

# $\alpha$ -AMYLASE FROM *BACILLUS SIMPLEX*- PRODUCTION, CHARACTERIZATION AND PARTIAL PURIFICATION

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## ABSTRACT

In this study, some bacteria were isolated from the soil obtained from Ergani Makam Mountain. The isolated bacteria were defined as *Bacillus simplex* with the biochemical tests and 16S rRNA analysis. The optimum conditions for bacterial growth were determined as the 32<sup>nd</sup> hour, 37°C and pH 7.0. While the maximum amylase activity was observed of the 72<sup>nd</sup> hour, the optimum temperature and pH of the enzyme were determined to be 37°C and 7.0 reciprocally. It was observed that the enzyme production increased with ammonium sulfate and ammonium nitrate from nitrogen sources and decreased with the addition of carbon sources. Amylase was partially purified with 70% ammonium sulfate precipitations and dialysis. When the effect of detergents was examined on the partially purified enzyme it was determined that SDS considerably inhibited and the other detergents increased the enzyme activity. It was also determined that while CaCl<sub>2</sub> increased the activity whereas FeCl<sub>2</sub>, CuCl<sub>2</sub>, ZnCl<sub>2</sub> and HgCl<sub>2</sub> considerably inhibited the enzyme activity.

## KEYWORDS:

*Bacillus simplex*;  $\alpha$ -amylase; isolation; production; partial purification; characterization

## INTRODUCTION

Microorganisms in particular have been regarded as treasure of useful enzymes [1]. The reason is that microorganism-based enzymes can easily be obtained, do not constitute unwanted waste product, are more stable and economic, can be obtained in high purity and high quantity [2,3]. Among microorganisms, mostly, some *Bacillus* species and subspecies that can be found widely in the nature are used.  $\alpha$ -Amylase,  $\beta$ -amylase, xylanase, alkaline phosphatase,  $\beta$ -glucanase (cellulase), glucose isomerase,  $\beta$ -lactamase, neutral protease and pullulanase can be counted among the primary enzymes synthesized by *Bacillus* [4].

The enzyme industry as we know it today is the result of a rapid development seen primarily over the past four decades thanks to the evolution of

modern biotechnology [5,6]. The first enzyme produced industrially was  $\alpha$ -amylase that was used for medicinal purposes.  $\alpha$ -Amylase (EC 3.2.1.1, 1,4- $\alpha$ -D-glucan-glucanohydrolase) is an extracellular enzyme that hydrolyzes starch and glycogen molecules [7]. It breaks the  $\alpha$ -1,4 bonds in starch molecule into glucose, maltose, maltotriose and  $\alpha$ -limit dextrine. It is primarily used in food industry for preparation of maltose syrup, purification and clarification of various drinks and production of bread and beer. Besides, it has a wide area of usage like paper, detergent and textile industries and pharmaceuticals [8,9,10,11].

$\alpha$ -Amylase, which has economical, industrial importance was produced from *Bacillus simplex* which is isolated from soil. Characterization of the partially purified enzyme has been performed.

## MATERIAL AND METHODS

**Microorganism and Culture Conditions.** *Bacillus simplex*, isolated from the soil was produced in 120 rpm, pH 7.0 37°C and NB environment for 72 hours.

**Morphological and Biochemical Tests.** A test for the morphological and physiological identification of the obtained isolation was conducted. Gram, and spore staining methods and motility tests were used in order to determine the characteristics of the bacterium. Through biochemical tests (starch, gelatin and casein hydrolysis, catalase, urease and lipase activities, etc.) some characteristics of the isolates were determined and comparison was made.

**$\alpha$ -Amylase Activity Assay.** The enzyme activity was measured by DNS according to the method described by Bernfeld using 0.5% starch dissolved in a 0.1 M Tris-HCl buffer pH 7.0, for 30 min at 37°C. One unit of amylase activity was defined as the amount of enzyme that released 1  $\mu$ mol of reducing end groups per minute at 37°C [12].

**Determination of Protein Content.** The protein content was determined by the method of Lowry [13].

**Effect of Temperature, pH and Incubation**

**Time on Microorganism and Amylase Production.** Temperature values increasing from 15°C to 55°C with 5°C intervals were tried to investigate the effect of temperature on the production of bacteria and enzyme. Samples obtained were measured in spectrophotometer. Bacteria and enzyme production was made in various pH ranges from pH 2.0 to pH 11.0 for pH effect. For the effect of incubation time on microorganism development and enzyme production; bacteria were cultivated in NB (Nutrient broth) and measured in spectrophotometer between 4<sup>th</sup> and 96<sup>th</sup> hours at 4 hours intervals.

**Effect of pH and Temperature on Enzyme Activity.** Incubated culture of *Bacillus simplex* in NB was centrifuged for the pH effect. To define the optimum pH of  $\alpha$ -amylase in the obtained supernatant; enzyme activity was measured with the usage of substrate prepared in 0.1 M citric acid (pH 4.0-6.0), 0.1 M Tris-HCl buffer (pH 7.0-9.0) and 0.1 M carbonate/bicarbonate (pH 10.0-11.0) buffers. Besides, the effect of temperature on amylase activity was investigated with enzyme activity measurement in various temperatures from 25°C to 55°C at 5°C intervals.

