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Full Length Research Paper

Production, purification and characterization of α-amylase from *Trichoderma harzianum* grown on mandarin peel

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The production, purification and characterization of α-amylase from *Trichoderma harzianum* grown on mandarin peel were investigated. The effect of incubation time and mandarin peel concentration on the production of α-amylase by *T. harzianum* was studied. α-Amylase A3 was purified from *T. harzianum* to electrophoretic homogeneity by using DEAE-Sepharose and Sephacryl S-200 columns. The enzyme had molecular weight of 70 kDa using gel filtration and SDS-PAGE. The affinity of the substrates toward A3 was in the order of amylopectin > glycogen > starch > β-cyclodextrin > dextrin > α-cyclodextrin. These findings tend to suggest that the enzyme has high affinity toward high-molecular mass substrates. The K_m and V_{max} values of the enzyme for hydrolyzing potato soluble starch and glycogen were 6.53, 4.5 mg/ml and 2 and 2.2 µmol reducing sugar/ml, respectively. The maximum activity of enzyme against soluble starch was determined at pH 4.5 and 40°C. α-Amylase A3 was stable up to 40°C for 30 min of incubation and retained 70 and 50% of its activity at 50 and 60°C, respectively. While all the examined metal cations were effective in inhibiting the enzyme, Ca^{2+} considerably enhanced the activity. The metal chelators, EDTA, sodium citrate and sodium oxalate had inhibitory effects on A3. The rate of breakdown of starch was higher than the rate of formation of reduced sugar indicating A3 is endo-acting enzyme. These properties of A3 with its remarkable activity meet the prerequisites needed for liquefaction and saccharification of starch industry.

Key words: *Trichoderma harzianum*, mandarin, α-amylase, purification, characterization.

INTRODUCTION

Amylases are widespread in animals, fungi, plants, and are also found in the unicellular eukaryotes, bacteria and archaea (da Lagea et al., 2007). Though plants and animals produce amylases, enzymes from microbial sources are generally used in industrial processes. This is due to a number of factors including productivity, thermostability of the enzyme as well as ease of cultivating microorganisms (Reddy et al., 1999). The major advantages of the enzymatic route are the selectivity with its associated high yield and exclusivity toward the desired product (Kim and Dale, 2004). Bacteria used in commercial production are the *Bacillus* spp. (Olafimihan and Akinyanju, 1999; Pandey et al., 2000; Gupta et al., 2003). Others, such as *Escherichia* spp, *Pseudomonas*, *Proteus*, *Serratia* and *Rhizobium* also yield appreciable quantity of the enzyme (Oliviera et

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al., 2007). *Aspergillus*, *Rhizopus, Mucor, Neurospora, Penicillium* and *Candida* are some of the fungi that also produce extracellular amylases of commercial value (Gupta et al., 2003). Plant sources had not been considered with enough significance as the source of these enzymes yet (Azad et al., 2009).

The utilization of agriculture waste materials serves two functions: reduction in pollution and upgrading of these materials. Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. These nutrient sources included pearl millet starch, orange waste, potato, corn, tapioca, wheat and rice as flours were used for α-amylase production (Djekrif-Dakhmouche et al., 2006; Haq et al., 2005).

α-Amylases are produced commercially in bulk from microorganisms and represent about 25-33% of the world enzyme market (Nguyen et al., 2002). They had numerous applications including liquefaction of starch in the traditional beverages, baking and textile industry for desizing of fabrics (Dauter et al., 1999; Hendriksen et al., 1999; Nielsen and Borchert, 2000). Moreover, they have been applied in paper manufacture, medical fields as digestives and as detergent additives (Bruinenberg et al., 1996; Mitidieri et al., 2006).

The aim of this study was to produce and purify appreciable level of α-amylase from *Trichoderma harzianum* cultured on mandarin peel as fruit waste. Displaying new characters of α-amylase which may meet the prerequisites needed for several applications especially starch industry.

MATERIALS AND METHODS

Organism

T. harzianum (CECT 2413) was obtained from Plant Pathology Department of the National Research Centre, Cairo, Egypt.

Chemicals

Potato starch, glycogen, dextrin, amylopectin, α-cyclodextrin, β-cyclodextrin, sodium dodecyl sulphate (SDS), molecular weight markers for gel filtration and all resins and reagents for electrophoresis were obtained from Sigma Chemical Co. (St Louis, Mo). Sephacryl S-200, diethylaminoethyl (DEAE)-Sepharose and molecular weight markers for SDS-polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals. Other chemicals were of analytical grade.

Cultivation and culture conditions

Medium

T. harzianum (CECT 2413) was cultivated and maintained on slants of potato dextrose agar (PDA 4.3%) powder from Defeco Company (USA). A liquid medium used for growing the organism was 5% mandarin peel.

Cultivation of organism

Conidia were scrapped from mycelia which were grown on slants for five days at 28°C and suspended by hand shaking in sterile distilled water. 1 ml aliquot of this suspension was used to inoculate under aseptic conditions, 250 ml Erlenmeyer flasks each containing 100 ml of sterile medium. The inoculated flasks were incubated at 30°C on a rotary shaker at 200 rpm for 5 to 7 days.

