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Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production

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HIGHLIGHTS

- *Myceliophthora thermophila* JCP 1-4 is a new enzymes source for biomass hydrolysis.
- A fast 8 h hydrolysis was enough for ozonated sugarcane bagasse saccharification.
- The fungus was highlighted as a glucose isomerase producer, releasing fructose.
- Ozonated sugarcane bagasse acted as enzymes activities activator during hydrolysis.
- Hydrolysate from ozonated bagasse was greatly fermented for bioethanol production.

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ABSTRACT

The saccharification of ozonated sugarcane bagasse (SCB) by enzymes from *Myceliophthora thermophila* JCP 1-4 was studied. Fungal enzymes provided slightly higher sugar release than commercial enzymes, working at 50 °C. Sugar release increased with temperature increase. Kinetic studies showed remarkable glucose release (4.99 g/L, 3% w/w dry matter) at 60 °C, 8 h of hydrolysis, using an enzyme load of 10 FPU (filter paper unit). FPase and β-glucosidase activities increased during saccharification (284% and 270%, respectively). No further significant improvement on glucose release was observed increasing the enzyme load above 7.5 FPU per g of cellulose. Higher dry matter contents increased sugars release, but not yields. The fermentation of hydrolysates by *Saccharomyces cerevisiae* provided glucose-to-ethanol conversions around to 63%.

1. Introduction

The use of lignocellulosic residues – such as SCB – as raw material for second generation fuels production is a promissory research field. Lignocellulosic wastes stand out as a renewable bioenergy resource to reduce environmental, economic and political problems related to the use of the traditional fossil fuels. These residues can be used as substrates for microbial enzymes depolymerization to produce monomeric sugars that can be converted into second generation and other biobased products.

Brazilian government established, in 1975, the *PRO-ÁLCOOL* program, a policy to reduce the dependence from another countries fuel. The program was based in the increase of SCB ethanol production to be used as gasoline substitute as well as to blend it in a proportion up to 24%. In Brazil, SCB is generated in large quantities as a residue of sugar production and first generation ethanol factories (more than 800 million tons of sugarcane processed in the 2014/2015′ crop) (UNICA, 2015). Considering this scenario and taking into account the environmental issues, scientific researches for new energy sources in Brazil have been focused on the use of SCB to obtain second generation ethanol and other value-add products.

For the production of second-generation ethanol from SCB, a step of pretreatment is necessary to remove or disrupt lignin and

Abbreviations: AIL, acid insoluble lignin; ASL, acid soluble lignin; CBU, cellobiohydrolase unit; FPU, filter paper unit; SCB, sugarcane bagasse; HPLC, high pressure liquid chromatography; SSF, solid state fermentation; TL, total lignin.

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to decrease cellulose crystallinity. Subsequently, carbohydrates polymers have to be chemical or enzymatically saccharified into glucose, which finally will be converted to ethanol by fermentative microorganisms (Tomás-Pejó et al., 2011).

Ozonolysis was studied decades ago as a chemical oxidative pretreatment of lignocellulosic materials (Neely, 1984) and has been rediscovered recently (García-Cubero et al., 2010, 2012; Li et al., 2015; Travaini et al., 2013). Ozonolysis pretreatment promotes kinetic-selective degradation of lignin, because its high quantity of electron-rich centers prone to ozone attach. Ozone converts acid insoluble lignin (AIL) into acid soluble lignin (ASL) and low molecular weight compounds, mainly the carboxylic acids oxalic, formic and acetic. Lignin removal increases enzymes accessibility to polysaccharides and sugar release (Travaini et al., 2015). Among the many advantages of ozonolysis on other pretreatments, these can be highlighted: high saccharification yields; low inhibitory compounds formation; the process occurs at ambient pressure and temperature; a single solid phase is generated, avoiding problems related to products dilution; ozone can be generated *in situ* and residual ozone can be easily destroyed, preventing environmental problems (Travaini et al., 2016).

Enzymatic saccharification of lignocellulosic materials to obtain glucose requires the use of microbial enzymes cocktails comprising at least three hydrolases: endoglucanase, exoglucanase and β-glucosidase (Arantes and Saddler, 2010). These enzymes are produced by many microorganisms, especially filamentous fungi (Maeda et al., 2013). Among them, thermophilic fungi stand out, since they can use lignocellulosic wastes as substrates to produce enzymes by solid state fermentation (SSF). In general, they better support temperature rise, common in SSF processes, and produce more active and stable enzymes under high temperatures, compared to mesophilic fungi (Gomes et al., 2007).

The search for new microbial cellulases that can be produced by SSF, using lignocellulosic residues as substrates, is interesting to reduce the costs of second-generation ethanol production, since enzymatic saccharification is one of the most expensive steps in the global process. These enzymes must have some characteristics, such as: high hydrolysis rate; activity and stability at high temperatures; and low inhibition by end-product and by secondary compounds that could be generated in the process (Mielenz, 2001). All the works available in scientific literature related to enzymatic saccharification of lignocellulosic material pretreated with ozone use commercial cellulases (García-Cubero et al., 2009; Li et al., 2015; Travaini et al., 2013).

