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Stimulation of thermal stability of α-amylase from *Bacillus icheniformis* ATCC 6346 by treating with cations

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ABSTRACT

α-Amylases (1,4-α-D-glucan glucanohydrolase; E.C.3.2.1.1) catalyze the cleavage of α-1,4-glucosidic linkages in starch, glycogen, and various oligosaccharides. Thermostable α-amylases from *Bacillus* species are of great industrial importance in the production of corn syrup or dextrose. In this study effect of different cations on the enhancement of stability of α-amylase from *Bacillus licheniformis* ATCC 6346 was examined. Optimal activity of the enzyme was at pH 7.0 and 85 ^{o}C. α-Amylase activity was strongly inhibited by Cu^{2+}, Hg^{2+} and Mn^{2+} but less affected by Mg^{2+} and Ba^{2+}. Ca^{2+} and Na^{+} stimulated the enzyme activity at 85 ^{o}C and at pH 7.0. Addition of 0.01 M Na^{+} enhanced the enzyme stability from 1-33% for 60 min at 85 ^{o}C and pH 7.0. With 0.1M Na^{+}, 100 % of initial enzyme activity was retained for 150 min and 70 min at 60 ^{o}C and 70 ^{o}C, respectively and 88% activity was retained at 80 ^{o}C, at pH 7.0 for 60 min. In the presence of 1 mM Ca^{2+}, no loss of activity was observed in 60 min, at 85 ^{o}C and pH 7.0. Combined addition of 1mM Ca^{2+} and 0.1 M Na^{+}, retained 17.3 % of the enzyme activity for180 min. But the enzyme in the presence of 1 mM Ca^{2+} and 0.1 M Na^{+} separately, lost its total activity in 120 min and 90 min, respectively at 95 ^{o}C and pH 7.0.

Key words: calcium, sodium, enzyme activity, enzyme stability, half life, starch

INTRODUCTION

Bacillus species produce a variety of extracellular enzymes, such as amylases, which have significant industrial importance (Cordeiro *et al*., 2003). Bacterial amylases are known to be more thermostable than fungal amylases (Eke and Oguntimehin, 1992). Amylases have attracted the world's enzyme market because of their wide application in starch based industries especially food, textile, paper, detergent and baking industries (Gregory and Woods, 1995). They represent 25 % of the world's market of enzymes (Niehaus *et al*., 1999; Asgher *et al*., 2007). Most of the commercially produced amylases are of microbial origin (Pandey *et al*., 2000). The enzyme α-Amylase (EC 3.2.1.1, 1,4-α-D glucanohydrolase, endoamylase) hydrolyses starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages. Amylase activity has been shown to be influenced by temperature, pH and presence of some chemicals (Swain and Ray, 2007). All known α-amylases have a conserved calcium binding site (Boel *et al*., 1990; Machius *et al*., 1998; Machius *et al*., 1995). The binding of Ca^{2+} ions has been shown to increase the α-helical structure of α-amylase leading to increased stability (Kim *et al*., 1991). Apart from calcium, other ions such as sodium and chloride have been implicated as allosteric activators of α-amylases (Kim *et al*., 1991). Sodium ions rather than calcium ions have retained the structure and function of Amy K38, an α-amylase from the *Bacillus sp.* strain KSM-K38 (Hagihara *et al.*, 2001; Nonaka *et al.*, 2003) and various other α-amylases (Vihinen *et al.*, 1990; Kobayashi *et al.*, 1992). Calcium ions have been implicated in the mechanisms involving thermal inactivation of *Bacillus* α-amylases, where it has been proposed that the first step involves the reversible dissociation of calcium ions from the native enzyme, followed by irreversible denaturation at high temperatures (Lecker *et al*., 1996; Tanaka *et al*., 2002). Almost all of the technical α-amylases however need a certain amount of calcium ions in the application, because their thermostability depends on the presence of structural calcium ions (Chiang *et al*., 1979). Therefore, the objective of this study was to improve the thermostability of α-amylase from *Bacillus licheniformis* ATCC 6346 by applying different cations.

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MATERIALS AND METHODS

Bacillus licheniformis ATCC 6346 from Heriot-Watt University U.K was used in this study.

