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Research Article

Optimization of Physical Factors Affecting the Production of the α-Amylase from a Newly Isolated Bacillus sp. M10 Strain

Yeni İzole Edilen Bacillus sp. M-10 Suşundan α-Amilazın Üretimini Etkileyen Fiziksel Faktörlerin Optimizasyonu

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Abstract

In this study, sixty bacteria isolated from soil samples that come from 15 different cities of Turkey. The four isolates which had a higher ratio of clearing zone were determined to be α-amylase positive, and were investegated for morphological and physiological. All of these were defined as *Bacillus*. Enzyme production capacity of these 4 strains have been tested. The bacterium which has the highest α-amylase activity was selected as a *Bacillus* strain and this strain was named as *Bacillus* sp. M-10. It's investigated that physical factor which is affecting the α-amylase production of *Bacillus* sp. M-10. For this purpose, temperature, pH, aeration, inoculum size and inoculum age have been tested as physical parameters. In starchy medium, 37˚C temperature, pH 7.0, 150 rpm for aeration, 2.5% (v/v) inoculation size and 2 days for inoculation age were the optimum rate for maximum α-amylase production of *Bacillus* sp. M-10 strain. In order to enhance the production of α-amylase, medium was modified by our, and the enzyme activity was found 30 U/mL at the hour of 48. In modified environment, enzyme activity increased about 42% rate.

Keywords: *Bacillus* sp., Isolation, Physical factors, α-amylase

Öz

Bu çalışmada Türkiye' nin 15 farklı ilinden temin edilen toprak örneklerinden 60 bakteri izole edilmiştir. α-Amilaz pozitif olarak belirlenen en geniş açık zon çapına sahip 4 bakterinin morfolojik ve fizyolojik özellikleri araştırılmış ve hepsinin *Bacillus* cinsine ait olduğu saptanmıştır. 4 suşun enzim üretim kapasiteleri test edilmiştir. En yüksek α-amilaz aktivitesine sahip *Bacillus* suşu seçilmiş ve bu suş, *Bacillus* sp. M-10 olarak adlandırılmıştır. *Bacillus* sp. M-10'un α-amilaz üretimini etkileyen bazı fiziksel faktörlerin etkisi araştırılmıştır. Bu amaçla, sıcaklık, pH, havalandırma, inokulüm miktarı ve inokulüm yaşı gibi fiziksel parametreler denenmiştir. *Bacillus* sp. M-10 suşunun maksimum α-amilaz üretimi, nişastalı ortamda, 37˚C sıcaklıkta, pH 7,0, havalandırma 150 rpm, inokülasyon miktarının %2.5 (v/v) ve inokülasyon yaşının 2 gün olduğu değerlerde optimum düzeydedir. Tarafımızdan modifiye edilen ortam, daha yüksek enzim aktivitesi elde etmek için en uygun ortam olup, enzim aktivitesi 48. saatte 30 U/ mL olarak bulunmuştur. Modifiye edilen ortamda enzim aktivitesinde yaklaşık % 42 oranında artış gözlenmiştir.

Anahtar Kelimeler: *Bacillus* sp., İzolasyon, Fiziksel faktörler, α-amilaz

1. Introduction

α-Amylase (1,4-a-D-glucan glucanohydrolase, EC 3.2.1.1) catalyses the hydrolysis of a-D-(1,4) glycosidic linkages in starch components and related carbohydrates. This enzyme is used in desizing fabrics, in the baking industry, pharmaceuticals and detergents (Pandey et al. 2000). Recent discoveries of starch degrading enzymes have led to increased application of amylases in various industrial processes. α-amylase is an extracellular enzyme which is widespread among higher plants, animals and microorganisms. Bacterial starins have been well known for the starch and cellulose degrading enzymes they naturally secrete (Abou-Zeid 1996). The commonly used *Bacillus* sp. is widely used for thermostable production to meet industrial. Because, *Bacillus* sp. is the organism of choice because of its ubiquitous nature, non fastidious nutritional requirements needs (Singh et al. 2012).

