ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Comparison of SHF and SSF Processes From Steam-Exploded Wheat Straw for Ethanol Production by Xylose-Fermenting and Robust Glucose-Fermenting *Saccharomyces cerevisiae* Strains

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ABSTRACT: In this study, bioethanol production from steam-exploded wheat straw using different process configurations was evaluated using two *Saccharomyces cerevisiae* strains, F12 and Red Star. The strain F12 has been engineerically modified to allow xylose consumption as cereal straw contain considerable amounts of pentoses. Red Star is a robust hexose-fermenting strain used for industrial fuel ethanol fermentations and it was used for comparative purposes. The highest ethanol concentration, 23.7 g/L, was reached using the whole slurry (10%, w/v) and the recombinant strain (F12) in an SSF process, it showed an ethanol yield on consumed sugars of 0.43 g/g and a volumetric ethanol productivity of 0.7 g/L h for the first 3 h. Ethanol concentrations obtained in SSF processes were in all cases higher than those from SHF at the same conditions. Furthermore, using the whole slurry, final ethanol concentration was improved in all tests due to the increase of potential fermentable sugars in the fermentation broth. Inhibitory compounds present in the pretreated wheat straw caused a significantly negative effect on the fermentation rate. However, it was found that the inhibitors furfural and HMF were completely metabolized by the yeast during SSF by metabolic redox reactions. An often encountered problem during xylose fermentation is considerable xylitol production that occurs due to metabolic redox imbalance. However, in our work this redox imbalance was counteracted by the detoxification reactions and no xylitol was produced.

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Introduction

Biofuels, which are obtained from biomass, are the only renewable products that can be easily integrated into current fuel distribution systems, and they are one of the few alternatives for short-term diversification in the transportation sector. Therefore, they represent a top priority in the European Union energy policies intended to enhance the security of supply and reduce greenhouse gas emissions (Directive, 2003/30/EC).

In order to get a competitive price compared to conventional fuels, production cost of biofuels needs to be reduced and cheap and abundant raw materials need to be used. In this context, the use of lignocellulosic material is glimpsed as a promising choice as a second generation bioethanol fuel.

Among different lignocellulosic raw materials, wheat straw is an abundant source of biomass, mainly in Europe, and it is available as agricultural waste. Wheat straw production in the European Union in 2006 was about 140 million of tonnes (EUROSTAT, 2006). Furthermore, because of its high cellulose content, it is a promising raw material for bioprocesses producing bioethanol from lignocellulosic biomass using enzymatic hydrolysis (EH) in combination with fermentation (Alfani et al., 2000; Olsson et al., 2006). The lignocellulosic nature of wheat straw makes the pretreatment a crucial step due to the physical and chemical barriers caused by the close association of the main components; cellulose, hemicellulose and lignin. During the pretreatement step, the enzyme accessibility to cellulose is enhanced, therefore, the efficiency of cellulases to release fermentable sugars is increased. High-pressure steam is a very suitable pretreatment method for bioethanol production from wheat straw biomass (Alfani et al., 2000;

Ballesteros et al., 2006; Öhgren et al., 2006). One of the main characteristics of steam explosion pretreatment is the solubilization of hemicellulosic sugars and most of hemicelluloses are removed from the water insoluble solid (WIS) fraction (Ballesteros et al., 2006).

After pretreatment, the following steps are hydrolysis and fermentation, which can be carried out separately or simultaneously. When separate hydrolysis and fermentation (SHF) is performed, EH and fermentation are run in two different vessels. In this process each step can be conducted at optimal conditions of pH and temperature. However, glucose and cellobiose accumulation in the hydrolysis step inhibits the activity of the cellulases (Stenberg et al., 2000; Xiao et al., 2004). In a simultaneous saccharification and fermentation (SSF) process, the EH and fermentation are run in the same vessel, thus, glucose released by the action of cellulases is converted directly to ethanol by the fermenting microorganism and this continuous removal of glucose from the medium minimizes the end-product inhibition on enzyme activity (Ballesteros et al., 2004; Olsson et al., 2006). Furthermore, the presence of ethanol in the culture broth helps to avoid undesired microbial contamination. These advantages result in an increased rate of saccharification compared with separate hydrolysis and total ethanol productivity in the SSF is notably higher than in the SHF process (Alfani et al., 2000; Wyman, 1996).

Although processes to obtain bioethanol from lignocellulose are promising methods to produce biofuels at low cost, one of the best opportunities to reduce the cost of cellulosic bioethanol is, so far, through enhancing sugar yields from cellulose and hemicellulose (Wyman, 2007). Therefore, because not only glucose is contained in the lignocellulosic pretreated material, microorganisms which could ferment other sugars such as xylose, mannose, arabinose, or galactose are required for an economically viable conversion from lignocellulose to ethanol (Zaldivar et al., 2005). It is well-known that one of the most successful microorganisms to produce ethanol is *Saccharomyces cerevisiae*, it shows high ethanol productivity, high tolerance to ethanol and tolerance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass (Olsson and Hahn-Hägerdal, 1993). However, wild type *S. cerevisiae* strains are unable to utilize pentoses and several research groups have metabolically engineered yeast to ferment xylose (Hahn-Hägerdal et al., 2007; Ho et al., 1998; Jeffries and Sin, 2004). *S. cerevisiae* strain F12 has been modified for this purpose using an industrial strain background (Sonderegger et al., 2004). Two genes from *Pichia stipitis* related with xylose utilization have been introduced in the yeast. These genes are *XYL1* and *XYL2* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, furthermore, over-expression of the endogenous gene *XKSI* encoding xylulokinase (XK) is also needed for improving xylose utilization (Eliasson et al., 2000; Hahn-Hägerdal et al., 2007; Ho et al., 1998).

