

Purification and Characterization of α -amylase from *Penicillium olsonii* under the Effect of Some Antioxidant Vitamins

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Abstract: The effect of four antioxidants, vitamin E, β -carotene, vitamin C and folic acid on the dry weight, biosynthesis of protein and amylolytic activity of *Penicillium olsonii* were studied. Five concentrations of each antioxidant were used. Vitamin C increased both accumulations of proteins and α -amylase biosynthesis followed by folic acid, but there was a remarkable decrease by either β -carotene or vitamin E. The purification and characterization of α -amylase from *Penicillium olsonii* under the effect of vitamin C was investigated. The crude enzyme was purified by acetone precipitation, column chromatography on Sephadex G-100 and DEAE-Cellulose. Maximal activities of the purified enzyme was recorded at pH 5.6, 30°C and 1.25% starch with Km value 0.556 mg/ml. The enzyme was found to have a good thermostability. The enzyme activity was enhanced in presence of Mn^{++} and Mg^{++} while, less stimulation was occurred in presence of Ca^{++} and Co^{++} . The enzyme activity couldn't change in presence of Zn^{++} , K^+ , Fe^{+++} and Na^+ . Strong inhibitory activity was detected by using Hg^{++} and Cu^{++} . The most injurious inhibitors were Potassium ferro-cyanide, L-Cysteine and Iodo-acetate.

Key words: Antioxidants • Vitamin E • β -carotene • Vitamin C • Folic acid • Amylolytic activity • *Penicillium olsonii* • Purification • Protein • Dry weight

INTRODUCTION

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents [1]. Although amylases can be obtained from several sources, such as plants and animals. The enzymes from microbial sources generally meet industrial demand [2]. Alpha amylase (1, 4- α -D-Glucan glucanohydrolase) is a glycoprotein with single polypeptide chain of 475 residues, two SH groups, and four disulfide bridges and contains a tightly bound Ca^{2+} . It exists in two forms (I and II) which have identical enzymatic properties, differing only in electrophoretic mobility [3, 4]. The enzyme was widely documented in the industries of starch sugar (glucose, maltose, dextrin, fructose, and oligosaccharide), alcohol, beer, monosodium glutamate, brewing, organic acid, textile, print, dyeing, paper making and other fermentation processes [2].

Vitamins have critical roles in enhancement the optimal enzyme activity by serving as co-enzymes [5]. Recently, some vitamins including , vitamin C

(Ascorbic acid), E and β -carotene were documented as antioxidants. The role of such compounds are known to be important for preventing oxidative damage in cellular and sub-cellular structures [6]. In addition, Carr and Frei [7] proved that vitamin C could reduce the metal ions which lead to the generation of free radicals through the Fenton reaction. Moreover, Duarte and Lunec [8] found that vitamin C has an antioxidant activity by reducing the oxidizing agents such as hydrogen peroxide.

Antioxidants are either soluble in water (hydrophilic) or in lipids (hydrophobic). Generally, hydrophilic antioxidants could react with oxidants in the cell cytoplasm, while hydrophobic antioxidants protect the cell membranes from lipid peroxidation [9]. Sometimes these compounds may be synthesized in the cells or supplied externally [10]. Although some antioxidants have inhibitory activities, they could stimulate few numbers of fungi and change the cellular structure of the treated fungus [11]. Antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged [12]. The aim of the present work was to investigate the purification and characterization of

α -Amylase from *Penicillium olsonii* under the effect of the most potent antioxidant vitamins that caused a maximal increase in α -Amylase production and protein accumulation.

MATERIALS AND METHODS

Micro-organism and Culture Medium: *Penicillium olsonii* used throughout this work was previously isolated by Houseny [13]. The culture was grown on Czepek's Dox broth [14] at 2% soluble starch instead of sucrose at 30°C for 7 days before use.

