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PRODUCTION AND CHARACTERIZATION OF THERMOPHILIC CARBOXYMETHYL CELLULASE SYNTHESIZED BY *Bacillus* sp. GROWING ON SUGARCANE BAGASSE IN SUBMERGED FERMENTATION

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Abstract - The production and characterization of cellulase from thermophilic strain *Bacillus* sp. C1AC5507 was studied. For enzyme production, sugarcane bagasse was used as carbon source. The produced carboxymethyl cellulase (CMCase) had a molecular weight around 55 kDa and its activity varied between 0.14 and 0.37 IU mL^{-1} in conditions predicted by Response Surface Methodology. The optimum temperature and pH for the CMCase production were 70 °C and 7.0, respectively. The enzyme activity was inhibited mostly by Cu^{+2} and activated mostly by Co^{+2}, Mn^{2+}, Ca^{+2} and Fe^{+3}. Our findings provide a contribution to the use of natural wastes such as sugarcane bagasse as substrate for growth and production of thermophilic CMCase. Further optimization to increase the production of cellulase enables the use in industrial applications.
Keywords: *Bacillus* sp.; CMCase; Sugarcane; Enzyme production; Thermophilic cellulase.

INTRODUCTION

Lignocellulosic biomass is the most abundant organic material in nature with high biotechnological potential (Rastogi *et al*., 2010). In Brazil, the largest producer of sugarcane in the world, 720 million tons were generated in 2012 (http://faostat.fao.org/default.aspx, Mar/2014). The large volume of lignocellulolytic waste could cause serious environmental pollution (Vivek *et al*., 2008).

The cellulose conversion to simple sugars by microorganisms plays an important role in the carbon cycle (Romano *et al*., 2013). A set of cellulases is required for complete hydrolysis of cellulosic material: endoglucanase (1,4-ß-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-ß-D-glucanglucohydrolase; EC 3.2.1.74) and ß- glucosidase (ß-D-glucosideglucohydrolase; EC3.2.1.21). The endoglucanase randomly hydrolyzes the ß-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit, showing a recurrent reaction from the chain extremity. Lastly, the cellobiose is converted to glucose by ß-glucosidase (Lynd *et al*., 2002). The enzymatic process to hydrolyze

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cellulosic materials could be accomplished through a complex reaction of these various enzymes (Lee *et al*., 2008). Cellulases can be utilized for applications in the textile and laundry, food and feed, pulp and paper, baking, alcohol from biomass, and waste treatment industries (Bhat, 2000).

Cellulases are produced by a wide range of organisms, including fungi, bacteria and invertebrates (Gautam *et al*., 2011). Most fungi are capable of degrading both amorphous and crystalline cellulose, while the majority of the bacterial strains effectively hydrolyze only amorphous cellulose (CMC, carboxymethylcellulose). Some bacterial genera such as *Bacillus* and *Paenibacillus* are capable of degrading both CMC and Avicel (Afzal *et al*., 2010), as well as lignocellulosic waste from the agro-industry (Macedo *et al*., 2013).

Many researchers have used low-cost agricultural residues such as sugarcane bagasse, wheat bran, rice bran and others, which have been found to be good sources of cellulase production (Techapun *et al.*, 2003). Moreover, various carbon sources in the growth medium influenced the bacterium's ability to produce cellulase (Techapun *et al*., 2003). Cellulose degradation under aerobic and thermophilic conditions offers many advantages, such as preventing contamination and because the products from aerobic cellulose degradation are simple sugars that have the versatility and flexibility to be transformed into many value-added commodities including, but not limited to, ethanol, butanol, hydrogen, and amino acids (Liang *et al*., 2009).

The search for new cellulases with properties more suitable for industrial application remains a constant effort (Lima *et al.*, 2005). In addition, new thermophilic cellulases are being exploited in order to make the relevant industrial processes more efficient and economical (Liang *et al*., 2009).

In this current study a cellulolytic bacteria of the *Bacillus* genus was isolated and identified from a soil sample and described the characterization of the thermophilic cellulase produced using sugarcane bagasse as a carbon source.