**Effect of Carbon and Nitrogen Sources on Enzyme and Bacteria Production.** Glucose, galactose, fructose, lactose, soluble starch, maltose and sucrose from the carbon sources at a concentration of 1% were added to 25 ml NB in 100 ml erlenmeyers. The effect of carbon sources on enzyme and bacteria production was determined by incubation in optimal conditions with spectrophotometric measurements.

After adding peptone, tryptone, urea, ammonium sulfate, ammonium chloride, ammonium nitrate, sodium nitrate, beef extract, yeast extract, it was incubated in optimal conditions and measured in spectrophotometer.

**Partial Purification of  $\alpha$ -Amylase.** Bacteria cultivation was made in NB and incubated in 37°C temperature for 72 hours. After the incubation, it was centrifuged in 10 000 rpm for 15 minutes. Two following processes were applied in the partial purification of the enzyme. The processes were precipitated with ammonium sulfate and dialysis.

**Ammonium Sulfate Precipitation.** Ammonium sulfate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] of 40% and 70% respectively added to the supernatant in a sterile beaker. Afterwards, the mixture was centrifuged in a refrigerative centrifuge in 10 000 rpm for 15 minutes.

**Dialysis.** Pellet was dissolved in 0.1 M pH 7.0 Tris-HCl buffer, transferred to the dialysis tube and dialyzed against the same buffer in +4°C for overnight.

**Determination of Thermal Stability and pH**

**Stability.** For the determination of pH stability of  $\alpha$ -amylase enzyme, 0.1 M citric, 0.1 M Tris-HCl, 0.1 M carbonate / bicarbonate (10.0 and 11.0) were prepared. The enzyme was pre-incubated in different buffers for 180 minutes. After the pre-incubation, substrate was added and the enzyme activity was measured under the experimental conditions.

To determine the temperature stability (thermal stability) of partially purified  $\alpha$ -amylase enzyme, enzyme alone was pre-incubated in 30°C, 40°C and 50°C temperature values for 15-120 minutes. After pre-incubation, enzyme activity specification was made.

**Effect of Some Metals on Enzyme Activity.** In order to determine the effect of some metals on enzyme activity, 1.5 mM concentration in overall volume from the 50 mM-stock solutions of CaCl<sub>2</sub>, CuCl<sub>2</sub>, ZnCl<sub>2</sub>, MgCl<sub>2</sub>, HgCl<sub>2</sub>, MnCl<sub>2</sub> ve FeCl<sub>2</sub> were prepared in 0.1 M pH 7.0 Tris-HCl buffer. After 30 minutes of pre-incubation, substrate was added and  $\alpha$ -amylase activity was tested.

**Effect of Some Detergents on Enzyme Activity.** To investigate the effect of some detergents on partially purified enzyme activity, in the ratio of %0.5 SDS, Tween-40, Tween-80 and TritonX-100 were used. These detergents were prepared in 0.1 M pH 7.0 Tris-HCl buffer and pre-incubated for 30 minutes. Then, substrate was added and left to  $\alpha$ -amylase activity.

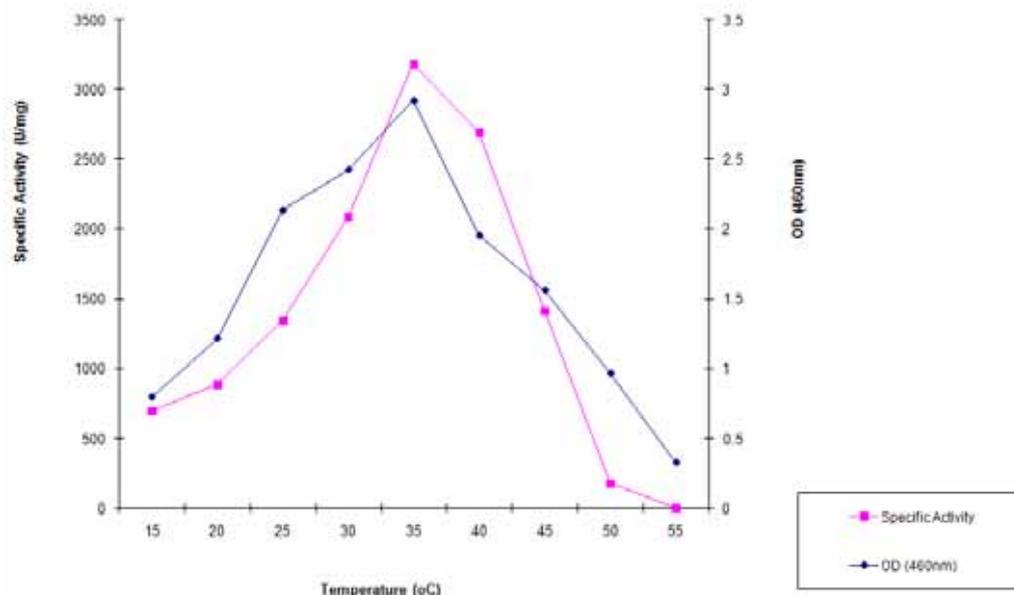
**TABLE 1**  
**Morphological, Physiological and Biochemical Tests**