Preparation of Antioxidants: Fat soluble vitamins (vitamin E and β -carotene) ; water soluble vitamins (vitamin C and folic acid) were used in five concentrations of each as follows: 10, 20, 30, 40 and 50 IU/100 ml medium for β -carotene & vitamin E and 10, 20, 30, 40 and 50 mg/100 ml medium for vitamin C & Folic acid. Triplicate sets of 250 ml Erlenmeyer flasks containing 50 ml of the broth Czepek's Dox with 2% soluble starch instead of sucrose were prepared. After sterilization at 120° for 15 min the vitamins were added at each concentration under aseptic conditions. One ml of spore suspension of *Penicillium olsonii* was inoculated under aseptic conditions. The flasks were then incubated at 28-30°C for seven days. Two Control media were used for the fat soluble vitamins, the first one was Czepek's Dox broth [14] with 2 % soluble starch instead of sucrose, without any additions of antioxidants. The second was the same as the first one except that Tween 80 was added for facilitating the solubility of fat soluble vitamins. While for the water soluble vitamins, the first control medium was only used.

Growth Measurement: Measuring the fungal growth was made according to the method described by Kane and Mullins [15]. Briefly, the flasks of each set were filtered off after the end of incubation period, and then the mats were washed carefully and dried up to a constant dry weight at 70-80°C for 24 hours.

Protein Assay: Extracellular protein in the culture filtrate of the fungus was measured according to the method of Lowry *et al.* [16].

α -amylase Assay: The assay mixture contained: 10 mg of soluble starch in citrate phosphate buffer (pH 6.0) and one ml of crude enzyme. After the incubation at 30°C for 30 minutes, the reducing sugars were determined according to Nelson-Somogyi modified method [17]. One

ml of heat-killed crude enzyme was used in another treatment as control . One unit of amylase enzyme is defined as the amount of enzyme which liberate 1 μ mol of reducing sugar (as glucose) per min.

Purification of α -amylase: Cell-free culture filtrates (CFC) were concentrated by lyophilization using freeze-dryer (Labconco, USA) and dialysed in Sigma dialyzing bags against sterilized distilled water for 24 hours at 4°C. The obtained cell-free dialysate (CFD) was precipitated by acetone (3:1). Then, the enzymatic protein was collected, dissolved in 0.2 M citrate phosphate buffer (pH 6.0) and fractionated through Sephadex G-100 column (Frac-100, Pharmacia-Fine Chemicals) (36 x 1.6 cm). Elution was carried out with the respective buffer at 1 ml/min. The protein content of each fraction was determined. The fractions which showed the highest amylolytic activities were thereafter pooled, concentrated and applied to a column of Diethylaminoethyl-cellulose (DEAE-C) pretreated with distilled water followed by 1N HCl and water till the pH of the suspension was about 6.0, then treated with 0.5 N NaOH until no more colour was removed. Elution takes place by linearly increased molarity of (0.0-0.8M) NaCl in the corresponding buffer at a flow rate of 0.4 ml/min. Fractions of five ml were obtained and assayed for protein and enzyme activity. High potent fractions were collected up and dialyzed once again to remove Na⁺ and Cl⁻ [18, 19]. This enzyme preparation was concentrated and stored at 0°C for further investigations.

Characterization of the Purified α -amylase

Effect of pH and pH stability: Different pH values (3.6, 4.0, 4.4, 4.8, 5.0, 5.2, 5.4, 5.6, 6.0, 6.4 and 6.8) were used. The amylolytic activities were determined after incubation at 30°C for 30 minutes. To determine pH stability, enzyme was incubated in presence of the previous pH values for two time intervals of 20 and 60 minutes. The original pH value was restored and the residual activity was estimated after incubation at 30°C for both intervals at 20 and 60 minutes. The results were expressed as relative activity (%) referred to the activity observed before incubation.

Effect of Temperature and Reaction Temperature: The reaction mixture (purified amylase with its substrate "starch") was incubated at different temperatures; 30, 35, 40, 45, 50, 55, 60 and 65°C for 30 minutes, then the amylolytic activity was assayed. The effect of temperature on enzyme reaction was assessed by keeping

the enzyme for both 20 and 60 minutes at the previously different temperatures. The estimation of the activities was carried out under optimal conditions. The results were expressed as relative activity (%) referred to the activity observed before incubation.

