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A N I N T E R N A T I O N A L J O U R N A L

Production and Properties of Xylanase from Thermophilic *Bacillus* sp.

Carlos Alberto Martins Cordeiro, Meire Lelis Leal Martins*, Angélica Bárbara Luciano and Roberta Freitas da Silva

Universidade Estadual do Norte Fluminense; CCTA/LTA; Av. Alberto Lamego, 2000; 28015-620; Horto; Campos dos Goytacazes - RJ - Brazil

ABSTRACT

An aerobic, thermophilic, xylanolytic bacterium was isolated from local soil. The results of 16S rRNA sequence comparisons indicated that the isolate was closely related to Bacillus caldoxylolyticus and Bacillus sp strain AK1. These organisms exhibited 94% levels of ribossomal DNA sequence homology. Studies on the xylanase characterisation from liquid cultures grown on beechwood xylan revealed that the enzyme retained 100% of activity for 2 hours at temperatures ranging from 30 to 50° C, while at 60, 70 and 100° C, 10%, 11% and 29% of the original activities were lost, respectively. The optimum pH of the enzyme was found to be between 6.5 and 7.0. After incubation of crude enzyme solution for 24 hours at 25° C and at pH 5.5 to 8.0, a decrease of about 12% of its original activity was observed.

Key-words: Xylanase, thermophilic bacterium, *Bacillus* sp.

INTRODUCTION

Biodegradation of xylan, a major component of plant cell walls, requires action of several enzymes, among which xylanases play a key role (Blanco et al., 1999). A wide variety of microorganisms are known to produce xylanases, that are involved in the hydrolysis of xylan (Dimitrov et al., 1997; Sunna and Antranikian, 1997; Sunna et al., 1997; Pham et al., 1998; Beg et al., 2000). Recently the interest in xylanases has markedly increased due to the potential applications in pulping and bleaching processes using cellulase free preparations, in the food and feed industry, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment (Van der Broeck et al., 1990; Gilbert et al., 1992; Godfrey and West, 1996; Mechaly et al., 1997; Wong et al., 1988). Most of these processes are carried out at high temperatures, so that thermostable enzymes would give an advantage (Sonnleitner and Fiechter, 1983). Therefore, thermophilic organisms are of special interest as a source of novel thermostable enzymes (Becker et al., 1997; Lee et al., 1999; Beg et al., 2000; Touzel et al., 2000). In the present study, the production of an thermostable xylanase by a Bacillus sp isolated from soil and properties of the crude enzyme are reported.

* Author for correspondence

MATERIALS AND METHODS

Culture medium. Agar plate A consisted of 2% Bacto-tryptone, 1% Bacto-yeast extract, 1% NaCl and 2% agar at pH 7.0. This was used for selection of thermophilic bacteria. Agar plate B contained 1% xylan, 0.2% yeast extract, 0.5 % peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at pH 7,0. This was used for screening xylanase-producing bacteria.

Isolation and screening. Soil suspensions in sterilised water were poured and spread onto agar plates A. These plates were incubated at 65 ^oC for 2 days. The colonies that were found on the plates were transferred onto agar plates B, which were again incubated at 65 ^{o} for 2 days. Several xylanase-producing bacterial colonies were selected after flooding the plates with 0,1% aqueous Congo red for 15 min followed by repeated washing with 1 mol L^{-1} NaCl (Gessesse and Gashe, 1997). All colonies showing a clear zone on agar plates were further screened by growing them in liquid medium and assaying enzyme activity from the cell-free culture supernatant fluid One strain, designated as *Bacillus* sp was selected for further experiments.

16S rRNA sequence analysis: The genomic DNA was extracted from the isolate and the amplification of the 16S rDNA was performed through PCR technique. The purified PCR products was sequenced on an automated DNA sequencer (ALFexpress, Pharmacia). The 16S rDNA sequence of isolate was aligned with the 16S rRNA gene sequences of various members of the genus *Bacillus* obtained from the Ribosomal RNA Database Project and from Genbank (Larsen et al., 1993). Matrices of evolutionary distances were computed from the sequences alignments by calculating a pair wise Jukes-Cantor (Jukes and Cantor, 1969). From these distances, phylogenetic trees were inferred by a neighbour-joining method (Saitou and Nei, 1987).

