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Production, Partial Purification and Characterization of α-Amylase from High Molecular Weight Polycyclic Aromatic Hydrocarbons (HMW-PAHs) Degrading Bacillus subtilis BMT4i (MTCC 9447)

[α-Amilazın, Yüksek Moleküler Ağırlıklı Polisiklik Aromatik Hidrokarbonları (HMW-PAHs) degrade eden Bacillus subtilis BMT4i (MTCC 9447)'dan Üretimi, Kısmi Saflaştırılması ve Karakterizasyonu]*

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ABSTRACT

Objective: The present study reports for the first time the production, purification and characterization of α-amylase from a known HMW-PAHs degrader *Bacillus subtilis* BMT4i.
Methods: Culture conditions for the production of α-amylase were optimized. The α-amylase was further purified partially by ammonium sulphate precipitation and kinetic characterization of the α-amylase was done.

Results: The observations demonstrated BMT4i as an efficient producer of α-amylase. that revealed maximum production at 72 h, pH 8.0, starch (20 g/l), peptone (10g/l) and $CaCl_2$ (0.2g/l). The α-amylase exhibited a specific activity of 1001.08 U/mg corresponding to 3.86 fold purification and 76.7% yield. The enzyme exhibited optimal activity at 40°C and pH 8.0. The enzyme was stable in the pH range of 4.0-8.0 and retained stability at 50°C for 2 h. The V_{max} and K_m of α-amylase was found to be 5000 U and 4.0 mg ml-1 respectively. The enzyme activity was strongly activated by Ca^{2+} and Fe^{3+}.
Conclusion: Our findings emphasize upon the prospect of the commercial production of α-amylase from *Bacillus subtilis* BMT4i that can be employed in various sectors such as food, pharmaceuticals, textiles, detergents, etc.

Key Words: Amylase, *Bacillus subtilis* BMT4i (MTCC 9447), enzyme activity, high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs), optimization, purification

Conflict of Interest: Authors declare no conflict of interest of any kind.

ÖZET

Amaç: Bu çalışma, ilk kez α-amilazın bilinen bir HMW-PAH degrade edici *Bacillus subtilis* BMT4i'den üretimi, saflaştırılması ve karakterizasyonu rapor etmektedir.

Yöntemler: α-Amilazın üretimi için kültür koşulları optimize edilmiştir. Devamında α-amilaz, amonyum sülfat presipitasyonu ile kısmen saflaştırılmış ve kinetik karakterizasyonu gerçekleştirilmiştir.

Bulgular: Elde edilen bulgular BMT4i'nin verimli bir α-amilaz üreticisi olduğunu göstermiştir. Maksimum üretimin 72. saatte, pH 8.0, nişasta (20 g/l), pepton (10g/l) ve $CaCl_2$ (0.2g/l) koşullarda elde edildiği saptanmıştır. α-Amilaz, 1001.08 U/mg spesifik aktivite ile 3.86 kat saflaştırılarak % 76.7 verim elde edilmiştir. Enzim optimum aktivitesini 40°C ve pH 8.0'de göstermiştir. Enzim pH 4.0-8.0 aralığında kararlıdır ve kararlılığını 50°C'de 2 saat süreyle korumuştur. α-Amilaz'ın V_{max} ve K_m değerleri sırasıyla 5000 U ve 4.0 $mg.ml^{-1}$ olarak saptanmıştır. Enzim aktivitesi Ca^{2+} and Fe^{3+} varlığında kuvvetli biçimde aktive olmuştur.
Sonuçlar: Bulgularımız, gıda, farmasötik, tekstil, deterjan, vb. Sektörlerde kullanılabilecek olan α-amilazın, *Bacillus subtilis* BMT4i'den ticari üretimi olasılığını vurgulamıştır.

Anahtar Kelimeler: amilaz, *Bacillus subtilis* BMT4i (MTCC 9447), enzim aktivitesi, yüksek molekül ağırlıklı polisiklik aromatik hidrokarbonlar (HMW-PAHs), optimizasyon, saflaştırma

Çıkar Çatışması: Yazarlar hiçbir çıkar çatışması bulunmadığını beyan eder..