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Cellular growth and its morphology are affected by genetic factors; inoculum size, type and age; medium shear; medium constituents; temperature and pressure; biosynthesis or addition of polymers, surfactants and chelators and the pH (Pazouki and Panda 2000, Papagianni 2004).

The present work was carried out to study certain physical factors (temperature, pH, agitation, inoculum size, and inoculum age) affecting amylases production by new isolate *Bacillus* sp. M-10 strain.

2. Material and Methods

2.1. Screening and Culture Conditions

Soil samples of 15 different cities of Turkey were collected. Soil suspensions in sterilised water were poured and spread onto starch nutrient agar plate. These plates were incubated at 37°C for 24 h. Amylase-producing bacterial strains were selected after flooding the plates with iodine solution. Starch degrading activities were detected as clear zones after exposure to iodine solution. The diameters of both the colony and the clear halo-zone around colonies were measured by millimeter ruler, which allow to calculate the ratio R (diameter of the colony / diameter halo-zone) (Djamel et al. 2009). The strains selected were those whose ratio is the most important. The four isolates which had a higher ratio (R) were selected for quantitative test of α-amylase. For determination of the genus of the selected strains were studied morphology and biochemical tests, and genus of the selected strain was identified as *Bacillus* (Gots et al. 1975).

Basal enzyme production medium for liquied culture consists of (%): starch, 1; peptone, 0.5; corn steep liquor, 0.5; $(NH_4)_2SO_4$, 0.8; $MgSO_4.7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.05; K_2HPO_4, 1.4; KH_2PO_4, 0.6, and the pH was adjusted to 7.0 before autoclaving (Dincbas and Demirkan, 2010). The precultures were cultivated in Luria-Bertani (LB) medium for 18 h. Then, overnight cultures with OD_{600} 0.3 were inoculated at 1% in enzyme production media (150 mL in 500 mL Erlenmeyer flasks) and incubated at 37 °C for 16, 24, 40, 48, 64, and 72 h in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (3461 xg, 10 min) and the supernatants were used for determination of α-amylase activity. Bacterial growth was determined by measuring optical density at 600 nm.

The strain that yielded a high level of α-amylase was selected for further experiments.

2.2. Enzyme Activity

α-Amylase activity was assayed by the starch-iodine method (Yoo et al. 1987). Five milliliters of substrate solution (0.1% w/v) is added to a test tube and maintained for 10 min at an incubation temperature in a water bath. Enzyme (0.5 mL) is added to the substrate solution and incubated under the test conditions. The digest is added to 5 mL stopping reagent (0.1 M HCI). After mixing, 0.5 mL of this mixture is added to 5.0 mL iodine solution. The absorbance of blue color is measured in a spectrophotometer (620 nm).

The activity of the enzyme is calculated from the formula, Activity (unit/mL) = D [(Ro-R)/Ro] X 100, where Ro is the absorbance of the substrate-iodine complex in the absence of enzyme; R is the absorbance of the digest; and D is the dilution factor of the enzyme. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 mg of starch in 10 min at 37 °C and pH 5.9 (unit/mL).

2.3. Effect of Some Physical Factors on α-Amylase Production

Some parameters (temperature, pH, agitation, inoculum size and inoculum age (days) were studied for its influence in α-amylase production in basal medium.

2.3.1. Effect of incubation temperature

The effect of temperature was evaluated by incubating the reaction mixtures at different temperatures such as 30, 35, 37 (control) 40, and 45 °C in production medium with optimal pH of 7.0. The α-Amylase activity and bacterial growth were performed after 48 h of incubation.

2.3.2. Effect of pH

pH in the range of 4.0–8.0 were examined for their effect on α-amylase production by the selected isolate grown in production medium. The pH of the medium was adjusted using 1 M HCl or 1 M NaOH. The flasks were incubated at 37 °C for 48 h. Samples were taken for α-amylase activity and bacterial growth.

2.3.4. Effect of agitation

To optimize the agitation rate for maximum α-amylase production, the inoculated production medium was agitated at different rotations per minute (rpm) such as 0, 50, 100, 150 (control), 200, and 250 rpm. The flasks were inoculated at 37 °C for 48 h. The culture filtrate was used to check the enzyme activity. Bacterial growth was also recorded.

