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CULTURE CONDITIONS FOR THE PRODUCTION OF THERMOSTABLE AMYLASE BY *BACILLUS* SP

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ABSTRACT

Studies on the α-amylase production were carried out with a bacterial strain isolated from a soil sample. The cells were cultivated in a mineral medium containing soluble starch as sole carbon source. The addition of calcium (10 mM) or peptone (1%) and yeast extract (0.5%) to the mineral medium shortened the lag period and improved the growth and α-amylase synthesis. The addition of glucose to the culture diminished greatly the synthesis of α-amylase, demonstrating that a classical glucose effect is operative in this organism. The optimum temperature and initial medium pH for amylase synthesis by the organism were 50°C and 7.0 respectively. The optimal pH and temperature for activity were 6.0 and 50°C respectively. The enzyme extract retained 100% activity when incubated for one hour at 90°C and 40% at 60°C for 24 h. The addition of glucose to the culture diminished greatly the synthesis of α-amylase.

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

INTRODUCTION

Recent discoveries of starch degrading enzymes have led to increased application of amylases in various industrial processes (1). The c-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyses α1,4 glucosidic linkages in starch and related substrates (5,18,23). This enzyme has extensive commercial applications in starch liquefaction, brewing, sizing in textile industries, and paper and detergent manufacturing processes (4,13,20,21). Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes. Recent research with thermostable α-amylases has concentrated on the enzymes of thermophiles and extreme thermophiles (2,3,7,8,16,17,20) and little is known about the properties of the enzymes produced by these organisms. The present study deals with the isolation and identification of a bacterium and describes the effects of culture conditions on the activity of α-amylase.

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% soluble starch, 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 bacteria capable of producing starch digesting enzymes.

The liquid medium contained (g/L): Soluble starch 10.0, $NaH_2PO_4.2H_2O$ 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 $2.5x10^{-3}$, $FeCl_3.6H_2O$ $2.7x10^{-2}$, $MnCl_2.4H_2O$ $1.0x\ 10^{-2}$, $CuCl_2.2H_2O$ $8.5x10^{-4}$, $CoCl_2.6H_2O$ $2.4x10^{-3}$, $NiCl_3.6H_2O$ $2.5x10^{-4}$, H_3BO_4 $3.0x10^{-4}$, Na_2MoO_4 $1.0x10^{-3}$ and Biotin $1.0x10^{-3}$. The pH was adjusted to 7.0 and the medium sterilised by autoclaving at 121°C and 15 psi for 30 min.

Isolation and determination of thermophilic bacterium producing α-amylase. Soil suspensions in sterilised water were poured and spread onto agar plates A. These plates were

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incubated at 55°C for 2 days. The colonies that were found on the plates were transferred onto agar plates B. These plates were incubated at 55°C for 2 days. Several amylase-producing bacterial colonies were selected after flooding the plates with iodine solution. The strain that yielded a high level of α-amylase was selected for further experiments.

Cultural conditions. The organism was germinated on agar plate B, as described by Liao *et al.* (19), and the plates were incubated at 55°C for 18 hours. Liquid medium (approximately 5 ml) was pipetted into the agar plates B and the cells scraped off using a sterile Pasteur pipette. Liquid medium (50 ml contained in a 250 ml Erlenmeyer flask) was inoculated with this suspension to give an initial absorbance at 470 nm of at least 0.1 and the cultures were incubated at 50°C with vigorous aeration in a shaker at 250 rpm for 96 hours. Before assay, the cells were separated by centrifugation at 5000 g. The clear supernatant was used as crude enzyme preparation.

Experiments to determine the effect of temperature and pH on the growth of the organism and α-amylase production were carried out at temperatures varying from 30°C to 60°C, and at various values of pH varying from 4.0 to 9.0 for 40 hours.