Purification of *T. harzianum* α-amylase

Preparation of cell-free broth

The culture filtrate of *T. harzianum* was concentrated by using lyophelization to concentrate large volumes of culture filtrate, then dialyzed against 50 mM Tris-HCl buffer, pH 7.2. The dialyzed filtrate was centrifuged at 10,000 rpm for 20 min at 4-7°C and the supernatant was pooled and designated as cell-free broth. The cell free broth was frozen at -20°C for further purification steps.

DEAE- Sepharose column

The cell-free broth was loaded on a DEAE- Sepharose CL-6B column (10 x 1.6 cm i.d.) equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting α-amylase activity were pooled in three peaks (A1, A2 and A3).

Sephacryl S-200 column

α-Amylase A3 containing the highest activity was concentrated through ultrafiltration membrane cut-off 10 kDa and loaded on Sephacryl S-200 column (90 x 1.6 cm i.d.) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 and developed at a flow rate of 30 ml/h and 3 ml fractions were collected.

Protein determination

Protein concentration was measured according to Bradford (1976) with bovine serum albumin as standard.

α-Amylase assays

Two assays were performed for determination of α-amylase activity. The first assay (dinitrosalicylic acid assay) was used for the experiments of purification and characterization of enzyme. The second assay (starch-iodine assay) was used for the mode of action of the enzyme.

Dinitrosalicylic acid assay

α-Amylase activity was determined by measurement of maltose released from starch according to the method of Miller (1959). The reaction mixture was incubated at 37°C for 30 min in tubes containing 5 mg soluble potato starch, 50 mM sodium acetate

buffer, pH 4.5, appropriate amount of enzyme solution and distilled water to give a final volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml dinitrosalicylic acid reagent, followed by incubation in a boiling water bath for 10 min followed by cooling. The absorbance was recorded at 560 nm. The enzymatically liberated reducing sugar was calculated from a standard curve using maltose. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol reducing sugar as maltose per hour under the standard assay conditions.

Starch-iodine assay

α-Amylase activity was measured by the decreasing of blue colour after action of the enzyme on soluble starch according the method of Jones and Varner (1967). The iodine stock solution was prepared by mixing 6 g of potassium iodide and 0.6 g of iodine in 100 ml of distilled water. 1 ml of stock solution was added to 99 ml of 0.05 N hydrochloric acid. This is used to stop the α-amylase activity and giving the colour reaction.

The reaction mixture was incubated at 37°C for 10 min in tubes containing 10 mg soluble potato starch, 50 mM sodium acetate buffer, pH 4.5, appropriate amount of enzyme solution and distilled water to give a final volume of 1 ml. The reaction was stopped and the colour was developed by addition of 1.0 ml of diluted iodine reagent. The absorbance was recorded spectrophotometrically at 620 nm.

Molecular weight determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 x 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and *β*-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and *α*-lactalbumin (14,200) were used for the calibration curve.

Characterization of α-amylase

K_m

The K_m values were determined from Lineweaver-Burk plots by using starch and glycogen concentrations from 1-10 mg/ml.

Optimum pH

α-Amylase activity was determined at various pH using different buffers, sodium acetate (pH 4.0-6.0) and Tris-HCl (6.5-8.5) at 50 mM concentration. The maximum activity was taken as 100% and percent relative activity plotted against different pH values.

Optimum temperature

α-Amylase activity was determined at a temperature range of 20-70°C. The maximum activity was taken as 100% and percent relative activity were plotted against different temperatures.

Thermal stability

The enzyme was incubated at a temperature range of 20-70°C for 30 min prior to substrate addition. The enzyme activity at zero time was taken as 100% and percent relative activity was plotted against different temperatures.

Effect of metal ions

The enzyme was incubated with 2 mM solution of Co^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Hg^{2+} for 15 min prior to substrate addition. The enzyme activity without metal ions was taken as 100% and percent relative activity was determined in the presence of metal ions.

Effect of metal chelators

α-Amylase activity was determined in the presence of metal chelators, EDTA, sodium citrate and sodium oxalate at a concentration of 2-10 mM. The enzyme activity without metal chelators was taken as 100% and percent relative activity was determined in the presence of metal chelators.

Mode of action

The enzyme (1 unit) was added to 10 ml reaction mixture containing 100 mg soluble starch and samples were removed at fixed time intervals. The starch-iodine colour and reducing sugar were determined accordingly.

RESULTS AND DISCUSSION

Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. In the present study, production, purification and characterization of α-amylase from *T. harzianum* cultured on mandarin peel were studied.

Production of α-amylase from *T. harzianum* cultured on mandarin

The results on the time-course studies on α-amylase production of *T. harzianum* grown on 5% mandarin peel are shown in Figure 1. α-Amylase production peak (84 units/ml) was obtained on fourth-day old culture and declined gradually up to seventh-day old culture. Similarly, α-amylase activity from *Asperigllus niger* (Hernandez et al., 2006) and *Asperigllus oryzae* (Tiwari et al., 2007) was obtained at 3-day of cultivation. The reason for the maximum activity obtained at 3-day of cultivation and then decrease in enzyme yields might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium (Ramachandran et al., 2004). The effect of concentration of mandarin peel on α-amylase production from *T. harzianum* was measured on fourth-day old culture (Figure 2). The highest level of enzyme activity was obtained at 5% mandarin peel (90 units/ml). The high concentration of mandarin peel repressed the enzyme

