Bioconversion of dilute-acid pretreated sorghum bagasse to ethanol by *Neurospora crassa*

Ioannis Dogaris, Olga Gkounta, Diomi Mamma, Dimitris Kekos*

Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Str., Zografou Campus, 157 80, Zografou, GREECE

*Correspondence to:
Dimitris Kekos
Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Str., Zografou Campus, 157 80, Zografou, GREECE.
Tel.: +30 2107723205; Fax: +30 2107723163
e-mail address: kekos@chemeng.ntua.gr

Abstract

Bioethanol production from sweet sorghum bagasse (SB), the lignocellulosic solid residue obtained after extraction of sugars from sorghum stalks, can further improve the energy yield of the crop. The aim of the present work was to evaluate a cost-efficient bioconversion of SB to ethanol at high solids loadings (16% at pretreatment and 8% at fermentation), low cellulase activities (1-7 FPU/g SB) and co-fermentation of hexoses and pentoses. The fungus *Neurospora crassa* DSM 1129 was used, which exhibits both depolymerase and co-fermentative ability, as well as mixed cultures with *Saccharomyces cerevisiae* 2541. A dilute-acid pretreatment (sulfuric acid 2 g/100g SB; 210 ^{o}C; 10 min) was implemented, with high hemicellulose decomposition and low inhibitor formation. The bioconversion efficiency of *N. crassa* was superior to *S. cerevisiae*, while their mixed cultures had negative effect on ethanol production. Supplementing the in situ produced *N. crassa* cellulolytic system (1.0 FPU/g SB) with commercial cellulase and *β*-glucosidase mixture at low activity (6.0 FPU/g SB) increased ethanol production to 27.6 g/l or 84.7% of theoretical yield (based on SB cellulose and hemicellulose sugar content). The combined dilute-acid pretreatment and bioconversion led to maximum cellulose and hemicellulose hydrolysis 73.3% and 89.6%, respectively.

Keywords

sorghum bagasse; ethanol; bioconversion; cellulase; filamentous fungi; yeast;

Introduction

The inevitable oil depletion, energy security concerns and the increasing problem of the CO_2 emissions has strengthened the interest in alternative, non-petroleum-based sources of energy. Sorghum is a promising C_4 energy plant because of its high photosynthetic efficiency, high biomass yield per hectare, increased drought resistance and low production costs (Li et al. 2010; Martinez et al. 2000; Xu et al. 2011). An approach to utilizing sweet sorghum is processing the harvested plant into juice and sorghum bagasse (SB), the lignocellulosic solid residue obtained after extraction of sugars. Sweet sorghum juice contains high concentrations of sugars that could be directly fermented to ethanol. Because a huge amount of SB will result from the large scale production of sweet sorghum juice, its utilization is could improve the economy of the global use of the crop (Li et al. 2010).

Ethanol production from lignocellulosic biomass comprises the following main steps: pretreatment, hydrolysis of cellulose and hemicellulose, sugar fermentation and finally recovery of ethanol (Cardona and Sánchez 2007; Wyman 2007). One option is to perform the enzymatic hydrolysis together with the fermentation, implementing a simultaneous saccharification and fermentation process (SSF). In order to reduce SSF process energy costs, a high substrate loading is required at the pretreatment step than will lead to high concentration of fermentable sugars and thus ethanol concentrations for efficient recovery (Alvira et al. 2010; Olofsson et al. 2008a). However, efficient ethanol production from lignocellulosic agro-industrial residues, such as sugarcane bagasse and corn stover, is reported mostly on either low solids loadings 2-5 % dry matter (Carrasco et al. 2010; da Silva et al. 2010; Ewanick and Bura 2011) or required high cellulase activities 15-30 FPU/g biomass (Carrasco et al. 2010; Mesa et al. 2011; Varga et al. 2004; Zhao and Xia 2009) and using hexose-only fermenting microorganisms or genetic engineered pentose-fermenting strains.

Our previous work demonstrated that the mesophilic filamentous fungus *Neurospora crassa* DSM 1129 has the ability to synthesize and secrete high levels of all three enzyme types involved in cellulose degradation as well as endoxylanase and β-xylosidase activities, when grown on inexpensive agricultural residues (Dogaris et al. 2009b). In addition, *N. crassa* has the ability to convert to ethanol xylose (Rao et al. 1983; Zhang et al. 2008), cellulose and hemicellulose (Deshpande et al. 1986) and brewer's spent grain (Xiros et al. 2008). However, our previous attempts to use *N. crassa* for the bioconversion of SB to ethanol without prior pretreatment resulted in low ethanol production (Dogaris et al. 2009b). Nevertheless, applying a hydrothermal pretreatment was reported to improve the enzymatic digestibility of SB by *N. crassa* enzyme system (Dogaris et al. 2009a).

The aim of the present work was to evaluate a cost-efficient bioconversion of SB to ethanol at high solids loadings (16% at pretreatment and 8% at fermentation), low cellulase activities (1-7 FPU/g SB) and co-fermentation of hexoses and pentoses by the fungus *N. crassa* DSM 1129. The addition of dilute-acid at the hydrothermal pretreatment was initially assessed. Pretreatment operating conditions were chosen so that most of SB hemicellulose fraction was hydrolyzed, while producing a low concentration of sugar decomposition compounds which may inhibit the ethanol fermentation step. We applied a SSF process without any separation or detoxification that may lead to additional process cost as well as possible loss of fermentable sugars (Olofsson et al. 2008a). In order to further improve ethanol yields and productivity from hexoses, the application of mixed cultures with *S. cerevisiae* was also studied.

Materials and Methods

Microorganisms

The fungal strain *N. crassa* DSM 1129 was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ, Germany). The yeast strain *S. cerevisiae* 2541 belongs to the culture collection of the Biotechnology Laboratory, School of Chemical Engineering, NTUA, and has been used in previous works (Lezinou et al. 1995; Mamma et al. 1996). Stock cultures were maintained on potato dextrose agar slants at 4°C.

Reagents and solid substrates

All chemicals were analytical grade. Wheat straw (WS) and wheat bran (WB) substrates for *N. crassa* aerated growth were prepared as described by Dogaris et al. (2009b). Fresh sweet sorghum stalks were chopped to less than 3mm diameter particles. Sorghum bagasse (SB) was prepared following double extraction of sugars from sweet sorghum in 15% (w/v) aqueous suspension at 50 ^{o}C for 45 min, filtration, extensive washing and drying at 70 ^{o}C overnight (Dogaris et al. 2009b). Commercial cellulase (Celluclast 1.5 L, from *Trichoderma reesei* ATCC 26921) and *β*-glucosidase (Novozyme 188, from *Aspergillus niger*) were kindly provided by Novozymes Corporation (Denmark).

