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ORIGINAL PAPER

Production and Partial Characterization of High Molecular Weight Extracellular α-amylase from *Thermoactinomyces vulgaris* Isolated from Egyptian Soil

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Abstract

Optimizing production of α-amylase production by *Thermoactinomyces vulgaris* isolated from Egyptian soil was studied. The optimum incubation period, temperature and initial pH of medium for organism growth and enzyme yield were around 24 h, 55°C and 7.0, respectively. Maximum α-amylase activity was observed in a medium containing starch as carbon source. The other tested carbohydrates (cellulose, glucose, galactose, xylose, arabinose, lactose and maltose) inhibited the enzyme production. Adding tryptone as a nitrogen source exhibited a maximum activity of α-amylase. Bactopeptone and yeast extract gave also high activity comparing to the other nitrogen sources (NH_4Cl, NH_4NO_3, $NaNO_3$, KNO_3, $CH_3CO_2NH_4$). Electrophoresis profile of the produced two α-amylase isozymes indicated that the same pattern at about 135–145 kDa under different conditions. The optimum pH and temperature of the enzyme activity were 8.0 and 60°C, respectively and enzyme was stable at 50°C over 6 hours. The enzyme was significantly inhibited by the addition of metal ions (Na^+, Co^{2+} and Ca^{2+}) whereas Cl^- seemed to act as activator. The enzyme was not affected by 0.1 mM EDTA while higher concentration (10 mM EDTA) totally inactivated the enzyme.

Key words: *Thermoactinomyces vulgaris*, high molecular weight α-amylase

Introduction

Enzymes produced by thermophilic bacteria have been highlighted for their potential as biocatalysts in biotechnology. The importance of this was referred to the general relationship between enzyme thermostability and the thermophilicity of the host bacterium (Herbert, 1992).

The α-amylase (EC 3.2.1.1) is a well-known endo-amylase that hydrolyzes starch by randomly cleaving internal a- 1,4 – glucosidic linkages. The spectrum of α-amylase applications has widely used in many fields, such as starch saccharification, textile, food, brewing, distilling industries, medical and analytical chemistries (Pandey *et al.*, 2000). Despite this, interest in new and improved α-amylase is growing vastly. Therefore, the search for thermostable Ca^{2+} independent α-amylase (Tonkova, 2006) is continuous. Enzymes of the thermophilic actinomycetes *Thermoactinomyces* species, especially their α-amylase (Ito *et al*., 2007), have attracted much interest because of their activity at high temperature. The advantages for using thermostable α-amylases in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external cooling. In addition, the stability of biocatalysts is often a limiting factor in the selection of enzymes for industrial applications due to the elevated temperature or extreme pH of many biotechnological processes.

The aim of the investigations was to determine the optimum conditions for production of highly active and industrial stable α-amylase by an Egyptian isolate of the thermophilic actinomycete *Thermoactinomyces vulgaris* and to investigate the enzyme properties.

Experimental

Materials and Methods

***T. vulgaris* strain.** *T. vulgaris* strain was isolated from fertile soil samples collected from Egypt at 50°C and identified according to Bergey's Manual of Systematic Bacteriology (Lacey and Cross, 1989). The culture was maintained on Czapek-yeast-casein (CYC) agar slants.

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Production of α-amylase. The organism was grown in conical flasks containing starch-nitrate medium (Waksman, 1959) as a basal medium with pH adjusted to 7.5. The medium (20 ml taken in 250 ml Erlenmeyer flasks) was inoculated with 1 ml of spore suspension of pure colonies of the organism and incubated at 50°C with shaking (150 rpm) for 72 hours.

The initial pH of the medium (4.0–10.0), temperature of incubation (30–60°C), and incubation period (12–96 h) were tested for α-amylase production. Different carbon sources (1%) and nitrogen sources (equimolecular nitrogen amounts equivalent to the nitrogen content of the basal medium) were used for optimization of nutritional factors. The various tested carbon sources were: starch, cellulose, glucose, galactose, xylose, arabinose, lactose and maltose. The nitrogen sources were included NH_4Cl, NH_4NO_3, $NaNO_3$, KNO_3, ammonium acetate, bactopeptone, yeast extract and tryptone were tested.