Bacterial dry weight. Dry weight of the cells was determined using the method of Herbert *et al.* (12). Duplicate samples (5 ml) were centrifuged at 3000 g for 10 min and washed once in distilled water. The cells were resuspended in distilled water (2 ml) and poured into pre-weighted foil thimbles. Additional aliquots of water (2x2 ml) ensured that all remaining cells were washed out into the thimbles. These were then, dried to constant weight in an oven at 105°C. The thimbles were cooled in a desiccator and weighted on an analytical balance sensitive to 0.1 mg.

Analytical procedure. The amylase assay was based on the reduction in blue colour intensity resulting from enzyme hydrolysis of starch (4). The reaction contained 1 ml enzyme (cell free supernatant) and 10 ml of 1% starch solution incubated at 50°C for 10 min. The reaction was stopped by adding 10 ml of 0.1N HCl. One millilitre of this acidified solution was added to 10 ml of 0.1N HCl. From this, 1 ml was added to 10 ml iodine solution (0.05% iodine in 0. 5% KI). The optical density of the blue-coloured solution was determined at 660 nm. The same procedure was repeated using 1 ml-distilled water instead of the enzyme sample in order to measure the optical density without the enzyme. One unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 1% reduction of blue colour intensity of starch-iodine solution a 50°C in 1 min.

Effect of temperature on enzyme activity. The enzyme's activity profile was obtained by measuring enzymatic activities in 0.1 M citric acid-sodium phosphate buffer, pH 6.0, between 30°C and 90°C.

Effect of pH on enzyme activity. The effect of pH on α-amylase activity was investigated by measuring enzyme activity at 50°C in different buffer solutions.

RESULTS AND DISCUSSION

Isolation and determination of thermophilic bacterium producing α-amylase. The strain was a Gram-positive bacillus, negative on the Voges-Proskauer test (at pH 7.2), and facultative anaerobic. It was actively motile, 2.5 to 3.0 μm long and approximately 0.6 μm wide, with central spores and predominantly unswollen cylindrical sporangia. The strain possessed the ability to hydrolyse both starch and gelatin. Catalase was positive. Indole was not formed, and acetoin formation was positive. Nitrates were reduced to nitrites. The final pH after growth in glucose-broth was about 5.5 and growth was obtained in nutrient broth containing 7% NaCl. The strain grew in nutrient broth at 30°C to 60°C with an optimum at 50°C for 24 h. From these results, the strain was identified as *Bacillus* sp by the criteria of Bergey's Manual of Systematic Bacteriology.

Enzymatic production. Measurements of the enzyme activity and cell growth of *Bacillus* sp at a number of time intervals are shown in Fig. 1. Initially, the organism was grown in the liquid medium (Fig.1a) and then, in the liquid medium supplemented with calcium (10 mM) (Fig. 1b). The addition of 10 mM calcium to the liquid medium improved the growth and amylase production. Since the enzyme is known to be a calcium metalloenzyme, it is possible that the results found were because of the more availability of calcium ion. These results are similar to the findings of Hewitt and Solomons (13), with cultures of *Bacillus amyloliquefaciens*.

The amylase synthesis by several microorganisms has been correlated to the presence or absence of various amino acids and complex nitrogenous sources in the culture medium (9, 13, 14). Indeed, the addition of yeast extract (0.5%) and peptone (1%) to the liquid medium shortened the lag period and increased both the dry weight of the cell and the enzyme synthesis (Fig. 2). Therefore, the result suggests that yeast extract and peptone is favoured for the growth and synthesis of amylase by the organism studied.

It has been reported that the synthesis of carbohydrate-degrading enzymes in most species of the genus *Bacillus* is subject to catabolic repression by readily metabolizable substrates such as glucose (20). Ours results are in good agreement with these findings. The addition of glucose (0.5%) to the culture diminished greatly the synthesis of α-amylase (Fig. 3). These results are similar to the findings of Haseltine *et al.* (10), who observed that glucose represses the production of α-amylase in the hyperthermophilic archaeon *Sulfolobus solfataricus*. According to them glucose prevented α-amylase gene expression and not merely secretion of preformed enzyme.

