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Production of α-amylase from Bacillus megaterium MD-1

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Bacillus megaterium, α-Amylase, Enzyme production, Carbon and nitrogen sources,

Abstract: The alpha-amylase is used extensively in many different industrial sectors and is renowned for modifying starch by rupturing 1-4 glycosidic bands. Depending on the intrinsic properties of the microorganism, several alpha-amylases with thermostable and halotolerant properties are expressed. In the current study, the bacteria were isolated from Ergani Makam Mountain. Identification and optimization of the isolated bacteria were performed. As a result of the 16S rRNA analysis, physiological, morphological and biochemical analyis were carried out for the identification of the isolated microorganism and consequently the bacterium was defined as *Bacillus megaterium* MD-1.

Following its identification, α-Amylase, was isolated from *B. megaterium*. Optimal conditions for bacteria and enzyme production were determined as 48 hours, 35°C and pH 7.0. Maximum enzyme activity was optained at 40°C and pH 8.0. The effects of various carbon and nitrogen sources on enzyme production were investigated by adding to the nutrient medium. Compared to the control regarding enzyme production, it was determined that carbon sources, particularly sucrose, fructose and lactose inhibited enzyme production by 75%, all carbon sources inhibited production. It was also observed that urea and sodium nitrate from nitrogen sources had an inhibitory effect on enzyme production whereas other nitrogen sources did not. The highest amylase production among nitrogen sources was obtained with peptone addition.

In our study, it was determined that an increase in amylase activity could be achieved by using the optimum values of physical parameters. These findings displayed that enzyme could be utilized in fruit juice industries for clarification of apple juice, textile industry and raw starch hydrolyzing.

Bacillus megaterium MD-1'den α-amilaz üretimi

Anahtar Kelimeler
Bacillus megaterium, α-Amylase, Enzim üretimi, Karbon ve azot kaynakları

Öz: Alfa-amilaz, birçok farklı endüstriyel sektörde yaygın olarak kullanılmaktadır ve 1-4 glikozidik bantları parçalayarak nişastayı modifiye etmesiyle ünlüdür. Mikroorganizmanın içsel özelliklerine bağlı olarak, termostabil ve halotolerant özelliklere sahip birkaç alfa-amilaz ifade edilir. Mevcut çalışmada, bakteriler Ergani Makam Dağı'ndan izole edilmiştir. İzole edilen bakterilerin tanımlanması ve optimizasyonu yapıldı. 16S rRNA analizi sonucunda izole edilen mikroorganizmanın tanımlanması için fizyolojik, morfolojik ve biyokimyasal analizler yapılmış ve sonuç olarak bakteri *Bacillus megaterium* MD-1 olarak tanımlanmıştır.

Tanımlanmasının ardından α-amilaz, *B. megaterium* MD-1'den izole edildi. Bakteri ve enzim üretimi için optimum koşullar 48 saat, 35°C ve pH 7.0 olarak belirlendi. Maksimum enzim aktivitesi 40°C'de ve pH 8.0'da elde edildi. Besin ortamına ilave edilerek çeşitli karbon ve azot kaynaklarının enzim üretimine etkileri araştırıldı. Enzim üretimi ile ilgili kontrol ile karşılaştırıldığında, başta sakaroz, fruktoz ve laktoz olmak üzere karbon kaynaklarının enzim

üretimini %75 oranında inhibe ettiği, buna karşın glukoz, nişasta ve galaktozda herhangi bir değişiklik olmadığı belirlendi. Azot kaynaklarından gelen üre ve sodyum nitratın enzim üretimi üzerinde inhibitör etkisi olduğu, diğer azot kaynaklarının ise olmadığı gözlemlendi. Azot kaynakları arasında en yüksek amilaz üretimi pepton ilavesiyle elde edilmiştir.

Çalışmamızda fiziksel parametrelerin optimum değerleri kullanılarak amilaz aktivitesinde artış sağlanabileceği belirlendi.

1. INTRODUCTION

The enzyme known as alpha-amylase (α-amylase; 1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) hydrolyses the alfa bonds in massive, alfa-linked polysaccharides such as starch and glycogen to form shorter chains, dextrins, and maltose [1]. Alpha-amylase can be produced by a broad variety of organisms, including micro-organisms such aquatic bacteria, fungi, actinomycetes, plants and mammals [2, 3]. Microbial organisms are the principal source of alpha-amylase, producing a large amount of the enzyme at a high ratio of growth and proliferation. Additionally, the microorganisms that have undergone genetic manipulation are made to produce α-amylase with unique properties such as thermostability. The microbes also produce a lot of enzyme, which may easily be improved using different techniques including response surface methodology [4-7]. Alpha amylases are used today in biotechnology-important industries such as paper, food, textile, detergent, and in alcohol fermentation, starch hydrolysis, silage production, clinical, pharmaceutical, medical, analytical chemistry. They are industrial enzymes that are widely used in the field [8, 9].

