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Enhanced Production and Extracellular Activity of Commercially Important Amylolytic Enzyme by a Newly Isolated Strain of Bacillus. sp. AS-1

[Ticari Öneme Sahip Amilolitik Enzimin Yeni İzole Edilmiş Bacillus. Türü As-1 Tarafından Arttırılmış Üretimi ve Hücre Dışı Aktivitesi]

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ABSTRACT

Studies on the optimum conditions for the production of α-amylase were carried out with a newly isolated bacterial strain of bacillus sp.AS-1. The optimum temperature for amylase production was detected as 35°C. α -Amylase production occurred at pH 5.0-9.0 with a maximum at pH 7.0. The optimal pH and temperature values for extracellular activity were 7.5 and 50°C respectively. Effect of different salts were noted and it was found that CaCl2 with concentration of 0.2g/l played an important role for optimum production and stability of alpha amylase in the fermentation medium. Starch with a concentration of 20 g/l was a good source for the enzyme synthesis. The levels of the α-amylase production detected in culture supernatants varied greatly with the type of carbon source used. Lactose, soluble starch and glucose stimulated α-amylase production. Effect of different nitrogen sources revealed that peptone increase the enzyme yield. The concentration of yeast extract was an important factor for the α-amylase synthesis by the isolate. The activity of the enzyme increased between 2 and 4 g/l yeast extract concentrations with an optimum of 4 g/l. The optimal concentration of peptone for the production of α-amylase was detected as 10g/l.

Key Words: α-Amylase, *Bacillus* sp., Starch, CaCl2, Nitrogen source.

ÖZET

Yeni izole edilen bakteri suşu Bacillus türü AS-1'in α-amilaz üretimi için optimum koşulları araştırıldı. Amilaz üretimi için optimum sıcaklık 35°C idi. α-amilaz, pH 7.0'de en çok olmak üzere pH 5.0-9.0 arasında üretildi. Hücre dışı aktivite için optimum pH ve sıcaklık sırasıyla 7.5 ve 50°C idi. Farklı tuzların etkileri araştırıldı ve fermentasyon ortamındaki 0.2g/l CaCl2'nin optimum alfa amilaz üretimi ve stabilitesi için önemli olduğu bulundu. Enzim sentezi için en uygun nişasta konsantrasyonu 20 g/l idi. Kültür üst sıvısında tesbit edilen α-amilaz düzeyi, kullanılan karbon kaynağına göre büyük değişiklik gösterdi. Laktoz, çözünür nişasta ve glukoz, α-amilaz üretimini uyardı. Farklı azot kaynakları arasında, peptonun enzim verimini arttırdığını gösterildi. İzolatın α-amilaz sentezlemesinde maya ekstresi önemli bir etkendi. Enzim aktivitesi 2 ve 4 g/l maya ekstresi konsantrasyonları arasında, 4g/l'de optimum olmak üzere, artış gösterdi. α- Amilaz üretimi için en uygun pepton konsantrasyonu 10 g/l olarak bulundu.

Anahtar Kelimeler: α-amilaz, *Bacillus* türü, nişasta, CaCl2, Azot kaynağı

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INTRODUCTION

Amylases [α-amylase, β-amylase and glucoamylase (GA)] are among the most important enzymes in present-day biotechnology. The enzymes of amylase family have great significance due to its wide area of potential application. The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistry. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (1, 2). Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (3, 4). Amylases are universally distributed throughout the animal, plant and microbial kingdoms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (2). Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries (5), bread making (6), glucose and fructose syrups, detergents, fuel ethanol from starches **(7)**, fruit juices (8), alcoholic beverages (9), sweeteners (10), digestive aid and spot remover in dry cleaning (11). Therefore, any improvement in the enzyme production, extracellular activity and thermo stability or activity will have a direct impact on the process performance, economics and feasibility. Alpha Amylase (E.C 3.2.1.1), hydrolyses starch, glycogen and related polysaccharides by randomly cleaving α-1→4 glucosidic linkages (12). Almost all microorganisms of the *Bacillus* genus synthesized alpha amylase, thus this genus has the potential to dominate the enzyme industry (13). The industrially important *Bacillus* strains, which are extensively used to produces alpha amylase are *B. amyloliquefaciens*, *B. licheniformis* (14), *B. stearothermophilus* (15), *B. subtilis* (16), *and B. megaterium* (17) and B. *circulans* (18).

The present study deals with the isolation and identification of a bacterium and effects of culture condition on the production of α-amylase.

