Simultaneous saccharification and fermentation by co-cultures of *Fusarium oxysporum* and *Saccharomyces cerevisiae* enhances ethanol production from liquefied wheat straw at high solid content

Thomas Paschos[a], Charilaos Xiros[b], Paul Christakopoulos[c],*

[a] Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Str., Zografou Campus, Athens 5780, Greece
[b] Division of Agriculture, School of Agriculture, Forestry and Food Science, Bern University of Applied Sciences, Längasse 85, 3052 Zollikofen, Switzerland
[c] Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, Luleå SE-971 87, Sweden

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ABSTRACT

A co-fermentation process involving *Saccharomyces cerevisiae* and *Fusarium oxysporum* was studied, using hydrothermally pretreated wheat straw as substrate. In the first step of the study, we examined lique-faction of the material in a free-fall reactor. Both the enzyme loading and the dry matter content affected severely the liquefaction efficiency. In the second step (simultaneous saccharification and fermentation (SSF) experiments), we found that the enzymatic system of *F. oxysporum* contributed significantly to sub-strate hydrolysis, while its metabolic system played a secondary role in fermentation. SSF in the presence of *F. oxysporum* cells and enzymes gave $62\,g\,L^{-1}$ ethanol. In the third step of the study, a semi-consolidated bioprocess was designed in which *F. oxysporum* culture (submerged or solid-state) was added at the SSF stage along with *S. cerevisiae*. The addition of solid *F. oxysporum* culture increased ethanol production by 19%, leading to a final ethanol concentration of $58\,g\,L^{-1}$. The present study proposes a semi-consolidated process combining two microorganisms for the fermentation at high solids concentration of a liquefied material using an in house free fall mixing reactor. The semi-consolidated process proposed not only increased the ethanol yields significantly, but could also lead to lower overall cost of the process by incorporating in-situ enzyme production.

1. Introduction

For some time ethanol production from lignocellulosic materials has shown promising industrial potential, but it is only now that commercial plants to produce second-generation ethanol are finally under construction. Until recently, the high operational cost of second-generation ethanol was one of the main constraints (Janssen et al., 2013). High-solids and very-high-solids bioethanol production processes gave the possibility of commercializing cellulosic ethanol, mainly because they could reach final ethanol concentrations higher than 4% w/w without achieving very high conversion yields. The high ethanol content reduced the distillation cost and brought second-generation ethanol nearer to commercial reality (Kawa-Rygielska and Pietrzak, 2014).

Until recently, the two most common strategies for ethanol production at high dry matter content (DM) were simultaneous saccharification and fermenatation (SSF) and separate hydrolysis and fermentation (SHF) (Tomas-Pejo et al., 2008). Both techniques have shown high efficiency and productivity, but the feasibility of each one depends mainly on the enzymatic system that is used (Cannella and Jorgensen, 2013). Older-generation commercial enzymes (Celluclast 1.5L — Novozyme 188) were found to work better in SSF, in contrast to the latest generation of enzymes (Cellic Ctec2), which have been shown to work equally well in SSF and SHF processes. This difference is probably due to different product inhibition of the enzymes. New-generation enzymes show less inhibition by the hydrolysis products, mainly glucose and cellobiose. This is why older-generation enzymes have been shown to work better in SSF processes, as the hydrolysis products do not accumulate but they are simultaneously fermented by the microorganisms (Cannella and Jorgensen, 2013; Viikari et al., 2012).

Working under high-gravity conditions (high DM) is associated with technical problems and challenges (Koppram et al., 2014). It

has been shown that lignocellulosic materials at concentrations above 20% DM not only give high viscosity, which makes mixing less efficient, but they also require very high power consumption when the process is performed in stirred-tank reactors (Viikari et al., 2012). This led to the need to create new mixing reactors that would overcome the problem (Jorgensen et al., 2007). Free-fall mixing reactors can reduce the power consumption and create better mixing conditions. They are based on gravitational mixing. The literature on this subject indicated that using this kind of reactor for liquefaction of lignocellulosic materials leads to sufficient hydrolysis of the substrate, reducing the viscosity of the slurry and thus improving the fermentation yield and productivity (Jorgensen et al., 2007). The process including a liquefaction step before an SSF process has been termed prehydrolysis, simultaneous saccharification, and fermentation (P-SSF) (Xiros and Olsson, 2014).

The ethanol yield can be increased by fermentation of hexoses and pentoses present in the hydrolysis broth (Erdei et al., 2013). Although wild-type Saccharomyses cerevisiae, commonly known as baker's yeast, is unable to ferment xylose into ethanol, it is the predominant microorganism used in large-scale processes due to the high ethanol yields and high productivities that can be achieved. Although solutions to the problem of pentose sugar fermentation have been proposed using different approaches (Demeke et al., 2013), there are still serious constraints regarding xylose fermentation. Co-cultivation of a pentose-fermenting microorganism with S. cerevisiae is an alternative approach to solving this issue (Chen, 2011).

Fusarium oxysporum is a filamentous fungus that is capable of producing ethanol both from pentose and hexose sugars (Panagiotou et al., 2005). Moreover, it is able to produce many cellulases and hemicellulases, making it capable of growing on many lignocellulosic substrates under submerged or solid-state conditions (Xiros et al., 2009, 2008a). This fungus is capable of degrading and fermenting a wide variety of different substrates under anaerobic or limited oxygen conditions. Moreover, not only its fermentative metabolism, but also its enzymatic system shows adequate tolerance to inhibitory compounds formed during the pretreatment of lignocellulosic materials (Xiros et al., 2011). The secretome of F. oxysporum was shown to be capable of hydrolyzing cellulose in the presence of high ethanol concentrations (Paschos et al., 2015). However, the low rate of ethanol production and in some cases the formation of significant amounts of acetic acid as a by-product, have been considered as obstacles for its industrial exploitation. Earlier, the ability of F. oxysporum and a recombinant S. cerevisiae strain to efficiently cooperate in degradation and fermentation of wheat straw polysaccharides to produce ethanol has been studied (Panagiotou et al., 2011). The potential of using pre-treated wheat straw (PWS) as raw material at high solids content for lignocellulolytic enzyme production and fuel ethanol production by fermentation with F. oxysporum and S. cerevisiae F12 has already been demonstrated. The levels of enzyme production obtained and the absence of free oligosaccharides in the fermentation medium throughout these experiments indicated that the lignocellulolytic enzyme system of F. oxysporum is very efficient and well-balanced (Panagiotou et al., 2011).

The pre-treatment of the raw material plays an important role for its conversion to ethanol. This step allows the depolymerisation of the lignocellulose, making it accessible to enzymes (Thomsen et al., 2006). But after the pre-treatment the material partly maintains its structure and the presence of long entangling particles along with high dry matter content poses challenges for the enzymatic hydrolysis of the material (Jorgensen et al., 2007).