Following the development of enzyme technology, the industrial enzymes have gained more attention in recent years. Majority of the enzymes (approximately 90%) used in industry are of microbial origin. Microorganisms are preferred for reasons such as being an important source of enzymes, biochemical diversity, being able to be produced by methods that will enable large-scale production and being suitable for genetic [10-13]. In addition, such bacteria are preferred in enzyme production due to their short fermentation cycle, stability, safe use, strong enzyme activity in stress situations and being environmentally friendly [14].

Among the bacterial enzymes, majority of the industrial enzymes are produced by different types of *Bacillus* strains [12]. Gram-positive *bacillus* and aerobic spore-forming *Bacillus megaterium* are among the largest group of the bacteria [15, 16]. Despite being utilised for industrial purposes over half century owing to its ability to produce key enzymes, it has recently been preferred in the field of biotechnology due to its capacity of recombinant protein production [17]. Amylases are the leading enzymes synthesized by *Bacillus*. Amylases are of 3 types; α-amylase, β-amylase and γ-amylase [18].

The aim of the current study was to isolate α-amylase, which constitutes the majority of the industrially important enzymes of the bacteria isolated from the soil. The second aim of our study was to increase the production of the enzyme obtained through optimization of the several parameters including temperature and pH.

2. MATERIAL AND METHOD

Morphological and biochemical analysis were carrıed out on *Bacillus megaterium* MD-1, a bacteria isolated from soil samples brought from Ergani Makam mountain. Optimum conditions for bacterial growth and α-amylase production were also determined within the scope of our work. To obtain the best amylase activity, optimum pH and temperature values were also investigated.

2.1. Isolation of Bacteria and its Phylogenetic Tree

Bacillus megaterium MD-1 was isolated from the soil sample in the Ergani Makam mountain, Diyarbakır in Turkey. Along with performing the morphological and biochemical investigations of the enzyme properties, we also performed 16s rRNA analysis of these bacteria were via Refgen. In order to collect the microbes from the soil, we took one gram of sample and added 9 ml of sterile water on the collected sample. Via application of that methodology, the sample was diluted 10 folds. A serial dilution procedure was applied and the sample was further diluted as follow: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}. For the purpose of obtaining a single colony, smear sowing procedure was applied to the serially diluted samples instead of Nutrient Agar (NA) and incubated at 37 ^{o}C.

2.2. Enzyme Production and Determination of Protein Amount

Bacillus megaterium MD-1 was regularly cultured through shaking at following experimental conditions: 120 rpm, pH 7.0 and 35 °C. After a 24-hour incubation period, the culture sample was centrifuged (Sigma Christ 2K15) for 10 min at 10 rpm. After centrifugation, the supernatant was used to measure the enzyme activity. The Lowry technique was used for protein quantification [19].

2.3. Determination of Enzyme Activity

The Bernfeld method [20] developed in 1955 was modified to determine the amylase activity. For 30 minutes at 70 °C, either a 100 µL crude enzyme solution or a 10 µL purified enzyme solution was added to a 200 µL 0.5% solvable starch solution (dissolved in a Tris-HCl buffer at 0.1 M pH 7.0). At the conclusion of this period, 400 µL of 3,5-dinitro salicylic acid (DNS) was added, and it was then heated in a water bath for 5 minutes to cease the substrate-enzyme reaction. The

decreasing sugar was calculated using DNS, which also creates color. After boiling, test tubes were held for chilling, and 3 mL of distilled water was added for dilution. UV-VIS spectrophotometric (SPD-20A UV) detection of the enzyme activity at 489 nm. For amylase activity, the amount of enzyme resulting the production of 1 µmole glucose per minute was defined as one unit.