Cellulase activity

Total cellulase activity (FPU) was measured against filter paper by the standard IUPAC method, as described by Ghose (1987). Experiments were carried out in duplicate.

Dilute-acid pretreatment of SB

Microwave digestion equipment (speed wave^{TM} MWS-2, Berghof Instruments GmBH, Germany) was employed for substrate pretreatment, as described elsewhere (Dogaris et al. 2009a). The microwave power was set at 700 W. Sorghum bagasse was pretreated at 160, 180, 200, and 210 ^{o}C for 10 min. The SB solids loading was 16% (w/v). For each operating temperature, the following sulfuric acid was added (g/100g SB): 0, 0.25, 0.5, 1, 2 and 4, (final acid concentrations: 0, 4, 8, 16, 33 and 65 mM, respectively).

After pretreatment experiments, the liquid fraction was separated from solids by filtration (0.45 μm) and used for sugar and inhibitor content analysis. The solid residue was extensively washed and dried at 70 ^{o}C overnight for subsequent structural carbohydrate analysis. Experiments were carried out in duplicate.

For the fermentation experiments the pretreatment slurry (both solid and liquid fractions) was aseptically neutralized with 1M NaOH and transferred to the fungal culture flasks to a final SB concentration of 8% (w/v).

Aerated growth

For solid state cultivation of *N. crassa* DSM 1129, a mixture 5:1 of WS and WB was used as carbon source, ammonium sulfate as nitrogen source, initial culture pH set at 5.0 and initial moisture at 70.5 % (w/w) (Dogaris et al. 2009b). The flasks were incubated at 30 ^{o}C for 6 days. *S. cerevisiae* 2541 cells were grown on (g/l): 5 $(NH_4)_2HPO_4$, 5 KH_2PO_4, 1 $MgSO_4 \cdot 7H_2O$ and 5 yeast extract, supplemented with 10 g/l sucrose (Mamma et al. 1996). The flasks were incubated in a rotary shaker operating at 250 rpm at 30 ^{o}C for 18 h.

Bioconversion of SB into ethanol

SB was fermented into ethanol applying a two-stage bioconversion, where enzyme production (first stage) was combined with SSF of the substrate (second stage) by *N. crassa* DSM 1129, as described elsewhere (Dogaris et al. 2009b) and presented in Fig. 1. Briefly, at the first stage, fungal cells were grown aerobically under the optimal conditions found for the production of the cellulolytic and hemicellulolytic system, as described in "Aerated growth" section. During the second (SSF) stage, fungal mycelia and the in situ produced cellulolytic and hemicellulolytic enzymatic systems were aseptically supplemented with citrate-phosphate buffer (50mM; pH 5.0) and SB (neutralized pretreatment slurry, both solid and liquid fractions) to a final concentration of 8% (w/v). Fermentation continued in a rotary shaker operating at 30 ^{o}C and 80 rpm using 250 ml Erlenmeyer flasks provided with needle-pierced rubber stoppers, which ensured micro-aerobic conditions and allowed the release of produced carbon dioxide. Experiments were carried out in duplicate.

In parallel, mixed-culture fermentations were performed using *S. cerevisiae* 2541 together with *N. crassa*, in order to evaluate their effect on ethanol production. Yeast biomass was inoculated in the beginning of the fermentation at a concentration of 10% v/v.

For external enzyme addition experiments, a mixture 5:1 (v/v) of commercial cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188) was added at the beginning of the fermentation. The cellulase activity of the added mixture was 6 FPU/g SB.

Analytical methods

Released sugars (glucose, xylose and arabinose), degradation products (5-hydroxymethyl furfural [HMF] and furfural) and organic acids (formic and acetic acid) formed during dilute-acid microwave pretreatment of SB were quantified by an HPLC system (Jasco PU987, USA) equipped with a refractive index detector (Waters 410) and: (a) an Aminex HPX-87P (Bio-Rad, USA) chromatography column for sugar analysis, or (b) an Aminex HPX-87H (Bio-Rad, USA) for degradation products and organic acids, according to the NREL protocol (Sluiter et al. 2005a).

The extracellular metabolites pattern (glycerol, xylitol, ethanol and acetic acid) of fungal cells grown on SB was analyzed by column liquid chromatography (HPLC) as described elsewhere (Dogaris et al. 2009b).

Structural carbohydrate content (cellulose and hemicellulose) of pretreated and fermented materials was determined by the NREL protocol (Sluiter et al. 2005b). For each sample, the carbohydrate content analysis was carried out in duplicate.

Calculations

Removal percentage (%) of each structural carbohydrate (cellulose or hemicellulose) accounts for the percentage change (loss) from each initial dry content - 40.4% (w/w) for cellulose and 35.5% (w/w) for hemicellulose - previously estimated for sorghum bagasse (Dogaris et al. 2009b). Cellulose was described as the estimated glucan percent content, while hemicellulose was estimated by the sum of xylan, arabinan, galactan and acetate content, based on previous results of structural carbohydrate analysis of SB (Dogaris et al. 2009a; Dogaris et al. 2009b).

Ethanol production from pretreated SB by *N. crassa* DSM 1129 could be efficiently described by a logistic model (Eq. 1), first proposed by Verhulst (1838) for population growth.

$$C(t) = \frac{C_{\max}}{1 + e^{-k(t-tm)}} \qquad \textbf{Eq. 1}$$

where C is the ethanol concentration, t is time, k is a constant that determines the curvature of the production pattern, t_m is the inflection point at which the production rate reaches its maximum value (Verhulst 1838).

Results

Dilute-acid pretreatment of SB

Sulfuric acid loadings above 1% (g/100g SB) significantly increased SB hemicellulose solubilization (Fig. 2d) on all pretreatment temperatures tested. SB decomposition seemed to depend more on temperature when acid loading increased above 1%. Hemicellulose removal reached 97.4% (based on initial SB hemicellulose content) at the most intense conditions (210 ^{o}C; 4g acid /100g SB), while cellulose decomposition was lower on all samples, reaching 26.6% (based on initial SB cellulose content) (Fig. 2d and 2a, respectively). Consequently, released glucose concentrations were low (Fig. 2b) while galactose was not detected at the pretreatment liquid. As far as pentose sugar (xylose and arabinose) release is concerned, a peak concentration of 20.0 g/l at 2% acid loading was followed by decrease in pentoses concentration (Fig. 2e), probably due to their further decomposition to furfural (Fig. 2f).