Enzyme production: The growth medium used for xylanase production contained (g/L): beech wood xylan 10.0, yeast extract 2.0, peptone 5.0, $MgSO_4$ 0.5, NaCl 0.5, $CaCl_2$ 0.15. The pH was adjusted to 7.5 and the medium was sterilised by autoclaving at 121 ^oC (15 psi) for 15 min. This medium (50 mL in 250 mL erlenmeyer flasks) was inoculated with 1 mL of an overnight culture and incubated at 50 ^oC with vigorous aeration in a shaker at 150 rpm for 144 hours. Before assay, the cells were separated by centrifugation at 4500g. The clear supernatant was used as crude enzyme preparation.

Enzyme assay: Xylanase activity was assayed by measuring the release of reducing sugar from beechwood xylan following the dinitrosalicylic acid (DNS) method (Miller 1959). To 1.8 mL of substrate in phosphate buffer, pH 6.5, 0.4 mL of culture supernatant was added and incubated at 90^o C. After 10 min, 2.0 mL of DNS solution was added to the reaction mixture and boiled for 10 min. Absorbance was measured at 540 nm against a reagent blank. One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol reducing sugar equivalent to xylose per min under the above assay conditions.

Effect of pH on activity and stability of xylanase: Effect of pH on the activity of xylanases was measured by incubating 0.4 mL of enzyme and 1.8 mL of buffers, adjusted to pH of 5.5 to 8.5, containing beechwood xylan (0.5%). The buffers used were:sodium acetate buffer, pH 5.5; phosphate buffer, pH 6.0 – 8.0; Tris-HCl buffer, pH 8.5. Stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 5.5 – 8.5 for 24 hours at 25^o C and then estimating the residual activity.

Effect of temperature on activity and stability of xylanase: The effect of temperature on the enzyme activity was determined by performing the standard assay procedure as mentionated earlier for 10 min at pH 6.5 within a temperature range of 40 –100^o C. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 40-100^o C for 2 hours. After treatment the residual enzyme activities were assayed.

RESULTS AND DISCUSSION

Identification of organism: The strain isolated was categorized as thermophilic since it required a temperature range to grow of 44 -70^o C. It was unable to grow outside this range.
An analysis of the 16S rRNA gene of isolate revealed that this organism was closely related

phylogenetically to members of the genus *Bacillus* rRNA group 5 (Rainey et al., 1994). This group includes *Bacillus stearothermophilus* and other thermophilic *Bacillus* spp. Further analysis indicated that the levels of similarity ranged from 90 and 91% from *B. stearothermophilus*, *B. thermocatenulatus*, *stearothermophilus*, *B. thermocatenulatus*, *B. thermoleovorans*, *B. denitrificans*, *B. flavothermus* and *B. thermoglucosidasius* to 94% for *B. caldoxylolyticus* and *Bacillus* sp Strain AK1 (Fig.1).

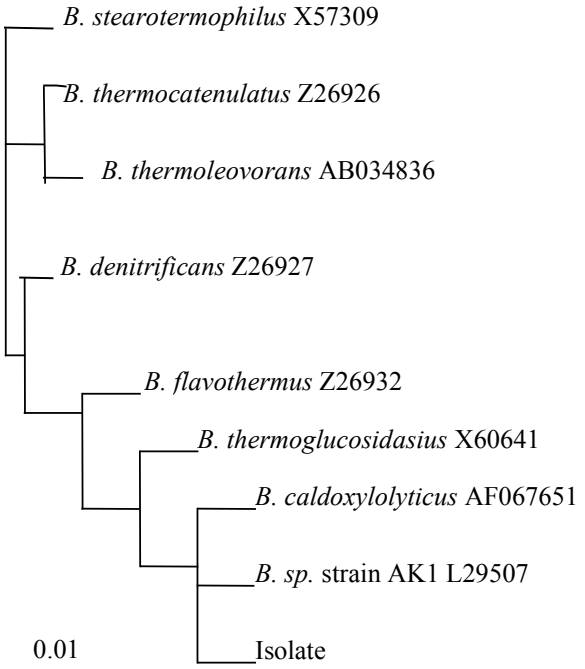

Figure 1 - Phylogenetic dendrogram based on 16SrDNA sequence data indicating the position of isolate among representatives of the genus *Bacillus*. All the sequences used in the analysis were obtained from the Ribosomal Database Project and Genbank. Scale bar indicates evolutionary distance.

The bacteria most closely related to strain *Bacillus* sp. AK1 (Maciver et al., 1998) for which 16S rRNA sequence data are available is *B. thermoglucosidasius*. This organism, described by Susuki et al., 1983, was isolated from soil and it was a obligate thermophile with an optimum temperature at 61-63^{o} C. Therefore, on the basis of phylogenetic studies we concluded that the isolate was closely related to the members of the *Bacillus* rRNA group 5 and was identified as *Bacillus* sp.