In this work, the enzymatic extract produced by SSF from the new isolated thermophilic fungus *Myceliophthora thermophila* JCP 1-4 was used to saccharify ozone pretreated SCB. This fungus shows significant production of cellulases when growing in a variety of lignocellulosic substrates, including avicelase, the enzyme responsible for hydrolysis of crystalline cellulose (Cassia Pereira et al., 2015). Glucose isomerase activity was also detected in the enzymatic extract, which is not usual in fungi. Commercial enzymes were also used for comparison purposes. The influence of time, temperature, enzyme and bagasse load in saccharification were evaluated. Enzyme activities were monitored along with saccharification, and the endurance of enzymes subjected to concentration using a rotary evaporator was also studied. Hydrolysates were fermented using a bakery strain of *Saccharomyces cerevisiae* to obtain ethanol.

2. Methods

2.1. Sugarcane bagasse

SCB was donated by Usina Vale, city of Onda Verde, São Paulo State, Brazil. It was washed with distilled water to remove sugar residues and particulate material, dried in a ventilated oven at 37 °C and ground in an agricultural crusher (Trapp Model TRF-400) to a size of 3–5 mm. *In natura* SCB chemical composition (%) was: 3.13 ± 0.04 of ASL, 19.54 ± 0.03 of AIL, 22.67 ± 0.07 of total lignin (TL), 20.86 ± 0.05 of xylan and 46.21 ± 0.10 of cellulose (Travaini et al., 2013).

2.2. Ozonolysis pretreatment

Pretreatment was carried out in a fixed bed reactor (glass column of 50 cm in height and 2.7 cm in diameter) provided with a porous glass diffuser, as described by Travaini et al. (2013). The optimal operation parameters found in the previously mentioned work were applied: moisture content of 80%, ozone/oxygen concentration of 3.44% (mol/mol) at a flow of 60 L/h, for 45 min. After pretreatment, SCB was washed with distilled water. Ozonated SCB chemical composition (%) was: 6.22 ± 0.07 of ASL, 12.99 ± 0.02 of AIL, 19.20 ± 0.09 of TL, 20.74 ± 0.12 of xylan and 44.86 ± 0.75 of cellulose (Travaini et al., 2013).

2.3. Microorganism

The fungus *M. thermophila* JCP 1-4 was isolated from SCB silage piles in Potirendaba, São Paulo State, Brazil. It was chosen for the present study, among 26 thermophilic fungi, as the best producer of cellulases and β-glucosidase, when cultivated by SSF, at 45 °C, using a variety of lignocellulosic materials as substrates (Cassia Pereira et al., 2015). β-glucosidase from *M. thermophila* JCP 1-4 is resistant to glucose inhibition (Cassia Pereira et al., 2015), an important characteristic for saccharification experiments. Stock cultures are maintained in cryo tubes, in 20% glycerol solution at −80 °C.

2.4. Enzymes production by solid state fermentation

To obtain enzymatic extract, *M. thermophila* JCP 1-4 was precultivated on Sabouraud agar plates for 72 h at 45 °C. Five mycelial disks (1 cm diameter) from plates were used as inoculum for each 250 mL Erlenmeyer flask, containing 2.5 g of *in natura* SCB and 2.5 g of soybean meal (both washed and oven dried at 37 °C). Soybean meal was donated by Trouw Nutrition, Mirassol, São Paulo State, Brazil. Each Erlenmeyer flask was moisturized with 11 mL of saline solution as described by Toyama and Ogawa (1978), providing an initial substrate moisture close to 70%. Erlenmeyer flasks with substrates and saline solution were autoclaved at 121 °C, 1 atm, during 20 min before inoculation. Erlenmeyer flasks were inoculated and incubated at 45 °C, for 96 h, time for a satisfactory cellulolytic enzymes production (Cassia Pereira et al., 2015). After this period, 50 mL of distilled water were added to each flask, the mixture was homogenized with a glass bar, stirred in an orbital shaker at 100 rpm for 1 h. Then, it was filtered through nylon cloth disks and centrifuged at 10,000*g*, for 15 min, at 5 °C. Supernatants were collected, lyophilized and stored. For use, the enzymes were resuspended in water in the same water ratio of the original extract, for reproduce the same conditions when crude enzymes can be used. The enzymatic activities in the reconstituted extract were 0.33 FPU/mL and 1.00 CBU/mL (1:3 FPU/CBU ratio, the same found in crude extract).

2.5. Enzyme concentration

Fungal enzymatic extract had to be concentrated for some of the saccharification experiments (those in which the influence of dry matter content was evaluated), in order to achieve the desired FPU per g of cellulose. Concentration was performed by rotaevaporation, the extract was concentrated by 5-fold (initial volume

divided by final volume) on a rotatory evaporator at 60 °C under 100 rpm in vacuum. Each concentration procedure took approximately 2 h.

2.6. Enzyme activities

FPase activity was determined as described by Ghose (1987) and expressed as PFU. β-glucosidase activity was assayed according to Leite et al. (2008) and expressed as CBU. Endoglucanase and exoglucanase (avicelase) activities were determined as described by Cassia Pereira et al. (2015).

Glucose isomerase activity was determined according to Zhang et al. (2013), with modifications. Reaction tubes were composed of: 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.2 mL of 1 M D-glucose, 0.1 mL of 0.1 M $MgSO_4 \cdot 7H_2O$, 0.1 mL of 0.01 M $CoCl_2 \cdot 6H_2O$, 0.9 mL of distilled water and 0.2 mL of crude enzymatic extract. Reaction tubes were incubated at 40, 50 or 60 °C, for 1 h, and then reaction was stopped by addition of 2 mL 0.5 M perchloric acid. One unit of glucose isomerase activity was defined as the amount of enzyme needed to produce 1 μmol of fructose per minute, under the assay conditions.