Effect of Enzyme Concentration: The purified α -amylase preparation was diluted to different concentrations; 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/ml. All concentrations of the enzyme were incubated with its substrate (starch) as previously mentioned, then the amylolytic activity was assayed.

Effect of Substrate Concentration: The purified amylase was incubated with its substrate at different concentrations; 0.25-1.50 g % under the optimum conditions of the enzyme, then the amylolytic activity was determined.

Effect of Incubation Period: The purified enzyme was incubated with its substrate at time intervals of 15, 30, 45, 60, 75 and 90 minutes at the optimum conditions, then the amylolytic activity was measured.

Effect of Some Modulators: The purified enzyme was incubated for five minutes with one nM and 10 nM of each of the following substances; KCl, NaCl, NaNO₃, CaCl₂, MnCl₂, HgI, MgSO₄, FeSO₄, CuSO₄, AgNO₃, ZnSO₄ and CoCl₂. Also, with EDTA (ethylene diamine tetra acetic acid), NaH₂ASO₄.7H₂O, NaN₃, I₂ C₂H₆O₅, L-cystein, KMNO₄ and K₄Fe(CN)₆. Then the amylolytic activity was assayed.

Data Analysis: Least significance difference (LSD) at 0.01 and 0.05 level was used to test difference between means using Spss version 14 soft ware.

RESULTS AND DISCUSSION

The effect of four antioxidants (vitamin E; β -carotene; vitamin C and folic acid) on the dry weight, biosynthesis of protein and the amylolytic activity of *Penicillium olsonii* were shown in Tables 1-4. Vitamin E and β -carotene in all concentrations were significantly inhibited the growth of *Penicillium olsonii* and its production of protein and α -Amylase (Tables 1 & 2). These results are in agreement with that obtained by Tamas *et al.* [20] who concluded that vitamin E could decrease dry cell mass, inhibit autolysis of *Emergilla nidulans* and has a negative effect on sporulation. In

this regard Gumustekin *et al.* [21] proved that the inhibitory activity of vitamin E is due to the inhibition of glucose 6-phosphate dehydrogenase activity which is important in stimulation of the cell growth. The results (Tables 3 & 4) showed that vitamin C and folic acid in all concentrations were significantly stimulated the same parameters of *Penicillium olsonii*. The optimum concentrations of vitamin C and folic acid for the highest protein and amylolytic activity were 20 and 40 IU/100 ml medium respectively. In this regard, Kundu [22] stated that vitamins have catalytic functions in the cell and are known to be precursors of coenzymes or constituent parts of enzymes. Moreover, Bjelakovic [23] reported that antioxidants are substances that can protect the living cells against the damage caused by unstable molecules known as free radicals. Similarly, Nakamura [24] stated that antioxidants interact with and stabilize free radicals to prevent some of the damage that may be caused by these molecules and work via various mechanisms including preventive antioxidants.

In the light of our results, vitamin C had the most pronounced effect on growth (0.255 g/100 ml medium), protein accumulation (0.743 mg/ml) and α -Amylase production (0.698 U/ml) with the specific activity 0.939 U/mg by *Penicillium olsonii* (Table 4), therefore, α -Amylase of this fungus was purified and subjected to further studies.

A summary of purification steps of α -Amylase produced by *Penicillium olsonii* was recorded in Table 5. The first step of purification was carried out by precipitation of protein from the cell free dialysate with acetone (3:1 cell free filtrate) and reach to 3.002 folds of purification with yield of 88.1% of the original activities. The same result was recorded by Watanabe and Fukimbara [25] who explained that this solvent act by reducing the dielectric constants of the medium and consequently reducing the solubility of protein by favoring protein-protein rather than protein-solvent interaction. The precipitated enzyme was then purified by gel filtration through Sephadex G-100 with 5.3 folds and yield of 73.9%. This result is in agreement with that reported by Shelby [26]. The purification procedure was completed by ion exchange chromatography on DEAE-Cellulose using a linear NaCl gradient with 25.14 folds obtaining a final specific activity of 23.45 U/mg protein. The same results about the purification of extracellular α -Amylase from *Fusarium moniliforme* and *Clostridium acetobutylicum* were recorded by Augustin *et al.* [27] and Veronique *et al.* [28], respectively.