On the compilation of the previous experiments, the cell-free enzyme supernatant was obtained by centrifugation at 8000×g for 20 min. Experiments were carried out in triplicate and the results were treated statistically and standard errors are shown.

α-Amylase assay. The amylase assay was based on the reduction in blue colour intensity resulting from starch hydrolysis (Palanivelu, 2001). The reaction mixture consisted of 0.4 ml of diluted enzyme, 0.5 ml of 0.1% soluble starch and 1 ml of phosphate buffer (0.1 M, pH 7) was incubated at 50°C for 10 minutes. The reaction was stopped by adding 0.5 ml of 0.1 N HCl and the colour was developed by adding 0.5 ml of (2% KI in 0.2% I_2) solution. The optical density (OD) of the blue colour solution was determined using a Unico 7200 SERIES spectrophotometer at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that caused 20% reduction of blue colour intensity of starch iodine solution at reaction incubation temperature in 1 min per ml.

Intracellular protein content of *T. vulgaris*. The harvested mycelia of *T. vulgaris* strain were used for determination of intracellular protein. Washed pellets were dissolved in 20 ml of NaOH (1 M), and boiled for 20 minutes. Dilution of clarified solution was used to determine the intracellular protein concentration using Bradford method (1976). Bovine serum albumin was used as standard.

Physicochemical properties of α-amylase. Enzyme activity at various temperatures and pH was studied by incubating reaction mixtures at different temperatures (30–80°C) and Tris-HCl buffer (pH 4.0–9.0). Enzyme stability at various temperatures was also studied by pre-incubating cell-free supernatants for different time (1–6 h) at various temperatures (50°C–80°C). The effect of metal salts (NaCl, $CoCl_2$ and $CaCl_2$) and EDTA on activity was determined by adding of different concentrations of each salt to the standard assay. Activities were expressed as a percentage of the maximal activity.

Ammonium sulphate precipitation of the enzyme. The supernatant of culture was brought to 70% ammonium sulphate saturation in an ice bath. The precipitated protein was collected by centrifugation at 3000×g at 4°C and dissolved in 1–2 pellet volumes of phosphate buffer (0.1 M, pH 7.0). The enzyme solution was dialyzed overnight at 4°C against the same buffer then concentrated over sucrose bed. The final enzyme solution was taken for polyacrylamide gel electrophoresis (PAGE).

Electrophoresis and molecular weight determination. Nondenaturing PAGE was carried out by omitting SDS from the method of Laemmli (1970) with 10% polyacrylamide. The reference pre-stained protein marker (Molecular weights 10 to 170 kDa, SM 0671, Fermentas) was used. Amylase activity of proteins was detected according to Garcia-Gonzalez *et al*., (1991) by incubating the gels at 50°C for 20 min in 0.2 M phosphate buffer (pH 7.0) containing 2% starch and then immersing in staining solution (KI 13 g/l and I_2 6 g/l). The gel was destained with distilled water. The stain was stable for only a few minutes.

Statistical analysis. Data were statistically analyzed for variance and the least significant difference (LSD at 0.01 level) using one-way analysis of variance (ANOVA). A software system SPSS version 15 was used.

Results

The production of α-amylase by *T. vulgaris* increased significantly during the growth of the organism, with the maximum production after 24 hours (1.03 ± 0.09 U/ml). After 36 hours the activity was reduced rapidly by 72.8% (Fig. 1). Although there was