Introduction

Alpha (α)-amylases (E.C.3.2.1.1) are the enzymes that are extra-cellular and hydrolyze internal 1, 4-glycosidic linkages in starch to yield low molecular weight products, such glucose, maltose and maltotriose units [1-3]. These are the most important class of industrial enzymes that are of great significance in biotechnology and occupy approximately 25% of the world enzyme market [3-4]. Amylases can be obtained from plant, animal and microbial sources. Currently, majority of microbial amylases are commercially available and in the starch processing industries, they have almost completely replaced chemical hydrolysis of starch. The wide applications of microbial amylases in the industries are endorsed to their superior stability in comparison to amylases of plant and animal origin [5]. The production of amylases using microorganisms has a major advantage of economic commercial production and easy manipulation of microbes for obtaining the enzymes of desired characteristics. The fungal and bacterial α-amylases have wide applications in the brewing, food, fermentation, textile, paper, detergent, and pharmaceutical industries in addition to many fields such as clinical, medicinal and analytical chemistry [1-2, 6].

The *Bacillus* genus has the potential to dominate the enzyme industry since its every bacterial species is capable of synthesizing amylase [7]. The extensively exploited *Bacillus* strains, for producing α-amylases include *B. amyloliquefaciens*, *B. licheniformis* [8], *B. stearothermophilus* [9], *B. subtilis* [10], *and B. megaterium* [11] and *B. circulans* [12]. The *Bacillus* species are very adaptable to the environment and a number of factors affect the enzyme production. The production of bacterial amylases is greatly affected by the composition of media and the culture conditions, which needs to be optimized in order to attain the maxima [13-15].

In view of the above, the present study reports the production, partial purification, and characterization of α-amylase from a novel benzo-a-pyrene (BaP) degrading *Bacillus subtilis* BMT4i (MTCC 9447) previously isolated from automobile contaminated soil that is capable of degrading high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) including BaP [16-18]. Production conditions were also optimized (time, pH, carbon source, nitrogen source and $CaCl_2$ concentration) to achieve high enzyme production and better enzyme activity.

Materials and methods

Bacillus subtilis strain: The *Bacillus subtilis* BMT4i (MTCC 9447), isolated from automobile contaminated soil is an efficient degrader of HMW-PAHs including BaP, a potent carcinogen [16-18] was used in this study. The culture was maintained on nutrient agar slants at 4°C.

Optimization of culture conditions for α-amylase production

The amylase production capability of BMT4i was evaluated by growing BMT4i for 48 h in fermentation media (1.0% starch, 0.5% yeast extract, 0.02% $CaCl_2$, 0.1% NaCl and 0.1% $MgSO_4$, pH 7.0) and presence of extracellular α-amylase in the fermentation media was checked by α-amylase assay. The culture conditions for optimum α-amylase production were standardized with respect to incubation time, pH, starch concentration, nitrogen source and $CaCl_2$ concentration. Effect of incubation time and pH on enzyme production was studied by adjusting the incubation time for varied time intervals (6, 8, 24, 48, 72, 96, 120, 144 and 168 h) and fermentation media pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0) keeping the temperature constant at 37°C throughout the experiment. In addition, the effects of starch (substrate) concentration, nitrogen sources and $CaCl_2$ concentration were evaluated. Different concentrations of starch (0.5, 1.0, 2.0, 3.0 and 4.0%), nitrogen sources (0.5% each of tryptose, beef extract, tryptone, yeast extract, peptone:yeast exrtact (1:1), urea, ammonium oxalate, sodium nitrate, ammonium sulfate, ammonium chloride and potassium nitrate) and $CaCl_2$ concentrations (0.005, 0.01, 0.015, 0.02, 0.025, 0.03 and 0.035%) were adjusted in the fermentation media. Varied concentrations of peptone (0.2, 0.5, 1.0, 1.5, 2.0 and 2.5%) were also tested and even concentration of NaCl (0.1%) and $MgSO_4$ (0.1%) was used in the fermentation media.

Partial purification of α-amylase

Bacillus subtilis BMT4i was grown under optimized conditions and filtrate broth (crude amylase) was collected and centrifuged at 8,000 rpm 4°C for 10 min to obtain cell free filtrate (CFF). Partial purification of amylase enzyme was achieved by ammonium sulphate precipitation followed by dialysis at 4°C. For that, 100 ml of CFF was saturated with ammonium sulphate up to 80%. The content was incubated over night and centrifuged at 5000 rpm for 20 min. Afterwards, the pellet was collected, dissolved in 50 mM phosphate buffer (pH 7.5), transferred in dialysis bag (dialysis membrane-50, HiMedia India) and dialyzed for 24 h at 4°C in phosphate buffer. The buffer was changed three times during the process in order to obtain salt free amylase preparation.