2.4. Effect of Temperature, pH and Incubation Time on Microorganism and Amylase Production

Bacteria were inoculated into 100 ml flasks. In order to determine the optimum bacterial and enzyme production values at 25-55 ºC temperature ranges, they were kept in a shaking water bath at 120 rpm and absorbance measurements were made in the spectrophotometer. Bacteria and enzymes were produced at different pHs between pH 4.0 and 11.0 in 0.5 increments in the prepared NB medium. To determine the effect of incubation time on microorganism growth and enzyme production; Absorbance measurements were made in spectrophotometer by taking samples at 4-hour intervals between 4-96 hours in NB medium.

2.5. Influence of temperature and pH on enzyme activity and stability

Using the aforementioned techniques, solvable starch was used as the substrate and temperatures ranging from 20 to 80°C were used to determine the purified α-amylase's optimal temperature. By preincubating the purified enzyme at various temperatures (40-70°C) for 0–180 min, the enzyme's thermostability was discovered. The Bernfeld approach was used to calculate the residual activity under ideal conditions [20].

During a 30-minute period at optimal temperature and a range of pH values (pH 3.0-11.0), the effect of pH on amylase activity was examined. Enzyme activity was determined for pH stability using the Bernfeld method [20] by maintaining the enzyme solution at various pH ranges (4.0-8.0) for 0-240 min.

2.6. Effect of Carbon and Nitrogen Sources on Amylase Production

After adding glucose, galactose, fructose, lactose, soluble starch and sucrose as carbon source to NB media at 1% concentration, bacteria were cultivated and incubated at 37°C for 48 hours. Nitrogen sources such as peptone, tryptone, urea, ammonium sulfate, ammonium chloride, ammonium nitrate, sodium nitrate and yeast extract were added to NB broths at the rate of 1% and incubated under optimal conditions for enzyme production.

3. RESULTS

B. megaterium MD-1 (YC2) was determined as the bacterium with 16 s rRNA analysis. The related phylogenetic tree is shown in Figure 1. 16S rRNA analysis to identify the bacterium was performed by Ref-Gen (METU Technocity/Ankara).

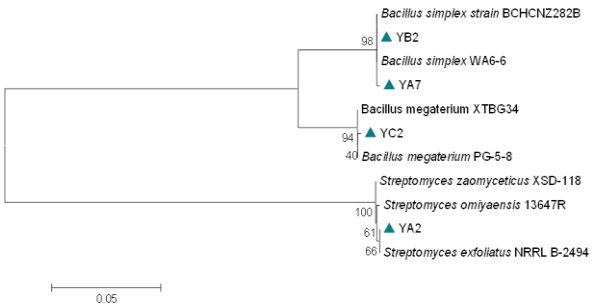

Figure 1. Phylogenetic tree of bacteria

In order to identify B. megaterium MD-1, biochemical tests such as starch, gelatin, catalase, casein, urease, lipase, hydrolysis, motility, indole, phosphatase, hemolysis, as well as morphological and physiological analyzes were performed. (Table 1).

Characters	***Bacillus megaterium***
Optimal pH	7
Hemolysis	-
Motile	+
Hydrolysis	
Starch	+
Casein	-
Gelatine	-
Activity	
Urease	+
Catalase	+
Lipase	+
Amylase	+
Indole	+
Phosphatase	+

Table 1: Morphological, Physiological and Biochemical Tests

To examine the effect of incubation time on enzyme production and growth of *B. megaterium* MD-1, bacterial growth curve was followed for 92 hours and enzyme activity was measured every 4 hours. As a result of the analysis, it was determined that bacterial growth and maximum enzyme production were at 48 hours (Figure 2).

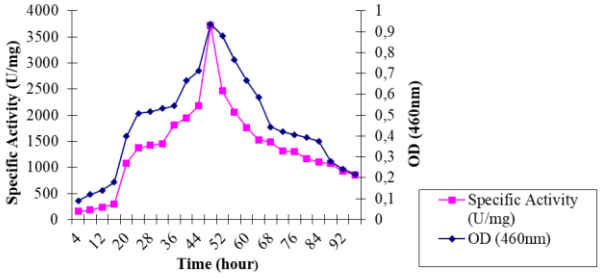

Figure 2. The effect of time on bacteria and amylase production

For the purpose of determination of the impact of temperature, bacteria culture was incubated in NB medium (pH 7.0) at 30 to 55°C for 48 hours. At the conclusion of the incubation time, it was discovered that the bacterial growth and enzyme synthesis were at their highest levels at 35°C. (Figure 3). A wide temperature range of 35°C to 80°C is given for optimum bacterial growth and α-amylase production in bacteria.