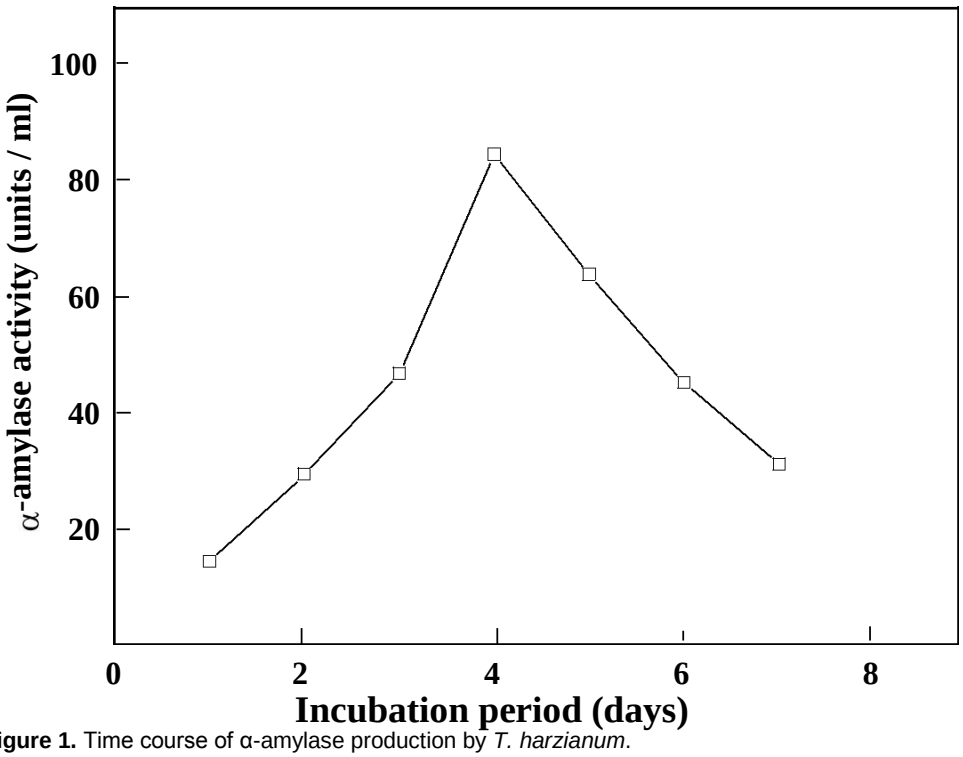

Figure 1. Time course of α-amylase production by *T. harzianum*.

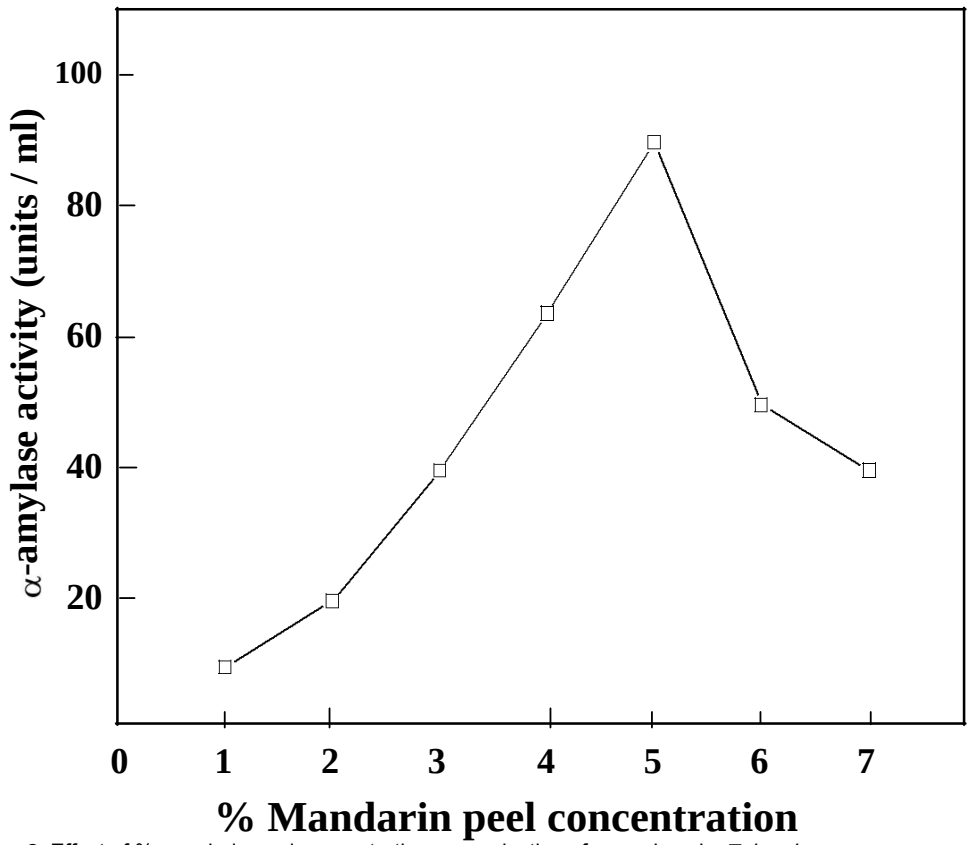

Figure 2. Effect of % mandarin peel concentration on production of α-amylase by *T. harzianum*.