2.7. Sugarcane bagasse enzymatic saccharification

Saccharification assays were performed in 100 mL Erlenmeyer flasks containing *in natura* or ozone pretreated SCB (3–10%, w/w dry basis) in sodium citrate buffer 0.05 M, pH 5.0 and the desired load of FPU per g of cellulose, provided by the enzymatic extract of *M. thermophila* JCP 1-4 or commercial enzymes, with 25 mL of final volume. The commercial enzymes used were Celluclast 1.5 L and Novozym 188 (Novozymes, Denmark). The first one was used as FPU source and the second as β-glucosidase source, to achieve the same FPU/CBU of 1:3 ratio found in the fungal enzymatic extract. Flasks were incubated on an orbital shaker, at different temperatures and 300 rpm, for up to 48 h. Substrate blank experiments were performed without enzyme addition, using only bagasse and buffer solution. Enzyme blank experiments were performed without bagasse, only with enzyme and buffer solution. At each time interval, a flask was taken, cooled in an ice bath, its content was filtered using 0.22 μm filters and sugars in the liquid fraction were determined and quantified by HPLC (high pressure liquid chromatography). The values of sugars (g/L) in blank experiments were subtracted from the values obtained in saccharification media. Yields were expressed as g of glucose divided per g of cellulose in the raw material, multiplied by 100 (expressed as a percentage). Experiments were performed as duplicates.

2.8. Statistical analysis

The effects of temperature, time, enzyme load and dry matter in ozonated SCB saccharification by *M. thermophila* JCP 1-4 enzymes were evaluated, using a one-factor-at-a-time (OFAT) design of experiments.

Statistical analysis of the data from saccharification experiments included a one-way ANOVA followed by Tukeýs test with a 5% significance level. All analyses were run in the STATGRAPHICS® Centurion XVI Version 16.2.04 (64-bit).

2.9. Alcoholic fermentation

For preculture production, a fresh bakery strain of *S. cerevisiae* acquired in a grocery shop was grown on commercial YEPD (Yeast Extract Peptone Dextrose) (1% yeast extract, 2% peptone and 2% glucose), aerobically, on a rotatory shaker at 30 °C, 175 rpm, for 24 h.

Hydrolysate slurries were transferred to 100 mL penicillin flasks, supplemented with 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4 \cdot 7H_2O$ and inoculated with 5.5% (v/v) of the preculture (Wanderley et al., 2013). Flasks were sealed and fermentation occurred with the oxygen present in the empty flask space, at 30 °C, under 175 rpm, for 24 h. The supernatants were filtrated and analyzed by HPLC.

Ethanol yield was calculated with respect to the maximum theoretical production. Ethanol content (g/L) was divided by the glucose content (g/L), multiplied by the stoichiometric factor 0.511, and then the value was multiplied by 100 to give the percentage.

2.10. Analytical methods

Ethanol analysis was performed using the same HPLC setup previously described by Toquero and Bolado (2014), equipped with a Biorad Aminex® HPX-87H column, with 0.6 mL/min sulfuric acid 5 mM as eluent, at 50 °C. For sugars analysis, the system was equipped with a Phenomenex® HPLC column Rezex™ RPM-Monosaccharide Pb^{+2} (8%), 300 × 7.8 mm, with 0.6 mL/min MilliQ water as eluent, at 80 °C.

3. Results and discussion

3.1. Saccharification of sugarcane bagasse using fungal or commercial enzymes

In order to compare the efficiency of *M. thermophila* JCP 1-4 enzymatic extract in relation to the commercial enzymes mixture, preliminary saccharification assays were performed using *in natura* or ozonated SCB (3% w/w – dry basis, at 50 °C, for 24 h). The enzyme load used was 10 FPU and 30 CBU per g of cellulose content in raw material, for both fungal and commercial enzymes. These conditions are commonly cited in studies about sugarcane enzymatic saccharification (Cassia Pereira et al., 2015; Travaini et al., 2013). Besides, 50 °C is the optimum temperature for the commercial enzymes activities, and the enzymatic extract from *M. thermophila* JCP 1-4 shows good activities of endoglucanase, exoglucanase, β-glucosidase and FPase under this temperature (55.16, 2.01, 1.02 U/mL and 0.51 FPU, respectively).

As shown in Table 1, for *in natura* SCB, fungal enzymatic extract provided a glucose release of approximately 1.4 folds higher than commercial enzymes. Xylose release from *in natura* SCB was also higher (3.9 folds) when using *M. thermophila* JCP 1-4 enzymes. Regarding cellobiose release from *in natura* bagasse, very close values were obtained when using fungal or commercial enzymes. A standard one-way ANOVA analysis indicated significant differences in glucose release when using MT or commercial enzymes to saccharify in natura SCB ($F = 213.2$ and p-value = 0.003).

Table 1
Sugars released from *in natura* and ozonated bagasse, in saccharification assays (3% – w/w dry basis, 10 FPU and 30 CBU per g of cellulose, 50 °C, 24 h) using *Myceliophthora thermophila* JCP 1-4 enzymatic extract (MT) or commercial enzymes*.

