Effects of alkaline and alkaline-oxidative chemical pretreatments of crop residues on enzymatichydrolysisbyfungalcellulases

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Abstract—Lignocellulosic materials such as crop residues arevery abundant and can be an important source of carbohydratesfor bioprocesses such as the production of bioethanol. Cellulose is a biopolymer formed by glucose units and cellulases enzymes,under specific physical and chemical conditions, can hydrolyse the cellulose chain producing fermentable sugars. Cellulases enzymes were produced by *Aspergillus niger* using sugarcane bagasse as the fermentation solid substrate. The cellulases were used for hydrolysing the sugarcane bagasse, corn straw and wheat strawas enzyme´s substrate,with and without chemical pretreatments. The pretreatments increase the cellulose content, modifies its structure and enhance the cellulase activity. The alkaline treatment usesNaOHsolution at 4% (w/v) and the alkaline-oxidative treatment uses 1% (v/v) H_2O_2/NaOHsolution at pH11.5. The aim of this work was to compare the effects of alkaline and alkaline-oxidative pretreatments of thesugarcane bagasse, corn straw and wheat straw on their cellulose content and on the enzymatic hydrolisis using cellulases produced by *Aspergillus niger*. It was concluded thatboth alkaline and alkaline-oxidative pretreatmentsincreases the cellulose content and improves the cellulases activity. The alkaline pretreatment provided higher cellulose content and the alkaline-oxidative pretreatment provided higher cellulase activity and was more effective for the straw type substrates.

Index Terms—cellulase, cellulose, enzyme,fermentation, corn straw,lignocellulosic biomass, sugarcane bagasse, wheat straw

1 INTRODUCTION

Lignocellulosic materials are produced by plants as part of their constitutive biomass being the most abundant agro-industrial residues in the world. Due to thehigh cellulose content they can be an important source of fermentable carbohydrates for manybioprocesses. Recently, lignocellulosic biomasses have gained an increasing research interests and special importance due to their renewable nature as a raw material for the production of bioethanol. The development of new fermentation processes utilizing lignocellulosic biomass as a raw material for biofuel production can minimize the world´s dependence on fossil fuels by providing a convenient and renewable source of glucose [1]. Brazil has a wide variety of agricultural and agroindustrialresidues and the processing of those wastes may be of great economic, social and environmental interest [2]. There are plenty of waste lignocellulosic materials derived from activities such as pulp and paper industries, wood processing, production of ethanol and sugar from sugarcane, agricultural crop production of cereals, fruits, among others [3].

Lignocellulosic materials are composed of cellulose, hemicellulose, lignin and minor amounts of extractives [4]. The celluloseis a biopolymer made of glucose units linked by β-(1-4) glycosidic bonds. The cellulase enzymes can release the glicose molecules from the cellulose chain by hydrolisis. The cellulose structure hassome crystalline regions that present more resistence to enzymatic hydrolysis. The less ordered and amorphous areas where the cellulose chains have random orientation are more susceptible to enzyme activity[5]. The adjacent cellulose chains form a set of aggregates, or elementary fibrils, that are associated with each other to form the cellulose crystallite. Subsequently, four of those aggregates are joined by a monolayer of hemicellulose and lignin. The natural compound that results from this association is called cellulose microfibril [6]. The association of different types of biopolymers that comprise the vegetable matter (cellulose, hemicellulose and lignin), the degree of crystallinity and the packaging caused by the complex structure of lignin form a rigid structural material that is naturally very resistant to the enzymatic hydrolisis. This characteristic makes being necessary applies some pretreatments already in the early stages of bioconversion of the lignocellulosic biomass to ethanol [5] and the pretreatments aim to remove the lignin and hemicellulose barriers to hydrolisis. In addition, the pretreatments reduce the cellulose crystallinity and increase the porosity of the lignocellulosic biomass and thereby exposing the cellulose to the enzymatic action. There are several types of physical and chemical pretreatments that can be applied in the lignocellulosic biomass and wich increase the susceptibility of cellulose to enzymatic hydrolysis [7]. An alkaline chemical pretreatment is performed using solutions of NaOH, $Ca(OH)_2$ or NH_3. It removes part of the lignin and part of the hemicellulose wich improves the accessibility of the cellulase enzymes. The alkaline pretreatment is considered being very effective in breaking the bonds between cellulose, hemicellulose and lignin, and promote the fragmentation of hemicellulose [8]. Compared to the alkaline, the alkaline-oxidative chemical pretreatment adds hydrogen peroxide (H_2O_2) to the alkaline solution andit-

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was used in several studies of enzymatic hydrolysis of lignocellulosic materials: Rabelo [9] evaluated the performance of pretreatment with alkaline hydrogen peroxide to the enzymatic hydrolysis of sugarcane bagasse. Krishna *et al.*[10] and[11] treated the lignocellulosic biomass with 1% H_2O_2 (v/v) solution at pH 11.5 and the saccharification obtained was near 100%.