Table 1. Purification scheme for *T. harzianum* α -amylase.

Step	Total protein (mg)	Total activity (units)*	S.A* (units/ mg protein)	Fold purification	Recovery (%)
Cell-free broth	1.7	860	505	1.0	100
Chromatography on DEAE- Sepharose					
0.05 M NaCl (A1)	0.03	30	1000	1.98	3.4
0.1 M NaCl (A2)	0.08	16	200	0.39	1.8
0.2 M NaCl (A3)	0.25	434	1736	3.43	50.4
Gel filtration on Sephacryl S-200					
α-Amylase (A3)	0.15	339	2260	4.47	39.4

* One unit of αamylase activity was defined as the amount of enzyme producing 1 μmol maltose per h under standard assay conditions.
* S.A: Specific activity.

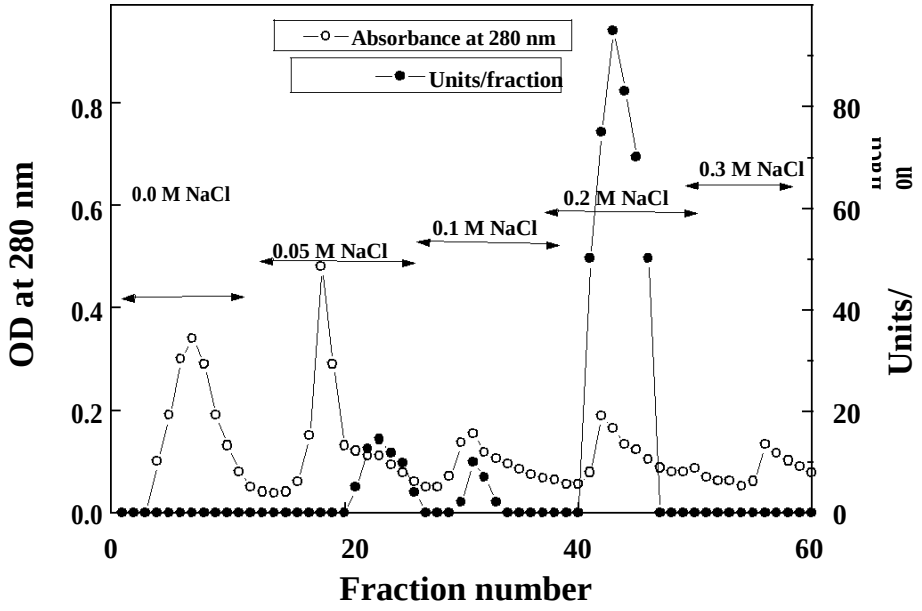

Figure 3. A typical elution profile for the chromatography of *T. harzianum*α-amylase on DEAE-Sepharose column (10 x 1.6 cm i.d.) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

production.

Purification of α-amylase from *T. harzianum*

The purification procedure of α-amylase from *T. harzianum* included chromatography on DEAE-Sepharose and Sephacryl S-200 columns. The purification was summarized in Table 1. The cell-free broth was applied onto DEAE-Sepharose column. From the elution profile of chromatography on DEAE-Sepharose column, three α-amylases A1, A2 and A3 were separated (Figure 3). The completion of purification was restricted on α-amylases A3 with highest α-amylase activity. A Sephacryl S-200 column was used to obtain A3 with the highest possible specific activities of 2260 units/mg protein which represented 4.47 fold purification over the cell-free broth with 39.4% recovery (Figure 4). Similar specific activity of αamylases from *Scytalidium thermophilum* (2450 units/ mg protein) was detected (Roy et al., 2000). The lowest specific activity ofα-amylases from *Acremonium sporosulcatum* (55.35 units/ mg protein) (Valaparla, 2010) and *Thermobifida fusca* (245 units/ mg mg protein) (Yang and Liu, 2004) was reported. α-Amylases from *Aspergillus flavus* (6471 units/mg protein) had highest specific activity (El-Safey and

