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# Purification and characterization of thermostable α-amylase from thermophilic *Anoxybacillus flavithermus*

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ABSTRACT

This study reports on the purification and characterization of thermostable α-amylase (α-1-4 D-glucan glucanohydrolase EC 3.2.1.1) from a newly isolated *Anoxybacillus flavithermus*. *A. flavithermus* was used, which was isolated from hot water springs of Ömer, Afyonkarahisar, Turkey. The gram-positive, spore-forming, motile, moderately thermophilic bacteria were found to be a strain of *A. flavithermus* analysed by 16S rRNA comparison. The optimal conditions for bacterial growth were determined to be at 20th h, 55 °C and pH 6.0. Maximum α-amylase activity was obtained at 55 °C at pH 7.0 after 24 h of incubation. Thermostable α-amylase from *A. flavithermus* was purified by 70% $(NH_4)_2SO_4$ and ion-exchange chromatography (5.2-fold; 65.8% yield). The molecular weight of α-amylase was 60 kDa, as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The α-amylase hydrolyzed soluble starch at 55 °C with $K_m$: 0.005 mM and $V_{max}$: 3.5 μmol $min^{-1}$.



## 1. Introduction

For several decades thermophilic bacteria have attracted the interest of many scientists not only out of scientific curiosity but also because of the biotechnological potential of these bacteria. There is also the potential for thermostable enzymes produced by these organisms to affect the quality of products. Research interest in these organisms has increased due to their biotechnological potential, especially as sources of thermostable enzymes (Lasa & Berenguer, 1993; Haki & Rakshit, 2003). Thermophilic microorganisms have been isolated from different habitats such as permanently cold habitats, deep ocean-basin cores, shallow marine environments, hot springs, petroleum reservoirs, deep-sea hydrothermal vents and the leachate of a waste pile from a canning factory (Szewzyk, Szewzyk, & Stenstrom, 1994; Adiguzel et al., 2009).

Enzymes involved in starch bioconversions are of major industrial interest and considerable attention has been focused on obtaining new enzymes with improved properties or new applications. Thermostable starch-hydrolyzing enzymes such as amylase, pullulanase and glucoamylase play an important role in the food, chemical and pharmaceutical industries (Mollania, Khajeh, Hosseinkhani, & Dabirmanesh, 2010).

α-Amylase (EC 3.2.1.1,1,4-α-D-glucan-glucanohydrolase) is one of the most important industrial enzymes that can be used in a number of industrial processes including brewing, baking, textiles and detergent and bioethanol production (Lama, Nicolaus, Di Donato, Poli, Toksoy, Oner, 2009). *Anoxybacillus* means "*Bacillus* without oxygen" and according to the authors, most of the species described grow well aerobically and for some species even anaerobic growth has been registered but only under certain conditions (Pikuta et al., 2000).

α-Amylases isolated from thermophilic bacteria are thermostable and active at high temperature. Bacillus species such as *Geobacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Anoxybacillus*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus globisporus* and *Bacillus alvei* are known for the production of starch-hydrolyzing enzymes (Agüloğlu, Ensari, Uyar, & Otludil, 2000).

In the present study, we report the optimum conditions for the production, purification and characterization of extracellular α-amylase from the newly isolated strain *Anoxybacillus flavithermus*.

## 2. Materials and methods

### 2.1. Material

*A. flavithermus* was used, which was isolated from hot water springs of Ömer, Afyonkarahisar, Turkey by Dr. Sadin Özdemir. The organism was identified by biochemical tests and 16S rRNA sequence. The 16S rRNA sequence 16S rRNA analyses of the

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obtained isolates were conducted by Ref-Gen (METU Technocity/Ankara).