Several microorganisms can producecellulasic enzymes. Cellulases are able to hydrolyze the cellulose chain producing low molecular weight sugars like glucose and cellobiose[12]. MuthuvelayudhamandViruthagiri [13] cultivated the fungus *Trichoderma reesei* to produce cellulases using sugarcane bagasse and rice straw as the fermentation substrates. Ojumu*et al*. [1] used the fungus *Aspergillus flavus* to produce cellulases using powder-saw, sugarcane bagasse and corncobs as the fermentation substrates.

The enzymes that are involved in the hydrolysis of cellulose is called cellulasic enzyme complex. According to [14] such complex is divided into three groups,depending to their place of actuation in the cellulosic substrate:

1) Endoglucanases: the enzymes that ramdomly hydrolyze the internal regions of the amorphous structure of the cellulose chain and produce oligosaccharides and, consequently, new reducing and non-reducing terminals;
2) Exoglucanases: the enzymes that are divided into cellobiohydrolases (CBHs) and glucanohidrolases (GHs). The CBHs are responsible for releasing cellobiose from the cellulose ends. The GHs are able to release glucose directly from the cellulose chain.
3) β-glycosidases: the enzymes the hydrolysecellobiose and the soluble oligosaccharides (with less than seven monomeric units) into glucose.

Aspergillus niger is a microfunguscommonly found in nature.It is grown in many fermentation processes for the production of some enzymes and organic acids under specific physical and chemical conditions.*Aspergillus niger* produces cellulases and It may be considered superior to other fungi that are known being good producers of the cellulasic complexes, such as *Trichoderma reesei* [15].

This study aimed to compare the effects of alkaline and alkaline-oxidative chemical pretreatments of crop residues on their cellulose content and in their enzymatic hydrolysis using *A.niger*cellulases. It was used sugarcane bagasse, corn straw and wheat straw as lignocellulosic substrates and the cellulase enzymes were produced by fermentation using *Aspergillus niger*.

2 Materialand Methods

2.1 Lignocellulosic substrates

It was used sugarcane bagasse, corn straw and wheat straw as lignocellulosic materials. Sugarcane bagasse was kindly supplied by COOPCANA (CooperativaAgrícola Regional de Produtores de Cana) located in Paraíso do Norte, PR, Brazil. Corn straw and wheat straw were collected in the plantation fields, soon after harvesting.

2.2 Physical pretreatments

The wheat straw and corn straws were sun dried and milled in a Trapp hammer mill, model TRF-400, and then sieved in No.4 mesh. The sugarcane bagasse was sun dried only. All dried and powdered lignocellulosic substrates were stored in sealed plastic bags and kept in the fridge for later use.

2.3 Chemical pretreatments

Alkaline treatment: the alkaline treatment was carried out according to the procedure described by Aguiar and Menezes [1]: after the physical pretreatment, the lignocellulosic substrates were immersed in a 4% (wt/wt) NaOH solution and autoclaved at 121 °C during 30 min. and then they were extensively washed with tap running water. Phosphoric acid was added until neutral pH was reached. The washed, now alkaline treated substrates were oven dried at 65 °C.

Alkaline-oxidative treatment: the alkaline-oxidative treatment was adapted from the methodology described by Krishna et al. [11]. The substrates were immersed in distilled water during a period of 4 hours for removing most of its soluble contents. After, they were oven dried at 50 °C and then transferred to Erlenmeyer flasks with 1% (v/v) H_2O_2 solution at pH 11.5 by addingNaOH. The flasks were shaken during 16h at 200rpm at room temperature. The insoluble fractions representing the alkaline-oxidative treated substrates were recovered by filtration and extensively washed until neutral pH and then oven dried at 50 °C.

2.4 Cellulases production by fermentation

The fermentation was carried out in 2000 mL Erlenmeyer flasks that were autoclaved at 120 ^{o}C during 20min. The fermentation solid substrate was the alkaline treated sugarcane bagasse (100 g/L) as the main carbon source and then using 1000mL of culture medium described by Mandels and Weber [16]. Tween 80 (1mL/L) was added to the culture medium andthe flasks were inoculated with 10mL of *Aspergillus niger*sporessuspension (about $1x10^6$ spores/mL)and incubated at 30^{o}C during 7 days as described by de Aguiar and de Lucena [17]. The fermentation broth was recovered by filtration and the liquid fraction containing the cellulases (enzyme extract) was used for the enzymatic hydrolysis of the cellulosic substrates.

2.5 Enzymatic hydrolisis of the cellulosic substrates

The enzymatic hydrolysis of the sugarcane bagasse, wheat straw and corn straw (cellulosic substrates) were based on the adapted methodology described by Ghose [18]. In triplicated analysis assay tubes were added 300mg of one chemically pretreated cellulosic substrate, 4mL of 50mM, pH 4.8 acetate buffer and 2mL of the enzyme extract. The tubes were incubated at 50 ^{o}C for 50 min.The total reducing sugars (TRS) released during hydrolysis were measured according the methodology described by Miller (1959) [19]. It was defined that one unit of cellulase activity (1U) releases 1μmol of TRS by 1 mL of the enzyme extract by 1 minute (1U = 1 μmol mL^{-1} min^{-1}) using the selected cellulosic substrate.

