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Full Length Research Paper

Purification and biochemical characterization of a Ca^{2+}-independent, thermostable and acidophilic α-amylase from *Bacillus* sp. RM16

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***Bacillus* sp. RM16 was isolated from a hot spring in Karachi and screened for the production of α-amylase. The enzyme was obtained after 72 h cultivation of strain in Luria broth containing 1% starch (w/v). Enzyme Amy RM16 was purified to electrophoretic homogeneity by a series of sequential steps including precipitation with ammonium sulfate at 70% saturation, Q-Sepharose, Phenyl Sepharose and reversed phase chromatography. The purified enzyme is made up of a single polypeptide chain of 66 kDa as established by a combination of SDS-PAGE and zymographic analysis. In our experimental conditions, a total yield of 1.35% with specific activity of 6380U/mg was obtained providing 17 fold final purification of the enzyme. Biochemical characterization of the Amy RM16 such as optimum temperature and pH, substrate specificity and enzymatic susceptibilities towards different metal ions and inhibitors were also performed. Results of these studies revealed that, the enzyme is active at wide temperature range with optimum activity at 80°C and retained 85% of the activity for 3 h at 50°C and around 50% of remaining activity for 1 h at 80°C. The enzyme showed optimum activity at pH 5.0. On the other hand, Ca^{+2} and EDTA (1 to 5 mM) did not significantly affect the enzyme activity. The main substrate for the enzyme was found to be starch but it could also hydrolyze raw starch, dextrin, γ-cyclodextrin and pullulan.**

Key words: Ca^{2+}-independent, *Bacillus* sp, thermostable α-amylases, low pH profile, enzyme, raw starch digestion, HPLC.

INTRODUCTION

Bacteria belonging to the genus *Bacillus* produce a large variety of extracellular enzymes like proteases, cellulases, lipases and amylases (Kamal et al., 1995; Hagihara et al., 2001; Eckert and Schneider, 2003; Setyorini et al., 2006; Asoodeh et al., 2010). Among these, proteases and amylases are of significant industrial importance. In particular, amylases comprise 30% of the world's industrial enzyme production. Amylases have diverse applications in a wide variety of industries such as detergent, textile, fermentation, paper, pharmaceutical, food and sugar industries (Gupta et al., 2003; Hmidet et al., 2010). Each application of these enzymes requires unique properties with respect to temperature, pH, specificity and stability (Reddy et al., 2003). Although, amylases can be derived from several sources such as microorganisms, plants and animals, microbial enzymes are generally considered to be the most suitable for industrial applications. Among the various *Bacillus* species, *Bacillus amyloliquefaciens*, *Bacillus licheniformis, Bacillus stearothermophilus* and *Bacillus subtilis* are most frequently used in the commercial production of thermostable amylase. Several amylolytic enzymes, with different molecular weight, optimum pH, temperature and specificities have also been reported recently (Arikan et al., 2003; Aiyer, 2005;

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Hashim et al., 2005; Asgher et al., 2007; Saxena et al., 2007; Arikan, 2008; Hmidet et al., 2008).

The natural pH of starch slurry in the starch processing industry is around 4.5 (Sivaramakrishnan et al., 2006), whereas the optimum pH for most industrial α-amylases are around 6 to 7 being less stable at low pH. Therefore, the starch liquefaction step in the industry is currently limited to the pH range of 5.5 to 6.5 and for this reason, improving the thermostability of the enzymes at low pH is necessary (Sajedi et al., 2005). In the food processing industry, acidophilic and thermostable α-amylases are widely used (Goyal et al., 2005; Sajedi et al., 2005; Arikan, 2008). During the last few years, there has been a worldwide interest in development of thermostable, acidophilic and raw starch-digesting amylases, which would be of value for efficiently simplifying the process of starch conversion. Fungi such as *Aspergillus* and *Rhizopus* species and yeast such as *Cryptococcus* sp. have been extensively reported as good producers of raw starch-digesting amylases capable of acting at low pH and high temperatures, while there are also some reports describing the characterization of raw starch degrading α-amylases from *Bacillus* sp. (Morita and Fujio, 2000; Matsubara et al., 2004; Sajedi et al., 2005; Liu and Xu, 2008). For example, Farouk and coworkers (2009) reported a novel highly thermostable alkaline amylase isolated from hyperthermophilic *Bacillus* strain HUTBS71 which exhibited maximum activity at 100°C and at pH 7.8. Also, in the starch processing industries, thermostable Ca^{2+}-independent α-amylases are required which have particularly low pH optima, so as to eliminate the need for addition and removal of $CaCl_2$ to economize the process. Many studies are being conducted to produce Ca^{2+}-independent thermostable enzymes through site directed mutagenesis (Hashida and Bisgaard-Frantzen, 2000). Thus, diversity in applications and the properties of enzyme encourage us to make special attempts in search for new α-amylases.

