Evaluation of sugarcane bagasse and flower stems as substrates for cellulase production by *Bacillus amyloliquefaciens C18 A*

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Abstract
Microbial cellulase production largely depends on the substrates and microorganisms used. Some lignocellulosic agroindustrial wastes can be utilized to produce these enzymes, thus avoiding environmental pollution. This study evaluated the production of cellulases by Bacillus amyloliquefaciens (isolate C18A) in culture media prepared from sugarcane bagasse and flower stem waste; the media contained 56.97% w/w and 58.54% w/w cellulose respectively. The media was incubated for 72 hours at 37 °C and samples were taken every 12 hours to evaluate the cellulose degradation and enzymatic activity in the crude extracts at pH 5.0 and pH 7.0. Positive control cellulases were produced in Mandels media using same conditions of incubation and microorganism.

The cellulolytic activity at pH 7.0 was higher (35.06 U/mL) for the cellulases produced in the flower stem medium, while the enzymatic activity at pH 5.0 was higher (39.09 U/mL) for the cellulases obtained from the bagasse medium. Cellulases produced in Mandels medium presented activities of 29.94 ± 0.05 U/mL at pH 7.0 and 31.72 ± 0.06 U/mL at pH 5.0 demonstrating that these wastes are sources of available cellulose that can be utilized for cellulase production by B. amyloliquefaciens C18A.

Keywords: Enzyme activity, cellulolytic activity, cellulose, lignin, agroindustrial wastes.

Introduction

Cellulose is an insoluble, high-molecular-weight biopolymer formed from glucose units linked by β-1,4 glycosidic bonds [1]. It is present in the fibers of plants such as cotton, jute, flax and hemp. Wood contains between 40 and 50% cellulose in its structure where it serves as support. Cellulose is the main component of plant cell walls [2].

Plants contain substantial amounts of cellulose that could be an important carbon source for the production of various industrial products; however, the bioconversion of cellulose to glucose is a pre-requisite for microbial fermentation. Cellulose can be degraded to D-glucose via enzymatic hydrolysis by cellulases from cellulolytic bacteria or fungi of the genera *Bacillus*, *Streptomyces*, *Aspergillus*, *Chaetomium* and *Trichoderma* [3]. Cellulases hydrolyze the β-1,4 glycoside bonds of cellulose; furthermore, unlike chemical hydrolysis, cellulases reduce biomass waste but do not generate polluted by sub-products[4].

Cellulases have an important direct role in generating products in the biofuel industry including the production of ethanol [5], solvents and organic acids in the food industry by improving the yield and of extraction of fruit and vegetable juices, in the pulp and paper industry as a co-additive in pulp bleaching and in the textiles industry in the biopolishing, biostoning and biofinishing of fabrics, among others, ranking third in significance in the enzyme market[6].

Corn fiber and stover, sugarcane bagasse, rice husks and straw, woody crops and forest residues are agroindustrial wastes that are considered renewable resources and have potential as sources of cheap and easily accessible lignocellulosic biomass[7]. Orange peel, coconut biomass, sawdust, paper pulp, other industrial wastes and even cellulosic municipal solid waste and ground paper sludge have been industrially used as lignocellulosic sources; additionally, the production of cellulases from agroindustrial wastes, including corn flour, wheat bran, corncob, rice husk, sesame oil cake, soybean meal, cotton seed meal and sugarcane bagasse, has been reported[8-10].

The objective of this study was to evaluate the production of cellulases by *Bacillus amyloliquefaciens* (isolate C18A), previously isolated from the soil of wetlands in Bogotá in liquid media formulated from sugarcane bagasse (SB) and flower stems (FS). A qualitative test was used to determine the residual cellulose in the culture media and a quantitative test was used to determine the enzymatic activity of the cellulases obtained from each evaluated media.

Material and Methods

Pre-treatment of wastes: FS waste was collected from the market square in Paloquemao, Bogotá, Colombia and SB was collected from a sugarcane mill in Tobia, Cundinamarca, Colombia. The wastes were washed with potable water, cut into pieces of between 0.5 and 1.0 cm in length, dried at 70 °C in a Binder FED 115 oven to a constant weight and stored at -80 °C until use.

Characterization of residues

Determination of moisture and ash: Five grams of untreated waste were weighed and the percentage of water was determined via gravimetry. Once the moisture of the wastes was quantified, the ash content was determined [11].