As well as improving sugar yields from cellulose and hemicellulose cost reduction should be attempted. One approach to reduce operational costs could be to avoid filtration step after the pretreatment. Furthermore, if the filtration step of the pretreated material is avoided and consequently the whole slurry is used, the amount of waste water is diminished and the concentration of the fermentable sugars will be higher due to the hemicellulosic sugars that have been solubilized during pretreatment. This will result in higher ethanol concentration in the fermentation broth prior to distillation. An ethanol concentration in fermentation broth of 4% (v/v) could be considered as a bench mark for an economically viable distillation (Zacchi and Axelsson, 1989). Whereupon, the whole slurry should preferably be used instead of the WIS fraction.

When the whole slurry is to be used, apart from sugars, inhibitors released during pretreatment are present and this may be determinant to the microbial activity (Klinke et al., 2004). Thus, it is necessary to employ a yeast that has a robust industrial strain background which can tolerate the inhibitors (Hahn-Hägerdal et al., 2007). The proportion of each inhibitory compound depends on the pretreatment employed and on the lignocellulosic feedstock that has been pretreated.

The aim of the present study was to investigate the bioethanol production from steam exploded wheat straw using both cellulosic and hemicellulosic sugars. For that reason, the main objective was to compare SSF performances when using a xylose-fermenting yeast and another unable to ferment xylose and determine whether any significant improvements could be observed when employing the recombinant strain. Furthermore, the fermentation performance on the whole slurry was compared with the one filtered (solid fraction).

Materials and Methods

Raw Material

Wheat straw (provided by Ecocarburantes de Castilla y León, Salamanca, Spain) having the following composition (% dry weight): cellulose, 30.2; hemicellulose, 22.3 (xylan, 18.7; arabinan, 2.8; and galactan, 0.8); acid insoluble lignin, 15.3; and acid soluble lignin, 1.7 (Ballesteros et al., 2006) was used as raw material in the present study.

The raw material was firstly sieved using a laboratory hammer mill in order to obtain a chip size between 2 and 10 mm and after which it was stored until use.

Steam Explosion Pretreatment

Pretreatment assays were performed using Masonite technology in a 2 L reactor equipped with a quick-opening ball valve and an electronic device programmed for accurate control of steam time and temperature (Fernández-Bolaños et al., 2001). The wheat straw was exploded at 210°C for 5 min without acid impregnation. These conditions were

chosen according to previous studies (data not published) and based on optimal EH yields and xylose recovery in the liquid fraction.

After pretreatment, the slurry was recovered. A certain portion of the slurry was vacuum filtered in order to obtain two fractions: (i) the WIS or solid fraction and (ii) prehydrolysate or liquid fraction. The WIS fraction was dried in a heater at 50°C for 90 h.

The slurry and WIS fraction were analyzed for carbohydrates and acid insoluble lignin and were used as substrate in SSF tests. Sugars and degradation compounds present in the prehydrolysate were also analyzed.

Microorganisms and Media

Two different strains were used and genotypes and references for both strains are listed in Table I. *S. cerevisiae* strain F12 is an engineered industrial strain which can produce ethanol from xylose. It has been modified with the endogenous gene encoding XK and genes for XR and XDH from *Pichia stipitis* (Sonderegger et al., 2004). Red Star is an industrial strain used in the fuel alcohol industry (Devantier et al., 2005), but it cannot consume xylose.

In order to prepare a preinoculum a colony of the respective strains was taken from a YPD-agar plate and inoculated into 2 L Erlenmeyer flask containing 500 mL Delft medium as follows: 40 g/L glucose, 7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2 mL/L trace metal solution, 1 mL/L vitamin solution, and 0.05 mL/L antifoam 289 (Sigma A-8436, St. Louis, MO). The preculture was grown on a rotarory shaker at 150 rpm, 30°C and 24 h (until the culture reached the late exponential growth phase). The preculture was centrifuged at 18,000*g* for 5 min at 4°C and the supernatant was discarded. The cells were washed twice with sterile 0.9% sodium chloride solution and centrifuged again. The supernatant was removed and the cells were weighed and diluted with sterile water to obtain an inoculum level of 1 g/L cells in the fermenter.

Analytical Methods

The pretreated material (WIS and slurry) was analyzed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, LAP-004, LAP-017, and LAP-019) (NREL, 2007). Dry matter was determined by drying samples at 105°C for 24 h (LAP-001).

Sugars, glycerol, xylitol, and ethanol were determined by high-performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatograph with a refractive index detector (Waters, Mildford, MA). An Aminex HPX-87P carbohydrate analysis column (Bio-Rad Labs, Hercules, CA) operated at 85°C with deionized water as mobile-phase (0.6 mL/min) was used for the separation.

Furfural, 5-hydroxymethylfurfural (HMF), coumaric acid, ferulic acid, vanillin, and syringaldehyde were analyzed by HPLC (Hewlett Packard, Palo Alto, CA), using an Aminex ion exclusion HPX-87H cation-exchange column (Bio-Rad Labs) at 55°C. As mobile phase 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL/min was used. For detection a 1040A Photodiode-Array detector (Agilent, Waldbronn, Germany) was employed.