MATERIALS AND METHODS

Isolation & screening of microorganism

The *Bacillus* sp. used in this study was isolated from environment. The primary screening was done by starch agar plate method (19). Strains capable of producing alpha amylase were screened by allowing them to grow for 24 hrs on nutrient-agar plates containing 1%(w/v) starch at 35°C. The plates were stained with Gram's iodine solution (2%I_2 and 0.2%potassium iodide), and largest halo-forming zone was considered as the most promising strain and was chosen for further investigation.

Culture Maintenance

The strain was maintained on nutrient agar slant and was stored at 4°C for further studies.

Media Composition

The growth medium used for amylase production was composed of (g/l): 20.0 Soluble Starch, 4.0 Yeast Extract, 10.0 Bacto Peptone, and 0.5 MgS_4.7H O, 0.5 NaCl, and 0.2 $CaCl_2$. The pH of the medium was adjusted to pH 7.0 with 1N NaOH and was autoclaved at 121°C for 15 minutes.

Production of Amylase

Five ml starch broth was inoculated with a loop- full of growing culture of *Bacillus* strain and was incubated at 35°C for 24 hrs. This 5 ml of 24 hrs old culture was then transferred into 45 ml of sterile starch broth medium and was incubated for 35°C for 24 hrs. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10,000 rpm for 10 min at 0°C and this Cell Free Filtrate (CFF) was stored at -20°C.

Enzyme Assay

The reaction mixture containing 0.1ml of crude enzyme and 1.0 ml (1.0%) solution of soluble starch in 50 mM Phosphate buffer (pH 7.5) was incubated at 50°C for 5 minutes. The reaction was stopped by addition of 1.0 ml of 1N NaOH. The level of amylase activity was determined by measuring the reducing sugar released from soluble starch (20). One unit of amylase activity was defined as the amount of enzyme which liberates 1µmol of reducing sugar as glucose per min under the conditions of the assay.

Protein determination

The protein concentration of the CFF was determined by the Lowry method (21), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Time course of growth and production of α- amylase

At different time courses the production of alpha amylase and cell mass are shown in Fig.1. Maximum amylase production was obtained at 24 hrs of incubation. After 24 hrs cell mass was increased but enzyme production declined, and after 72 hrs no activity was observed.

Effect of substrate concentration on α- amylase production

Bajpai et al (22) reported that carbon source greatly influence amylase production and the most commonly used substrate is starch. In this research, the effect of different concentrations of soluble starch on amylase production was studied (Fig.3). It was reported earlier that starch concentration beyond 1%in fermentation medium did not increase the enzyme production (23) but our strain showed that the 2%starch concentration in medium can also increases enzyme production while 3%starch in the medium decreased the enzyme production.

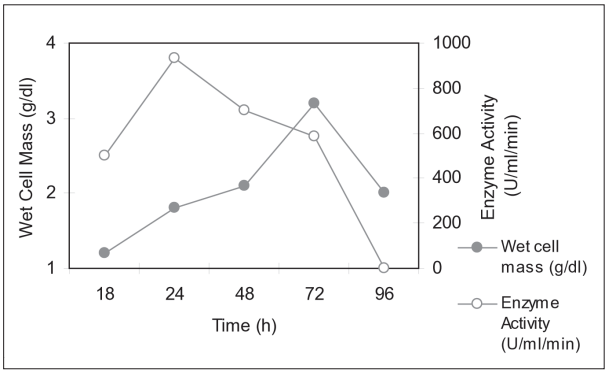

Figure 1. Effect of incubation time on α-amylase production.

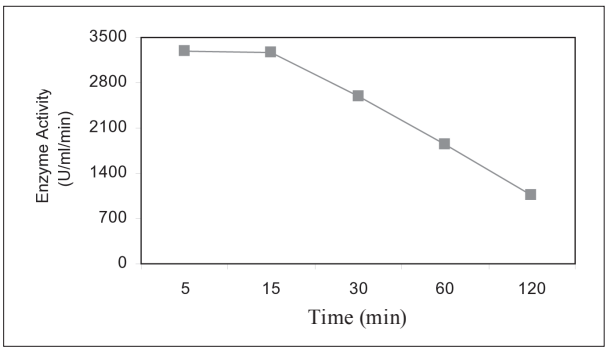

Figure 2. Effect of incubation time on extracellular α-amylase activity.