Quantification of cellulose and lignin: Cellulose and lignin were quantified according to the method proposed by Kürschner-Hanack, cited by Kulić and Radojičić [12]. Each type of previously frozen agroindustrial waste was placed directly in a Binder FED 115 oven at 70 °C for 1 hour. Once dried, 20.5 g of FS and 21.8 g of SB were weighed and then added separately to an ethanol-hexane (1:1) solution. The mixture was boiled in an open reflux system for one hour to extract tannins, waxes, gums and resins. Subsequently, the residues were recovered via vacuum filtration, washed with 100 mL of ethanol and dried in an oven at 105 °C to a constant weight. Cellulose and lignin were extracted by boiling in an open reflux system with a mixture of acetic acid-nitric acid (10:1) for one hour, the residue was vacuum filtered and washed with distilled water and the fibers were transferred to a beaker to which water was added and neutralized with 40% w/v sodium hydroxide (NaOH) solution.

The neutralized residues were vacuum filtered, washed with distilled water and dried at 105 °C to a constant weight. Based on this final weight, the amounts of cellulose and lignin in the samples were determined. To extract the lignin from the previously treated residues, the residues were boiled in an open reflux system for two hours with NaOH at 17.5% w/v, vacuum filtered and washed in distilled water.

The pH of the fibers was neutralized with a concentrated hydrochloric acid solution and the residue was again vacuum filtered, washed with distilled water and dried in an oven at 105 °C to a constant weight. The final weight after drying corresponded to the cellulose content of the samples. The extraction and quantification of lignin and cellulose were performed in triplicate for each waste type.

Culture medium with SB: The treated and characterized SB stored at -80 °C was dried at 105 °C for one hour. Approximately 1.755 g of dried SB was sterilized in an autoclave and sterile standard culture medium (SCM) (0.14% w/v ammonium sulfate, 0.15% w/v potassium dihydrogen phosphate, 0.03% w/v urea, 0.04% w/v calcium chloride, 0.03% w/v magnesium sulfate and 0.075% w/v peptone) was added to a final volume of 100 mL with a cellulose concentration of 1.0% w/v.

Culture medium with FS: The treated and characterized FS at -80 °C was dried at 105 °C for one hour. Approximately 1.708 g of dried FS was sterilized in an autoclave and mixed with SCM to a final volume of 100 mL for a cellulose concentration of 1.00% (w/v). For the positive and negative controls, 100 mL Mandels broth composed of SCM and 1.0% carboxymethylcellulose (CMC)[13] was prepared.

Production of cellulases: Cultures were performed using a strain of *B. amyloliquefaciens* C18 previously isolated from wetland soil from Bogotá and stored in 1mL cryovials at -80 °C [14]. To reactivate the microorganism, the cryovial contents were added to 100 mL of nutrient medium and kept at 37 °C for 24 hours. A 10 mL aliquot was taken from this culture, added to 90 mL of Mandels broth and incubated at 37 °C for 24 hours. From this culture, 10 mL was inoculated at a concentration of $6.0x10^8$ cells/mL in 100 mL of SB culture medium at pH 6.0, FS culture medium at pH 6.0 or Mandels broth, which was used as a positive control. Uninoculated Mandels broth was used as a negative control. All media including controls were incubated for 72 hours at 37 °C and 120 rpm in a New Brunswick Innova 40 orbital shaking incubator. For each culture medium, 2 cultures were used.

Obtaining enzymatic extracts: During the 72 hours of culturing the SB and FS media and Mandels broth, 4 mL samples were taken every 12 hours and centrifuged at 3000 rpm for 30 minutes at 7 °C in a Hettich Rotanta 460R centrifuge. The supernatant or enzymatic extract was stored at -20 °C until the presence of cellulose and cellulolytic activity was evaluated.

Evaluation of cellulose hydrolysis: To determine the hydrolysis of cellulose during the 72 hours of culture, a drop of 1.0% w/w Congo red reagent was added to 3 mL of enzymatic extract [8,15]. The presence of cellulose was evaluated based on the intensity of the red color of the extract after the addition of the reagent; the lower is the intensity, the lower is the cellulose concentration.