Acetic and formic acid were quantified by HPLC (Waters) with a 410 Waters refractive index detector (Waters). A Bio-Rad Aminex HPX-87H (Bio-Rad Labs) column maintained at 65°C and a flow rate of 0.6 mL/min mobile phase (5mM H_2SO_4) were used.

Fermentation

Two fermentations using synthetic medium (as described below) and prehydrolysate were performed to determine the ability to ferment xylose by *S. cerevisiae* F12. The fermentations were run in 1 L fermenters, which were sterilized before adding 500 mL of media and incubated at 32°C for 50 h. The pH was controlled at 5.0 by automatic addition of 2 M NaOH.

Prehydrolysate was obtained after filtration of the slurry. Most of the sugar present in the liquid fraction were oligomers. Because of that, a mild acid post-hydrolysis (4% [v/v] H_2SO_4, 120°C and 30 min) was needed with the purpose of determining the sugar composition (24.7 g/L xylose, 5.7 g/L glucose, 1.1 g/L galactose, 1.6 g/L arabinose, and 0.6 g/L mannose) and making monomeric sugars available to the yeast. The pH reached after the post-hydrolysis was around 1.0 and was adjusted to 5.0 using 2 M NaOH. This prehydrolysate corresponded to a slurry with 13.5% of WIS (w/v) and was diluted to 10% with sterilized water before fermentation test.

The other medium employed was a synthetic medium having the same sugar concentration as the prehydrolysate and containing 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 mL/L trace metal solution, 1 mL/L vitamins solution and 0.05 mL/L antifoam. The media were supplemented with 1 mL/L of ergosterol to favor the synthesis of unsaturated fatty acids and sterols during anaerobic growth of *S. cerevisiae* (Deytieux et al., 2005).

Table I. *Saccharomyces cerevisiae* strains used in the present study.

Strain	Relevant genotype	Origin/reference
F12	*S. cerevisiae* F *HIS3*::YIploxZEO overexpressing XR, XDH, and XK	Sonderegger et al. (2004)
Red Star	Industrial strain, wild type	Ethanol Red™, Lesaffre, Milwaukee, WI

Simultaneous Saccharification and Fermentation Processes

Slurry and dried WIS were used as SSF media having a dry matter content of 10% (w/v). All the tests were carried out in 1 L fermenters with 500 mL of media. The fermenter and salts were sterilized before adding the slurry or WIS and vitamin solution and ergosterol were also added after sterilization.

The cellulase mixture supplied by Novozymes A/S (Bagsværd, Denmark) consisted of Celluclast 1.5 L (60 FPU/mL) supplemented with ß-glucosidase NS50010 (900 IU/mL). The enzyme loading was 15 FPU/g cellulose and 15 IU/g cellulose, respectively.

All SSF tests were preceded by a prehydrolysis step for 6–8 h carried out in 2 L shake flask in a water bath at 50°C and 200 rpm.

Enzymatic Hydrolysis

EH was performed at the same conditions as used for the SSF process. First, a prehydrolysis step for 6–8 h at 50°C (corresponding to prehydrolsis step in case of SSF) was performed in 2 L shake flask in a water bath at 200 rpm. After that, in order to estimate the amount of fermentable sugars available during SSF, the conditions were changed and fixed at the same conditions used for SSF processes (pH 5.0, 32°C, 500 rpm for 120 h). This second step was carried out in in-house manufactured 1 L fermenters. WIS or slurry (10%, w/v) were used as substrate and together with sterile water were added into a sterile fermenter to a volume of 500 mL. Furthermore, the enzyme loading used was the same as in the SSF processes (15 FPU/g cellulose and 15 IU/g cellulose) using Celluclast 1.5 L and NS50010, respectively. Glucose and xylose were analyzed by HPLC as previously described.

Calculations

EH yields were calculated as the ratio of glucose and xylose released at 120 h divided by glucose and xylose content in WIS fraction.

The volumetric ethanol productivity was calculated in all cases based on ethanol concentration at certain time (3 and 48 h) divided by number of hours.

The ethanol yield on total consumed sugar ($Y_{EtOH/CS}$) was calculated as produced ethanol divided by the consumed amount of fermentable sugars.

Results and Discussion

Pretreatment

A slurry with total solids content of 18.5% (w/v) resulted after steam explosion pretreatment of the wheat straw. The WIS content of the slurry after pretreatment was 13.5% (w/v) and as a result the water soluble solids content was 5% (w/v). Total solid recovery value after steam explosion pretreatment (expressed as insoluble solids remaining after pretreatment divided by dry raw material) was about 51% (w/w).

The composition of the pretreated wheat straw (WIS) after pretreatment is stated in Table II. As can be noticed, glucose proportion (57.5%) after pretreatment increased in relation to untreated material (33.2%) due to the solubilization of the hemicellulosic sugars. Taking into account the glucose content in the raw material and glucose recovery after pretreatment, 91% of glucose remained in the solid fraction after steam explosion pretreatment.

The sugar composition as well as the concentrations of toxic compounds from the prehydrolysate are shown in Table II. It can be concluded that most of the hemicellulosic sugars were solubilized during pretreatment and they were mostly present in the prehydrolysate in oligomeric form and therefore a mild acid post-hydrolysis was carried out in order to obtain monomeric sugars.

