EVALUATION OF COMMERCIAL CELLULOLYTIC ENZYMES FOR SUGARCANE BAGASSE HYDROLYSIS

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Conversion of plant biomass to glucose requires the synergistic effort of cellulolytic enzymes. Commercial cellulolytic enzymes (HPL, CL, P1 and P4) were used for enzymatic hydrolysis of sugarcane bagasse and the amount of total released sugars was quantified. Total cellulolytic activity and the required enzyme concentration were determined. Then, the enzymes were tested with regard to their efficiency in pretreating sugarcane bagasse in acidic and alkaline solutions, followed by a steam autoclave treatment. Upon total sugars quantification, it was possible to conclude that the most efficient bagasse treatment was an acidic pretreatment procedure with commercial enzyme P4.

Keywords: biomass, cellulose, total sugars, cellulases, bagasse

INTRODUCTION

Sugarcane is considered one of the main agricultural commodities in Brazil with an estimated production of 684.77 million tons for the 2016/2017 crop season – a 2.9% increase from the previous year.[1] From this total amount, 39.96 million tons were destined to sugar production, while over 39 billion liters of ethanol (hydrous and anhydrous) were produced from the rest.[1]

Among the by-products of sugar and alcohol production, special attention is given to bagasse, vinasse, filter cake and yeast. These by-products have been extensively studied for reuse, seeking to reduce production costs or for further development of commercial products.[2] This is the common case of the yeast, which is used in animal feed as probiotics.[3-5] The bagasse from distilleries and sugar mills can also be reutilized in the manufacture of pulp and paper products. However, it is mostly used as a feedstock for the generation of steam, which in turn is used as energy source for the entire sugar mill and the surplus generation is exported to the local power grids. The energetic yield from a ton of sugarcane, on average, comes from 250 kg of humid bagasse (560.000 kcal), 70 liters of alcohol (392.000 kcal) and from 11.830 liters of biogas from vinasse digestion (60.000 kcal). Therefore, it is possible to conclude that there is more energy in sugarcane by-products than just the alcohol alone. Silalertruksa *et al.*[6] have successively proved, in a sugarcane refinery in Thailand, the applicability of these co-products as alternative energy sources, while emphasizing the commercial potential for these feedstocks.

The material composition of the feedstock is crucial to the correct application.[7] As one of the main sugarcane by-products, bagasse can be basically defined as a lignocellulosic material composed of cellulose (32-48%), hemicellulose (19-24%) and lignin (23-32%). Lignin is a macromolecule composed of phenylpropene units, which enables water transportation at the xylem

and increases cell wall resistance and rigidity. Also, lignin restricts cellulose saccharification by acid pretreatment and enzymatic hydrolysis. Basically, in sugarcane, lignin deposits shift spatially and temporally between plant parts, tissues and cells.[8] Hemicellulose is one of the most common natural carbohydrate polymers found in all the layers of the plant cell wall. Hemicelluloses are concentrated in the primary and secondary layers associated with cellulose and lignin. These polymers are chemically heterogeneous, differing from the homogeneous cellulose, composed of xylose, galactose, mannose, arabinose and other sugars.[9] As for cellulose, a structural polysaccharide, its direct correlation to the hydrolysis rate can be associated with several factors, such as crystallinity index and polymerization degree, among others.[6] The amount of lignin-hemicellulose surrounding the cellulosic portion of the biomass is considered the main burden for enzymatic hydrolysis. This motivates extensive research to reduce the input cost for these reactions and facilitate the access to the homogeneous polymer, thus improving hydrolysis.[10-13]

Menon and Rao[14] state that the main challenge in obtaining ethanol from lignocellulosic biomass is the glucose yield from cellulose hydrolysis and the high cost of this process. Bagasse is mostly composed of cellulose and hemicellulose, with variations in structural and physical-chemical properties (morphology, molecular orientation, chemical and mechanical resistance *etc*.). These are key factors to improve hydrolysis and optimize the glucose yield.

Before lignocellulosic biomasses can be used, a pretreatment is usually necessary to allow better activity of enzymes in the fibers. This is a crucial step since low efficiency of enzymes increase the final product cost. Pretreatments influences the entire downstream processes: the input of energy and water; effluents generated and recovered; the formation of by-products and CO_2 emission and overall efficiency.[15] Several by-products obtained from biomass pretreatments are sometimes the main inhibitory agent associated with loss in enzymatic activity.[16] Some of these by-products are: acetic acid formed by acetyl hydrolysis in the hemicellulose fraction; formic and levulinic acids from sugar degradation products; phenolic compounds formed from partial lignin degradation; furam aldehydes formed from the degradation of pentoses. When oxidative methods are applied, aldonic, aldaric, furoic, phenolic and acetic acids are formed.[16]

