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Effect of the Medium Composition on Formation of Amylase by *Bacillus sp.*

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ABSTRACT

Studies on the α-amylase synthesis was carried out with a moderately thermophilic, facultatively anaerobic Bacillus sp, isolated from soil samples. The cells were cultivated in a complex medium containing soluble starch or maltose as carbon source. The levels of the α-amylase activity detected in culture supernatants varied greatly with the type of carbon source used. Maltose, soluble starch and citrate stimulated α-amylase formation. Addition of exogenous glucose repressed formation of α-amylase, demonstrating that a classical glucose effect was operative in this organism. The concentration of yeast extract was found to be important factor in the α-amylase synthesis by the isolate. The activity of the enzyme increased between 2 and 5 g/L yeast extract concentration and then fell very rapidly beyond this point. The best concentration of peptone to α-amylase formation was found to be around 10g/L.

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

INTRODUCTION

Starch is an important renewable biological resource. Bacterial ∝-amylases have several applications in the food industry and are potentially useful in the pharmaceutical and fine chemical industries if enzymes with suitable properties can be found (Roychoudhury et al., 1975; Hewitt and Solomons, Hillier et al., 1996; 1996; Abou-Zeid, 1997; Igarashi et al., 1998; Lin et al., 1998). The starch processing industry requires the use of amylolytic enzymes at high temperatures (Uguru et al., 1997; Bolton et al., 1997). Thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes (Chandra et al., 1980; McMahon et al., 1997; McMahon et al., 1999). The advantages for using thermostable ∝-amylases in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external cooling (Lin et al., 1998). With the more widespread use of ∝-amylases, it has become essential to isolate new hyperproducing microbial strains. Almost all microorganisms of the *Bacillus* genus synthesised ∝-amylase, thus this genus has the potential to dominate the enzyme industry (Pretorius et al., 1986). The present study deals with the isolation and characterisation of a moderately thermophilic *Bacillus* and describes the effects of medium composition on the production of ∝-amylase.

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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): $NaH_2PO_4.2H_2O$ 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_3 $3.0x10^{-4}$, Na_2MoO_4 $1.0x10^{-3}$, bacto-tryptone 10.0, yeast extract 5.0 and desired amounts of soluble starch or maltose.

Screening of microorganism. Soil suspensions in sterilised water were poured and spread onto agar plates A. These plates were incubated at 55 ^oC for two days. The colonies that were found on the plates were transferred onto agar plates B. These plates were incubated at 55 ^{o} 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 $\propto$-amylase was selected for further experiments.

The taxonomic study of strain isolated was made until the genus (*Bacillus* sp) according to the Bergey's Manual of Determinative Bacteriology (Sneath, 1986).

Cultural conditions: The organism was germinated on agar plate B, as described by Liao et al.(1986), and the plates were incubated at 50° 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 ^oC with vigorous aeration in a shaker at 250 rpm for 96 hours. At time intervals, samples of the culture were with draw for turbidimetric measurements at 470 nm. Before assay, the cells were separed by centrifugation at 5000g. 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 0.01M phosphate buffer, pH 6.5. After incubation at 90° C 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 (Miller, 1959). An enzyme unit is defined as the amount of enzyme releasing 1 μmole of glucose from the substrate in 1 min at 90 ^oC.

RESULTS AND DISCUSSION

Screening of microorganism. The isolated bacteria was gram-positive, negative on the Voges-Proskauer test (at pH 7.2), and facultatively 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. No growth was obtained in nutrient broth containing more than 7 % NaCl. The strain grew in nutrient broth at 30-60 ^oC with a optimum at 50 ^oC. On the basis of these characteristics, the bacterium was classified in the genus *Bacillus* (Sneath, 1986) and denominated *Bacillus* sp.

Enzymatic production. The growth pattern of *Bacillus* sp and α-amylase activity was observed for four days in the liquid medium containing 1% soluble starch or 1% maltose as a carbon source in 250 mL Erlenmeyer flask (Fig.1).