Acetic acid (5.1 g/L) was released due to solubilization of acetyl groups present in hemicellulose and it was the predominant inhibitory compound in the hydrolysate. Furfural (1.4 g/L) was formed due to the degradation of pentoses (mainly xylose) and 5-hydroxymethylfurfural (HMF) was originated from hexoses. HMF was found in a very low concentration (<0.1 g/L), because hemicellulosic hexoses are in minority in herbaceous biomass, and only a low amount of cellulosic glucose is solubilized during pretreatment (91% recovered in the solid fraction). Furthermore, furfural and HMF could be degraded into other inhibitors such as formic acid (Oliva et al., 2003; Palmqvist and Hahn-Hägerdal, 2000a) and 1.3 g/L formic acid were found in the prehydrolysate. Beside these inhibitors, some phenolic compounds were detected. These compounds were ferulic acid (<0.1 g/L) and coumaric acid (<0.01 g/L), both derived from cinnamic acid. These acids are characteristic of herbaceous materials where they are esterified acting as a bond between lignin and hemicellulose. Moreover, in case of ferulic acid they can exert ether linkages between different plant cell wall components (García-Aparicio et al., 2006; Sun and Cheng, 2002). Vanillin and syringaldehyde are aldehydes compounds derived from lignin. Both were found in concentrations lower than

Table II. Pretreated wheat straw.

WIS		Prehydrolysate			
Component (% dry weight, w/v)		Monosaccharides (g/L)		Inhibitors (g/L)	
Glucose	57.5 ± 0.5	Glucose	5.7 ± 0.3	Furfural	1.4 ± 0.07
Xylose	11.9 ± 0.3	Xylose	24.7 ± 1.2	HMF	0.1 ± 0.01
Lignin	28.6 ± 0.5	Arabinose	1.6 ± 0.08	Acetic acid	5.1 ± 0.2
Ashes	1.8 ± 0.1	Galactose	1.1 ± 0.05	Formic acid	1.3 ± 0.06
		Mannose	0.6 ± 0.03	Coumaric acid	<0.01
				Ferulic acid	<0.1

0.001 g/L (data not shown in Table II). The first one is formed by degradation of guaiacyl propane units of lignin and syringaldehyde is produced by degradation of syringylpropane units of lignin (García-Aparicio et al., 2006), both present in herbaceous biomass.

The mentioned inhibitors compounds have been identified previously by some authors in prehydrolysates from wheat straw (Oliva et al., 2003), corn stover (Öhgren et al., 2005), barley straw (García-Aparicio et al., 2006), or cardoon biomass (Ballesteros et al., 2007). The above-mentioned prehydrolysates came from slurries with similar WIS content to those obtained in this work. Therefore, similar range of inhibitor concentrations was found in all cases.

Fermentation

Fermentation test with a synthetic medium with the same composition of sugars as the prehydrolysate (corresponding to 13.5%, w/v, of WIS) was performed to determine the fermentative behavior of the yeast in a medium with high amount of xylose and low concentration of glucose in a broth without inhibitors. Previous studies pointed out that fermentability of a real prehydrolysate differs from fermentability of a synthetic medium with the same amount of the major hydrolysate inhibitors due to the importance of inhibitors that cannot be identified which indicate synergistic effects between several inhibitory compounds (Oliva et al., 2003; Palmqvist and Hahn-Hägerdal, 2000b).

Figure 1a shows cell growth, sugars consumption, by-products formation and ethanol production with synthetic medium without inhibitors. The maximum ethanol concentration was 6.6 g/L. All the glucose was exhausted during the first 5 h and xylose consumption started after glucose depletion. Xylose was consumed with a linear rate, slightly decreasing by time and xylose was not completely consumed by F12, 7.3 g/L remained as residual xylose in the broth after 50 h of fermentation. Taking into account the amount of consumed sugars and ethanol yields of F12 on glucose and xylose (Sonderegger et al., 2004), 92% of theoretical ethanol concentration was obtained with synthetic media after 50 h of fermentation. Furthermore, 62% of the theoretical ethanol concentration from xylose was obtained. Xylitol formation (7.4 g/L) could explain the suboptimal ethanol yield on xylose. Xylitol started to be produced when the xylose consumption started and it is formed due to a redox imbalance as a consequence of different redox dependence during the initial xylose conversion steps (Hahn-Hägerdal et al., 2007; Sonderegger et al., 2004).

There was a low amount of glucose present in the hydrolysate and only 0.7 g/L of glycerol were formed, reflecting that glucose was mainly used for ethanol production. It is known that during fermentation processes, major amounts of glycerol are produced during glucose consumption as a consequence of biomass production (Zaldivar et al., 2005) and no further glycerol production took place during the stationary phase (no further cell growth). Acetate levels in the broth after fermentation were low (0.2 g/L) and previous studies have also reported low acetate yields resulting from strain F12 fermentations on glucose and xylose (Sonderegger et al., 2004).

Cells ceased to grow and reached stationary phase after 5 h when glucose was completely consumed and as can be observed, most of the ethanol was produced during that stationary phase.

During the prehydrolysate fermentation, the inhibitory compounds affected the fermentation performance of F12 significantly, almost half of the xylose (13.6 g/L) present in the medium was not consumed during the 50 h long fermentation, whereas all the glucose was exhausted. Glucose consumption was slower than in case of the synthetic medium. Due to the low amount of consumed sugars, the resulting ethanol concentration was low, 3.3 g/L.