In the present paper, we propose a novel process design for the efficient conversion of PWS straw to ethanol by exploiting the properties of F. oxysporum. In comparison with stirred reactors, traditionally used in biotechnological applications, a gravitational mixing reactor enables the efficient mixing with small power inputs at high dry matters content. Due to its mixing principle it can easily be scaled up (Larsen et al., 2008). These kinds of reactors have been already used for enzymatic liquefaction of lignocellulose replacing conventional stirred reactors (Jorgensen et al., 2007; Larsen et al., 2008). Therefore, a free-fall mixing reactor was manufactured for the liquefaction of hydrothermally treated wheat straw using the commercial enzyme mixture Celluclast 1.5 L and Novozyme 188. Moreover, SSF experiments at 26% DM content were performed in order to explore the contribution not only of the enzymatic system but also of the metabolic system of F. oxysporum, to cooperate with S. cerevisiae in enhancing ethanol production from liquefied wheat straw. Finally, F. oxysporum grown in submerged or solid-state culture was added to the liquefied PWS along with dry baker's yeast. We evaluated both submerged and solid-state culture techniques for the enzyme production stage by F. oxysporum. The enzyme production was coupled to the P-SSF process and the contributions of the metabolic and enzymatic systems of the fungus to the final ethanol concentration were investigated. The study demonstrates the possibility of significantly reducing the amount of commercial enzymes used while at the same time increasing the final concentration and yield of ethanol.

2. Materials and methods

2.1. Microorganisms

F. oxysporum F3 isolated from cumin, as described by Christakopoulos et al. (1989), was used in the present study. The fungus was grown on potato-dextrose-agar (PDA) slants for 5 days at 30 °C. The slants were maintained as a stock culture at 4 °C. Commercial dry baker's yeast (Yiotis, Athens, Greece) was used for the PWS fermentations. Yeast was used in its commercial form as dried pellets.

2.2. Carbon sources and chemicals

Pretreated wheat straw (PWS) (Triticum aestivum L.) was used as raw material for ethanol production. The pretreatment of the straw was performed in the Inbicon pilot plant in Skærbæk, Denmark. The composition of the raw material was 30.4% cellulose, 21.3% hemicellulose, and 19.4% lignin. The residence time set point in the reactor was 12 min and the reactor temperature was maintained at 190 °C by injection of steam (Thomsen et al., 2006). The severity factor (SF) was determined to be 3.73 according to the following equation (Garrote et al., 1999):

$$SF = \log(R) = \log\left(t \times e^{\frac{T-100}{14.75}}\right)$$

where t is pretreatment time and T is pretreatment temperature. The heating and cooling time were not considered for the calculation of SF. By the design of the whole pre-treatment process the heating and cooling times are eliminated. The pretreatment resulted in approximately 63% recovery of solids (g of fiber fraction per 100 g of raw material) at the fiber fraction while the recovery of glucan can be up to 100% (Larsen et al., 2008).

Polysaccharides content of PWS in% was 50.2 ± 1.1 cellulose and 3.8 ± 0.4 hemicellulose. There were 0.7 (± 0.1 g free sugars per 100 g dry material. Structural carbohydrate content (cellulose and hemicellulose) was determined following the NREL protocol (Sluiter et al., 2005).

Brewer's grain (BG) and corn cobs (CC) were supplied by Athens Brewery S.A. and the Agricultural University of Athens, respectively. BG and CC were used as substrate in F. oxysporum enzyme production and aerobic solid state cultures. The compositions of BG

and CCs were described in Xiros et al. (2008b) and Katapodis and Christakopoulos (2007), respectively.

All chemicals and reagents were provided by Sigma–Aldrich (USA).

The commercial enzymes Celluclast 1.5 L and Novozyme 188 were provided by Novozymes (Denmark). The commercial enzymes were used in a ratio of 5:1 (v/v).

2.3. Inoculum

For production of inoculum, spores were extracted from the stock slants using 5 mL of sterile distilled water. The fungus was then cultivated in 250-mL Erlenmeyer flasks containing 100 mL of the following mineral medium (in g L^{-1}): 1.00 KH$_2$PO$_4$, 0.30 CaCl$_2$·2H$_2$O, 0.30 MgSO$_4$·7H$_2$O, 10.00 (NH$_4$)$_2$HPO$_4$, 6.94 NaH$_2$PO$_4$·2H$_2$O, and 9.52 Na$_2$HPO$_4$·2H$_2$O, supplemented with either 40 g L^{-1} BG–CC mixture (2:1) or 20 g L^{-1} glucose, depending on the experimental procedure. The pH was adjusted to 6.0. For mycelium production, the flasks were incubated at 30 °C for 2 days in an orbital shaker at 200 rpm (Zhicheng ZHWY-211C, China).

2.4. Aerobic submerged culture

Aerobic submerged cultures were prepared in 250 mL Erlenmeyer flasks containing 2 g of glucose or 4 g of BG–CC mixture (2:1) and 100 mL of the mineral medium described above (pH 6.0). Medium and substrate were sterilized separately at 109 °C for 40 min. The culture medium was inoculated with 10 mL of 48 h-old inoculum (prepared as described above). The flasks were incubated at 30 °C for 5 days under fully aerobic conditions.

2.5. Aerobic solid-state culture

Solid-state culture was carried out in 3 L Erlenmeyer flasks containing 700 g of BG–CC mixture and the mineral medium described above at 33% DM concentration. Medium and substrate were sterilized separately at 109 °C for 40 min. The cultures were inoculated with 48 h-old inoculum (prepared as described above). The flasks were incubated at 30 °C for 7 days under fully aerobic conditions.

2.6. Cell mass production

Fungal cells for SSF experiments were produced in 3 L Erlenmeyer flasks. For *F. oxysporum* mycelium production, each flask (sterilized at 110 °C for 40 min) containing 1000 mL of the appropriate mineral medium and 20 g glucose and xylose (at a 1:1 ratio (w/w)) as carbon source was incubated at 30 °C for 3 days in an orbital shaker (200 rpm). At the end of cell growth, the culture was centrifuged (10,000 rpm at 4 °C for 10 min) and the precipitated cells were collected under aseptic conditions and added to the SSF stage. At different time intervals, aliquots were withdrawn for cell mass estimation.

2.7. Cell mass estimation

Samples of the aerobic cultures where glucose was used as carbon source were filtered using 0.2 μm pore size filter paper (Millipore, USA). The cell mass content was measured by weighing dried samples.

In aerobic cultures where BG and CC were used as carbon sources, the cell mass content was measured by the colorimetric method of Scotti et al. (2001), based on estimation of glucosamine in the fungal cell wall.

