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# Extracellular production of avicelase by the thermophilic soil bacterium *Bacillus* sp. SMIA-2

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**ABSTRACT.** Nowadays, the isolation of new bacterial strains that produce enzymes with novel properties is a subject of great relevance to the scientific community. This study, in order to search for producers of new cellulase strains, investigated the avicelase production by thermophilic *Bacillus* sp. strain SMIA-2. The best avicelase activity was observed in a culture medium containing 0.5% (w $v^{-1}$) avicel and 0.5% (w $v^{-1}$) corn steep liquor with initial pH 7.5-8.0 incubated at 50ºC. When avicel was replaced in the medium by the treated sugarcane bagasse (0.5%, w $v^{-1}$) the avicelase activity levels were not affected. Studies on the avicelase characterization revealed that the optimum pH of the enzyme was found to be 8.5 and the enzyme retained more than 80% of its activity after incubation at room temperature for 2h at pH 6.5-8.5. The optimum temperature of this enzyme was 70ºC and the enzyme retained 67% of the original activity after 20 min. of heat treatment at 70ºC. Avicelase was stimulated by $Mn^{2+}$ and $Co^{2+}$, whereas $Hg^{2+}$ greatly inhibited the enzyme activity.

**Keywords:** Avicelase, corn steep liquor, sugarcane bagasse, *Bacillus* sp.

## Produção de avicelase extracelular pela bactéria termofílica *Bacillus* sp. SMIA-2

**RESUMO.** Atualmente, o isolamento de estirpes de bactérias que produzem enzimas com novas propriedades é um tema de grande relevância para a comunidade científica. Este trabalho, buscando por novas cepas produtoras de celulases, investigou a produção de avicelases pelo termofílico *Bacillus* sp. cepa SMIA-2. A melhor atividade da enzima foi obtida em uma cultura contendo 0,5% (p $v^{-1}$) avicel e 0,5% (p $v^{-1}$) água de maceração de milho com pH inicial de pH 7,5-8,0 e incubada a 50ºC. A substituição da avicel no meio de cultura pelo bagaço de cana- de- açúcar tratado (0,5%, p $v^{-1}$) não afetou os níveis de atividade da avicelase. Estudos sobre a caracterização da avicelase revelaram que o pH para atividade ótima da enzima foi 8,5 e que a mesma reteve mais de 80% de sua atividade após ser incubada à temperatura ambiente por 2 h a pH 6,5-8,5. A temperatura ótima da avicelase foi 70ºC e a enzima reteve 67% da sua atividade original após 20 min. de incubação a 70ºC. A avicelase foi estimulada pelos íons $Mn^{2+}$ e $Co^{2+}$, ao passo que $Hg^{2+}$ inibiu a atividade da enzima.

**Palavras-chave:** Avicelase, água de maceração de milho, bagaço de cana-de-açúcar, *Bacillus* sp.

## Introduction

Cellulose, a polymer of β -1,4-linked glucose units, is a major polysaccharide constituent of plant cell walls. It has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources (YIN et al., 2010). The complete enzymatic hydrolysis of cellulosic materials needs at least three different types of cellulases: endoglucanase (CM cellulase, EC 3.2.1.4); exoglucanase (Avicelase, EC 3.2.1.91); and β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) (SINGHANIA et al., 2010). Among these types of cellulases, the exoglucanases appear to catalyze most of the bond-cleavages in the saccharification of crystalline cellulose and are usually the major component of cellulase preparations, especially in the case of current fungus-derived commercial enzymes (LIU et al., 2011).

Cellulases are industrially important enzymes that are sold in large volumes (SINGHANIA et al., 2010) for their usage in different industrial applications (KARMAKAR; RAY, 2011; SHI et al., 2011). Thermostable cellulases are advantageous for some applications due to the fact higher processing temperatures can be employed, with consequent faster reaction rates, improved hydrolysis of cellulosic substrates, and reduced incidence of

microbial contamination from mesophilic organisms (RASTOGI et al., 2011; TURNER et al., 2007). Thus, thermo-stable cellulases, produced from thermophilic bacteria, are of considerable interest in a range of commercial applications (SZIJÁRTÓ et al., 2008; VIIKARI et al., 2007; YANG et al., 2010).

Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes (ASGHER et al., 2007). The ability of different species to ferment in acid, neutral, and alkaline pH ranges, combined with the presence of thermophiles in the genus, has lead to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address several specific applications (SCHALLMEY et al., 2004). Among some species reported as cellulases producers are *Bacillus brevis* (LIANG et al., 2010a), *B. subtilis* (SHABEB et al., 2010), *B. pumilus* (KOTCHONI et al., 2003), *B. cereus* (KUMAR et al., 2012), and *Paenibacillus polymyxa* (WANG et al., 2008).

A fairly common observation has been that bacilli lack the complete cellulase system, whose main activity is carboxymethylcellulase (endoglucanase), which does not hydrolyze crystalline cellulose (MAWADZA et al., 2000). However, two distinct avicelase activities in *B. circulans* were observed (KIM, 1995). In addition, *Caldibacillus cellulovorans*, a thermophilic aerobic bacterium, was reported to produce avicelase (HUANG; MONK, 2004). Recently, a thermophilic *Geobacillus stearothermophilus* that was isolated from soil was capable of producing avicelase (MAKKY, 2009).

Although a number of microorganisms were reported to produce cellulases, in comparison to that of endoglucanase and β-glucosidase, the report of exoglucanase or avicelase production is remarkably scanty. Hence, extensive research has been made to isolate new microbial sources capable of producing exoglucanase (MUKHERJEE et al., 2011). The aim of the present study was to identify some culture conditions that support avicelase production by *Bacillus* sp. strain SMIA-2, along with some biochemical properties of the enzyme for the purposes of exploiting its potential to industrial applications.

## Material and methods

Organism: The bacterial strain used in this study was a thermophilic *Bacillus* sp. strain SMIA-2, previously isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro State, Brazil (SOUZA; MARTINS, 2001).

Enzyme production: The culture medium used in this work for avicelase production contained (g $L^{-1}$ of distilled water): Avicel PH-101 (Sigma-cell 20)-5.0, corn steep liquor-0.5, KCl-0.3, $MgSO_4$-0.5, $K_2HPO_4$-0.87, $CaCl_2$-0.29, ZnO-$2.03x10^{-3}$, $FeCl_3.6H_2O$-$2.7x10^{-2}$, $MnCl_2.4H_2O$-$1.0x10^{-2}$, $CuCl_2.2H_2O$-$8.5x10^{-4}$, $CoCl_2.6H_2O$-$2.4x10^{-3}$, $NiCl_3.6H_2O$-$2.5x10^{-4}$, $H_3BO_3$-$3.0x10^{-4}$. The pH was adjusted to 7.0 with 1.0M NaOH, and this basal medium was sterilized by steam-autoclaving at 121°C, 1 atm for 15 minutes. The medium (50 in 250 mL Erlenmeyer flasks) was inoculated with 1mL of a standard overnight culture and incubated at 50°C in an orbital shaker (Thermo Forma, Ohio, USA), operated at 150 rpm for 192 hours. Triplicate flasks were withdrawn at regular intervals, and the turbidity of the cultures was determined by measuring the increase in optical density at 600 nm with a Hitachi Model U-2000 spectrophotometer. The flasks were allowed to settle for approximately 15min. before the suspension was taken for measurement, considering the insoluble characteristic of cellulose. Before starting the assay, the cells were separated by centrifugation in a centrifuged HERMLEZ 382K (Wehingen, Germany), operated at 15.500 g for 15 min. at 4°C, and the clear supernatant was used as crude enzyme preparation.