The formation of sugar decomposition compounds, such as furfural, 5-hydroxymethyl furfural (HMF) and formic acid, increased substantially at acid loadings above 1% (Fig. 2f, 2c, and 2h, respectively). In addition, acetic acid was also released (Fig. 2g), due to de-acetylation of acetyl groups present in the hemicellulose during the pretreatment process (Palmqvist and Hahn-Hägerdal 2000). Maximum concentrations of inhibitors were measured at the most intense pretreatment conditions (4 g acid /100g SB; 210 ^{o}C; 10 min) and reached 10.08, 2.52, 0.56 and 2.23 g/l for acetic acid, formic acid, HMF and furfural respectively.

Different SB pretreatment conditions led to different materials in terms of cellulose-hemicellulose content, sugar release and inhibitor formation (Fig. 2). In order to solubilize most of the hemicellulose fraction while keeping the formed inhibitor levels low, the sulfuric acid loading was set at 2% (g/100g SB) and four different pretreatment temperatures (160, 180, 200 and 210 ^{o}C) were applied in the subsequent bioconversion experiments.

Bioconversion of pretreated SB by *N. crassa*

Pretreated SB (whole slurry, liquid and solids fractions) was subjected to fermentation by *N. crassa* DSM 1129, without any separation or detoxification. *N. crassa* was able to ferment to ethanol all pretreated SB samples (Fig. 3). Increasing pretreatment temperature along with 2% (g/100g SB) acid loading resulted in increased ethanol production (Fig. 3b). Maximum ethanol production reached 8.10 g/l or 24.8% of theoretical yield (based on SB cellulose and hemicellulose sugar content) after pretreatment at 210 ^{o}C and 2% acid loading (Table 1). Ethanol production during fermentations without sulfuric acid at pretreatment (controls) was at low levels, regardless of the pretreatment temperature (Fig. 3a).

The production of metabolic by-products (xylitol, glycerol and acetic acid) was kept at low levels. The initial acetic acid concentration - formed during SB pretreatment - decreased to zero during fermentation (Fig. 3b). No glucose was present at the end of fermentation step, while the residual xylose concentrations were higher after bioconversion of SB pretreated at temperatures 200 - 210 ^{o}C and 2% sulfuric acid loading (Table 1).

Inhibiting compounds (HMF, furfural and acetic acid) were not detected on samples pretreated without sulfuric acid (controls) (Fig. 3a). Monitoring the time course of the bioconversion of dilute-acid pretreated samples showed that the ethanol production phase starts after the substantial decrease of inhibitor concentrations (HMF and furfural) in the fermentation broth, especially on samples pretreated at higher temperatures (Fig. 3b). The formed inhibitors did not seem to affect the final ethanol production by the fungus but led to increased fermentation times (Fig. 3b) and therefore low productivity levels.

Ethanol production from dilute-acid pretreated samples could be efficiently described by a logistic model (Eq. 1 and Fig. 4a) and the estimated parameters are shown on Table 2. As it can be seen from Eq. 1, the ethanol concentration at t_m is half of its maximum value, C_{max}. Increasing the pretreatment temperature led to higher initial inhibitor concentrations (furfural, HMF and acetic acid) and also higher t_m values (Fig. 4b), depicting a longer lag phase. At 160 ^oC furfural and HMF were not present, while acetic acid concentration was 1.01 g/l and the model parameter t_m estimated at 49 h (Table 2). At higher temperatures, the 2- to 3- fold increase of initial furfural and HMF concentrations seemed to have a more significant effect on t_m than acetic acid, which started relatively high and elevated at lower rate (Fig. 4b). After statistical analysis, the parameter t_m was found to have a strong linear correlation with furfural concentrations and less with HMF, present at the beginning of the fermentation step. So, the parameter t_m (time for half C_{max}) could be estimated from initial furfural concentration by Eq. 2.

$$t_m = 49.7 + (587.9 \cdot F) \quad \textbf{Eq. 2}$$
$$(R^2=0.999,\ standard\ error\ of\ estimates=1.4,\ P<0.0001)$$

where t_m is the time when the ethanol concentration is half of its maximum value (as described in Eq. 1) and F is the initial furfural concentration (g/l).

The analysis of the fermentation residual solids was conducted in order to evaluate the effect of SB decomposition performance of the combined dilute-acid pretreatment and bioconversion. Without acid addition at pretreatment step, total (pretreatment and bioconversion) cellulose hydrolysis remained below 10% regardless the pretreatment temperature. Increasing the acid loading to 2% led to higher cellulose hydrolysis by *N. crassa* cellulases, showing that the SB cellulose structure was more accessible to enzymatic hydrolysis when pretreated with dilute acid (Table 1). Total hemicellulose removal also increased at 2% acid loading, by 3-fold at lower temperatures (160 and 180 ^oC) and by 18% at higher temperatures (200 and 210 ^oC) by the combined pretreatment and enzymatic hydrolysis of the in situ produced *N. crassa* hemicellulolytic system (Table 1).

Effect of mixed cultures with *S. cerevisiae* and cellulase supplementation

In order to improve the sugar assimilation rate, mixed cultures of *N. crassa* DSM 1129 with the yeast *S. cerevisiae* 2541 were applied with the same bioconversion setup, where SB was pretreated at 210 ^oC in the presence of 2% sulfuric acid (g/100g SB) and fermentation was conducted as described above. The application of mixed cultures of *S. cerevisiae* with *N. crassa* had negative effect on ethanol production (Fig. 5a), leading to almost half ethanol production, 4.5 g/l, compared to *N. crassa* single cultures (Table 1). However, less time (48 h) was required for the mixed microbial system to assimilate or transform furfurals, compared to *N. crassa* single cultures (96 h)

(Fig. 3b). Increased residual xylose was present, 5.09 g/l, while no glucose was detected at the end of fermentation (Table 1).

The single and mixed microbial systems were supplemented with a mixture 5:1 (v/v) of commercial cellulase and *β*-glucosidase at low activity (6 FPU/g SB), to elucidate whether the in situ produced cellulolytic system by *N. crassa* (1.0 FPU/g SB) is limiting the enzymatic hydrolysis rate. Enhancing the cellulolytic enzyme load led to significant increase in final ethanol production (Fig. 5b, 5c and 5d). The single culture of *N. crassa* produced more ethanol than the mixed system with *S. cerevisiae*, followed by *S. cerevisiae* single cultures (Table 1). Maximum ethanol production by *N. crassa* reached 27.6 g/l or 84.7% of theoretical yield, while *S. cerevisiae* produced 14.9 g/l or 49.9% of theoretical yield. Some xylitol accumulation was observed in all cellulase-supplemented cultures, ranging from 1.40 to 2.92 g/l (Table 1). Residual pentose sugars (xylose and arabinose) were present in all cultures, but lower values were observed during fermentations by *N. crassa* single cultures.