MATERIALS AND METHODS

Organism and growth conditions

The organism used in this study was *Bacillus* sp. RM16. This culture was originally isolated from a hot spring located in Karachi, Pakistan. The strain was Gram positive spore former and hence, it was identified as *Bacillus* sp. The strain was further examined for morphological, physiological and biochemical characterization with reference to Bergey's manual of systemic bacteriology (Sneath, 1986). The isolate was grown in Luria broth supplemented with 1% starch (w/v) at 37°C for 72 h at 150 rpm in an orbital shaker. Culture was then harvested and cells were separated by centrifugation at 9500 rpm for 20 min at 4°C.

Purification of the enzyme

Cell-free broth was used for further purification. Proteins were precipitated from cell-free broth using ammonium sulfate at 70% saturation. Precipitates were separated by centrifugation at 9500 rpm for 20 min at 4°C, dissolved in the minimum volume of 10 mM Tris-HCl buffer at pH 8.0, containing 5 mM $CaCl_2$ and dialyzed against the same buffer overnight. The dialyzed sample was applied to Q-Sepharose column (1.5 x 10 cm, Sigma-Aldrich) previously equilibrated with the same buffer. Proteins were eluted using a linear gradient of 0 to 0.5 M NaCl. Flow rate was maintained at 20 ml/h and the eluate was monitored at 280 nm. The active fractions were pooled and the buffer was exchanged to 20 mM Tris-HCl buffer (pH 7.4) using Amicon ultrafiltration assembly (Millipore, USA). The sample was adjusted to 0.4 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl buffer at pH 7.4 and applied to phenyl sepharose column (1 x 5 cm, Sigma-Aldrich) previously equilibrated with 0.4 M $(NH)_2SO_4$ in 20 mM Tris-HCl buffer (pH 7.4). The enzyme was eluted with descending gradient of 0.4 to 0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl buffer at pH 7.4 at a constant flow rate of 20 ml/h. The UV absorbance of the eluate was monitored at 280 nm. Fractions showing amylase activity were pooled and concentrated by Amicon, ultrafiltration assembly. Final purification of the enzyme was achieved by subjecting the enzyme active fraction on PROTEIN-C4 (5 µm, 46 mm x 250 mm; Vydac, The Separation Group, Inc., USA) reversed phase column. The elution was performed under the following conditions: eluent A, 0.1% TFA in water (v/v), eluent B, 100% acetonitrile and 0.05% TFA. Gradient program was 5% B for 5 min, 0 to 60% B in 20 min followed by 100% B for 25 min. The flow rate was maintained at 1 ml/min and absorbance was monitored at 230 nm. Total protein concentration in the eluted fractions as well as the purified enzyme was determined using Bradford (1976) method. Bovine serum albumin was used as a standard.

SDS-PAGE and zymography

The purity and molecular mass of the different fractions as well as purified enzyme was established by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Likewise, zymographic determination of the amylase activity was established by incorporating 1% starch in the gel as described by Lacks and Springhorn (1980). Briefly after electrophoresis, the gel was washed with 2.5% Triton-X 100 (3 x 20 min) to remove SDS followed by washing with water and incubation for 1 h at 37°C in activation buffer (10 mM Tris-HCl buffer of appropriate pH). Finally, the gel was stained with 1% iodine solution to visualize the clear bands of catalysis against the dark background.

Enzyme assay

Amylase activity was assayed by adding 100 µl of enzyme fraction to 100 µl 1% (w/v) starch dissolved in 50 mM sodium acetate buffer (pH 5.0). The reaction mixture was incubated at 80°C for 3 min. The reducing sugar was then determined by the method described by Bernfeld (1955). One unit of amylase activity was defined as the amount of enzyme that releases one µmol of reducing sugar per minute. Maltose was used to construct a standard curve.