2.3.5. Effect of inoculum sizes and age

The bacterium was grown in basal medium until OD_{600} of 0.3, and different inocula sizes of culture including 0.5,

1 (control), 2, 2.5, and 3% (v/v) were applied. The flasks were incubated at 37 ºC for 48 h. Samples were taken for α-amylase activity and bacterial growth.The α-Amylase activity and bacterial growth was measured by incubating production medium seeded with different inoculum age (18 h (control), 1, 2 and 3 days) at 37 ºC.

A new medium including the best contitions with physical factors for α-amylase production were improved, and the novel *Bacillus* sp. isolate was grown in this modified medium for 48 h. Bacterial growth yield and α-amylase was recorded, and compared with basal medium.

Results presented in this study are means of three independent determinations.

3. Results

3.1. Screening and Isolation of Bacteria

60 bacteria isolated from soil samples that come from 15 different cities of Turkey. Among these bacteria, 18 *Bacillus* sp. strains were determined on the basis of morphological and biochemical characteristics (Table 1).

Total 18 *Bacillus* sp. strains were found as extracellular α-amylase producer. Among *Bacillus* sp. strains, four isolates were showed higher ratio (R=1 and 1.25) than others (Figure 1).

These isolates were named as *Bacillus* sp. M-10, M-11, M-13, and M-18. Four isolates were checked for quantitative test of α-amylase in the basal enzyme production medium. The amount of α-amylase produced was investigated after every 16 h up to 72 h. The result indicates that incubation time affected α-amylase production very significantly. Enzyme activities of M-10, M-11, M-13, and M-18 were 21.2 IU/mL, 16.3 IU/mL, 11.3 IU/mL, and 12.7 IU/mL at 48 h for all, respectively, and bacterial growth was 0.8, 0.7, 0.7, and 0.8 at 40 h for all, respectively (Table 2). Maximum enzyme activity was obtained at the stationary phase of growth phase, and continued during the stationary phase. Isolate M-10 showing highest α- amylase activity was selected, and used for further experiments. Maximum enzyme activity was obtained at the stationary phase of growth phase, and continued during the stationary phase. The enzyme activity by *Bacillus* sp. M-10 was not in parallel with bacterial growth.

3.2. Effect of Some Physical Factors on the Production of α-Amylase

The environmental parameters are showing great influence in the growth of the organisms and the production of enzymes. The present study was mainly focused on the production of α-amylase form new isolate *Bacillus sp.* M-10 by optimizing various physical parameters such as temperature, pH, agitation, inoculum size, and inoculum age.

3.2.1. The effects of temperature on the production of α-amylase

Temperature is a highly sensitive parameter for α-amylase productivity. Figure 2 shows the effect of various temperatures on α-amylase production. The maximum production of α-amylase was obtained at 37˚C. Biosynthesis of α-amylase was significantly decreased with the increase in the incubation temperature above 40ºC. The production of the enzyme was greatly inhibited at 45˚C. But, differences in bacterial growth were observed. While bacterial growth was increased at 40˚C. Thus, the temperature of incubation at 37 ˚C was selected for maximum production of enzyme.

Table 1. Morphological and biochemical characteristics of *Bacillus* genus

Test	Result
Shape	Rods
Gram staining	+
Spore formation	+
Motility	+
Starch hydrolysis	+
Catalase	+
Indol Production	-
Nitrate Reduction	+
Gas Production from Glucose	-
Acid Production from Glucose	+

Figure 1. Starch agar plate with α-amylase activity showing clear zone formation.

