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Characterization of Thermostable α-amylase from Thermophilic and Alkaliphilic *Bacillus* sp. Isolate DM-15

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Abstract: *Bacillus* sp. DM-15 was isolated from Çiftehan Thermal Spring, Turkey produced thermostable α-amylase at 55°C at pH 9. Analysis of the enzyme for molecular mass and amylolytic activity carried out by SDS-Starch-PAGE electrophoresis revealed a single band with high molecular mass 126 kDa. After extraction, the enzyme remained stable in a range of temperature and pH between 40-100°C and 4.5-10, respectively. The optimum enzyme activity was displayed at 60°C and pH 5.5-6.0. α-amylase production by thermophilic *Bacillus* sp. strain cultivated in liquid media reached a maximum at 12 h with levels of 27.5 μmol mg^{-1} protein min^{-1}. The enzyme was stable for 15 min at 60°C while 24% of the original activity was lost at 100°C. Enzyme activity was increased in the presence of 5 mM $CaCl_2$, Na_2SO_3 and KCl (105%) and inhibited in the presence of 5 mM EDTA, $CuSO_4$, $FeSO_4$, SDS (1%), Urea (8 M), Co up to 98, 79, 57, 36, 33 and 10%, respectively. The DM-15 α-amylase may be suitable for in liquefaction of starch in detergent and textile industries and in other industrial applications.

Key words: Thermostable α-amylase, thermophilic *Bacillus*, isolation, characterization, molecular mass, Turkey

INTRODUCTION

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Yamashita, 1994; Glazer and Nikaido, 1995; Nielsen and Borchert, 2000; Reddy *et al*., 2003; Asgher *et al*., 2007). Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end products (Yamagata and Udaka, 1994; Bertoldo and Antranikian, 2002; Haki and Rakshit, 2003). α-amylase (EC 3.2.1.1; α-4-glucan glucano-hydrolase) is an endo-type enzyme that catalyzes the hydrolysis of α-1,4-glucosidic linkages (Yamashita, 1994; Yamagata and Udaka, 1994; Haki and Rakshit, 2003). Thermostable α-amylases have extensive commercial applications in starch processing, brewing and sugar production (Leveque *et al*., 2000) designing in textile industries (Hendriksen *et al*., 1999; Asgher *et al*., 2007) and in detergent manufacturing processes (Hewitt and Solomons, 1996; Lin *et al*., 1998; Asgher *et al*., 2007). Among micro-organisms *Bacillus* sp. produce various extracellular enzymes of industrial importance (Priest, 1977; Aygan and Arikan, 2008) like amylases. The great diversity of thermophilic bacteria related to the genus *Bacillus* has frequently been discussed. Only a few of the isolates have properly been identified mainly due to the great genotypic and phenotypic variability that characterize strains belonging to the thermophilic *Bacillus* sp. (Mora *et al*., 1998).

Nazina *et al*. (2001) grouped the aerobic thermophilic bacilli *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* into the new genus *Geobacillus* and described two new species *Geobacillus subterraneus* and *Geobacillus uzenensis*. Natural habitats of the thermophilic bacteria are all types of terrestrial and marine hot environments including natural and man-made environments such as hot springs and spas. Thermophilic

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organisms grow optimally between 50 and 80°C (Vieille and Zeikus, 2001). There are hot springs all over the earth on every continent and even under the oceans and seas (Ibrahim and El-diwany, 2007). Çiftehan Thermal Spring is one of the hot springs in Turkey which has been not yet explored in details from the microbiological point of view. In this research, researchers isolated thermostable microorganism, *Bacillus* sp. DM-15 from the thermal spring in Turkey producing thermostable α-amylase and described partial characterization of the enzyme.