Characteristics	<i>Bacillus simplex</i>
Gram painting	+
Cell form	rod shape
Temperature range (°C)	20-45
Optimum temperature (°C)	35
pH range	5.0-10.0
Optimum pH	7.0
Hemolysis	+
Motility	-
Hydrolysis of:	
Starch	++
Activity of:	
Urease	+
Catalase	+

+, positive result or growth; -, negative result or no growth.

## RESULTS AND DISCUSSION

In this study, *Bacillus simplex* was isolated from the soil obtained from Ergani Makam Mountain and  $\alpha$ -amylase production, partial purification and characterization were performed.



**FIGURE 1**  
Effect of temperature on bacterial growth and enzyme activity

**Morphological and Biochemical Tests.** It was determined that the obtained isolate is gram-positive and has rod-shaped cells with the ability to form spores (Table 1).

The microorganism was identified by biochemical tests and 16S rRNA sequence. The 16S rRNA analyses of the obtained isolates were conducted by Ref-Gen (METU Technocity/Ankara). The 16S rRNA sequence of this strain is:

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CCCACTGGCGGCGTGCTATACTGCAGT
CGAGCGAATCGATGGGAGCTTGCTCCCTGA
GATTATTCGGCGGACGGGTGAGTAACACGT
GGGCAACCTGCCTATAAGACTGGGATAACT
TCGGGAAACCGGAGCTAATACCGGATACGT
TCTTTTCTCGCATGAGAGAAGATGGAAAGA
CGGTTTACGCTGTCACTTATAGATGGGCC
GCGGCGCATTAGCTAGTTGGTGAGGTAATG
GTCACCAAGGCGACGATGCGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAACGAAGA
AGGCCTTCGGGTCGTAAAGTTCTTTGTTAGG
GAAGAACAAGTACCAGAGTAACTGCTGGTA
CCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGTGGTTCCTTAA
GTCTGATGTGAAAGCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGGAACCTTGAG
TGCAGAAGAGGAAAGTGGAAATCCAAGTGT
AGCGGTGAAATGCGTAGAGATTTGGAGGAA
CACCAGTGGCGAAGGCGACTTTCTGGTCTG
TAACTGACACTGAGGCGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAG

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AGGGTTTCCGCCCTTTAGTGCTGCAGCTAAC
GCATTAAGCACTCCGCTGGGGAGTACGGC
CGCAAGGCTGAAACTCAAAGGAATTGACGG
GGGCCCCGACAAGCGGTTGGAGCATGGTGG
ATTTAATTTCGAAGCAACGCGAAGAACCTTA
CAGGTCTTGACATCCTCTGACAACCCTAGA
GATAGGCTTCCCTTCGGGGACAGAGTGAC
AGGTGGTGCATGTTGTCGTCAGCTCTGTTCT
GGAATGTTGGGTTAAGTCCCCACGAGCCAC
CTTGATCTTAGTTGCAACATTCAGTTGGCAC
TCTAAGGTGACTGGCTGTGACAACGGAGAA
AGGTGGATAGACGTCATCTTCATGCCTTT
GACTGGCTAACCGGTCTAATGATGTTAAA
GGTTGCAACCTGCAGTAGGATCCTTAAGCA
C

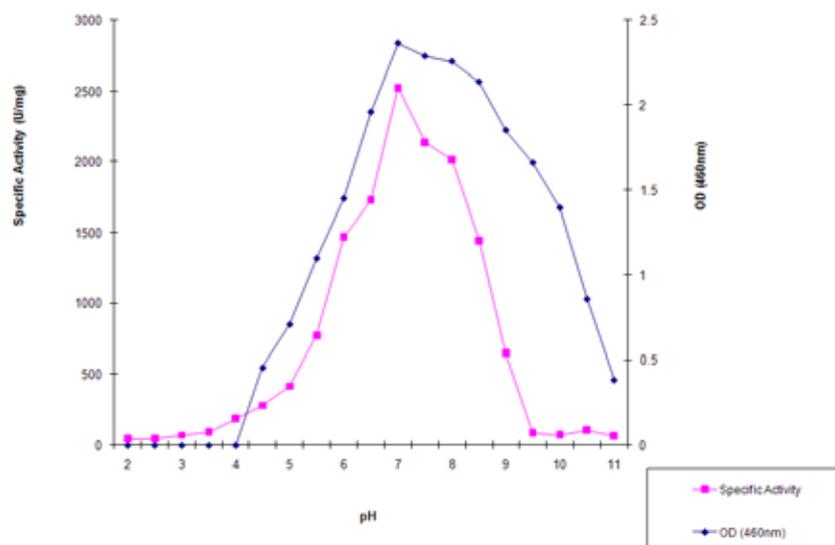
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**Effect of Temperature, pH and Incubation Time on Microorganism and Amylase Production.** To investigate the effect of temperature on bacteria reproduction and enzyme production, it was incubated between 15°C and 55°C. It was determined that maximum bacteria reproduction and enzyme production were observed at 37°C (Fig.1).