The application of mixed cultures *N. crassa* - *S. cerevisiae* without external cellulase supplementation had no significant effect on SB decomposition, as cellulose and hemicellulose removal were at the same levels as after *N. crassa* SSF (Table 1). On the other hand, when the single and mixed cultures were supplemented with external (commercial) cellulases at low enzyme activity loading (6 FPU/g SB) cellulose hydrolysis was doubled (Table 1). Maximum SB cellulose hydrolysis was 73.3% and hemicellulose removal 89.6%, after combined pretreatment at 210 ^{o}C with 2% (g/100g SB) sulfuric acid and bioconversion by *N. crassa* single cultures (Table 1).

Discussion

The addition of dilute sulfuric acid at the hydrothermal pretreatment of sorghum bagasse led to a high degree of hemicellulose hydrolysis to its constituent pentose sugars, while the cellulosic fraction was less affected. Results obtained during SB pretreatment are comparable with those reported by Chen et al. (2007), where increasing the acid concentration from 0.5 to 2% led to significant increase in xylan hydrolysis during hydrothermal pretreatment (121 °C; 15 psi; 60 min) of sweet sorghum straw. Steam pretreatment of forage sorghum in the presence of 2% H_2SO_4 (140 °C; 30 min) led to maximum pentose yield of 93%, while no hexoses were released (Corredor et al. 2009). Increasing the pretreatment temperature and acid concentration leads to increased formation of inhibitors, such as acetic acid, formic acid, furfural and 5-hydroxymethyl furfural (HMF). Nevertheless, the concentrations of the inhibiting compounds detected in the present study are lower than those reported to inhibit the bioconversion of lignocellulosics to ethanol (Martinez et al. 2000).

The applied dilute-acid pretreatment combined with the SSF by *N. crassa* and the in situ produced enzymes led to a 3-fold increase in ethanol production compared to our previous results on the same but untreated substrate (Dogaris et al. 2009b). Monitoring the time course of the fermentations, ethanol production phase starts after the substantial decrease in inhibitor concentrations (HMF and furfural) in the culture broth. This pattern indicates that metabolic activity of the fungus *N. crassa* halts until furfural levels drop enough, either by assimilating or transforming the inhibitors into less toxic compounds, such as furfuryl alchohol and furoic acid (Eilers and Sussman 1970), a phenomenon that has also been observed on fermentations by *S. cerevisiae* (Almeida et al. 2007; Almeida et al. 2009). The initial furfural concentrations seemed to account more for the observed lag phases (as depicted by the model parameter t_m, time for half maximum ethanol production) than HMF or acetic acid. Palmqvist *et al* (1996) reported that furfural is more inhibitory to *S. cerevisiae* fermentations than HMF. No toxicity levels have been reported so far for *N. crassa*, while Xiros et al. (2011) observed that furfural had the most severe inhibitory effects than HMF on growth of the fungus *Fusarium oxysporum* F3 and ethanol fermentation is inhibited by 20-50% at concentrations above 3.0 g/l furfural and 3.2 g/l HMF. Generally, acetic acid is inhibitory to yeast when its concentration is between 4 and 10 g/l (Cardona et al. 2010). Low acid concentrations (<6 g/l) were shown to increase the ethanol yield at pH 5.5, whereas the yield decreased at higher concentrations (Palmqvist and Hahn-Hägerdal 2000). Lower inhibitor concentrations were estimated during the present study, but synergistic inhibition effects between furfural and HMF have been previously reported (Taherzadeh et al. 2000).

Apart from inhibition phenomena, the bioconversion efficiency of *N. crassa* could be limited by the rate of sugar assimilation, enzymatic hydrolysis or both. *S. cerevisiae* is known for its high glucose assimilation rates. Utilization of co-cultures for ethanol production appears to have advantages over single culture since there is potential for synergistic action of the metabolic pathways of all involved strains (Bader et al. 2010). However, the application of mixed cultures of *S. cerevisiae* with *N. crassa* in the present study had negative effect on ethanol production. Pentose sugar assimilation appeared problematic by the mixed cultures, resulting in increased residual pentose concentrations and xylitol accumulation. Negative interactions could happen when two microorganisms compete for the same resource, such as space or a limiting nutrient (Bader et al. 2010).

The bioconversion efficiency of *N. crassa* DSM 1129 was found superior to *S. cerevisiae* 2541. *N. crassa* fermented both glucose and pentose sugars present in SB cellulose and hemicellulose,

reaching almost double ethanol production than *S. cerevisiae*. Ethanol production from SB by *N. crassa* (supplemented with cellulase at 6 FPU/g SB) during the present study, 27.6 g/l or 34.5 g/100g SB, was higher than those reported by Ballesteros et al. (2004), 16.2 g/l or 16.2 g/100g SB with the addition of double cellulase activities. Li et al. (2010) reached higher ethanol concentration (42.3 g/l) from AFEX pretreated sweet sorghum bagasse by applying separate hydrolysis and fermentation, but overall yield was 15.9 g/100g bagasse. Xu et al. (2011) reported ethanol production 21.9 g/l (20 g/100g sorghum) by SSF of sorghum's both soluble and insoluble sugars at 4% solids loading. In another study by Salvi et al. (2010) ethanol production from dilute-ammonia pretreated sorghum fibers with high enzyme loadings (60 FPU/g glucan) reached 24 g/l with a theoretical ethanol yield of 84%. Generally, the yields in the present work were comparable with SSF processes reported for other lignocellulosic substrates (Olofsson et al. 2008a; Olofsson et al. 2008b).

In the present work we demonstrated an alternative cost-efficient bioconversion of SB to ethanol by the fungus *N. crassa* DSM 1129 at high solids loadings (16% at pretreatment and 8% at fermentation), low external cellulase loadings (6 FPU/g SB) and co-fermentation of hexoses and pentoses. Although in order to increase the system's efficiency some process aspects must be further optimized, such as increasing sugar assimilation rates and inducing a more active cellulolytic system production which will reduce or remove the need for external cellulases.

Acknowledgements

This work has been funded by the project PENED 2003. The project is cofinanced 80% of public expenditure through EC-European Social Fund, 20% of public expenditure through Ministry of Development - General Secretariat of Research and Technology and through private sector, under measure 8.3 of OPERATIONAL PROGRAMME "COMPETITIVENESS" in the 3rd Community Support Programme.

The authors would like to thank Novozymes Corporation for generously providing the cellulase enzyme samples.

