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Production and characterization of endoglucanase secreted by *Streptomyces capoamus* isolated from Caatinga

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Cellulases are hydrolases of great importance to industries, especially due to their ability to produce ethanol via hydrolysis of cellulolytic materials. Actinomycetes are the producers of these enzymes, particularly the genus *Streptomyces* sp. The present study is the first report on the production and characterization of cellulolytic complex secreted by *Streptomyces capoamus*, isolated from the rhizosphere soil of Caatinga. In selecting the microbial producers of cellulolytic complex in qualitative tests, 171x microorganism showed the most expressive enzymatic index. Regarding the production time of the complex, fermentation was done for 7 days, with aliquots being taken every 24 h. Peak production was obtained during 48 h fermentation. It was done at 37ºC and under an agitation of 180 rpm. It was noted also that the 171x micro-organism produced the enzyme in greater quantity. The experiment was done with the most significant actinomycetes (171x), optimal substrate concentration (carboximeticellulose), cultivation temperature and pH of initial output. The results showed that a higher cellulolytic complex was obtained with 2% substrate, 45°C temperature and initial pH 4.0. The microorganism was identified at genus level by microculture method; and with molecular identification method, it was identified as *S. capoamus* UFPEDA-3410. In optimal culture conditions, this strain produced 0.309 U/mL cellulose, a good production for a thermostable endoglucanase stable in a broad range of pH and stable temperature. It has potential applications in a wide range of industries. Industrial processes are generally carried out at elevated temperatures. Therefore enzymes with a high optima temperature and stability are desired for such applications.

Key words: Cellulase activity, actinomycetes, fermentation, carboxymethyl cellulose.

INTRODUCTION

Cellulose is the most abundant biological compound in terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar et al., 2011). It is the dominant waste material from agricultural industries in the form of stalks, stems and husk. There has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababu et al., 2012). Cellulose is composed of D-glucose units linked together to form linear chain via ß-1,4-glycosidic linkages (Salmon and Hudson, 1997).

Cellulose is commonly degraded by cellulase. Cellulolytic enzyme system is a complex mixture of enzyme proteins with different specificities, which act synergistically to hydrolyze glycosidic bonds. The three major cellulase enzyme activities are: Endocellulase or 1,4-β-D-glucanglucanohydrolases (EC 3.2.1.4.); exocellulase or 1,4-β-D-glucancellobiohydrolase (EC 3.2.1.91) and beta-glucosidase or β-D-glucosideglucohydrolases (EC 3.2.1.21) (Nishida et al., 2007).

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulases are widely used in food, feed, textile and pulp industries (Nakari and Penttila, 1995). The bioconversion of cellulosic materials is now a subject of intensive research as a contribution to the development of large scale conversion process beneficial to mankind (Sreeja et al., 2013).

Cellulolytics enzymes are produced from plant, animal and microbial sources. For commercial production, microbial enzymes have the enormous advantage of being scalable to high-capacity production by established fermentation techniques (Tahtamouni et al., 2006).

Different authors report that actinomycetes are potential cellulose producers and help considerably in recycling nutrients in the biosphere. Also, they are involved in the primary degradation of organic matter in compost and related materials (Goodfellow and Williams, 1983; Jang and Chen, 2003; Prasad and Sethi, 2013; Mohanta, 2014).

The rise of enzyme and advancement in biotechnology industries have led to the research and selection of the sources and production processes of enzyme, since it is a promising alternative to reduce the waste arising from other industries. Enzyme production, characterization and application are an enduring, fundamental and vital area of current research.

Caatinga, a dry tropical deciduous vegetation, composed of small trees, bushes and grasses, xerophiles and deciduous plants, is the largest vegetation type. It covers an approximately 845, 000 km^2 area, in the semi-arid region of Northeast of Brazil. It has a rhizosphere rich in microorganisms that produce enzymes with biotechnological potential thus the overall objective of the research is to produce and characterize endoglucanase secreted by Streptomyces capoamus isolated Caatinga.

MATERIALS AND METHODS

Screening of cellulose

To select microorganisms capable of producing cellulases, qualitative assays were performed. 87 actinomycetes strains were inoculated into agar medium containing 1% carboxymethylcellulose (CMC), and subsequently incubated for up to 7 days at 37°C. To visualize the hydrolysis zone, plates were developed using a 0.1% Congo red solution and then washed with a 1 M NaCl solution (Pratima et al., 2012). The ratio of the diameters of the hydrolysis zone and the diameter of the colony were calculated in order to select the largest producer of cellulase. The highest proportion was assumed to greater activity (Ariffin et al., 2006).