Enzyme source	SCB	Sugar (g/L)**		
		Glucose***	Xylose	Cellobiose
MT	*In natura*	1.40 ± 0.02	0.50 ± 0.02	0.15 ± 0.04
Commercial		0.95 ± 0.20	0.13 ± 0.16	0.18 ± 0.01
MT	Ozonated	4.19 ± 0.02	1.94 ± 0.02	0.23 ± 0.05
Commercial		4.13 ± 0.20	1.08 ± 0.44	0.04 ± 0.00

* Mixture of Celluclast 1.5 L and Novozym 188.
** No sugars were detected in the substrate blank and enzyme blank under the HPLC method limit of detection (>0.01 g/L).
*** No significant difference found on sugar released applying one-way ANOVA analysis ($p < 0.005$), using ozonated SCB saccharified with MT or commercial enzymes.

However, there was no significant difference in glucose release from ozonated SCB when using MT or commercial enzymes ($F = 0.106$ and p-value = 0.8).

Regarding ozonated SCB, the use of fungal enzymes provided a glucose release very close to that observed when using the commercial ones, and release of xylose and cellobiose 44% and 83% higher, respectively. These data clearly show the efficiency of fungal enzymes to saccharify ozonated SCB. Furthermore, ozone pretreatment improved the releasing of reducing sugars in saccharification for both enzymes.

From these results, further ozone pretreated SCB saccharification experiments were performed using the enzymatic extract from *M. thermophila* JCP 1-4. Works dealing with the use of non-commercial microbial enzymes to saccharify ozonated SCB or other pretreated lignocellulosic materials are scarce in literature. Besides, the use of new microbial enzymes with appropriate characteristics for lignocellulosic material saccharification can greatly cheapen biorefineries processes.

3.2. Influence of temperature and time on saccharification of ozonated bagasse

Saccharification assays were performed at 40, 50 and 60 °C (temperatures around the optimal for commercial enzymes), using 3% (w/w, dry basis) ozonated SCB. *M. thermophila* JCP 1-4 enzymatic extract was loaded for 10 FPU and 30 CBU per g of cellulose content in the raw material and samples were taken at different time intervals.

Glucose release increased with the increasing of temperature (Fig. 1a). The higher release of this sugar (5.38 g/L; 39% yield) was observed at 48 h and 60 °C. Additionally, at this temperature, a glucose release of 4.99 g/L (36% yield) was obtained at only 8 h of saccharification, which can be considered a satisfactory sugar release in a short hydrolysis time, indicating that it is not advantageous to extend the experiment. The profiles of glucose releasing at 40 and 50 °C were similar, and the amount of glucose release tends to stabilize after 16 h of saccharification (around 4.0 g/L). A very close glucose yield (41.79%) was obtained by Travaini et al. (2013) with ozonated SCB produced in identical conditions of this work, using the commercial enzymes NS50013 and NS50010 (from Novozymes) during 24 h saccharification at 50 °C. Souza-Correa et al. (2013, 2014), working with ozonated SCB achieved cellulose-to-glucose conversions between 65% and 78.8%; however they used different ozonolysis conditions, 20 FPU per g of substrate and worked with just 1% of substrate concentration, during 24 h at 50 °C.

Surprisingly, fructose was also detected in hydrolysates (Fig. 1b) but not in the fungal enzymatic extract and even in substrate chemical composition, which was considered an indicative that *M. thermophila* JCP 1-4 produced a glucose isomerase. To confirm this hypothesis, glucose isomerase activity in the enzymatic extract was determined at 40, 50 and 60 °C. It is a rare characteristic, since glucose isomerase (responsible for glucose isomerization into fructose) is hardly found among fungi. The traditional glucose isomerase source are bacteria, and its production was also reported for some basidiomycetes (Horwath and Irbe, 1984), but there is no report in literature about this enzyme production by other fungi.

Relationship between temperature of saccharification and fructose release was opposite to that observed for glucose (Fig. 1b). The highest amount of fructose (1.40 g/L) was obtained after 48 h, at 40 °C. Again, from 8 h on very close amounts were obtained. Glucose isomerase activity was measured in fungal enzymatic extract. From these data it can be inferred that, during the saccharification, part of the glucose content was converted to fructose by the action of glucose isomerase, avoiding β-glucosidase inhibition and, consequently, increasing the conversion of cellobiose to glucose (Fig. 1).

Enzyme activities at 40, 50 and 60 °C were 0.36, 0.31 and 0.23 U/mL, respectively. Thus, fructose production is directly related to glucose isomerase enzymatic activity, since both decreased with the increasing of temperature. It is worth to note that in the chemical characterization of SCB it was not observed the presence of fructose, which can only have arisen from glucose conversion.

Cellobiose concentration initially increased, increasing the kinetic with temperature, but decreased after around 16 h saccharification time (Fig. 1c).

After defining the most suitable condition for saccharification, based on glucose release (8 h and 60 °C), new experiments were performed, in order to evaluate the efficiency of commercial enzymes after 8 h saccharification at 60 °C and at the optimum temperature of the commercial enzymes (50 °C). At 60 °C, *M. thermophila* JCP 1-4 enzymes provided a glucose release around 30% higher when compared to commercial enzymes mixture (4.99 and 3.50 g/L, respectively). It is interesting to observe that the saccharification efficiency of commercial enzymes at 50 and 60 °C was very close (glucose release of 3.62 and 3.50 g/L, respectively), which indicates that at 60 °C their activity is not being considerably reduced.