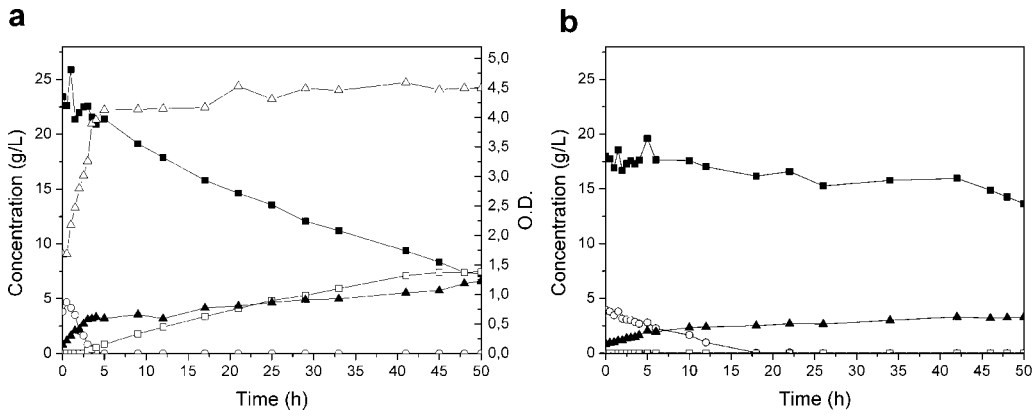

Figure 1. **a**: Ethanol (▲) production; glucose (○) and xylose (■) consumption; xylitol (□) formation and cell growth (Δ) by *S. cerevisiae* F12 during fermentation of synthetic medium having the same sugar concentration as the prehydrolysate (13.5%, w/v, of WIS) without inhibitors added. **b**: Ethanol (▲) production; glucose (○) and xylose (■) consumption; xylitol (□) formation by *S. cerevisiae* F12 during fermentation of the prehydrolysate obtained after filtration of the slurry.

It is worth to mention that inhibitors present in the medium influence the cells significantly as a lag phase in cell growth has been observed (Taherzadeh et al., 2000) and furthermore, the sugar consumption was slowed down and delayed (Fig. 1b). Thus, glucose exhaustion took place at 20 h. Analysis of residual inhibitors after fermentation of prehydrolysate showed that furfural and HMF were fully metabolized. Yeast are capable to reduce these aldehydes to their corresponding alcohols and furfural and HMF were not detected after SSF or fermentation. Some authors have demonstrated that a complete assimilation of aldehydes is necessary before growth and ethanol production is started resulting in a long lag phase (Liu et al., 2004; Taherzadeh et al., 2000). On the other hand, no significant consumption of the other inhibitors (acetic, formic, coumaric, and ferulic acid) was found and consequently they retained their inhibitory action till the end of the process, showing that acids were not metabolized by yeast.

During fermentation of prehydrolysate, no net production of acetate resulted. At the start of the fermentation 3.8 g/L of acetate were present (resulting from the pretreatment; Table II) and the concentration of acetate present in the medium at the end of the fermentation was 3.5 g/L.

It is known that XR and XDH have different co-factors preferences and xylitol is formed in order to maintain a redox balance (Sonderegger et al., 2004; Zaldivar et al., 2005). During fermentation of the prehydrolysate, no xylitol production was detected and only very low amount of glycerol (<0.1 g/L) was found. These results agree with previous studies, in which the presence of external electron acceptors such as furfural reduced xylitol formation (Karhumaa et al., 2007). Consequently, the presence of HMF and furfural can contribute to increase the ethanol yield on xylose. These by-products are reduced by the yeast and act as external electron acceptors during anaerobic fermentation of xylose, which supplies NAD+ for the XDH reaction and directs product formation toward ethanol production at expense of xylitol (Wahlbom and Hahn-Hägerdal, 2002).

Athough inhibitors in the medium make fermentation of prehydrolysate delayed, using a pentose fementing yeast, ethanol yield on consumed sugars was found to be as high as 0.37 g/g.

Simultaneous Saccharification and Fermentation Processes

In order to compare ethanol production in the SSF process with an industrial strain used in the fuel alcohol industry (Red Star) and the recombinant strain (F12), respectively, different SSF tests on slurry and WIS fraction were performed (Figs. 2 and 3). Substrate loading of 10% (w/v) was chosen due to the well-known limiting effect of elevated substrate loadings for SSF, caused by difficulties in stirring the viscous medium. Previous studies have pointed out the feasibility of using 10% (w/v) substrate concentration in SSF processes (Ballesteros et al., 2004; Linde et al., 2007; Öhgren et al., 2006). All the SSF tests were preceded by a prehydrolysis step (6–8 h at 50°C) allowing a period of time where enzymes are acting before the yeast inoculation. Furthermore, this step made the slurry or WIS mixture more fluid and easier to handle.

The highest ethanol concentration in the WIS SSF tests (15.2 g/L) was obtained using strain F12 as fermenting yeast, resulting in 25% more ethanol than produced by Red Star (Fig. 2a and b). Glucose was entirely consumed by both yeasts, but F12 consumed it faster than Red Star and consequently presented higher volumetric ethanol productivity at 3 h (Table III).