Quantification of cellulolytic activity: Cellulolytic activity was quantified at pH 5 and 7. To evaluate the activity at pH 7, 1 mL of enzymatic extract was mixed with 1 mL of 1.0% w/v CMC phosphate buffer solution and for evaluation at pH 5, 1.0% w/v of CMC solution in citric acid buffer was used. As a negative control, an enzymatic blank was prepared by mixing 1 mL of 1% w/v CMC solution at pH 5.0 or 7.0 with 1 mL of distilled water. The mixtures were heated at 60 °C for one hour in a Barnstead Lab-Line water bath. The enzymatic reaction was stopped by cooling in an ice bath for 15 minutes and centrifuged at 4600 rpm for 20 minutes at 7 °C. The supernatant was recovered to determine the glucose produced during the enzymatic reaction. Glucose was quantified using the 3-5 dinitrosalicylic acid (DNS) technique according to Olanbiwoninu and Fasiku [16].

The absorbance of the samples was read at 540 nm and the sugar concentration was calculated based on a calibration curve prepared with glucose solutions of 0.08 to 0.43 mg/mL. The glucose concentration detected in the negative control was subtracted from the concentration found in the samples. The enzymatic activity was determined in U/mL (enzymatic units) i.e. the amount of enzyme responsible for releasing one µmol of glucose in one minute per mL. To determine significant differences between the cellulolytic activities of the enzymatic extracts from the evaluated culture media at pH 5 and pH 7 (Mandels broth and SB and FS media), one-way ANOVA was performed at a significance level of $\alpha = 0.05$.

Results and Discussion

Characterization of sugarcane bagasse and flower stem wastes: The moisture, lignin and cellulose contents (dry basis) of the SB and FS wastes are shown in table 1. To quantify cellulose and lignin, 20.52 g of FS and 21.78 g of SB were weighed and after the second treatment with acids, 16.81 g and 17.53 g were recovered respectively after the third treatment with sodium hydroxide solution to hydrolyze the lignin, 12.01 g and 12.41 g were obtained respectively (Table 1). These analyses defined the amount of each waste required to formulate the culture media at a final cellulose concentration of 1.0% w/v: 1,755 g of SB and 1,708 g of FS were used per 100 mL of medium.

Qualitative determination of cellulose in each culture medium: Cellulase hydrolysis was used as an indicator of cellulase production. After adding Congo red to the samples, the reddish color became fainter over time in culture. At culture time zero in SB and FS media and Mandels broth, cellulose was detected based on the red color. After 12 and 24 hours, all the supernatants from the three culture media showed a less-intense reddish color, evidencing cellulose hydrolysis in the media and the production of enzymes.

At 36, 48 and 72 hours, all the supernatants became reddish-orange indicating a decrease in the amount of cellulose in the medium via the action of the produced cellulases. The supernatant of the control medium prepared with CMC which was not inoculated with the microorganism, presented the same reddish color at all tested times.

Quantification of cellulolytic activity: The enzymatic activity of the cellulases produced in the SB and FS media was evaluated over 72 hours and the cellulases produced in each of the media had the highest activity at 72 hours. The enzymatic extract obtained from the FS medium showed the highest cellulolytic activity (35.06 ± 0.14 U/mL) at pH 7.0 and 60 °C (Figure 1) while in the cellulases produced in the SB medium, the highest activity was detected at pH 5 and 60 °C with an activity of 39.09 ± 0.17 U/mL (Figure 2).

In Mandels medium, the enzymatic activities were 29.94 ± 0.05 U/mL at pH 7.0 and 31.72 ± 0.06 U/mL at pH 5.0; these activities were between 5 and 7 units less than those of the enzymes produced in the media prepared with the agroindustrial wastes (Figures 1 and 2).

The enzymatic extracts obtained from the three evaluated culture media showed a gradual increase in cellulolytic activity over the first 36 hours of fermentation with 63 and 80% of the total enzymatic activity recorded at 72 hours.

