 nm using a Perkin-Elmer Spectrophotometer. Dry matter content was determined according to a calibration line at 700 nm (Nedalco standard procedure): (dry weight [DW] (g/L) = $281[OD_{700}]^2 + 187.29[OD_{700}] + 9.822$).
- Sugars, ethanol, lactic acid, acetic acid, and glycerol were analyzed by Shimadzu HPLC on a Bio Rad column (HPX-87H), and detected by a refractive index (RI) detector. The working temperature was 65°C. H_2SO_4 (0.25 *M*) was used as eluent at a flow rate of 0.55 mL/min. Before HPLC samples were passed through a 0.2 µm pore size filter.
- Production of carbon dioxide was monitored online using a BAM-6 module (HaloteC, The Netherlands).

Results and Discussion

The analytical characteristics of the pretreated materials and the HHs are summarized in Table 1. The HH consisted of soluble hemicellulose quantified as monomers (glucose, xylose, arabinose, and mannose) in different concentrations. Differences between softwood (spruce) and hardwood (willow) is noticeable in chemical composition. Hemicellulose of hardwood is rich in xylan polymers and contain small amounts of mannan, whereas mannose is a predominant sugar originating mainly from softwood *(5)*. As xylose and mannose could not be separated on the applied HPLC column, therefore carbohydrate of pretreated spruce was assumed to be mannose, whereas at pretreated corn stover and willow, the sugar peak was interpreted as xylose (Table 1). The concentration of glucose in spruce HH is higher compared with the other pretreated substrates, demonstrating an easily degradable glucose containing biopolymer in spruce. Pretreatment of willow was carried out with and without SO_2 (which is used to improve hemicellulose recovery) in order to test the effect of SO_2 present in the HH on ethanol fermentation.

During steam explosion carbohydrate degradation products (furfural and HMF) and carboxylic acids (acetic, formic, and levulinic acids) were formed. Because hemicellulose of hardwood (willow) is more acetylated, pretreated willow resulted in the highest acetic acid concentration (Table 1). Overall the highest concentration of inhibitors were observed in pretreated spruce and it was the only pretreated material wherein levulinic acid formation was noticed as well. Presence of SO_2 did not dramatically affect the concentration of inhibitors in pretreated willow. Compared with pretreated woody substrates significantly lower inhibitor concentration was measured in hemicellulose hydrolysate of pretreated corn stover (originating from Lund) as shown in Table 1, wherein inhibitors are presented as w/w (%) of pretreated material (because of the structure of corn stover pretreated at ENEA, complete and accurate analysis could not be carried out). Lignin degradation products were not determined from HHs.

The effect of inhibitors present in steam pretreated lignocellulosic substrates was investigated using an inhibitor resistant yeast strain, which was previously selected by screening to have a resistance to inhibitory compounds generated during steam pretreatment. Of all tested strains *S. cerevisiae* ATCC 26602 seemed to grow best on toxic materials (data not shown). For fermentation studies pretreated materials were separated by means of centrifugation and the liquid part (HH) was used for fermentation. To test the fermentability of these pretreated lignocellulosic materials glucose was added to the HH (as described in Materials and Methods section) assuming that all cellulose (Table 1) could be hydrolyzed.

Fermentation of lignocellulosic substrates is usually carried out at pH 5.0–5.5. Running a fermentation under sterile conditions on large scale is not economical, and therefore it is important to keep the pH low (<4.5) to

prevent bacterial contamination and more organic acid formation with more inhibited process. Preliminary fermentation studies were carried out earlier (unpublished data) to test the effect of pH in the range of 4.8–3.8. Based on these preliminary investigations and on industrial considerations, fermentation of different steam exploded hydrolysate samples were tested at pH 4.0.

The fermentability of steam pretreated samples was tested under the same circumstances, only the ratio of hydrolysate was varied in a range of 30–94 vol% in the medium, containing minerals, vitamins, and trace elements (*see* Materials and Methods section). The results of ethanol fermentation on HHs are presented in Fig. 1A–D, wherein gas production rates (mL/min) are plotted vs fermentation time as a measure of actual volumetric ethanol production rate during the course of the process. In general, the CO_2 production profile showed the same curve with differences in lag phase. By increasing the ratio of HH in the medium the lag phase became longer indicating that the yeast requires an adaptation period because of inhibitors present in the fermentation broth.