2.8. Enzyme production

Production of *F. oxysporum* crude enzyme extract was carried out in 3 L Erlenmeyer flasks containing 40 g of carbon source (BG–CC mixture in a 2:1 ratio) and 1 L of mineral medium. Before sterilization, the initial pH of the medium was adjusted to 6.0. The medium was sterilized at 121 °C for 20 min and was inoculated with 100 mL of 72 h-old inoculum (prepared as described above). The flasks were incubated at 30 °C for 5 days with shaking at 200 rpm. At the end of the enzyme production stage, the culture was centrifuged (14,000 rpm at 4 °C for 40 min) and the clarified supernatant was concentrated using ultrafiltration membranes (10,000 kDa; Millipore). There was no addition of buffer during the ultrafiltration process.

2.9. Enzyme assays

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of product per min, at assay temperature.

Filter paper activity (FPA) was determined as described by Wood and Bhat (1988). In a capped 2 mL Eppendorf tube, 1 mL of buffer solution, 0.5 mL of enzyme (with the appropriate dilution so as to release less than 1 mg product per mL) and a piece of filter paper (Whatman no. 1, 1 × 6 cm, approximately 50 mg) were added. The mixture was incubated at 50 °C for 1 h with shaking at 1000 rpm.

The β-1,4-D-endoxylanase activity was determined by incubating the enzyme with 1% birchwood xylan for 10 min at 50 °C (Bailey et al., 1992).

The reducing sugars released were measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959).

2.10. Liquefaction of hydrothermaly treated wheat straw

For the liquefaction and saccharification of PWS, an in-house reactor (Fig. 1a) was manufactured as described by Jorgensen et al. (2007). The reactor consisted of a cylindrical drum 25 cm wide and 60 cm in diameter, with a rotating shaft for mixing the material. A 0.55 kW motor was used to rotate the shaft. The rotation speed could be controlled from 0 to 20 rpm, and the motor could be programmed to shift its rotation clockwise and anticlockwise. An external jacket filled with oil was used for temperature control up to 90 °C. The experiments were executed at 50 °C, at 20%, 26%, and 30% DM of PWS supplemented with buffer (phosphate–citrate, 50 mM, pH 5.5) and an enzyme mixture of 3, 5, or 7 FPU g^{-1} DM, consisting of Celluclast 1.5 L and Novozym 188 (both from Novozymes A/S, Bagsværd, Denmark) at a ratio of 5:1 (v/v). The activity of this mixture was measured 81 FPU mL^{-1} by the filter paper assay. The mixing speed was 7 rpm, shifting clockwise and anticlockwise twice a minute. The duration of the liquefaction was 6 h or 8 h. At the end of the process, samples were withdrawn for viscosity measurement and sugar determination. For estimation of the sugar concentration of the liquefied material, the sugars present in the PWS prior to liquefaction were subtracted.

2.11. Fermentations

All experiments were carried out in submerged cultures at 30 °C, at pH 5.5 in 250 mL Erlenmeyer flasks provided with needle-pierced rubber stoppers, which ensured micro-aerobic conditions and allowed the release of produced carbon dioxide. After each sampling, nitrogen (0.1 vvm) was flushed in the cultures for 10 min to assure limited oxygen conditions. At different time intervals, aliquots were aseptically withdrawn and used for estimation of ethanol production. Table 1, presents a conclusive list of the con-
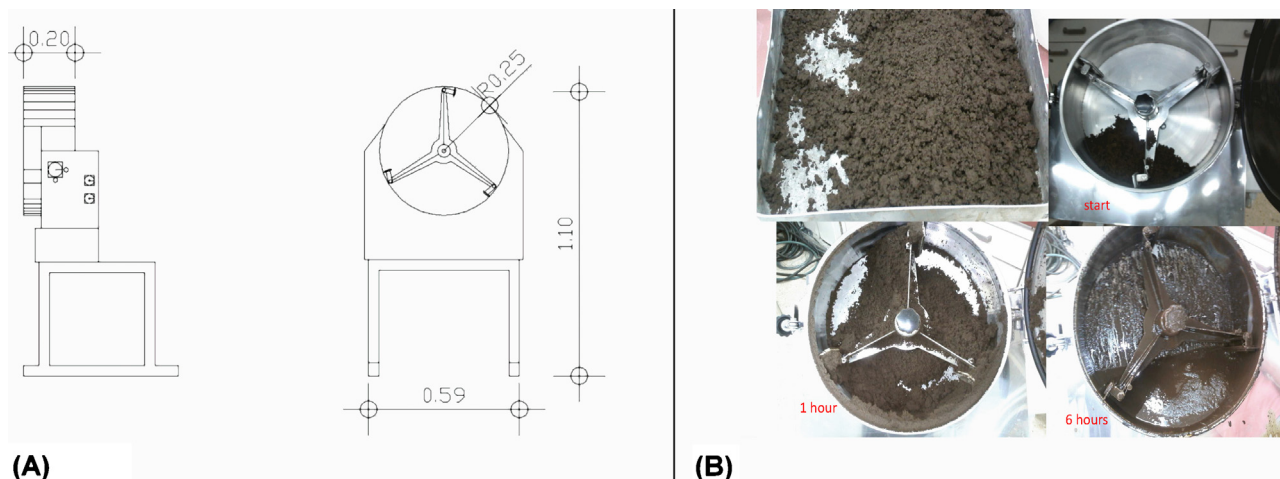
Fig. 1. Free-fall mixing reactor manufactured for the PWS liquefaction. (A) A CAD (computer-aided design) sketch of the liquefaction reactor. (B) The progress of the liquefaction process. The liquefaction of PWS at 26% DM, with enzyme loading of 5 FPU g^{-1} DM, is presented. Photographs were taken at the beginning, after 1 h, and at the end of the process (after 6 h).

Table 1
Fermentation experiments of *F. oxysporum* addition in the SSF process.

Experiment	Liquefaction	Additions in fermentation			
		S. cerevisiae	*F.oxysporum* enzymes (concentrated) up to 6 FPU g^{-1} DM	*F. oxysporum* cell mass (centrifuged) up to 70 mg g^{-1} DM	*F. oxysporum* submerged or solid culture (without any treatment)
Fermentations with *S. cerevisiae* (as control experiments)	6 h, 50 °C, 5 FPU g^{-1} DM commercial enzymes	Yes	No	No	No
Fermentations adding *F. oxysporum* enzymes	6 h, 50 °C, 5 FPU g^{-1} DM commercial enzymes	Yes	Yes	No	No
Fermentations adding *F. oxysporum* cell mass	6 h, 50 °C, 5 FPU g^{-1} DM commercial enzymes	Yes	No	Yes	No
Fermentations adding *F. oxysporum* enzymes and cell mass	6 h, 50 °C, 5 FPU g^{-1} DM commercial enzymes	Yes	Yes	Yes	No
Fermentations adding *F. oxysporum* cultures	6 h, 50 °C, 7 FPU g^{-1} DM commercial enzymes	Yes	No	No	Yes (includes both enzymes and cell mass)

ducted experiments concerning *F. oxysporum* addition in the SSF process.