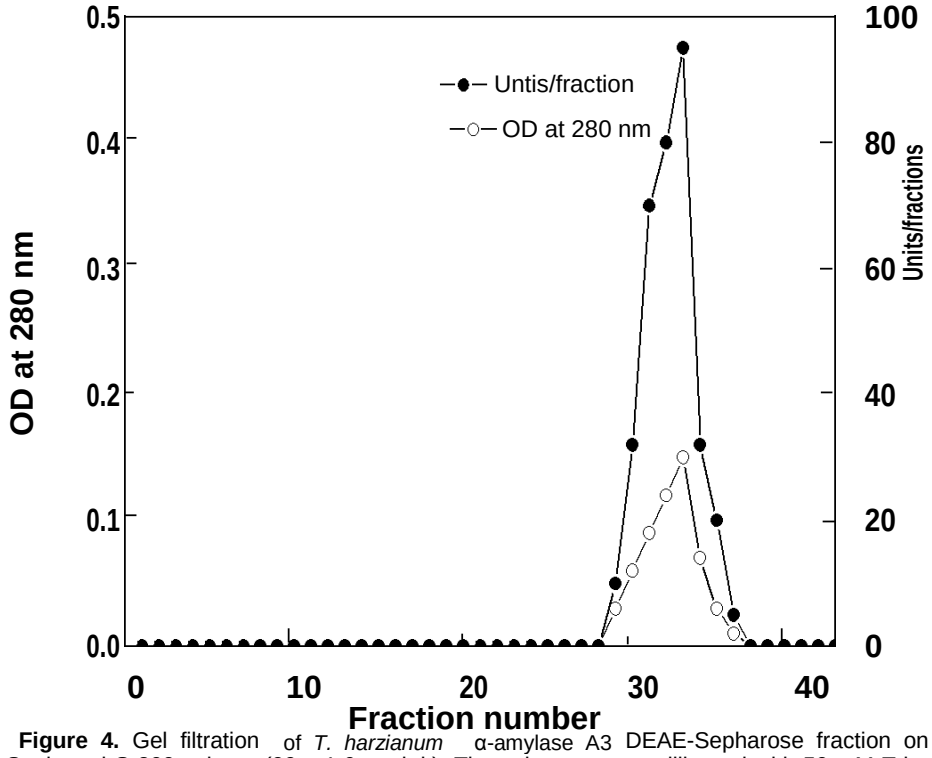

Figure 4. Gel filtration of *T. harzianum* α-amylase A3 DEAE-Sepharose fraction on Sephacryl S-200 column (90 x 1.6 cm i.d.). The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions.

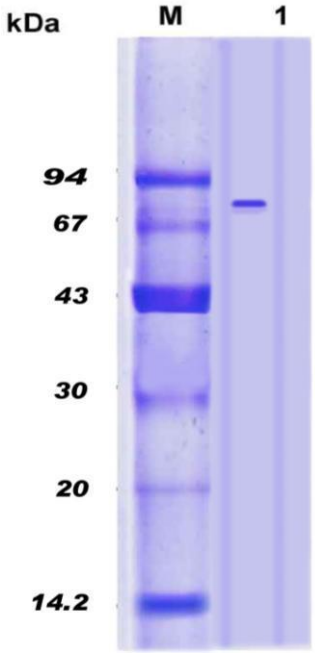

Figure 5. SDS-PAGE for homogeneity and molecular weight determination of *T. harzianum* α-amylase A3. M-Protein markers; 1- Sephacryl S-200 A3.

Ammar 2004). The pooled active fractions of Sephacryl S-200 column were concentrated by dialysis against solid sucrose before examining the homogeneity. The homogeneity of the purified A3 was detected by SDS-PAGE. A3 was demonstrated by the presence of one single protein band (Figure 5).

Molecular weights of α-amylases vary from about 10 to 210 kDa. The lowest value, 10 kDa for *Bacillus caldolyticus* (Grootegoed et al., 1973) and the highest of 210 kDa for *Chloroflexus aurantiacus* (Ratanakhanokchai et al., 1992) had been reported. The native molecular weight of *T. harzianum*α-amylase A3 was determined by gel filtration and found to be 70 kDa. This value was confirmed by SDS-PAGE, and appeared as single subunits (Figure 5). Similar molecular weights were reported for α-amylases from *Aspergillus chevalieri* (68 kDa) (Olutiola and Nwaogwugwu, 1982) and *perfringens* (76 KDa) (Shih and Labbe, 1995).

Characterization of α-amylase A3

The substrate specificity of α-amylase varies from microorganism to another. In the present study, *T. harzianum* α-amylase A3 hydrolyzed soluble starch (Glu α 1,4 Glu-Glu 1, 6 Glu), amylopectin (Glu α1,4 Glu-Glu 1, 6 Glu), glycogen (Glu α1, 4Glu-Glu 1, 6 Glu),

Table 2. Relative activities of *T. harzianum* -amylase A3 toward different substrates.

Substrate	% Relative activity
Potato starch	100
Amylopectin	450
Glycogen	274
β-Cyclodextrin	28
Dextrin	22
α-Cyclodextrin	4

Each value represents the average of two experiments.

β-cyclodextrin, dextrin and α-cyclodextrin (Glu α 1,4 Glu) at a relative rate of 100, 450 and 274, 28, 22 and 4%, respectively (Table 2). These findings tend to suggest that the enzyme had high affinity toward high-molecular mass substrates (starch, amylopectin and glycogen) containing α-1,4 and α-1,6 linkages. However, alkaliphilic *Bacillus* -amylase hydrolyzed soluble starch, amylopectin, glycogen and dextrin at a relative rate of 100, 114, 37 and 7, respectively (Igarashi et al., 1998). Paquet et al. (1991) reported that the best substrates for *Clostridium acetobutylicum* -amylase were amylopectin and amylose, while glycogen and pullulan were hydrolyzed at a lower rate and the enzyme hydrolyzed neither dextran nor cyclodextrins. In contrast, the substrate specificity of amylases from *Bacillus amyloliquefaciens* showed maltopentaose (low-molecular mass substrate) as the best substrate and α-amylase of the mutant type showed difference from the wild type and this amylase was more active against large substrates such as amylopectin and soluble starch containing α-1,6 and 1,4 glycosidic linkages (Demirkan et al., 2005).