MATERIALS AND METHODS

Organisms and cultivation conditions: *Bacillus* sp. DM-15 was isolated from coast sediment samples collected from the thermal spring in Çiftehan, Nigde, Turkey. The samples were incubated at 80°C for 10 min for selection of gram-positive spore forming bacteria, *Bacillus* sp. The isolates screened for α-amylase production on starch-agar plates containing (g L^{-1}) peptone 10, yeast extract 5, NaCl 5, starch 5, agar 15 at 55°C. The pH was adjusted to 7.5 with 1N NaOH. α-amylase positive colonies were selected with iodine staining.

Enzyme production: Strain DM-15 was grown up in LB medium at 55°C with shaking at 200 rpm for 24 h. After removal of cells by centrifugation (Hettich Universal EBA12) (5,000 rpm, 10 min), the supernatant was used for enzyme assay.

Enzyme assay: α-amylase activity was assayed by adding 0.5 mL enzyme to 0.5 mL soluble starch (2%, wt/v) in 0.1M phosphate buffer, pH 6.5 and incubating at 55°C for 30 min. The reaction was stopped by addition of 2 mL of 3,5-dinitrosalicylic acid reagent and $A_{540\ nm}$ was measured in a Pharmacia spectrophotometer (Miller, 1959). An enzyme unit is defined as the amount of enzyme releasing 1 mmol of glucose from the substrate in 1 min at 55°C.

Effect of pH and temperature on activity and stability: Temperature and pH effects on enzyme activity were assayed at different temperatures ranging from 40-100°C and at pH values ranging from 4-10 for 30 min. Following buffers were used in the reactions: 100 mM Na-acetate (pH 4-6), 100 mM Na-phosphate (pH 6-7) and 100 mM Tris (pH 7-10) (Burhan *et al*., 2003). For the measurement of thermal stability, the enzyme was pre-incubated at temperatures between 40-100°C for 30 min at optimum pH. The enzyme activity was determined under standard enzyme assay condition.

Effect of various metal ions, surfactants, chelating agents and inhibitors on activity: The effect of metal ions, chelating agents, surfactants and inhibitors on the activity of α-amylase were determined by pre-incubating the enzyme in the presence of EDTA (5 mM), SDS (1%), $CaCl_2$ (5 mM), Na_2SO_3 (5 mM), $MgCl_2$ (5 mM), PMSF (5 mM), Kcl (5 mM), $FeSO_4$ (5 mM), $CuSO_4$ (5 mM), Co (5 mM), β-mercaptoethanol (1%), TritonX-100 (1%) and Urea (8 M) for 30 min at 55°C before adding the substrate. Subsequently relative α-amylase activities were measured at optimum temperature (Egas *et al*., 1998; Lo *et al*., 2001). The control (without any additive) was taken as having 100% activity.

SDS-PAGE and zymogram analysis: SDS-PAGE and SDS-Starch-PAGE (0.2% starch) were done as described by Laemmli (1970) with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 h with Coomassie blue R 250 dye in methanol-acetic acid water solution (4:1:5 by volume) and destained in the same solution without dye (Burhan *et al*., 2003). For activity staining (zymogram) of α-amylase activity by SDS-Starch-PAGE, SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 20% for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C. Gel was then transferred onto a glass plate, sealed with film and incubated at 55°C for 4 h. Gel was stained in a solution of iodine (iodine 5 g L^{-1}, KI 50 g L^{-1}) for 30 min, clear band indicate the presence of amylase activity (Lee *et al*., 1994; Burhan *et al*., 2003; Ikeda *et al*., 1992). Molecular weight of thermostable α-amylase was measured using BioCapt MW software (Vilber Lourmat).

Identification of products: Thin-Layer Chromatography (TLC) was used to analyze product resulting from the action of thermostable α-amylase on starch. Cell extracts (1 mL) were incubated with 1 mL of 2% substrate in water at 55°C for 48 h. Digestion products were separated on Silica Gel 60 (Whatman) plates by 10:5:4.5:0.4 (by volume) toluene-ethyl acetate-methanol-water solvent (Wójciak-Kosior and Oniszczuk, 2008). The sugars were detected by dipping the plate in 0.5% (wt/v) α-naphthol-5% (v/v) H_2SO_4 in ethanol and heating to 120°C (Simpson and Russell, 1998).