Effect of culture conditions on avicelase production*:* The effect of the level of avicel on the enzyme formation was studied first. Its amount was varied in the culture medium from 0.25 to 1.0% (w $v^{-1}$). To evaluate the effect of different concentrations of corn steep liquor to avicelase production, its amount was varied in the culture medium from 0.1% to 0.7% (w $v^{-1}$), with avicel level at 0.5% (w $v^{-1}$). The effect of temperature on avicelase formation was investigated by incubating culture flasks at 42, 45, 50 and 60°C in an orbital incubator shaker for up to a period of 120h. The effect of initial medium pH on avicelase secretion was studied in shake flasks by varying pH (7.0-9.0) and maintaining optimum temperature and other growth supporting conditions. The pH of the medium was adjusted with 10% (w $v^{-1}$) sodium carbonate. In order to investigate the possibility of replacing avicel with complex lignocellulosic biomass for the production of avicelase, *Bacillus* sp. SMIA-2 was grown on sugarcane bagasse over a concentration range (2.5-20 g $L^{-1}$) (w $v^{-1}$). Sugarcane bagasse was provided by a local sugar factory and

was washed first with tap water followed by distilled water to remove the adhered surface dust particles. The material was oven dried at 60º C and grinded in an electric grinder to attain 0.5 mm size of mesh powder. Thereafter, 100 g of this material were treated with 2000 mL solution containing 4% (w $v^{-1}$) $Ca(OH)_2$ and autoclaved at 121ºC for 30 min. The material recovered by filtration on Bucnher funnel, using Whatman no. 1 paper, was washed with distilled water until pH 7.0 and dried in an oven at 65ºC to constant weight.

All experiments were carried out in duplicates, and results were expressed as average values. Statistical tools were carried out to value the significance of the experiments.

Enzymatic assays: The activity of avicelase (EC 3.2.1.91) was assayed in triplicate by incubating 0.5 mL of the crude enzyme with 0.5 mL avicel (1.0%, w $v^{-1}$) prepared in 0.05M Tris-HCl buffer, pH 8.5 at 70ºC. Enzyme and reagent blanks were also simultaneously incubated with the test samples. After 10 min. of reaction, 2 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5min to stop the reaction. The resulting samples were then cooled to room temperature, and the absorbance was measured at 540 nm (MILLER, 1959). One unit (U) of avicelase activity was defined as the amount of enzyme releasing 1µmol of glucose from avicel within 1min. of reaction at 70ºC.

Partial crude enzyme characterization: The optimum pH of crude avicelase secreted by *Bacillus* sp SMIA-2 cultivated on medium containing avicel (0.5%, w $v^{-1}$) and corn steep liquor (0.5%, w $v^{-1}$) was estimated in the pH range of 5.0-10.0 using different assay buffers. The enzyme assays were conducted with avicel 1% (w $v^{-1}$) as substrate, dissolved in different buffers (citrate phosphate, pH 5.0-5.5, sodium phosphate, pH 6.0-7.5, Tris-HCl, pH 8.0-9.0 and glycine NaOH, pH 9.5-10.0). The enzyme activity obtained at the optimum pH was used to calculate the relative percentage enzyme activity at other pH values. The effect of pH on enzyme stability was determined by pre-incubating the crude avicelase preparation without substrate at different pH values (5.0-10.0) for 2h at room temperature, and measuring the residual enzyme activity under standard assay conditions at optimum pH. The effect of temperature on the avicelase activity was determined by performing the standard assay procedure at pH 8.5 within a temperature range of 40-100ºC. The enzyme activity obtained at the temperature optimum was used to calculate the relative percentage enzyme activity at other temperature values. The thermal stability of crude avicelase secreted by *Bacillus* sp. SMIA-2 was tested by determining the enzyme activity remaining after incubation of the crude avicelase at 70, 80 and 90ºC for a period of 2h in a constant-temperature water bath. The residual activities were determined under optimum pH and temperature conditions as described above. The initial activity was assumed to be 100% and used to calculate the enzyme activities as percentages of the initial activity during the incubation period. The effects of metal ions like $K^+$ (KCl), $Na^+$ (NaCl), $Hg^{2+}$ ($HgCl_2$), $Ca^{2+}$ ($CaCl_2$), $Mn^{2+}$ ($MnCl_2$), $Mg^{2+}$ ($MgCl_2$), $Co^{2+}$ ($CoCl_2$) and $Zn^{2+}$ ($ZnCl_2$) on the avicelase activity were determined. The enzyme was pre-incubated with final concentration of 1mM of the corresponding ion in Tris-HCl buffer, pH 8.5 at 40ºC for 15min., and the reaction was carried out at standard assay conditions. The relative activity was measured.