The organism did not grow in the culture media adjusted to pH 4.0, 5.0, 6.0 and 10.0 (Fig.4). In those media where bacterial growth occurred, pH increased after 18 hours of fermentation, never reaching values greater than 9.0. The

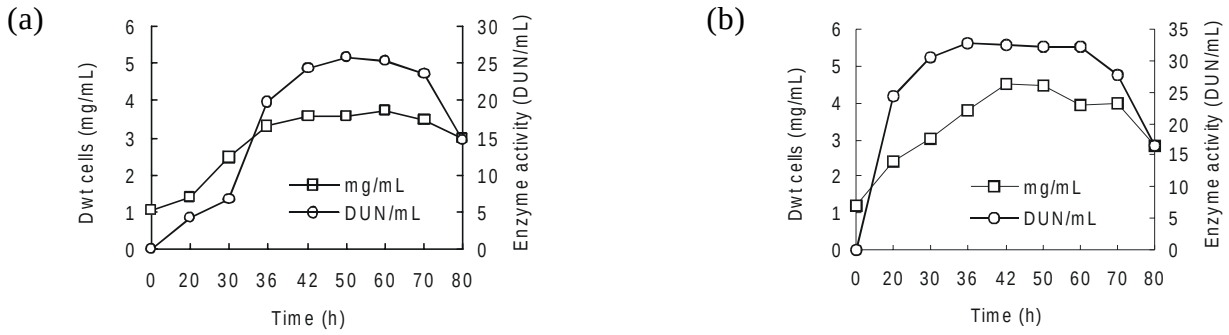

Figure 1. Time course of growth and α-amylase production by *Bacillus* sp in liquid medium (a) and in liquid medium supplemented with calcium (b).

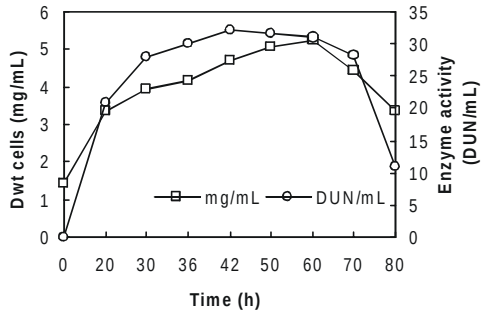

Figure 2. Time course of growth and α-amylase production by *Bacillus* sp in liquid medium supplemented with yeast extract and peptone.

enzyme activity of the broth increased during 72 hours of incubation at all pH values.

These results suggest that there is a stimulation of enzyme synthesis at pH 7.0 and that the higher enzyme production at this pH was a result of increased cell growth. The optimum pH for enzyme activity was between 6.0 and 6.5. There was a nearly 73% reduction in maximum activity at pH 8.5 or 9.0. Regarding to *Bacillus* genus they produced enzymes with optimum activities at pH values as low as 3.5 or as high as 10.6 (15, 11).

Growth and enzyme production both increased with temperature within the range of 30°C to 50°C with an optimum of 50°C (Fig. 5a). Thus, the results indicated that the optimum temperatures for amylase synthesis and growth were the same. Other investigators also reported that maximum amylase production occurred at the optimum growth temperature (4,20,22).

The optimum temperature for enzyme activity was between 45°C and 55°C. A reduction in enzyme activity was observed at values above 60°C (Fig. 5b).

According to the results presented the optimal conditions for the cell growth were adequate for enzyme production. These results contrast with the findings found by Chandra *et al.* (6) to *Bacillus licheniformis* CUM 305. This organism did not produce α-amylase at 30°C although it grew very well at this temperature. In addition, Saito and Yamamoto (22), studied a *Bacillus licheniformis* which produced a-amylase at temperatures around 50°C and never produced the enzyme at temperatures lower than 45°C.

