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PRODUCTION AND PROPERTIES OF α-AMYLASE FROM THERMOPHILIC *BACILLUS* SP.

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ABSTRACT

α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) production by thermophilic *Bacillus* sp strain SMIA-2 cultivated in liquid media containing soluble starch reached a maximum at 48h, with levels of 57U/mL. Studies on the α-amylase characterization revealed that the optimum temperature for activity was 70ºC. The enzyme was stable for 2h at 50ºC, while at 60ºC, 70ºC and 90ºC, 4%, 13% and 38% of the original activities were lost, respectively. The optimum pH of the enzyme was 7.5. After incubation of crude enzyme solution for 24h at pH 7.5, a decrease of about 5% of its original activity was observed. The enzyme was strongly inhibited by Co^{2+}, Cu^{2+} and Ba^{2+}, but less affected by Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+} and Mn^{2+}. The enzyme in 1M and 5M NaCl solutions the enzyme retained 70% and 47% of the original activity after 24h of incubation at 4ºC, respectively.

Key-words: α-amylase, thermophilic bacterium, *Bacillus* sp.

INTRODUCTION

Thermophilic and extremely thermophilic microorganisms have gained a great deal of attention recently (2,3,11,21). Enzymes from these microorganisms are of special interest since they are not usually denatured by high temperatures and are even active at elevated temperatures (1,6,10,23,24). The genus *Bacillus* produces a large variety of extracellular enzymes, some of which such as the amylases are of significant industrial importance (4). Among these enzymes, the thermostable varieties are more versatile with respect to industrial significance. Thermostable α-amylases have had many commercial applications for several decades. These enzymes are used in the textile and paper industries, food, adhesive, and sugar production (12,15,16,18,21,22).

The α-amylases produced by different *Bacillus* species vary not only in their types (saccharifying or liquefying) but also in the range of pH and temperature for their optimal activity. The bacterial source of the enzyme is usually from either *Bacillus amyloliquefaciens or Bacillus licheniformis*, the latter now being of greater industrial importance (5).

In this article the production of thermostable α-amylase by thermophilic *Bacillus* sp strain SMIA-2, previously isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro, Brazil, is reported.

MATERIALS AND METHODS

Organism

The bacterial strain used in this study was a thermophilic *Bacillus* sp strain SMIA-2 (19), previously isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro, Brazil. Phylogenetic analysis showed that this strain is a member of the *Bacillus* rRNA group 5. This group includes *Bacillus stearothermophilus* and other thermophilic *Bacillus* spp. The optimum temperature and pH for growth of this organism were around 55ºC and pH 7.0, respectively. The organism was found to produce α-amylase on culture medium composed of 1% Soluble starch, 0.2% Yeast extract, 0.5% Peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at 55ºC (pH 7.0).

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Enzyme production

The culture medium used in this work for α-amylase production contained (g/L): $NaH_2PO_4.2H_2O$-1.56, NH_4Cl-5.35, KCl-0.745, $Na_2SO_4.10H_2O$-0.644, Citric acid-0.42, $MgCl_2.6H_2O$-0.25, $CaCl_2$-$2.2x10^{-3}$, ZnO-$2.5x10^{-3}$, $FeCl_3.6H_2O$-$2.7x10^{-2}$, $MnCl_2.4H_2O$-$1.0x10^{-2}$, $CuCl_2.2H_2O$-$8.5x10^{-4}$, $CoCl_2.6H_2O$-$2.4x10^{-3}$, $NiCl_3.6H_2O$-$2.5x10^{-4}$, H_3BO_3-$3.0x10^{-4}$, Na_2MoO_4-$1.0x10^{-3}$, Bacto-tryptone-10.0, Yeast extract-2.5 and Soluble starch-5. The pH was adjusted to 6.9-7.0 with 1.0 M NaOH and this basal medium was sterilised by autoclaving at 121ºC for 15min. Yeast extract, Bacto-tryptone and Soluble starch were sterilised separately and aseptically added to the flasks containing the liquid medium, after cooling. The above medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of an overnight culture and incubated at 55ºC with vigorous aeration in a rotary shaker at 150 rpm for 144h. At time intervals, the turbidity of the cultures was determined by measuring the optical density at 470 nm in a Hitachi Model U-2000 spectrophotometer. Before assay, the cells were separed by centrifugation at 13.000 rpm for 15 min and the clear supernatant was used as crude enzyme preparation.