2.11.1. Fermentations of liquefied wheat straw with S. cerevisiae

The material, liquefied for 6 h at 26% DM, was used for ethanol production. *S. cerevisiae* (dry baker's yeast) at 5 mg g^{-1} DM was used as the fermentative microorganism. These fermentations were considered to be the control experiments.

2.11.2. Addition of F. oxysporum enzymes in fermentation of liquefied wheat straw

The material that was liquefied, for 6 h at 50 °C, with 5 FPU g^{-1} DM of commercial enzymes was used as carbon source for the fermentations at 26% DM. *F. oxysporum* enzymes at up to 5 FPU g^{-1} DM were added at the start of fermentation, along with 5 mg g^{-1} DM dry baker's yeast. In these experiments, no *F. oxysporum* cell mass was added.

2.11.3. Addition of F. oxysporum cell mass in fermentation of liquefied wheat straw

The material that was liquefied for 6 h at 50 °C with 5 FPU g^{-1} DM of commercial enzymes was used as carbon source for the fermentations at 26% DM. *F. oxysporum* cell mass (previously grown on glucose for 4 days under submerged culture) at up to 46 mg g^{-1} DM was added at the start of fermentation along with 5 mg g^{-1} DM dry baker's yeast. There was no enzyme addition at this stage.

2.11.4. Evaluation of the enzymatic and microbial potential of F. oxysporum during the co-fermentation process

The material that was liquefied for 6 h at 50 °C with 5 FPU g^{-1} DM of commercial enzymes was used as carbon source for the fermentations at 26% DM. *F. oxysporum* enzymes from 0 to 6 FPU g^{-1} DM and centrifuged *F. oxysporum* cells at from 0 to 70 mg g^{-1} DM were added at the start of fermentation along with dry baker's yeast (5 mg g^{-1} DM).

2.11.5. Addition of submerged and solid-state culture of F. oxysporum in fermentation of liquefied wheat straw

The material that was liquefied, for 6 h at 50 °C, with 7 FPU g^{-1} DM of commercial enzymes was used as carbon source for the fermentations at 20, 26, and 30% DM. Before the fermentation stage, the liquefied material was condensed slightly at 50 °C under vacuum so that the addition of *F. oxysporum* cultures would not affect the PWS concentration. Submerged or solid-state cultures of *F. oxysporum* were added at the start of fermentation along with 5 mg g^{-1} DM dry baker's yeast.

2.12. Analytical methods

The concentration of reducing sugars was determined according to the dinitro 3,5-salicilic acid (DNS) method (Miller, 1959), and glucose was measured using D-glucose GOD/PAP Assay Kit (Biosis, Greece). The measurement is based on glucose oxidase/peroxidase assay. Ethanol was analyzed using an HPLC system (Szimadju) equipped with an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm, particle size 9 μm) using a refractive index (RI) detector. The mobile phase was 5 mM H$_2$SO$_4$ in HPLC-grade water at a flow rate of 0.6 mL/min, the column temperature was 40 °C, the injection volume was 50 μl, and the total runtime was 30 min All samples were filtered (0.2 μm; Macherey–Nagel) before the analysis.

2.13. Viscosity measurements

The viscosity of liquefied material was measured using a computer-controlled rotary viscometer (RHEOTEST RC1; Medingen GmbH, Radeburg, Germany) consisting of an electronic unit with standard DIN coaxial cylinder measuring systems of different viscosity ranges, a temperature-measuring sensor (Pt100), a thermostat device (FTK-CC; −10–90 °C), and RHEO 2000 software. The measuring system used was a double-gap cylinder MS-CC25 DIN/FTK. Viscosity was measured and recorded at a shear rate of 44.45 s^{-1}. All measurements were conducted in duplicate at room temperature (25 °C). Viscosity measurements are presented in cP (1 cP = 1000 Pa.s).

3. Results and discussion

3.1. Optimization of liquefaction

The liquefaction step is the first step of the P-SSF process. The main goal of the liquefaction step was to reduce the viscosity of the material in order to increase the mixing efficiency, and thus to make the material accessible to the enzymes and the microorganisms during the SSF stage. During this step, the paste-like wheat straw fibers are transformed into a pumpable material containing significant amounts of soluble monosaccharides and oligosaccharides. The custom-made reactor (designed in-house), based on the principle of free-fall mixing (Fig. 1a), allowed adequate mixing of the slurry, and thus reduced the heat and mass transfer limitations. The effects of initial DM content, enzyme loading, and liquefaction time were studied with respect to the saccharification yield and to the decrease in viscosity.

PWS concentrations of 20%, 26%, and 30% DM were tested. During this stage, the viscosity of the material was reduced due to the action of lignocellulolytic enzymes, which partially hydrolyzed the wheat straw fibers. As shown in Table 2, reduction of viscosity was evident with all the different enzyme loadings tested, and also with the different liquefaction times. The initial DM content played an important role in determining liquefaction efficiency. The higher the DM content, the lower the decrease in viscosity

that was observed. This was probably due to reduced mixing efficiency, which hindered the action of hydrolytic enzymes. Moreover, although insoluble solids are not directly linked to the saccharification yield, it has been shown that high DM content negatively affects enzymatic activities due to low availability of water, caused mainly by the soluble sugars released (Selig et al., 2012). The DM content (w/w) had a slight negative effect on the liquefaction, up to 26% DM. However, this effect was more severe as the DM content increased. As shown in Table 2, at 30% DM (w/w), the final viscosity could not reach lower than 2000 cP, regardless of the liquefaction time or the enzyme loading used. As expected, the reduction in viscosity was higher when higher enzyme loadings were used. 5 FPU g^{-1} DM were enough to reduce the final viscosity to lower than 2000 cP at 26% DM, while more than 7 FPU g^{-1} DM would be needed to achieve the same reduction in viscosity at 30% DM (w/w). Although no linear correlation was observed, the results indicate that the enzyme loading is the decisive factor for the liquefaction efficiency, regarding the reduction in viscosity. In Fig. 1b, the shape and condition of the PWS at 26% DM with 5 FPU g^{-1} DM enzyme loading is shown. There was a visible change in the form of the material, indicating the effect of the liquefaction process.

The amounts of reducing sugars released at the end of the liquefaction stage were also affected by DM content and enzyme loading. As shown in Fig. 2a,b, as expected, enzyme loading is the decisive factor for the release of sugars from wheat straw fiber. The levels of diluted sugars were not increased at all when the initial DM content was increased above 26% w/w, which was also reflected by the lower saccharification yields obtained from slurries above 26% DM (Fig. 2c,d). Apart from the reasons mentioned above, end-product inhibition is another reason for the lower saccharification yields at high DM content. At 30% DM (w/w), sugar concentrations from 58 to 60 g L^{-1} were released. As previously shown by researchers who studied the same combination of commercial enzymes, as the one used in the present study, glucose concentrations above 50 g L^{-1} significantly inhibit cellulolytic enzymes (Andric et al., 2010).

