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PRODUCTION OF CELLULOSE-DEGRADING ENZYME ON SISAL AND OTHER AGRO-INDUSTRIAL RESIDUES USING A NEW BRAZILIAN ACTINOBACTERIA STRAIN *Streptomyces* sp. SLBA-08

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Abstract - Several types of lignocellulosic raw materials including wheat straw, sisal bagasse and sugarcane bagasse have different contents of cellulosic components. In our studies, aimed at isolating cellulose-degrading actinobacteria from Brazilian soils, a promising strain was selected and identified as *Streptomyces* sp. SLBA-08. This strain was tested for cellulase production under submerged fermentation in a mineral medium using different carbon sources (sisal bagasse, sugarcane bagasse and straw), as well as ammonium sulphate in different concentrations as nitrogen source. The results showed that medium containing 2.4% (*w/v*) sisal bagasse and 0.3% (*w/v*) ammonium sulphate resulted in the highest production of carboxymethylcellulase (1.11 U mL^{-1}), after 48 hours. The pH and temperature profile showed optimal activity at pH 6.0 and 50 °C. As for thermostability, carboxymethylcellulases were tolerant at 50 °C, retaining 70% of the maximal activity even after 2h of incubation. The results obtained indicate that *Streptomyces* sp. SLBA-08 was capable of producing CMCase using lignocellulosic residues, especially sisal bagasse.
Keywords: *Streptomyces* sp. SLBA-08; Cellulose-degrading enzyme; Sisal bagasse; Sugarcane straw; Sugarcane bagasse.

INTRODUCTION

Actinobacteria are Gram positive filamentous bacteria abundantly found in soil, considered the most economically important and biotechnologically valuable prokaryotes. They account for the production of about half of the discovered bioactive secondary metabolites (Bérdy, 2005), notably enzymes (Goodfellow *et al*., 1988). The *Streptomyces* are the most important genus in this group, able to produce and excrete a large variety of enzymes, including those involved in the degradation of cellulose, hemicellulose and lignin. Since the microbiology of Brazilian tropical soils is largely unknown, these soils can represent an excellent source for the search for new species and enzymes, especially cellulases (Grigorevski-Lima

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et al., 2005; Nascimento *et al*., 2009; Da Vinha *et al*., 2011). These cellulose degrading enzymes are related to industrial processes, like food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper industries, as well as research and development technologies (Bhat, 2000; Jang and Chen, 2003; Coelho and Nascimento, 2011).

The utilization of agricultural by-products and other natural compounds for growing microorganisms may constitute an interesting alternative for enzyme production with lower costs. In Brazil, the bagasse portion of sugarcane comprises one of the largest cellulosic agro-industrial residues, and contains approximately 50% cellulose and 25% hemicellulose and lignin, while the straw is composed of 37.4% cellulose, 30% hemicellulose and 18.5% lignin (Pandey *et al*., 2000). Both substrates have already been tested as the main carbon source for the production of several enzymes including cellulases (Pandey *et al*., 2000; Adsul *et al*., 2004; Sukumaran *et al*., 2009; Maeda *et al*., 2010; Barros *et al*., 2010). As to sisal (*Agaveae sisalana*), this is the main worldwide plant used for fiber extraction, Brazil being the world's largest producer (Andrade, 2011). Sisal fiber contains about 65% cellulose, 12% hemicellulose, 10% lignin and 10% starch, and may represent an important alternative source for enzymatic processes and biofuel production. However, it has not been reported yet as a substrate for enzyme production or other biotechnological applications.

Studies dealing with cellulase (CMCase) production by actinobacteria using low-cost agro-industrial residues are scarce in literature, however our group has been isolating cellulolytic strains from Brazilian tropical soils for some time, obtaining promising results (Grigorevski-Lima *et al*., 2005; Nascimento *et al*., 2009; Da Vinha *et al*., 2011; Nascimento and Coelho, 2011).