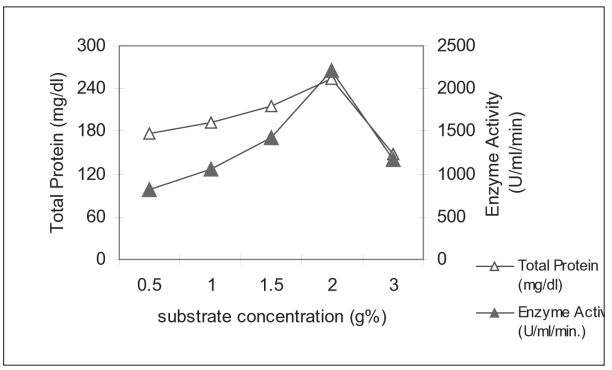

Figure 3. Effect of substrate concentration on α-amylase production

Effect of temperature on α-amylase production

The effect of temperature on bacterial growth and α-amylase production from *Bacillus* strain AS-1 was studied. The production of enzyme and bacterial growth was determined at different temperatures ranging from 25°C to 45°C and optimum enzyme production was observed at 35°C (Fig. 4). After 35°C both growth and amylase production were decreased, which indicated that the optimum temperature for maximum bacterial growth and amylase production were the same. Other investigators also reported that maximum amylase production occurred at an optimum growth temperature (22, 24, 25). But for *Bacillus licheniformis* CUM 305 although the maximum growth was observed at 30°C, no enzyme production was reported. This organism did not produce α- amylase at 30°C although it grew very well at this temperature (26).

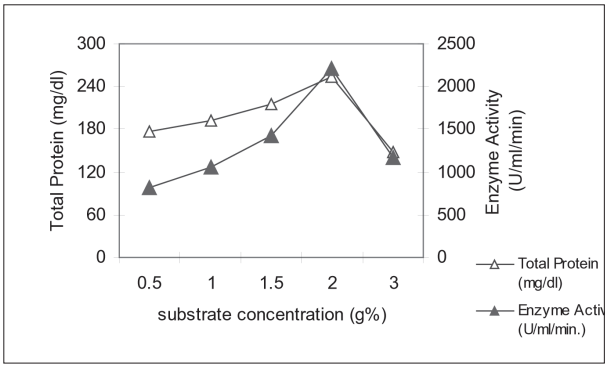

Figure 4. Effect of temperature on α-amylase production.

In addition, Saito and Yamamoto (25) studied a *Bacillus licheniformis* strain which produced α-amylase at temperatures around 50°C and never produced the enzyme at temperatures below 45°C. The optimum temperature for extracellular enzyme activity was 50°C. A reduction in enzyme activity was observed at values above 50°C (Fig. 5)

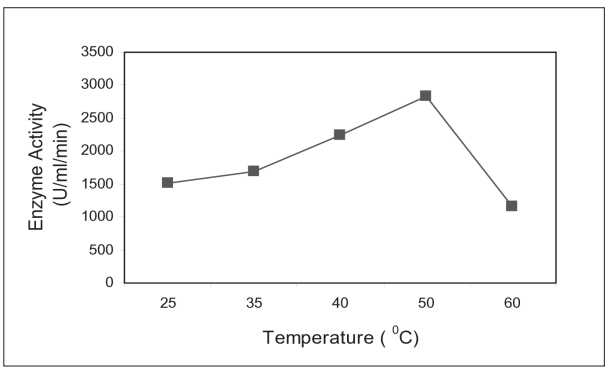

Figure 5. Effect of temperature on extracellular α-amylase activity.