All experiments were conducted in duplicates, and the results were expressed as average values.

## Results and discussion

Culture conditions for enzyme production: The data presented in Figure 1 revealed that *Bacillus* sp. SMIA-2 was able to synthesize avicelase in submerged cultures containing insoluble crystalline cellulose avicel and corn steep liquor. Increasing avicel concentration in the medium to 0.5% (w $v^{-1}$) improved the enzyme secretion. At higher avicel concentrations, enzyme activity was comparatively lower. Regarding the influence of corn steep liquor concentration in the medium, the activity of the avicelase increased between 0.1 and 0.5% (w $v^{-1}$) corn steep liquor concentration and then fell beyond this point (Figure 2b). Corn steep liquor (CLS), a major by-product of the corn wet-milling industry, is an inexpensive substrate available in large scale (PAREKH et al. 1999), capable to provide an additional nitrogen source by providing peptides and amino acids made readily available for cell metabolism. This cheap residue has been successfully used in some culture media for a variety of fermentations such as cellulase (ABDEL-FATTAH et al., 2007; JO et al., 2008; LIMA et al., 2005; SINGH et al., 2001) and protease production (DE AZEREDO et al., 2006). In a previous study performed in our laboratory, we have shown the advantage of using CSL as a rich nitrogen source for proteases and amylases production by *Bacillus* sp. SMIA-2 (CORRÊA et al., 2011) and in this wok it was successfully used for avicelase production. Our

findings are coherent to previous reports of several researchers who have used CSL in the culture medium for the production of cellulases (ABDEL-FATTAH et al., 2007; JO et al., 2008; LIMA et al., 2005; SINGH et al., 2001).

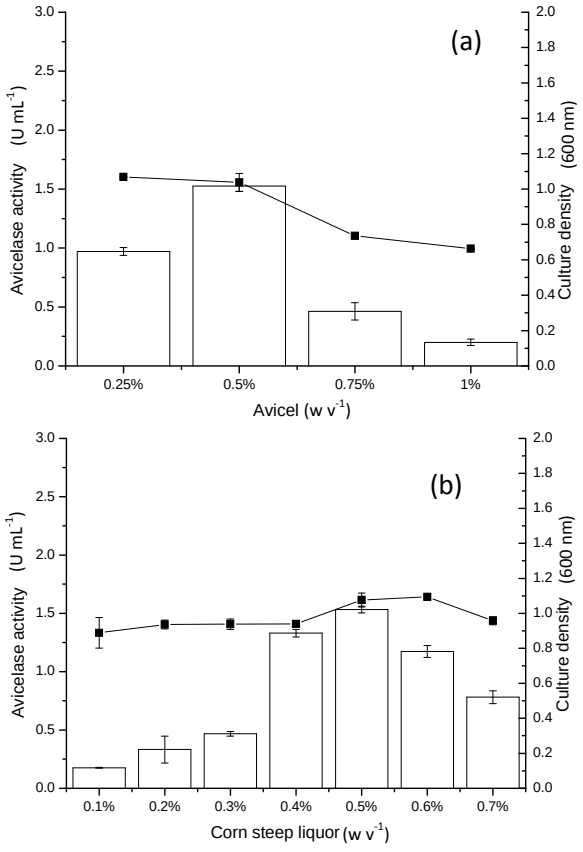


**Figure 1.** Maximal avicelase activity and growth (-■-) of *Bacillus* sp. cultivated in the liquid medium containing different concentrations of avicel (a) and corn steep liquor (b) during 120h at 50ºC.