MATERIALS AND METHODS

Isolation of the Microorganism Producing Cellulase

To isolate microorganisms, a sample of soil (25 g) in a sugarcane plantation field in Brazil (6.59 S 35.1 W) was used as a screening source. The soil was suspended with 225 mL of Ringer solution containing (g/L): NaCl 7.2; $CaCl_2$ 0.17 and KCl 0.37; at pH 7.0. The suspension was stirred at 200 rpm for 20 minutes. Suitably diluted samples were spread onto a solid medium containing (g/L): CMC 1.0; NaNO3 0.5; K2HPO4 1.0; MgSO4.7H2O 0.5; FeSO4.7H2O 0.01; yeast extract 1.0; and agar 15.0; at pH 7.0 (Teather and Wood, 1982). Plates were incubated at 55 °C for 3 days. Isolates were transferred to this CMC-agar medium and incubated at 55 °C for 3 days. Microorganisms with cellulolytic activity were detected by the formation of clear zones around colonies through the Congo red overlay method (Teather and Wood, 1982). The isolate C1AC5507 was chosen for subsequent studies.

Analysis of 16S rDNA Gene Sequence

Genomic DNA from the isolate was extracted as described by Cheng and Jiang (2006), with slight modifications. The genomic DNA was diluted appropriately and used as a template in PCR reactions using universal eubacterial primers 27F and 1525R. The PCR reaction was run for 25 cycles with the following thermal profile for the PCR: 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min. The final cycle included extension for 10 min at 72 °C to ensure full extension of the products. The DNA sequencing reaction of PCR amplification was carried out using an ABI BigDye Terminator v3.1 cycle sequencing kit and an ABI 3730XL DNA analyzer (Applied Biosystems, USA).

The 16S rRNA gene sequences of related taxa were obtained from GenBank. Phylogenetic trees were constructed using the neighbor-joining method with the program MEGA 5.0 (Tamura *et al*., 2011). The partial 16S rDNA sequence was submitted to GenBank under accession no. JX437692.

Production of Cellulase

Firstly, sugarcane bagasse samples were dried at 60 °C for 24 h. Then they were ground to a powder in a blender (Waring, USA). The ground bagasse was sieved and it was autoclaved twice (121 °C/0.1 MPa for 15 min). The medium used for production of the cellulase contained the following components (g/L): sugarcane bagasse powder 10; $NaNO_3$ 0.5; $K2HPO_4$ 1.0; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.01; and yeast extract 1.0 at pH 7.0. The inoculum medium was the same medium with 1% lactose added. Cellulase production was carried out in 250 mL conical flasks containing 100 mL of the described medium. These were then inoculated with 1% (v/v) of inoculum culture and incubated for 18 h at 37 ºC under shaking at 150 rpm. After 72 h of incubation, the contents of the fermented flasks were followed by centrifugation

at 9,000 *g* for 15 min at 4 °C, and the clear cell-free supernatant (crude extract) was collected and stored at 4 °C before enzymatic assay. Using the same method, other carbon sources (lactose and CMC) were evaluated.

Enzymatic Assay

CMCase activity was determined according to the method of Ghose (1987), with some modifications. This method measures at proper temperature the release of the reducing sugars produced in 10 min from a mixture of enzyme solution (0.25 mL) and 0.25 mL of a 4.5% CMC (Sigma, USA) solution made in a specific buffer. After incubation the concentration of reducing sugars was determined using the dinitrosalicylic acid (DNS) method described by Miller (1959). One unit of CMCase was defined as the amount of enzyme that catalyzes the release of 1μmol of reducing sugar glucose equivalent per minute under the specified assay conditions. All activity measurements were repeated three times.

Effect of pH, Temperature and Additives on Cellulasic Activity

Through the enzymatic assay performed with the crude extract, it was possible to evaluate the effect of temperature and pH on cellulosic activity by response surface methodology (RSM) using 2^2 central composite designs with 5 coded levels, leading to 11 sets of experiments, 8 unique combinations, and 3 replicates at the central point (Table 1). Various buffer salts were used for determination of the optimal pH, 100 mM citrate (pH 4.2–5), sodium phosphate (pH 7–8), glycine–NaOH (pH 9–9.8), and appropriate temperatures (ranging from 36 to 70 ºC). The data analysis was carried out using STATISTICA 7.0 software (StatSoft Inc., Tulsa, USA).

Table 1: Experimental range and levels of each variable studied using RMS.

Independent Variables	Code Level of Variables				
	-α	-1	0	1	+α
Temperature (°C)	30	36	50	64	70
pH	4.2	5.0	7.0	9.0	9.8

The effects of additives on enzyme activity were assayed using various metal salts such as $CaCl_2$, $CuCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $CdCl_2$, $NiCl_2$, $NaCl_2$ and EDTA. Each reagent was added to the enzyme solution at a final concentration of 5 mM, and Tween 20 (0.25%) at room temperature for 10 min. Relative CMCase activity was determined.