Amylase Assay

The activity of α-amylase was assayed by incubating 0.3 mL enzyme with 0.5 mL Soluble starch (1%, w/v) prepared in 0.05M Phosphate buffer, pH 6.5. After incubation at 90ºC for 10 min the reaction was stopped and the reducing sugars released were assayed colorimetrically by the addition of 1 mL of 3-5-dinitrosalicylic acid reagent (17). An enzyme unit is defined as the amount of enzyme releasing 1 mmole of glucose from the substrate in 1 min at 90ºC.

Effect of pH on activity and stability of α-amylase

Effect of pH on the activity of α-amylase was measured by incubating 0.3 mL of enzyme and 0.5 mL of buffers, adjusted to pH of 5.5 to 8.5, containing Soluble starch (0.5%). The buffers used were: sodium acetate pH 5.5; phosphate pH 6.0 – 8.0; Tris-HCl 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 24h and then estimating the residual activity.

Effect of temperature on activity and stability of α-amylase

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure as mentioned earlier for 10 min at pH 6.5 within a temperature range of 40 –100ºC. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 40-100ºC for 2h in a constant-temperature water bath . After treatment the residual enzyme activities were assayed.

Effect of metal ions

The effect of different metal ions on α-amylase activity was determined by the addition of the corresponding ion at a final concentration of 1mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Cs^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}, Ag^{1+} chlorides, Pb^{2+}acetate, and Cu^{2+}sulphate.

Salt tolerance test

Enzyme was incubated in 10 mM Phosphate buffer (pH 7.0) containing various NaCl concentrations (0.05 to 5M) for 24h at 4ºC and in each case activity of the enzyme was measured in the same way as mentioned earlier.

RESULTS AND DISCUSSION

Enzymatic production

Fig. 1 reports the time-course of α-amylase production by *Bacillus* sp. strain SMIA-2 grown in basal medium supplemented with 0.5% Soluble starch. α-amylase production reached a maximum at 48h, with levels of 57U/mL. Subsequently, α-amylase levels remained more or less constant up to 96h and after 144h n dropped to 23U/mL. It was observed that maximum α-amylase production occurred when the cell population reached the peak, suggesting that this organism may be unusually sensitive to metabolite repression. Effective induction may not occur until the stationary phase has been reached and the readily available carbon source was depleted.

Effect of pH on activity and stability of α-amylase

The effect of pH on α-amylase activity is shown in Fig.2. Optimum pH was found to be 7.5. The enzyme activity at pH 5.5 and 10.0 were 73% and 55% of that at pH 7.5, respectively. After incubation of crude enzyme solution for 24h at pH 5.0–10, a decrease of about 5% of its original activity at pH 7.5 was

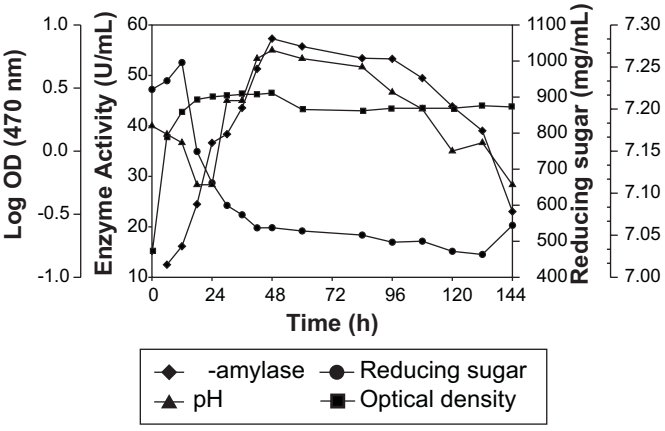

Figure 1. Time course of α-amylase production by *Bacillus* sp. strain SMIA-2 grown at 55ºC on 0.5% soluble starch in shake flasks.