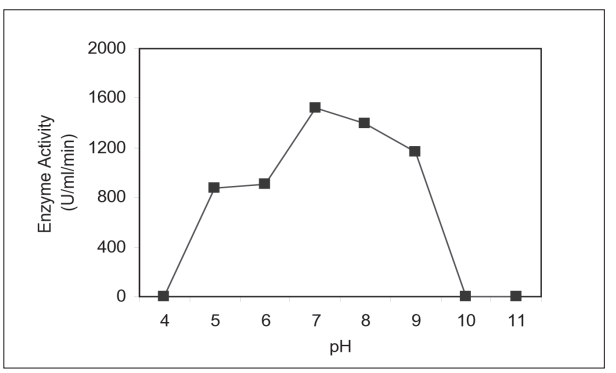

Figure 6. Effect of media pH on amylase Production

Effect of pH on α-amylase production

Enzyme synthesis and bacterial growth of *Bacillus* sp. AS-1 was observed between pH 4.0 to 11.0 (Fig.6). The results suggest that there is a stimulation of enzyme synthesis at pH 7.0 and the higher enzyme production at this pH was concluded as the result of increased cell growth. The organism did not grow at pH 4.0, 10.0 and 11.0. In acidic medium results are insignificant. This may be due to the fact that bacteria required slightly alkaline pH for the production of α-amylase. Increasing the initial pH of the medium up to pH 9.0 resulted in a decrease of the amylase production. Bajpai et al (22) reported that growth of *Bacillus licheniformis* TCRDC-B13 occurred at pH 3 to 11, although bacterial growth start decreasing as the pH increases. They also found out that optimum enzyme production was obtained at pH 6.0 to pH 9.0. The effect of pH on extracellular amylase activity was determined by using 50 mM phosphate buffer in a pH range of 6.0 to 8.0. As shown in Fig.7 the optimum pH was pH 7.5.

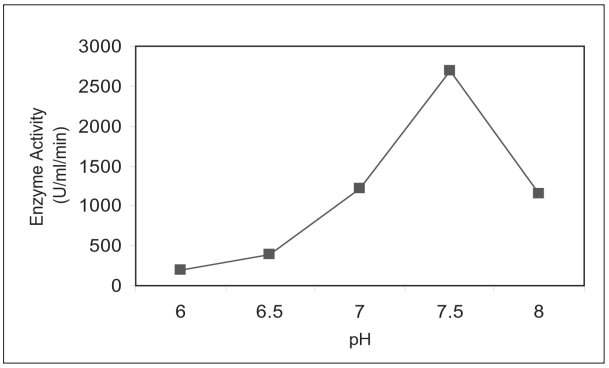

Figure 7. Effect of pH on extracellular α-amylase activity

Effect of carbon source on α-amylase production

To investigate the effects of various carbon sources on α-amylase production, *Bacillus* sp. AS-1 strain was grown in different media containing starch, galactose, lactose, dextran, fructose, sucrose, glucose and maltose as carbon sources. Starch is a generally accepted nutritional component for induction of amylolytic enzymes. This material was applied as a reference. Fig.8 shows that highest amylase production was obtained in medium containing lactose. It was also observed that starch, fructose and glucose favored α-amylase production, whereas sucrose inhibited α-amylase synthesis. In case of *B. flavothermus* the highest α-amylase activity with maximum biomass was obtained when lactose was used as a carbon source; but presence of sucrose, fructose and glucose in the media gave rise only to good bacterial growth with little or no amylase production (27).

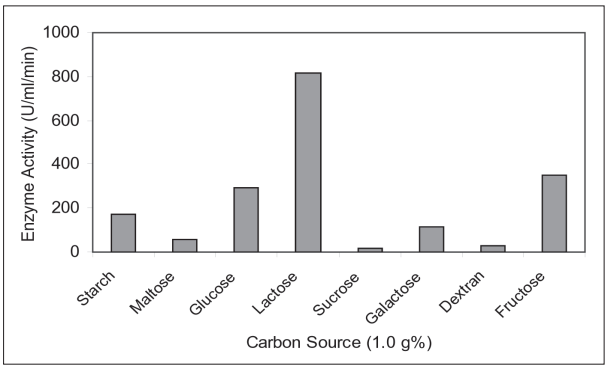

Figure 8. Effect of supplemented carbon source on α-amylase production

It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose (24).

Effect of nitrogen source on α-amylase production

The influence of organic and inorganic nitrogen sources on amylase production was determined (Fig.9). It has been reported that more amylase was produced when organic nitrogen compounds were used. Maximum enzyme production was found with peptone as the nitrogen source (24, 22). It has also been reported that the optimum production of α-amylase for *Bacillus* sp. was found when yeast extract was used (23). Our results suggested that optimum peptone concentration for α- amylase production was 1.0%(Fig. 10) This finding is in accordance with Bajpai et al (22). Yeast extract also seems to be suitable as well. Inorganic sources inhibit amylase