2.6 Cellulose contentdetermination

The cellulose content of the sugarcane bagasse, wheat straw and corn straw (with and without the chemical pretreatments) was determined in triplicated samples analysis according to Van Soest methodology described by Silva and Queiroz (2002)[20].

3 Results and Discussions

The results of the chemical pretreatments on the enzymatic hydrolysis are shown in fig.1 and table 1, respectively. After the physical treatments the sugarcane bagasse, wheat straw and corn straw were submitted to the chemical treatments before effecting the hydrolysis by cellulases. The fig.1 shows the results of the enzyme activitywhen using the cellulosic substrates with no chemical treatment, with alkaline treatment and with alkaline-oxidative treatments.

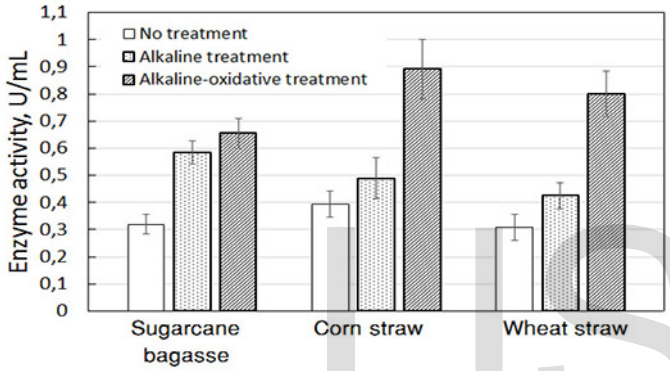

Fig.1.Effect of alkaline and alkaline-oxidative treatments of the lignocellulosic substrates on cellulase activity.

Table 1shows the cellulose contentof the lignocellulosic residues after applying the chemical treatments comparedwith no treatment applied.

TABLE 1
Cellulose content in dry-basis of the lignocellulosic residuesaccording the treatment type.

Lignocellulosic residues	Treatment type	Cellulose content (%, Dry Wt.)
Sugarcane bagasse	no treatment	63,4±0,4
	alkaline	71,2±0,5
	alkaline-oxidative	65,5 ±0,1
Corn straw	no treatment	40,3 ± 0,3
	alkaline	77,4 ± 0,2
	alkaline-oxidative	66,7 ±1,7
Wheat straw	no treatment	44,9 ± 2,9
	alkaline	76,0 ± 0,1
	alkaline-oxidative	70,5 ± 0,8

By comparing the results shownin fig.1,all the chemical treatments that were applied have a positive effect on the enzyme activity when using the cellulases produced by*Aspergillus niger*. There was an increase in the cellulase activity when compared to the enzyme activity that was obtained by usingsubstrates that had no chemical treatments. These results were expected because the chemical treatments modify the microstructures of the cellulosic substrates and increase the cellulose content, as shown in table 1. The highest enzymatic activities were obtained for the residues that were submitted to the alkaline-oxidative treatment. It is also observed that the alkaline-oxidative treatment is very effective for the straw type residues in such a way as almost to double the cellulase activity when compared with the enzyme activities obtained for the substrates submitted to the alkaline treatment. According to Gupta (2008) [5], the alkaline-oxidative treatments have been used to dissolve the components of the lignocellulosic matrix, to decrease cellulose crystallinity and to expose the cellulose chains in such a way that effectively enhances the enzymatic hydrolysis. Pitarelo (2007) [6] also states that the alkali tends to reduce the crystallinity of the cellulose chain, which improves the action of cellulases. Table 1 shows that the cellulose content increased after applying the alkaline and alkaline oxidative treatments to the lignocellulosic residues. This change in compositionoccurrs because those chemical treatments solubilize and removepart of the lignin and hemicellulose and thus increase the cellulose content in the solid fraction of the residue. The alkaline treatment usingNaOH only was slightly more effective in increasing the cellulose fraction compared to the alkaline-oxidative treatment that uses H_2O_2/NaOH; however, that higher cellulose content did not yield higher cellulase activity meaning that the alkaline-oxidative treatment is better in leaving the cellulose chains more susceptible to the enzyme activity. Ifthe enzimatichydrolisis of a lignocellulosic residue is the goal, then the alkaline-oxidative pretreatment should be applied instead of applying the alkaline pretreatment. The alcaline-oxidative pretreatmentis more effective in modifying the cellulose chains by making them more susceptible to the enzymatic hydrolisis, specially the straw type residues.

4 Conclusion

The alkaline and alkaline-oxidative treatments were very effective and provided higher cellulase activity and higher cellulose content when compared to those results that were obtained by using substrates with no previous chemical treatments. The alkaline pretreatment provided higher cellulose content for all evaluated lignocellulosic residues and the alkaline-oxidative pretreatment provided higher cellulase activity.In addition,the alkaline-oxidative treatment is even more effective for treating the straw type residues that were submitted toenzymatic hydrolisis.

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