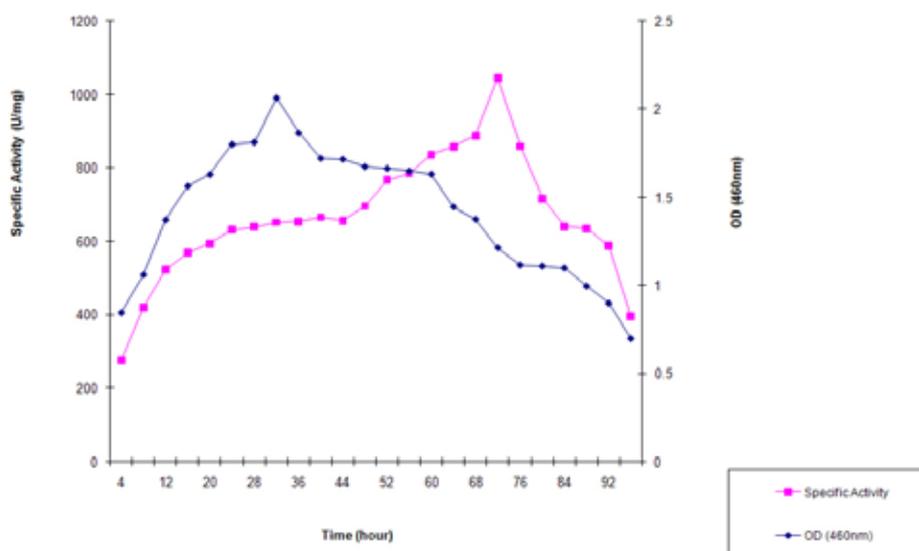
Temperature is related with microorganism growth and therefore,  $\alpha$ -amylase production. Wide temperature range (35-80°C) was given for the optimum bacterial growth and  $\alpha$ -amylase production by many researchers.

In the bacteria culture produced in various pH at 37°C, the best bacteria reproduction and amylase production was determined at pH 7.0 (Fig. 2).

pH of the growth environment among the physical parameters has an important role for the morphological changes of microorganism, therefore the increase of enzyme syntheses.



**FIGURE 2**  
Effect of pH on bacterial growth and enzyme activity



**FIGURE 3**  
Effect of time on bacterial growth and enzyme production

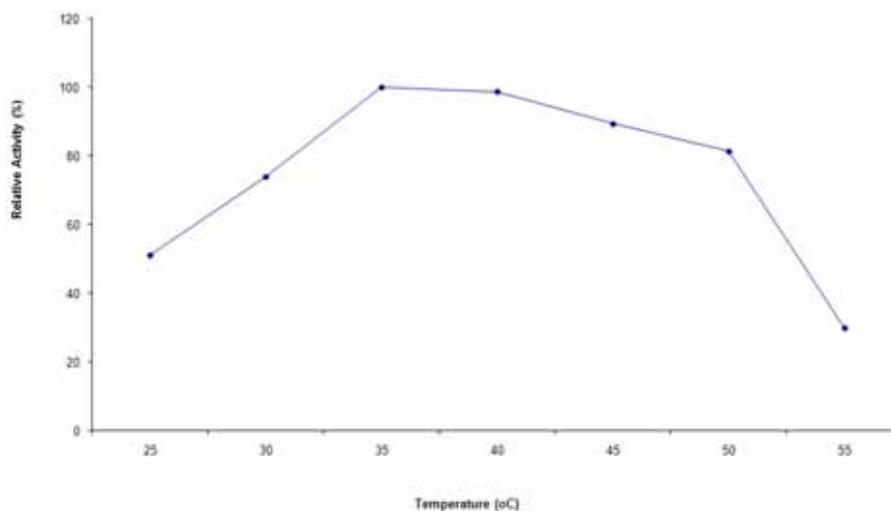
For the effect of incubation time on microorganism development and enzyme production, a sample was taken at 4 hour intervals from the bacteria culture that was produced in 37°C NB, between 4<sup>th</sup> and 96<sup>th</sup> hours. As seen in the Fig. 3, the optimum incubation time for *Bacillus simplex* was determined to be 72 hours.

Hamilton et al. [14] determined the optimum incubation time at 41 hours, and temperature to be 40°C for *Bacillus sp.* IMD 43 production. Asgher et al.[15] determined incubation time to be 48 hours, temperature to be 50°C and pH to be 7.0 of thermophilic *Bacillus subtilis* JS-2004.