In the present work we have isolated an actinobacteria strain from a Brazilian soil under sisal cultivation, and tested it qualitatively for cellulase production. The CMCase production in submerged fermentation was then evaluated using three different agro-industrial residues as the main carbon source, sugarcane bagasse, sugarcane straw and sisal bagasse.

MATERIALS AND METHODS

Microorganism Isolation, Maintenance and Preliminary Test for Cellulolytic Activity

Actinobacteria SLBA-08 strain (CBMAI-1473) was isolated from a soil sample under *Agave sisalana* (sisal) cultivation in the semiarid region of Brazil, using the dilution plate technique and starch-casein medium (Kuster and Williams, 1964). After incubation at 28 °C for 7-14 days, colonies with actinobacterial characteristics were isolated and tested qualitatively for cellulolytic activity. The first test was performed after growth on solid medium containing CMC and using Congo red to reveal CMC-degrading zones (Sazci, 1986); in a second one, after growth on cellulose-azure medium, liberation of azure dye was observed followed by the degradation of cellulose-azure (Plant *et al*., 1988). Stock cultures were maintained on yeast extract-malt extract-agar plates (Shirling and Gottlieb, 1966) and the spore suspension was prepared according to Hopwood *et al*. (1985) after cultivation (28 °C/15 days) in this same medium. Spores were maintained in 30% glycerol (*v/v*) at -18 °C.

Submerged Fermentation

Cells were cultivated in a mineral medium (Da Vinha *et al*., 2011) containing (g L^{-1}): KH_2PO_4, 6.0; K_2HPO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.05; $MnSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001. The medium was supplemented with three different carbon sources [sugarcane bagasse (SCB), sugarcane straw (SCS) and sisal bagasse (SB)] in two concentrations [0.8 and 2.4 % (*w/v*)], and a nitrogen source [ammonium sulphate (AS)] also in two concentrations [0.3% (*w/v*) and 1.3 % (*w/v*], generating 4 runs for each carbon source (Table 1).

Table 1: Medium composition used in the different submerged fermentation conditions.

Culture Medium	Carbon Sources %(*w/v*)	Nitrogen Source % (*w/v*)
1	0.8	0.3
2	2.4	0.3
3	0.8	1.3
4	2.4	1.3

All media were supplemented with a mineral solution (see Materials and Methods)

Fermentations were performed in 250-mL Erlenmeyer flasks containing 50 mL of the culture medium (initial pH 6.8). Each medium was inoculated with 50 µL of spore suspension (1.61 x 10^8 spores mL^{-1}) of strain SLBA-08 to a final concentration of 10^5 spores mL^{-1}. Cells were incubated at 30 °C, under agitation in an orbital shaker (150 rev min^{-1}), for 5 days. On each day, the whole contents of the shake flasks were centrifuged at 2,038 g and 4 °C for 15 min, filtered through glass microfiber filters (Whatman GF/A), and the crude supernatants used for enzymatic assays. All experiments were carried

out in duplicate, and results were expressed as average values. Statistical tools were employed to evaluate the regression analysis and the significance of the experiments (standard deviation).

Enzymatic Assay

Carboxymethylcellulase (CMCase) activity was assayed by measuring the release of reducing sugars in a reaction mixture of 1.0 mL of the crude extract and 1.0 mL of 2.0% (*w/v*) carboxymethylcellulose (CMC) sodium salt (SIGMA®, St Louis, MO, USA) solution in 50 mM sodium citrate buffer (pH 4.8) incubated at 50 °C for 20 min. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method (Miller 1959). One unit (U) of CMCase activity corresponded to 1 μmol of glucose equivalent released per minute under the assay conditions (Ghose, 1987).