The nutrient agar medium used in the study contained (L^-) 25.0 g nutrient agar, 3.0 g soluble starch. The activation medium used in the study contained (L^-) 25.0 g nutrient broth, and 3.0 g soluble starch at pH 7.0. The fermentation medium contained (L^-) 4.0 g soluble starch, 5.0 g $(NH_4)_2SO_4$, 6 g peptone; 0.01g $FeCl_3$; 0.01 g $MgCl_2.6H_2O$; 0.01g $CaCl_2.2H_2O$; 4.0 g of KH_2PO_4, and 7.5 g of K_2HPO_4 at pH 7.0.

A loopful of *Bacillus licheniformis* ATCC 6346 grown in nutrient agar slants with 0.3 % soluble starch at 37 ^oC for 24 h was transferred to 10 ml activation medium and incubated at 42 ^oC in a rotary shaker (100 rpm) for 12 h and used as the inoculum. The fermentation medium was inoculated with 20% (v/v) inoculum and the inoculated flasks were incubated for 48 h at 42 ^oC and spun at 100 rpm. The culture filtrate was used as the source of α-amylase.

To determine the effect of temperature on the stability of α-amylase, α-amylase in pH 7.0 buffer was pre-incubated at 75 and 85 ^oC and the enzyme activity was monitored (Miller, 1959). The half-life of the enzyme was taken as the time taken for its activity to be reduced to half of the original activity.

Effect of cations on the activity and stability of α-amylase

To partially purify the crude α-amylase, to 10 ml of the crude enzyme extract 2.91 g of solid ammonium sulphate was added to bring the concentration of ammonium sulphate to 50%. The precipitate was allowed to settle and collected by centrifugation (refrigerated centrifuge, 3000 rpm for 30 min at 4 ^oC). The precipitate was dissolved in 2 ml distilled water and dialyzed against distilled water. The dialyzed α-amylase was used to study the effect of cations on the activity and stability of α-amylase.

To determine the effect of 2 mM cations such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^+, Hg^{2+}, and Cu^{2+} on the activity of α-amylase produced by *B. licheniformis* ATCC 6346. Some cations and phosphate ions present in the spent medium were removed by ammonium sulphate precipitation and dialysis before using because Ca^{2+}, Ba^{2+}, Mg^{2+} and Hg^{2+} ions would precipitate with phosphate ions (Ca^{2+}, Mg^{2+}, Na^+, Hg^{2+} and Ba^{2+} in the form of chlorides and 2 mM Cu^{2+} and Mn^{2+} in the form of sulphate).

To determine the effect of Na^+ on the stability of α-amylase, α-amylase was incubated at 85 ^oC in pH 7.0 buffer containing different concentrations of Na^+ (0.05 to 0.4 M) and the enzyme activity was monitored.

To determine the thermal stability of α-amylase containing Na^+ at different temperatures, α-amylase was pre-incubated at temperatures 60, 70, 80 and 85 ^oC in pH 7.0 buffer containing 0.1 M Na^+ and the α-amylase activity was monitored.

To determine the effect of Ca^{2+} on the stability of the enzyme, α-amylase from *Bacillus licheniformis* ATCC 6346 was pre-incubated at 85 ^oC in pH 7.0 buffer containing different concentrations of Ca^{2+} (1 to 0.05 mM) and the α-amylase activity was monitored.

To determine the stability of α-amylase in presence of both Na^+ and $Ca^{2+,}$ at different temperatures, α-amylase was incubated in pH 7.0 buffer containing 0.1 M Na^+ and 1mM Ca^{2+} at temperatures 85, 90 and 95 ^oC and the activity of α-amylase was monitored.

RESULTS

Effect of temperature on the stability of α-amylase

When the crude α-amylase extract was pre-incubated at 85 ^oC it retained 60% of its original activity for 10 min and lost all its activity at 60 min (Fig. 1). When the crude α-amylase was pre- incubated at 75 ^oC, 88 % of its activity was retained for 10 min and 81% of its original activity for 60 min. Thus, the stability of α-amylase was longer at 75 ^oC than at 85 ^oC.

Effect of cations on the activity of α-amylase

The effect of 2 mM Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Na^+, Hg^{2+} and Cu^{2+} on α-amylase activity are presented in Table 1. In the presence of Ca^{2+} and Na^+, the enzyme showed higher activity at 85 ^oC and pH 7.0. A slight inhibition in enzyme activity was produced by Mg^{2+} and Ba^{2+} and a stronger inhibition by Cu^{2+}, Mn^{2+} and Hg^{2+} (Table 1).