Organism and growth

The identification of actinobacteria (171x) was carried out by members of the genetics laboratory and collection of micro-organisms/UFPEDA. *S. capoamus*, UFPEDA-3410, a new single producer of cellulase was used in this study. This was identified and deposited in microorganisms culture collection (UFPEDA) at the Department of Antibiotics, Federal University of Pernambuco. The actinobacteria were cultured in ISP-2 solid at 37°C for 7 days to do colony purity check.

The pre-inoculum was obtained by culturing in 48 h fermentation medium containing 50 ml of ISP2 liquid in which 5 agar blocks of 9 mm diameter was inoculated and incubated at 37°C under agitation of 120 rpm. Subsequently, 5 ml of this pre-inoculum was added to 50 mL of ISP2 liquid medium containing 1% carboxymethyl cellulose as the only carbon source. It was incubated at 37°C for 96 h under stirring at 120 rpm, and then filtered to obtain the enzyme complex. All assays were performed in triplicate.

Cellulase assay

The tested filtrate was subjected to dosages of enzyme and protein activity. The cellulolytic activity was determined using carboxymethyl cellulose as a substrate. The reaction mixture is composed of 0.5 ml of 1% (w / v) substrate, 0.1 M sodium acetate buffer (50 mM, pH 5.6) and 0.5 ml of the culture supernatant. The mixture was incubated at 50°C for 30 min. The reducing sugar released was measured by DNS method. A cellulase activity unit was defined as the amount of enzyme which liberates 1 µmol of glucose per minute. In all cases, the specific activity was expressed as units of activity per milligram of protein.

Determination of protein

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

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Enzyme production from various substrates

To select the source of inducing carbon for the production of the enzyme, *S. capoamus* strain was inoculated into Erlenmeyer flasks (125 mL) containing 100 ml of liquid medium ISP2. 1% of each substrate (carboxymethylcellulose, passion fruit residue, and corn cobs) was incubated at 37°C, and pH 6.5 under stirring (120 rpm) for up to 168 h. Aliquots were taken every 24 h. The various enzyme complexes were subjected to enzymatic dosages, based on the method previously described. All assays were performed in triplicate.

Effect of substrate concentration, pH and temperature on cellulase production

Different substrate concentrations (0.5, 1 and 2%) were tested for optimizing the production of cellulolytic complex secreted by *S. capoamus* fermented under the conditions specified in the previous steps. The effect of initial pH (4.0 to 8.0) and temperature of 30 to 55°C on the production of cellulolytic complex was studied. After the incubation period, each test was submitted to enzymatic and protein measurements. All assays were performed in triplicate.

Enzyme characterization

Optimum pH and temperature for cellulolytic complex activity

The best pre-established production conditions for obtaining the cellulolytic complex were characterized as optimal pH and temperature. The enzymatic activity was determined at different pH values using McIlvaine buffer of 2.5 to 8.0 at 50°C. The optimum temperature test reaction was performed at the optimum pH and cellulolytic complex was incubated between 30 and 90°C (Silva and Carmona, 2008).

Stability of cellulolytic complex at different temperatures and pH

In the characterization of the stability of the cellulolytic complex at different pH, the enzymatic complex was diluted (1: 1) in McIlvaine buffer (pH 3.0 to 9.0) and maintained at 25°C for 24 h. Then residual enzymatic activity was determined. In the detection of the thermal stability of the cellulolytic complex, the enzyme was incubated at different temperatures (50, 60, 80) and time intervals of 10, 20, 30, 60, 90 and 192 min. The residual activity was determined with the optimum conditions used for the said enzyme (Silva and Camona, 2008). All assays were performed in triplicate.

Molecular identification

DNA extraction was performed through the culture grown in a liquid medium of ISP-2 for 48 h at 37ºC. Subsequently, the sample was centrifuged and DNA extraction was performed using the Wizard Genomic DNA Purification kit (Promega), according to manufacturer's instructions. The DNA sequence was assessed by electrophoresis on agarose gel. Then, amplification of the 16S rRNA gene was performed through the technique of polymerase chain reaction (PCR), using universal oligonucleotides (fD15´-AGAGTTTGATCCTGGCTCAG-3`; rD15´-AAGGAGGTGATCCAGCC–3´). The reaction consisted of the following: a mixture of 50 ng of DNA, 10 pmoles of each oligonucleotide, 200 mM of dNTP, 1.5 mM of $MgCl_2$, 1X buffer, 1 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), in a final volume of 25 µL. The reaction conditions were: 5 min of denaturation at 94°C, followed by 25 cycles of 1 min at 94°C, 30 s at 52°C and 2 min at 72°C, a final extension of 10 min at 72°C. The amplification product was analyzed by electrophoresis in 1.2% agarose gel and, subsequently, the sample was sent for sequencing. The sample was sequenced by Macrogen and this sequence was compared to all sequences in Genbank, using the software Blast of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The sequence was aligned with the software Clustal and the phylogenetic tree was constructed using Mega 5.5. Topology was assessed by analyzing bootstrap (1,000 resampling).