Table 1: Effect of vitamin E on dry weight, protein and amylolytic activity of *Penicillium olsonii*

Fungal Item	Controls		β-carotene (conc. = IU/100 ml medium)					L.S.D.	
	Media without β-carotene	Media+Tween 80 without β-carotene							
			10	20	30	40	50	0.01 =	0.05 =
Growth (g/100 ml medium)	0.256±0.003	0.258±0.002	0.250±0.002 -HS	0.191±0.001 -HS	0.190±0.002 -HS	0.181±0.002 -HS	0.172±0.003 -HS	0.007	0.01
Protein (mg/ml)	0.791±0.2	0.798±0.3	0.790±0.2 -HS	0.788±0.2 -HS	0.779±0.3 -HS	0.765±0.2 -HS	0.754±0.2 -HS	0.003	0.004
Enzyme activity (U/ml)	0.595±0.1	0.597±0.2	0.492±0.3 -S	0.484±0.2 -HS	0.462±0.2 -HS	0.450±0.1 -HS	0.440±0.2 -HS	0.012	0.017
Specific activity (U/mg)	0.725	0.748	0.622 -HS	0.614 -HS	0.593 -HS	0.588 -HS	0.583 -HS	0.012	0.017

HS =Highly Significant

Table 2: Effect of β-carotene on dry weight, protein and amylolytic activity of *Penicillium olsonii*

Fungal Item	Controls		β-carotene (conc. = IU/100 ml medium)					L.S.D.	
	Media without β-carotene	Media+Tween 80 without β-carotene							
			10	20	30	40	50	0.01 =	0.05 =
Growth (g/100 ml medium)	0.256±0.003	0.258±0.002	0.250±0.002 -HS	0.191±0.001 -HS	0.190±0.002 -HS	0.181±0.002 -HS	0.172±0.003 -HS	0.007	0.01
Protein (mg/ml)	0.791±0.2	0.798±0.3	0.790±0.2 -HS	0.788±0.2 -HS	0.779±0.3 -HS	0.765±0.2 -HS	0.754±0.2 -HS	0.003	0.004
Enzyme activity (U/ml)	0.595±0.1	0.597±0.2	0.492±0.3 -S	0.484±0.2 -HS	0.462±0.2 -HS	0.450±0.1 -HS	0.440±0.2 -HS	0.012	0.017
Specific activity (U/mg)	0.725	0.748	0.622 -HS	0.614 -HS	0.593 -HS	0.588 -HS	0.583 -HS	0.012	0.017

Table 3: Effect of vitamin C on dry weight, protein and amylolytic activity of *Penicillium olsonii*

Fungal Item	Control	vitamin C (conc. =mg/100 ml medium)					L.S.D.	
	Media without vitamin C	10	20	30	40	50	0.01=	0.05 =
Growth (g/100 ml medium)	0.131±0.001	0.242±0.001 HS+	0.255±0.003 HS+	0.210±0.005 +HS	0.144±0.006 +HS	0.141±0.003 +HS	0.009	0.013
Protein (mg/ml)	0.511±0.3	0.539±0.4 +HS	0.743±0.2 +HS	0.574±0.4 +HS	0.565±0.1 +HS	0.546±0.1 +HS	0.013	0.019
Enzyme activity (U/ml)	0.279±0.3	0.461±0.1 +HS	0.698±0.6 +HS	0.452±0.2 +HS	0.342±0.4 +HS	0.300±0.1 +HS	0.024	0.036
Specific activity (U/mg)	0.545	0.855 +HS	0.939 +HS	0.787 +HS	0.605 +HS	0.549 +HS	0.027	0.038