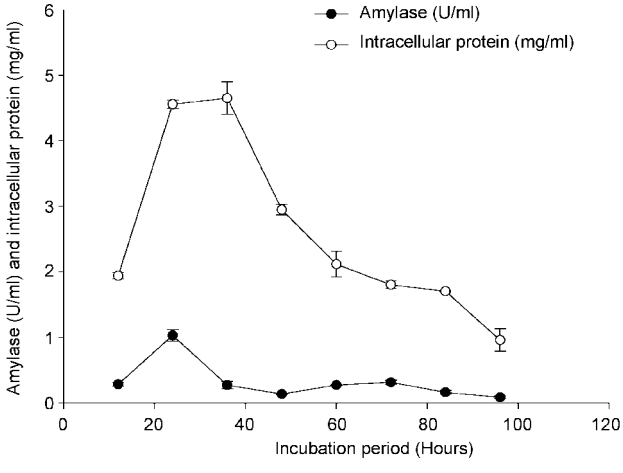

Fig. 1. Effect of incubation period on growth and α-amylase production by *T. vulgaris* grown in starch-nitrate medium containing 1.0 % starch.

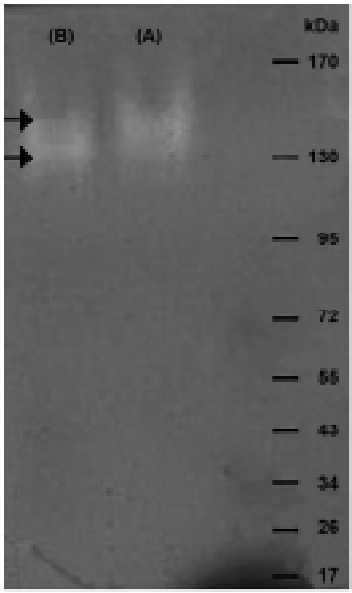

Fig. 2. Active-PAGE of α-amylase profile of *T. vulgaris* grown in starch-nitrate medium containing 1.0 % starch after 24 hours (A), and 72 hours (B).
Arrows indicate the position of the two α-amylase isozymes of approximate size between 135–145 kDa.

a significant increase in the production after 72 hours, the α-amylase profile; on active PAGE didn't differ from that at 24 hours (Fig. 2) as they produce two α-amylase isozymes with approximate molecular weight 135–145 kDa.

The optimum temperatures for the production of α-amylase were in the range 45°C to 55°C with non significant difference in this range. Below 45°C, the organism could grow weakly but no activity could be detected (Fig. 3).

Maximum production occurred in the pH range of 6.0 to 7.0 (3.5 U/ml), increasing the pH above 7.0 induced a significant decrease in the yield. At pH 10.0

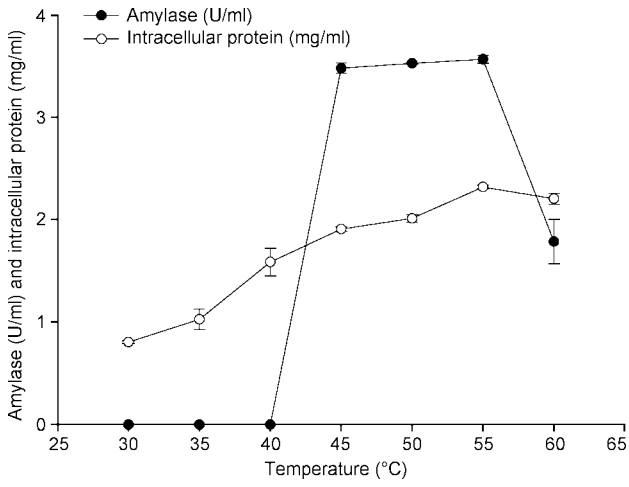

Fig. 3. Effect of temperature on growth and α-amylase production by *T. vulgaris* grown in starch-nitrate medium containing 1.0% starch.