α-Amylase assay

The reaction mixture containing 0.1 ml of crude enzyme and 1.0 ml (1.0%) solution of soluble starch in 50 mM phosphate buffer (pH 7.5) was incubated at 37°C for 10 minutes. The reaction was stopped by adding 1.0 ml of 1N NaOH. Further, 1.0 ml of 3, 5-dinitrosalicylic acid (DNS) was added to the tube and kept at boiling water bath for 10 min. The amount of reduced DNS (orange colored compound) that is proportional to the reducing sugar released from the hydrolysis of starch by α-amylase was measured at 540 nm [19]. One unit (U) of

amylase activity was defined as the amount of enzyme which liberates 1µmol of reducing sugar as glucose per min under the standard conditions of the assay.

Protein determination

The protein concentration in the dialyzed enzyme preparation was determined by the Lowry method [20], using bovine serum albumin (BSA) as standard.

Determination of the specific activity of α-amylase

The specific activity of the α-amylase protein was expressed in terms of units/mg protein according the following equation:

Specific activity = enzyme activity / protein content (mg)

Enzyme Characterization

Effect of temperature on the activity and stability of α-amylase

The effect of temperature on purified enzyme activity was investigated at temperatures between 30 to 80°C at pH 7.5. In order to determine the thermal stability of the α-amylase, the purified enzyme was pre-incubated at 30 to 80°C for 10 min to 2 h respectively. Thermal stability was expressed as percent residual activity, taking the initial enzyme activity at each temperature considered as 100%.

Effect of pH on the activity and stability of α-amylase

The optimum pH of the enzyme preparation was investigated in the pH range of 4.0 to 12.0 by using the following buffer systems: 0.1 M sodium acetate (pH 4.0-5.5); 0.1 M sodium phosphate (pH 6.0-7.5); 0.1 M Tris–HCl (pH 8.0-9.0); 0.1 M glycine NaOH (pH 9.5-12.0). The enzyme assay was performed at substrate concentration of 2.0 mg/ml under optimum temperature. In addition, pH stability of the α-amylase was also determined by pre-incubating the purified enzyme in the buffers of pH 4.0 to 12.0 for 24 h respectively. The pH stability was expressed as percent residual activity, taking the initial enzyme activity at each pH considered as 100%.

Progress curve of α-amylase

In order to determine the effect of incubation time on α-amylase activity, the enzyme was assayed under standard conditions at varied time durations ranging from 0 to 120 min.

Kinetic analysis of α-amylase

The reaction rate of α-amylase was determined at different starch concentration ranging from 0.39 to 3.07 mg/ml of starch under optimum conditions of pH, temperature and incubation time. The α-amylase velocity (enzyme activity per unit time) was determined at each substrate concentration and the values of K_m and V_{max} were determined by plotting Lineweaver–Burk plot.

Effect of metal ions on α-amylase activity

For determining the effect of metal ions ($CaCl_2$, $Co(NO_3)_2$, $FeCl_3$, $MgCl_2$, $PbNO_3$, and $SnCl_2$) on amylase activity, enzyme assays were performed in the presence of the metal ions at final concentration of 2 mM in 50 mM phosphate buffer (pH 7.5) using starch as a substrate. The relative enzyme activity was measured under standard assay conditions.

Results and discussion

Effect of incubation time on α-amylase production

The effect of incubation time on the α-amylase production by *Bacillus subtilis* BMT4i revealed that α-amylase synthesis started within 6 h of growth achieving maxima at 72 h (Table 1). Further incubation from 96 h to 168 h resulted in sharp decrease in total α-amylase production. Similar observation has been reported previously by Qader et al. in *Bacillus* sp. AS-1 [21].

Table 1: Effect of incubation time on α-amylase production

Time (h)	Enzyme activity (U)
6	50
8	48
24	137
48	238
72	400
96	272
120	182
144	180
168	110

Effect of pH on α-amylase production

Effect of fermentation medium pH range 4.0 to 12.0 on the total enzyme production is represented in Table 2. A steady increase in the total amylase production was observed in the pH range 4.0 to 8.0 achieving maxima at pH 8.0, thereafter it declined sharply. This might be attributed to the requirement of slightly alkaline pH by bacteria for the production of α-amylase. Our findings are in accordance with the earlier reports [21-22]. Bajpai and Bajpai reported the growth of *Bacillus licheniformis* TCRDC-B13 in the pH range 3.0 to 11.0 with optimum amylase production in the pH range 6.0 to pH 9.0. In another report, Qader et al. demonstrated optimum amylase production by *Bacillus* sp. AS-1 at pH 7.5.