Table 1. Screening of actinomycetes strains with cellulolytic activity.

Microorganisms	Ratio of zone size and colony size
757	2.636 ± 0.600
818B	2.076 ± 0.400
178	4.666 ± 0.090
171X	3.961 ± 0.054
753A	0.692 ± 0.005
800X	2.65 ± 0.050
247	1.531 ± 0.240
875A	2.04 ± 0.180
874	1.609 ± 0.210
871	1.977 ± 0.090
869	1.833 ± 0.320
867	1.857 ± 0.110
868	1.921 ± 0.120
763	2.214 ± 0.500
4T	2.300 ± 0.280
20G	2.950 ± 0.040
5M	2.300 ± 0.060
7N	2.960 ± 0.025
16I	1.730 ± 0.110
12Q	1.660 ± 0.500

RESULTS AND DISCUSSION

Cellulase activity of actinomycetes was carried out using preliminary screening method by hydrolyzing the substrate incorporated in the basal salt medium. The present study is a preliminary characterization of cellulase activities of 87 strains of actinomycetes isolated from the soil samples of different locations. A total of 20 (23%) actinomycetes isolates found to be positive on screening media (cellulose Congo-Red agar) producing clear zone (Table 1) indicated the cellulase enzyme activity. 4 were isolated and as a result, an H/C value greater than 2.9 was obtained (H: hydrolysis halo diameter; C: colony diameter (Table 1). This shows that the strains could produce cellulases with high activities, which might have the potential to liberate glucose from cellulose. Previous studies have shown that, screening of

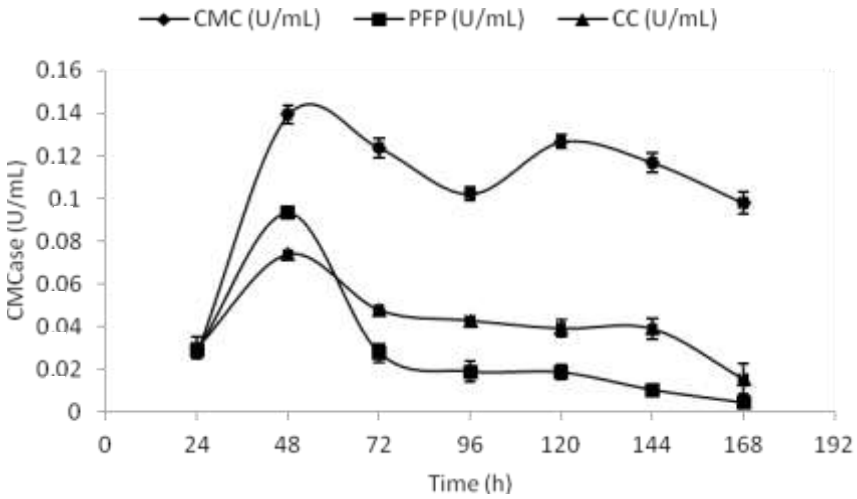

Figure 1. Effect of different substrate carboxymethylcellulose (CMC), passion fruit residue (PFP), and corn cobs (CC) on cellulase production by *S. capoamus*.

cellulases was carried out by carboxymethylcellulose agar plate assay method. Hydrolysis zones were visualized by staining of cellulose-agar media with Congo-Red solution after the growth of microorganisms (Selvam et al., 2011; Bui, 2014; Lin et al., 2012). The 171X strains of actinomycetes were selected to study their ability to secrete the cellulolytic complex in the fermentation liquid medium. The 171X strain had better use when grown on CMC compared to the other substrates tested (Figure 1). The morphological and molecular features of the isolate were identified. DNA extraction was performed using the Wizard Genomic DNA Purification kit (Promega). The strain was deposited in UFPEDA microbial culture collection center in Brazil as UFPEDA-3410. It was preserved at Culture Collection UFPEDA, Department of Antibiotics, Federal University of Pernambuco. Among well-established species of the genus *Streptomyces,* 171X strain having the closest sequence (99%) with type strains is identified as *S. capoamus.*

Cellulase production was found to be dependent upon the nature of the source used in culture media. The effect of several carbon sources on the cellulase production was investigated using different substrates (carboxymethyl cellulose, corn cobs and passion fruit residue). The choice of the cheapest and appropriate substrate is of great importance for the successful production of enzymes (Sadhu et al., 2013). The highest levels of extracellular cellulase activities were detected in medium supplemented with carboxymethyl cellulose (Figure 1).