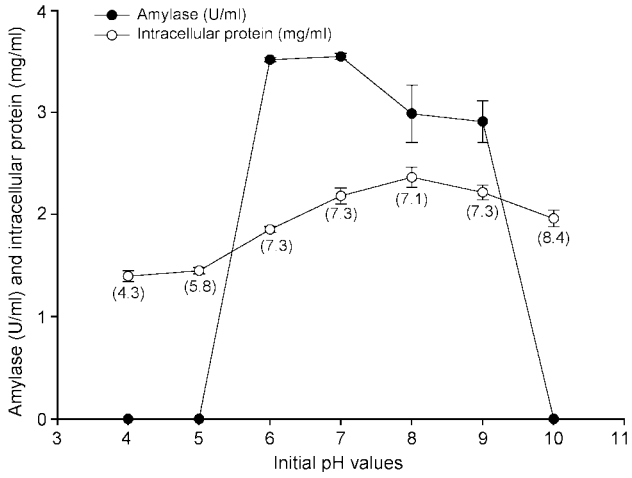

Fig. 4. Effect of pH on growth and α-amylase production by *T. vulgaris* grown in starch-nitrate medium containing 1.0 % starch
( ) denotes the final pH.

and below pH 6, the production of the enzyme was completely inhibited (Fig. 4).

T. vulgaris was able to grow well using different carbon sources (1% w/v), namely, starch, cellulose, glucose, galactose, xylose, arabinose, lactose and maltose. However, the used carbon sources other than starch induced an extremely significant decrease in the enzyme yield (Table I). Although different carbon sources affected the quantity of the enzyme production, it didn't affect its isozymal pattern as they produced the same two α-amylase isozymes of approximate molecular weight 135–145 kDa (data not shown).

Tryptone, bactopeptone, yeast extract and NH_4Cl caused a highly significant increasing in enzyme production comparing with KNO_3 of the basal medium (Table II). The highest production of α-amylase (93.81 ± 0.20 U/ml) was recorded with tryptone and the lowest (1.86±0.16) was recorded with $NaNO_3$. The different used nitrogen sources (NH_4Cl, NH_4NO_3, $NaNO_3$, KNO_3, ammonium acetate, bactopeptone, yeast

Table I
Effect of carbon sources on growth and α-amylase production by *T. vulgaris* grown in starch-nitrate medium containing different carbon sources instead of starch

Carbon sources (1.0%) (w/v)	Amylase (U/ml)	Intracellular protein (mg/ml)
Starch	3.43 ± 0.02	1.06 ± 0.04
Glucose	0.04 ± 0.02	0.78 ± 0.02
Galactose	0.02 ± 0.01	0.86 ± 0.08
Xylose	0.10 ± 0.06	0.76 ± 0.02
Arabinose	0.07 ± 0.02	0.76 ± 0.01
Lactose	0.11 ± 0.02	0.63 ± 0.02
Maltose	0.36 ± 0.05	0.71 ± 0.04
Cellulose	0.05 ± 0.03	0.52 ± 0.01

± standard error

Table II
Effect of nitrogen sources on growth and α-amylase production by *T. vulgaris* grown in starch-nitrate medium containing different nitrogen sources instead of KNO_3

Nitrogen sources	Final PH	Amylase (U /ml)	Intracellular protein (mg/ml)
Ammonium acetate	6.71	1.06 ± 0.15	6.46 ± 0.11
Tryptone	6.75	1.10 ± 0.12	93.81 ± 0.20
Yeast extract	6.87	0.72 ± 0.03	45.29 ± 0.08
Bactopeptone	6.64	0.93 ± 0.07	63.05 ± 0.63
NH_4Cl	6.46	0.66 ± 0.06	21.56 ± 3.64
NH_4NO_3	6.40	0.95 ± 0.03	6.34 ± 0.17
$NaNO_3$	6.81	0.80 ± 0.01	1.86 ± 0.16
KNO_3	6.73	0.53 ± 0.02	3.54 ± 0.01

± standard error

extract and tryptone) didn't have any effect on the enzyme electrophoretic profile. The α-amylase profile had the same previous pattern of two α-amylase isozymes at about 135–145 kDa (data not shown).

Physicochemical properties of α-amylase. The α-amylase activity couldn't be detected at pH 4.0 but it increased significantly with the increase of the pH until it reaches its optimum point at pH 8.0 (Fig. 5). The optimum activity occurred at temperature range between 50°C and 60°C, with an optimum point of 60°C (Fig. 5). The minimum level of the activity (54.4 ± 2.3 U/ml) was occurred at 80°C.