Effect of starch concentration on α-amylase production

To determine the best concentration of starch as the carbon source in the fermentation media for α-amylase production, BMT4i was cultivated for 72 h in the fermentation media containing different concentrations

of starch (0.5, 1.0, 2.0, 3.0 and 4.0%) and the enzyme activity was measured. The relative amounts of α-amylase produced in the presence of different concentrations of starch are depicted in Table 3. The results showed that maximum amylase production occurred in the presence of 2.0% starch in the fermentation media. Therefore, 2.0% starch was selected as the best concentration of starch as the carbon source for α-amylase production by strain BMT4i. Starch has been reported to enhance amylase production in many strains such as *B. subtilis* IMG22, *Bacillus* sp. PS-7, *Bacillus* sp. I-3, *B. stearothermophilus* and *B. subtilis* [5, 13, 23-25]. Santos and Martins reported negligible increment in amylase production in the presence of more than 1% starch in the media [26]. However, BMT4i showed maximum amylase production in 2% starch concentration, beyond which amylase production decreased. Our findings are consistent with previous report that demonstrated 2% starch as the optimum concentration for amylase production by *Bacillus* sp. AS-1 [21].

Table 2: Effect of pH on α-amylase production

pH	Enzyme activity (U)
4.0	72
5.0	114
6.0	152
7.0	181
8.0	205
9.0	132
10.0	112
11.0	150
12.0	11

Table 3: Effect of starch concentration on α-amylase production

Starch (%)	Enzyme activity (U)
0.5	103
1.0	142
1.5	165
2.0	336
3.0	256
4.0	251

Effect of nitrogen source on α-amylase production

In order to determine the best nitrogen source for α-amylase production, different organic and inorganic nitrogen sources were tested in the media with starch as the carbon source. As shown in Table 4, reduction of amylase production by BMT4i was observed on the addition of inorganic nitrogen such as ammonium chloride, ammonium oxalate, ammonium sulfate, potassium nitrate and sodium nitrate. However, enhancement in amylase production was observed in the presence of the organic nitrogen sources. Our data is in consonance with the finding of Gupta et al. who reported that organic nitrogen sources enhance amylase production [1]. Amongst the varied organic nitrogen tested, maximum α-amylase production was achieved with peptone supplement as the nitrogen source (Table 5). In addition, the best concentration of peptone as the nitrogen supplement was determined using different concentrations in the range of 0.2 to 2.5%. The data showed maximum α-amylase production at 1.0% peptone (Table 5). Among the nitrogen sources, peptone has been previously reported to maximize the production of amylase [21, 22, 25, 27, 28]. Maximum amylase production has been reported in *B. amylolyticus* and *B. stearothermophilus* strains under vigorous shaking in the presence of peptone, yeast extract, and maltose in the medium [29].

Table 4: Effect of nitrogen source on α-amylase production

Nitrogen Source (0.5%)	Enzyme activity (U)
Tryptose	177
Beef extract	171
Tryptone	177
Yeast extract	199
Peptone	222
Peptone:Yeast(1:1)	183
Urea	68
Ammonium oxalate	137
Sodium nitrate	56
Ammonium sulfate	46
Ammonium chloride	57
Potassium nitrate	169

Table 5: Effect of peptone (%) on α-amylase production

Peptone (%)	Enzyme activity (U)
0.2	80
0.5	114
1.0	1154
1.5	80
2.0	330
2.5	273

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

To determine the ideal $CaCl_2$ concentration for amylase production, different concentrations of $CaCl_2$ in the fermentation media are tested. The production of α -amylase was found to be Ca^{+2} dependent attaining maximum amylase production at 0.02% $CaCl_2$ (Table 6). It has been demonstrated that, induction of *Bacillus licheniformis* with calcium salt in the medium increase the α-amylase production [30]. Moreover, the stability of α-amylase has been reported to be calcium dependent [31].

The production of α-amylase by *Bacillus* sp. AS-1 has been reported to be maximum in the presence of 0.02% $CaCl_2$ [21]. Our finding is in agreement with the previous studies on *Bacillus amyloliquefaciens* and *Bacillus subtilis* cultures with respect to α-amylase activity [32, 33]. These observations may also be supported by the fact that amylase is a calcium metalloenzyme and increment in the Ca^{2+} concentration up to 0.02% increases the bioavailability of Ca^{2+} to the saturation leading to enhancement in enzyme production.