### 2.2. Culture condition

The growth medium for liquid culture consisted of (g/l): beef extract 10; peptone 10; NaCl, 5 (with tap water) and the pH was adjusted to 7.0 before autoclaving. Amylase production was carried out by inoculating 25 ml of the medium in a 100 ml flask with 1 ml of a 1-day-old culture. Cultures were incubated at 55 °C on a shaker at 160 rpm. Flasks were removed at regular intervals and the contents were centrifuged at 10 000 rpm for 10 min at 4 °C to remove the bacteria. The supernatant was used for amylase activity. Bacterial growth was monitored by measuring absorbance at 470 nm in a spectrophotometer (UV-6450; Jenway, UK).

### 2.3. Morphological, biochemical and physiological 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.

### 2.4. Effect of time, temperature and pH on bacterial growth and amylase production

The effect of time, temperature and pH on enzyme production was investigated by cultivating the organism at different times, different temperatures, and different pH values using appropriate buffers at concentrations of 0.1 M (3.0–6.0, sodium citrate; 7.0–8.0, Tris–HCl; 9.0–11.0, glycine–NaOH). The amylolytic activity was measured after 24 h of incubation.

### 2.5. Effect of carbon and nitrogen sources on bacterial growth and amylase production

The effect of different carbon (0.5%, 1%) and nitrogen (1%) sources was studied. The various carbon sources (glucose, lactose, maltose galactose, fructose, sucrose and starch) and the nitrogen sources (peptone, tryptone, beef extract, yeast extract, ammonium chloride, ammonium sulfate, casein and urea) were tested.

### 2.6. α-Amylase activity assay

The α-amylase activity was measured by DNS according to the method described by Bernfeld (1955) using 0.5% starch dissolved in a 0.1 M Tris–HCl buffer pH 7.0 at 55 °C. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute at 55 °C. D-Maltose was used as the standard of reducing end sugar.

### 2.7. Enzyme purification

The crude culture was precipitated with ammonium sulphate (at 70% saturation) by slow continuous stirring at 4 °C. The precipitate protein was collected by centrifugation (7000 × *g* for 15 min) at 4 °C, dissolving the pellet in a minimum volume of 0.1 M potassium phosphate buffer (pH 8.0), and then it was dialyzed against the same buffer. The dialyzed crude enzyme was concentrated in a lyophilizer and applied on a DEAE-cellulose (diethylaminoethyl cellulose, DE 32) column (1.5 cm × 20 cm glass column, flow rate 15 mL $h^{-1}$) equilibrated with 0.01 M phosphate buffer (pH 8.0). The protein was then eluted with a linear gradient of NaCl (0.1–1 M) in the same buffer. The active fractions were pooled, dialyzed and lyophilized. This purified enzyme was used for further biochemical characterization. The active fractions were collected and dialyzed overnight in distilled water.

### 2.8. Protein determination

The protein concentration was determined by the method of Lowry, Roserbrough, Farr, and Randall (1951) using bovine serum albumin as the standard in the final step and during the purification procedure.

### 2.9. Electrophoresis

The molecular weight of the purified α-amylase was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The purified enzyme (1 μg) was loaded onto 1 mm thick 10% polyacrylamide gel together with molecular size markers. The SDS-PAGE molecular weight markers (Sigma) were β-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). After completion of electrophoresis, the protein gel was stained with Coomassie Brilliant Blue R-250.

Discontinuous native-PAGE was performed in 10% acrylamide gel with Bio-Rad mini gel electrophoresis. All the buffer used for native-PAGE was prepared without SDS. After electrophoresis, the gel was incubated at 55 °C in soluble starch solution (3%) prepared in 0.1 M sodium acetate buffer (pH 6.0). Subsequently the gel was stained with iodine reagent (KI-$I_2$ solution). The α-amylase bands were visualized as transparent bands on a dark blue background.

### 2.10. Determination of kinetic parameter

The $K_m$ and $V_{max}$ were determined to use soluble starch as the substrate. The enzyme was assayed at varying substrate concentrations from 0.5% to 2%. The $K_m$ and $V_{max}$ were estimated from the reciprocal plot of substrate concentration ($S$) versus velocity ($V$).