Enzymatic production: The growth pattern of *Bacillus* sp , xylanase activity and pH change were observed for five days in the liquid medium containing 0.5% xylan as a carbon source in 250 mL Erlenmeyer flask (Fig.2). The pH of the culture medium increased from 6.7 at the beginning to 7.5 in 36 h and then dropped to 6.8 at the and of fermentation. The organism grew logarithmically up to 36h and there was no obvious correlation between growth and extracellular xylanase activity. The formation of xylanase started from 18h and reached a maximum at 72h. Low amounts of extracellular xylanases were detected during the logarithmic growth phase of the *Bacillus* SSP-34 (Subramaniyan, Prema, Ramaktisna, 1997) and *Bacillus* sp (Nakamura et al., 1993).The increase in xylanase activity during later stages of growth might be due to the release of small amounts of xylanases from the aged cells entering into autolysis (Espinar et al., 1992) and also due to the scarcity of insoluble xylan particles in the medium which if present in the culture broth might bind the xylanases (Subramaniyan, Prema, Ramaktisna, 1997).

Effect of pH on activity and stability of xylanase: A pH range between 5.5 and 8.5 was used to study the effect of pH on xylanase activity (Fig.3). Optimum pH was found to be between 6.5 and 7.0. The enzyme activity at pH 6, 7 and 8.5 were 87, 97 and 64% of that at pH 6.5 respectively. After incubation of crude enzyme solution for 24 hours at pH 5.5 –8.0 there was a decrease of about 12% of its original activity and at pH 8.5 the decrease was of 32%.

Effect of temperature on activity and stability of xylanase: The supernatant xylanolytic activities were assayed at different temperatures ranging from 30^{o} C-100^{o} C at a constant pH of 6.5 and a substrate concentration of 0.5% (Fig.4). Xylanase from the isolate exhibited a temperature profile with a sharp peak of maximal activity at 90^{o} C and showing activity between 40-100^{o} C. The residual activities of crude xylanase incubated at different temperatures for a period of 2 hours were estimated at optimum temperature The enzyme was stable for 2 hours at temperatures ranging from 30-50^{o} C while at 60^{o} C, 70^{o} C and 100^{o} C, 10%, 11% and 29% of the original activities were lost respectively.

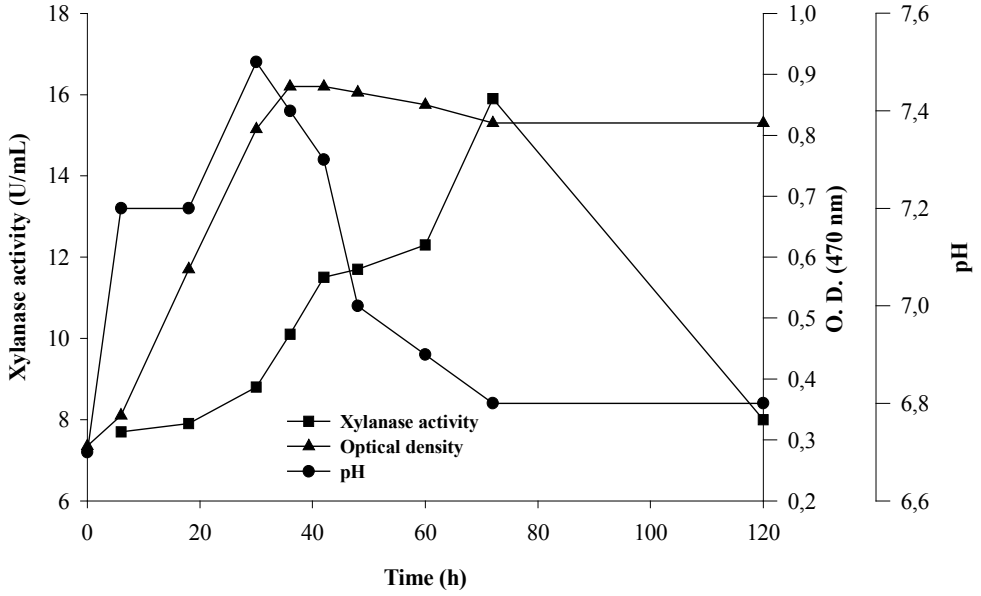

Figure 2 - Time course of xylanase production by *Bacillus* sp at 50° C on 0.5% beechwood xylan in shake flasks.