Effect of temperature and pH

The effect of temperature was determined by incubating the enzyme reaction mixture at different temperatures from 37 to 100°C and activity was measured as standard assay procedure described earlier. The effect of pH on amylase activity was determined by testing enzyme activity with various buffers of different pH values. The buffers used were 50 mM Glycine-HCl buffer (pH 3.0), sodium acetate buffer (pH 4.0 to 5.0), sodium phosphate buffer (pH 6.0 to 7.0), Tris-HCl buffer (pH 8.0 to 9.0) and Glycine-NaOH buffer

(pH 10.0 to 11.0).

Thermal stability

Thermal stability of the purified enzyme was determined by incubating the enzyme at different temperatures for various time intervals and activity was measured by the method described earlier.

Effect of metal ions and chemical agents

The effect of metal ions on amylolytic activity was determined by incubating the enzyme with different concentrations (1 to 5 mM) for 30 min at 50°C. The residual activity was then measured by assaying at optimum conditions. Likewise, the effect of different inhibitors and chelating agent was tested by incubating the enzyme with respective concentrations of different chemical agents at 50°C for 30 min. The residual activity was measured at optimum assay conditions as described earlier.

Substrate specificity

Substrate specificity of the purified enzyme was determined by assaying with different substrates using 1% (w/v) concentration under optimum assay conditions as described earlier. Likewise, the digestion of raw starch was also determined by assaying under optimum assay conditions using 1% (w/v) raw starch as substrate.

RESULTS AND DISCUSSION

Purification and properties of α-amylase RM-16

Bacillus sp. RM16 was isolated from a hot spring near Karachi and subjected to screening for the production of industrially important enzyme (α-amylase). The extracellular enzyme was obtained by cultivation of the strain for 72 h in Luria broth containing 1% starch (w/v). Enzyme Amy RM16 was purified to electrophoretic homogeneity from the cell-free broth by a series of sequential steps including ammonium sulfate precipitation at 70% saturation, Q-Sepharose, phenyl-sepharose and reversed phase HPLC (Figure 1a). The purified enzyme was found to be a single polypeptide chain of 66 kDa as established by a combination of SDS-PAGE and zymographic analysis (Figure 1b). Table 1 summarizes the activity and percent yield of Amy RM16 at each step of purification. A total yield of 1.35% with specific activity of 6380 U/mg was obtained, which provided the final purification of enzyme up to 17 folds. Our results are quite comparable with the very recently reported data on acidophilic α-amylase from a thermophilic *Bacillus* sp Ferdowsicous by Asoodeh et al. (2010).

Optimum temperature and pH of the enzyme

The purified enzyme was found to be active over a wide range of temperature that is, from 37 to 100°C (Figure 2a) showing the optimum activity at 80°C; 85% of the activity was still retained at 100°C.

The optimum pH of Amy RM16 was found to be 5.0, while it showed activity at broad pH range as depicted in Figure 2b. Although, the optimum activity was at pH 5.0, the enzyme was found to be significantly active at pH 8.0 and 9.0 as it demonstrated 75 and 61% of enzyme activity, respectively. To date various amylases have been reported for their pH profile in acidic range like α-amylase from *Bacillus* sp. Ferdowsicous having optimum pH of 4.5 (Asoodeh et al., 2010), α-amylase from *B. subtilis* showing optimum activity at pH 6.0 (Liu et al., 2010), amylase from *Bacillus* sp KR8014 showing pH optima at 4.0 to 6.0 (Sajedi et al., 2005) and another amylase from *Bacillus* sp YX-1 (Liu and Xu, 2008) having optimum activity at pH 5.0.

Thermal stability

The stability towards increased temperature was evaluated by incubating the purified enzyme at various temperatures. The aliquots were taken at regular intervals and residual activity was measured at 80°C in sodium acetate buffer at pH 5.0. Figure 3 indicates that, the enzyme retained its 85% activity after 3 h of incubation at 50°C. At the optimum temperature (80°C), 50% of its activity was lost after 1 h of incubation (Figure 3). On the other hand, when the enzyme was incubated with 1% starch, it retained 100% of its activity after 3 h of incubation at 70°C. The results are in good agreement with the previous reports that the presence of substrate confers some resistance against thermal inactivation (Lo et al., 2001). Similar observation was also reported by Hashim et al. (2005) suggesting that, the enzyme was more sensitive to thermal inactivation in the absence of substrate.