### 2.11. Some properties of the purified α-amylase

#### 2.11.1. Thermal stability

Heat stability was measured by incubating the enzyme at 50–70 °C for 15–120 min in 0.1 M Tris–HCl buffer, pH 7.0. After heat treatment, the enzyme solution was cooled and the residual activity was assayed under standard assay conditions.

#### 2.11.2. pH stability

The stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the enzyme at pH 4.0–11 for 1 h at 37 °C.

#### 2.11.3. Effects of detergents on amylase activity

The effects of detergents 0.5% SDS, Tween-40, Tween-80 and TritonX-100 on the enzyme activity were studied. For this purpose, the enzyme was incubated in the presence of the above-mentioned agents for 30 min at optimum temperature and pH conditions and then assayed for the remaining activity.

#### 2.11.4. Effects of inhibitors on amylase activity

The effects of various inhibitors on the enzyme activity were investigated by pre-incubating the enzyme with inhibitors in 0.1 M Tris–HCl buffer solution (pH 7.0) for 30 min at 37 °C followed by measuring the residual amylase activity.

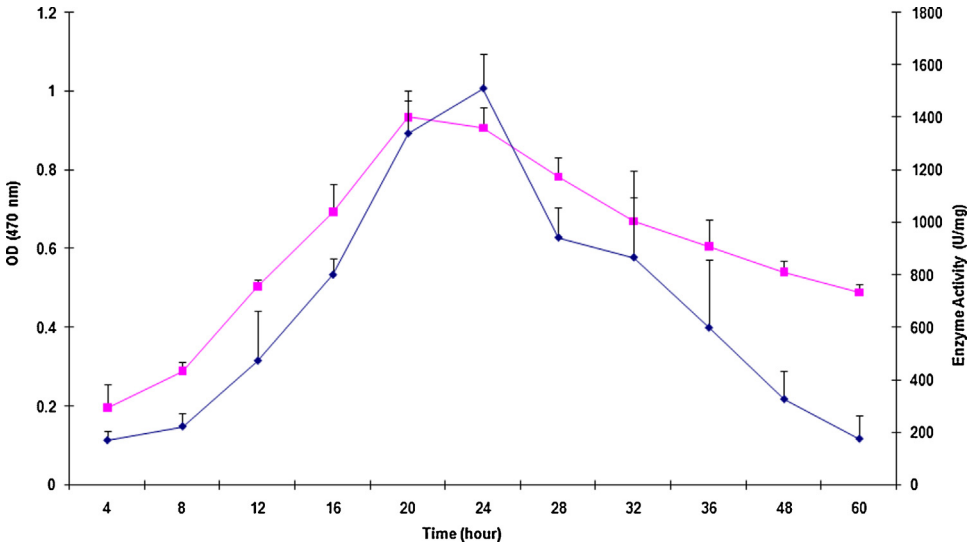


**Fig. 1.** Effect of time on bacterial growth and enzyme production.

*2.11.5. Effects of metal ions on amylase activity*

Enzyme assays were performed in the presence of different metal ions, all at 1.5 mM final concentration. The relative activity of the enzyme was compared in 0.1 M of Tris–HCl buffer. $CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$, $HgCl_2$, $MnCl_2$, $CoCl_2$ and $FeCl_2$ were used.

## 3. Results and discussion

### 3.1. Biochemical and molecular identification

It was determined that the obtained isolate is gram-positive and has rod-shaped cells with the ability to form spores. The isolate from the spring water was confirmed to be a member of the genus *Anoxybacillus*. 16S rDNA gene product with approximately 1418 bp was sequenced for the isolated microorganism. As a result of 16S rRNA analysis, comparison of 16S rDNA sequence of this strain with other related bacteria shows that the 16S rDNA sequence of this strain has high similarity with *A. flavithermus*. The phylogenetic position of the rDNA sequences was determined by the construction of a phylogenetic tree. The 16S rRNA sequence of this strain (1418 nucleotide sequences) is given below:

TCTTTGCCCCCTTCGGCGGCTGGCTCCCGTAAGGGTTACCTCACCG-

ACTTCGGGTGTTGCAAACTCTCGTGGTGTGACGGGCGGTGTGTACAA-
GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG-
CGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGA-
GAGCGGCTTTTTGGGATTGGCTCCCCCTCGCGGGTTCGCAACCCTTTG-

TACCGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGA-
TGATTTGACGTCATCCCCACCTTCCTCCGACTTTTAGCCGGCAGTCAC-
CTTAGAGTGCCCGACTTGACTCGCTGGCAACTAAGGTCGAGGGTTGC-
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGAC-
AACCATGCACCACCTGTCACCCTGTCCCCCCGAAGGGGGAACGCCCG-
ATCTCTCGGGTTGTCAGGGGATGTCAAGACCTGGTAAGGTTCTTCGC-
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGT-

CAATTCCTTTGAGTTTCACTCTTGCGAGCGTACTCCCCAGGCGGAGTG-

CTTAATGCGTTAGCTACAGCACTAAAGGGTGGATACCCTCTAACACT-
TAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT-
TGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGC-
CGCCTTCGCCACTGGTGTTCCT. . ..

### 3.2. Effect of time, temperature and pH on bacterial growth and enzyme production

It is known that most of the *Bacillus* strains used commercially for the production of bacterial α-amylases by SmF have an optimum pH between 6.0 and 7.0 for growth and enzyme production. The optimum growth time of *A. flavithermus* was determined to be the 24th h (Fig. 1), the optimal temperature as 55 °C (Fig. 2) and the optimal pH as 6.0. The extracellular α-amylase production of the bacterium was studied using the SmF method. The highest α-amylase production was obtained at the 24th h (1509.3 U/ml). Since it is important to produce enzyme in a short time in the field of biotechnology, a 24-h incubation time is also important in terms of biotechnology. Liu and Xu (2008) obtained maximum amylase production at the 24th h. Similar findings have been reported on *Bacillus amyloliquefaciens* (Hillier, Wase, Emery, & Solomons, 1997), *Bacillus flavothermus* (Kelly, Bolton, & Fogarty, 1997) and *Bacillus* sp. ANT-6 (Burhan et al., 2003).

The optimum enzyme activity was determined as 55 °C and pH as 7.0.According to this study, it can be concluded that *A. flavithermus* is able to produce high levels of thermostable α-amylase of industrial importance in economic culture medium by SmF. The organism showed poor growth in the culture media adjusted to pH 5.0, 6.0, and 10.0. There was a stimulation of enzyme synthesis with an increase in pH from 5.0 to 7.0 and higher enzyme synthesis at pH 7.0 was a result of enhanced bacterial growth. Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. Agüloğlu Fincan (2008) reported an optimum pH of 7.0 and optimum temperature of 65 °C for α-amylase from *Bacillus subtilis*. The optimal pH and temperature for α-amylase activity in the ranges are reported for α-amylase from some species of *Bacillus* (Asoodeh, Chamani, & Lagzian, 2010; Wang, Liang, & Liang, 2011; Özdemir, Güven, Baysal, & Uyar, 2009). It has been reported that most amylases of different species of *Bacillus* have an optimum temperature of around 40–70 °C (Bernhardsdotter, Ng, Garriott, & Pusey, 2005; Cordeiro, Martins, & Luciano, 2002).

### 3.3. Effect of C/N sources on bacterial growth and enzyme production

The effects of carbon and nitrogen sources on the growth and production of enzyme were investigated. It was observed