As has been pointed out by some authors (García-Aparicio et al., 2006; Sørensen et al., 2005), some xylose is also released from the hemicellulosic fraction by the action of Celluclast 1.5 L mixture in an SSF process and xylose released from the WIS fraction was almost entirely

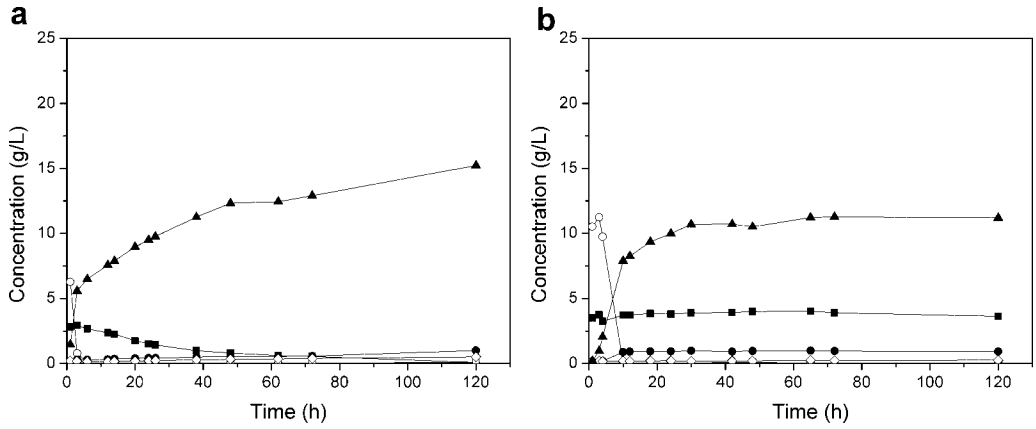

Figure 2. **a**: Time course for SSF process with 10% (w/v) of WIS substrate loading with *S. cerevisiae* F12. Symbols used: (▲) ethanol, (○) glucose, (■) xylose, (□) xylitol, (●) glycerol, and (◇) acetate. **b**: Time course for SSF process with 10% (w/v) of WIS substrate loading with *S. cerevisiae* Red Star. Symbols used: (▲) ethanol, (○) glucose, (■) xylose, (□) xylitol, (●) glycerol, and (◇) acetate.

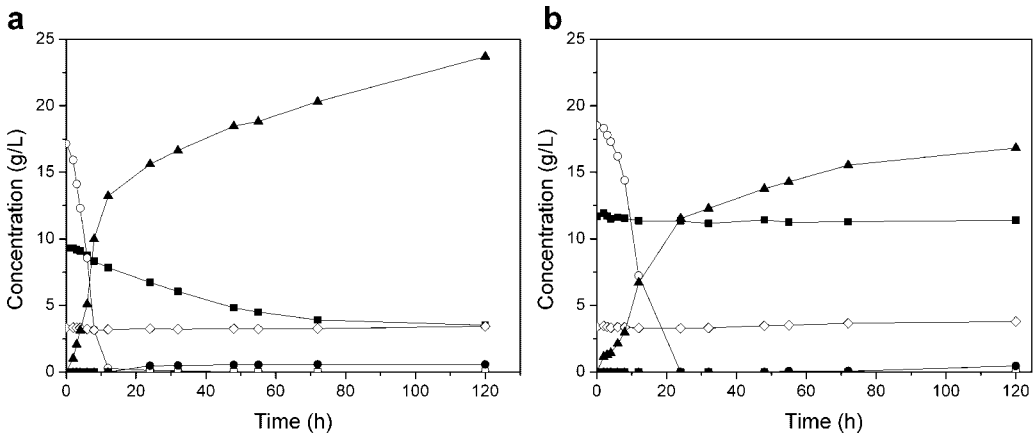

Figure 3. **a**: Time course for SSF process with slurry at 10% (w/v) of WIS substrate loading with *S. cerevisiae* F12. Symbols used: (▲) ethanol, (○) glucose, (■) xylose, (□) xylitol, (●) glycerol, and (◇) acetate. **b**: Time course for SSF process with slurry at 10% (w/v) of WIS substrate loading with *S. cerevisiae* Red Star. Symbols used: (▲) ethanol, (○) glucose, (■) xylose, (□) xylitol, (●) glycerol, and (◇) acetate.

consumed by F12 during SSF. Because of that, very low amount of residual xylose (<0.1 g/L) was detected in the F12-SSF broth compared with residual xylose in case of Red Star (3.6 g/L).

Red Star reached its maximum ethanol concentration (11.2 g/L) at 40 h when the EH was practically ceased. Nevertheless, owing to its xylose fermenting ability, the F12 fermentation reached higher ethanol concentration after xylose depletion (120 h).

Figure 3a and b shows time courses for SSF with slurry using F12 and Red Star strains, respectively. Ethanol production with strain F12 reached 23.7 g/L after 120 h SSF process while only 16.8 g/L were obtained by Red Star. Furthermore, F12 consumed glucose faster than Red Star, and it was clearly reflected in higher volumetric ethanol productivities at 3 h (Table III).

Ethanol concentrations resulting from the whole slurry fermentations were in all cases higher than those obtained in WIS fermentations. It proved the fact that using the whole slurry, the amount of potential fermentable sugars was increased, because of the sugar present in the liquid fraction. In the slurry fermentations, in addition to glucose released from cellulose, solubilized glucose (4.2 g/L) was present in the liquid fraction, due to the release during pretreatment. As a result, Red Star also yielded higher ethanol concentrations from slurry than from WIS.

In the same way as in SSF process with WIS, most of the xylose was consumed by F12 and all the xylose available was detected as residual monosaccharide in case of Red Star. Despite these differences on xylose fermentation, both strains showed similar ethanol yields on consumed sugars (0.43 and 0.44 g/g, respectively). It is worth to comment that very low amount of glycerol was detected in all cases (both strains and both SSF media) and it is remarkable the fact that when furfural was present in the media (slurry and prehydrolysate) less glycerol was detected in the fermentation broth after SSF or separate fermentation (Table III). It is known that glycerol is formed in order to maintain the redox

Table III. Ethanol production and productivity, residual monosaccharides and by-products formation during fermentation and SSF performance with two *S. cerevisiae* strains.