Nowadays, the most common pretreatment methods are acid-based,[17,18] biological,[5] hydrothermal processing,[19] mild alkaline,[20] oxidative[21,22] and alternative solvent processes.[23] Alkaline pretreatment has been considered a promising method due to several features[2], such as the use of non-corrosive chemicals (*e.g.* ammonia, sodium hydroxide, calcium hydroxide), reaction requiring milder temperatures and the selectivity of alkaline reagents, which react primarily with lignin, producing highly pure lignin polymers.[24] On the other hand, acid pretreatment is commonly associated with hemicellulose release by the breakage of strong chemical bonds under high temperatures.[12,17,18,25,26] This distinct behavior in alkaline and acid media have been understood as complementary, suggesting approaches of high efficiency when combining the co-extraction of hemicellulose and lignin polymers, affecting cellulose crystallinity.[17]

Many microorganisms in nature, mostly bacteria and fungi, are capable of producing biomass-degrading enzymes. Among the cellulolytic enzymes produced by several microorganisms, the one secreted by *Trichoderma reesei* is the most widely studied.[26-31] This organism possesses at least 5 endoglucanases (EG I, II, III, IV and V) where, from the total protein content, 5% corresponds to EG I and 0.5% to EG II, while the remaining endoglucanase production is minimal. Cellobiohydrolases are the main secreted enzyme with CBH I, accounting for 50 to 60% of the total protein, and CBH II – from 10 to 15%. However, CBH II has highly specific activity toward crystalline cellulose, when compared to the remaining cellobiohydrolases.[32] Cellulases act synergistically with hemicellulases in breaking down the plant cell wall material.

Cellulases have been commercially available for more than 30 years, and these enzymes represent a target for both academic and industrial research. Basic and applied studies on cellulolytic enzymes have demonstrated their biotechnological potential in various industries, including food, agriculture, biomass refining, textile, pulp and paper. However, the optimal combination of enzymes to influence hydrolysis depends on the nature of the substrate and the interactions between the individual enzymes.

Given the importance and the desired cost

efficiency of adequate pretreatments, this report seeks to select commercial cellulolytic enzymes by determining their total cellulolytic activity and efficiency in saccharification in pretreated sugarcane bagasse.

EXPERIMENTAL

Sugarcane bagasse pretreatment

Sugarcane bagasse was supplied by Raizen Sugar Mill, Piracicaba, SP – Brazil. The bagasse was sieved between 5.6 mm (tyler 14) and 1.19 mm (tyler 3.5) grids, air dried and placed in Erlenmeyer flasks containing the treatment solution in a 1:5 (w/v) ratio. For acid pretreatment, 0.05 M sulfuric acid was used, and for alkaline pretreatment – 0.4 M calcium hydroxide solution. The control treatment consisted of only distilled water. The material was homogenized in an autoclave at 121 °C for 30 minutes. Following the pretreatment, the pH was adjusted to 4.5 to optimize enzyme activity.

Commercial enzymes and cellulolytic activity

Four commercial enzymes were kindly donated by AB enzymes: HPL (pH 4.5-6.0; optimal temperature 50-60 °C); CL (pH 4.0-5.0; optimal temperature 60-65 °C); P1 (pH 4.5-5.5; optimal temperature 40-60 °C); P4 (pH 4.5-5.5; optimal temperature 40-60 °C). All the enzymes were obtained from lineages of *Trichoderma reesei*, and total cellulolytic activity in filter paper and enzyme concentrations were determined according to Adney and Baker,[33] Miller[34] and Ghose.[35] Using a glucose standard curve, the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes was designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC (2016)[36] and consequently the enzyme concentrations.

Lignin, cellulose and hemicellulose determination

The content of lignin, cellulose and hemicellulose in pretreated bagasse was determined according to the methodology proposed by Goering and Van Soest,[37] Van Soest,[38] Silva.[39]

Enzymatic saccharification

Following the pH adjustment of pretreated bagasse, 0.05 M citrate buffer (3:1 v/v), 100 ppm cyclohexamine and commercial enzyme solution (2:1 v/v) were added for enzymatic hydrolysis. The flasks were incubated in an orbital shaker at 40 °C for 65 hours. After incubation, the samples were collected, vacuum filtered (0.22 µm) and boiled in a water bath for 30 minutes for enzyme inactivation. The samples were filtered again, and total sugar determination was carried out by the DNS (3,5-dinitrosalicylic acid) methodology, according to Adney and Baker.[33] The samples were analyzed in triplicate, using the SAS statistical software.

RESULTS AND DISCUSSION

The acidic pretreatment with sulfuric acid presented the most distinct results, with a reduction by 12 times in the hemicellulose fraction, when compared to the control sample (bagasse *in natura*). The fractions of cellulose and lignin increased 1.3- and 1.7-fold, respectively (Table 1).

The increase in the amount of cellulose and lignin through the acidic treatment is due to the breakage of lignin and cellulose fibers located in the inner layer of the bagasse. According to literature, the variation in hemicellulose fractions occurs because the acid promotes rupture in hemicellulose fibers, generating other sugars, such as xylose and arabinose.[22,31]

Using the calcium hydroxide pretreatment, a lower reduction in hemicellulose was obtained (by 2.1 times) when compared to the control. The lignin percentage remained relatively the same (~10%), compared to that of bagasse *in natura*, and the cellulose fraction increased to only 62.38%.