Increase in the liquefaction time from 6 h to 8 h did not lead to a significant increase in the sugars released; nor did it have any substantial influence on the viscosity. Of the three process parameters studied, the initial DM content and the enzyme loading had the most significant effects on viscosity and saccharification. The optimum initial DM content of the slurry was not found to be related to the amount of enzymes used, and was shown to be 26%. Regarding the enzyme loading, for all DM contents studied, it was found that 3 FPU g^{-1} DM were not enough to ensure low viscosity and high levels of sugars after 6 h or 8 h of liquefaction. The choice of the optimum enzyme dose is a trade-off between process cost and saccharification yield. In all the cases studied here, it was found that a linear increase in sugars released followed the increase in enzymes added. However, this was only true because enzyme loading was kept at moderate levels in order to be in the range of loading used in large-scale applications. As shown previously (Xiros et al., 2009), for enzyme doses above 20 FPU g^{-1} dry PWS, no linear increase in sugars released can be observed.

3.2. Contribution of the enzymatic system of F. oxysporum during co-cultivation SSF with S. cerevisiae

To evaluate the contribution of the enzymatic system of *F. oxysporum*, enzyme loadings up to 5 FPU g^{-1} DM were added at the start-up of SSF along with 5 mg g^{-1} DM dry baker's yeast. It was found that the enzymes of *F. oxysporum* had a positive effect on the fermentation yields. As shown in Fig. 3a, addition of 5 FPU g^{-1} DM resulted in a 33% increase in the final concentration of ethanol. The fermentation was also enhanced by adding the *F. oxysporum* enzymatic system at 1–4 FPU, as 2.5%, 10%, 18%, and 25% increases in ethanol production, respectively, were measured.

Table 2
Viscosity of liquefied PWS at high DM content. The effects of reaction time and DM content on the viscosity of PWS. All experiments were carried out in duplicate. Error levels are presented.

Time(h)	DM(%w/w)	Enzymes(FPUnits g $^{-1}$ DM)	Initial viscosity(cP)	Final viscosity(cP)
6	20	3	15112 ± 116	3545 ± 105
		5	15052 ± 108	1136 ± 85
		7	15113 ± 122	754 ± 91
	26	3	15508 ± 181	4123 ± 112
		5	15543 ± 142	1626 ± 93
		7	15446 ± 125	1042 ± 86
	30	3	15821 ± 184	8510 ± 110
		5	15813 ± 175	3123 ± 102
		7	15762 ± 203	2200 ± 105
8	20	3	15091 ± 122	3103 ± 94
		5	15119 ± 125	954 ± 82
		7	15052 ± 113	710 ± 64
	26	3	15541 ± 154	3803 ± 121
		5	15503 ± 162	1205 ± 103
		7	15532 ± 146	904 ± 74
	30	3	15904 ± 210	7854 ± 130
		5	15836 ± 198	3125 ± 112
		7	15784 ± 194	2023 ± 91
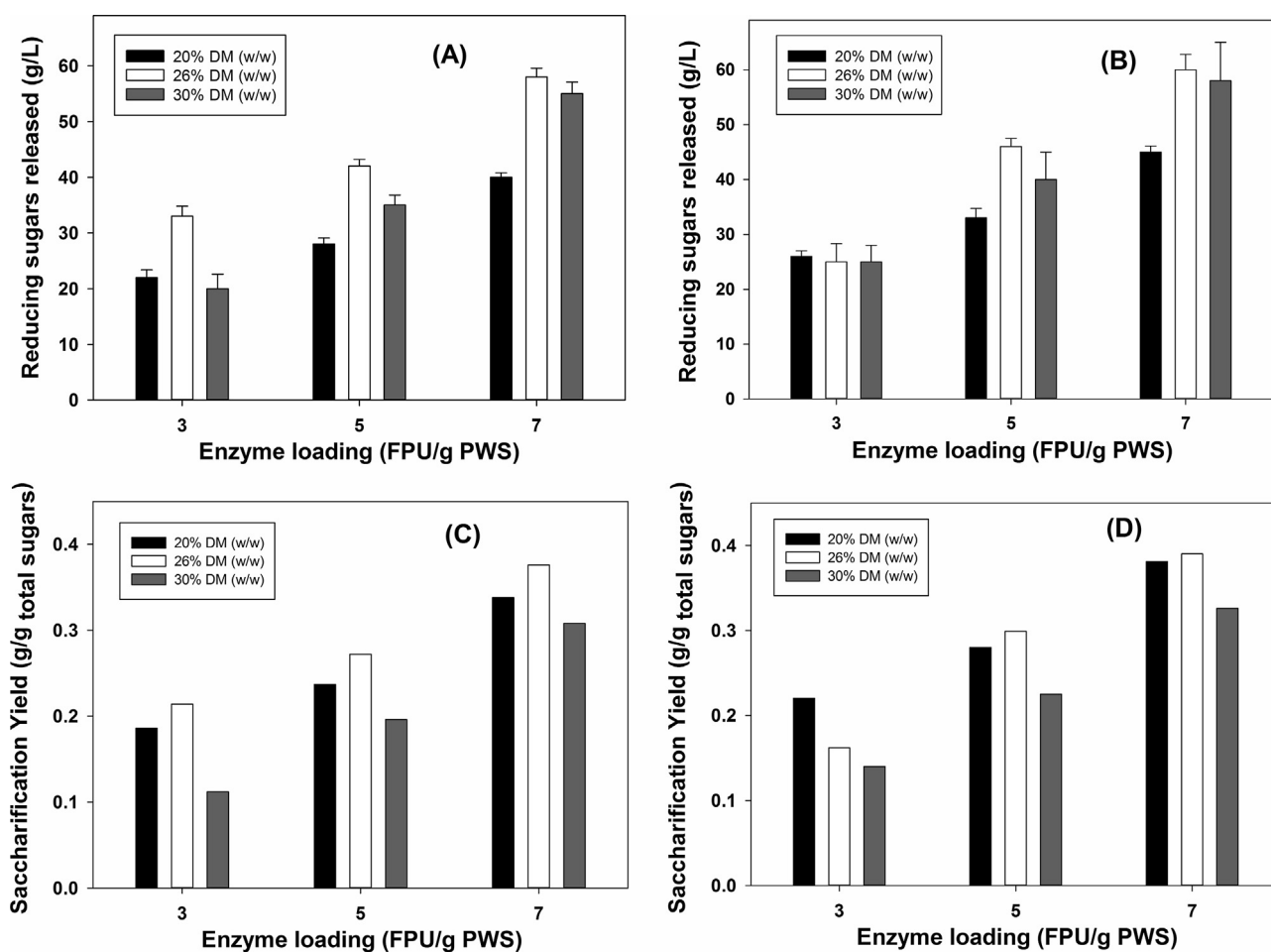
Fig. 2. Total reducing sugars released (A, B) and saccharification yields (C, D) after liquefaction. Liquefaction of PWS was carried out in a free-fall mixing reactor, at a solids content of 20% DM (black), 26% DM (light gray), or 30% DM (dark gray). Enzyme loadings of 3, 5, and 7 FPU g $^{-1}$ DM were tested. The duration of the process was 6 h (A, C) or 8 h (B), (D). All experiments were carried out in duplicate; vertical bars indicate the error levels.