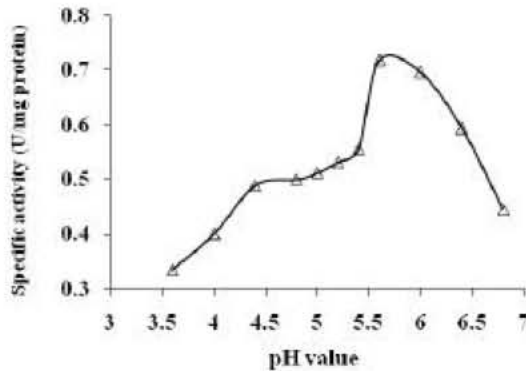
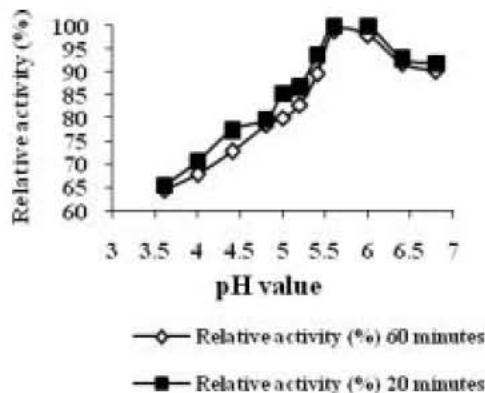
Table 4: Effect of folic acid on dry weight, protein and amylolytic activity of *Penicillium olsonii*

Fungal Item	Control	Folic acid (conc. =mg/100 ml medium)					L.S.D.	
	Media without folic acid	10	20	30	40	50	0.01 =	0.05 =
Growth (g/100 ml medium)	0.200±0.001	0.210±0.002 +HS	0.250±0.004 +HS	0.290±0.002 +HS	0.310±0.004 HS+	0.280±0.004 +HS	0.007	0.01
Protein (mg/ml)	0.616±0.2	0.685±0.6 +HS	0.706±0.4 +HS	0.754±0.2 +HS	0.795±0.1 HS+	0.708±0.5 +HS	0.01	0.014
Enzyme activity (U/ml)	0.270±0.1	0.361±0.1 +HS	0.378±0.3 +HS	0.406±0.4 HS+	0.453±0.1 +HS	0.390±0.1 +HS	0.01	0.014
Specific activity (U/mg)	0.438	0.527 +HS	0.535 +HS	0.538 +HS	0.569 +HS	0.55 HS+	0.007	0.01

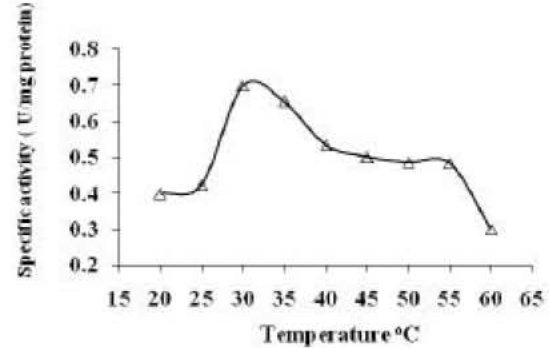
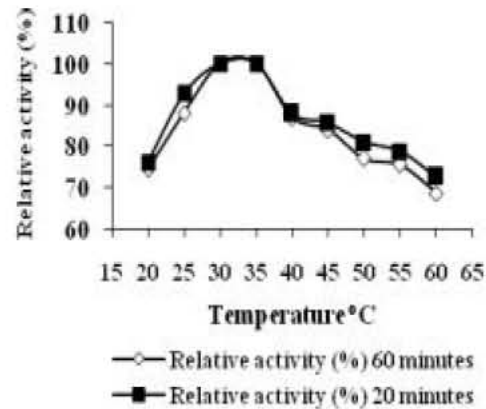
HS =Highly Significant

Table 5: A summary of purification steps of α -amylase produced by *Penicillium olsonii*

Treatment	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Overall Recovery (%)	Purification Fold (fold)
Cell-Free Filtrate (CFF)	34.900	37.15	0.933	100	1.000
Cell-Free Dialysate (CFD)	31.500	36.12	0.872	90.2	0.930
Precipitation (Acetone 3:1)	30.760	10.98	2.801	88.1	3.002
Gel filtration Sephadex G-100	25.808	5.210	4.954	73.9	5.304
Ion-exchange chromatography (DEAE-cellulose)	12.783	0.545	23.455	36.5	25.14