Thermostability of α-amylases greatly varies between different *Bacillus* species. Recently, Farouk and coworkers (2009) reported a novel highly thermostable alkalophilic amylase showing maximum activity at 100°C which retained 60% of its activity at 100°C after 1 h of heat treatment. Studies by Hmidet and coworkers (2008) show thermostability at 70°C for 1 h in the absence of substrate. Thermostability for 4 h at 100°C has also been reported from *B. licheniformis* CUMC 305 (Krishnan and Chandra, 1983). Likewise, Asgher and coworkers (2007) reported an α-amylase which retained its activity at 80°C for 1 h. Amy RM16 showed considerable thermostability at pH 5.0 with the reports on amylases. On the other hand, thermostable acidophilic α-amylase with half life of 48 min at 80°C has also been reported by Asoodeh et al. (2010). Liu and Xu (2008) reported a raw starch digesting α-amylase showing decreased thermostability at and above 50°C.

Effect of metal ions and inhibitors

The effects of various metal ions were tested on amylolytic activity of Amy RM16 by incubating the

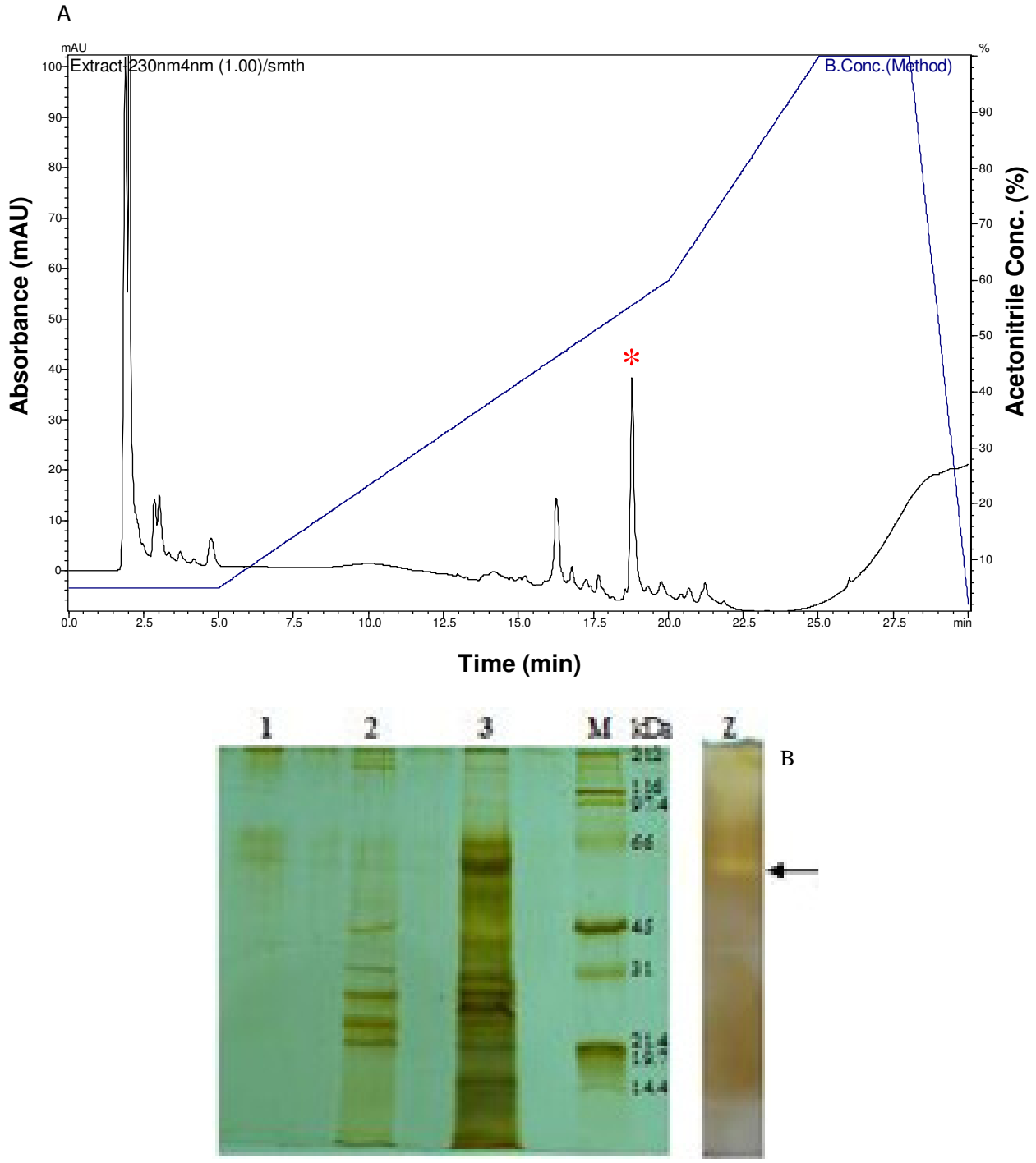

Figure 1. (A) Final purification profile of phenyl sepharose active fraction on C4 reversed phase HPLC column; (B) 10% SDS PAGE profile of Amy RM16 at different purification steps. Lane 1, purified Amy Rm16 