The enzyme extract retained 100% activity when incubated for one hour at 90°C (Fig. 6a). After this time the activity decreased drastically. After 24 hours at 50°C and 60°C, the enzyme retained 65% and 62% of its initial activity (Fig. 6b) and was inactivated on incubation at 95°C for 10 min.

A temperature stable α-amylase from *Bacillus licheniformis* 584 was reported by Saito and Yamamoto (22). This enzyme rapidly lost activity at temperatures above 76°C. According to the results presented in this article, the α-amylase from the *Bacillus* sp isolated, is heat stable, although it showed optimum activity lower than that described for the α-amylase produced by *Bacillus* sp (4, 20).

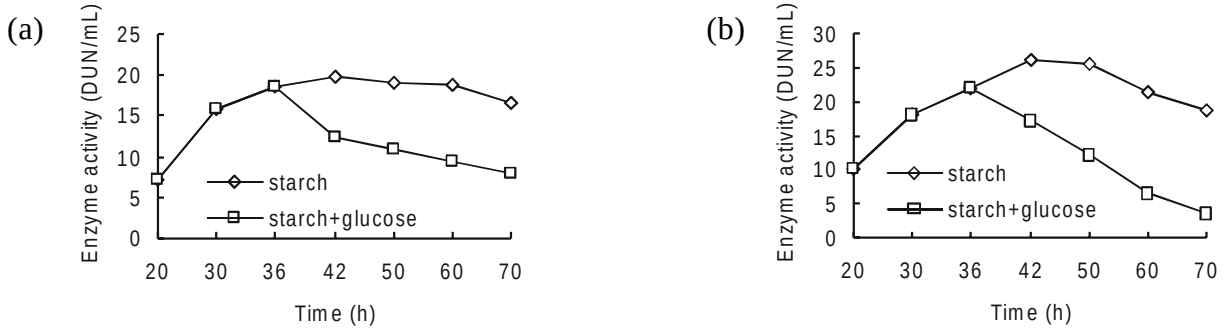

Figure 3. Glucose repression of α-amylase production. Cells of *Bacillus* sp were growth in the liquid medium with 0.5% (a) and 1.0% (b) of starch.

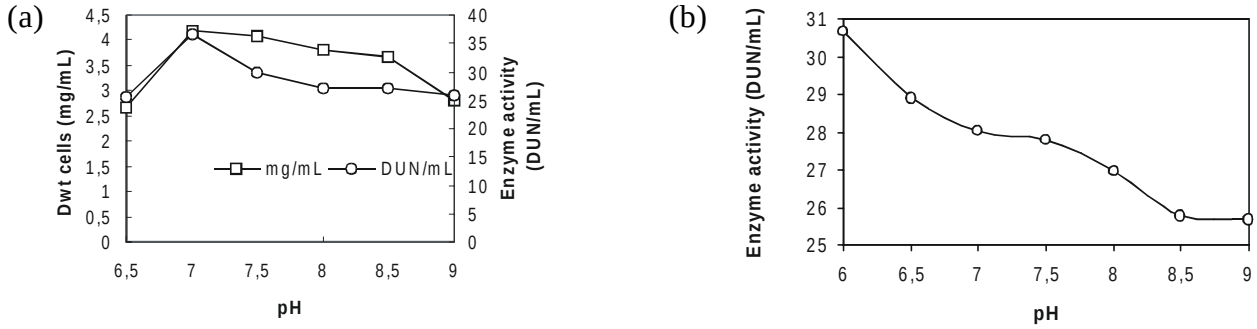

Figure 4. Effect of the initial pH of the culture medium on growth and α-amylase production by *Bacillus* sp (a) and effect of the pH on the a-amylase activity at 50°C (b).