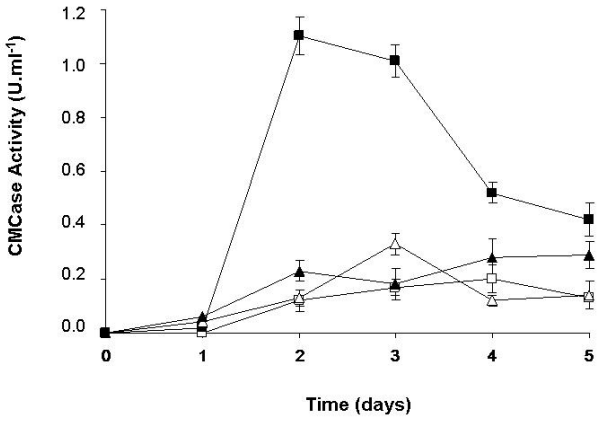
Partial Crude Enzyme Characterization: Influence of Temperature, pH Effect and Thermostability

Supernatants from strain SLBA-08 cultivated in the best conditions (SB 2.4% and AS 0.3%) were used as crude enzyme. The temperature profile for CMCase activity, assayed as described above, was determined by varying the incubation temperature between 20 °C and 90 °C at pH 4.8. In the same way, CMCase activity was determined in the pH range of 2.0–10.0, with the following buffers (50 mM) incubated at 50 °C: glycine-HCl for pH 2.0–3.0, sodium citrate for pH 3.0–6.0, sodium phosphate for pH 6.0–8.0, Tris–HCl for pH 8.0–9.0 and glycine–NaOH for pH 9.0–10.0. To study the CMCase thermal stability, the supernatant was pre-incubated at 50 °C for 0.5, 1, 2, 4, 6, 8 and 16 hours (pH 4.8). The residual activity was measured by usual enzyme activity determination procedures as described above. For comparison, we used the commercial cellulase Indiage Super L (Genencor). All the experiments were performed in duplicate, and results expressed as average values.

RESULTS AND DISCUSSION

Several actinobacterial strains were obtained from soil under sisal plantations in a semi-arid region of Brazil. However, preliminary tests (Congo-red and cellulose-azure) showed that strain SLBA-08 was the most promising and worthy of a more detailed study. This strain was further identified within the genus *Streptomyces* sp., based on a 16S RNA sequence analysis and spore chain morphology (data not show). The strain *Streptomyces* sp. SLBA-08 (number code 1473) was deposited in the "Coleção Brasileira de Microrganismos de Ambiente e Indústria (CBMAI)".

The studies performed under submerged fermentation showed that the strain was capable of producing cellulases using sisal bagasse as the sole organic substrate. However, two other substrates (sugarcane bagasse and sugarcane straw) were also good sources for cellulase production. The kinetic profiles of enzyme production in the four media tested (Table 1) for each substrate, obtained after five days of cultivation under agitation at 30 °C, are described in Fig. 1. The best CMCase activity (1.11 U mL^{-1}) occurred after two days of fermentation, when sisal bagasse was used in medium 02 [2.4% (*w/v*) of SB and 0.3% (*w/v*) AS] (Fig. 1(a)). However, very similar CMCase titers (1.01 U mL^{-1}) were observed when sugarcane straw was the carbon source, after one day fermentation (Fig. 1(b)), at these same C and N source concentrations (medium 02). *Streptomyces* sp SLBA-08 was also able to produce cellulase using sugarcane bagasse as the sole organic source, with maximal levels of CMCase of 0.66 U mL^{-1}, obtained after three days fermentation, also on the same medium (Fig 1(c)).