from phenyl sepharose column; lane 2, active fraction of Q-sepharose; lane 3, crude ammonium sulfate precipitates; lane M, known molecular weight markers (Z) starch hydrolysis by Amy RM16 established by zymography.

enzyme with metal ions for 30 min at 50°C. The residual activity was measured by taking the activity of enzyme without any ion as 100%. As summarized in Table 2, the enzyme was found to be inhibited by Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+}, while Ca^{2+}, Ba^{2+} and Mg^{2+} did not affect the enzyme activity, whereas, enzyme activity was rather enhanced in the presence of Co^{2+} which was quite consistent with the previous report of Hashim et al. (2005). On the other

Table 1. Purification steps of extracellular α-amylase from *Bacillus* sp.RM16.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	37	13620	368	100	1
Q-Sepharose	3.0	10235	3412	8	9
Phenyl-Sepharose	0.3	4680	4254	2.09	12
RP-HPLC	0.5	319	6380	1.35	17

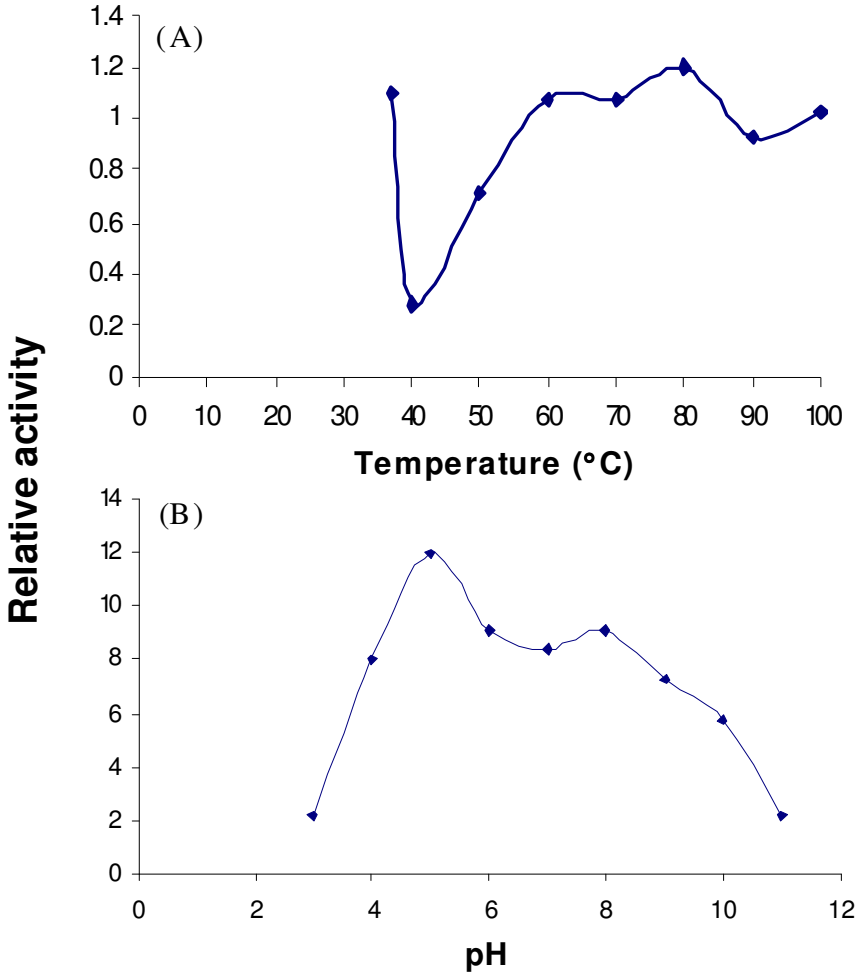

Figure 2. Optimum temperature on enzyme activity and pH profile of Amy RM16. (A), The activity, as function of temperature, was determined under standard assay conditions in the range of 37 to 100°C; (B), effect of pH on enzyme activity in the presence of starch as substrate was assayed at different pH values ranging from 3.0 to 11.0.