Thermostability was first investigated by incubating the enzyme at 20, 30, 40, 50, 60, 70, 80, and 90 °C for 30, 60, and 120 minutes. The residual enzyme activity was determined with 4.5% CMC in a 100 mM sodium phosphate buffer with pH 7.0, at 70 °C (value optimized by RSM analysis).

The pH stability was analyzed by incubating the enzyme in 100 mM buffers with variable pH values at 25 °C for 30, 60, and 120 minutes, and then measuring the residual enzyme activity in 4.5% CMC at pH 7.0 within a 100 mM sodium phosphate buffer at 70 °C. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad software, San Diego, USA) by 1-way Analysis of Variance (ANOVA), followed by Tukey posttest.

Cellulase Molecular Weight Determination

Crude extract was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to the method described by Laemmli (1970). After electrophoresis, the gels were stained with a solution of 0.1% (w/v) Coomassie blue, 30% (v/v) methanol, and 10% (v/v) acetic acid. The molecular mass markers used were those for low molecular weight ranges (GE, USA) including phosphorylaseb (97kDa), serum albumin (66kDa), ovalbumin (45 kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20.1 kDa), and α-Lactalbumin (14.4 kDa). For detection of cellulase activity, 0.1% CMC was included in gels before polymerization. The enzymes are separated in denaturing conditions (SDS). After electrophoresis, gels were incubated in Triton (to remove the SDS), then washed in 50 mM phosphate buffer, pH 7.0, for 30 min, and incubated at 37 ºC for 30 min in the same buffer. Gels were then stained with 0.1% Congo Red for 15 min and washed with 1 M NaCl until the cellulase band became visible. Gels were then immersed in 5% (v/v) acetic acid and photographed.

RESULTS AND DISCUSSION

During isolation and screening of cellulase-producing strains, one active organism was identified by its morphological and physicochemical characteristics to be a *Bacillus* species according to the methods described in Bergey's Manual of Systematic Bacteriology (Vos *et al*., 1984). The genus *Bacillus* is comprised of Gram positive bacteria whose species have been isolated from different environments (Bruce *et al*., 2010; Maughan and Van der Auwera, 2011). Woo and colleagues (2013) argue that physical and

chemical properties of soil could explain the presence of potentially novel and efficient lignocellulolytic bacteria in tropical forests. However, studies of bacterial isolation from South American Atlantic Rainforest soil are scarce; nevertheless, this environment represents a vast source of novel bacteria (Bruce *et al*., 2010; Faoro *et al*., 2010).

The partial sequence of 16S rDNA was obtained with a length of 829 bp. The 16S rRNA gene sequence was compared with those of others in the GenBank databases using the NCBI BLAST tool (http://www.ncbi.n1m.nih.gov/blast/Blast.cgi). Based on the 16S rRNA gene sequence similarity (98%), C1AC5507 was closely related to *Bacillus safensis*, which was first isolated by Venkateswaran and colleages (2001) after being collected from a spacecraft assembly facility. Further studies are needed to determine whether C1AC5507 can be classified as *Bacillus safensis* or as a new species. A phylogenetic tree illustrating the relationship of strain C1AC5507 to other *Bacillus* species is depicted in Figure 1.

Several cellulases have been found in different members of the genus *Bacillus* (Rawat and Tewari, 2012). *Bacillus* sp. strain C1AC5507 showed clear zones on CMC agar plates followed by staining with 1% Congo red solution, indicating that it secretes CMCase. The CMCase production by *Bacillus* sp. strain C1AC5507 was high when the cell population entered into the stationary phase, suggesting that enzyme secretion is not growth-associated (Figure 2). CMCase activity was highest at 72 h of incubation and declined on further increase in the time of incubation. Experiments were conducted to determine the effect of different carbon sources on CMCase production. Overall, sugarcane had the highest CMCase activity; i.e., 0.38 IU mL^{-1} at 72 h (Figure 3).