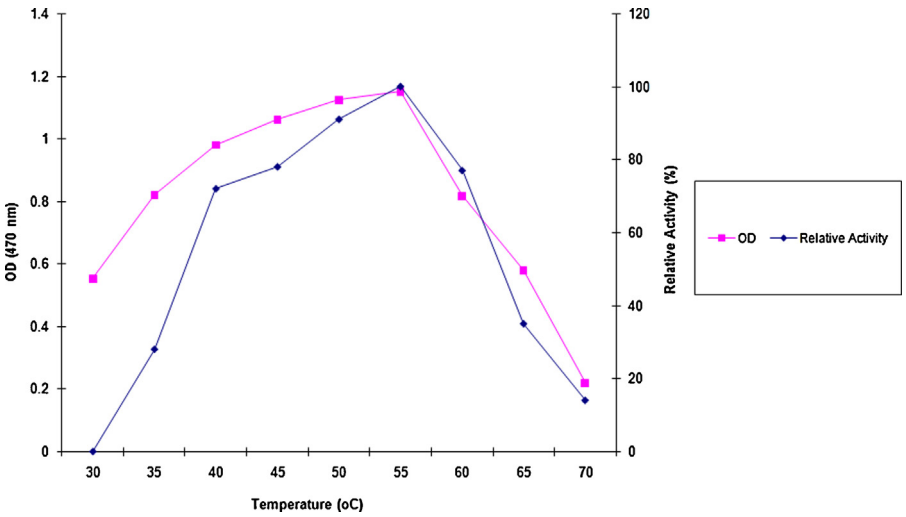


**Fig. 2.** Effect of temperature on bacterial growth and enzyme activity.

**Table 1**
Purification of amylase from *A. flavithermus*.

| Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold |
|---|---|---|---|---|---|
| Crude extract | 734.5 | 0.233 | 3.155 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation/dialysis | 600.4 | 0.144 | 4.157 | 81.7 | 1.2 |
| DEAE-cellulose | 483.6 | 0.030 | 16.263 | 65.8 | 5.2 |

that glucose and sucrose declined α-amylase production, whereas starch did not influence the enzyme production. Among the carbon sources tested, starch was found to support α-amylase synthesis, whereas dextrose, lactose and sucrose showed a repressive effect on α-amylase production (Kıran, Çömlekçioglu, & Arıkan, 2005). It is well known that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subjected to catabolic repression by glucose and sucrose (Qader, Bano, Aman, Syed, & Azhar, 2006). In the study nitrogen sources repressed α-amylase production. Of the other nitrogen sources (ammonium sulphate, ammonium chloride and caseine) these inhibited bacterial growth, whereas nitrogen sources (peptone, tryptone, beef extract, yeast extract and urea) did not influence bacterial growth. It has also been reported that the optimum production of α-amylase for *Bacillus* sp. was found when yeast extract was used (Qader et al., 2006).

### 3.4. Enzyme purification

The enzyme was purified by ammonium sulfate precipitation (70%), DEAE-cellulose ion-exchange chromatography. An overall purification of about 5.2-fold with an activity yield of 65.8% was achieved. The purification of the enzyme is summarized in Table 1. Other folds and yields for different amylases have been reported:

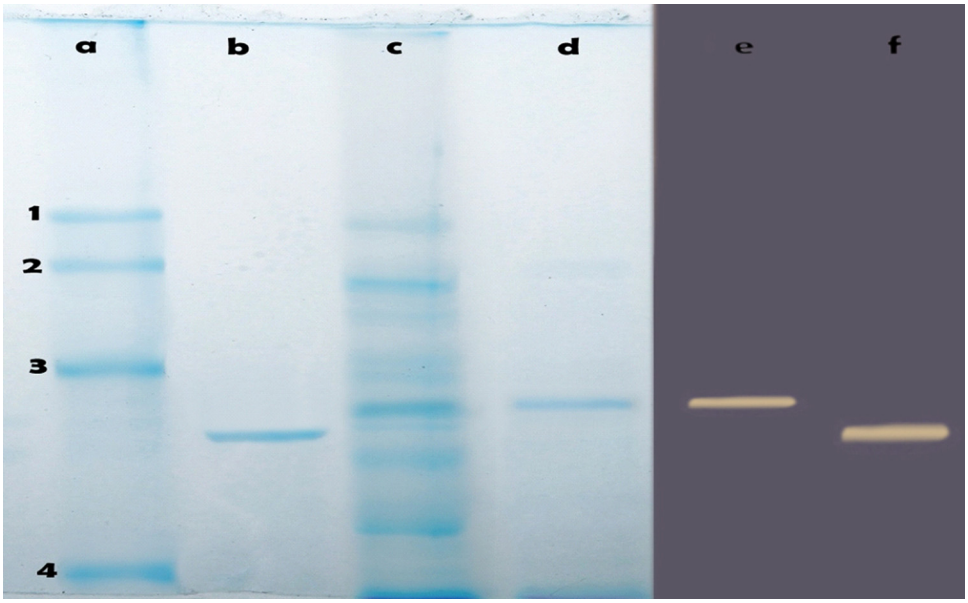


**Fig. 3.** The α-amylase showed a molecular mass. SDS-PAGE Lane a: Standard protein; Lane b: standard α-amylase; Lane c: dialyse; Lane d: purified enzyme from DEAE cellulose; native-PAGE (using iodine stain) Lane e: purified enzyme from DEAE cellulose; Lane f: standard α-amylase.

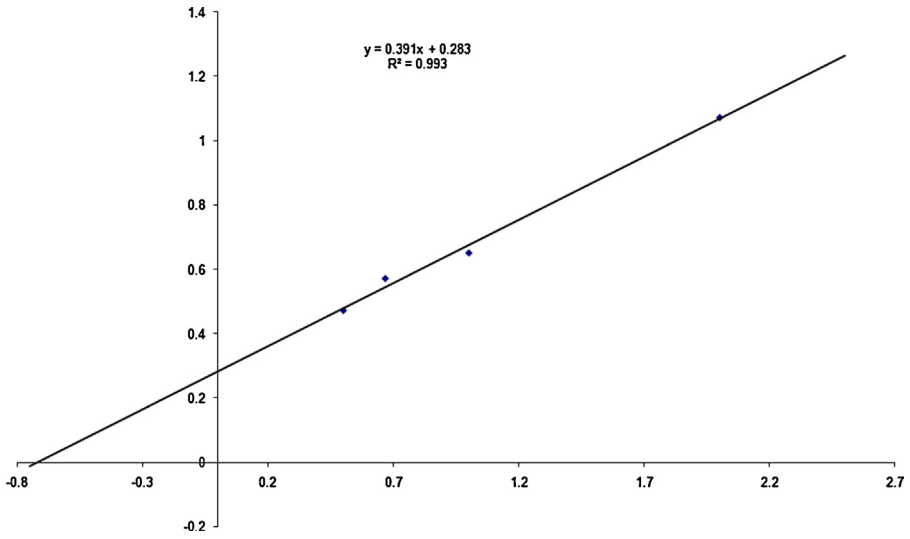


**Fig. 4.** Lineweaver–Burk plot of purified α-amylase from *A. flavithermus*.

The 6.0-fold purification was achieved with a yield of 38% (Božić, Ruiz, Santín, & Vujčić, 2011). The purification steps were combined to give an overall purification of about 5-fold (Özdemir et al., 2009). The overall activity yield of the purified amylase was 11%. The α-amylase secreted by *Geobacillus thermodenitrificans* HRO10 was purified to homogeneity (13.6-fold and 11.5% yield, respectively) through a series of steps (Ezeji & Bahl, 2006).

### 3.5. Molecular mass determination

The molecular mass of amylase was calculated to be 60 kDa by SDS-PAGE (Fig. 3). The molecular weight of α-amylase was 58 kDa, as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ezeji & Bahl, 2006). SDS-PAGE and zymogram activity staining showed a single band equal to molecular mass of 66 kDa (Chakraborty, Khopade, Kokare, Mahadik, & Chopade, 2009). Other molecular masses for different amylases have been reported: 62 kDa for *Bacillus licheniformis* NCIB 6346 (Morgan & Priest, 1981); 53 kDa for *Bacillus* sp. *Ferdowsicous* (Asoodeh et al., 2010); 42 kDa for *Bacillus* sp. TS-23 (Lin, Chyau, & Hsu, 1998); 58 kDa for *Bacillus licheniformis* (Ivanova, Dopreva, & Emanuilova, 1993) and 56 kDa for *Bacillus* sp. YX-1 (Liu & Xu, 2008).