	Strain					
	F12				Red Star	
Substrate	Prehydrolysate	Synthetic prehydrolysate	SSF WIS	SSF slurry	SSF WIS	SSF slurry
Residual monosaccharides						
Glucose (g/L)	—	—	—	—	—	—
Xylose (g/L)	13.6	7.3	<0.1	3.5	3.6	11.4
$Ethanol_{120\ h}$ (g/L)	3.3	6.6	15.2	23.7	11.2	16.8
$Glycerol_{120\ h}$ (g/L)	<0.1	0.7	1.0	0.6	0.9	0.5
$Xylitol_{120\ h}$ (g/L)	—	7.4	0.1	<0.1	<0.1	<0.1
$Acetate_{120\ h}$ (g/L)	3.5	0.2	0.5	3.4	0.3	3.8
Volumetric ethanol productivity $(g/L\,h)_{3\ h}$	0.5	1.0	1.9	0.7	0.3	0.4
Volumetric ethanol productivity $(g/L\,h)_{48\ h}$	<0.1	0.1	0.3	0.4	0.2	0.3
$Y_{E/CS}$ (g/g)	0.38	0.29	0.50	0.43	0.47	0.44

imbalance during anaerobic growth of yeast, but when furfural is present in the broth, furfural reduction to furfuryl alcohol is preferred to glycerol production as redox balance product. Thus, furfural reduction caused less glycerol production and more glucose to be available for ethanol production (Palmqvist et al., 1999). Furfural and HMF were completely metabolized by both strains in the SSF processes, but no differences were detected in the concentration of the other inhibitors (data not shown). Also in this case, the acetate present in slurry SSF could come from the acetic acid present in the hydrolysate after pretreatment because no netto formation was observed neither with F12 (3.4 g/L) nor Red Star (3.8 g/L). It is also worthy to state that no xylitol was found in any SSF process. In the same way that occurred in the fermentation of the prehydrolysate, the HMF and furfural act as external electron acceptors during anaerobic fermentation of xylose and diminish xylitol formation (Wahlbom and Hahn-Hägerdal, 2002).

Enzymatic Hydrolysis

The main objective of carrying out the EH was to estimate the amount of sugars available for the yeast during the SSF process. Consequently, the hydrolysis was performed at the same conditions as used in the SSF. Figure 4a and b represents EH processes with 10% of WIS (w/v) and with the whole slurry, respectively.

A prehydrolysis for 6–8 h at 50°C was carried out in all cases and this first stage of hydrolysis allows some monosaccharides to be released in the medium and these will be available when yeast is inoculated to the SSF process. Sugars released during the prehydrolysis, when the WIS (10%, w/v) was used as a substrate, were 4.4 g/L of xylose and 12.8 g/L of glucose. On the other hand, 13.6 g/L of xylose and 20.1 g/L of glucose were the initial amount of sugars after prehydrolysis using the whole slurry (10%, w/v, of WIS).

Higher amounts of sugars are suspected to be found in the slurry after this prehydrolysis due to the sugars that are solubilized in the liquid fraction after pretreatment. Nevertheless, the glucose available in the slurry after prehydrolysis (20.1 g/L) is 15% higher than the expected level due to the solubilized glucose (4.2 g/L) during pretreatment.

Although, some researchers have pointed out lower saccharificaton yields with cellulases using the whole slurry due to the presence of toxic compounds (García-Aparicio et al., 2006; Panagiotou and Olsson, 2006), higher saccharification yields were obtained in this work using the whole slurry. These higher yields could be due to several factors that affect cellulase digestibility, being drying after pretreatment one of them. After filtration, WIS fraction was dried completely in a heater and some researchers have pointed out that this kind of performance could alter slightly cellulose accessibility (Esteghlalian et al., 2001) and was reflected in lower hydrolysis yields.

The final sugar concentrations after 120 h of EH of the WIS were 23.9 g/L of glucose and 6.6 g/L of xylose and in case of the whole slurry these amounts were 37.5 and 20.7 g/L, respectively. These concentrations were taken into account with the purpose to calculate ethanol yields on consumed sugars in the SSF. They could be underestimated, because it is known that in an SSF process the amount of released sugars by enzyme is higher than in an EH, as in an SSF process glucose released is converted quickly to ethanol by yeast and this continuous removal of sugars minimizes the end-product inhibition on enzyme activity.

EH yield on glucose was 41.7% in case of WIS and 57.9% in case of slurry. As have been mentioned before, celullases mixture Celluclast 1.5 L contains mainly cellulase activity, but as has been observed its utilization also results in high