Table 1
Results obtained in the characterization of the SB and FS wastes

Analysis	Sugarcane bagasse	Flower stems
Moisture	87.620% w/w	87.503% w/w
Lignin	23.50% w/w (db)	24.40% w/w (db)
Cellulose	56.97% w/w (db)	58.54% w/w (db)

db = dry basis

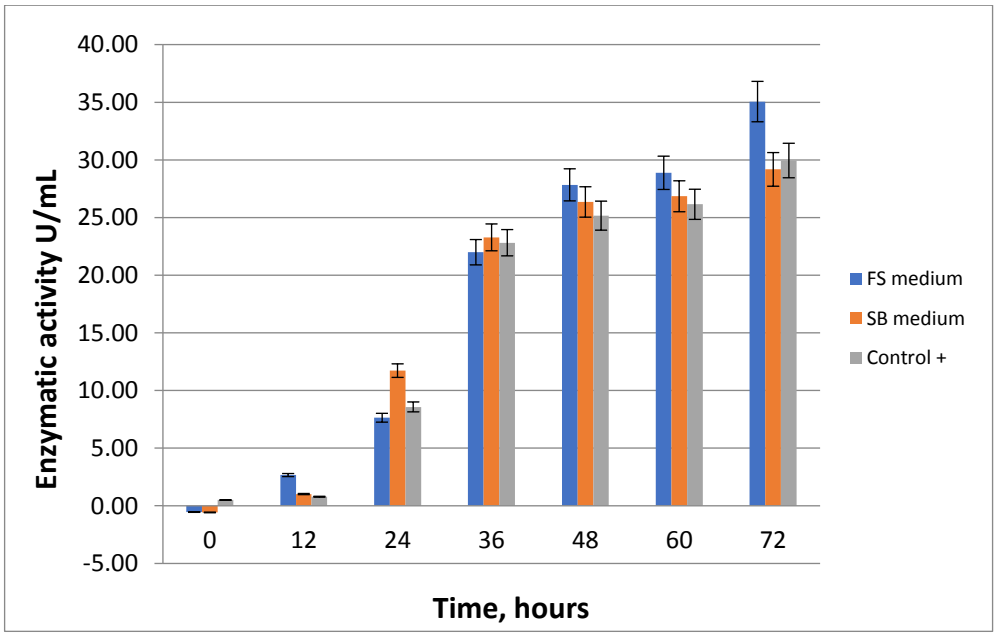

Figure 1: Enzymatic activity quantified in CMC broth (phosphate buffer, pH 7, 60 °C) cultured with SB or FS and in the positive control

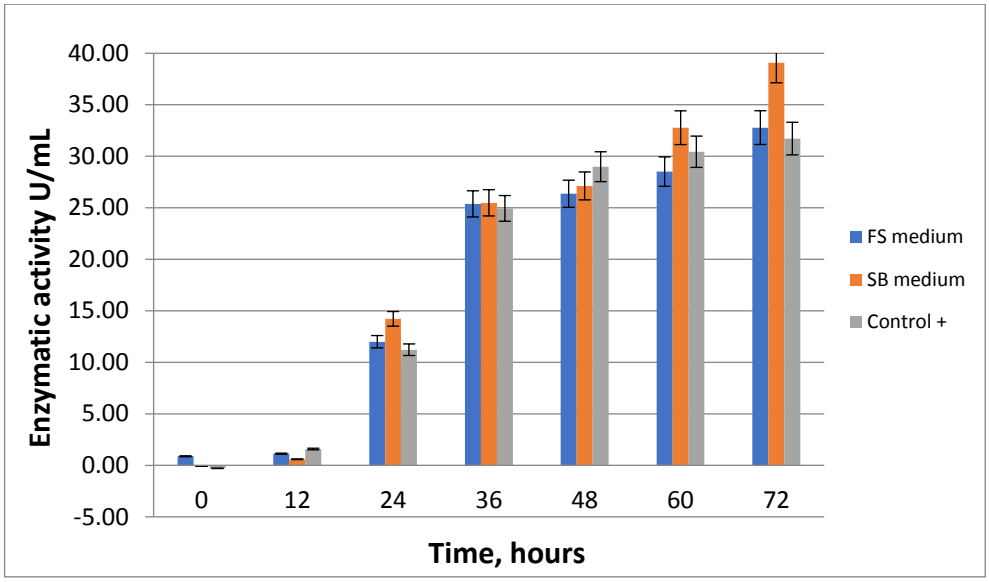

Figure 2: Enzymatic activity quantified in CMC broth (citrate buffer, pH 5, 60 °C) cultured with SB or FS and in the positive control

During these first 36 hours, cellulose hydrolysis was also observed; after this period, cellulose availability decreased and cellulolytic activity increased by only 20%. These results suggest that fermentation can be stopped after 36 hours, reducing the fermentation time and increasing the profitability of obtaining celluloses.