The kinetic parameters of *T. harzianum* α-amylase A3 were studied on starch and glycogen as substrates. The K_m and V_{max} values of the enzyme for hydrolyzing potato soluble starch and glycogen were 6.53, 4.5 mg/ml and 2 and 2.2 µmol reducing sugar/ml, respectively (Figures 6a and b). Low K_m values were reported for α-amylases from *Thermobifida fusca* (0.88 mg/ml) (Yang and Liu, 2004) and *Bacillus subtilis* (0.22 mg/ml) (Uyar et al., 2003). Ezeji and Bahi (2006) detected high value of K_m of α-amylase from *Geobacillus thermodenitrificans* (3.05 mg /ml).

T. harzianum α-amylase A3 exhibited temperature optimum at 40°C, and the enzyme showed 92 and 50% activity at 50 and 60°C, respectively (Figure 7). The same temperature optimum was reported for α-amylases from *Aspergillus awamori*, *A. chevalieri* and *A. oryzae* (Perevozchenko and Tsyperovich, 1972; Olutiola and Nwaogwugwu, 1982). The lowest temperature optimum was reported to be 25-30°C for *Fusarium oxysporum* amylase (Chary and Reddy, 1985). The highest temperature optima were recorded for amylases from *Thermococcus profundus* (80ºC) (Chung et al., 1995) and

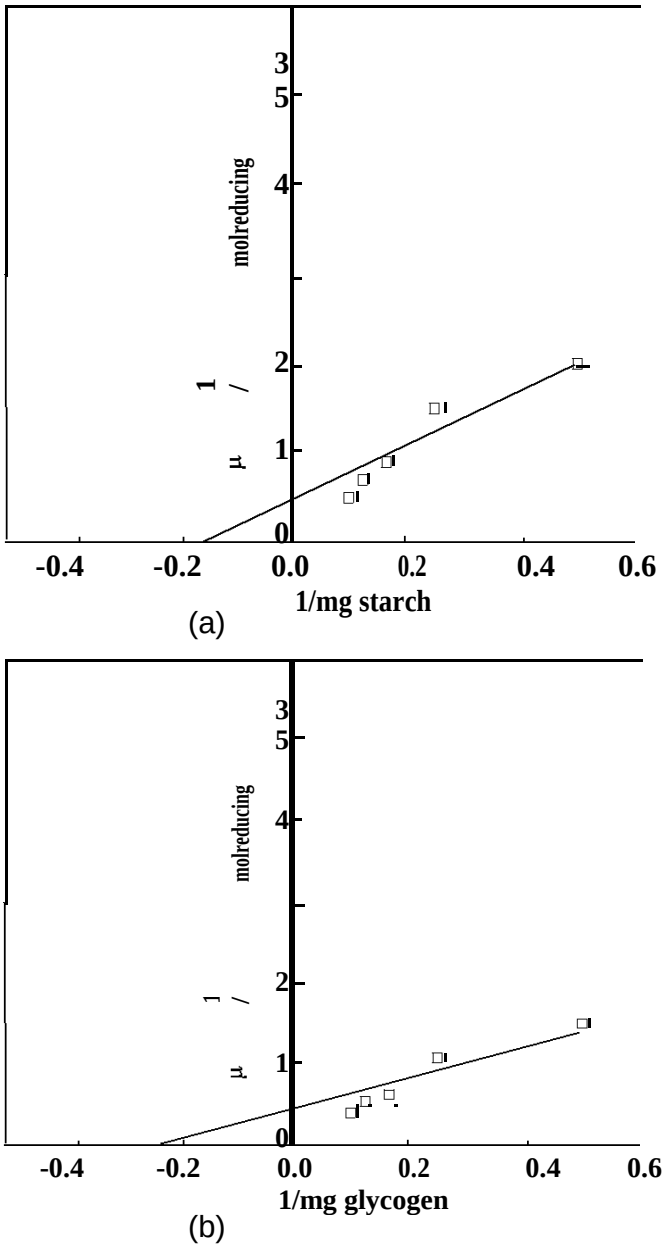

Figure 6. Lineweaver-Burk plot relating *T. harzianum* α-amylase A3 reaction velocities to (a) starch and (b) glycogen as substarte concentrations. The reaction mixture contained in 1 ml: 50 mM sodium acetate buffer, pH 4.5, suitable amount of enzyme and concentrations of substrate ranging from 1 mg to 10. Each point represents the average of two experiments.

Pyrococcus furiosus (100ºC) (Jorgensen et al., 1997). *T. harzianum* α-amylase A3 was stable up to 40°C after 30 min of incubation prior to substrate addition (Figure 8). The enzyme retained 70 and 50% of activity at 50 and 60°C, respectively. *Thermus* sp. α-amylase showed relatively high thermostability and retained about 80% of its original activity after heating at 60°C for 15 min (Shaw et al., 1995). Amylolytic activity of *Rhizopus microsporus*

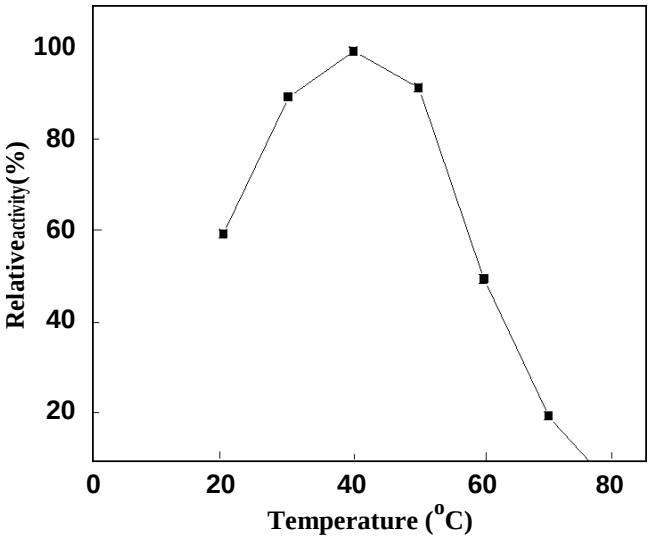

Figure 7. Temperature optimum of *T. harzianum*α-amylase A3. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments.