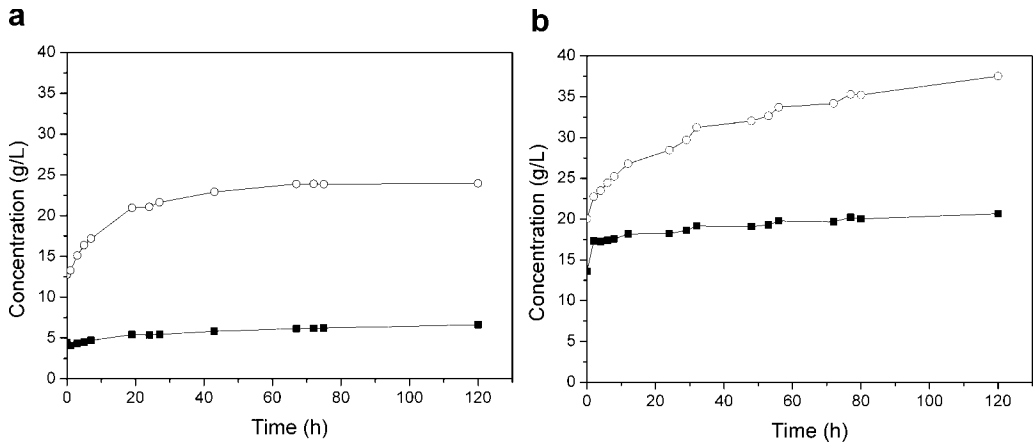

Figure 4. **a**: Xylose (■) and glucose (○) released in EH process with 10% WIS (w/v) as substrate. Cellulase mixture consisted of Celluclast 1.5 L (15 FPU/g cellulose) and NS50010 (15 IU/g cellulose). **b**: Xylose (■) and glucose (○) released in EH process with the whole slurry as subtrate. Cellulase mixture consisted of Celluclast 1.5 L (15 FPU/g cellulose) and NS50010 (15 IU/g cellulose).

yields on xylose (García-Aparicio et al., 2006; Sørensen et al., 2005). In case of slurry, xylose comes from the solid fraction as well as prehydrolysate (sugars from liquid fraction are mainly oligomers but can easily be hydrolysed). EH yield of xylose in the case of slurry without considering xylose from liquid fraction was found to be 20.3%.

The major part of the EH were conducted at 32°C, while it is known that the optimal temperature for cellulases is about 50°C. EH temperature was fixed at 32°C, the temperature at which all SSF fermentations were carried out. Moreover, previous studies have shown that reaction rates diminish gradually throughout EH processes and enzyme stability dropped at temperatures above 38°C when EH were carried out for more than 144 h (Tengborg et al., 2001), however, it is not expected that the short prehydrolysis at 50°C have influenced the enzyme stability.

Table IV shows theoretical ethanol concentrations in SHF processes and those obtained in SSF processes. Taking into account the released sugars during the EH and assuming previously mentioned yields for ethanol production on glucose and xylose by F12 (0.46 and 0.26 g/g, respectively), lower ethanol concentrations would be obtained in SHF processes. These results agree with those obtained by some authors working with wheat straw (Alfani et al., 2000), wheat hemicellulose substrates (Olsson et al., 2006), and corn stover (Öhgren et al., 2007). All mentioned researchers have demonstrated higher ethanol productivities and higher ethanol concentrations using SSF processes instead of SHF.

Furthermore, SSF must be carried out at high substrate loading in order to obtain higher ethanol concentration in the fermentation broth and thereby decreasing the energy demand in the distillation step. Moreover, an elevated substrate loading may lead to difficulties in stirring or mixing. In this context, some authors have referred the fact that saccharification step at high substrate levels improved the enzyme performance (Jørgensen et al., 2007).

Conclusions

Bioethanol production from lignocellulosic biomass requires a fermenting microorganism that converts all types of monosaccharides present in the raw material with high yields in presence of fermentation inhibitors formed during pretreatment and hydrolysis.

In this work the highest ethanol concentration (23.7 g/L) was obtained with the modified *S. cerevisiae* strain F12 capable to ferment xylose and the whole slurry as substrate, showing the potential of pentose fermentation on real media in improving lignocellulosic ethanol production. Furthermore, it is remarkable that no xylitol was formed during fermentation of the lignocellulosic hydrolysate or slurry SSF with strain F12. Xylitol formation occurs at the expense of ethanol production, due to the need of redox balancing as the two first steps of xylose conversion use different redox factors. However, in the lignocellulosic substrates used in this study, redox reactions took place as part of converting the inhibitory compounds and this allows a balancing of the redox metabolism in the cells. These inhibitors direct the reaction toward ethanol production and diminish xylitol formation.

It was estimated that *S. cerevisiae* F12, produced 12.7 and 22.6 g/L of ethanol from WIS and slurry, respectively, using a SHF process configuration. Hence, comparing these concentrations with those obtained using SSF process configuration, 15.2 and 23.7 g/L, respectively, were produced. Consequently, it can be concluded that bioethanol production was improved carrying out the SSF processes.

Moreover, it is preferable to include the liquid fraction resulting after pretreatment in the fermentation broth because it increases the amount of fermentable sugars, that could be reflected by higher final ethanol concentrations. In addition, handling of the pretreated material is easier and the capital cost of filtration and washing steps can be excluded.

Table IV. Theoretical ethanol production in an SHF and real ethanol production in an SSF process with strains F12 and Red Star.

	Strain F12		Strain Red Star	
	WIS	Slurry	WIS	Slurry
Available glucose (g/L)	23.9	37.5	23.9	37.5
Available xylose (g/L)	6.6	20.7	—	—
Ethanol concentration in SSF (g/L)	15.2	23.7	11.2	16.8
Theoretical ethanol concentration in SHF (g/L)[a]	12.7	22.6	11.0	17.2

[a]Calculated as the amount of sugars released during the enzymatic hydrolysis and assuming ethanol yields on glucose and xylose by F12 [0.46 and 0.26 g/g, respectively (Sonderegger et al., 2004)].

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