RESULTS

Bacillus sp. DM-15 was gram positive, rod shaped, spore forming and aerobic. It was identified as *Bacillus* sp. depending on various morphological and biochemical

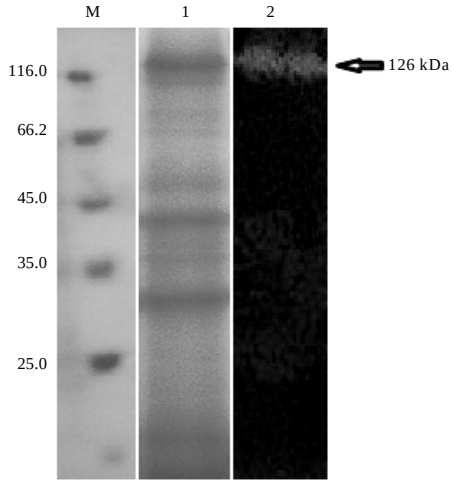

Fig. 1: Zymogram analysis of α-amylase on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassie Brillant Blue staining (Lane: M, 1) and the activity of enzyme revealed by iodine (Lane: 2)

characteristics. The growth was observed between pH 6 and 10 in LB medium at 55°C. The maximum enzyme synthesis occurred 12 h later from inoculation in LB medium at 55°C.

Determination of molecular mass: Molecular mass determined by SDS-Starch-PAGE electrophoresis revealed single band showing α-amylase activity in gel using BioCapt MW software. The molecular mass of this band was 126 kDa (Fig. 1).

Enzyme properties: The enzyme had a broad temperature range between 40-90°C and the optimum activity was observed at 60°C. The mean enzyme activities were 59, 87, 85, 72 and 67% at 40, 50, 70, 80 and 90°C, respectively, whereas only 17% activity was retained at 100°C for 30 min (Fig. 2a). The enzyme also showed a significant relative activity (73%) between pH 4.5 and 10 with an optimum pH 5.5 (Fig. 2b). For thermal stability estimation, the retaining activity was determined at optimum pH and temperature (Fig. 2c). The retained original enzyme activity was obtained from 40-100°C 85% for 15 min. The enzyme was stable for 15 min at 60°C while at 70, 80, 90 and 100°C, 21, 24, 24 and 24% of the original activities were lost, respectively.

Effect on some metal ions, surfactants, chelating agents and inhibitors on enzyme activity: The enzyme was pre-incubated at 60°C for 30 min at different concentration of the metal ions and various chemicals prior to standard