The original publication is available at www.springerlink.com
http://link.springer.com/article/10.1007%2Fs00253-012-4113-1

Figures

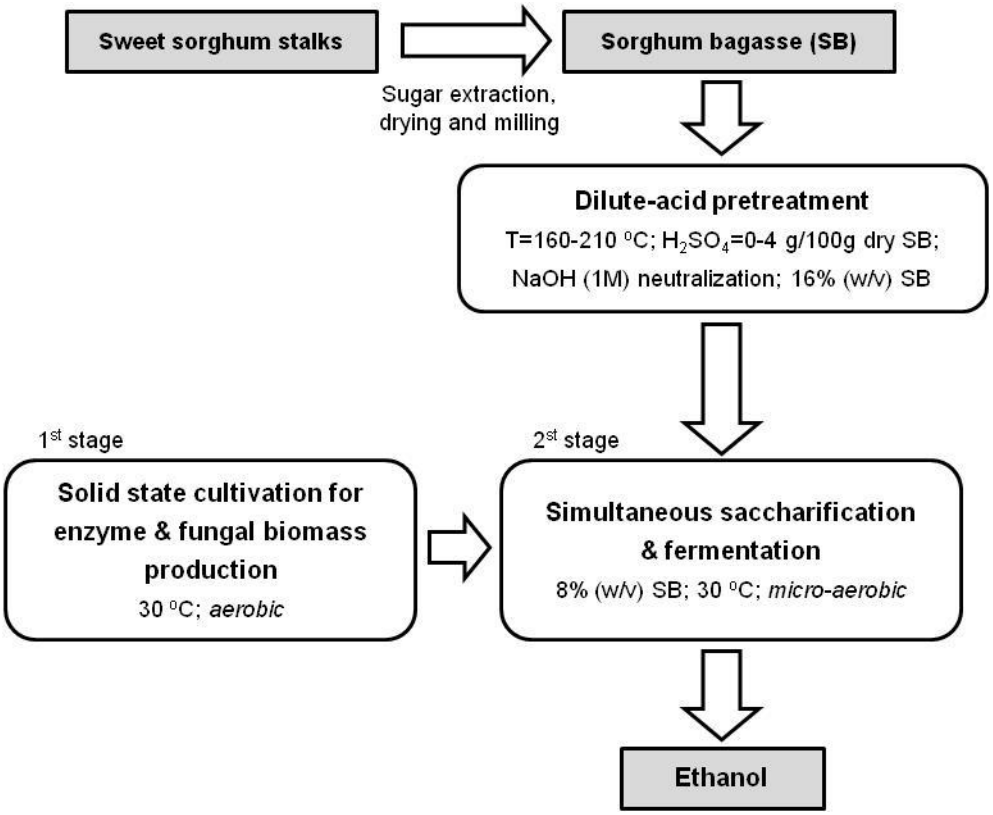

Fig. 1 General process scheme for the bioconversion of dilute-acid pretreated SB into bioethanol

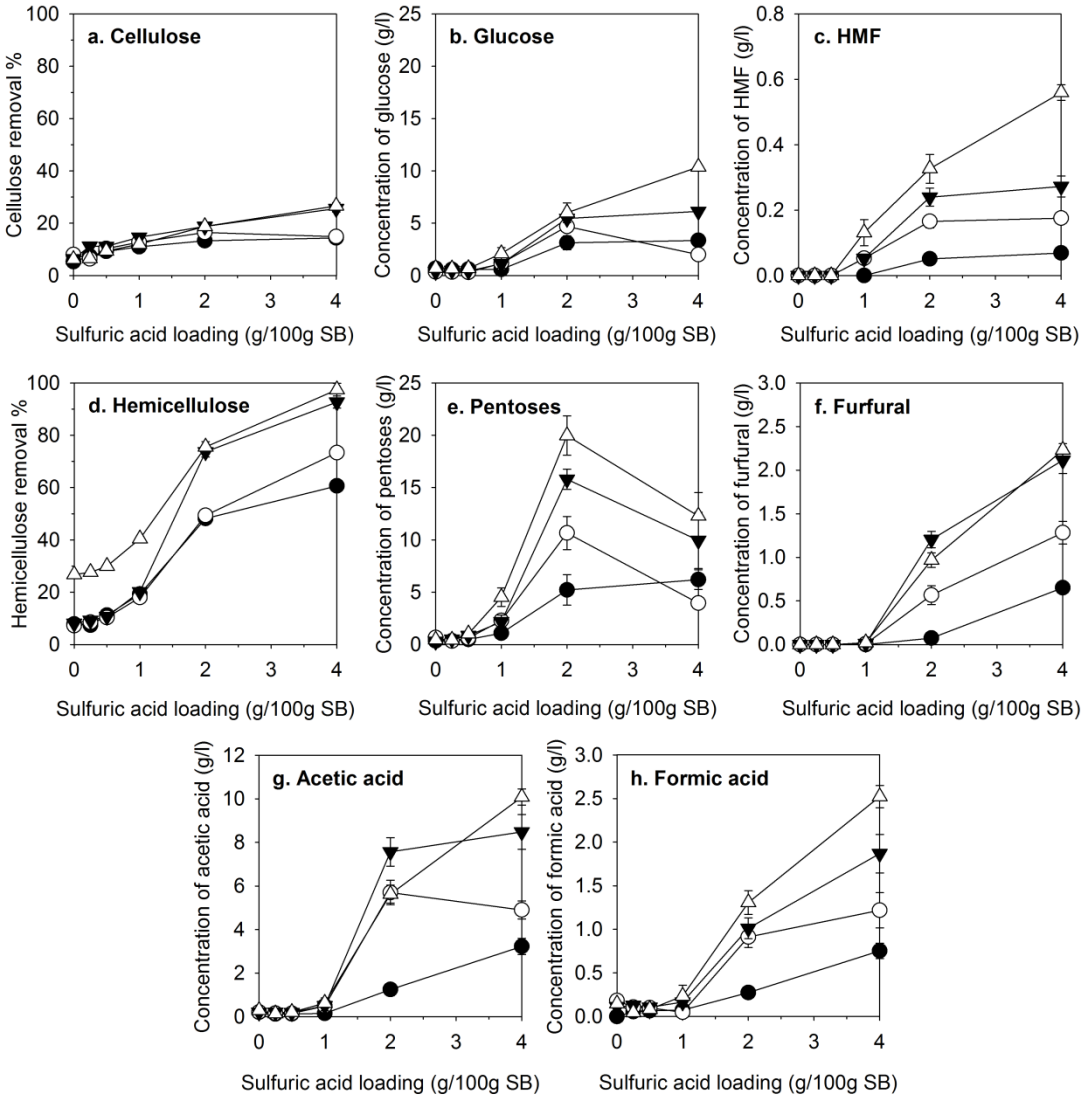

Fig. 2 Effect of sulfuric acid loading on (a) cellulose removal, (b) glucose release, (c) HMF formation, (d) hemicellulose removal, (e) pentose release, (f) furfural formation, (g) acetic and (h) formic acid formation after pretreatment of SB at different temperatures for 10 min. *Symbols*: (●) 160 ^{o}C, (○) 180 ^{o}C, (▼) 200 ^{o}C and (△) 210 ^{o}C. *Error bars*: standard error of two replicates