3.3. Monitoring fungal enzymes activities during enzymatic saccharification

FPase and β-glucosidase activities of fungal extract were monitored during saccharification experiments. Results discussed below are presented as percentage relative to the enzymatic activity measured at zero time in the saccharification media. Enzymatic extract was loaded at saccharification media to achieve 0.14 FPU and 0.42 CBU/mL in the final volume (corresponding to 10 FPU and 30 CBU by g of cellulose content in raw SCB). Identical or very similar enzymatic activities were obtained from the measurements at time zero in the saccharification media (ANOVA analysis showed no-significant differences, and so, these data are not shown).

Initially, an experiment using *in natura* SCB at 3% (w/w, dry basis) as substrate and an enzyme blank experiment containing only buffer and enzymatic extract were conducted, at 50 °C (enzyme load as described in Section 3.2). In the enzyme blank experiment, FPase activity decreased clearly with time, retaining around 70% of its original activity between 16 and 24 h (Fig. 2a). Regarding β-glucosidase activity (Fig. 2b), a slight increase was observed during the first 8 h (around 5%) and the enzyme retained 100% of its original activity until 16 h. At 24 h, β-glucosidase activity decreased only 7.5%, indicating that this enzyme is more stable than FPase. Activities of FPase and β-glucosidase were improved slightly in the experiment with *in natura* bagasse (11% and 10% higher when compared to their original activities, at 8 and 24 h of saccharification, respectively) (Fig. 2a and b). Bagasse seems to weakly stimulate the enzymatic activity of both enzymes from the fungal extract.

FPase and β-glucosidase activities were also monitored in the saccharification experiment using ozonated bagasse (described in Section 3.2). A remarkable increase of enzymatic activities was observed during the saccharification course. The most outstanding increases in FPase activity were observed in 48 h at 50 and 60 °C (284% and 274%, respectively) (Fig. 2c). Concerning β-glucosidase, it can be highlighted a maximum increase of 269.93% in 48 h, at 50 °C (Fig. 2d). This considerable increase in enzyme activities was not related to an increase in glucose release. Glucose release increased very slightly after 8 h of saccharification, probably because there were no more polysaccharides suitable for conversion or due to some complex enzymatic inhibition process. From these results, lignocellulose structure breakdown by pretreatment seems to be the limiting step of the global process. Nevertheless,