Table 2. The comparison of enzyme activity of four *Bacillus* sp. strains in the basal medium

	M-10		M-11		M-13		M-18	
Incubation Period (h)	Enzyme Activity (U/mL)	OD (600 nm)	Enzyme Activity (U/mL)	OD (600 nm)	Enzyme Activity (U/mL)	OD (600 nm)	Enzyme Activity (U/mL)	OD (600 nm)
16	12	0.5	0	0.4	0	0.4	0	0.5
24	14.5	0.6	0	0.5	3.9	0.5	0	0.6
40	16	0.8	9.7	0.7	8.75	0.7	9.1	0.8
48	21.2	0.9	16.3	0.7	11.3	0.6	12.7	0.8
64	18.7	0.6	16	0.6	6.2	0.5	8.3	0.7
72	16.2	0.6	0	0.5	0	0.4	0	0.5

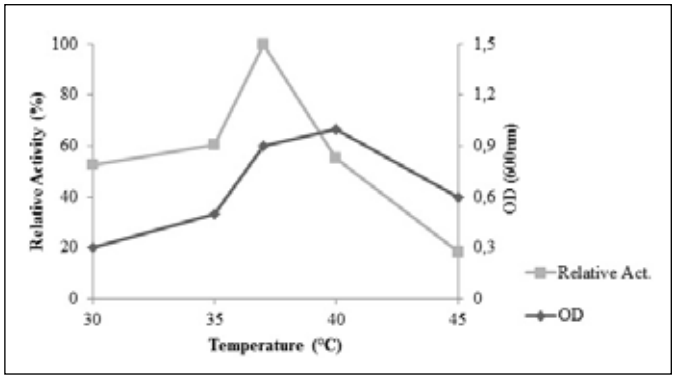

Figure 2. Effect of temperature on α-amylase production by *Bacillus* sp. M-10.

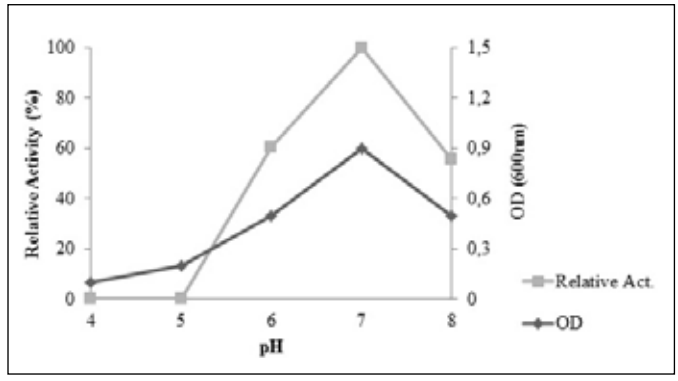

Figure 3. Effect of pH on α-amylase production by *Bacillus* sp. M-10.

3.2.2. The effects of pH on the production of α-amylase

The optimization of pH was carried with different pH (4, 5, 6, 7, and 8). Maximum α-amylase production was achieved at neutral pH 7.0 (Figure 3). The α-amylase activity was not recorded at pH 4.0 and 5.0, and bacterial growth was also too low. Increasing the pH of the medium up to pH 8.0 resulted in a reduction in α-amylase production, but gowth was increased at the same pH. Maksimum bacterial growth was also obtained at pH 7.0 (Figure 3). Thus, the pH 7.0 was selected for maximum production of enzyme.

3.2.3. The effects of agitation on the production of α-amylase

The effects of agitation condition on the production of α-amylase and bacterial growth were investigated. Figure 4 shows that the maximum α-amylase production and bacteria growth were obtained when agitated at 150 rpm.

Although the production of α-amylase was found to decrease when shaken at 200 rpm, the static condition almost inhibited its production. The lowest α-amylase activity and bacterial growth were observed at 50 rpm. Thus, 150 rpm was selected for maximum production of enzyme.

3.2.4. The effects of inoculum levels and ages on the production of α-amylase

Different inoculum levels (0.5, 1, 2, 2.5, and 3% v/v) and different inoculum ages (1, 2, and 3 days) were tried to investigate their effect on α-amylase production so as to achieve an optimum inoculum level and age. The studies shown that the optimum level of inoculum size and age were 2.5% v/v and 2 days, respectively (Figure 4). On the other hand, the minimum amount of α-amylase production was observed at 0.5% inoculum level, and observed 3 days for inoculum age. As the level of inoculum was further increased, the productivity of α-amylase was decreased. Thus, the inoculum level of 2.5 % v/v and 2 days of inoculum age were selected for maximum production of enzyme.