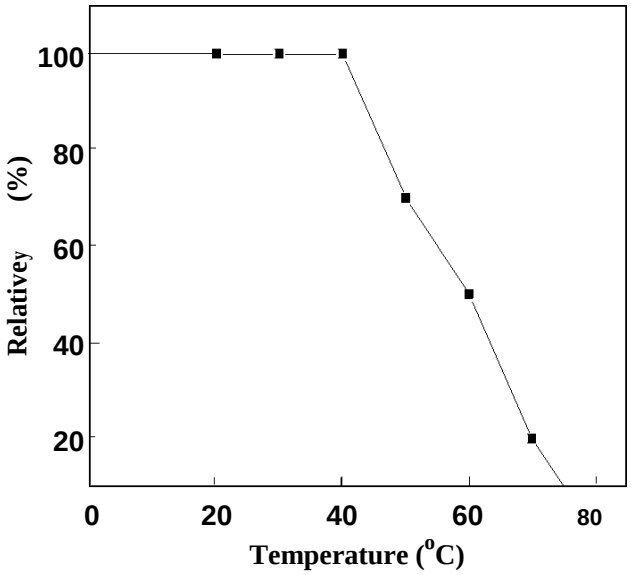

Figure 8. Effect of temperature on the thermal stability of *T. harzianum* α-amylase A3. The reaction mixture contained in 0.5 ml: 50 mM sodium acetate buffer, pH 4.5 and suitable amount of enzyme. The reaction mixture was preincubated at various temperatures for 30 min prior to substrate addition. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments.

was also rather stable, maintaining 50% activity after 120 min at 60°C (Peixoto et al., 2003). α-Amylases from most bacteria and fungi have pH optima in the acidic to neutral range (Vihinen and Mantsala, 1989). *T. harzianum*α-amylase A3 exhibited acidic Ph optimum at 4.5

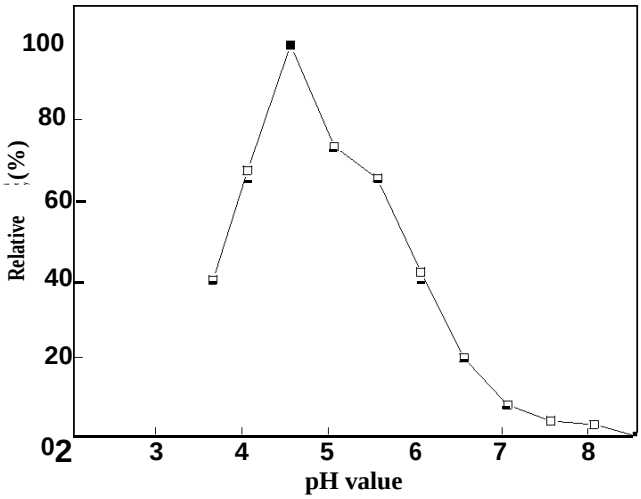

Figure 9. pH optimum of *T. harzianum*α-amylase A3. The reaction mixture contained in 0.5 ml: 1% starch, suitable amount of enzyme and 50 mM sodium acetate buffer (pH 3.6 - 6.0), 50 mM Trsi-HCl buffer (6.5 - 8.5). Each point represents the average of two experiments.

Table 3. Effect of metal cations on *T. harzianum* α-amylase A3.

Metal cation	Relative activity (%)
Ca^{+2}	145
Zn^{+2}	83
Ni^{+2}	77
Co^{+2}	26
Hg^{+2}	0.0
Cu^{+2}	0.0

Enzyme was preincubated for 15 min at room temperature with 2 mM of listed metal ions as final concentration prior to substrate addition. Activity in absence of metal cations was taken as 100% activity. Each value represents the average of two experiments.

(Figure 9). Similar pH optima were reported for α-amylases from *Lipomyces kononenkoae* (pH 4.0) (Prieto et al., 1995), *Aspergillus kawachii* (pH 5.0) (Sudo et al., 1994) and *Rhizopus microsporus* (pH 5.0) (Peixoto et al., 2003). As evident from the present results, *T. harzianum* α-amylase A3 had been highly active in acidic range, which is a good indication for its use in starch industry (Negi and Banerjee, 2009).

Stability of an enzyme in presence of metal salts plays a vital role in their application in industries. More than 75% of enzymes require the presence of metal ion activators to express their full catalytic activity (Tunga et al., 1999). In the present study, Ca^{2+} was found to enhance the activity of *T. harzianum* α-amylase A3 by 145% (Table 3). Amylolytic enzymes are metalloenzymes with up to six Ca^{2+} atoms at the active site whose catalytic activity can be affected by mono- and divalent metals (Normurodova et al., 2007). Ca^{2+} enhanced the

Table 4. Effect of chelating agents, EDTA, sodium citrate and sodium oxalate on *T. harzianum* α-amylase A3.