Effect of Na^+ on the stability of α-amylase

When α-amylase containing different concentrations of Na^+ (0.05-0.4 M) was incubated at pH 7.0 and 85 ^oC, in the presence of all concentrations Na^+, α-amylase showed a higher stability than in the absence of Na^+ (Fig. 2). The enzyme in the presence of 0.1 M $Na^{+,}$ lost 67 % of its initial activity in 60 min (Fig. 2). When α-amylase was pre-incubated in the presence of 0.05, 0.2, 0.3, 0.4 M and in the absence of Na^+, the enzyme activity was retained

at 29.5, 29.4, 28, 20.5 and 0.0 % respectively at 60 min (Fig. 2). Half-life of α-amylase in the presence of 0.1 M Na^+ was 42.8 min while in the absence of Na^+ it was 13.8 min (Table 2). In the presence of 0.1 M $Na^{+,}$ α-amylase showed a 1.9 fold increase in half-life in comparison to 0.4 M Na^+ (Table 2).

Stability of α-amylase in the presence of Na^+ at different temperatures

When α-amylase was pre-incubated in the presence of 0.1 M Na^+ at 60 ^oC, 98% of its initial activity was retained for 120 min. At 70, 80 and 85 ^oC; 9, 29 and 96% of its initial activity was lost (Fig. 3) respectively at 120 min and pH 7.0. When the pre-incubation temperature was decreased from 85 to 60 ^oC, half-life of 0.1 M Na^+ containing α-amylase was increased by 19.6 fold at pH 7.0 (Table 3).

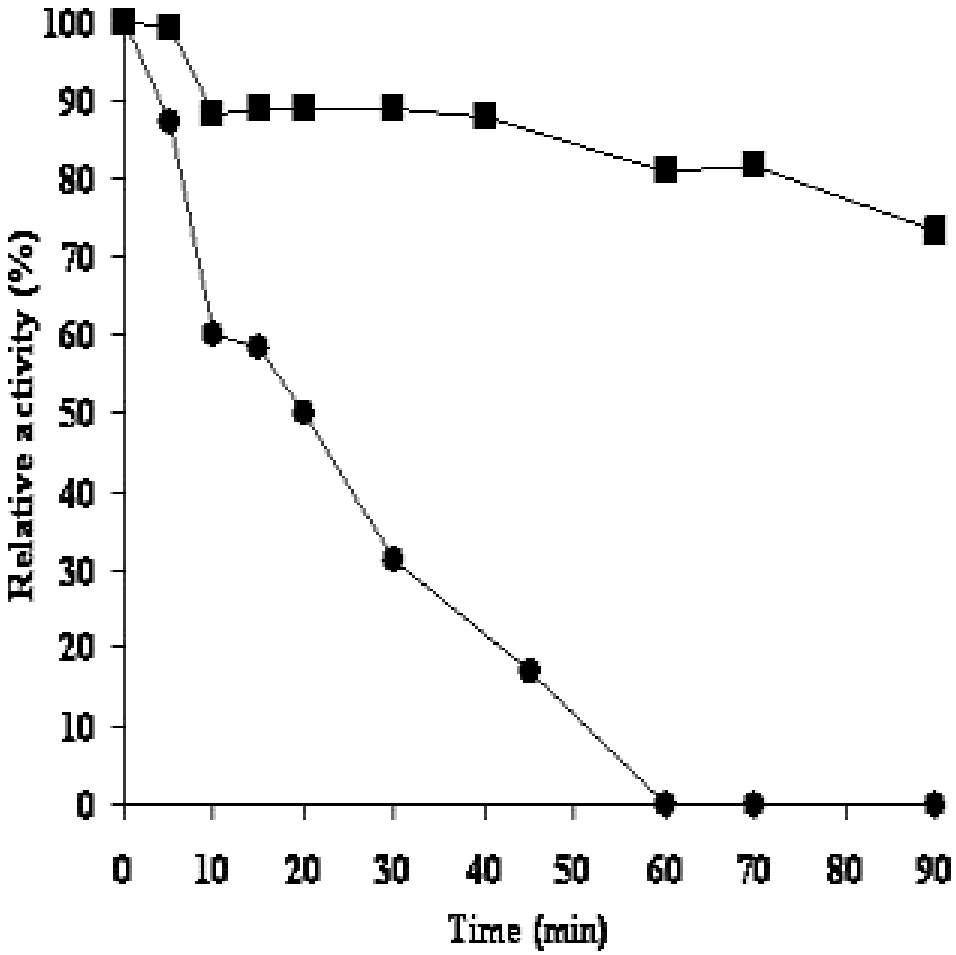

Figure 1. Stability of crude α-amylase at 75 ^oC (■) and 85 ^oC (●). α-amylase activity was measured using 20 gL^{-1} starch as substrate and incubating for 5 min at pH 7.0.

