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Production of Lignocellulolytic Enzymes and Application in Waste Hidrolysis Using Mixture Design

Vanessa Elisa Pinheiro[1], Lummy Maria Oliveira Monteiro[1], Paula Zaghetto de Almeida[1], Maria de Lourdes Teixeira de Moraes Polizeli[2]

[1]Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo – Departamento de Bioquímica Ribeirão Preto – SP - E-mail: vanessa.well.usp@gmail.com
[2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto – Universidade de São Paulo – Departamento de Biologia – Ribeirão Preto - SP

ABSTRACT

Industries generate tons of lignocellulosic wastes, as used paper, sugar cane and barley bagasse. These wastes may be recycler in many processes, once they serve as a raw material for the biotechnological production of various compounds. The waste biodegradation by enzymes is a viable alternative since they can efficiently act and replace the alkali and acid pre-treatments, which may be harmful to the equipment and environment. The objective of this work was to produce a powerful enzymatic extract for the agroindustrial waste hydrolysis from the filamentous fungus Aspergillus versicolor*, using barley bagasse, as carbon source. The enzymatic extract produced was applied in the hydrolysis of barley, sugarcane bagasse, and recycled paper, using an interesting mixture design, where was checked which waste the enzymes could act more efficiently, and improve cellulose pulp production for paper industries. It was determined that the enzyme extract mainly acted on barley bagasse, using methods of dosage of reducing sugar, thin layer chromatography and Statistic analysis.*

Keywords: agroindustrial wastes, enzymes, mixture design, enzymatic hydrolysis, TLC

INTRODUCTION

The agroindustrial production, as sugar cane and barley, generates tons of lignocellulosic wastes throughout the processing, such as sugar cane and barley bagasses. Many of these residues, which apparently have no function can be used in many other industrial processes, serving as raw material for sustainable production of ethanol, organic acids, enzymes, paper (Soccol *et al*., 2003). The production of recycled paper is another industrial production aimed to cooperate with the environment, once the paper used and discarded (waste) can be collected and generate a new paper (Pinheiro *et al*., 2013).

Countries are trying to adapt to the reuse and recycling of products, in order to reduce the generation of pollutants and waste from industrial activities. The industry of beer in Brazil is the most important in South American market and it is one of the largest in the world, which means that this industry generates a significant amount of waste, mainly barley bagasse (Sindicerv, 2011). This waste is composed by hemicellulose, lignin, cellulose, protein, extractives and ash (Cabral-Filho *et al.*, 2007). Then, biochemical studies indicate that the modifications of the plant fibers require the presence of microbial xylanolytic, cellulolytic, and/or amylolytic systems (Ibarra *et al*., 2012). Due the large quantity of barley bagasse generated in the industry and its composition in carbon source that can induce the lignocellulosic enzyme production, this work aimed the lignocellulolytic enzyme production from *Aspergillus versicolor*, selected by a previous screening for the agroindustrial waste hydrolysis, which destroy the fiber integrity and then facilitates the biobleaching, drainage and retting of the cellulose pulp and helps the paper production through these materials.

MATERIAL AND METHODS

Crude extract (CE) production: A. versicolor was the fungus selected by a previous screening using 15 fungal strains from mycology collection of Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo. The submerged fermentation was performed by inoculating 1 mL of spores solution (10^9 spores), into 125 mL Erlenmeyer flasks containing 25 mL of M5 medium (Peralta *et al*., 1990). The medium was supplemented with 1% (w/v) of barley bagasse (waste) as carbon source, incubated at 30°C, under static condition, for 144 h. The culture was vacuum filtered using Büchner funnel and Whatman nº 1 filter paper, for later use.

Enzymatic and Protein assays: Amylase, xylanase, cellulases, arabinases, β-glucanases, mannanase and pectinase activities were determined by reducing sugar (Miller, 1959). Besides, was also determined β-glucosidases, arabinofuranosidases, cellobiohydrolases, lipases and β-xylosidase activities, using synthetic substrates based on generation of p-nitrophenol. The assays were incubated at 50°C, in 100 mM sodium acetate buffer, pH 5.5 with time and final volume of reaction ranging for each enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme equivalent to release 1 µmol of product per minute, in the assay conditions. The protein quantification was performed according to Bradford (1976) using bovine serum albumin as standard and the absorbance was measure at 595 nm. Protein was expressed as mg/mL.