The fiber fraction of wheat straw generated after the pretreatment (PWS) was used in this study. The liquefied PWS was used as the substrate for the SSF stage. Although PWS is mainly rich in cellulose, it also contains hemicellulose. After the liquefaction, 37% of the carbohydrate content was released as fermentable soluble sugars, i.e., 63% of the total sugar content remained in the form of cellulose

and hemicellulose. This result is in accordance with other studies on the liquefaction of PWS, where 32% and 49% of cellulose and hemicellulose were found to be converted to monomeric and oligomeric sugars after 24 h of liquefaction (Jorgensen et al., 2007). As stated in the same work, the xylose concentration remained constant during the SSF process, so the hemicellulose was not hydrolyzed further
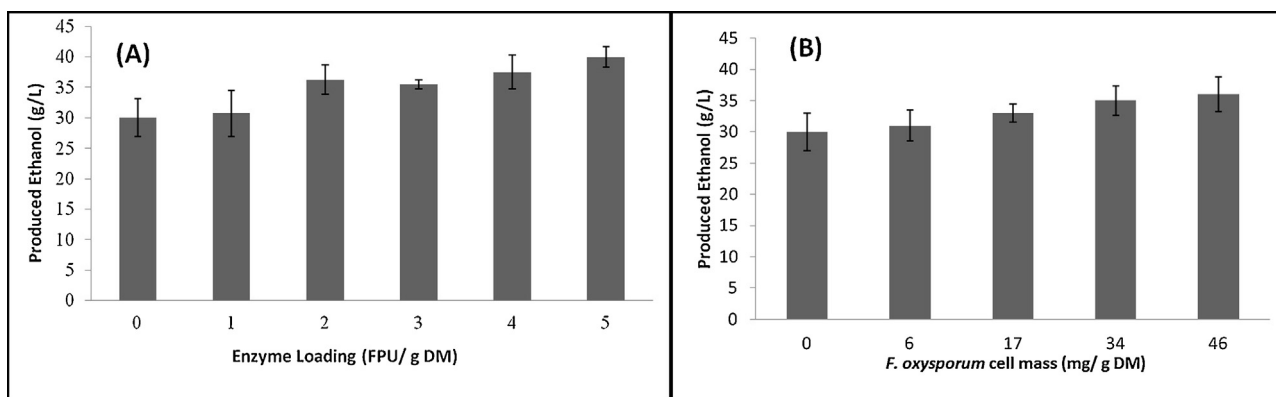
Fig. 3. Effect of additions of crude enzyme extract (A) and cell mass (B) from *F. oxysporum* in liquefied PWS fermentation. (A) Enzyme loadings up to 5 FPU g^{-1} DM, of concentrated *F. oxysporum* enzymes, were added at the start of the fermentation stage. *S. cerevisiae* (5 mg g^{-1} DM) was the fermenting microorganism. There was no addition of *F. oxysporum* cell mass. (B) *F. oxysporum* cell mass of up to 46 mg g^{-1} DM was added at the start of the fermentation process along with 5 mg g^{-1} DM *S. cerevisiae*. There was no further addition of enzymes in the fermentation process. PWS liquefied with 5 FPU g^{-1} DM of commercial enzymes was used as carbon source at 26% DM. All experiments were carried out at 30 °C in duplicate. Vertical bars indicate error levels.

during that stage. In order to exploit PWS in a more efficient way, an enzymatic system containing both cellulases and hemicellulases is needed. The overall ethanol yield of the process would increase not only because hemicellulose would also be converted to ethanol, but also because the presence of xylan inhibits cellulose hydrolysis (Viikari et al., 2012). The enzymatic hydrolysis rate of cellulose has been found to be negatively affected even by low amounts of residual xylans in lignocellulosic materials after pretreatment (Viikari et al., 2012). Using PWS as raw material, Zhang et al., (2011) found that xylanase supplementation of cellulases resulted in synergistic enhancement of cellulose hydrolysis. Xylanases are required to expose cellulose in microfibrils covered by hemicelluloses (Zhang et al., 2011).

The enzymatic system of *F. oxysporum* is a multi-enzyme system rich in cellulases and hemicellulases that would be able to enhance the saccharification of lignocellulose during the SSF process. The system is rich in xylanase activity: In a crude extract representing 1 FPU, the xylanase activity was measured to be 65 U, while the correponding xylanase activity for 1 FPU of the mixture of Celluclast 1.5 L and Novozyme 188 used in the liquefaction step was found to be 10 U (García-Aparicio et al., 2007). Furthermore, the enzymatic system of *F. oxysporum* contains many auxiliary xylanolytic and cellulolytic activities that are necessary for the degradation of oligosaccharides to monomeric sugars (Viikari et al., 2012). This lignocellulolytic system is more active at the fermentation temperature (30 °C) than the commercial enzymes used in this study, as a consequence of its relatively low optimum temperature (40 °C) (Xiros et al., 2009). The effect of ethanol on *F. oxysporum* enzymes were studied recently (Paschos et al., 2015) and they were found to be affected by ethanol at 30 °C as much as the commercial enzymes.

3.3. Contribution of the metabolic system of F. oxysporum during co-cultivation SSF with S. cerevisiae

Many scientific reports have described the inhibitory effects of monomer sugars (mostly glucose) on the cellulases of *F. oxysporum* and *T. reesei* (Xiros et al., 2009; Andric et al., 2010). Although recently developed commercial enzyme preparations have not been found to be significantly affected by end-product inhibition (Xiros and Olsson, 2014), SSF was for a long time the preferred fermentation process, due to reduced inhibitory effects of end-products on the cellulolytic enzymes. The quick removal of monomer sugars (by conversion to ethanol) during the SSF process ensures that the enzymes will keep up the hydrolysis during fermentation. *S. cerevisiae* can metabolize glucose to ethanol

at high rates, removing the inhibitory effect of glucose from the process. As wild type *S. cerevisiae* strains cannot ferment pentoses, there is a need for a xylose-fermenting metabolic system to enhance the fermentation yield by converting the hydrolyzed xylose to ethanol. Apart from metabolic engineering and evolutionary engineering approaches, which both involve development of xylose-fermenting *S. cerevisiae* strains (Van Leeuwenhoek, 2006), an alternative approach would be a process configuration based on co-culture of a xylose-fermenting strain with *S. cerevisiae*. Such systems have been studied previously, revealing that mixed co-cultures of fungi and yeast can lead to better exploitation of lignocellulosic materials (Chandel et al., 2011). On this theoretical basis, an experiment was designed to investigate the ability of *F. oxysporum* to cooperate with *S. cerevisiae* in the fermentation of previously hydrolyzed wheat straw.