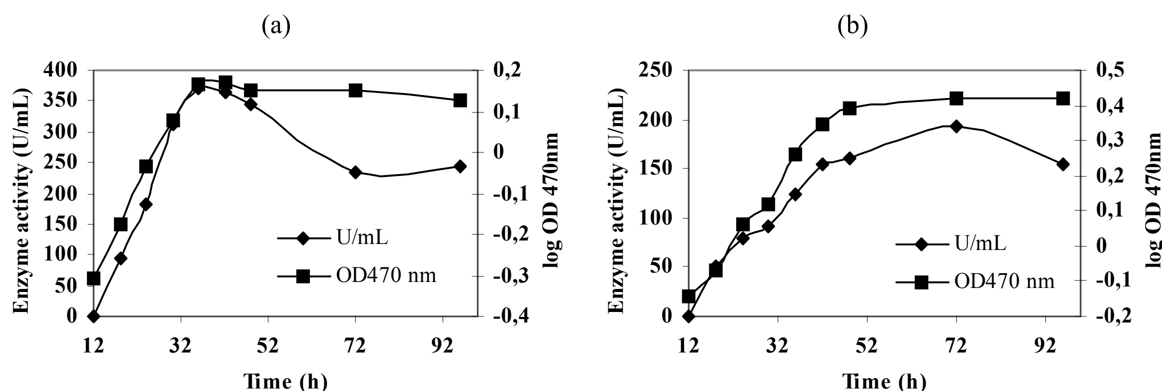

Figure 1 - Growth and α-amylase activity of *Bacillus* sp. in liquid medium containing 1% peptone, 0.5% yeast extract and 1% soluble starch (a) or 1% maltose (b).

Regardless of the carbohydrate used, the enzyme was not detected before 12 hours of fermentation and was formed in parallel with the growth . In general, the α-amylase concentration in the culture broth increased between 18 and 48 hours. In *B. subtilis* (Stephenson et al., 1998) and *B. licheniformis* TCRDC-B13 (Bajpai and Bajpai, 1989) also, amylases are formed during the logarithmic growth phase in parallel with cell-mass growth.

The effects of different concentrations of soluble starch on α-amylase production were studied (Fig.2a). Increasing starch concentration in the medium beyond 1%, enzyme activity did not increase. At higher starch concentrations, enzyme production was comparatively lower and the time required to reach the maximum enzyme level was longer. Similar results were found when maltose was used as a carbon source (Fig.2b). At 1% maltose, the highest α-amylase activity was observed.

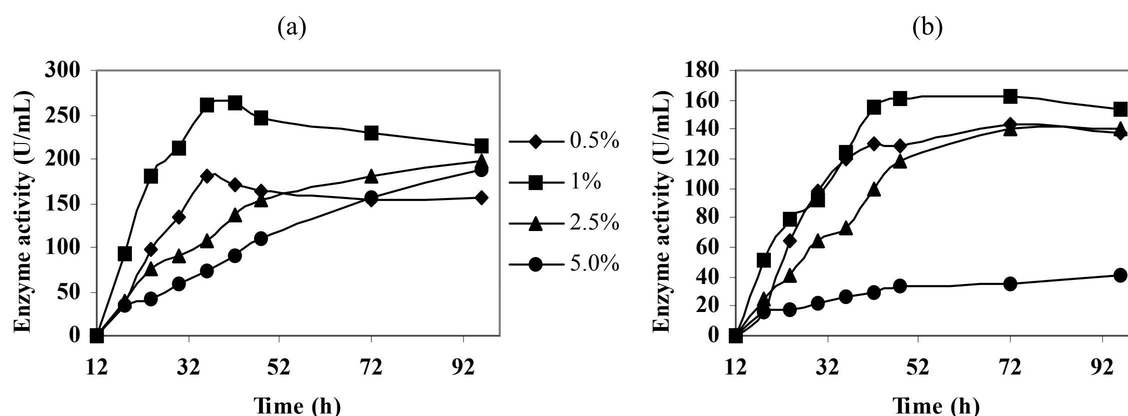

Figure 2 - α-amylase activity of *Bacillus* sp in liquid medium containing 1% peptone, 0.5% yeast extract and soluble starch (a) or maltose (b) as a carbon source.

Carbon source greatly influences α-amylase production and the most commonly used substrate is starch (Bajpai and Bajpai, 1989). The Figure 3 shows that with some carbon sources an inverse relationship exists between the growth and the amount of α-amylase produced. Maltose, starch and citrate did not conform to this relationship in that these substrates stimulated α-amylase formation. The enzyme formation with the other carbon sources were much lower compared to that

with starch and maltose. In fact, 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 and fructose (Lin et al., 1998). In *B. flavothermus* the highest activity of α-amylase was obtained with lactose as carbon source as was maximum biomass. Utilisation of carbon sources such as sucrose, fructose and glucose gave rise to good growth with concomitant reduction in amylase production (Kelly et al., 1997).