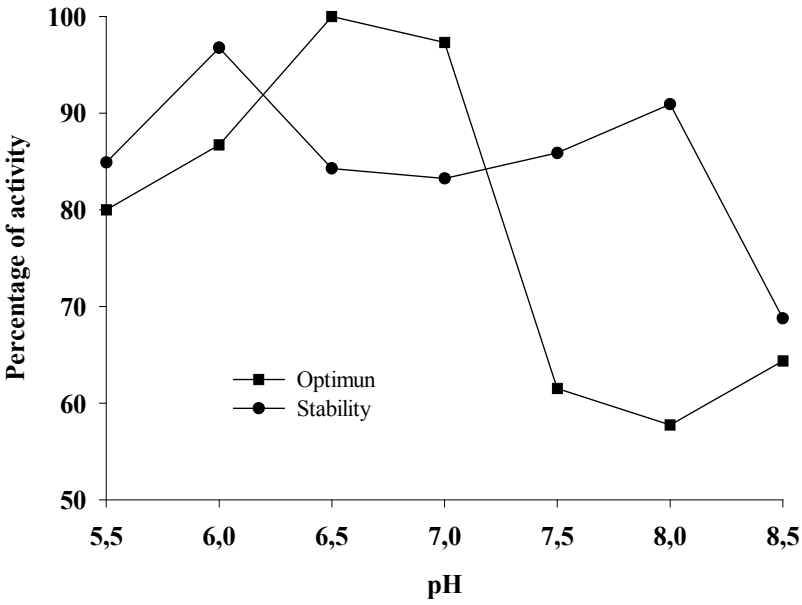

Figure 3 - Optimum pH and stability pH of *Bacillus* sp crude xylanase.

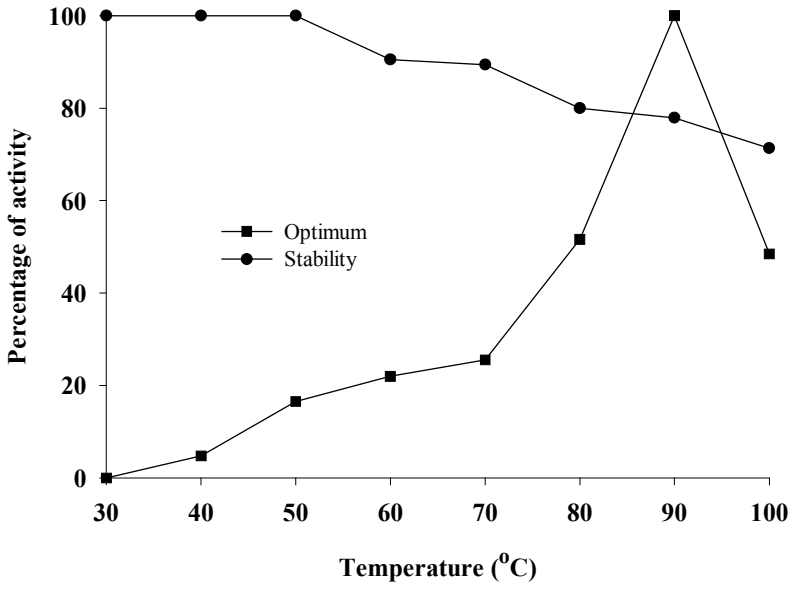

Figure 4 - Optimum temperature and stability temperature of *Bacillus* sp crude xylanase.

The temperature and pH stable xylanase from *Bacillus* sp 3M has been reported by Marques et al., (1998). The enzyme showed optimum activity at 60° C and retained 100% of activity for at least 3 d at 55° C. At 80° C, it retains 47% of its maximal activity.

Since thermal stability of xylanase is a very important propertie due to its potential applications in several industrial processes the strain isolated by us could be a good candidate for biotechnological applications.

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RESUMO

Uma bactéria xilanolítica, termofílica e aeróbica foi isolada de amostras de solo local. Os resultados da comparação de seqüências de 16S rRNA indicaram que o isolado foi proximamente relacionado com o *Bacillus caldoxylolyticus* and Bacillus sp AK1 e estes três organismos exibiram níveis de homologia de 94% nas seqüências de DNA ribossomal. Estudos sobre a caracterização de xilanase de culturas líquidas cultivadas em xilana (beechwood), revelaram que a enzima permaneceu 100% ativa por 2 horas a temperaturas variando de 30° C a 50° C, enquanto a 60° C, 70° C e 100 ° C, 10%, 11% e 29% de sua atividade original foi perdida. O pH ótimo da enzima foi encontrado estar entre 6,5 e 7,0. Após a incubação da solução enzimática bruta por 24 horas a 25° C e a valores de pH variando de 5,5 a 8,0, foi observado um decréscimo em torno de 12% de sua atividade original.

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