Therefore, with the data obtained the optimum physical and chemical conditions for α-amylase production by strain BMT4i were considered to be 72 h of incubation, pH 8.0 of the fermentation media, 2.0% starch as the carbon source, 1.0% peptone as the nitrogen supplement and 0.02% $CaCl_2$.

Table 6: Effect of Ca^{2+}on α-amylase production

$CaCl_2$ (%)	Enzyme activity (U)
0.005	159
0.01	182
0.015	251
0.02	335
0.025	305
0.03	239
0.035	148

Partial purification of α-amylase

Bacillus subtilis BMT4i was grown under optimized conditions and the α-amylase produced was partially purified by 80% ammonium sulphate precipitation. Partially purified α-amylase exhibited a specific activity of 1001.08 U/mg that corresponds to 3.86 fold purification and 76.7% yield (Table 7). Our purification strategy is in accordance with the earlier reports [34-35].

Enzyme Characterization

Effect of temperature on the activity and stability of α-amylase

The optimum temperature for α-amylase activity was found to be 40°C (Figure 1 A). The relative activities of the α-amylase at 30 and 37°C were found to be 83 and 93%, respectively. At temperatures above 40°C, the amylase activity showed a drastic decrease. As shown in Figure 1 B, the residual amylase activity was found to be 96, 57 and 33% at 30, 40 and 50°C for 2 h respectively.

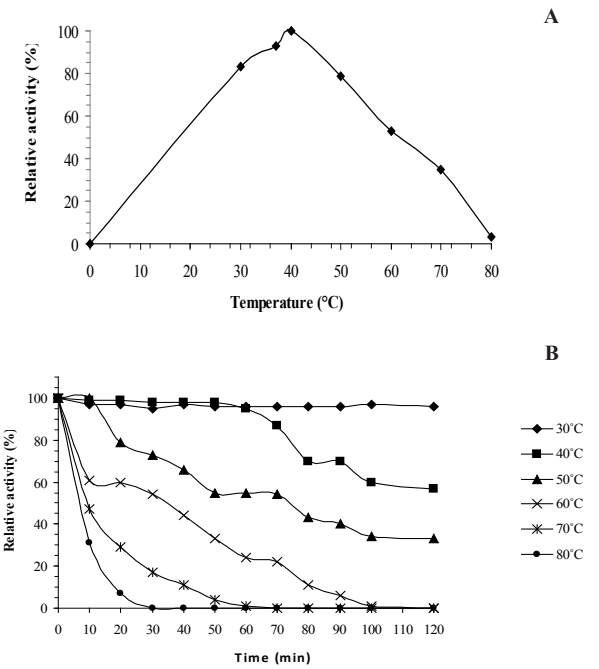

Figure 1. Effect of temperature on the (A) activity and (B) stability of α-amylase

However, at temperatures above 50°C the amylase activity was lost. The BMT4i amylase exhibited good stability below 50°C. Our results are in consonance with the previous reports [3, 36-38]. Generally, for α-amylase from most of the *Bacillus subtilis* strains, the optimum temperature and stability has been reported to be in the range of 37 to 50°C [3, 36-38].

Effect of pH on the activity and stability of α-amylase

The maximum activity of α-amylase was established at pH 8.0, however it was found to be most stable at pH 7.0 at 40°C (Figure 2). The relative activities at pH 4.0, 6.0, 7.0 and 8.0 were determined to be 16, 81, 97 and 100%, respectively, of that measured at pH 7.5. At pH above 8.0, the amylase activity decreased rapidly. The amylase from *Bacillus subtilis* BMT4i was stable in a range of pH 4.0-8.0 for 24 h and at pH 10.0 approximately 45% of its activity was retained (Figure 2). For most *Bacillus subtilis* strains, the pH optima and stability of α-amylase has been reported to be the range of pH 6.0 to 7.0 [3, 36, 37-39]. Our results are consistent with these findings.

Progress curve of α-amylase

The results presented in Figure 3 indicate that after 10 min, the product formed was found to be 1920

Table 7: Purification results

Sample	Protein (mg/ml)	Activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	1.2	311	259	1	100
80%	0.92	921	1001	3.86	76.7

µmole, which increased to 10060 µmole after 45 min of incubation. After 45 min, the product formation remained almost constant that may be due to substrate limitation and product inhibition.