Cells of *F. oxysporum* (previously grown in submerged culture using glucose and xylose as a carbon source) were added at the start of SSF along with 5 mg g^{-1} DM dry baker's yeast. At this point, up to 46 mg g^{-1} DM of *F. oxysporum* cell mass was used. It was clear from the experimental results (Fig. 3b) that this amount of biomass could only give a slight enhancement in ethanol production, as an increase of about 16.5% was observed when 46 mg g^{-1} DM of *F. oxysporum* cells was added. These results showed that the contribution of the xylose-fermenting ability of *F. oxysporum* to the final ethanol production was not as high as the contribution of the enzymatic system of the fungus, and appeared only when high cell concentrations were added to the fermentation medium. At this point the *F. oxysporum* cell mass added is 9 times higher than that of *S. cerevisiae*. The presence of low amounts of hemicellulolytic activity in the commercial enzymes—which did not lead to sufficient release of pentose sugars from hemicellulose—could be the reason for these observations. To check this hypothesis, another experiment was designed, with combined addition of *F. oxysporum* cells and enzymes at the start of the SSF stage.

3.4. Combined contribution of the enzymatic and metabolic systems of F. oxysporum to ethanol production during P-SSF

The combined effect of addition of *F. oxysporum* enzymes and cell mass on ethanol production in the SSF process was investigated (Fig. 4). The addition of *F. oxysporum* enzymes and cell mass in the SSF led to increased ethanol production. An ethanol concentration of 62 g L^{-1} was achieved, corresponding to a yield of 0.44 g ethanol g^{-1} cellulose and hemicelluloses (80% of the theoretical, based on the cellulose and hemicellulose content of the
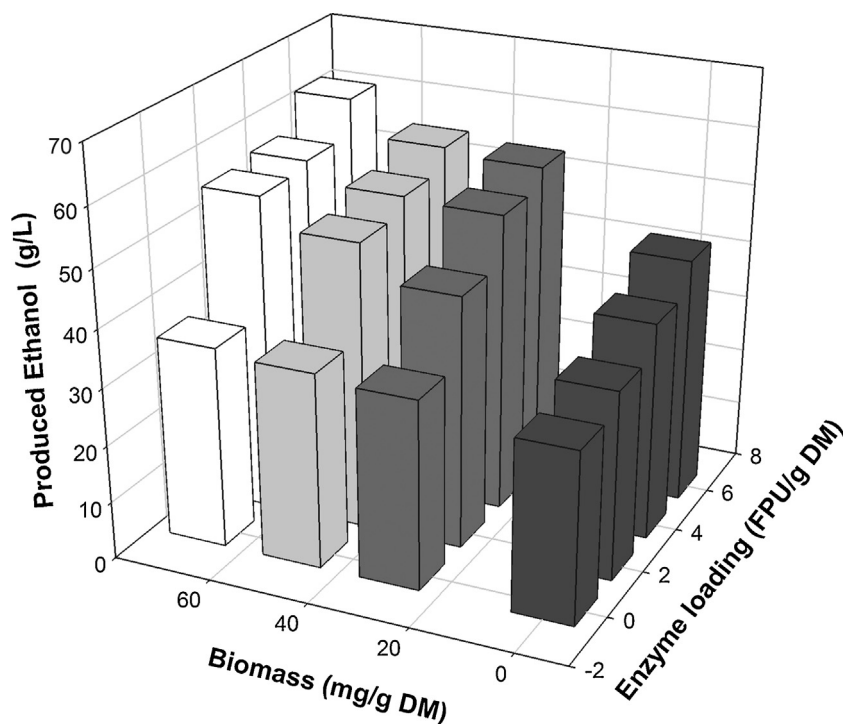
Fig. 4. Effect of combined addition of *F. oxysporum* enzymes and cell mass in liquefied PWS fermentation. *F. oxysporum* cell mass of up to 70 mg g^{-1} DM and enzymes up to 6 FPU g^{-1} DM were added at the start of the fermentation process, along with *S. cerevisiae* (5 mg g^{-1} DM). PWS liquefied with commercial enzymes (5 FPU g^{-1} DM) was used as carbon source at 26% DM. All P-SSF experiments were carried out at 30 °C in duplicate. Error levels were less than 5% in all cases.

material). Fig. 4 shows that both cells and enzymes of *F. oxysporum*, when added together, have a positive effect on the ethanol production. Another study by Olofsson et al. (2010), using wheat straw as raw material and proposing fed-batch enzyme loading, measured ethanol production yields of 0.35 g g^{-1}. As shown previously (Fig 3b) when no *F. oxysporum* enzymes were added, *F. oxysporum* cells did not have adequate effect on the ethanol production. It should be mentioned that at the previous experiment there was no enzyme addition during the fermentation stage. This can be explained by the fact that fungal cells have glucose consumption rates much lower than that of *S. cerevisiae*, and therefore, could not contribute to fermentation of glucose to ethanol. It can therefore be suggested that the positive effect we saw in the case of combined addition (Fig. 4) was mainly due to the release and consumption of xylose by the enzymatic and metabolic systems, respectively, of *F. oxysporum*. This experiment demonstrated the usefulness of the exceptional combined ability of *F. oxysporum* to produce and secrete xylanolytic enzymes (under aerobic growth) in order to hydrolyze arabinoxylan and ferment the xylose that was released to ethanol.
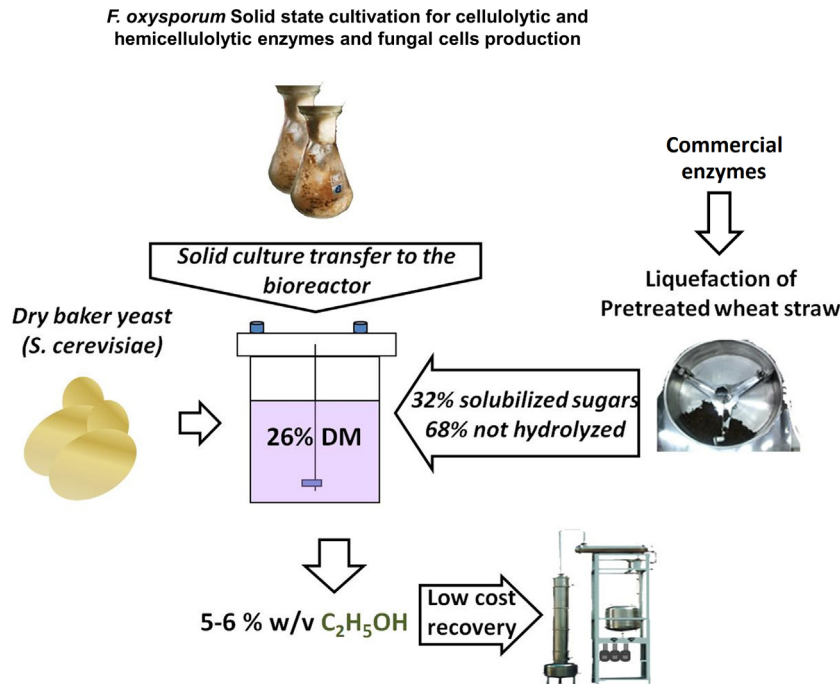
3.5. Simultaneous saccharification and co-fermentation of PWS for ethanol production by co-culture of S. cerevisiae and F. oxysporum