The enzyme was stable at 50°C retaining about 80% of its activity over 6 hours incubation period, but at higher temperature the activity of the enzyme declined (Fig. 6). At 60°C and 70°C, the enzyme has a half-life of about one hour. However, at 80°C it retained more than 20% of its activity up to 4 hours. At 90°C the enzyme lost its activity rapidly.

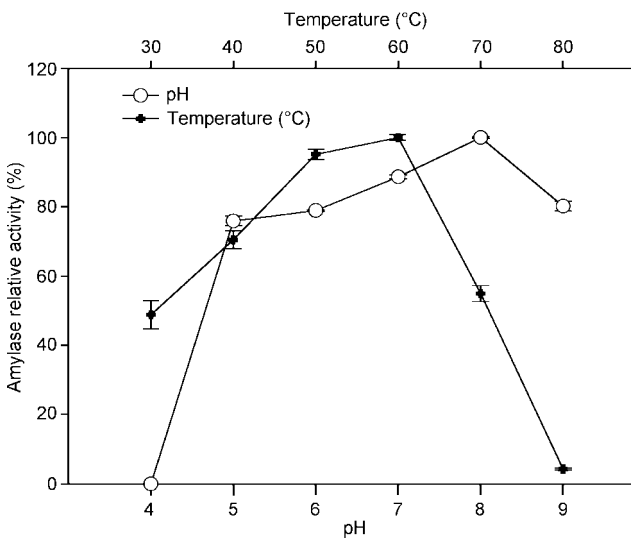

Fig. 5. Effect of temperature and pH on α-amylase activity.
The enzyme activities are represented relative to the maximal value.

Table III
Effect of various EDTA concentrations on α-amylase activity. The enzyme activities are represented relative to the control activity

Concentration of EDTA (mM)	Amylase relative activity (%)
0.001	99.62 ± 4.46
0.01	104.36 ± 1.70
0.1	108.85 ± 0.10
0.5	53.59 ± 0.52
1	18.81 ± 5.78
5	1.12 ± 0.70
10	0.00 ± 0

± standard error

The enzyme was significantly influenced by the different metal salts including NaCl, $CoCl_2$ and $CaCl_2$ (Fig. 7). $CoCl_2$ induced significant inhibition of the enzyme activity. An amount of 1 mM of NaCl and $CaCl_2$ caused extremely significant decrease in enzyme

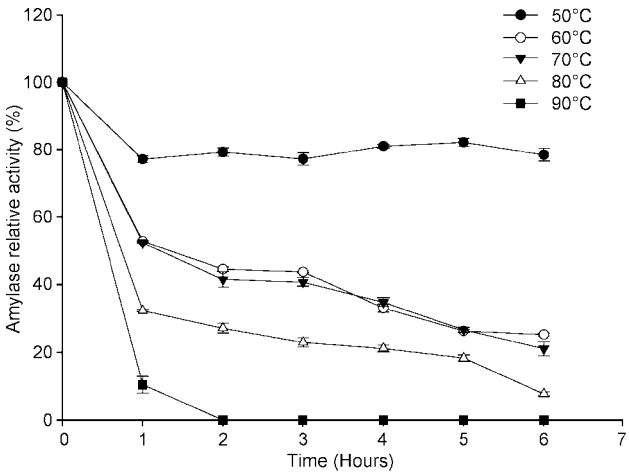

Fig. 6. Effect of temperature on the stability of α-amylase.
The enzyme activities are represented relative to the maximal value.