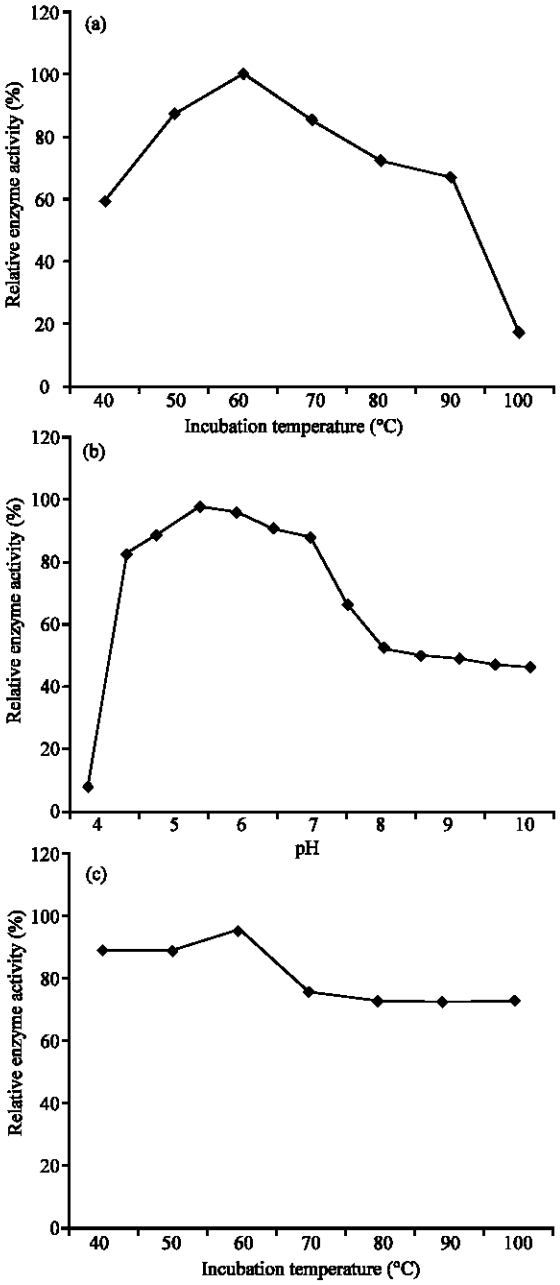

Fig. 2: Enzyme properties of *Bacillus* sp. DM-15 α-amylase (a): Effect of temperature; b): Effect of pH; c): Thermal stability

enzyme activity assay. The residual enzyme activity results have shown in Table 1. The relative activity of enzyme was slightly increased to 105% by $CaCl_2$, Na_2SO_3 and KCl (5 mM each).

On the other hand, partial inhibition of original enzyme activity was obtained with $CuSO_4$ (5 mM), $FeSO_4$ (5 mM), SDS(1%), urea (8 M) and Co (5 mM)

Table 1: Effect of different metal ions, surfactans, chelating agents and inhibitors on α-amylase from *Bacillus* sp. DM-15

Chemicals	Concentrations	Relative enzyme activity (%)
Control	None	100
$CaCl_2$	5 mM	105
EDTA	5 mM	2
SDS	1%	64
Triton X-100	1%	97
β-Mercaptoethanol	1%	95
PMSF	5 mM	98
Na_2SO_3	5 mM	105
KCl	5 mM	105
Urea	8 M	67
$MgCl_2$	5 mM	99
$FeSO_4$	5 mM	43
Co	5 mM	90
$CuSO_4$	5 mM	21

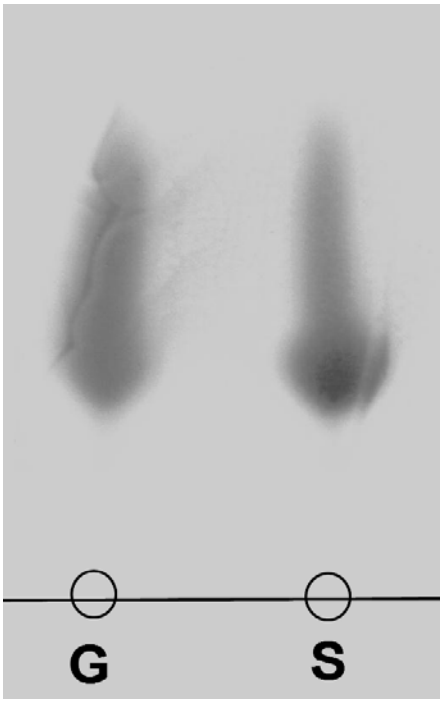

Fig. 3: TLC analysis of starch digested by cell extracts for 48 h. (Lane G: glucose, lane S: starch digested by *Bacillus* sp. DM-15 thermostable α-amylase)

up to 79, 57, 36, 33 and 10%, respectively. DM-15 α-amylase enzyme activity was strongly reduced to 98% by EDTA (5 mM) (Table 1).

