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

Bahri Devrim Ozcan, Makbule Baylan, Numan Ozcan and Dilek Tekdal
1Department of Biology, Faculty of Arts and Sciences, Osmaniye Korkut Ata University, Osmaniye, Turkey
2Department of Basic Science, Faculty of Fisheries, Cukurova University, Adana, Turkey
3Department of Animal Science, Faculty of Agriculture, Cukurova University, Adana, Turkey
4Department of Biological Sciences and Bioengineering, Faculty of Engineering and Natural Sciences, Sabanci University, Tuzla, Istanbul, Turkey

Abstract: Bacillus sp. DM-15 was isolated from Çilehvan Thermal Spring, Turkey. This produced thermostable α-amylase at 55°C at pH 9. Analysis of the enzyme for molecular mass and amyloytic 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 CaCl₂, Na₂SO₄, and KCl (105%) and inhibited in the presence of 5 mM EDTA, CuSO₄, FeSO₄, 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 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 dextrans 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 glucanohydrolase) 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-amylase 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 (Priefer, 1977; Ayyag and Arikan, 2008) like amylase. 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).

Nunana 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 azorenensis. 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

Corresponding Author: Bahri Devrim Ozcan, Department of Biology, Faculty of Arts and Sciences, Osmaniye Korkut Ata University, 8000 Osmaniye, Turkey
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 thermotolerant 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, Niğde, 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%, w/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 A₅₄₀nm 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%), CaCl₂ (5 mM), Na₃SO₄ (5 mM), MgCl₂ (5 mM), PMSF (5 mM), KCl (5 mM), FeSO₄ (5 mM), CuSO₄ (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 Na₃HPO₄, 50 mM NaH₂PO₄ (pH 7.2), isopropanol 20% for 1 h and 50 mM Na₃HPO₄, 50 mM NaH₂PO₄ (pH 7.2) for 1 h, respectively. Renaturation of enzyme was carried out by keeping the gel overnight in a solution containing 50 mM Na₃HPO₄, 50 mM NaH₂PO₄ (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; Ikele 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 (Wojsiek-Kosior and Oniszczuk, 2008). The sugars were detected by dipping the plate in 0.5% (w/v) α-naphthol-5% (v/v) H₂SO₄ 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
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 enzyme activity assay. The residual enzyme activity results have shown in Table 1. The relative activity of enzyme was slightly increased to 105% by CaCl₂, Na₂SO₄, and KCl (5 mM each).

On the other hand, partial inhibition of original enzyme activity was obtained with CuSO₄ (5 mM), FeSO₄ (5 mM), SDS(1%), urea (8 M) and Co (5 mM)
Table 1: Effect of different metal ions, surfactants, chelating agents and inhibitors on α-amylase from Bacillus sp. DM-15

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentrations</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5 mM</td>
<td>105</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>2</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
<td>64</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
<td>97</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>1%</td>
<td>95</td>
</tr>
<tr>
<td>PMSF</td>
<td>5 mM</td>
<td>98</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>5 mM</td>
<td>105</td>
</tr>
<tr>
<td>KCl</td>
<td>5 mM</td>
<td>105</td>
</tr>
<tr>
<td>Urea</td>
<td>8M</td>
<td>67</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5 mM</td>
<td>99</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>5 mM</td>
<td>43</td>
</tr>
<tr>
<td>Co</td>
<td>5 mM</td>
<td>90</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>5 mM</td>
<td>21</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Many Bacillus species that can produce starch-degrading enzymes are known. They include species such as Bacillus steaerotherophilus, B. amyloquefaciens, 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. steaerotherophilus 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 Manttala, 1989).

Molecular weight of DM-15 thermostable α-amylase was 126 kDa. Molecular weights of α-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. steaerotherophilus 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 Manttala, 1989) and generally α-amylases were stable over a wide range of pH from
4-11 (Fogarty and Kelly, 1979; Khoo et al., 1994; Hamilton 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; Pfueeler and Elliot, 1969, Ogashara 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; Pfueeler and Elliot, 1969; Ogashara 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 (Wend 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 dextrans released from raw starch might be rapidly hydrolyzed by unbound enzyme molecules into smaller fragments (Mamo and Gesesse, 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 CaCl₂, Na₂SO₄ and KCl (105%) (Table 1). The stimulatory effect of Ca²⁺ ion presence on the amylases from B. subtilis IS-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 Ca²⁺ 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 Ca²⁺ concentration (3 mM) (15%). There are also reports that Ca²⁺ 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%), MgCl₂ (1%) and Co (10%). The enzyme was also inhibited at proportion 36, 33, 57 and 79% by SDS (1%), Urea (8 M), FeSO₄ (5 mM) and CuSO₄ (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 FeSO₄ (5 mM). The effect of Fe²⁺ 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 pond stimulatory effect (102% with 1 mM) on the amylase from P. profundus (Leveque et al., 2000). The effect of Co²⁺ 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 (12%) with 1 mM) on the amylase from P. woesei (Leveque et al., 2000). But Co²⁺ 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.

ACKNOWLEDGEMENTS

This research was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) through Short-Term R and D Funding Programme (1002, Project 108T666).

REFERENCES