The enzymatic hydrolysis of the cellulosic feedstock has several advantages over the chemical processes because of its potential saccharification efficiency and lower energy consumption (Saratale et al., 2008, 2010). During the fermentation process, the biomass-degrading microorganisms secrete extracellular enzymes (Santos, 2013). The organic residues may be used in this process not only as the carbon source, but as solid support (Pandey et al., 2000).

In this study, using carboxymethyl cellulose (CMC), passion fruit residue (PFP) and corn cobs (CC) as substrate, actinobacteria *S. capoamus* (171X) produced highest CMCase enzyme in 48 h, with a peak enzymatic activity of 0.139 U/ml for the CMC (Figure 1). From the data obtained, it was observed that the low cost substrates tested favored the production of cellulolytic complex. These results are similar to those reported by Ferreira et al. (2012) and Santos et al. (2011) who worked on filamentous fungi. In both research, peak production was obtained in 72 h culture. Ramirez and Coha (2003) reported the production of cellulases by 10 strains of *Streptomyces* at 72 h. Maximum cellulolytic activity values were also achieved in 72 h by other strains of *Streptomyces* (Ishaque and Kluepfel, 1980). Considering the time the actinomycetes used in producing the cellulolytic complex, the tested specimen can be considered as more promising for industrial use; however, this is just one of the evaluation criteria. Similar results were observed in other works such as Hsu et al. (2011), Alani et al. (2008), and Li and Strohl (1996).

CMC was further tested at different concentrations for the production of cellulase by *S. capoamus* strain. CMCase production increased with increase in substrate (carboxymethylcellulose). 2% (0.237 U/mL) was optimum for cellulase production (Figure 2). Similar study on cellulotic enzymes has been demonstrated in many organisms (Narasimba et al., 2006; Kumar et al., 2012).

pH is an important parameter in the production of enzymes. For CMCase production, pH 4.0 was found to be optimum (0.2766 U/mL), as shown in Figure 3a. This result clearly indicates the acidophilic nature of

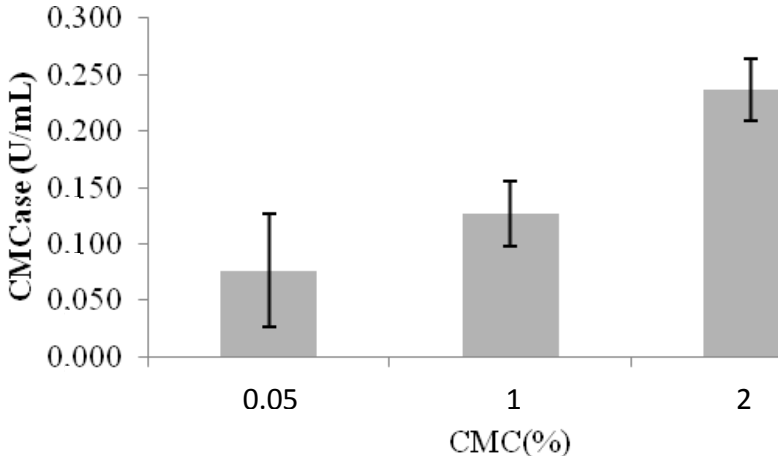

Figure 2. Effect of different carboxymethylcellulose concentrations for cellulase production by *Streptomyces capoamus*.