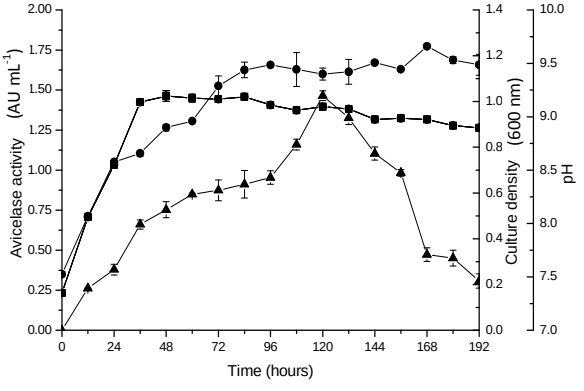


**Figure 2.** Avicelase production as a function of cultivation time by *Bacillus* sp grown on avicel (0.5%, w v$^{-1}$) and corn steep liquor (0.5%, w v$^{-1}$) in shake flasks at initial pH 7.2 and at 50ºC. Culture density (■); Avicelase activity (▲); pH (●).

Figure 2 reports the time-course of avicelase production by *Bacillus* sp. SMI-2 grown in liquid medium containing avicel (0.5%, w v$^{-1}$) as a carbon source and supplemented with corn steep liquor (0.5%, w v$^{-1}$) in 250 mL Erlenmeyer flask. Avicelase production reached a maximum at 120h cultivation (1.5 U mL$^{-1}$), with a rapid decrease after this period, possibly due to protein hydrolysis by protease secreted by the organism, when there was a lack of essential nutrients in the medium (NASCIMENTO; MARTINS, 2004; ROMERO et al., 2009). The medium pH increased from 7.35 to 9.13 within 36h and thereafter remained more or less constant until the end of fermentation. This increase in medium pH suggests that organic nitrogen was being consumed. ABDEL-FATTAH et al. (2007) described avicelase production by a thermophilic *Geobacillus* isolate growing in a optimized medium. The maximal enzyme activity expressed in this medium was 0.8 U mL$^{-1}$. According to these authors, the concentrations of avicel, yeast extract and ammonium sulphate were the most significant factors affecting the process of enzyme production. There are others reports on avicelase production by *Bacillus* sp. (KIM, 1995; KIM; KIM, 1995, BEUKES; PLETSCHKE, 2006; LEE; KIM, 1999), however these studies utilized purified avicelase to assay the avicelase activities. Our data on avicelase activity from *Bacillus* sp. SMIA-2 was generated from crude culture supernatants and therefore a detailed comparison with published literature was difficult to establish. In fact, the difficulty in comparison between cellulase(s) activities depends on several factors including the assay determination and conditions of production (HECK et al., 2002).

It has been shown that the growth of medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane (LIANG et al., 2010a). The avicelase secretion was maximum at 50ºC (Figure 3a) and was less at high (60ºC) and low (42ºC) temperatures. At higher temperature, the organism has to spend a lot of energy for maintenance purposes and at temperatures lower than the optimum temperature, transport of nutrients is hindered (PIRT, 1975). Results illustrated in Figure 3b show that the pH 7.5-8.0 was more suitable for optimization of avicelase production by *Bacillus* sp SMIA-2. These results are close to those of Ray et al. (2007), who found that the cellulase activity of *B. subtilis* and *B. circulans* was higher at pH 7.0-7.5.