Fig. 1: Effect of pH values on the specific activity of the purified α -amylase from *Penicillium olsonii*Fig. 2: Effect of pH stability on the relative activity of the purified α -amylase from *Penicillium olsonii*

The properties of purified α -Amylase from *Penicillium olsonii* were studied. Figure 1 shows the effect of different pH values on the activity of this enzyme. It had a maximum activity at pH 5.6. A decline in the reaction velocity was recorded on either side of pH 5.6. Incubation of this enzyme at different pH values for either 20 or 60 min demonstrated that the enzyme was almost unaffected by incubation in the pH range 5.6-6.0 (Fig. 2). These results agree with those obtained by Mizuho *et al.* [29] who recorded that α -amylase from *Trichoderma matsutake* was the most active at pH 5.0-6.0 and was stable within the broad pH range 4.0-10.0.

Fig. 3: Effect of different temperatures on the specific activity of the purified α -amylase from *Penicillium olsonii*.Fig. 4: Effect of reaction temp. on the relative activity of the purified α -amylase from *Penicillium olsonii*

Moreover, Alva *et al.* [2] stated that the optimum pH of amylase enzyme isolated from *Aspergillus* sp. was 5.8.

Concerning the effect of temperature on the enzyme activity, the results showed that α -amylase achieved a high hydrolyzing effect at 30°C followed by gradual decrease with temperature increase (Fig. 3). Likewise, Milada [30] obtained a similar conclusion. The results accumulated on the thermal stability of this enzyme for 20 or 60 minutes are represented in Fig. 4. Apparently the activity of α -amylase was affected largely by exposing to temperature above 30°C. The enzyme lost about 24.6-31.5% of its activity at temperature ranging between

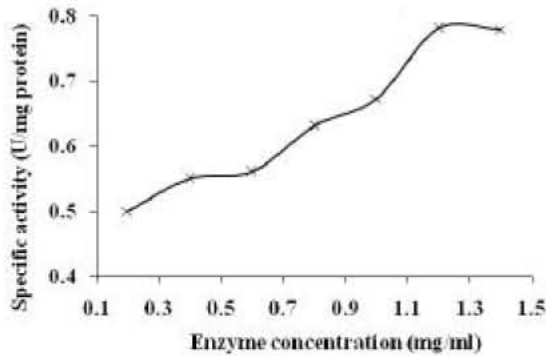


Fig. 5: Effect of enzyme protein concentrations on the specific activity of the purified α -amylase from *Penicillium olsonii*

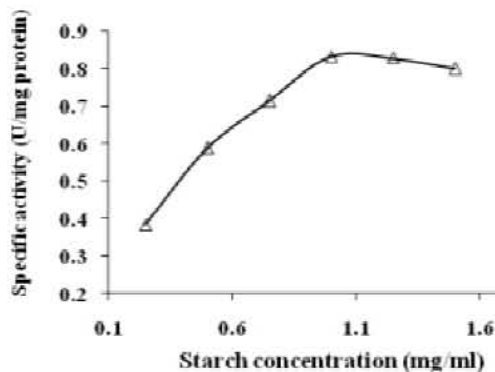


Fig. 6: Effect of starch concentrations on the specific activity of the purified α -amylase from *Penicillium olsonii*

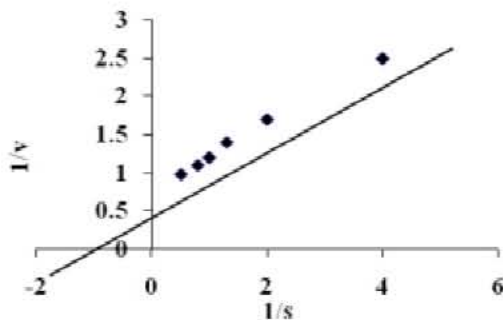


Fig. 7: Lineweaver-Burk plot of the reciprocal of initial velocities and starch concentration

55-60°C for one hour. The high temperature inactivation may be due to incorrect conformation due to hydrolysis of the peptide chain, destruction of amino acid, or aggregation [31].