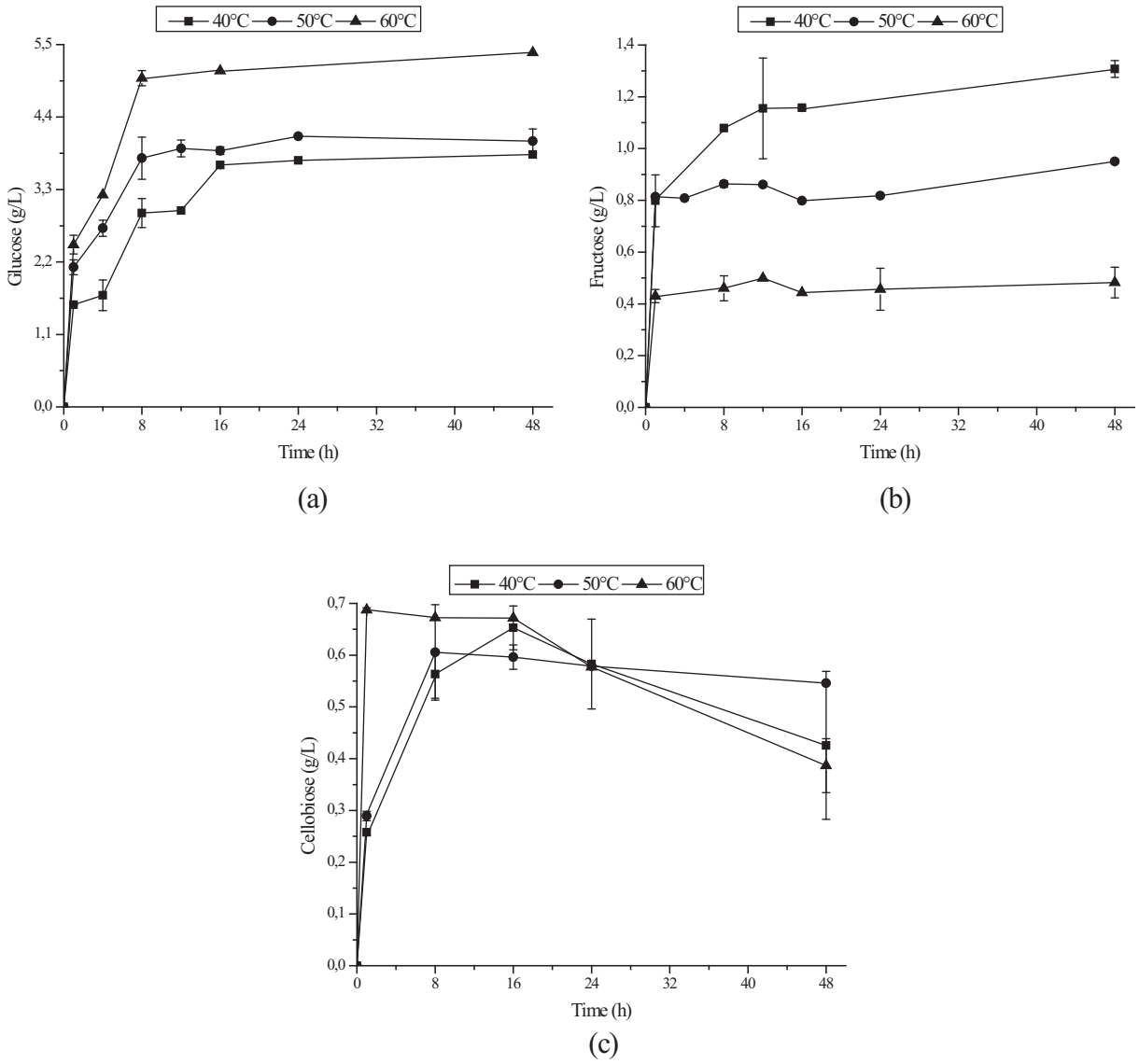

Fig. 1. Glucose (a), fructose (b) and cellobiose (c) release during saccharification of ozonated sugarcane bagasse (3% w/w, dry basis, 10 FPU and 30 CBU per g of cellulose) at different temperatures. Enzymes provided from *Myceliophthora thermophile* JCP 1-4.

the enzymatic activity increases could be very interesting when using other pretreatments.

Fructose has been described as a stabilizer of β-glucosidase activity (Weijers and Van't Riet, 1992). Since fructose was produced during saccharification by the action of glucose isomerase, the activities of FPase β-glucosidase in the enzymatic extract from *M. thermophila* JCP 1-4 were measured, at 50 °C, in the presence of this sugar. Fructose was added to the assay tubes at 0.95 g/L (the concentration found when the highest FPase and β-glucosidase activities increases were observed). In this assays, practically no increase was found on FPase activity. On the other hand, β-glucosidase activity was increased by 180% in the presence of fructose. These results suggest that fructose exerts specific activation effect on β-glucosidase activity. However, the increase in β-glucosidase activity may improve FPase action in a global view, since it removes cellobiose from media, reducing its inhibitory effect on cellulases.

The fructose effect is not enough to explain the observed increase on FPase and β-glucosidase activities during ozonated bagasse saccharification experiments. Increase on enzymatic activities are higher for ozonated SCB than for in natura SCB in all the experiments. Enzymatic activities decreased since time zero, as expected, for enzyme blank experiments without substrate. The lower increase in enzyme activation and activity retention in time found for in natura SCB can be attributed to enzymes active site protection and protein rearrangement (Kokkinidis et al., 2012). Since the increase on these enzymes activities was low during *in natura* bagasse saccharification experiments, we can infer that some compound(s) produced or released after ozonolysis pretreatment could have a more expressive effect on their activities. Apart from fructose, other factors may contribute to enzymatic activation, such as ions, which can act as cofactors helping to maintain the structure of polymeric proteins and also stabilizing active sites (Hernández-Salas et al., 2009; Iyer and Ananthanarayan, 2008).

3.4. Influence of enzyme load in ozonated SCB saccharification

Working with the best conditions of time and temperature (8 h at 60 °C) for enzymatic ozonated SCB (3% w/w, dry basis) saccharification found in Section 3.2, the influence of enzyme load was