hand, Ca^{+2} ions had varied effects on the amylases of different *Bacillus* species. Most of the amylases reported earlier show Ca^{2+} dependence (Kim et al., 1995; Nielson et al., 2003) while, some Ca^{2+} independent amylases have also been reported (Arikan, 2008; Asoodeh et al., 2010). Amy RM16 was found to be a Ca^{2+} independent enzyme. The earlier reports showed that Hg^{+2} and Cu^{+2} had inhibitory effects on α-amylases (Lo et al., 2001). For example, α-amylase from *Bacillus* sp. Ferdowsicous also showed inhibition with Hg^{2+} (Asoodeh et al., 2010). Inhibition of thermostable α-amylases by Zn^{2+} was also reported earlier (Lin et al., 1998; Arikan, 2008). Similarly, Amy RM16 was found to be affected by Zn^{2+}.

Different chemical agents were also tested for their

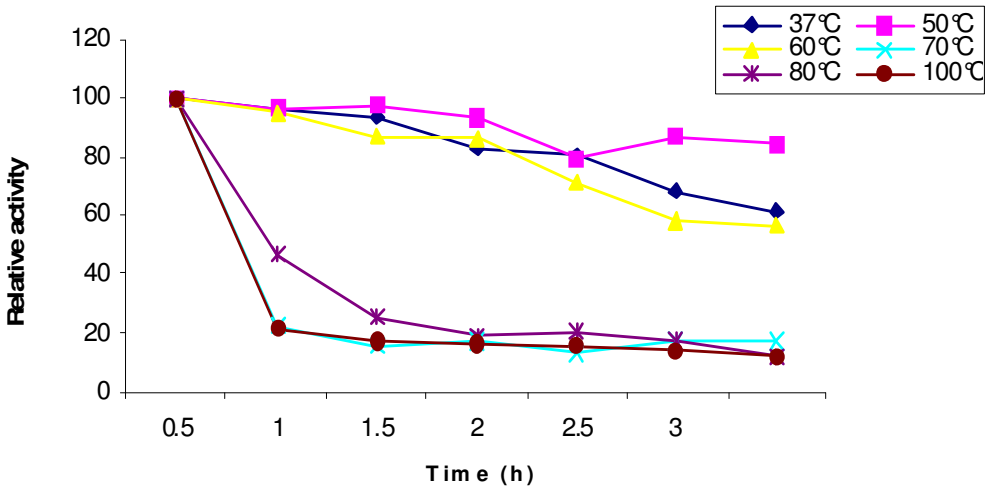

Figure 3. Thermostability of extracellular α-amylase Amy RM16 from *Bacillus* sp. RM16. The enzyme was incubated for 3 h at different temperatures, ranging from 37 to 100℃; aliquots were taken after every 30 min and cooled on ice. The remaining activity was determined under standard assay conditions.

Table 2. Effect of metal ions on α-amylase Amy RM16.

Metal ion	Residual activity (%)
Control	100
$CoCl_2$	129
$MgCl_2$	107
KCl	107
$CaCl_2$	99
$ZnCl_2$	46
$CdCl_2$	10
$BaCl_2$	10
$CuCl_2$	ND
$HgCl_2$	ND

ND, Not detectable. The Amy RM16 was pre-incubated with various metal ions for 30 min at 50℃ and then residual activities were determined under standard assay conditions. Enzyme activity incubated in the absence of any metal ion was taken as 100%. The data represents the average values of three measurements.

effect on enzymatic activity by incubating with the enzyme at 50℃ for 30 min and residual activity was measured thereafter (Table 3). Among the chemical inhibitors tested, PMSF and N-bromosuccinimide proved to be inhibitors of Amy RM16 in very low concentrations. Although, there is no report of inhibition of acidophilic α-amylases by PMSF in the literature, inhibition of alkaline α-amylases were very much known but without any established mechanism (Lin et al., 1998; Das et al., 2004). On the other hand, N-bromosuccinimide is known to iodize tryptophan residues which are involved in starch binding and/or catalysis by various starch hydrolyzing enzymes (Igarashi et al., 1998). In contrast, Amy RM16 showed relative activity of 21% at 1 mM concentration of N-bromosuccinimide after treating enzyme at 50℃ for 30 min. In addition, 28% of the activity was lost when 5 mM concentration of N-ethylmaleimide was used. Iodoacetate did not significantly affect the enzyme activity and only 12% of the activity was lost when 1 mM iodoacetate was used. Furthermore, in the presence of 1% sodium dodecyl sulfate, the enzyme activity was retained to 85% but at higher concentration (10%), the enzyme activity was totally abolished. Amy RM16 was also quite stable at high concentrations of EDTA and retained its activity up to 80% at 10 mM concentrations (Table 3).