A one-way ANOVA at the 0.05 significance level showed no significant differences among the cellulolytic activities of the enzymatic extracts from the SB, FS and Mandels media ($p = 0.1022$) at any of the evaluated pH values and time periods. The similarity between the enzymatic activities may be due to the cellulose concentrations in the wastes which was 56.97% w/w for SB and 58.54% w/w for FS (Table 1).

Other studies have also reported the use of SB as the sole carbon source for cellulase production using strains of *Bacillus* sp. However, the cellulolytic activities reported were lower than those presented in this study. Padilha et al[9] obtained cellulases with an activity of 0.37 U/mL at pH 7.0 and 50 °C after 72 hours of culture at 70 °C while Ladeira, Cruz, Delatorre, Barbosa and Martins [17] reported a CMCase activity of 0.29 U/mL at pH 7.5 and 70 °C which was produced after 168 hours of incubation at pH 8.0 and 50 °C.

Another study reported cellulase activities of 0.34 U/mL produced by *Bacillus licheniformis* after 48 hours of fermentation at 37 °C [8]. The cellulases reported in those studies had between 79 and 100 times less cellulolytic activity than the enzymes produced by *B. amyloliquefaciens* C18A after 12 to 72 hours of fermentation and evaluation at pH 7.0 and at 60 °C (Figures 1 and 2).

The cellulolytic activity of the enzymes produced in the FS medium is 300 times higher than that of the cellulases (0.06 U/mL) obtained by Gaitán and Pérez[18]; their cellulases were derived from *Bacillus* sp. and *Streptomyces* sp. acting on chrysanthemum waste. This result suggests that *B. amyloliquefaciens* strain C18A produces enzymes with high cellulolytic activity.

The enzymatic activities of the cellulases produced by *B. amyloliquefaciens* C18A in FS and SB media were 32.79 ± 0.06 and 39.09 ± 0.17 U/mL at pH 5.0, comparable to the activity of the enzymes produced in Mandels media used as positive control for cellulases production (Figures 1 and 2). In addition, the enzymes produced in FS and SB presented similar activities to that reported for the commercial enzyme produced by *Aspergillus niger* Biocellulase A [5] which was 32 U/mL at pH 4.5 and 55 ºC. These data suggest that the enzymes produced by *B. amyloliquefaciens* C18A in media formulated with agroindustrial wastes have potential uses in industrial processes; they have the advantage of being produced from a microorganism that grows quickly and is easy to culture and able to use the cellulase present in SB and FS decreasing both the production costs and environmental impacts.

The objective of the present study was to evaluate cellulase production by *B. amyloliquefaciens* C18A from cellulose-rich agroindustrial wastes termed SB and FS. The results indicate that this microorganism can produce cellulases with activities of between 33 ± 0.06 and 39 ± 0.17 U/mL from SB and of 29 ± 0.05 to 35 ± 0.14 U/mL from FS after 72 hours of fermentation. As a result, *B. amyloliquefaciens* C18A is a promissory strain to produce cellulases from agroindustrial wastes such as SB and FS. Although the cellulolytic activities in the two evaluated wastes are similar, the cellulases produced in SB medium have higher activity at pH 5.0, while those produced in FS medium have higher activity at pH 7.0. Thus, the SB medium can be used to produce enzymes for processes requiring a pH of 5.0 and when enzymes need to be applied in processes conducted at pH

values close to 7.0, the cellulases can be produced in FS medium.

Future studies should be performed to redesign the formulation of the media prepared from FS and SB, taking into account other variables such as pH, nitrogen content or salt content, to improve yield in cellulase production. Likewise, the enzymatic activity can be improved by conducting studies aimed at optimizing fermentation conditions and studies on the purification and characterization of these cellulases should be conducted.

Conclusion

The SB and FS are readily available sources of cellulose without requirements of further treatment that can be used as substrates in cellulase production from *B. amyloliquefaciens* C18A, isolated from soil of wetlands of Bogota. The cellulases produced from agroindustrial wastes presented similar activity to enzymes obtained in media Mandels and others from previous studies. In addition, the enzymes from SB and FS had comparable activity to commercial enzymes. Thus, the substrate and the bacteria evaluated in this study might be an alternative to produce industrial cellulases.

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