Rothstein et al. (1986) reported that cells grown in a medium containing a carbon source other than sugars (citrate or glutamate) produced more enzyme than cells grown in a medium with starch. They also showed that cells utilising citrate or glutamate initiate a considerable number of α-amylase transcripts, consistent with a strong expression of the α-amylase gene.

To determine if the amylolytic system was subject to catabolic repression, the *Bacillus* sp was grown in the liquid medium with soluble starch or maltose as a carbon source, at 50° C and glucose was then added to cultures of the organism after 30 hours growth (Fig.4). In the absence of glucose, enzyme production increased rapidly after 18 hours, with the highest enzyme activity being obtained after 48 h. On supplementation of the culture with glucose, there was an initial repression of amylase synthesis. This repression was reversed after 72 h growth, which could correlated to a depletion of glucose, demonstrating that the amylolytic system of *Bacillus* sp was subject to catabolic repression.

Similar results were found to the hyperthermophilic archaeon *Sulfolobus solfataricus* in that glucose repressed production of α-amylase, demonstrating that a classical glucose effect was operative in this organism (Haseltine et al., 1996). The concentration of yeast extract was found to be important factor in the α-amylase synthesis by several organisms (Alam et al., 1989) and thus the influence of this compound on α-amylase synthesis by *Bacillus* sp was investigated, varying its concentration in the medium between 2 and 10 g/L (Fig. 5a). The activity of the enzyme increased between 2 and 5 g/L yeast extract concentration and then fell very rapidly beyond this point. In *B. amyloliquefaciens* (Alam et al., 1989) a strict pH control was required in complex media containing high levels of yeast extract for studying the α-amylase synthesis. Increasing the concentration of yeast extract to a level of 5.0g/L lowed the pH significantly and this resulted in the complete repression of the enzyme. In our study was observed that the pH of the broth increased from 7.0 at the beginning to around 7.5 at the end of fermentation to all yeast extract concentrations utilised.

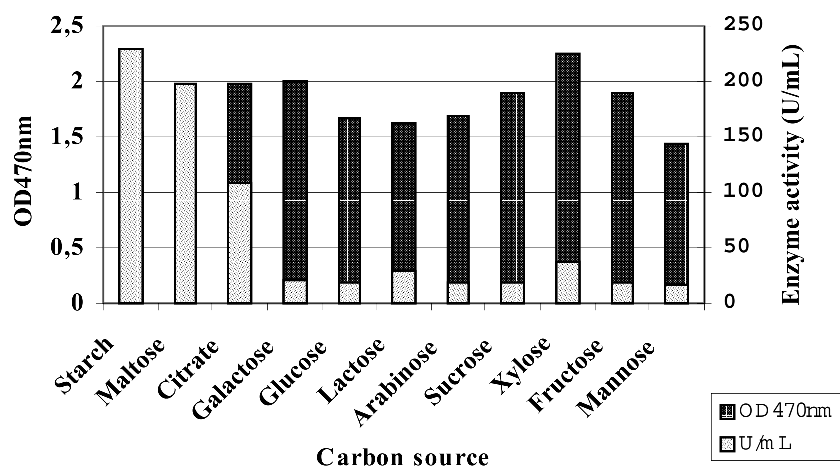

Figure 3 - Effect of the carbon source on growth and α-amylase activity by *Bacillus* sp.