**Effect of Temperature and pH on Enzyme Activity.** To investigate the effect of temperature on  $\alpha$ -amylase enzyme secreted by *Bacillus simplex*; bacteria were incubated in NB (pH 7.0) at 37°C that

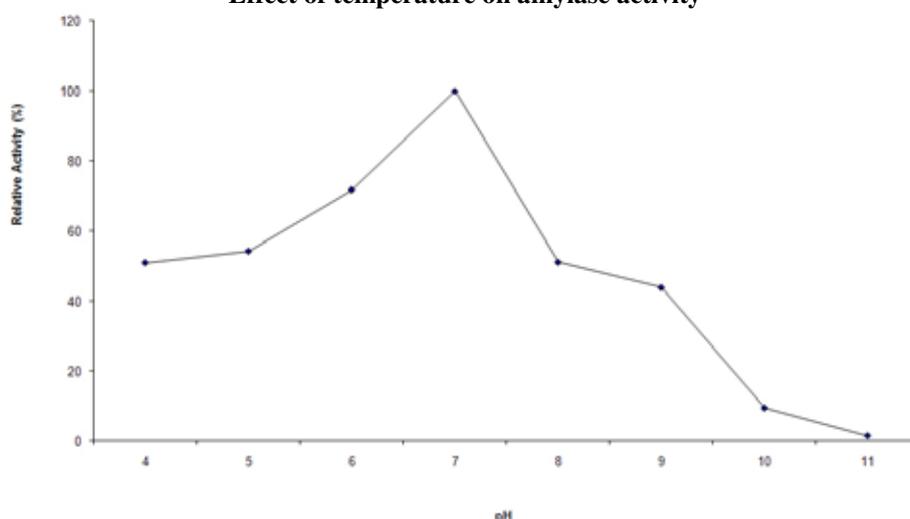
was the optimum incubation temperature for 72 hours that was the optimum incubation time.  $\alpha$ -Amylase activity was measured with temperature increase in the obtained supernatant from 25 °C to 55°C, according to Bernfeld method [12]. The highest  $\alpha$ -amylase activity of *Bacillus simplex* was determined between 35°C and 40°C, the optimum temperature of the enzyme was determined at 37°C (Fig. 4).

It was determined that the enzyme obtained from the bacteria produced in optimum conditions were active between pH 5.0 and pH 11.0. Increase of the enzyme activity was observed starting from pH 5.0 whereas decrease of the enzyme activity was determined up to 7.0. As seen in the Figure 5, the optimum pH of  $\alpha$ -amylase enzyme was determined to be 7.0.



**FIGURE 4**

**Effect of temperature on amylase activity**



**FIGURE 5**

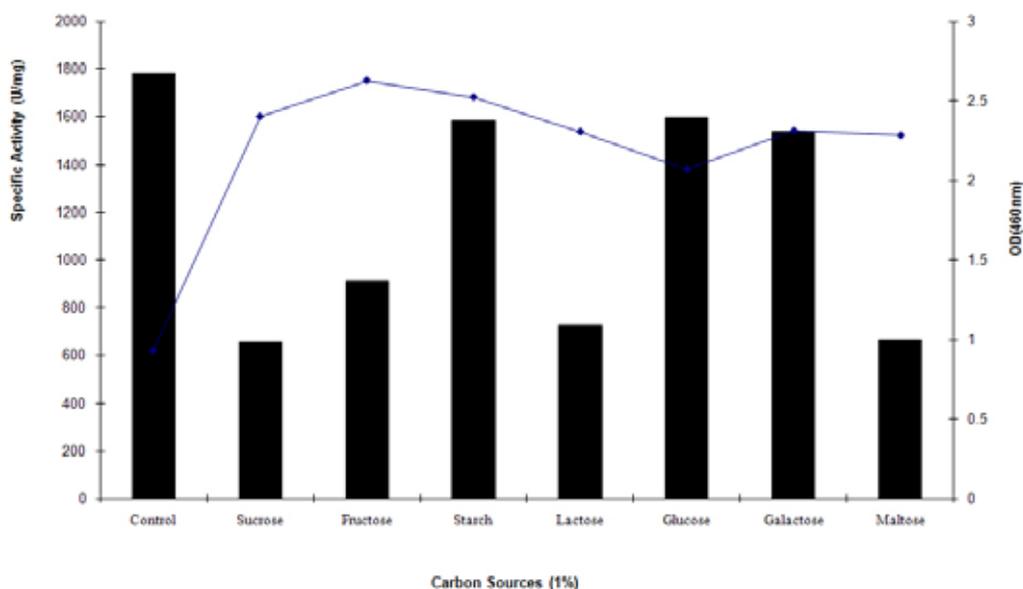
**Effect of pH on amylase activity**

Similar results were also implied by some other researchers. Noman et al. [16] studied on the purification of  $\alpha$ -amylase enzyme from *Pachyrhizus erosus* L. They determined the optimum pH of the enzyme at 7.3 and temperature to be 37°C. Sudharhsan et al. [17] investigated the psychical and nutritional factors that affect the amylase production in the *Bacillus* species isolated from rotten food garbage, analyzed the effects of temperature and pH factors psychically and stated that the maximum enzyme activity was observed at pH 7.0 and 37°C.