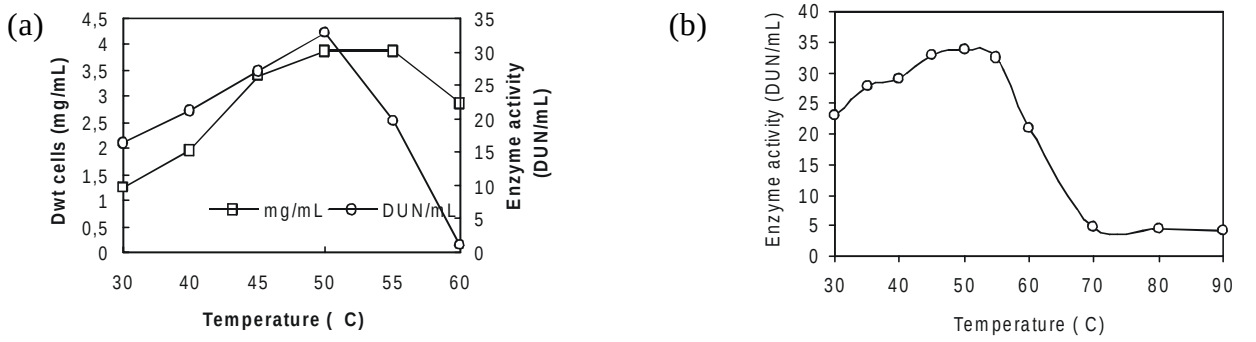

Figure 5. Effect of the temperature on the growth and α-amylase activity of *Bacillus* sp (a) and effect of temperature on α-amylase activity (b).

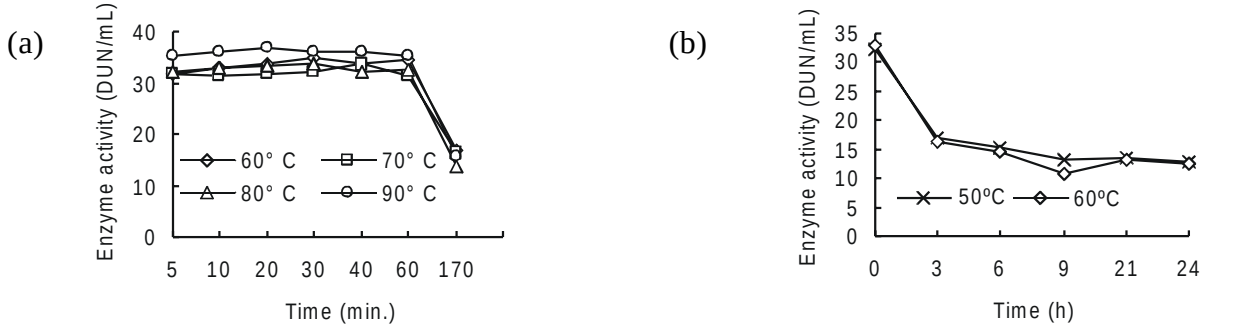

Figure 6. Effect of temperature on the stability of α-amylase.

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RESUMO

Condições de cultura para produção de amilase termoestável por *Bacillus* sp

Estudos sobre a produção de α-amilase foram realizados com uma bactéria isolada a partir de amostras de solo. As células foram cultivadas em um meio mineral contendo amido como única fonte de carbono. A adição de cálcio (10mM) ou peptona (1,0%) e extrato de levedura (0,5%) ao meio mineral, encurtou o período lag, melhorou o crescimento e a síntese de α-amilase. A adição de glicose a cultura diminuiu grandemente a síntese de α-amilase, demonstrando que o efeito glicose ocorre neste microrganismo. A temperatura ótima e o pH inicial ótimo para a síntese da amilase pelo organismo foram 50ºC e 7,0 respectivamente. O pH e temperatura ótima para atividade foram 6,0 e 50ºC respectivamente. O extrato enzimático reteve 100% de atividade quando incubado por uma hora a 90ºC e 40% a 60ºC por 24 horas. A adição de glicose ao meio de cultura diminuiu grandemente a síntese de α-amilase.

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

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