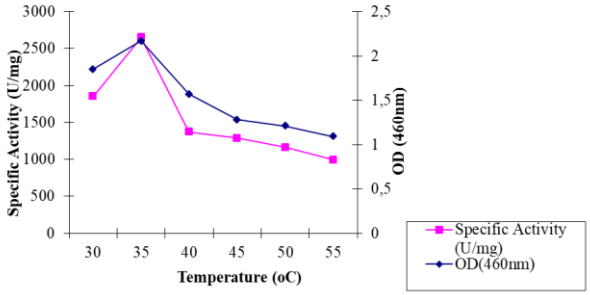

Figure 3. The effect of temperature on bacteria and amylase production

On the other hand, in order to determine the effect of pH on amylase production and bacteria growth, the bacteria culture was grown in the presence of different pH ranging between 4.0 to11.0 and the optimum pH value was determined as pH 7.0 for both analysis (Figure 4).

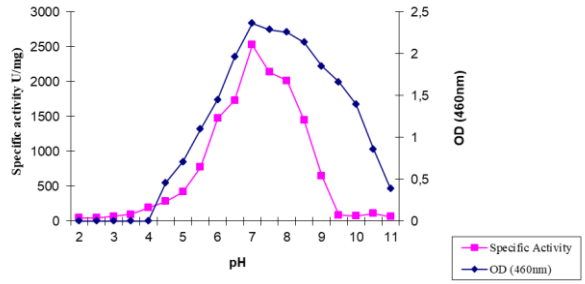

Figure 4. The effect of pH on bacteria and amylase production

The impacts of heat and pH on α-amylase activity isolated from *B. megaterium* MD-1 were also studied within the scope of current. In order to determine the effect of temperature on enzyme activity, it was determined that the maximum α-amylase activity was at 40°C in the experiment performed between 30-55°C temperature ranges. (Figure 5).

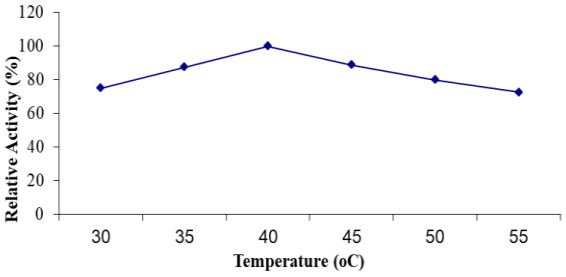

Figure 5. Effect of temperature on α-amylase

When the effect of pH on enzyme activity was studied, it was found out that the maximum α-amylase activity was achieved at pH 8.0. (Figure. 6).

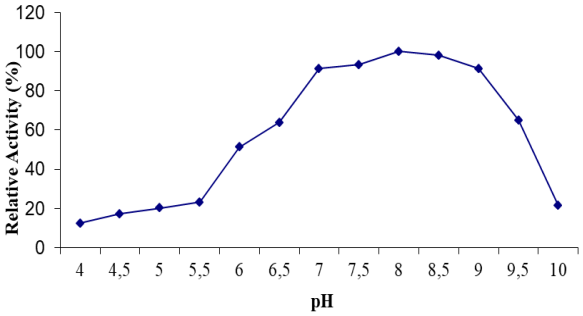

Figure 6. Effect of pH on α-amylase

The effect of carbon sources on amylase activity was also studied. Our results demonstrated that, the activity of the control for which no extra carbon sources added into the growth media was 1728 Umg-1 while after adding more glucose and starch added to the media the activity was 1577 Umg-1 and 1584 Umg-1 suggesting that the activity of the enzyme was not significantly changed in the presence of these carbon sources. The enzyme activity was decreased in the presence of sucrose, galactose, lactose and fructose (Figure 7).

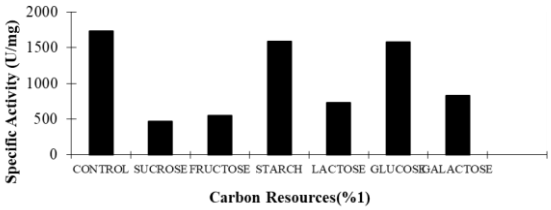

Figure 7. Effect of Nitrogen Sources on enzyme production

The effect of nitrogen sources on amylase activity revealed following results: When compared to the control (2227 Umg-1), suppression of amylase production was observed in media containing nitrogen sources urea (798 Umg-1), sodium nitrate (883 Umg-1) and yeast extract (896 Umg-1) (Figure 8).