### 3.6. Kinetic studies

Using soluble starch as substrate for α-amylase, the $K_m$ and $V_{max}$ values at 55 °C were estimated to be 0.005 mM and 3.5 μmol $min^{-1}$, respectively. Values for $K_m$ and $V_{max}$ were obtained from Lineweaver–Burk plots (Fig. 4).

### 3.7. Effects of temperature and pH on α-amylase stability

For thermal stability estimations, after pre-incubation of the enzyme for 15–120 min at temperatures of 40, 45, 50 and 55 °C. The remaining activity was measured under enzyme assay conditions. It was determined that the purified enzyme was stable at 40 and 45 °C (Fig. 5). There are reports on thermal stable amylase enzyme from bacteria belonging to genus *Bacillus* (Wang et al., 2011).

The pH stability of the enzyme was determined by incubating at 37 °C for 1 h at different pHs (pH 3–11.0) and the relative activity was measured by the standard assay method. The enzyme

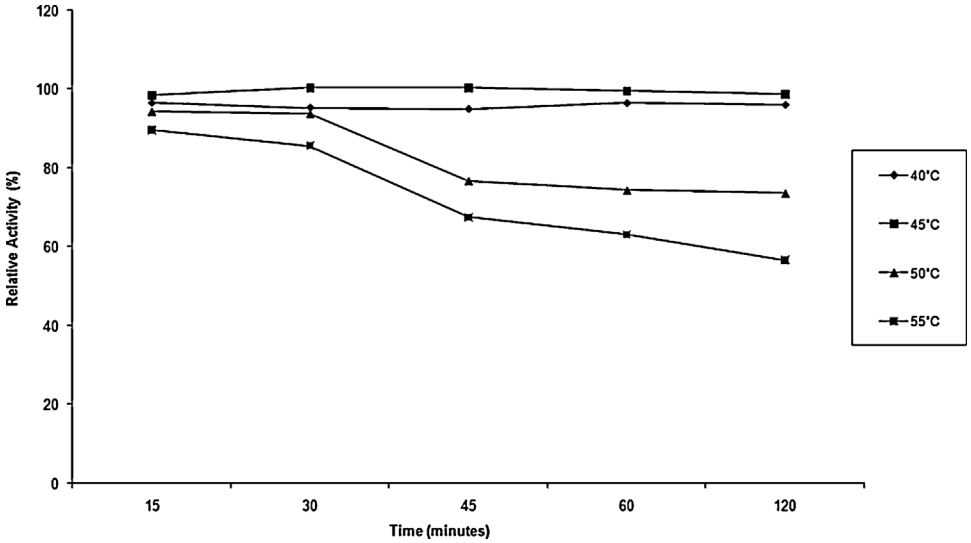


**Fig. 5.** Effect of temperature on α-amylase stability.

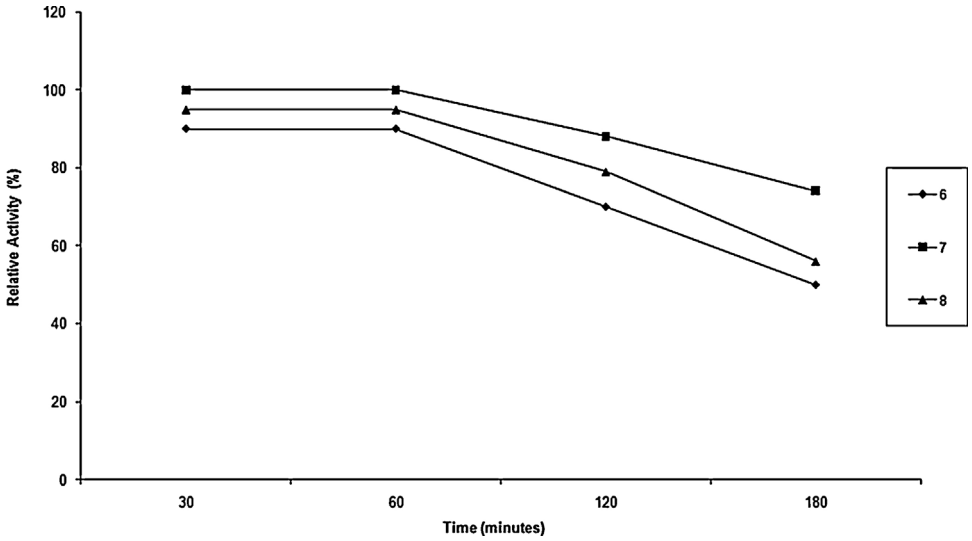


**Fig. 6.** Effect of pH on α-amylase stability.

showed pH 7.0 stability after treatment at 37 °C for 1 h (Fig. 6). Similar behaviour has been reported for bacteria belonging to genus *Bacillus* (Behal, Sharma, Puri, Singh, & Batra, 2006; Asoodeh et al., 2010).

### 3.8. Effects of metal ions and inhibitors on amylase activity

The effect of various cations on enzyme activity was investigated. The enzyme was activated by $Co^{2+}$ (102%). It was strongly inhibited by $Cu^{2+}$(33%), $Zn^{2+}$ (67%) and completely inhibited by $Hg^{2+}$ but less affected by $Ca^{2+}$ (11%), $Mn^{2+}$ (3%), $Fe^{2+}$ (4%) and $Mg^{2+}$ (3%). The supplementation of liquid culture medium with 10 mM calcium stimulated bacterial growth and enhanced α-amylase production.