Analysis of products: TLC analysis was used to determine the substrate which the α-amylase activity was able to hydrolyze. Cell extracts of *Bacillus* sp. DM-15 containing the thermostable α-amylase, hydrolyzed soluble starch. The product of hydrolysis was glucose (Fig. 3). Cell extracts of *Bacillus* sp. DM-15 produced the same pattern of product from starch.

DISCUSSION

Many *Bacillus* species that can produce starch-degrading enzymes are known. They include species such as *Bacillus stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, *B. cereus*, *B. circulans*, *B. megaterium*, *B. thuringiensis*, *B. coagulans*, *B. globisporus*, *B. alvei* and *B. macerans* (Claus and Berkeley, 1986). However, among these microorganisms, only few are known to produce thermostable α-amylase. *B. stearothermophilus* and *B. licheniformis* α-amylases are well characterized and are heavily used in the starch processing industry. Since, thermostability is an important feature for use of amylolytic enzymes in starch processing, thermophilic and hyperthermophilic microorganisms have special role as a source of novel thermostable enzymes (Burhan *et al*., 2003).

Bacillus sp. DM-15 wild type strain producing thermostable α-amylase was isolated and the enzyme was partially characterized. *Bacillus* sp. DM-15 showed growth at a wide range of pH from 6-10 with an optimum pH of 9. The bacterium is called typically alkaliphilic as it grows optimally at pH values above 8 and grows poorly under neutral pH value of 7 (Horikoshi, 1999). The optimum temperature for enzyme production and growth was 55°C. Vieille and Zeikus (2001) reported that thermophilic organisms grow optimally between 50 and 80°C. This suggests that strain *Bacillus* sp. DM-15 could be called thermophilic. It was also reported that the temperature optimum for the activity of α-amylase is related to the growth of the microorganism (Vihinen and Mantsala, 1989).

Molecular weight of DM-15 thermostable α-amylase was 126 kDa. Molecular weights of á-amylases are usually between 50-60 kDa. On the other hand, it has been reported that molecular weights of α-amylases vary from about 10-210 kDa (Gupta *et al*., 2003). The highest value, 210 kDa for *Chloroflexus aurantiacus* has been reported (Ratanakhanokchai *et al*., 1992). These differences of molecular weights of α-amylases result from the genes corresponding to the organism (Sidhu *et al*., 1997). Among *Bacillus* species, several high-molecular-weight amylases have been reported. *Bacillus* sp. TS-23 (Lin *et al*., 1998), *B. clausii* (Duedahl-Olesen *et al*., 2000) and *B. stearothermophilus* DSM 2358 (Egelseer *et al*., 1995) amylases with molecular weights of 150, 101 and 184 kDa, respectively have been reported.

The pH activity of the α-amylase was in the range 4.5-10 with an optimum pH 5.5 (Fig. 2b). It has been reported that the pH optima of α-amylases was vary from 2-12 (Vihinen and Mantsala, 1989) and generally α-amylases were stable over a wide range of pH from

4-11 (Fogarty and Kelly, 1979; Khoo *et al*., 1994; Hamitton *et al*., 1999; Reddy *et al*., 2003). On the other hand, slightly acidic pH optima have been reported for α-amylases from various *B. stearothermophilus* strains as well (Vihinen and Mantsala, 1989; Campbell, 1955; Pfueller and Elliot, 1969; Ogasahara *et al*., 1970).

The present results showed that the optimum temperature of the enzyme were 60°C (Fig. 2a). Temperature optima of *B. stearothermophilus* α-amylases are generally from 50-70°C (Campbell, 1955; Pfueller and Elliot, 1969; Ogasahara *et al*., 1970; Kim *et al*., 1989). These suggest that strain *Bacillus* sp. DM-15 could be a strain of *B. stearothermophilus*. But to make sure exactly, it is confirmed with the API 50 CHB test which tests the ability of a microorganisms to utilize a preselected panel of 50 different carbon sources (Wind *et al*., 1994). The end product of raw starch hydrolysis by DM-15 α-amylase is glucose. However, it is difficult to conclude that this is the only product of raw starch hydrolysis by α-amylase because dextrins released from raw starch might be rapidly hydrolyzed by unbound enzyme molecules into smaller fragments (Mamo and Gessesse, 1999).