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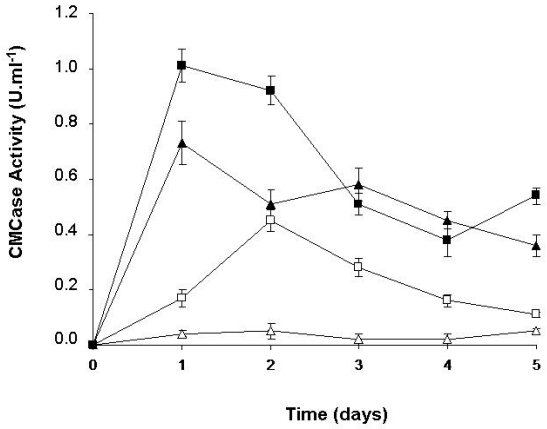

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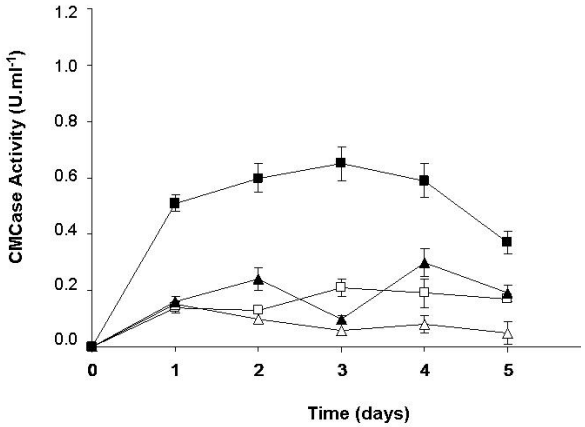


(c)

Figure 1: Fermentations time-course for CMCase production by *Streptomyces* sp. SLBA-08 at 30 °C in culture medium containing (a) sisal bagasse, (b) sugarcane straw and (c) sugarcane bagasse as carbon source at different concentrations, and ammonium sulphate as nitrogen source, as described in Table 1. Medium 1 (-□-), Medium 2 (-■-), Medium 3 (-△-), Medium 4 (-▲-).

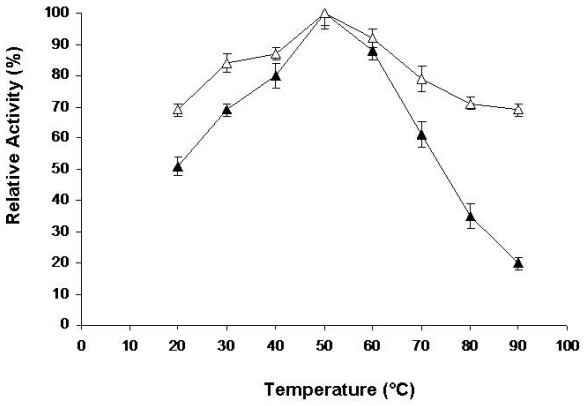
The use of low-cost residues from agro-industries as the main substrates for research aimed at enzyme production by microorganisms, including cellulase production (Adsul *et al*., 2004), has increased in the last few years, since it significantly reduces costs. Our group has been studying this subject for some time, and interesting results have already been described for xylanase and protease production by actinobacteria using different residues (Nascimento *et al*., 2002; Nascimento *et al*., 2005; De Azeredo *et al*., 2006). We also studied cellulase production by several *Streptomyces* strains isolated from Brazilian soils growing on distilled dried grains (DDG), wheat bran, brewer's spent grains and sugarcane bagasse in different concentrations and using corn steep liquor, which is also a low-cost residue from agriculture, as the main nitrogen source. *Streptomyces drozdowiczii* grown in 1.0% (*w/v*) DDG and 0.3% (*w/v*) corn steep liquor was able to produce around 0.15 U mL^{-1} CMCase in the crude supernatant, whereas when using wheat bran in the same conditions an activity of 0.22 U mL^{-1} was obtained (Grigorevski-Lima *et al*., 2005). Nascimento *et al*. (2009) obtained values of 0.72 U mL^{-1} as maximum activity for CMCase when growing *Streptomyces malaysiensis* AMT-3 on 0.5% (*w/v*) brewer's spent grains and 1.2% (*w/v*) corn steep liquor, after four days of fermentation. More recently, even better results were obtained by Da Vinha *et al*. (2011), who detected a high CMCase production (2.00 U mL^{-1}) by *Streptomyces viridobrunneus* SCPE-09 cultivated on 2.0% (*w/v*) wheat bran and 0.19% (*w/v*) corn steep liquor after five fermentation days. Very few other reports are described besides those reported by our group. Tuncer *et al*. (2004), for instance, studied the production of endoglucanase, among other enzymes, with *Streptomyces* sp. F262 grown with 1.2% (*w/v*) ball-milled wheat straw supplemented with yeast extract and observed a very low production. However, it must be emphasized that a comparison between results described in the literature is difficult, since the conditions for cellulase activity and enzyme production are not always the same. Nonetheless, the results presented here suggest that *Streptomyces* sp. SLBA-08 is a good producer of CMCase using lignocellulosic wastes as carbon source, especially sisal bagasse or sugarcane straw. At the present time, as far as we are concerned, there is no citation in the literature describing the use of sisal bagasse for the production of hydrolytic enzymes like cellulases, xylanases or amylases.