Mixture design: A mixture design was performed with 12 assays, which ranged in concentrations of the barley bagasse, sugar cane bagasse and magazine paper. The enzyme extract (20 mL) were mixed with 40 mL of 100 mM sodium acetate buffer, pH 5.5 (with 15 mM sodium azide to prevent bacterial growth). Each essay was performed with 5 mL of this mixture and 50 mg of substrate total. Three repetitions of the central point also were carried out at 50°C, in a dry bath, with stirring (500 rpm), for 24 h. The hydrolysis was stopped by heating of the samples in boiling water for 5 min. The reducing sugars were detected according to Miller (1959) and the data were analyzed by Statistica Software 12.

Analysis of hydrolysis products and protein profile: The waste hydrolysis products using CE were detected by thin layer chromatography - TLC (DC-Alufolien Kieselgel 60, Merck). It was used 1 µL of standards – (i) 0.1% (w/v) glucose, maltose, maltotriose, maltotetraose and maltopentaose, and (ii) xylose, xylobiose, xylotriose and xilotetraose, which are possible products due to enzymatic hydrolysis of large chain saccharides. After elution, hydrolysis products were revealed with 0.2% orcinol in n-butanol/distilled water:acetic acid (5:3:2) after incubation at 100°C, for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10% (Laemmli 1970) was used to identify an enzyme profile present in CE. The protein was stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

The CE showed various lignocellulosic enzymes activities (Figs. 1A,B). The presence of a large number of lignocellulosic enzymes in the CE contributes to the implementation of an enzymatic consortium, once it can lead to a complete degradation of the biomass, composed of natural polymers – arrangement between cellulose and hemicelluloses linked and fixed to the lignin, providing strength and rigidity to the plant cell wall (Salvador *et al.*, 2013). Then, in cellulose pulp production, cellulases smooth the fibers, increase drainage, and stimulate ink removal; xylanases and ligninases help to reduce the amount of chemicals for bleaching and lipases reduce pitch. The protein profile of the CE is shown by SDS-PAGE (Fig. 1C).

A mixture design was carried out in order to determine the waste (barley bagasse, sugar cane bagasse and magazine paper) that had been more hydrolysable by studied enzymes. The ANOVA of the linear model demonstrated that the model is highly significant, as is evidenced

from the Fisher's *F*-test. The computed *F*-value (148.72) is much higher than the tabular *F*-value (10.92 at the 10% level). The R^2 value of 0.98 indicates that the model could explain 98% of the variability in the response. The linear effects of components were found to be significant model terms for reducing sugar formation, as is evident from its *p*-value.

The equation found was: Reducing sugar (mg/mL) = 1.479A + 0.297B + 0.310C. Validation of the experimental model was carried out by conducting the batch experiment under the conditions of 66.66% barley bagasse, 33.33% sugar cane bagasse and 0% paper. The experiments were performed in triplicate and the results were compared. The condition led to formation of 1.088 ± 0.08 mg/mL of reducing sugars, which are comparable to the model predicted data (1.131 mg/mL).

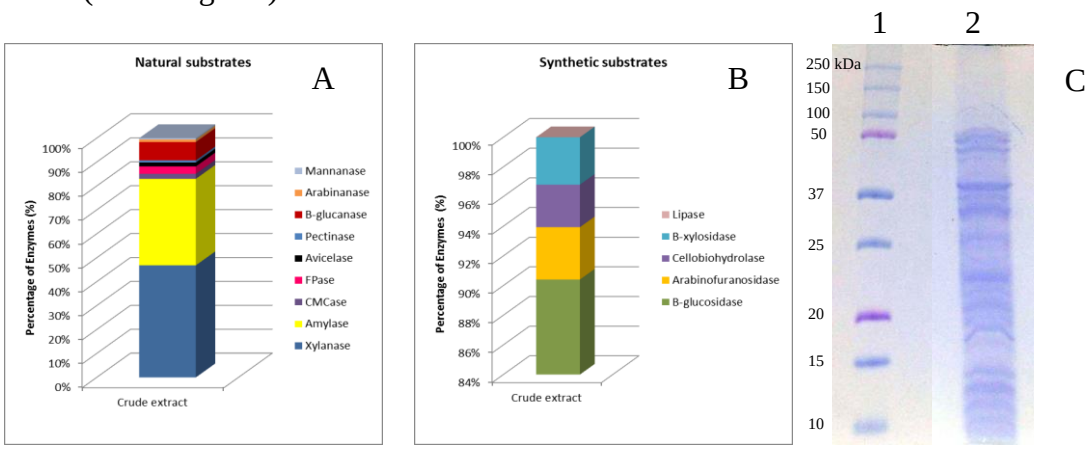

Figure 1. Percentage of enzyme activities **(A and B)** and protein profile on SDS-PAGE of the CE **(C). (A)** Activities determined by Miller method (1959); **(B)** Activities determined by synthetic substrates, as described in Methods; **(C)** SDS-PAGE of CE, where lane **1:** molecular weight marker; lane **2** CE.