The effect of metal ions, surfactants, chelating agents and inhibitors on enzyme activity have been investigated. Although, most α-amylases are not or less affected by various EDTA concentrations in the presence of 5 mM EDTA, DM-15 α-amylase was almost completely inactivated (98%). In the presence of 1 mM EDTA, AmyK 38 protected full of its activity (Hagihara *et al*., 2001), while Egas *et al*. (1998) and Burhan *et al*. (2003) reported 88 and 95% activity, respectively with 10 mM EDTA. On the other hand, a stimulated activity was observed in the presence of $CaCl_2$, Na_2SO_3 and KCl (105%) (Table 1). The stimulatory effect of Ca^{2+} ion presence on the amylases from *B. subtilis* JS-2004 (Asgher *et al*., 2007), *Bacillus* sp. ANT-6 (Burhan *et al*., 2003), *Pyrococcus woesei*, *Thermococcus hydrothermalis* and *T. profundus* (Leveque *et al*., 2000), *Bacillus* sp. TS-23 (Lin *et al*., 1998) and *B. stearothermophilus* (Srivastava, 1987) have been reported. Nevertheless, it was reported that Ca^{2+} can bind some catalytic residues (Boel *et al*., 1990) and therefore can inhibit α-amylase activity (Leveque *et al*., 2000). Leveque *et al*. (2000) reported that *P. woesei* α-amylase was poorly inhibited at high Ca^{2+} concentration (3 mM) (15%). There are also reports were Ca^{2+} didn't have any effect on the amylase (Landerman *et al*., 1993).

Slightly inhibition of the *Bacillus* sp. DM-15 α-amylase was observed with Triton X-100 (3%), β-mercaptoethanol (5%), PMSF (2%), $MgCl_2$ (1%) and Co (10%). The enzyme was also inhibited at proportion 36, 33, 57 and 79% by SDS (1%), Urea (8 M), $FeSO_4$ (5 mM) and $CuSO_4$ (5 mM), respectively (Table 1). PMSF slightly produce an inhibitory effect on DM-15 amylase activity (98% with 5 mM). Similar finding was also reported by Lin *et al*. (1998) (97% with 10 mM).

The findings show that DM-15 α-amylase inhibited at portion 57% by $FeSO_4$ (5 mM). The effect of Fe^{2+} varied between amylases. For instance, it had a strong inhibitory effect (15% with 3 mM) on the amylase from *P. woesei*, whereas it could poor inhibitory effect (with 2 mM) on the amylase from *P. furiosus* and poor stimulatory effect (102% with 1 mM) on the amylase from *P. profundus* (Leveque *et al*., 2000). The effect of Co^{2+} also varied between amylases. For instance, it had a strong inhibitory effect (52% with 1 mM) on the amylase from *Pyrococcus* sp. KOD1, whereas it had stimulatory effect (120% with 1 mM) on the amylase from *P. woesei* (Leveque *et al*., 2000). But Co^{2+} produced poor inhibitory effect on DM-15 α-amylase activity (90% with 5 mM) and similar finding (80% 3 mM) was also reported by Leveque *et al*. (2000).

CONCLUSION

The *Bacillus* sp. DM-15 strain produced high levels of thermostable α-amylase. The DM-15 α-amylase is a slightly acidophilic with wide range of pH (4.5-10), thermostable and resistant to most of chelators and metal ions. Most of thermophiles, thermostable and inhibitors resistant enzymes suitable for application in starch processing, other food, laundry and textile industries. The DM-15 α-amylase production process can be commercialized after further optimization for enhanced enzyme production.

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