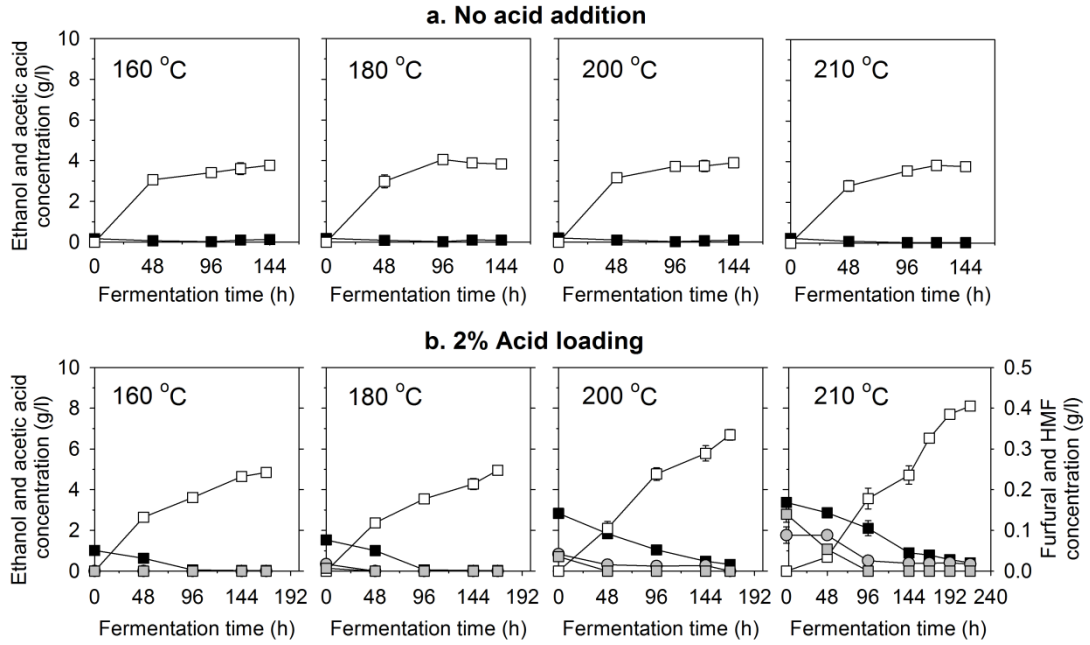

Fig. 3 Ethanol, acetic acid, furfural and HMF concentrations during bioconversion of pretreated SB by *N. crassa* DSM 1129. *Pretreatment conditions*: temperature 160, 180, 200 and 210 °C, 10 min and (a) no acid addition, (b) 2% (g/100g SB) sulfuric acid loading. *Symbols*: (□) ethanol, (■) acetic acid, (●) HMF, (■) furfural. *Error bars*: standard error of two replicates

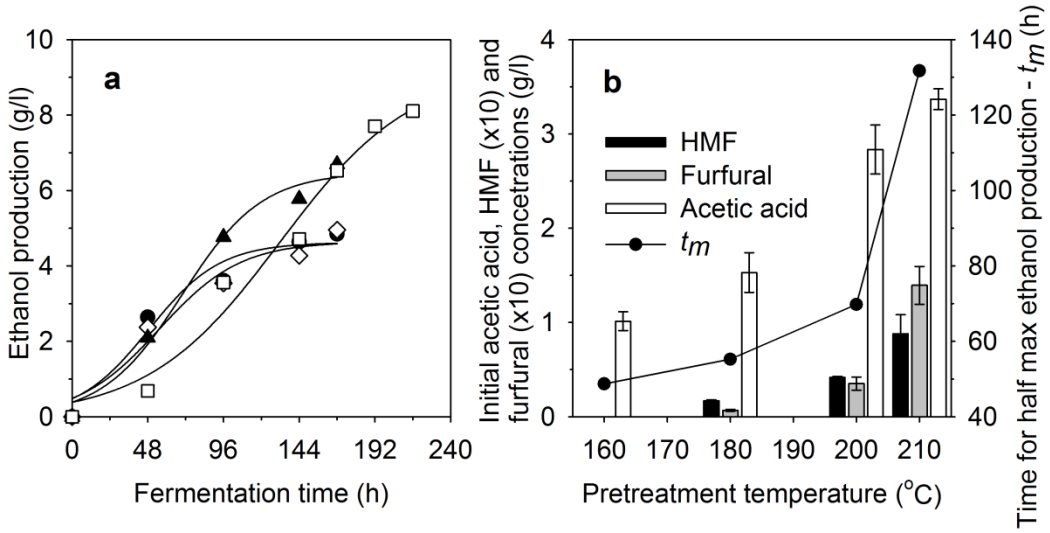

Fig. 4 (a) Regression curves (Eq. 1, Table 2) of ethanol production from SB dilute-acid pretreated at different temperatures. *Pretreatment conditions*: temperature (●) 160 °C , (◇) 180 °C, (▲) 200 °C and (□) 210 °C, 10 min, 2g acid/100g SB. (b) Effect of initial inhibitor concentration on fermentation time for half maximum ethanol production, as estimated by the logistic model parameter t_m from Eq. 1 for each dilute-acid pretreated SB fermentations. *Error bars*: standard error of two replicates

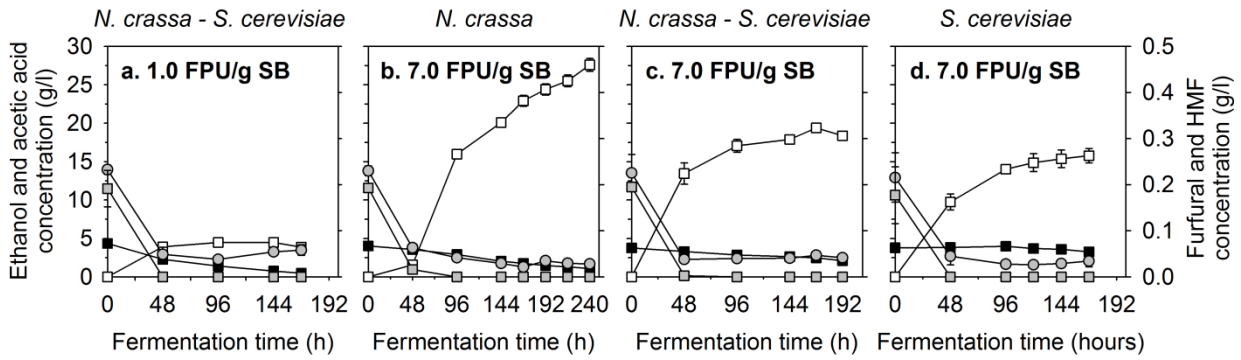

Fig. 5 Ethanol, acetic acid and inhibitor concentrations during bioconversion of pretreated SB by *N. crassa* and mixed cultures with *S. cerevisiae*. Total cellulase activity (FPU/g SB): (a) 1.0 (in situ produced), (b) 7.0, (c) 7.0 (1.0 in situ produced and 6.0 added from commercial enzyme mixture), and (d) 7.0 (commercial enzyme mixture). *Pretreatment conditions*: 210 ^{o}C, 10 min, 2g sulfuric acid/100g SB. *Symbols*: (□) ethanol, (■) acetic acid, (■) furfural, (●) HMF. *Error bars*: standard error of two replicates