According to the National Supply Company (CONAB, Brazil), the Brazilian production of dry sisal fiber was estimated to be 139,700 tons in 2004, at the same time that 489,000 tons of sisal bagasse were discarded. These values represent a significant amount (almost 3 times the fiber production weight) and should somehow be recovered. Indeed, in Brazil this waste is habitually abandoned in the field, and only a small portion is used as fertilizer or animal feed (Andrade, 2011).

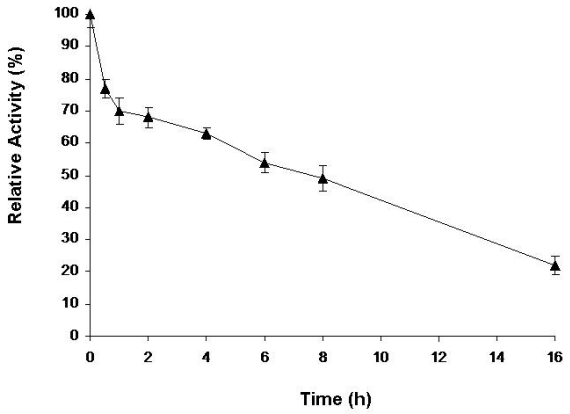
Thus, the use of sisal bagasse may represent a new alternative for the production of bioactive substances such as lignocellulolytic enzymes from residual plant biomass, particularly cellulases. Also considering bioethanol in the present international scenario, it is evident that second generation ethanol production will soon become a reality. In this sense, sisal becomes as an excellent option for the cheap production of cellulase.

The supernatant from *Streptomyces* sp. SLBA-08 grown on sisal bagasse in the best conditions was significantly active in the temperature range tested (Fig. 2(a)). Maximal activities (more than 90% relative activities) were observed within the range of 50–60 °C but activity levels of 30% were still detected at 80 °C. These results are similar to those reported in the literature by other authors for other *Streptomyces* strains (Schrempf and Walter, 1995; Hoshiro *et al*., 1999; George *et al*., 2001; Jang and Cheng, 2003; Grigorevski-Lima *et al*., 2005, Nascimento *et al*., 2009). In our previous studies, we observed that the CMCase produced by *S. malaysiensis* AMT-3 was thermophilic, with residual activity of around 100%

at temperatures between 40° and 60 °C (Nascimento *et al*., 2009). Thermal stability experiments are shown in Fig. 2(b). Crude enzyme was able to retain 70% residual activity at 50 °C for 2 h, the half-life being 6 h at this temperature. Half-lives of 24 h at 40 °C or 8 h at 50 °C have been cited in the literature for some *Streptomyces* strains (Grigorevski-Lima *et al*., 2005; Nascimento *et al*., 2009; Da Vinha *et al*., 2011).