In this study, a modified medium was obtained by optimizing of the physical parameters. In this medium, enzyme activity

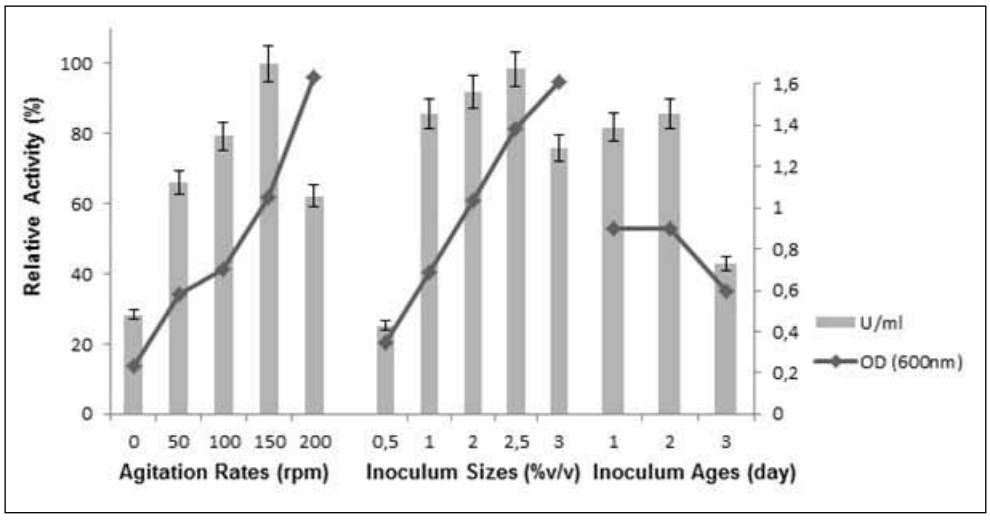

Figure 4. Effects of agitation rate, inoculum size and inoculum age on α-amylase production by *Bacillus* sp. M-10.

was found as 30 U/mL, while control was 21.2 U/mL. An increase was determined about 42% in α-amylase production when compared with the control.

4. Discussion

Nowadays the potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms (Abd-Elhalem et al. 2015). Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta et al. 2003, Wanderley et al. 2004)

In the present study, a total of 60 bacteria from soils have been screened for the presence of α-amylase production on starch plates. Eighteen strains were identified as *Bacillus* sp. on the basis of morphological and biochemical characteristics, and were α-amylase producers by zone of hydrolysis around the colonies. Four isolates were showed higher ratio (R), and quantified their activity. In α-amylase assay, M-10 showed highest production compared to other strains and used for α-amylase optimization studies.

The optimization of various physical and nutritional growth parameters caused an increase in the enzyme activities; the important physical factors that determine the bioprocess are temperature, pH, agitation, inoculum size and inoculum age (Ashokkumar et al. 2001). Therefore, in this study, the effects of physical parameters were investigated for the optimum production of extracellular enzyme α-amylase from *Bacillus* sp. M-10.

Temperature is a critical parameter which needs to be controlled and this is usually varied from organism to another (Sivakumar et al. 2011). Temperature can affect an enzyme in two ways. One is a direct influence on the reaction rate constant, and the other is in thermal denaturation of the enzyme at elevated temperatures (Park and Zipp 2000).

In this study, activity of α-amylase from was the highest about 37˚C. Biosynthesis of α-amylase was significantly decreased with the increase in the incubation temperature above 40ºC. Maximum bacterial growth was found at 40°C.

The same phenomenon of incubation temperature has also been reported (Sudharhsan et al. 2007, Padhiar and Kommu 2016). Similar incubation temperature for α-amylase production by *Aspergillus niger* and *Rhizopus stolonifer* was reported (Saleem and Ebrahim 2014). Low temperature at 35˚C has been reported for α-amylase production by *Bacillus* sp. The maximum production of α-amylase was obtained at 40 ˚C for *Bacillus* sp. and *Bacillus cereus* (Mukhtar and Ikram ul 2012, Singh et al. 2015, Sivakumar et al. 2011). The higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability (Goes and Sheppard 1999).