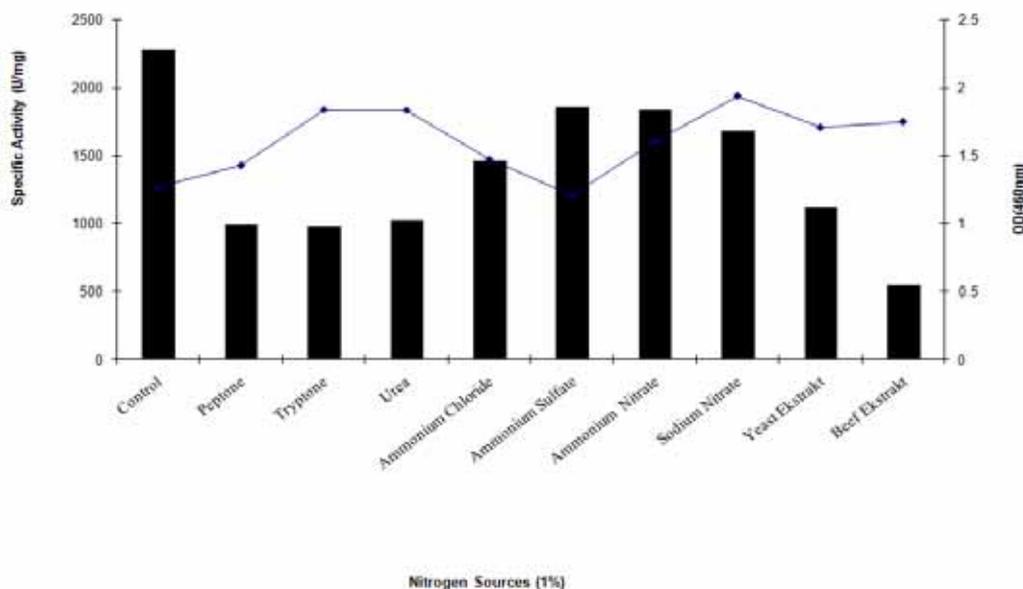
The optimum pH of extracellular  $\alpha$ -amylases varies between 3.0 and 10. In many studies, the optimum pH of  $\alpha$ -amylases obtained from bacteria and fungi are stated as acidic and neutral [2]. Due to its usage in the areas such as liquefaction of starch, food industry and dry cleaning, the neutral pH is essential in such processes. The fact that  $\alpha$ -amylase of *Bacillus simplex* exhibit maximum activity in neutral conditions that means it is usable in industrial processes.

**Effect of Carbon and Nitrogen Sources on Enzyme and Bacteria Production.** In the conducted research, when control was compared with other carbon sources, it was determined that glucose and galactose showed close activity to control while the amylase activity importantly reduced in other carbon sources. For bacteria reproduction, when control was compared to other carbon sources, it was determined that reproduction in all carbon sources was higher than control (Fig. 6).

As seen in the Fig. 7, when control was compared with other nitrogen sources, lower amylase activity was obtained in all nitrogen sources than control. The highest specific activity among nitrogen sources was obtained in the culture environment with ammonium nitrate and ammonium sulfate. In bacterial reproduction, an increase was observed in all nitrogen sources with regards to control.



**FIGURE 6**  
Effect of carbon sources on bacterial growth and amylase production



**FIGURE 7**  
Effect of nitrogen sources on bacterial growth and amylase production

Behal et al. [18] produced  $\alpha$ -amylase from *Bacillus* sp. AB 04 in nutrient media that has 1% fructose of carbon sources and %1 beef extract of nitrogen sources. Saxena et al. [19] acquired maximum specific activity in nutrient media that has (%) 0.6 starch, and 0.5 peptone 0.3 yeast extract for high thermostable amylase produced from *Bacillus* sp. PN5. Carvalho et al. [20] implemented the maximum production from thermophilic *Bacillus* sp. SMIA-2 in nutrient media with peptone as the nitrogen source and soluble starch as the carbon source. Prakash et al. [21] determined the best  $\alpha$ -amylase production from halophilic *Chromohalobacter* sp. TVSP 101 bacteria in the media with tripton as the nitrogen source.

**Partial Purification of Amylase.** After the determination of optimum conditions of *Bacillus simplex*  $\alpha$ -amylase, enzyme was partially purified. Bacteria culture produced in optimum conditions was centrifuged in a frigorific centrifuge in 10 000 rpm for 10 minutes. Ammonium sulfate  $[(NH_4)_2SO_4]$  in 70% saturation level was added with stirring to the supernatant in a sterile beaker. Subsequently the mixture was centrifuged in 10 000 rpm for 15 minutes. Obtained pellet was dissolved in 0.1 M pH 7.0 Tris-HCl buffer, transferred to the dialysis tube and was dialyzed over night.