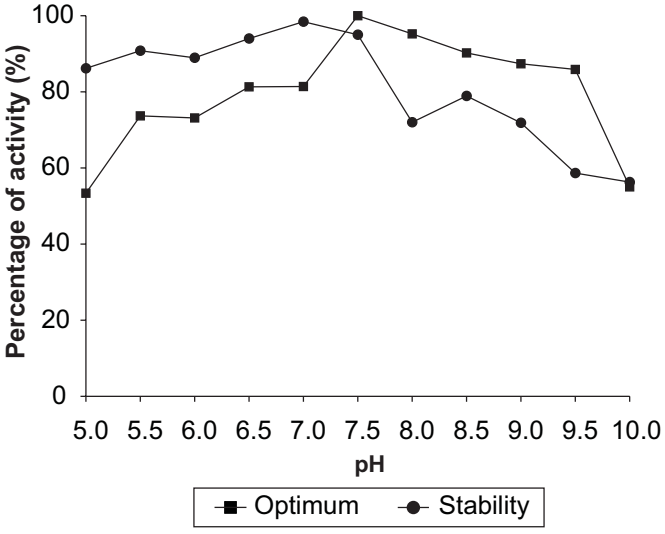

Figure 2. Optimum pH and stability pH of α-amylase produced by *Bacillus* sp. strain SMIA-2 grown at 55ºC for 48h. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 92U/mL).

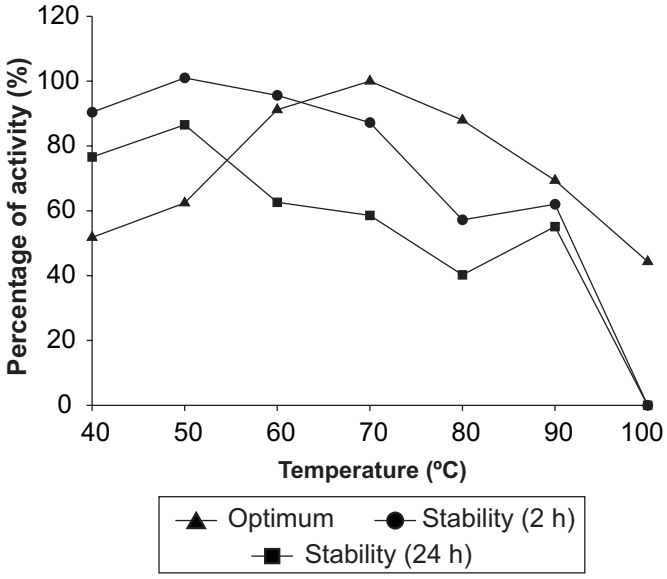

Figure 3. Optimum temperature and stability temperature of α-amylase produced by *Bacillus* sp. strain SMIA-2 grown at 55ºC for 48h. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 78U/mL).

observed. At pH 10.0, the decrease was of 44%. Thus, α-amylase of *Bacillus* sp. strain SMIA-2 strain seems to be active in very broad pH range.

Effect of temperature on activity and stability of α-amylase

The supernatant amylolytic activity were assayed at different temperatures ranging from 40ºC-100ºC at a constant pH of 7.5 and a substrate concentration of 0.5% as shown in Fig. 3. Enzyme activity increased with temperature within the range of 40ºC to 70ºC. A reduction in enzyme activity was observed at values above 70ºC. The optimum temperature of this α-amylase was 70ºC, which was higher or similar to that described for other *Bacillus* α-amylases (4,7,8,13). The residual activity of crude α-amylase incubated at different temperatures for a period of 2h and 24h was estimated at optimum temperature. The enzyme was stable for 2h at temperatures ranging from 40-50ºC while at 60ºC, 70ºC and 90ºC, 4%, 13% and 38% of the original activities were lost respectively.

Effect of metal ions

Because metal ions could be generated from equipment corrosion, specially when subject to acid hydrolysis, the effect of some metal ions at the concentration of 1 mM in the activity of α-amylase was investigated. As can be observed in Table 1, the α-amylase did not require any specific ion for catalytic activity. A slight activity inhibition was produced in the activity by Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+} and Mn^{2+}, and a stronger inhibitory effect was observed in the presence of Co^{2+}, Cu^{2+} and Ba^{2+}. Some amylases are metalloenzymes, containing a