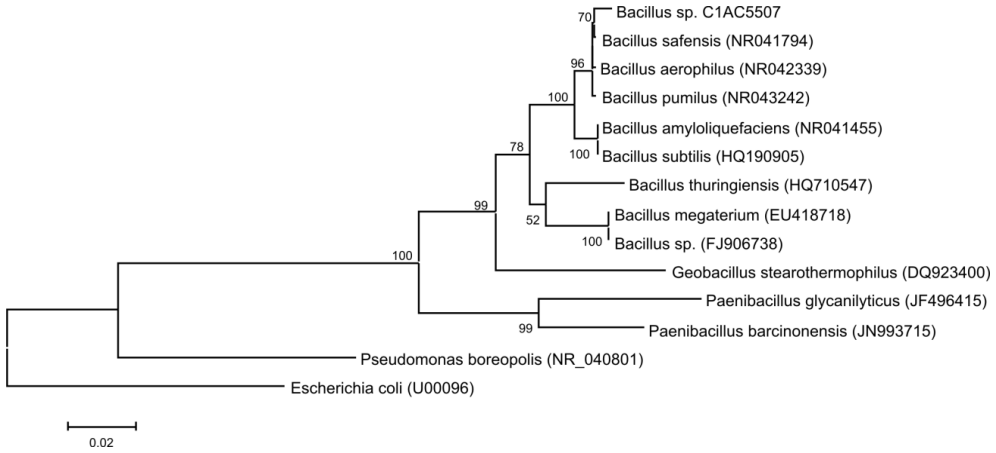

Figure 1: Phylogenic relationship of 16S rRNA sequences of *Bacillus* sp. strain C1AC5507 with other types of strains. The *E. coli* sequence was used as an outgroup model. Numbers at nodes are percentage bootstrap values based on 1000 re-sampled datasets. The bar shown in the figure represents 0.02% sequence dissimilarity per nucleotide position. Accession numbers of the 16S rRNA gene sequence are listed at the right.

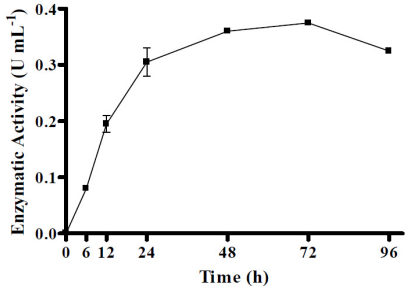

Figure 2: The time course of fermentation for CMCase production by *Bacillus* sp. strain C1AC5507 at 37 °C, 150 rpm, in culture medium containing sugarcane bagasse. The enzyme activity was determined in a 100 mM sodium phosphate buffer at 50 °C, pH 7.0. Values are the mean ± SD of two experiments in replicates.

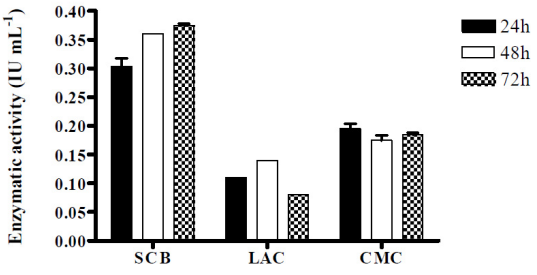

Figure 3: The effect of carbon source on CMCase productivity (fermentation using 1% carbon source, SCB – Sugarcane-bagasse; LAC – Lactose; CMC – carboxymethylcellulose, initial pH of 7.0, and temperature of 37 °C, 150 rpm). The enzyme activity was determined in a 100 mM sodium phosphate buffer at pH 7.0 and 50 °C. Values are the mean ±SD of two experiments in replicates.

The crude extract obtained from sugarcane bagasse in submerged fermentation was analyzed by RSM in terms of CMCase activity. The results of the experimental data and predicted values are depicted in Table 2. The activity varied between 0.14 and 0.37 IU mL^{-1} in the tested conditions, as required by RSM design. A second-order polynomial equation was used to explain the production:

$$AE = 0.297(\pm 0.007) + 0.069(\pm 0.004) * X$$

$$-0.042(\pm 0.006) * X * Y - 0.061\ (\pm 0.005) * Y^2$$

$$-0.021(\pm 0.005) * X^2,$$

where AE is CMCase activity, X represents temperature, and Y represents the pH. The proposed model equation illustrates the interaction between the two factors.

Table 2: Two-level factorial design for CMCase production by *Bacillus* sp. C1AC55-07 howing observed and predicted responses.