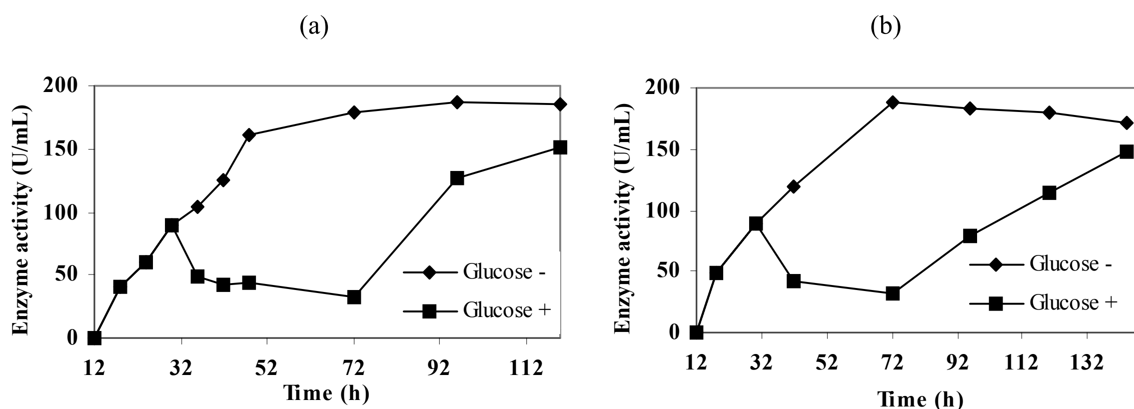

Figure 4 - Repression of α-amylase formation by glucose. *Bacillus* sp was grown in the presence of 1% soluble starch (a) and 1% maltose (b); 0.5% glucose was additionated after 30 h of growth.

The effect of peptone on α-amylase synthesis by *Bacillus* sp was also investigated (Fig. 5b). A 1% peptone concentration was optimum for maximum amylase synthesis. These results are similar to the findings to Bajpai and Bajpai (1989) to *B. licheniformis* TCRDC-B13. However these authors demonstrated that among different nitrogen sources tried, peptone was found to be best followed meat extract and yeast extract. We found that the best activity of α-amylase to *Bacillus* sp was found when yeast extract was used.

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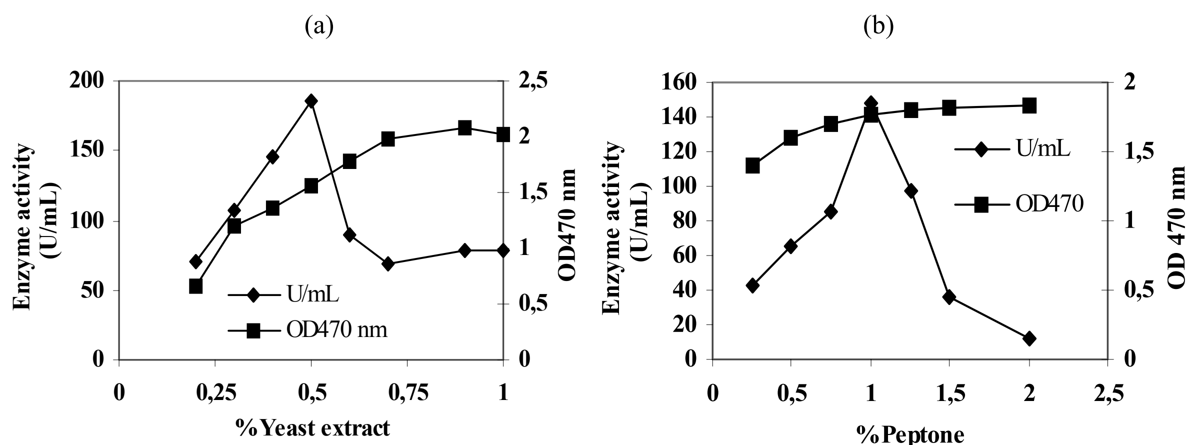

Figure 5 - Effect of yeast extract concentration (a) and peptone concentration (b) on growth and α-amylase activity of *Bacillus* sp cultivated in the liquid medium containing 1% soluble starch as carbon source.

RESUMO

Estudos sobre a síntese de α-amilase foram realizados com uma bactéria termofílica moderada e facultativa anaeróbica, isolada de amostras de solo. As células foram cultivadas em um meio complexo contendo amido solúvel ou maltose como fonte de carbono. Os níveis da atividade de α-amilase detectados no sobrenadante da cultura variaram grandemente com o tipo da fonte de

carbono utilizada. Amido solúvel, maltose e citrato estimularam a formação de α-amilase. A adição de glicose as culturas reprimiu a formação da α-amilase, demonstrando que o clássico efeito glicose foi operativo neste organismo. A concentração de extrato de levedura foi um fator importante na formação de α-amilase pelo isolado. A atividade da enzima aumentou entre concentrações de 2 a 5 g/L e então caiu muito rapidamente em torno deste ponto. A melhor concentração de peptona para a formação da α-amilase foi em torno de 10 g/L.

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