One triangular surface response graph was constructed (Fig. 2A). The barley bagasse was the one that stood out in the formation of simple sugars by action of synergistic enzymes in the extract. This fact could be related to the pretreatment received in the beer industry (fermentation process), which can have modified the cell wall's waste and made the bagasse available to enzymes. In addition, analyzing the literature about the cellulose composition in sugar cane and barley bagasses, the second one has lower amount of cellulose (167g/Kg) (Mussatto & Roberto, 2006) than to sugar cane bagasse (352 g/Kg) (Rezende *et al*., 2011). This cellulosic fraction is hydrolyzed into glucose and short-chain oligosaccharides by cellulases, which are not in high levels in the enzymatic consortium. On the other hand, the barley bagasse has larger amount of hemicelluloses (567g/Kg) (Mussatto & Roberto, 2006) than sugar cane bagasse (245g/Kg) (Rezende *et al*., 2011), and the CE has a large number of hemicellulases, mainly xilanases, which can act upon this polymer hydrolyzing xylan into xylose and short-chain oligosaccharides. For this reason a waste composed of more hemicellulose than cellulose, should be more hydrolysable due to the enzymatic consortium in the CE. The synergic action of enzymes, mainly hemicellulases, can act breaking the chains of hemicellulose and then releasing the lignin, providing less strength and rigidity to the plant cell wall which will collaborate with the paper production like less water used, less chemical products used in the production process, more whiteness paper and softer paper. Under magazine paper (*couché* paper) the CE had presented a low hydrolysis due to its composition, ink, additives, calcium carbonate, latex, kaolin and starch in the coating (Grupo ROYAL, 2016), that difficult the enzymatic access into the cellulose composition. TLC was carried out to detect the saccharides formation by CE hydrolysis and in the first three samples (zero time assay) can be observed the presence of

residual sugars traces before hydrolysis (Fig. 2B). However, after enzymatic hydrolysis by 24 hours of the barley bagasse (lane A1), sugar cane bagasse (lane A2) and magazine paper (lane A3), there are the formation of significant amount of mono- and short-chain oligosaccharides as result of enzymatic consortium present in the CE, like glucose and short-chain oligosaccharides as maltose, maltotriose, maltotetraose and maltopentaose resulted by mainly activity of cellulases and amylases, and xylose and short-chain oligosaccharides as xylobiose, xylotriose, xilotetraose by hemicellulases, mainly xylanases.

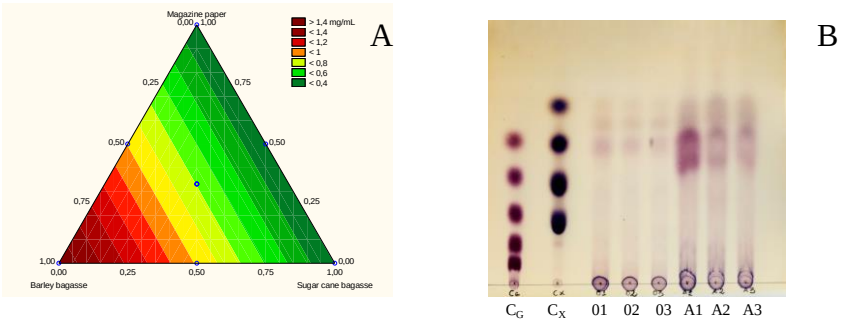

Figure 2. Triangular surface response **(A)** and TLC **(B)** related to the performance of the mixture design. **(A)** Triangular surface response of barley bagasse, sugar cane bagasse and magazine paper. **(B)** TLC, where **(C_G)** is the glucose standard constituted by (up to low): glucose, maltose, maltotriose, maltotetraose and maltopentaose, **(C_X)** is the xylose standard (up to low): Xylose, xylobiose, xylotriose, xilotetraose; Lanes 1, 2 and 3 represent the zero time of hydrolysis; Lanes A1, A2 and A3 are the end products result of 24 hours hydrolysis of barley bagasse, sugar cane bagasse and magazine paper, respectively.

CONCLUSION

The CE produced by *A.versicolor* is able to hydrolyze, in 24 hours, complex sugars present in industrial waste tested into monosaccharides and short-chain oligosaccharides due to the enzymatic consortium present in the CE. This result evidences the enzymes importance in waste hydrolysis, improving the paper making and thus is considered as a "green" product, once they are natural compounds, with little adverse impact on the environment.

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