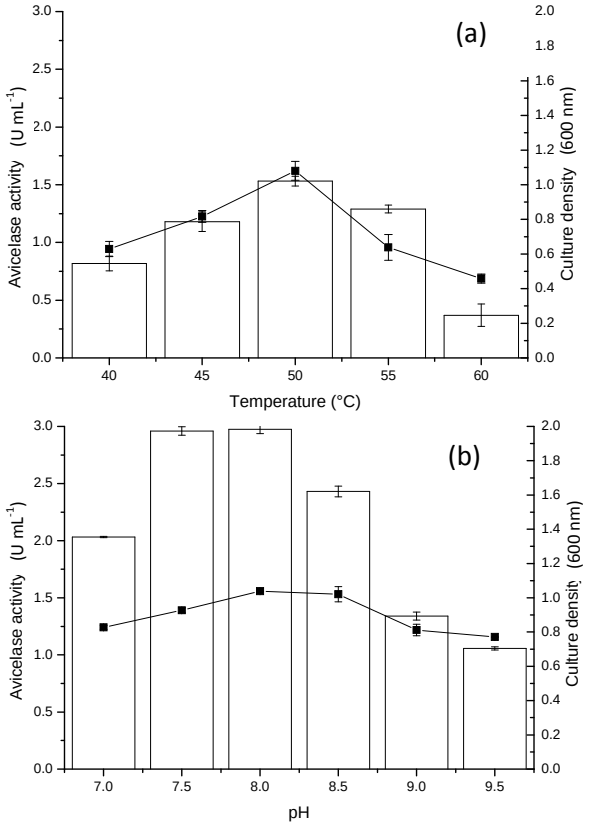


**Figure 3.** Effect of temperature (a) and initial pH (b) on growth (-■-) and avicelase activity of *Bacillus* sp. cultivated in the liquid medium containing avicel (0.5%, w $v^{-1}$) and corn steep liquor (0.5%, w $v^{-1}$), during 120h at 50°C.

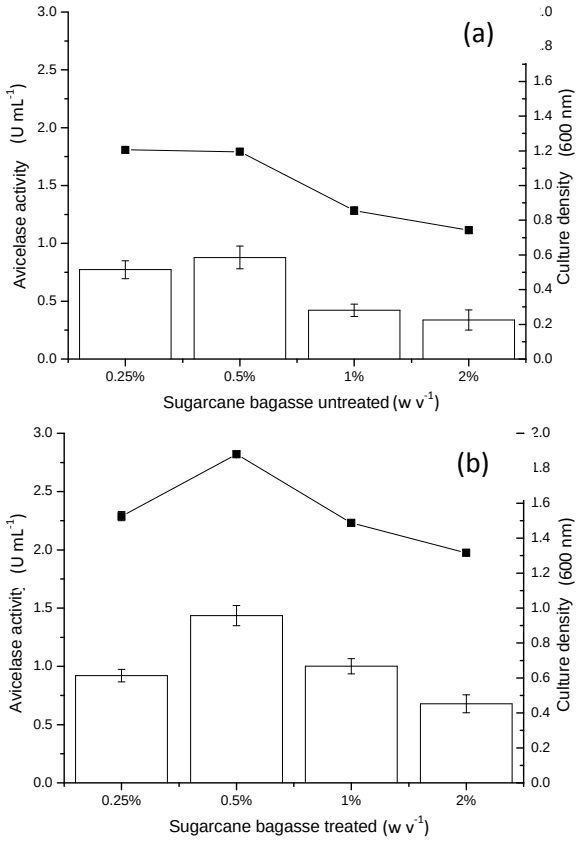


**Figure 4.** Effect of initial pH (a) and temperature (b) on growth (-■-) and avicelase activity of *Bacillus* sp. cultivated in the liquid medium containing avicel (0.5%, w $v^{-1}$) and corn steep liquor (0.5%, w $v^{-1}$), during 120h at 50°C.

Pre-treatment of sugarcane bagasse offers very digestible cellulose and potentially less inhibition (HUANG; MONK, 2004). The growth pattern of *Bacillus* sp. SMI-2 and avicelase production was observed for 192 hours in liquid medium containing treated sugarcane bagasse (0.5%, w $v^{-1}$) and supplemented with corn steep liquor (0.5%, w $v^{-1}$) in 250 mL Erlenmeyer flask (Figure 5).

One of the important criteria taken into account for the choice of thermophilic industrially important producer strain is its ability to secrete enzymes on cheap and local substrates (MAKKY, 2009). Sugarcane bagasse is abundantly and cheaply available as a byproduct from sugar industry in Brazil (CASTRO; PEREIRA, 2010). *Bacillus* sp. SMIA-2 was capable of using sugarcane bagasse as carbon source for avicelase secretion. Among the various sugarcane bagasse concentrations used in the medium, the presence of 0.25-0.5% (w $v^{-1}$) supported maximal avicelase activity (Figure 4a). However, this activity (0.88 U $mL^{-1}$) was lower when compared to cultures growing in avicel. The pre-treatment of sugarcane bagasse resulted in an increase in avicelase activity (Figure 4b). The maximum level (1.44 U $mL^{-1}$) attained was at 0.5% (w $v^{-1}$).