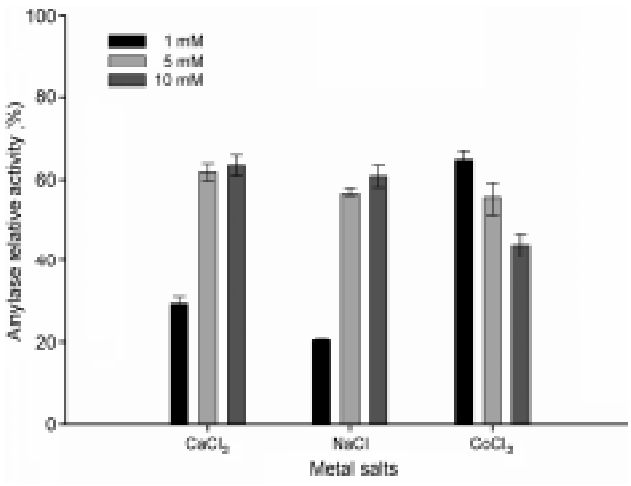

Fig. 7. Effect of various metal salts concentrations on α-amylase activity.
The enzyme activities are represented relative to the control activity.

activity by about 80% and 70% respectively. However, increasing of their concentration up to 10 mM of NaCl and $CaCl_2$ resulted in retaining 60.8% and 63.4% of its activity respectively. The enzyme activity was not affected by 0.001, 0.01 and 0.1 mM EDTA but it was totally inactivated by 10 mM EDTA (Table III). On the other hand, 0.5 mM EDTA was found to decrease the activity to 53.6% comparing with the control (96.6 Uml^{-1}).

Discussion

α-Amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α-amylase, microbial sources are used for the industrial production. This is due to their advantages such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization (Lonsane and Ramesh, 1990). Growth expressed as intracellular protein and α-amylase production by the thermophilic actinomycete *Thermoactinomyces vulgaris* reached maximum values after 24 hrs, after which, the activity was reduced rapidly by 72.8%. This might be corresponding to the rapid autolysis of *Thermoactinomyces* species (Lacey, 1971). However, it increased significantly at 60 and 72 hours. After 72 hrs, the production decreased gradually and reached its minimum recorded level at 96 hours. As the isozymal pattern was the same at 24 hrs and 72 hrs, the observed peaking and troughing of the production can be attributed to differential inhibition by products of substrate hydrolysis rather than the isozymes down or over expression. In support, studies on both glucoamylase and α-amylase indicate that inhibition does not occur below a critical concentration of product (Wang *et al*., 2006). These results indicated that the production of extracellular α-amylase by *T. vulgaris* was growth associated and this is in agreement with other investigators (Kuo and Hartman, 1966; Shimizu *et al.*, 1978; Murthy *et al*., 2009; Asoodeh *et al.*, 2010).

The influence of temperature on amylase production is related to the growth of the organism. The present results revealed an optimum yield of α-amylase at 45°C to 55°C. In spite of the relative good growth of the organism at 40°C, α-amylase activity couldn't be detected. This referred to the action of protease; that is rapidly inactivated at higher temperature (Behnke *et al*., 1982), which suppresses the amylolytic activity.

The pH change of the growth medium not only affected the growth and α-amylase secretion of *T. vulgaris* but also influence the enzyme stability in the medium. Therefore, the difference of the final pH of the medium from pH 10.0 to pH 6.0 could explain the complete inhibition of the enzyme activity at pH 10.0 in contrast to the optimum production at pH 6.0 in spite of the same ability of the organism to grow.

The results confirms the inducibility nature of the α-amylase when different carbon sources were used and compared. In support, starch is known to induce amylase production in different bacterial strains (Aiyer, 2004; Ryan *et al*., 2006; Asoodeh *et al.*, 2010). The α-amylase production is also appeared to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Bhella and Altosaar, 1985; Morkeberg *et al.*, 1995). Other carbon sources have been found to be strongly repressive although they supported good growth.

Among nitrogen sources, organic nitrogen sources have been preferred for the production of α-amylase, with ammonium acetate as an exception. It was recorded that organic nitrogen sources supported maximum α-amylase production by various bacteria and fungi due to their high nutritional amino acids and vitamins content (Gupta *et al*., 2003). The role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms have been reported to be highly variable (Gupta *et al*., 2003). Tryptone was the best nitrogen source that increased the productivity of α-amylase by 26.5 fold comparing to KNO_3. Ammonium chloride induced a significant increase in the enzyme yield that is higher than that of the ammonium acetate. Similar effect of ammonium chloride was obtained for *B. subtilis* DM-03 (Das *et al.*, 2004). Although the different nitrogen sources had a variable effect on α-amylase activity, they did not produce different isozymal profile. This indicate that they have no effect on the isozymal α-amylase over expression.