**Effect of Metals.** When the effect of some metals on partially purified enzyme activity was analyzed, it was determined that  $CaCl_2$  (113%)

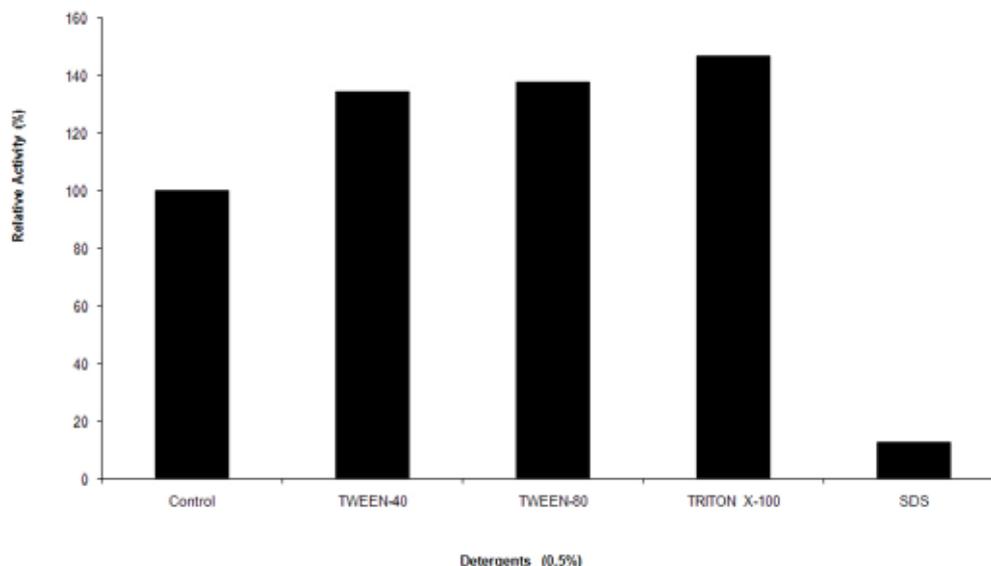


FIGURE 8

### Effect of detergents on amylase activity

increased the activity; no change was observed after adding  $MnCl_2$  (100%) and  $MgCl_2$  (92%);  $FeCl_2$  (61%) partially inhibited and  $CuCl_2$  (22%),  $ZnCl_2$  (24%),  $HgCl_2$  (16%) strongly inhibited the enzyme activity (Table 2).

TABLE 2

#### Effect of cations on $\alpha$ -amylase activity

Effectors (1,5 mM)	Relative enzyme activity (%)
Control	100
$Mn^{+2}$	100
$Fe^{+2}$	61
$Zn^{+2}$	24
$Mg^{+2}$	92
$Hg^{+2}$	16
$Cu^{+2}$	22
$Ca^{+2}$	113

Shafiei et al. [22] biochemically characterized the amylase enzyme excreted from *Nesterenkonia* sp. F strain. They determined that  $Zn^{2+}$ ,  $Fe^{3+}$ ,  $Cu^{2+}$  and  $Al^{3+}$  ions inhibited the amylase activity while  $Ca^{2+}$  increased the it. Noman et al. [16] determined that  $Zn^{2+}$ ,  $Fe^{2+}$  and  $Cu^{2+}$  ions make inhibition effect in high rate while  $Li^{+}$ ,  $Hg^{+}$  and  $Cd^{2+}$ ,  $Ag^{+}$ ,  $Mg^{2+}$  and  $K^{+}$  ions have low effects on activity. Srivastava [23] determined that  $Cu^{2+}$ ,  $Fe^{3+}$ ,  $Ni^{2+}$ ,  $Hg^{2+}$ ,  $Pb^{2+}$  and  $Ag^{1+}$  ions completely inhibited the activity of purified amylase, but  $Ca^{2+}$ ,  $Ba^{2+}$ ,  $Sr^{2+}$  and  $K^{+}$  ions increased it. The results obtained from conducted studies support the findings we obtained from our study.

**Effect of Detergents.** To investigate the effect of some detergents on partially purified  $\alpha$ -amylase enzyme activity, SDS in the ratio of 0.5%, Tween-40, Tween-80 and TritonX-100 were used. Rest of the enzymes were compared with the control and their relative activities were calculated. According to