(a)



(b)

Figure 2: Effect of temperature (a) and thermal stability (b) at 50 ºC on the activity (pH 4.8) of CMCase produced by *Streptomyces* sp. SLBA-08 (▲) grown on 2.7% (*w/v*) sisal bagasse and 0.8% (*w/v*) ammonium sulphate and the commercial enzyme Indi-age Super L (△). Residual activity is expressed as a percentage of the original activity. Error bars represent the standard deviation of each experimental point (n=2).

The pH activity profile of the crude extract obtained under the best growth conditions (Fig. 3) shows that 80% CMCase activity was maintained over the pH range 4.0–7.0, with optimal activity occurring at pH 6.0. Although commercial Indi-Age cellulase is superior at pH values below 4 or above 8, our strain showed promising results in the pH range from 4 to 8.

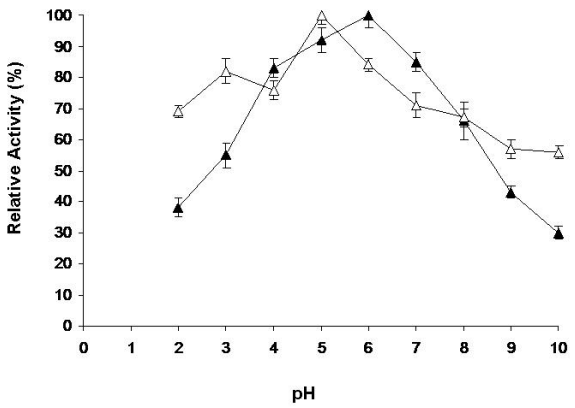


Figure 3: Effect of pH on activity (50 ºC) of CMCase produced by *Streptomyces* sp. SLBA-08 (▲) grown in 2.7% (*w/v*) sisal bagasse and 0.8% (*w/v*) ammonium sulphate and 0.25% (*w/v*) peptone and the commercial enzyme Indi-age Super L (△). The ionic strength for all buffers was 50mM. Residual activity is expressed as a percentage of the original activity. Error bars represent the standard deviation of each experimental point (n=2).

Da Vinha *et al*. (2011) described a similar profile (above 80% of relative activity) for *S. viridobrunneus* SCPE-09 in the pH range 4.0–7.0, with optimal activity around 5.0. CMCase activity in this pH range (4.0–8.0) was also detected by Grigorevski-Lima *et al*. (2005) for CMCase of *S. drozwdowiczii*. Nascimento *et al*. (2009) also observed a similar profile for CMCase of *Streptomyces malaysiensis* AMT-3.

CONCLUSIONS

In the present research we were able to isolate from a Brazilian soil under sisal cultivation an actinobacterial strain, *Streptomyces* sp SLBA-08, that was able to grow and produce cellulases (CMCases) in a cultivation medium containing solely a mineral salt solution and very cheap agriculture residues, either sisal bagasse, sugarcane straw or sugarcane bagasse as carbon source. Values of up to 1.11 U mL^{-1} of enzyme activity were obtained after only two days fermentation when sisal bagasse was used. The crude extract thus obtained gave maximal activity in an acidic pH range (4.0-7.0) and temperatures around 50-60 °C, with a half life of 6h at 50 °C.

Considering the low medium cost, the high titers of CMCase and the pH and temperature characteris-

tics, these results indicate the possibility of using these enzymes in biotechnological processes. As already stressed, sisal bagasse is a very abundant agricultural residue in Brazil, being almost entirely left in the field, and so could be easily recovered for enzyme production. In fact, the present work reports, for the first time, the use of sisal bagasse as a carbon source for cellulase production by an actinobacteria strain. However, it can represent an important alternative lignocellulosic source for the production of many other enzymes of biotechnological importance.

Our results also confirm the importance of searching for new biotechnological microbial sources in different ecosystems. Brazilian soils have shown a great microbial biodiversity with different enzyme profiles, especially cellulases, xylanases, and proteinases, and may have a great potential for the discovery of new strains with novel characteristics.

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