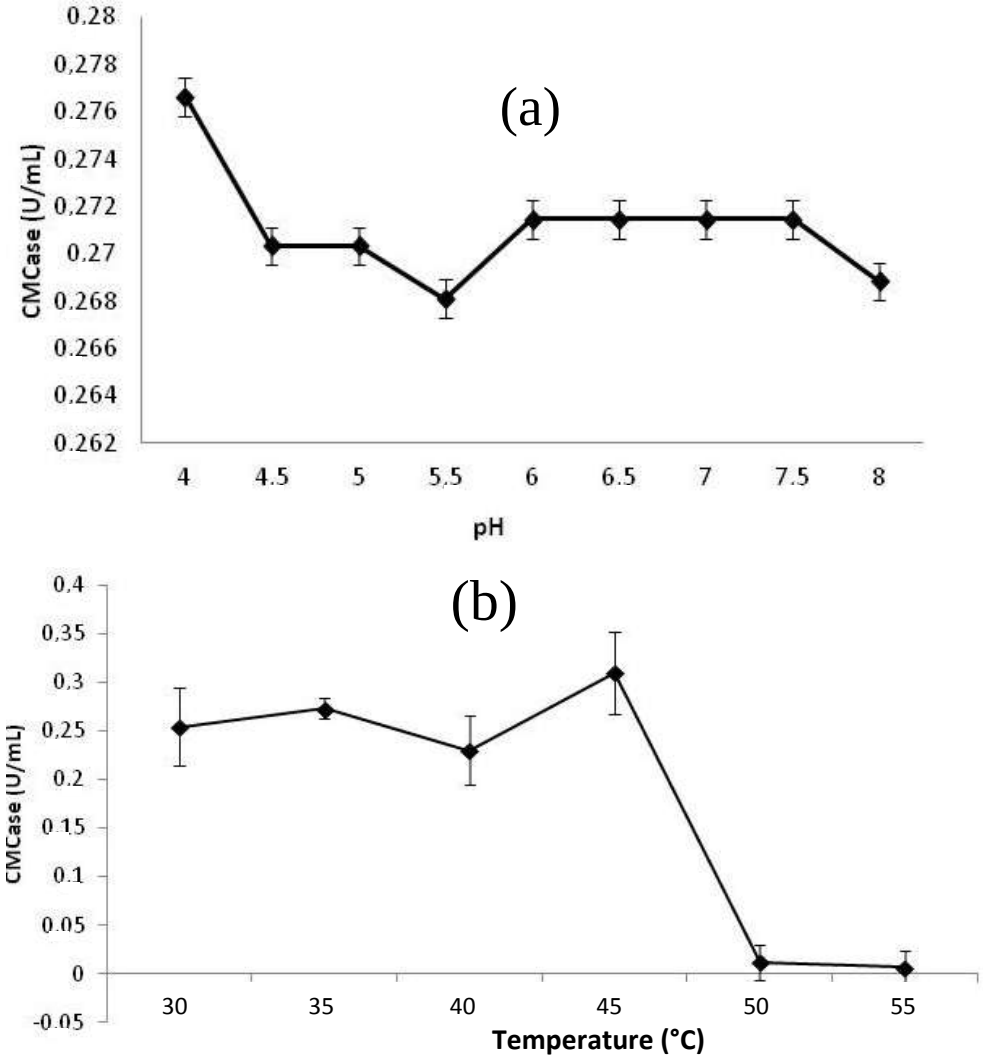

Figure 3. Effects of culture conditions on cellulaose production by *S. capoamusin*: (a) Culture initial pH effect (b) Temperature.

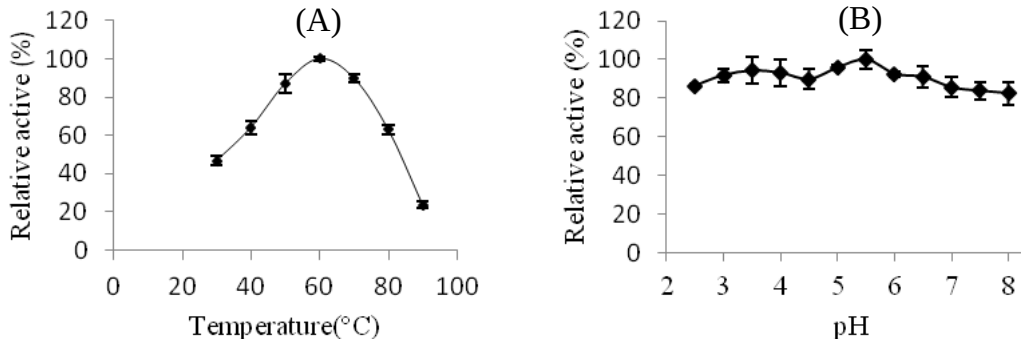

Figure 4. Temperature (a) and pH (b) influence on extracellular cellulase activity from *S. capoamus*.