Table 1 Ethanol production, by-product formation, residual sugars and structural carbohydrates removal percent after bioconversion of pretreated SB by *N. crassa* DSM 1129 and mixed cultures with *S. cerevisiae* 2541

Microbial system	SB pretreatment		Cellulase loading (FPU/g SB)	Ethanol yield (% of theoretical) *	Extracellular metabolites concentrations (g/l)				Residual sugar concentrations (g/l)		Removal % after pretreatment and bioconversion	
	Temperature (^{o}C)	Acid loading (g/100g SB)			C_{ethan}	C_{acet}	C_{glyc}	C_{xylit}	C_{xylo}	C_{ara}	Cellulose	Hemicellulose
N. crassa	160	0	1.0 [a]	8.7±0.0	3.4±0.0	0.03±0.00	n.d.	0.04±0.02	n.d	n.d	5.9±0.3	22.0±0.4
	180			10.4±0.0	4.1±0.0	0.01±0.00	n.d.	0.08±0.03	n.d	n.d	7.5±0.6	24.8±1.2
	200			9.5±0.2	3.7±0.1	0.02±0.00	n.d.	0.04±0.02	n.d	n.d	6.7±0.4	66.1±1.2
	210			9.8±0.3	3.8±0.1	0.03±0.00	n.d.	0.01±0.00	n.d	n.d	7.0±1.1	67.6±2.3
N. crassa	160	2	1.0 [a]	14.8±0.0	4.84±0.01	0.03±0.00	n.d.	0.18±0.00	0.91±0.10	0.59±0.09	20.7±0.5	69.5±3.5
	180			15.2±0.3	4.96±0.09	0.03±0.00	0.04±0.00	0.14±0.01	0.79±0.10	0.61±0.01	18.6±5.0	77.8±0.9
	200			20.5±0.1	6.69±0.27	0.32±0.06	0.30±0.07	0.49±0.01	1.88±0.18	0.63±0.00	30.2±3.2	80.2±1.6
	210			24.8±0.4	8.10±0.12	0.40±0.10	0.32±0.01	0.83±0.14	2.67±0.34	0.54±0.08	33.5±0.3	81.5±1.0
N. crassa - S. cerevisiae	210	2	1.0 [a]	13.0±0.8	4.5±0.3	1.40±0.10	n.d.	0.97±0.02	5.09±0.17	0.55±0.03	40.4±0.4	75.4±1.6
N. crassa	210	2	7.0 [b]	84.7±2.4	27.6±0.8	1.08±0.05	0.46±0.02	1.40±0.07	1.16±0.06	0.70±0.03	73.3±2.4	89.6±0.9
N. crassa - S. cerevisiae	210	2	7.0 [b]	59.4±4.0	19.4±0.6	2.48±0.08	0.45±0.03	2.92±0.21	3.23±0.19	0.39±0.01	68.7±0.4	87.5±1.4
S. cerevisiae	210	2	7.0 [b]	49.9±3.9	14.9±1.2	3.7±0.4	0.15±0.05	1.40±0.40	4.13±0.43	0.89±0.04	72.9±0.3	75.1±2.1

[a] *in situ* produced cellulase activity,

[b] total *in situ* produced and added cellulase activity,

* based on initial SB sugar content in cellulose and hemicellulose.

n.d. - not detected, *ethan* - ethanol, *acet* - acetic acid, *glyc* - glycerol, *xylit* - xylitol, *xylo* - xylose, *ara* – arabinose

Table 2 Regression (Eq. 1) statistic analysis and parameters estimation of fermentation of dilute-acid pretreated SB to ethanol by *N. crassa* DSM 1129.

SB pretreatment conditions	C_{max} *(g/l)*	k *(h^{-1})*	t_m *(h)*
160 °C; 10 min; acid 2g/100g SB	4.6237	0.0444	48.6912
	(R^2=0.9554, Standard Error of Estimate = 0.5878, *P*=0.0446)		
180 °C; 10 min; acid 2g/100g SB	4.6453	0.0388	55.2557
	(R^2=0.9564, Standard Error of Estimate = 0.5745, *P*=0.0436)		
200 °C; 10 min; acid 2g/100g SB	6.4800	0.0395	69.7973
	(R^2=0.9857, Standard Error of Estimate = 0.4680, *P*=0.0143)		
210 °C; 10 min; acid 2g/100g SB	9.2522	0.0238	131.7412
	(R^2=0.9785, Standard Error of Estimate = 0.5822, *P*=0.0005)		

C_{max}, k and t_m - as described in Eq. 1

References

Almeida JR, Modig T, Petersson A, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340-349. doi: 10.1002/jctb.1676

Almeida JRM, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G (2009) Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl Microbiol Biotechnol 82(4):625-638. doi: 10.1007/s00253-009-1875-1

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technol 101:4851-4861. doi: 10.1016/j.biortech.2009.11.093

Bader J, Mast-Gerlach E, Popović MK, Bajpai R, Stahl U (2010) Relevance of microbial coculture fermentations in biotechnology. J Appl Microbiol 109:371-387. doi: 10.1111/j.1365-2672.2009.04659.x

Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I (2004) Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochem 39:1843-1848. doi: 10.1016/j.procbio.2003.09.011

Cardona CA, Quintero JA, Paz IC (2010) Production of bioethanol from sugarcane bagasse: Status and perspectives. Bioresource Technol 101:4754-4766. doi: 10.1016/j.biortech.2009.10.097

Cardona CA, Sánchez OJ (2007) Fuel ethanol production: Process design trends and integration opportunities. Bioresource Technol 98(12):2415-2457. doi: 10.1016/j.biortech.2007.01.002