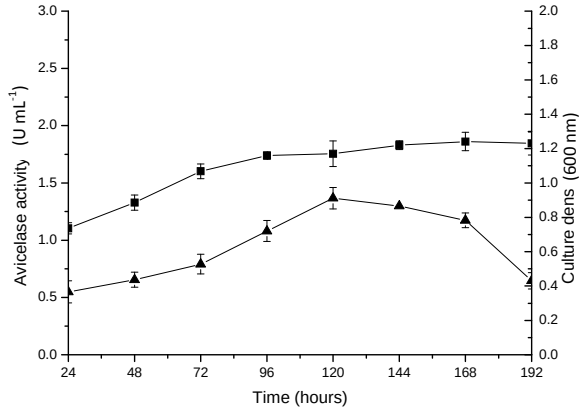


**Figure 5.** Avicelase production as a function of cultivation time by *Bacillus* sp. grown on treated sugarcane bagasse (0.5%, w $v^{-1}$). Culture density (■); Avicelase activity (▲).

The cellular growth profile and the maximum level of avicelase activity (1.37 U $mL^{-1}$) were not very affected in comparison to cultures growing on avicel. In addition, the enzyme deactivation, which was observed in a media containing avicel, was delayed, which is important to minimize the loss of enzyme during the removal of the

medium. Therefore, the utilization of sugarcane bagasse along with corn steep liquor seems to be a very attractive combination to avicelase production by *Bacillus* sp. SMIA-2, making this strain and these low-cost product worthy for further investigation, and potentially feasible for biotechnological applications in different areas.

Partial crude enzyme characterization: Avicelase activity was assayed at different temperatures ranging from 40-100ºC at a constant pH of 8.5. Enzyme activity increased with temperature within the range of 40 to 70ºC (Figure 6a). A reduction in enzyme activity was observed at values above 70ºC. The optimum temperature of this enzyme was 70ºC, which was higher or similar to that described for other *Bacillus* cellulases (LIANG et al., 2010b; MAWADZA et al., 2000). The thermostability of the avicelase was examined by measuring the remaining activities at 70ºC, after incubation of the enzyme without substrate at temperatures of 70, 80 and 90ºC for 2h.

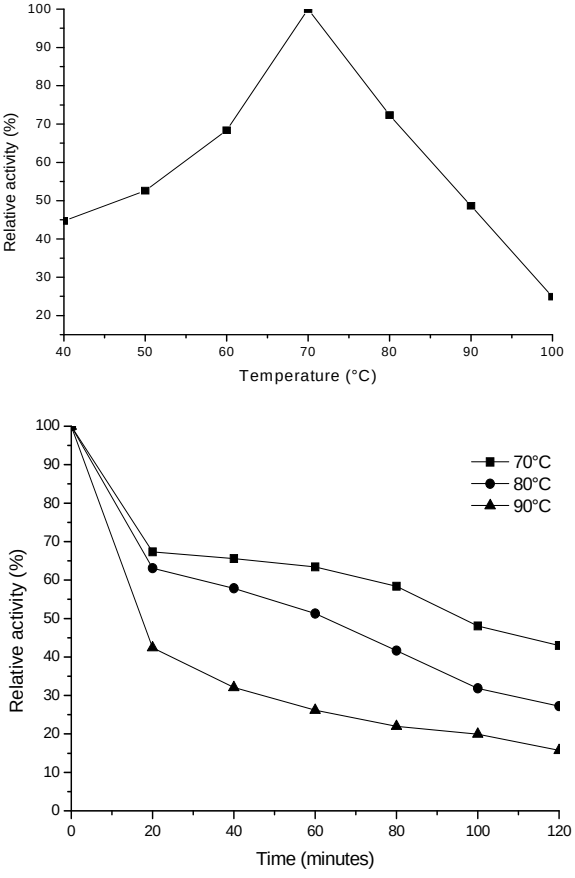


**Figure 6.** Optimum temperature (a) and stability (b) of avicelase produced by *Bacillus* sp grown on avicel (0.5%, w $v^{-1}$) and corn steep liquor (0.5%, w $v^{-1}$). (100% of enzyme activity = 1.58 U $mL^{-1}$).