Substrate specificity

The enzyme was tested for their ability to hydrolyze various carbohydrates under optimum assay conditions. The enzyme was able to effectively hydrolyze starch and dextrin. Among cyclodextrins, Amy RM16 could easily hydrolyze γ-cylodextrin but could not hydrolyze α and β-cyclodextrin. These results are consistent with the similar observation reported earlier (Hashim et al., 2005). The hydrolysis of γ-cyclodextrin also suggests the endo-acting mode of action of Amy RM16 as γ-cyclodextrin has cyclic structure. It is also worth mentioning here that, the enzyme was also able to hydrolyze pullulan but at a much slower rate (Table 4). This property is unique when compared with the previous reports and could lead to new findings related to enzyme structure and function. Amy RM16 was also able to degrade raw starch very effectively and the enzyme showed 98% activity with raw starch.

As stated earlier, all previously reported Ca^{2+}- indepen-

Table 3. Effect of different chemical agent on α-amylase Amy RM16.

Chemical agent	Relative activity (%)
PMSF	
1 mM	8±2
5 mM	ND*
Iodoacetate	
0.5 mM	90±4
1 mM	87±3
N-Bromosuccinimide	
0.1 mM	24±1
1 mM	21±2
N-Ethylmaleimide	
1 mM	66±2
5 mM	72±1
SDS	
1%	85±3
5%	ND*
EDTA	
1 mM	98±1
5 mM	84±3
10 mM	80±2

ND, Not detectable. PMSF, Phenyl methyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetra acetic acid. The Amy RM16 was pre-incubated with various enzyme inhibitors for 30 min at 50°C and then residual activities were determined under standard assay conditions. Enzyme activity incubated in the absence of any additive was taken as 100%. The data represents the average values of three measurements.

Table 4. Substrate specificity of α-amylase Amy RM16.

Substrate	Relative activity (%)
Starch	100
Dextrin	81 ± 3
Pullulan	12 ± 2
α-cyclodextrin	ND
β- cyclodextrin	ND
γ-cyclodextrin	58 ± 4
Raw starch	98 ± 1

ND = Not detectable. Substrate specificity was checked by assaying enzyme with 1% (w/v) of substrates in 50 mM sodium acetate buffer (pH 5.0) at 80°C. The data represents the average values of three measurements.

dent and/or acid stable amylases (Sajedi et al., 2005; Liu and Xu, 2008) had lower optimal temperature and thermostability than Amy RM16. For example, α-amylase reported by Asoodeh et al. (2010) had optimum temperature of 70°C at pH 4.5. Likewise, a thermostable and Ca^{2+}-independent α-amylase was also reported but with optimum pH in alkaline range (Malhotra et al., 2000). In contrast, Amy RM16 had thermostability, Ca^{2+}-independency and low optimum pH and also stability in wide pH range. The reported novel properties of Amy RM16 make it a very suitable candidate for starch processing industry. Formation of calcium oxalate upon addition of $CaCl_2$ is a major concern of α-amylases, which may block the processing pipes, heat exchangers and could accumulate in some products which are undesirable (Haki and Rakshit, 2003). Thus, the potential utilization of this enzyme in starch industry to eliminate the addition of $CaCl_2$ and its removal by ion exchange step would certainly benefit the overall efficiency and cost of the process.

Conclusions

Exotic locations like hot springs, deep sea and desserts are recognized as a potential source of novel bacterial isolates for new enzymes with new and unique characteristics which can be utilized in harsh chemical environment of industries. In this study, we isolated *Bacillus* sp. RM16 from the hot spring located in Karachi. Amy RM16, the enzyme isolated and purified from this bacterial strain is a natural, Ca^{2+}-independent amylase having low pH profile along with higher thermostability. The enzyme showed a broad substrate utility and was also capable of digesting raw starch. The overall biochemical characteristics of the enzyme indicate that, it could be a better candidate for use in starch processing industry.

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