Carrasco C, Baudel HM, Sendelius J, Modig T, Roslander C, Galbe M, Hahn-Hägerdal B, Zacchi G, Lidén G (2010) SO_2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse. Enzyme Microb Technol 46:64-73. doi: 10.1016/j.enzmictec.2009.10.016

Chen Y, Sharma-Shivappa RR, Keshwani D, Chen C (2007) Potential of agricultural residues and hay for bioethanol production. Appl Biochem Biotech 142(3):276-290. doi: 10.1007/s12010-007-0026-3

Corredor DY, Salazar JM, Hohn KL, Bean S, Bean B, Wang D (2009) Evaluation and characterization of forage sorghum as feedstock for fermentable sugar production. Appl Biochem Biotech 158(1):164-179. doi: 10.1007/s12010-008-8340-y

da Silva ASA, Inoue H, Endo T, Yano S, Bon EPS (2010) Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. Bioresource Technol 101:7402-7409. doi: 10.1016/j.biortech.2010.05.008

Deshpande V, Keskar S, Mishra C, Rao M (1986) Direct conversion of cellulose/hemicellulose to ethanol by *Neurospora crassa*. Enzyme Microb Technol 8(3):149-152. doi: 10.1016/0141-0229(86)90103-1

Dogaris I, Karapati S, Mamma D, Kalogeris E, Kekos D (2009a) Hydrothermal processing and enzymatic hydrolysis of sorghum bagasse for fermentable carbohydrates production. Bioresource Technol 100(24):6543-6549. doi: 10.1016/j.biortech.2009.07.046

Dogaris I, Vakontios G, Kalogeris E, Mamma D, Kekos D (2009b) Induction of cellulases and hemicellulases from *Neurospora crassa* under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. Ind Crop Prod 29(2-3):404-411. doi: 10.1016/j.indcrop.2008.07.008

Eilers FI, Sussman AS (1970) Conversion of furfural to furoic acid and furfuryl alcohol by *Neurospora* ascospores. Planta (Berl.) 94:253-264.

Ewanick S, Bura R (2011) The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse. Bioresource Technol 102:2651-2658. doi: 10.1016/j.biortech.2010.10.117

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59(2):257-268. doi: 10.1351/pac198759020257

Lezinou V, Christakopoulos P, Li L-W, Kekos D, Macris BJ (1995) Study of a single and mixed culture for the direct bio-conversion of sorghum carbohydrates to ethanol. Appl Microbiol Biotechnol 43(3):412-415. doi: 10.1007/s002530050427

Li B-Z, Balan V, Yuan Y-J, Dale BE (2010) Process optimization to convert forage and sweet sorghum bagasse to ethanol based on ammonia fiber expansion (AFEX) pretreatment. Bioresource Technol 101(4):1285-1292. doi: 10.1016/j.biortech.2009.09.044

Mamma D, Koullas D, Fountoukidis G, Kekos D, Macris BJ, Koukios E (1996) Bioethanol from sweet sorghum: Simultaneous saccharification and fermentation of carbohydrates by a mixed microbial culture. Process Biochem 31(4):377-381. doi: 10.1016/0032-9592(95)00075-5

Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO (2000) Effects of $Ca(OH)_2$ treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnol Bioeng 69:526-536. doi: 10.1002/1097-0290(20000905)69:5<526::AID-BIT7>3.0.CO;2-E

Mesa L, González E, Romero I, Ruiz E, Cara C, Castro E (2011) Comparison of process configurations for ethanol production from two-step pretreated sugarcane bagasse. Chem Eng J 175:185-191. doi: 10.1016/j.cej.2011.09.092

Olofsson K, Bertilsson M, Lidén G (2008a) A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1:7. doi: 10.1186/1754-6834-1-7

Olofsson K, Rudolf A, Lidén G (2008b) Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*. J Biotechnol 134:112-120. doi: 10.1016/j.jbiotec.2008.01.004

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technol 74:25-33. doi: 10.1016/S0960-8524(99)00161-3

Palmqvist E, Hahn-Hägerdal B, Galbe M, Zacchi G (1996) The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. Enzyme Microb Technol 19:470-476. doi: 10.1016/S0141-0229(95)00234-0

Rao M, Deshpande V, Keskar S, Srinivasan MC (1983) Cellulase and ethanol production from cellulose by *Neurospora crassa* Enzyme Microb Technol 5:133-136. doi: 10.1016/0141-0229(83)90048-0

Salvi DA, Aita GM, Robert D, Bazan V (2010) Dilute ammonia pretreatment of sorghum and its effectiveness on enzyme hydrolysis and ethanol fermentation. Appl Biochem Biotechnol 161:67-74. doi: 10.1007/s12010-010-8932-1

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D (2005a) Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Laboratory Analytical Protocol. National Renewable Energy Laboratory, Golden, CO

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D (2005b) Determination of structural carbohydrates and lignin biomass. Laboratory Analytical Protocol. National Renewable Energy Laboratory, Golden, CO

Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G (2000) Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:701-708. doi: 10.1007/s002530000328

Varga E, Klinke HB, Réczey K, Thomsen AB (2004) High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnol Bioeng 88:567-574. doi: 10.1002/bit.20222

Verhulst PF (1838) A note on population growth. Corr Math et Phys 10:113-121 (in French).

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153-157. doi: 10.1016/j.tibtech.2007.02.009

Xiros C, Topakas E, Katapodis P, Christakopoulos P (2008) Hydrolysis and fermentation of brewer's spent grain by *Neurospora crassa*. Bioresource Technol 99:5427-5435. doi: 10.1016/j.biortech.2007.11.010

Xiros C, Vafiadi C, Paschos T, Christakopoulos P (2011) Toxicity tolerance of *Fusarium oxysporum* towards inhibitory compounds formed during pretreatment of lignocellulosic materials. J Chem Technol Biotechnol 86:223-230. doi: 10.1002/jctb.2499

Xu F, Shi Y-C, Wu X, Theerarattananoon K, Staggenborg S, Wang D (2011) Sulfuric acid pretreatment and enzymatic hydrolysis of photoperiod sensitive sorghum for ethanol production. Bioprocess Biosyst Eng 34:485-492. doi: 10.1007/s00449-010-0492-9

Zhang Z, Qu Y, Zhang X, Lin J (2008) Effects of oxygen limitation on xylose fermentation, intracellular metabolites, and key enzymes of *Neurospora crassa* AS3.1602. Appl Biochem Biotechnol 145:39-51. doi: 10.1007/s12010-007-8038-6

Zhao J, Xia L (2009) Simultaneous saccharification and fermentation of alkaline-pretreated corn stover to ethanol using a recombinant yeast strain. Fuel Process Technol 90:1193-1197. doi: 10.1016/j.fuproc.2009.05.018.