Thermostability profile indicated that the enzyme retained 67, 63 and 42% of the original activity after 20 min. with heat treatment at 70, 80 and 90ºC, respectively (Figure 6b).

The avicelase from *Geobacillus stearothermophilus* supported maximal avicelase activities at 50ºC when this organism was grown in sugarcane bagasse treated and untreated. In addition, the enzyme showed good temperature stability between 30 and 80º for both treated and untreated sugarcane bagasse (MAKKY, 2009). A *Bacillus* sp. isolated from hot springs has been shown to produce thermo-stable cellulases; however, the cellulase secreted by this bacterium lost 30% activity within 30 min. of incubation at 70ºC (LI et al., 2008). A pH range between 5.0 and 10.0 was used to study the effect of pH on avicelase activity (Figure 7). The optimum pH was found to be 8.5 and the enzyme retained more than 80% of its activity after incubation at room temperature for 2h at pH 6.5-8.5. The avicelase from *Geobacillus stearothermophilus* showed an optimum pH 7.0 and presented good pH stability between 5-8 (MAKKY, 2009).

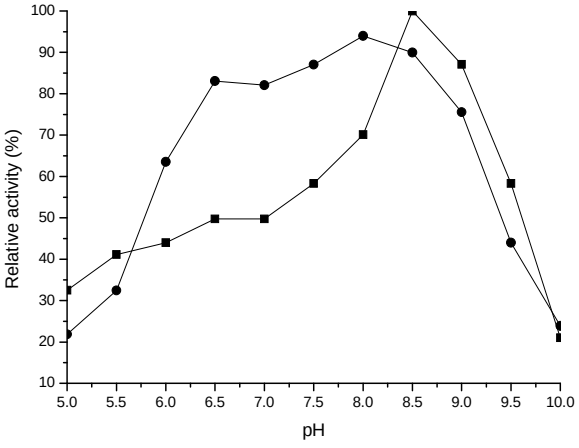


**Figure 7.** Optimum pH (■) and stability (●) of avicelase produced by *Bacillus* sp grown at 50ºC for 120h (100% of enzyme activity = 1.58 U $mL^{-1}$).

Microbial extracellular enzymes are generally known to require divalent cations for activity and stabilization. Avicelase was stimulated by $Mn^{2+}$ and $Co^{2+}$ (Table 1). $Ca^{2+}$ and $Zn^{2+}$ had a slight stimulatory effect and $Hg^{2+}$ greatly inhibited the avicelase activity, probably through the destruction of the active site by the denaturing of the present thiol groups (MUKHERJEE et al., 2011).

**Table 1.** Effect of some metal ions on the activity of avicelase produced by *Bacillus* sp. grown at 50ºC for 120h (Relative activity is expressed as a percentage of control. 100% of enzyme activity = 1.5 U $mL^{-1}$).

| Metal ions | Relative activity (%)⋆ |
|---|---|
| Control | 100 |
| $Co^{2+}$ ($CoCl_2$) | 128 |
| $K^+$ (KCl) | 100 |
| $Na^{2+}$ (NaCl) | 97 |
| $Ca^{2+}$ ($CaCl_2$) | 113 |
| $Mn^{2+}$ ($MnCl_2$) | 138 |
| $Hg^{2+}$ ($HgCl_2$) | 39 |
| $Mg^{2+}$ ($MgCl_2$) | 103 |
| $Zn^{2+}$ ($ZnCl_2$) | 116 |

⋆(Relative activity is expressed as a percentage of control. 100% of enzyme activity = 1.5 U $mL^{-1}$).

## Conclusion

In conclusion, *Bacillus* sp. SMIA-2 showed ability to secrete extra cellular avicelase in cultures containing avicel or sugarcane bagasse as substrate and supplemented with corn steep liquor. The comparative study displayed that the levels of enzyme produced from avicel and treated sugarcane were similar. The broad pH range and temperature stability may make the enzyme useful for industrial applications.

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