	Independent Variables		CMCase activity (IU ml^{-1})	
Run	pH	T (°C)	Experimental	Predicted
1	5.0	36	0.16	0.11
2	5.0	64	0.36	0.33
3	9.0	36	0.15	0.17
4	9.0	64	0.18	0.22
5	7.0	50	0.29	0.29
6	7.0	50	0.31	0.29
7	7.0	50	0.29	0.29
8	7.0	30	0.14	0.15
9	7.0	70	0.37	0.35
10	4.2	50	0.14	0.19
11	9.8	50	0.21	0.15

The analysis of variance of the regression for CMCase activity is summarized in Table 3. The optimum values of temperature and pH were determined. To investigate the interaction between the two factors researched on CMCase production, RSM was used and three-dimensional plots were made between the two factors and CMCase production. The response surface plots between temperature and pH are shown in Figure 4. The conditions that led to the greatest activity were 65-70 °C and pH 5.0 to 7.0. Figure 3 shows 3D plots obtained after simultaneously investigating the influences of pH and temperature on production of CMCase. CMCase production over a broad pH range is considered characteristic of cellulases secreted by *Bacillus* sp. (Mawadza *et al*., 2000). Cellulase production observed at 70 °C is obviously different from other *Bacillus* strains (Mawadza *et al*., 2000; Li *et al*., 2006; Rastogi *et al.*, 2010).

The thermal stability of CMCase was also determined at various temperatures ranging from 40 to 90 °C at pH 7.0, as shown in Figure 5(a). More than 80% of the original CMCase activity was maintained at temperatures ranging from 40 to 60 °C after 120 minutes of incubation at pH 7.0, whereas less than 25% of the original CMCase activity was observed at 70 to 90 °C after 30 minutes of incubation.

The pH stability of the CMCase was also examined at various pH levels ranging from pH 4.2 to pH 9.8, as shown in Figure 5(b). The original CMCase activity was maintained at a wide range of pHs ranging from 5.0 to 9.0 after 120 minutes of incubation at 25 °C. The pH stability of the CMCase produced by *Bacillus* sp. strain C1AC5507 over a broad pH range seems to be a common characteristic of many *Bacillus* CMCases (Ko *et al*., 2010).

The molecular mass of the CMCase from *Bacillus* sp. strain C1AC5507 was estimated to be about 55 kDa (Figure 6). In this study, the molecular weight of the enzyme is close that of to Lee *et al*. (2008), who also reported a molecular mass of purified CMCase at around 53 kDa, produced by *B. amyloliquefaciens*. Cellulases from *Bacillus* species exhibit a variable molecular mass ranging from 24.4-185 kDa (Qiao *et al*., 2009; Rawat and Tewari, 2012). The effect of various metal ions and other compounds on the cellulase activity was examined by performing the assay with the addition of additives. The activity measured with no additional reagent was taken as 100%.

Table 3: Analysis of variance (ANOVA) of the second order polynomial model for ptimization of production of CMCase.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio (Model SIgnificance)	*p*-value
pH (Quadratic)	0.021330	1	0.021330	159.9724	0.006193*
Temperature (Quadratic)	0.002600	1	0.002600	19.5018	0.047643*
Temperature (Linear)	0.038540	1	0.038540	289.0536	0.003442*
1L x 2L	0.007225	1	0.007225	54.1875	0.017959*
Lack of fit	0.012622	4	0.003156	23.6663	0.040952*
Pure error	0.000267	2	0.000133	-	-

* Significant factors ($p < 0.05$); R^2 (coefficient of determination) = 0.84; Adjusted R^2=0.7316.

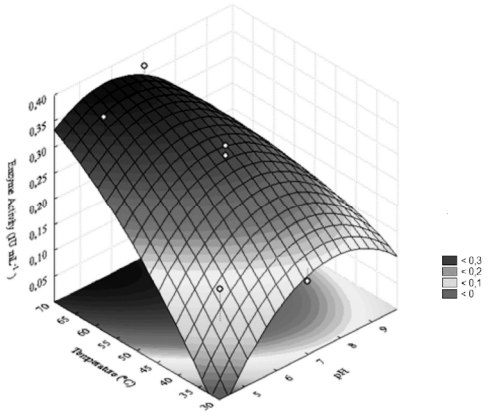

Figure 4: Response surface plot showing the effect of temperature and pH and their mutual interaction on production of CMCase from *Bacillus* sp. strain C1AC5507. The dots represent the averages of three replicates.