Comparison of fermentation results represented by different steam-exploded lignocellulosic raw materials is in good agreement with inhibitor concentration of samples. The most inhibitory sample (Fig. 1A) has resulted at the highest dilution ratio, which was necessary to avoid the effect of inhibitors. Obtained ethanol concentrations on different HHs at the highest concentration wherein fermentation was not blocked by inhibitors, are summarized in Table 2. The ethanol yield was calculated from the ethanol concentration determined by HPLC at the end of the fermentation based on the potential fermentable sugars (glucose and mannose) concentration. Xylose concentration in the liquid fraction was also high (Table 1), but according to our knowledge this strain is not able to ferment xylose.

Even though rather high final ethanol concentration was achieved on pretreated spruce and willow, the concentration of HH could not be further increased because of inhibitors. Although corn stover could be fermented almost pure, the final ethanol concentration with 2.7 vol% is too low to be commercially interesting. However, with further increase (35%) in the dry matter content of pretreated corn stover (ENEA) the economically profitable final ethanol concentration was achieved (Table 2). Ethanol yields were comparable with data obtained in the literature on different pretreated and hydrolyzed lignocellulosic substrates: on spruce approx 0.40 g/g *(22)*, on wheat straw 0.43–0.46 g/g *(16)*, on willow 0.41–0.46 g/g *(10,23)* ethanol yield based on fermentable sugars have been reported.

By increasing the concentration of pretreated materials in the medium the molar ratio of ethanol/glycerol has been increased (data not shown). This tendency was observed on all substrates; however, the degree of change has differed. Glycerol is nontoxic to the yeast even at very high concentration. It plays a role under anaerobic condition in the maintenance of intracellular redox balance and acts as an osmotic regulator of the