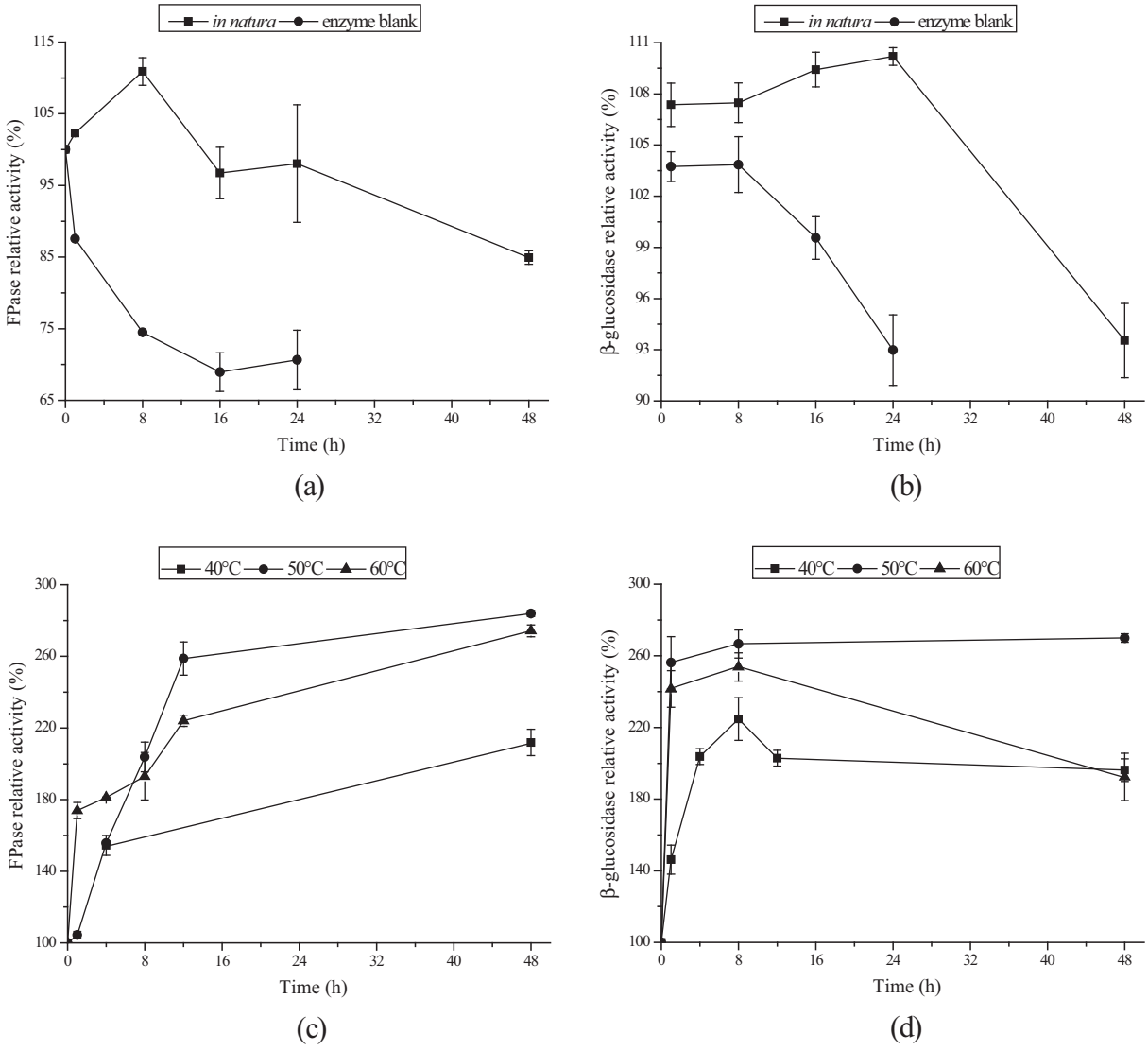

Fig. 2. *Myceliophthora thermophila* JCP 1-4 enzymes relative activities during saccharification experiments. FPase (a) and β-glucosidase (b) activities for control *in natura* bagasse (3% w/w dry basis, 50 °C, 10 FPU and 30 CBU per g of cellulose) and enzyme blank experiments (50 °C); FPase (c) and β-glucosidase (d) activities during ozonated sugarcane bagasse saccharification at different temperatures (3% – w/w dry basis, 10 FPU and 30 CBU per g of cellulose). Relative enzymatic activities compared to the activities at zero time in saccharification media (0.14 FPU and 0.42 CBU/mL, in the final volume to achieve 10 FPU and 30 CBU per g of cellulose content in SCB).

evaluated using the enzymatic extract from *M. thermophila* JCP 1-4 with basis on FPU. Enzyme loads in the range from 2.5 to 15 FPU - per g of cellulose were used. Glucose release markedly increased with enzyme loads up to 7.5 FPU per g of cellulose (4.86 g/L) (Fig. 3). Higher enzyme loads provided very slight increases on glucose release (5.21 g/L using 15 FPU per g of glucose). This enzyme load (7.5 FPU per g of cellulose) is lower than other cited by scientific literature for SCB saccharification: 10 FPU per g of cellulose (Travaini et al., 2013; Wanderley et al., 2013); 15 FPU per g of cellulose (Aguiar Souza et al., 2013; Mesa et al., 2011); 20 FPU per g of cellulose (Hongdan et al., 2013); and even values notably higher, such as 60 and 65 FPU per g of cellulose (Benjamin et al., 2013; Jiang et al., 2013).

It is well known that high enzyme loads increase glucose release during saccharification, however this is one of the most expensive steps in second-generation fuels production and in biorefineries factories. Thus, studies involving new microbial enzymes that present characteristics suitable for lignocellulosic material saccharification (high saccharification efficiency with low enzyme load, low product inhibition and stability during saccharification) are necessary.

3.5. Influence of dry matter content in ozonated SCB enzymatic saccharification and hydrolysates fermentation

The amount of dry matter is an important parameter in the study of enzymatic saccharification, since it refers to the amount of substrate to be converted by enzymes which, in turns, is related to the final hydrolysate sugar concentration, including fermentable sugars.

To achieve the desired FPU per g of cellulose in experiments with high dry matter concentration, a previous fungal enzymatic extract concentration was required. For this purpose, the fungal extract was concentrated 5 times by rotaevaporation (60 °C and 100 rpm, in vacuum, for 2 h). FPase and β-glucosidase activities were measured after concentration. Enzymes retained 90% and 64% of their original activities, respectively, providing a FBU/CBU ratio of 1:2.15 in the concentrated extract.

Saccharification experiments were performed at 60 °C, 8 h and 7.5 FPU per g of cellulose (the best conditions found in Sections 3.2 and 3.4), varying the amount of ozonated SCB in the range from 3% to 10% (w/w, dry basis). Results, in terms of glucose release and yield, are shown in Fig. 4. As expected, 10% of bagasse provided the