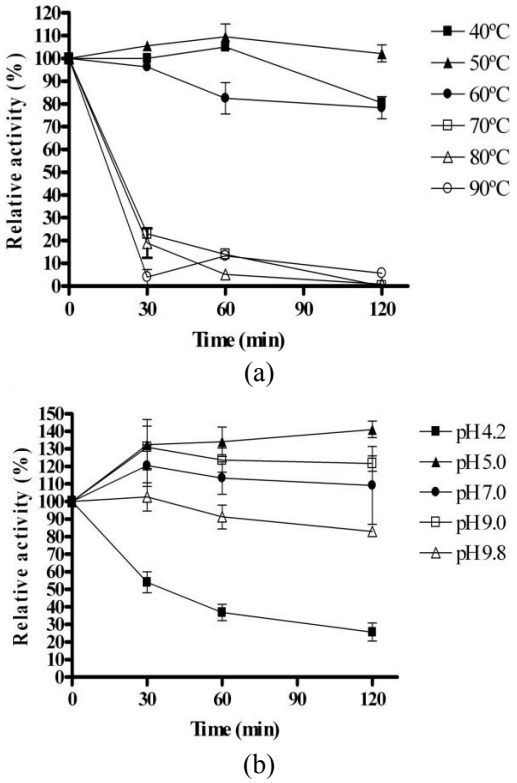

Figure 5: Effect of temperature and pH on the activity and stability of CMCase produced by *Bacillus sp.* strain C1AC5507. a – thermal stability at 40 °C (■), 50 °C (▲), 60 °C (●), 70 °C (□), 80 °C (△), and 90 °C (○). b – pH stability: Enzyme was analyzed first by incubating it in 100 mM buffers with the following pH: 4.2 (■), 5.0 (▲), 7.0 (●), 9.0 (□), and 9.8 (△) for 120 minutes. Values are the mean ±SD of three experiments in replicates.

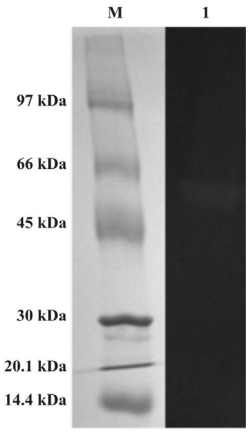

Figure 6: Zymogram analysis of CMCase produced by *Bacillus* sp. strain C1AC5507. The right panel shows a polyacrylamide gel containing CMC after zymogram activity staining. Lanes: molecular weight markers (M); CMCase obtained from crude extract (1).

As shown in Figure 7, CMCase activity was strongly stimulated by Co^{+2}, Mn^{+2}, Ca^{+2}, Fe^{+3} and Tween 20, and more mildly by Na^{+}, Cd^{2+}, Ni^{+2} and Mg^{2+}. EDTA slightly inhibited the CMCase activity, while Cu^{2+} strongly inhibited the enzyme activity. The results of additive effects indicate the presence of binding sites to stabilize the conformation of the CMCase with an increased affinity for the substrate (Fu *et al*., 2010).

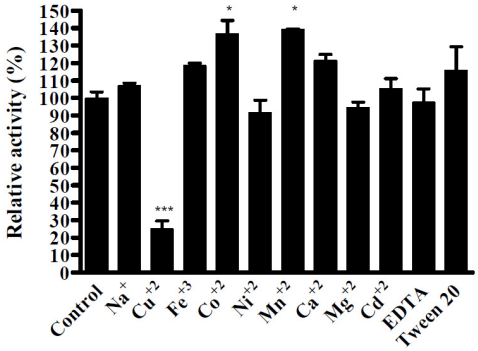

Figure 7: Effect of various additives on CMCase activity. Values are the mean ±SD of three experiments in replicates. Significant differences are indicated by * P<0.05 and *** P<0.0001.

Furthermore, enzymatic activity was not strongly inhibited by EDTA, indicating a resistance to the removal of divalent cations. Cu^{2+} strongly inhibited the activity of CMCase, which may be ascribed to the breakdown of the disulphide bonds. Although salt tolerance of the endoglucanase produced by *B.*

subtilis A-53 is one of distinctive characteristics of marine microorganisms (Kim *et al*., 2009), CMCase of soil microorganisms has demonstrated considerable salt tolerance. These properties confirm the essential requirements for use of the enzyme in industrial applications.

CONCLUSION

In the present study we isolated a *Bacillus* sp. strain from a Brazilian tropical soil producing CMCase with sugarcane bagasse in submerged fermentation. Values of up to 0.37 IU mL^{-1} were obtained when Response Surface Methodology was used. The optimum temperature and pH for the CMCase production were 70 °C and 7.0, respectively. On the other hand, the enzyme maintained more than 80% stability at 60 °C during 2 hours. Thermostable cellulases are a key for industrial application of cellulose hydrolysis. Taken together, our findings on the production of cellulose-degrading enzymes under unoptimized medium and culture conditions represent a strong contribution to the current knowledge on the use of natural wastes such as sugarcane bagasse as substrates for production of thermophilic CMCase. Further optimization of conditions to increase the production of cellulase could enable its use in industrial applications.

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