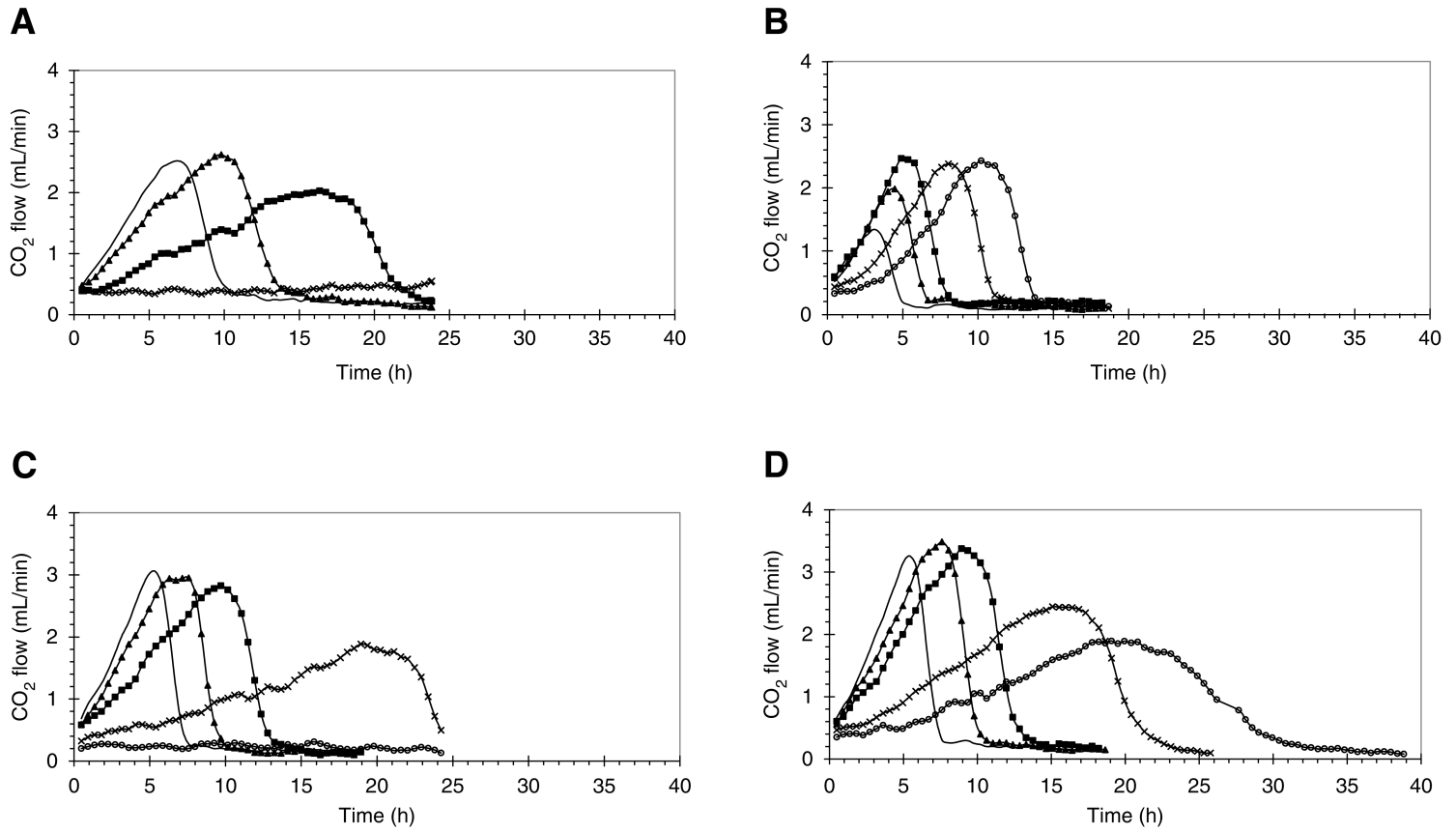

Fig. 1. (A) Fermentation of spruce HH at different vol% by *S. cerevisiae* ATCC 26602. 30% (–), 40% (-▲-); 50% (-■-), 55% (-×-). **(B)** Fermentation of corn stover HH at different vol% by *S. cerevisiae* ATCC 26602. 30% (–), 50% (-▲-); 70% (-■-), 85% (-×-), 94% (-○-). **(C)** Fermentation of willow HH (pretreated without SO_2 impregnation) at different vol% by *S. cerevisiae* ATCC 26602. 35% (–), 45% (-▲-); 55% (-■-), 64% (-×-), 67% (-○-). **(D)** Fermentation of willow HH (pretreated with SO_2 impregnation) at different vol% by *S. cerevisiae* ATCC 26602. 30% (–), 40% (-▲-); 50% (-■-), 59% (-×-), 67% (-○-).

Table 2
Ethanol Fermentation Results on Steam Pretreated Spruce, Corn Stover, and Willow With *S. cerevisiae* ATCC 26602

	Substrate			
Name	Maximum volume (%) of HH	Dry matter (%)[a]	Final ethanol (vol [%])	Gross yield (%)
Spruce	50	11.9	4.53	0.42
Corn stover	94	9.4	2.73	0.44
Corn stover[b]	75	15.8	5.03	0.49
Willow (–SO_2)[c]	64	11.9	4.09	0.48
Willow (+SO_2)[c]	65	16.6	5.74	0.50

[a]Corresponding to the concentration of HH.
[b]Pretreated at ENEA.
[c]Pretreated with (+) or without (–) SO_2.

cell *(24,25)*. Some of the inhibitors like acetic acid, furfural, and HMF were found to be a stimulator in the conversion of glucose to ethanol by *S. cerevisiae* in a certain extent rendering both a higher ethanol yield and lower byproduct (glycerol) yield *(26)*. Acetic acid has shown effect on growth energetic, leading to an increased ethanol yield *(15)*. Palmqvist et al. *(13)* have shown competition between furfural reduction and glycerol production in favor for furfural reduction, thus causing more sugar availability for ethanol production. Furfural and HMF are known to be reduced by yeast mainly to furfuryl alcohol *(13)*, 5-hydroxymethylfurfuryl alcohol, and slightly to 5-hydroxymethyl furan carboxylic *(12,13)*. During our fermentations, concentrations of HMF and furfural decreased and probably have been reduced by yeast to furfuryl alcohol and 5-hydroxymethylfurfuryl alcohol.

The fermentability of *S. cerevisiae* ATCC 26602 yeast strain was improved by adaptation to toxic components present in the pretreated lignocellulosic materials. Adaptation procedures were also performed on HH with added glucose in BAM6 module, followed by online measuring of CO_2 production (*see* Materials and Methods section). The simplified procedure for a rapid and reliable adaptation assay on spruce is shown in Fig. 2. During the procedure concentration (vol%) the HH was increased continuously (from 60% to 65%) in the fermentation broth. When the adaptation was succeeded at higher concentration, cells were separated at growing phase and reused in the next adaptation step.