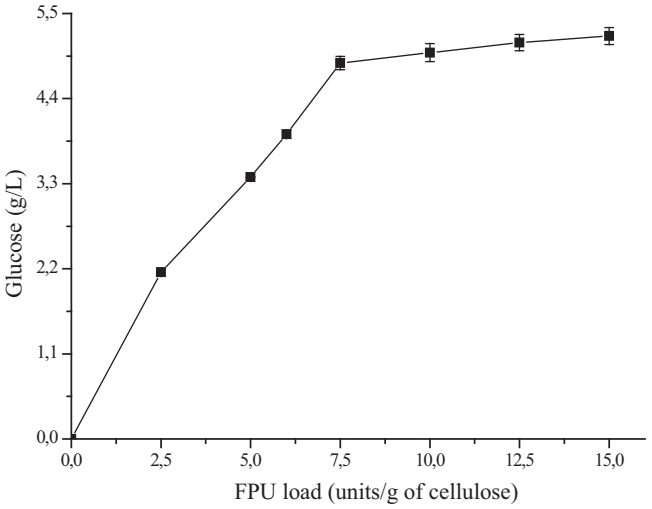

Fig. 3. Influence of enzyme load (FPU per g of cellulose) on glucose release from ozonated sugarcane bagasse in saccharification at 60 °C, 3% (w/w dry basis) during 8 h. Enzymes provided from *Myceliophthora thermophila* JCP 1-4, with a ratio FPU: CBU of 1:3.

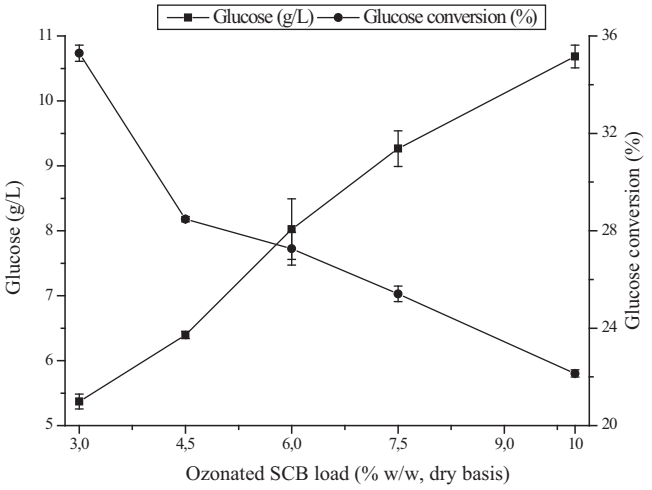

Fig. 4. Influence of dry matter content (% w/w dry basis) on glucose release (g/L) and glucose conversion from ozonated sugarcane bagasse in saccharification at 60 °C, 7.5 FPU and 15.1 CBU per g of cellulose, at 8 h. Enzymes provided from *Myceliophthora thermophila* JCP 1-4.

highest glucose release (10.19 g/L). Nevertheless, in terms of saccharification yield, the best result (30.11%) was obtained with 3% of ozonated SCB. Maitan-Alfenas et al. (2015) worked with 8% (w/v, dry basis) acid and basic pretreated SCB for saccharification with 5-fold concentrated fungal extract of *Chrysophorte cubensis*. They achieved 5.32 g/L (12.5% yield) and 2.94 g/L (7.7% yield) of glucose, respectively, for each pretreatment. However, they used 10 FPU per g of dry pretreated SCB and 72 h of saccharification at 50 °C.

High solid loads in saccharification may confer advantages and disadvantages and require an accurate analysis. A higher solid load provides higher glucose concentration after hydrolysis, increasing fermentation yield and reducing the costs related to the hydrolysate concentration. However, high solid loads usually bring lower enzymatic hydrolysis yields, due to some main factors: (1) increase on the dry matter concentration hinders the mass transfer and enzymes accessibility to biomass, being necessary higher stirring power and (2) high dry matter concentration leads to liquid absorption, resulting in a decrease of free water, reducing medium viscosity. Water is essential for hydrolysis reactions and necessary for solubilization. So, the increase on dry matter concentration can increase the process energy requirements and the production costs (Modenbach and Nokes, 2013).

3.6. Alcoholic fermentation

There is no previously published data about alcoholic fermentation of hydrolysates obtained from the saccharification of ozonated SCB. In order to test its fermentability, hydrolysates produced with fungal enzymes were fermented. Whole slurry hydrolysates from saccharification assays performed with 7.5 and 10% (w/w – dry basis) ozonated SCB were fermented with *S. cerevisiae* bakery strain to obtain ethanol. The 7.5% (w/w) dry matter hydrolysate provided 2.81 g/L of ethanol from 8.77 g/L of glucose (63% of the maximum theoretical conversion), whereas the 10% (w/w) dry matter hydrolysate gave 3.15 g/L of ethanol from 10.19 g/L (61% of the maximum theoretical conversion). Both fermentation yields are really close, indicating the absence of inhibitory compounds that could have been accumulated by high solids content. Wanderley et al. (2013) obtained 46.47% (5.33 g/L) of ethanol efficiency using enzymatic hydrolysates in the same conditions of biomass load, but using commercial enzymes.

4. Conclusions

M. thermophila JCP 1-4 is a promising fungus for bioethanol production. Its enzymes efficiency to saccharify ozonated SCB was higher than that of the commercial enzymes used and increased with temperature. Fructose was found in hydrolysates, indicating glucose isomerase production (rare among fungi). Ozonated bagasse increased FPase and β-glucosidase activities during saccharification, suggesting that pretreatment is the limiting step. The evaluated saccharification parameters influenced sugars release: optimal results were obtained at 60 °C, with low enzyme load (7.5 FPU), in only 8 h. Glucose released was converted into ethanol with a satisfactory yield, indicating no fermentation inhibition.

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