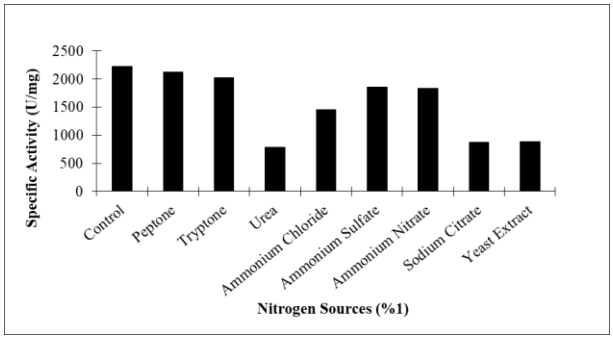

Figure 8. Effect of Nitrogen Sources on enzyme production

4. DISCUSSION AND CONCLUSION

Agüloğlu Fincan et al. [9] isolated a new thermo-tolerant bacteria named as *Bacillus licheniformis* SO-B3 from the mud sample of Şırnak-Meyrem deresi thermal spring and isolated amylase from the bacteria. 35°C and a pH of 7.0 were found to be the best conditions for enzyme generation. Özdemir et al. [21] found the maximum α-amylase production conditions from *Bacillus mojavensis* SO-10 bacteria to be 36 hour, 35°C and 7.0. Al Johani et al. [22] achieved maximum amylase production from *B.subtilis* at 45°C and pH 8.5 in agitated environment. Ortakaya et al. [23] isolated amylase from *Bacillus simplex*. While they determined the most suitable conditions for bacterial growth as 32. hour, 37°C and pH 7.0, they reported that they obtained the highest enzyme production at 72. hours, 37°C and 7.0. Agüloğlu Fincan and Enez [24] purified and characterized *Geobacillus stearotermophilus* α-amylase and they reported that maximum enzyme production was reached at pH 7.0 and 55°C at 24 hours and the enzyme was active in a wide temperature range between 50°C and 80°C.

α-Amylase's activity at alkaline pH is crucial in terms of its use in important areas such as detergent, food industry, liquefaction of starch and dry cleaning. Rakaz et al. [25] defined two species isolated from Omdurman Toti Island as *Bacillus cereus* and *Bacillus licheniformis*. They stated that *B. cereus* and *B. licheniformis*, which are alkalophilic and thermophilic, gave the best amylase activity at pH 8.0 and at 45°C and 65°C, respectively. Najafi et al. [26] determined the optimum temperature of *Bacillus subtilis* AX20 α-amylase, which they isolated from the soil, as 55°C and the optimum pH as 6.0. Kannan and Kanagaraj [27] named the bacteria they isolated from soil samples as *Bacillus licheniformis* and stated that they obtained the maximum amylase activity at pH 7.0 and 35°C after incubation at 48th hour. The results obtained in the studies carried out support the findings of our study.

Behal et al. [28] studied the effect of carbon and nitrogen sources on the production of α-amylase from *Bacillus sp*. AB 04, and obtained the maximum enzyme production by adding fructose as carbon source and meat extract as nitrogen source. Saxena et al. [29] *Bacillus sp*. maximum specific activity for PN5 thermostable amylase was obtained in media containing starch, peptone and yeast extract. Carvalho et al. [30] *Bacillus sp*. They achieved maximum enzyme production from SMIA-2 with peptone and soluble starch as carbon and nitrogen sources. Prakash et al. [30] obtained the maximum α-amylase production by adding tryptone. Agüloğlu Fincan and Enez [24] achieved the highest specific activity with ammonium nitrate among the nitrogen sources they used. Du [12] achieved maximum amylase production when they used peptone as N source and glucose as C source from *B. amyloliquefaciens* BH1. Asgher et al. [32] observed that 1% glucose suppressed amylase production from *Bacillus subtilis*. Al-Johani et al. [22] achieved maximum amylase production in culture medium supplemented with peptone and starch from B. subtilis. The findings we obtained in our study carbon and nitrogen sources are in agreement with the results of other researchers.

In our study, it was determined that temperature, pH and incubation time were effective on amylase activity and an increase in amylase activity could be achieved by using the optimum values of these physical parameters The maximum activity of the α-amylase we obtained at 40ºC will help especially in the washing sector. In addition, by adding the enzyme to detergents, a significant amount of energy will be saved as a result of maximum cleaning at low temperatures, and heat-induced wear will be prevented and the service life of the clothes will be extended.

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