To control the procedure of adaptation the fermentation ability of adapted and nonadapted yeast was tested on increased concentration of spruce HH. As can be seen from Fig. 3, *S. cerevisiae* ATCC 26602 could be adapted to higher concentration of inhibitors on spruce matrix with 5.2 vol% final ethanol concentration; however, this topic needs to be addressed in more detail in future studies.

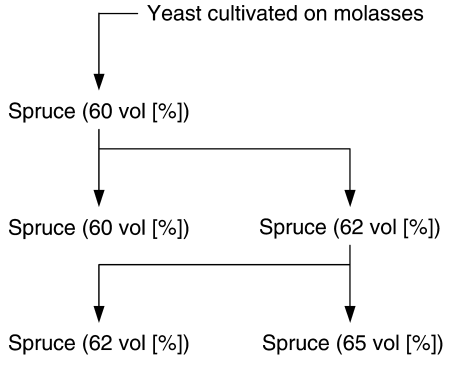

Fig. 2. Scheme of the adaptation procedure.

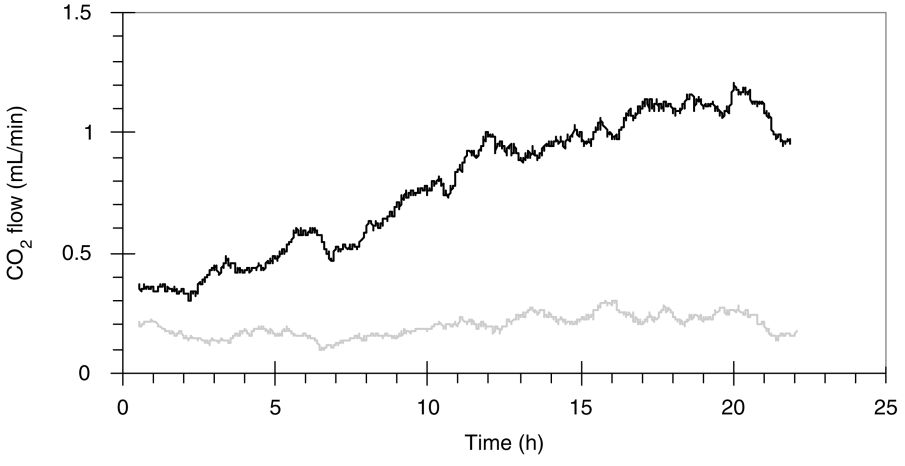

Fig. 3. Result of adaptation of *S. cerevisiae* ATCC 26602 on spruce HH at 65 vol%. Not adapted yeast (–), adapted yeast (–).

Conclusions

This study aimed to test the fermentability of different steam pretreated lignocellulosic raw materials at low pH by the inhibitor resistant *S. cerevisiae* ATCC 26602 yeast. *S. cerevisiae* ATCC 26602 was found to be capable of ethanol fermentation at low initial pH (pH 4.0), which is necessary to avoid bacterial contamination. After steam pretreatment all tested lignocellulosic materials (pretreated willow, spruce, and corn stover) seem to be a possible substrate for economically profitable industrial ethanol production with final ethanol concentration of 5 vol%. Impregnation with SO_2 did not affect the applied microorganism, but was found to be necessary to improve the recovery of hemicellulose. Steam pretreated corn stover seems to be a promising substrate for bioethanol production. The main advantage of this agricultural waste (produced in huge amounts in Hungary) is that

the concentration of toxic compounds formed during pretreatment does not reach the critical level, at which fermentation of yeast would be blocked. The inhibitor tolerance of the selected *S. cerevisiae* ATCC 26602 was improved with continuous adaptation on steam pretreated spruce matrix. Under the same circumstances and inhibitor concentrations, the adapted yeast was able to ferment 5 vol% ethanol, the original, nonadapted yeast strain was incapable of ethanol fermentation.

Ethanol yields were rather high (in all cases above 0.42 g ethanol/g fermentable sugars) considering that none of these experiments were made on real fibrous substrate, only on filtrate, using added glucose as sugar component (according to cellulose content). In order to test the real process, the glucose should be derived from enzymatic treatment of the pretreated fiber material as performed in simultaneous saccharification and fermentation on the whole slurry. To obtain the least 5% (v/v) ethanol concentration rather high (approx 20%) dry matter concentration is needed, whereas further increasing the substrate concentration more than 15% of dry matter would result in reduced ethanol yield owing to insufficient mass transfer *(27)*.

Acknowledgments

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