Most raw starch degrading enzymes had optimum pH in the acidic to neutral range (Pandey *et al.*, 2000; Sun *et al.*, 2010). In the present work, α-amylase from *T. vulgaris* showed a pH activity profile with a flat top which retaining more than 75% of the enzyme activity in the pH range 5.0–9.0, despite it was completely inhibited at pH 4.0. This pH profile could be attributed to the limitation of the enzyme catalysis by protonation of the nucleophile at low pH and by deprotonation of the hydrogen donor at high pH values (Nielsen *et al.*, 2001). At reaction temperature of 60°C, the enzyme expresses maximum activity whereas it showed less than 50% of its activity at 30°C. This reduced activity might be attributed to the reduced molecular flexibility of the thermophilic protein under mesophilic conditions. Although the enzyme showed only 4% of its activity at 80°C, it showed more than 20% of its activity at 50°C after being kept at 80°C for 4 hrs indicating inhibition of the enzyme catalysis

at 80°C rather than inactivation of the enzyme. In general, α-amylase from *T. vulgaris*, in the present work, is fairly stable at 50°C over 6 hrs and with a half-life of about one hour at both 60°C and 70°C.

It is proposed that α-amylases belong to a new class of metallo-enzymes characterized by a prosthetic group, *i.e.*, an alkaline-earth metal rather than a transition element, and which plays primarily a structural role (Prakash and Jaiswal, 2010). At different concentration of $CoCl_2$, a significant inhibition in the enzyme activity was recorded. The Co^{2+} effect was explained by Leveque *et al.*, (2000) to be a result of competition between the exogenous cations and the protein-associated cation. In spite of the important role of both calcium and sodium ions to retain the structure and function of α-amylases (Prakash and Jaiswal, 2010), α-amylase in the present study was strongly inhibited by both 1 mM $CaCl_2$ and NaCl. Similar inhibitory effect of $CaCl_2$ was reported to amylase of *Aspergillus oryzae* EI 212 (Kundu *et al.*, 1973). Calcium independent α-amylase is suitable for the manufacture of fructose syrup, where Ca^{2+} is an inhibitor of glucose isomerase (Tonkova, 2006).

Increasing the concentration of $CaCl_2$ and NaCl was found to retain the enzyme activity, assuming that Cl^- ion has a stabilizing role. Chloride ions have been found mainly in the active site of mammalian α-amylases, which have been shown to enhance the catalytic efficiency of the enzyme (Prakash and Jaiswal, 2010). In accordance with retaining the enzyme activity at high concentration of $CaCl_2$ and NaCl, the enzyme yield when using ammonium chloride was found to be 3.3 fold of that using other sources of ammonium *i.e.*, NH_4NO_3 and ammonium acetate. Regarding to the effect of EDTA, the enzyme retained almost 100% activity when 0.1 mM EDTA was added to the reaction mixture. But 0.5 mM EDTA inhibited the enzyme activity retaining only 53% of its activity. The inhibitory effect of EDTA was also documented by many studies (Gupta *et al*., 2003). Although the molecular weights of microbial α-amylases are usually range between 50 to 60 kDa (Vihinen and Mantsala, 1989), the present result revealed a highly molecular weight of two α-amylase isozymes (135–145 kDa). In support, *Thermoactinomyces vulgaris* R-47 was reported to produce two α-amylases, TVAI and TVAII with molecular weights of 71 kDa and 67.5 kDa respectively (Ohtaki *et al.*, 2003). However, molecular weight of some α-amylases was found to rise owing to carbohydrate moieties (Gupta *et al*., 2003). In conclusion, the present results indicated a new active highly molecular weight, thermostable and calcium independent α-amylase of *T. vulgaris* which could be of importance for the starch-processing industries. Further work is in progress to purify the α-amylase of *T vulgaris* and characterize the purified enzyme.

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