The inhibition by $Hg^{2+}$ may indicate the importance in enzyme function as has been demonstrated for other microbial α-amylases (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003). The inhibition of *B. subtilis* JS-2004 α-amylase by $Co^{2+}$ ion could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity (Leveque, Janecek, Haye, & Belarbi, 2000). The effects of metal ions on the activity of α-amylase in *Bacillus* sp. strain KSM–1378, were investigated by Igarashi et al. (1998). $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ ions strongly inhibited the enzymatic activity by 82%, 91%, 100% and 100%, respectively.

The effect of some inhibitors such as EDTA, PMSF, DTT and β-mercaptoethanol on purified enzyme was studied. At the end of this study, the highest inhibitory effect 1 mM EDTA (79%) and 10 mM EDTA (87%) was obtained. It was determined that the inhibitory effect on enzyme activity increased in parallel with the increase in the concentration (Table 2). Similar results were also reported by (Hmidet et al., 2008; Azad et al., 2009). Among all inhibitors tested the chelating agent EDTA inactivated the enzyme indicating that α-amylase is a metalloenzyme.

### 3.9. Effects of detergents on amylase activity

The stability was also studied by incubating the enzyme in the presence of detergents (SDS, Tween 40, Tween 80, Triton X-100) for 30 min at 37 °C. It was found that α-amylase retained 76%, 90%, 82%, 93% and 88% of its original activity after 30 min incubation at 37 °C in the presence of 0.5% SDS, Tween-40, Tween-80 and Triton X-100. Similar results were also reported by Hmidet et al. (2008).

**Table 2**
Activity remaining after incubation for 30 min at 37 °C.

| Agent | Concentration (mM) | Relative activity (%) of α-amylase |
|---|---|---|
| PMSF | 1 | 91 |
| | 2 | 87 |
| | 4 | 74 |
| | 10 | 45 |
| DTT | 1 | 96 |
| | 2 | 87 |
| | 4 | 71 |
| | 10 | 73 |
| β-Mercaptoethanol | 1 | 98 |
| | 2 | 95 |
| | 4 | 92 |
| | 10 | 88 |
| EDTA | 1 | 21 |
| | 2 | 20 |
| | 4 | 18 |
| | 10 | 13 |

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