The cellulase activity of commercial enzymes was measured, so as to determine the exact amount of enzyme to be used in the following treatments. In filter paper (FPU) assays, the following activities were obtained: HPL (92.5 FPU/mL), CL (52.24 FPU/mL), P1 (185 FPU/mL) and P4 (444 FPU/mL). Therefore, the concentrations of the enzymes used for the pretreated bagasse were as follows: HPL (0.0048 g/L), CL (0.0085 g/L), P1 (0.0024 g/L) and P4 (0.001 g/L).

The treatment with the alkaline solution seems to be relatively milder than the acidic treatment (Table 1). Although no reduction in the lignin fraction was seen, this alkaline pretreatment is crucial when working with lignocellulosic materials, since alkaline environments modify the crystallinity and accessibility of cellulose fibers to the enzymes.[24]

Considering the total sugar release illustrated in Figure 1, P4 presented a higher efficiency considering the lower concentration used – of 0.0010 g/L. This result could be related to the high enzyme activity of P4 (444 FPU/mL). This trend has been also observed when investigating cellulases and xylanases to ferment wheat straw biomass into sugars.[40]

Table 1
Compositional analysis of sugarcane bagasse *in natura*, after acidic and alkaline pretreatments

Samples	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Bagasse *in natura*	10.44	54.55	26.75
Bagasse + H_2SO_4	17.61	69.77	2.24
Bagasse + $Ca(OH)_2$	9.96	62.38	12.50

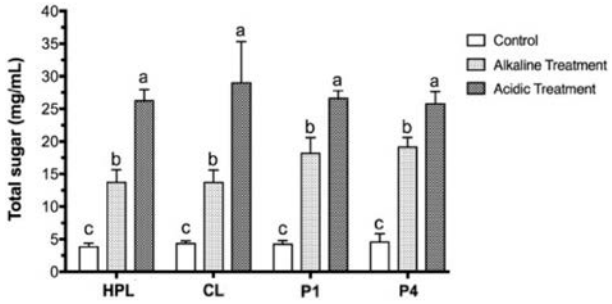

Figure 1: Total sugars (mg/mL) released by commercial enzymes, following pretreatment of bagasse; different letters indicate statistically significant differences between enzymes and treatments (Tukey's test, $p < 0.05$)

The acidic treatment yielded an increased total sugar release for all the commercial enzymes used, from 25.88 to 29.11 mg/mL (Fig. 1). Also, the alkaline pretreatment could generate a fair amount of sugars when compared to the control (ranging from 13.81 to 19.22 mg/mL). This combined action (pretreatment + enzyme) is significantly higher for the enzymes under acid pretreatment – up to 6.5- and 1.9-fold, when compared to the control and alkaline pretreatment, respectively. Nonetheless, no statistical difference was obtained for the sample treatments among the enzymes independently. From a cost-effectiveness standpoint, the enzyme with the lowest concentration used is considered more efficient, in this case, P4 with only 0.001 g/L.

Different possible pretreatment methods are known. These can include physical, biological and chemical, as well as combinations of those, *e.g.* physical treatment (high pressure/temperature) followed by a chemical or enzymatic treatment, which are often more effective. The goal of pretreatment is to prepare the feedstock for enzymatic hydrolysis, resulting in an increase in the sugar conversion.[13,22] The composition of different kinds of biomass varies. The digestibility of a given feedstock depends on properties, such as lignin content, the accessibility of cellulose and its crystallinity. Other important factors that will determine the digestibility are the degree of polymerization of cellulose, porosity,[40] hemicelluloses covering cellulose and fiber strength.

An ideal pretreatment results in a disrupted biomass structure, making it ready for hydrolysis, but does not lead to the formation of sugar degradation products or compounds that inhibit the fermentation. The pretreatments performed in this study disrupt and break the bonds between the lignin and the carbohydrates. In the case of the pretreatment involving high temperature, energy consumption is an important factor in the economic analysis of the entire process, as in any other available treatment, such as the hydrothermal and/or organosolv one.[15,6,17] Regarding the latter, common solvents, such as ethanol, methanol, acetone and glycols, are all mixed with water. Then, the removal of the solvents is necessary, since they can act as inhibitors in the downstream process. The recovery of the solvents is an important aspect regarding the economics of the process and major costs may be associated to this recovery step.[40]

CONCLUSION

This report successfully demonstrated that a pretreatment of the raw material is an essential step for the complete sugarcane bagasse hydrolysis, since it contributes to breaking down bagasse fibers, thus, facilitating the access of cellulolytic enzymes. Among the pretreatments tested, the one involving 0.05 M sulfuric acid was the most efficient, and in combination with low amounts of the commercial enzyme P4 yielded the highest amount of total sugars. Further, it was shown that the enzyme actions on their own are

not sufficient for a complete hydrolysis. Also, no statistical differences were found among the four enzymes tested for sugarcane bagasse saccharification.

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