Concentration mM	% Relative activity		
	EDTA	Sodium citrate	Sodium oxalate
2	48	98	70
4	33	80	53
6	24	62	44
8	18	52	33
10	15	40	25

Activity in absence of metal chelators was taken as 100% activity. Each value represents the average of two experiments.

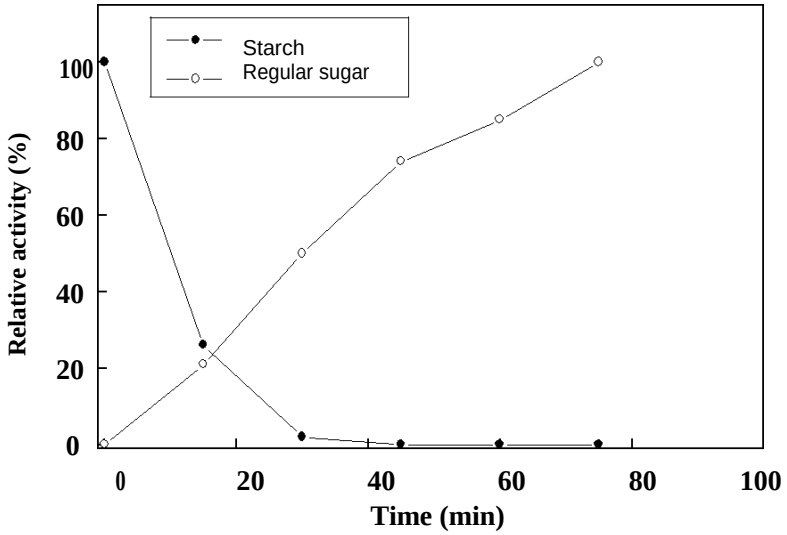

Figure 10. Pattern of starch disappearance and reducing sugar formation during the hydrolysis of starch by *T. harzianum* α-amylase A3. Each point represents the average of two experiments.

activity of α-amylases from *C. perfringens* (Shih and Labbe, 1995), *B. subtilis* (Asgher et al., 2007) and *Acremonium sporosulcatum* (Valaparla, 2010). *T. harzianum* α-amylase A3 was partially inhibited by Zn^{2+} and Ni^{2+}, while Co^{2+} had strong inhibitory effect. Complete loss of activity was detected by Hg^{2+} and Cu^{2+}. Yang and Liu (2004) showed that *T. fusca* amylase activity was completely inhibited by 1 mM Hg^{2+} indicating that sulfhydryl groups were concerned with the catalytic function of enzyme. The inhibition of *B. subtilis* JS-2004 α-amylase by Co^{2+}, Cu^{2+}, and Ba^{2+} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity (Leveque et al., 2000). Hernandez et al. (2006) reported that amylase activity was strongly inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+}.

Metal chelators caused inhibition effect for *T. harzianum* α-amylase A3 (Table 4). The order of inhibition effect was EDTA > sodium oxalate > sodium citrate. Most studies were concentrated on the effect of EDTA on the α-amylases. EDTA had inhibitory effect on α-amylase from *Geobacillus thermodenitrificans* (Ezeji and Bahi, 2006) and *P. rugulosum* (Tiwari et al. 2007). At 10 mM EDTA, no effect on *B. subtilis* amylase was detected (Najafi et al., 2005).

The action mode of the enzyme (exo- or endo- acting amylase) was examined by incubation of A3 with potato soluble starch for complete digestion. While the breakdown of starch started immediately revealing the disappearance of almost 95% starch, the rate of reducing sugar formation was slow during the first 30 min of incubation and increased gradually with incubation time. The results indicated that the degradation of starch was rapid than the formation of reducing sugars suggesting endo-acting enzyme (Figure 10). This result could classify the enzyme as α-amylase with ability for liquefaction and saccharification. Similar results were reported by Shih and Labbe (1995) which found that the degradation of starch by purified *C. perfringens* amylase yielded products with a high degree of starch hydrolysis but low levels of reducing sugars indicating an endo-hydrolysis pattern. As expected, exo-acting enzymes like

amylase and amyloglucosidase gave high amounts of reducing sugar but low degrees of starch hydrolysis. Low concentration of the *B. subtilis* amylase could convert soluble starch to low molecular oligosaccharides with rapid reduction in blue color and appearance of reducing sugar, suggesting an endo mode of action (Najafi et al., 2005).

Conclusion

This study developed and optimized a fermentation process for the production of α-amylase from *T. harzianum,* which has long history of safe use in food industry, cultured on mandarin peel as fruit waste. The use of fruit wastes lead to reduction in pollution and upgrading of these materials. *T. harzianum* α-amylase A3 characterized by acidic pH optimum at 4.5 (glycosidic bond of starch is stable at high pH but hydrolyzes at low pH), high affinity toward high-molecular mass substrates, retained 70 and 50% of its activity at 50 and 60°C, respectively and had endo-action mode. These findings may meet the prerequisites needed for starch liquefaction and saccharification industry.

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