Based on the results presented in the previous paragraphs, a process was designed (Fig. 5) where we took advantage of all the beneficial properties of *F. oxysporum*. Combined with the high fermentation rate of *S. cerevisiae*, we achieved very high ethanol yields and concentrations. Taking into account the levels of enzymatic activities achieved in submerged and solid-state cultures of *F. oxysporum*, and in order to keep the same overall enzyme loadings as in the previous experiments, 7 FPU of commercial enzymes were used. In the case of liquid culture addition, and in order to keep

the DM content at 26%, the liquefied PWS was condensed before the fermentation stage. The two approaches (addition of liquid cultures and solid cultures of *F. oxysporum*) were compared regarding their effects on final ethanol production and yield. The solid-state culture of *F. oxysporum* allows the production of higher concentrations of enzymes and fungal biomass (Xiros et al., 2008a). Thus, the addition of *F. oxysporum* solid culture technically permitted the addition of a greater amount of enzymes and a significantly greater amount of cells, as shown in Table 3. When submerged *F. oxysporum* culture was used, 0.7 FPU g^{-1} DM and 16 mg g^{-1} DM were the highest amounts of enzymes and cells, respectively, that could be added, while in the case of the solid culture, 1.23 FPU g^{-1} DM and 154 mg g^{-1} DM biomass could be transferred in the SSF reactor, always ensuring that the desirable high concentration of PWS (26% w/w DM) during SSF would not be reduced (Xiros et al., 2008a). Enhanced ethanol production was observed in both cases. At 26% w/w DM and after 120 h of fermentation, 11% and 19% increases in ethanol production were observed when submerged or solid-state cultures, respectively, were added, compared to fermentations without the addition of *F. oxysporum*.

The ethanol concentration reported in the case of *F. oxysporum* grown under solid-state culture (58 g L^{-1}), corresponding to 0.41 g ethanol g^{-1} total cellulose and hemicellulose content, is quite high compared to other high-solids fermentations in the literature using wheat straw as raw material and similar enzyme loadings (Table 4). Cannella and Jorgensen (2013) achieved 55 g kg^{-1} ethanol production at 30% DM using a similar technique of P-SSF (with a much longer liquefaction stage: 24 h) and the up-to-date commercial enzyme mixture (Cellic Ctec 2; 7.5 FPU g^{-1} DM) (Table 4). Comparison of these data confirms that the liquefaction time is not such an important parameter as long as the enzymes present during liquefaction and SSF are capable of efficiently hydrolyzing the substrate. On the other hand, in most cases saccharification yields drop significantly for DM contents above 30%. The presence

F. oxysporum Solid state cultivation for cellulolytic and hemicellulolytic enzymes and fungal cells production

Solid culture transfer to the bioreactor

Commercial enzymes

Liquefaction of Pretreated wheat straw

Dry baker yeast (*S. cerevisiae*)

26% DM

32% solubilized sugars
68% not hydrolyzed

5-6 % w/v C$_2$H$_5$OH   Low cost recovery

Fig. 5. Consolidated process of simultaneous saccharification and co-fermentation of PWS for ethanol production by co-culture of *S. cerevisiae* and *F. oxysporum*.

Table 3
Effect of the addition of solid-state or submerged cultures of *F. oxysporum* in liquefied PWS fermentation.

DM content (%)	Growth technique	*F. oxysporum* enzymes added (FPU/g DM)	*F. oxysporum* cell mass added (mg/g DM)	Ethanol production (144 h)			
				Ethanol produced % w/w	Control experiment	% increase	% theoretical
26	Submerged	0.70	15.9	5.2	4.6	+11	67
26	Solid State	1.23	154	5.8	4.9	+19	75

Solid or submerged cultures of *F. oxysporum* were added at the start of the fermentation process along with *S. cerevisiae* (5 mg g^{-1} DM). Control experiments had fermentations with *S. cerevisiae* without any addition of *F. oxysporum*. Liquefied PWS (7 FPU g^{-1} DM and 30% DM) was used as carbon source at 26% DM. All experiments were carried out in duplicate. Error levels were less than 5% in all cases.

Table 4
Comparative study of liquefied pretreated wheat straw fermentation.

DM	Liquefaction time (h)	Enzyme dosage in liquefaction (FPU g^{-1} DM)	Enzyme addition to SSF (FPU g^{-1} DM)	Ethanol (g/kg)	% Maximum theoretical ethanol	Reference
26	7	7CN	1.23 (*F. oxysporum*)	58	75[a]	Present study
25	24	7CN	no	33	46[b]	Jorgensen et al. (2007)
35	8	7CN	no	48	47[b]	Jorgensen et al. (2007)
20	24	7.5CN	no	38	65[b]	Cannella and Jorgensen (2013)
30	24	7.5CN	no	34	38[b]	Cannella and Jorgensen (2013)
20	24	7.5Ctec2	no	55	84[b]	Cannella and Jorgensen (2013)
30	24	7.5Ctec 2	no	50	62[b]	Cannella and Jorgensen (2013)

Abbreviations: CN, Celluclast 1.5 L and Novozyme188; Ctec2, Cellic Ctec2. All studies reported use same kind of hydrothermal pre-treatment.
[a] Based on cellulose and hemicellulose content.
[b] Based on cellulose content.

of a multi-enzyme system, like the one from *F. oxysporum*, that is rich in a variety of lignocellulolytic activities plays a crucial role in conversion of lignocellulose to ethanol.

4. Conclusions

In HG fermentations, the liquefaction process is of high importance for sufficient mixing of the slurry. The drop in viscosity of the material was crucial in order to make the substrate accessible to enzymes and microorganisms. Both the amount and the types of the enzymes used were important factors for the decrease in viscosity. The addition of *F. oxysporum* enzymes at the start of the fermentation significantly increased the ethanol levels produced. The semi-consolidated process proposed not only increased the ethanol yields significantly, but could also lead to lower overall cost of the process by incorporating in-situ enzyme production.

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