Different enzymes have different optimum pH values. This is the pH value at which the bonds within them are influenced by H^+ and OH^- ions in such a way that the shape of their active site is the most complementary to the shape of their substrate. At the optimum pH, the rate of reaction is optimum. Any change in pH above or below the optimum will quickly cause a decreasein the rate of reaction, since more of the enzyme molecules will have active sites whose shapes are not (or at least are less) complementary

to the shape of their substrate (Day 2015). In this study, maximum α-amylase production was found to be pH 7.0. Similar result has been recorded for *Bacillus* sp. and *Brevibacillus borstelensis* R1 (Singh et al. 2012, Suribabu et al. 2014). The production optimization studies showed that maximum enzyme production by *Penicillium notatum* IBGE 03 was obtained at pH 5.5 (Ahmed et al. 2015). In neutral conditions (pH 6.5-7.5) the α-amylase production was reported in *Bacillus* sp.(Asgher et al. 2007). In alkaline conditions (pH 7.5 - 11) the α-amylase production was reported in *Bacillus* sp.(Saxena et al. 2007).

It was stated that at high pH, the metabolic action of bacterium may be suppressed and thus it inhibits the enzyme production (Ellaiah et al. 2002). The bacteria did not grow at pH 4.0, and 5.0. This may be due to the fact that bacteria required alkaline pH for the production of α-amylase.

Agitation is another important parameter that affects enzyme production in bacteria. Micro-organisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks, and also effect the nutrient availability (Nascimento and Martins 2004). On the other hand, it has been reported that enzymes are also susceptible to mechanical force, which may disturb the elaborate shape of complex molecules to such a degree that denaturation occurs (Gupta et al. 2002). In this study, the maximum α-amylase production was observed at 150 rpm. However, enzyme production was reduced by further increases in the agitation rate. Higher agitation rates could increase the oxygen pressure of the system, but did not provide the increase in enzyme production, probably because at a high agitation rate, the structure of enzyme would be altered (Roychoudhury et al. 1989). A similar profile was determined for the effect of agitation speed on α-amylase production by *Bacillus subtilis* (Mukhtar and Ikram ul 2012). Some researchers reported various agitation rates (100-200 rpm) for enzymes production by different microorganisms (Dahot 1986, Mamma et al. 2008, Sharma and Satyanarayana 2011). But, the highest α-amylase production was obtained at 300 rpm from *Bacillus lichenifomis* ATCC 6346 in the fermenter (Vengadaramana et al. 2012).

The effect of inoculum size and age was also investigated. The finite volume of a culture medium means that it can only contain limited nutrients for the micro-organism. Furthermore, the consumption of the nutrients is largely dependent on the population of bacteria (Abusham et al. 2009). In the present study, optimal inoculum size and age for enzyme production were 2.5 % (v/v) and 2 days. Lower or higher inoculum size and age did not support higher activity.

The inoculum level of 2% (v/w) was found to be optimum for α- amylase synthesis and the highest α-amylase production was observed by *Bacillus cereus* at 5% inoculum size (Sivakumar et al. 2012, Suribabu et al. 2014). Maximum α-amylase production was observed with 0.5% of inoculum size and 6 days old inoculum age (Gupta et al. 2015). In earlier studies, 4% inoculum was used for α-amylase production from source (Jogezai et al. 2011). Whereas, optimal enzyme productivity by *Bacillus amyloliquefaciens* IIB-14 was 20% inoculum size and inoculum age of 24 h old (Zar and Ul Haq 2012).

In this study combination of physical factors was used for maximum α-amylase production. By optimizing the incubation conditions of α-amylase production from this strain was enhanced about 42% enzyme yield as compared to control. Enzyme activities in modified medium and control medium were 30 U/mL and 21.2 U/mL, respectively. The results obtained in this study indicate that optimization of culture conditions played a central role in improving yield through the fermentation process. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta et al. 2003). For large scale production of the enzyme with certain nutritional sources, purification and characterization are in progress.

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