The results in Fig. 5 showed that the increase of enzyme protein concentration was associated with an

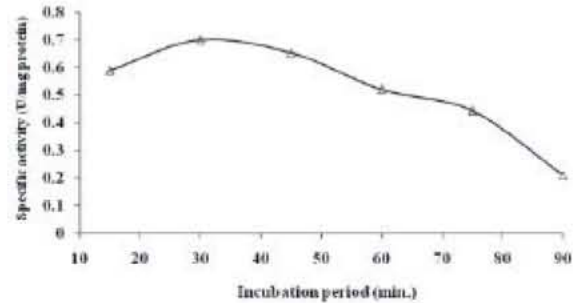


Fig. 8: Effect of incubation periods on the specific activity of the purified α -amylase from *Penicillium olsonii*

increase in α -amylase activity up to 1.0-1.2 U/ml at which the enzyme showed maximum activity then a decrease in the activity was observed.

With respect to the effect of different concentrations of starch on the enzyme activity, the results in Fig. 6 showed that the activity of α -amylase increased with the increase of starch up to 1.00 mg/ml followed by slight decrease in the activity. From the lineweaver-Burk plot of the reciprocal of initial velocities and substrate concentrations (Fig. 7), K_m value was calculated to be 0.556 mg/ml. This is equal to K_m value of amylase produced from *Bacillus stearothermophilus* [32]. The results in Fig. 8 showed that α -amylase activity was increased by increasing the reaction time up to 30 minutes (0.700 U/mg protein). Further extension of the reaction time led to decrease in its rate.

Concerning the effect of some metal ions on the specific activity of purified α -amylase from *Penicillium olsonii*, the results (Table 6) demonstrated that Mn^{2+} (1 mM) and Mg^{2+} (1 mM) enhanced the enzyme activity by values ranged between 23.0 and 12.0%. Less stimulation was occurred in presence of Ca^{2+} (1 mM). In this trend, Mahmoud *et al.* [33] found that metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme. The results are also showed that Co^{2+} , Zn^{2+} , K^+ , Fe^{3+} and Na^+ couldn't affect the enzyme activity at all. These results did not agree with that obtained from Haifeng *et al.* [34] who stated that α -amylase from the marine yeast *Aureobasidium pullulans* was activated by Ca^{2+} , Cu^{2+} , Ba^{2+} , Na^+ , Mg^{2+} and Co^{2+} . On the other hand, Ag^+ and Cu^{2+} (1 mM) suppressed to a small extent the α -amylase activity of *Penicillium olsonii*. Moreover, Hg^{2+} and Cu^{2+} (10 mM) produced a strong inhibitory effect on the enzyme activity. The metal ions may be

Table 6: Effect of some metal ions on the specific activity of the purified α -amylase enzyme produced by *Penicillium olsonii*

Metal ions	Molarity (mM)	Relative Activity (%)
0	0	100.0
K ⁺	1	100.0
	10	100.0
Na ⁺	1	100.0
	10	100.0
Ca ⁺⁺	1	102.0
	10	101.0
Mn ⁺⁺	1	123.0
	10	100.0
Hg ⁺⁺	1	50.5
	10	40.0
Mg ⁺	1	112.0
	10	101.0
Fe ⁺⁺⁺	1	100.0
	10	100.0
Cu ⁺⁺	1	87.0
	10	58.0
Ag ⁺	1	86.2
	10	84.8
Zn ⁺⁺	1	100.0
	10	100.0
Co ⁺⁺	1	100.0
	10	100.0

change the enzyme activity via change in electrostatic bonding which could change the tertiary structure of enzymes [35]. This may be due to the possible participation of sulfhydryl groups in the active site of the enzyme. The results in Table 9 showed different rates of inhibition in presence of enzyme inhibitors. The most injurious inhibitors were Potassium ferro-cyanide, L- Cysteine and Iodo-acetate. In contrast with the results obtained by Haifeng *et al.* [34], they stated that α -Amylase was inhibited by EDTA but was not inhibited by Iodo-acetic acid.

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