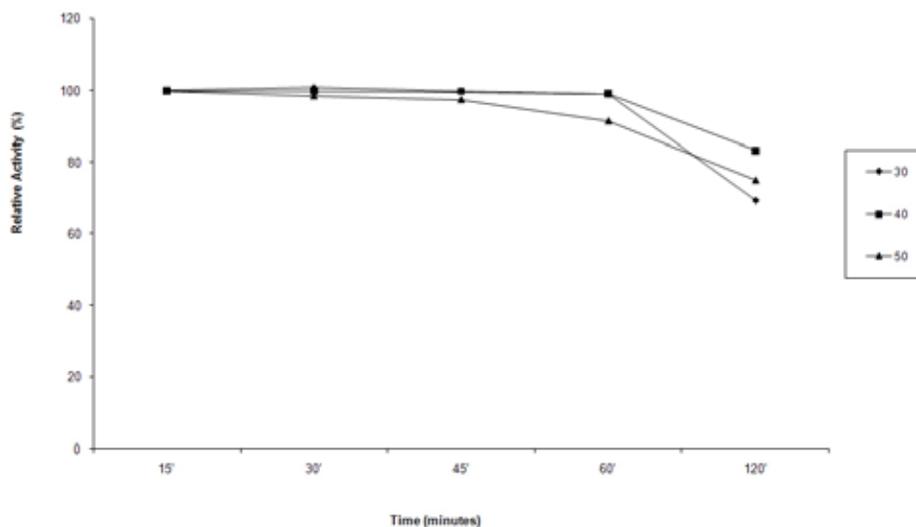
the control, the amount of rest of the enzymes were determined to be; SDS 12%, Tween-40 134%, Tween-80 138% and TritonX-100 140% (Fig. 8). While the inhibition was observed only with the SDS effect, the inhibition having not been observed with other detergents shows the the resistance to detergents.

Asoodeh et al. [24] determined that  $\alpha$ -amylase activity they purified from thermophilic *Bacillus sp. ferdowsicus* increased with Triton X-100. Shafiei et al. [22] determined that amylase was considerably stable to SDS, Triton X-100, Tween 80 and Tween 20 detergents.

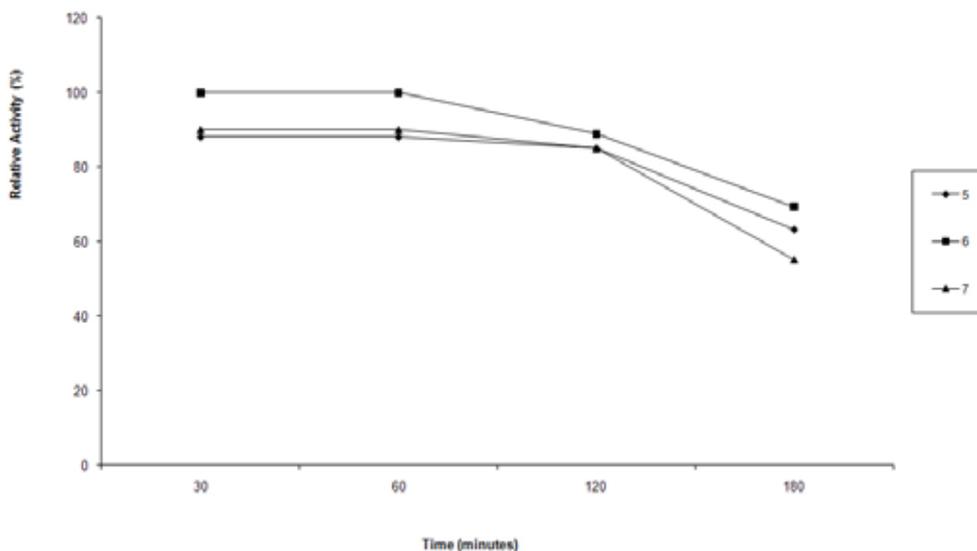
In the literature, because disulfide bonds are found in the enzyme and becoming unstable to oxidation due to the amino acids undergoing a change by oxidation; it is stated that the thermostability of the enzyme increases and thus the enzyme is not affected by the oxidizing agents.

This result obtained suggests that the enzyme is not affected by oxidant agents, the amino acids change with heat and cause resistance.

**pH and Temperature Stability.** 25  $\mu$ l enzyme and various buffers prepared in 50  $\mu$ l were left and pre-incubation was done for 60 minutes.  $\alpha$ -Amylase activity specification was made with substrate adding after pre-incubation. It was observed that pH stability of enzyme activity increased after pH 5.0 and started to decrease after pH 7.0. As seen in the Fig. 9, pH stability was found to be 7.0. To determine the temperature stability of partially purified enzyme, enzyme was pre-incubated in 30°C, 40°C and 50°C temperature values for 15, 30, 45, 60 and 120 minutes. After pre-incubation, enzyme activity specification was made. Partially purified enzyme was determined to be stable in 30°C, 40°C and 50°C after 2 hours (Fig.10).



**FIGURE 9**  
Effects of pH on amylase stability



**FIGURE 10**  
Effects of temperature on amylase stability

Bernhardsdotter et al. [25] detected that when the isolated amylase was pre-incubated in 37°C, between pH 7.0 and 11.0 for 1 hour, it lost the 20% percent of its original activity.

Wang et al. [26] detected that the purified amylase's pH stability was between 6.0 and 11.0 and its temperature stability was under 60°C

## CONCLUSION

Studies about industrial enzymes are becoming more important since the enzyme technology is developing. Therefore, in our study, amylase which has a great industrial importance was produced easily and economic processes and was partially purified in optimum conditions.

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