Kinetic Analysis of α-amylase

For determination of K_m and V_{max} of α-amylase, the reaction was carried out at different starch concentration ranging from 0.39 to 3.07 mg/ml of starch under optimum conditions of pH (8.0), temperature (40°C) and incubation time (45 min). The enzyme showed Michaelis-Menten kinetics while hydrolyzing starch. Based on the Lineweaver-Burk equation, the V_{max} value obtained for purified α-amylase was 5000 U, whereas K_m of purified enzyme was 4.0 mg ml^{-1} substrate (Figure 4).

Effect of metal ions on α-amylase activity

As indicated in Table 8, $Ca^{2+}$and Fe^{3+} activated the α-amylase from *Bacillus subtilis* BMT4i whereas Sn^{2+}, CO^{2+} and Pb^{2+} drastically inhibited its activity. There are several studies demonstrating the effects of metal ions on bacterial and fungal α-amylases. Although, any specific ion is not required for the catalytic activity of amylase but there are several reports on the Ca^{2+} dependent α-amylase from *Bacillus* spp [25, 40-43]. It has been reported that α-amylase is a metalloenzyme containing at least one activating Ca^{2+} ion. In contrast to any other ion, Ca^{2+} has much stronger affinity to α-amylase [1]. The enhanced activity of amylase in the presence of Ca^{2+} and Fe^{3+} could be attributed to their interaction with negatively charged amino acid residues including aspartic and glutamic acid, which could stabilize the enzyme conformation [44].

The present study is the first report on the optimization of culture conditions, purification and characterization of the activity of an efficient α-amylase from a HMW-PAH degrading bacterial strain *Bacillus subtilis* BMT4i (MTCC 9447) isolated from hydrocarbon contaminated soil which could be employed for industrial applications.

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Conflict of Interest: Authors declare no conflict of interest of any kind.

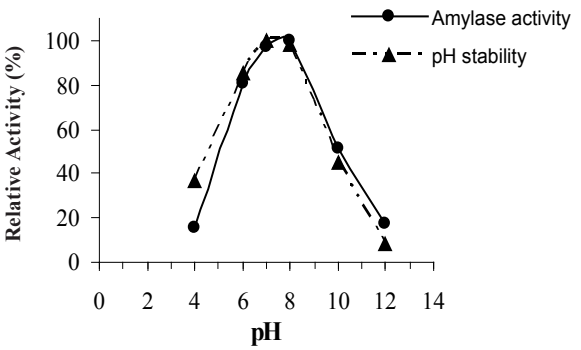

Figure 2. Effect of pH on activity and stability of α-amylase

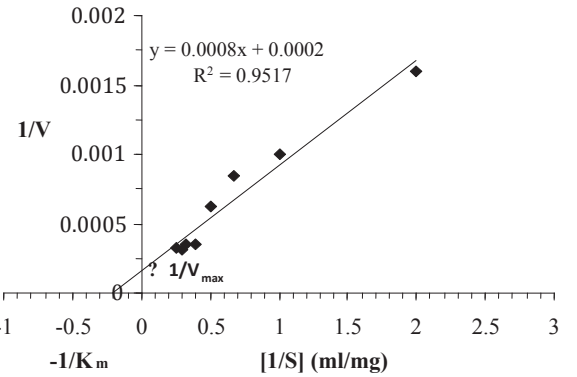

Figure 4. Line-Weaver Burk plot of α-amylase

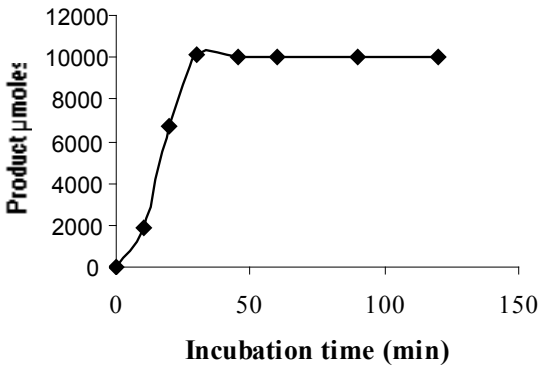

Figure 3. Progress curve of α-amylase

Table 8: Effect of metal ions on α-amylase activity

Metal ions (2mM)	Relative activity (%)
Control	100
$CaCl_2$	113
$FeCl_3$	103
$Co(NO_3)_2$	18
$SnCl_2$	25
$PbNO_3$	7
$MgCl_2$	87

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