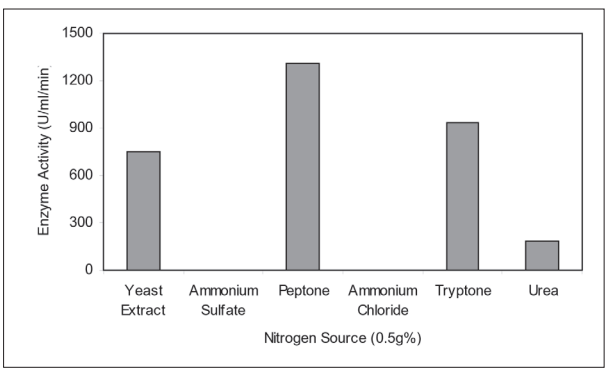

Figure 9. Effect of supplemented nitrogen sources on α-amylase production

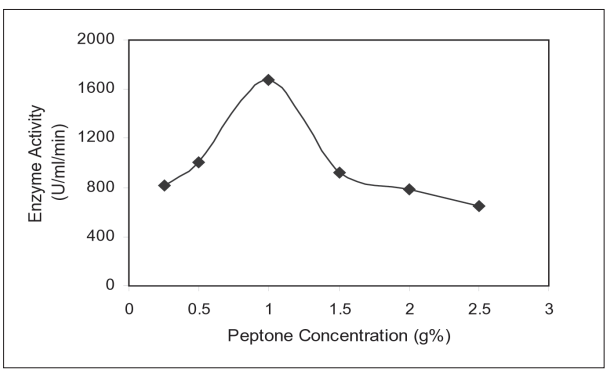

Figure 10. Effect of peptone concentration on α-amylase production

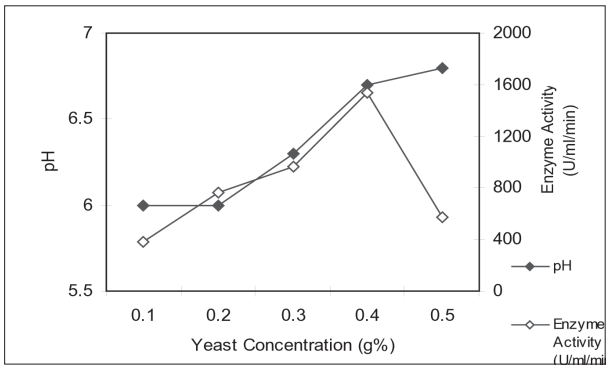

Figure 11. Effect Yeast Concentration of on α-amylase Production

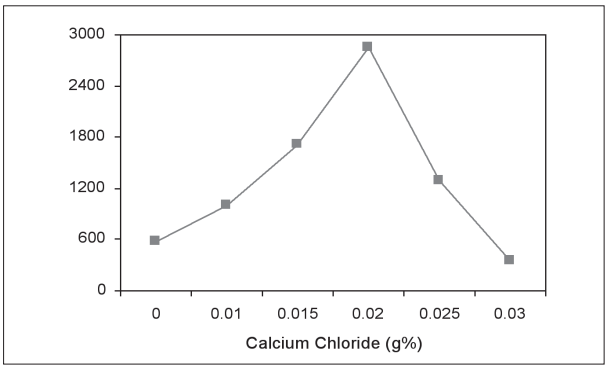

Figure 12. Effect of $CaCl_2$ of on α-amylase Production

synthesis.

Effect of yeast concentration on α- amylase production

The concentration of yeast extract was found to be important factor in the α-amylase synthesis by several organisms (28) and thus the influence of this compound on α-amylase synthesis by *Bacillus* sp. was investigated and 4g/l was found to be the optimum concentration (Fig.11). It has been reported that increasing the concentration of yeast extract to a level of 5.0 g/l lowered the pH significantly and this resulted in the complete repression of the enzyme (28). In our study it was observed that the pH of the broth increased from 6.0 to 6.8 at the end of the fermentation. This finding was also reported by Santos et al (23).

Effect of Ca^{+2} ions on α-amylase production

The production of α -amylase is Ca^{+2} dependent. Allan et al (29) reported that in case of *Bacillus Licheniformis* induction of calcium salt in the medium increased the α-amylase production. The stability of α-amylase is calcium dependent (30). In present study different concentration of $CaCl_2$ were evaluated. Fig.12 shows that 0.02%was found to be optimum for the production of α -amylase. With the increase in calcium ions there was a slight reduction in enzyme production. When calcium ions were not added in the medium, the results were insignificant. This may be due to the fact that calcium ion was the best binder, stabilizer and activator of α-amylase. Therefore the efficiency of enzyme was enhanced when the calcium ion was present in the medium. This finding is in accordance with the work reported by Suisheng et al (31). These results may also be due to the increasing availability of the calcium ion, since the enzyme is known to be a calcium metalloenzyme. These results are similar to the findings of Hewitt and Solomons (32) who worked with the culture of *Bacillus amyloliquefaciens.*

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