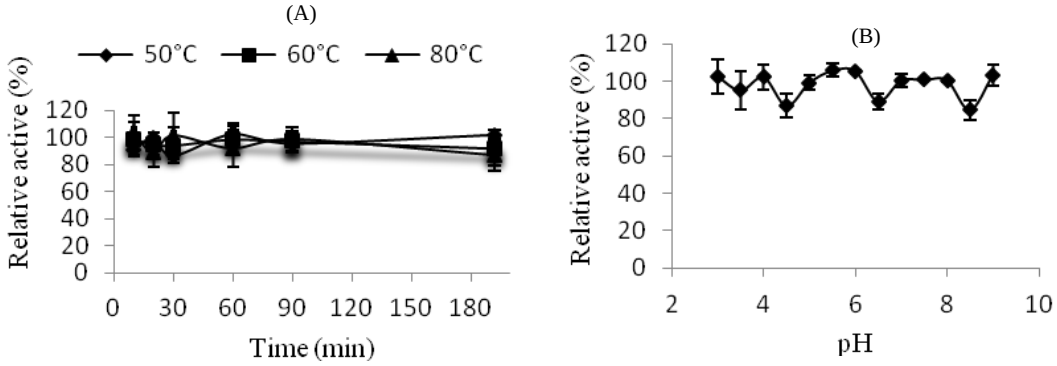

Figure 5. Temperature (a) and pH (b) influence on extracellular cellulase activity from *S. capoamus*.

actinomycete. George et al. (2001) and Mawadza et al. (2000) have reported pH 4.0 as optimum for maximum cellulase production by Thermomonospora sp. *and Bacillus* sp., respectively.

Maximum extracellular cellulase production under shaking condition (Figure 3b) was observed only at 45°C for 48 h. Extracellular cellulase activity values were 0.309 U/mL. Optimum temperature for CMCase production was 50°C (Jang and Chen, 2003; De Lima et al., 2005; Arunachalam et al., 2010).

The properties of extracellular crude cellulase are shown in Figure 4. The profile of cellulases activity obtained from *S. capoamus in relation to* temperature (Figure 4a) showed that the optimum temperature was 60°C.

Many studies have reported that the maximum activity of cellulases produced by bacteria has its peak in temperature ranging between 50 and 55°C, and it is interesting that in all the works cited, an activity above 55°C falls below 40% of residual activity (Ishaque and Kluepfel, 1980; Li and Gao, 1996; George et al., 2001; Saha et al., 2006; Prasad and Sethi, 2013). Even in studies with actinomycetes of the genus *Streptomyces* (*S. halstedii, S. thermodiastaticus* and *S. reticuli*) production peaks have shown a great cellulase activity at a temperature of 55°C (Crawford and Mccoy, 1972; Garda et al., 1997; Ramirez and Coha, 2003).

The favorable pH range used for the production of cellulase *by S. capoamus* was from 2.5 to 8.0; optimum pH was 5.5 (Figure 4b). In this range, there were significant activities (above 80%).

Studies have shown that *Streptomyces* produced maximum cellulase activities at pH 7.0 (Ishaque and Kluepfel, 1980; Li and Gal, 1998; George et al., 2001; Saha et al., 2006; Prasad and Sethi, 2013). Similar results with those found in this study were reported by Garda et al. (1997), where *S. halstedii* presented maximum cellulase activity at pH 6.0. It is shown that other genus of actinomycetes, *Thermomonospora* sp., used a tolerable pH of 5.0 for the production of cellulase and a wide pH range of 4.0 to 10.0 was used for residual enzymes above 80% (Ishaque and Kluepfel, 1980; George et al., 2001; Saha et al., 2006; Prasad and Sethi, 2013).

Besides optima temperature and pH, thermal stability constitutes a very important property for industrial enzymes (Gattinger et al., 1990). The thermal stability of the extracellular crude cellulase obtained from *Streptomyces capoamus* without substrate at 50, 60 and 80°C (Figure 5a) is quite thermostable, while the residual activity was above 80%.

The pH stability of the cellulolytic complex of *S. capoamus* was assayed from 3.0 to 9.0 (Figure 5b). It showed high stability (above 80 %) at pH 3.0 - 9.0.

This is the first report on the production of extracellular cellulase from *S. capoamus*. In optimal culture conditions this strain produced 0.309 U/mL cellulase, a good production for a thermostable endoglucanase with a broad range of pH and temperature stability. Industrial processes are generally carried out at elevated temperatures; therefore enzymes with high optima temperature and stability are desired for applications (Viikari et al., 2007). The purification of the main components of this complex and its physicochemical characterization is being developed in our lab.

Conflict of Interests

The authors have not declared any conflict of interests.

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