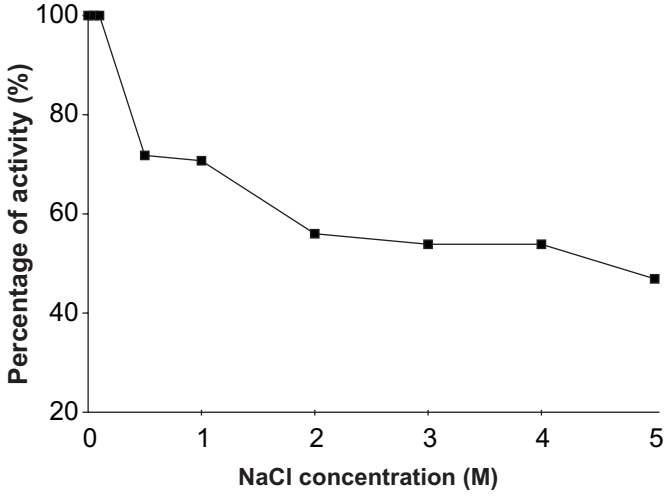

Figure 4. Effect of NaCl concentration on α-amylase produced by *Bacillus* sp. strain SMIA-2 grown at 55ºC for 48h. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 79 U/mL).

metal ion for catalytic activity. The inhibition of *Bacillus* sp. strain SMIA-2 α-amylase by Co^{2+}, Cu^{2+} and Ba^{2+} ions could be due to competition between the exogenous cations and the protein-associated cation, resulting in decreased metalloenzyme activity (12).

Table 1. Effect of different ions on α-amylase activity produced by *Bacillus* sp. strain SMIA-2 grown at 55ºC during 48h.

Ion	Percentage of activity (%) 1 mM
Control (no addition)	100
Ca^{2+}	89
Mg^{2+}	97
Fe^{2+}	75
Fe^{3+}	59
Co^{2+}	26
Zn^{2+}	73
Mn^{2+}	93
Hg^{2+}	45
Cu^{2+}	87
Cs^{2+}	101
Ni^{2+}	90
Sr^{2+}	91
Pb^{2+}	101
Cu^{2+}	26
Ba^{2+}	38
Ag^{1+}	88

The percentage of activity is expressed as a percentage of the control (100% of enzyme activity = 42U/mL).

The effects of metal ions on the activity of α-amylase in *Bacillus* sp. strain KSM-1378, a relative of *Bacillus firmus*, was investigated by Igarashi *et al.* (7). Ni^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+} ions strongly inhibited the enzymatic activity by 82, 91, 100, and 100% respectively. On the other hand, in *Bacillus* sp. TS-23, Ni^{2+} and Cd^{2+} slightly inhibited amylase activity.

Salt tolerance test

This test is important in treatment of effluent with high salinity containing starch or cellulosic residues in pollution control mechanism. The enzyme in 1.0 M and 5M NaCl solution retained 70% and 47% of the original activity after 24h at 4ºC, respectively. The α-amylase produced by *Bacillus* sp. MD 124 (9) was stable in 5M NaCl solution and retained 75% of its original activity after 24h.

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RESUMO

Produção e propriedades de α-amilase de *Bacillus* sp. termofílico

A produção de α-amilase (1,4-α-D-glicano glicanohidrolase, EC 3.2.1.1) por um *Bacillus* sp cepa SMIA-2 cultivado em meios líquidos contendo amido solúvel, alcançou o máximo em 48h com níveis de 57U/mL. Estudos sobre a caracterização de α-amilase revelaram que a temperatura ótima de atividade desta enzima foi 70ºC. A enzima foi estável por 2h a 50ºC, enquanto que a 60ºC, 70ºC e 90ºC, 4%, 13% e 38% da atividade original foram perdidas, respectivamente. O pH ótimo da enzima foi 7,5. Após a incubação da enzima bruta por 24h a pH 7,5 observou-se um decréscimo em torno de 5% de sua atividade original. A enzima foi fortemente inibida por Co^{2+}, Cu^{2+} e Ba^{2+}, mas foi menos afetada por Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+} e Mn^{2+}. Em solução de NaCl 1M e 5M, a enzima reteve 70% e 47% da sua atividade original após 24h a 4ºC, respectivamente.

Palavras-chave: α-amilase, bactéria termofílica, *Bacillus* sp.

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