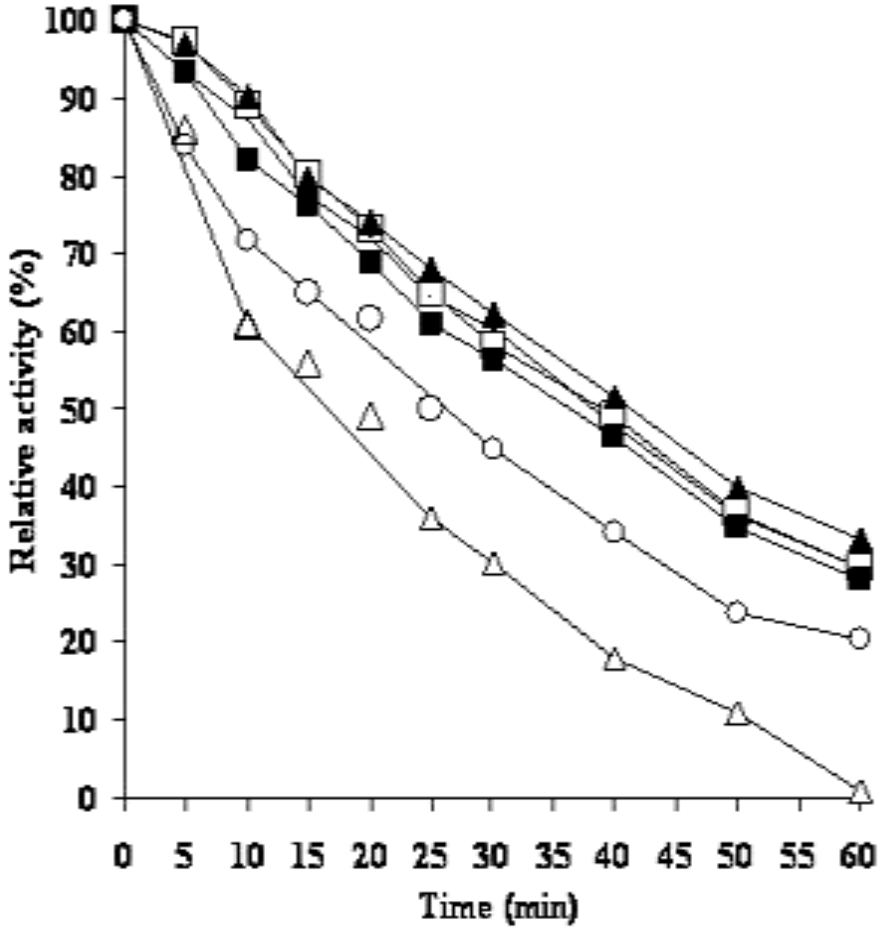

Figure 2. Effect of different concentrations of Na^+; 0 (Δ); 0.05 M (□); 0.1 M (▲); 0.2 M (●); 0.3 M (■) and 0.4 M Na^+ (O), on the stability of α-amylase from *Bacillus licheniformis* ATCC 6346. α-amylase activity was measured at 85 ^oC using 20 gL^{-1} starch as substrate and incubating for 5 min pH at 7.0.

Table 1. Effect of different cations (2 mM) on the activity of α-amylase produced by *Bacillus licheniformis* ATCC 6346. α-amylase activity was determined at 85 ^oC and pH 7.0 using 20 g L^{-1} starch as substrate by incubating for at 5 min.

Cations (2 mM)	**Relative α-amylase activity (%)**
Control*	100
Ca^{2+}	108
Ba^{2+}	93
Mg^{2+}	97
Mn^{2+}	39
Na^+	103
Hg^{2+}	0
Cu^{2+}	0.75

* Buffered α-amylase solution

Table 2. Half-life of α-amylase in the presence of different concentrations of Na^+ at 85 ^oC and pH 7.0.

Na^+ (M)	**Half-life (min)**
0	13.8
0.05	38.4
0.1	42.8
0.2	41.2
0.3	36.3
0.4	22.2

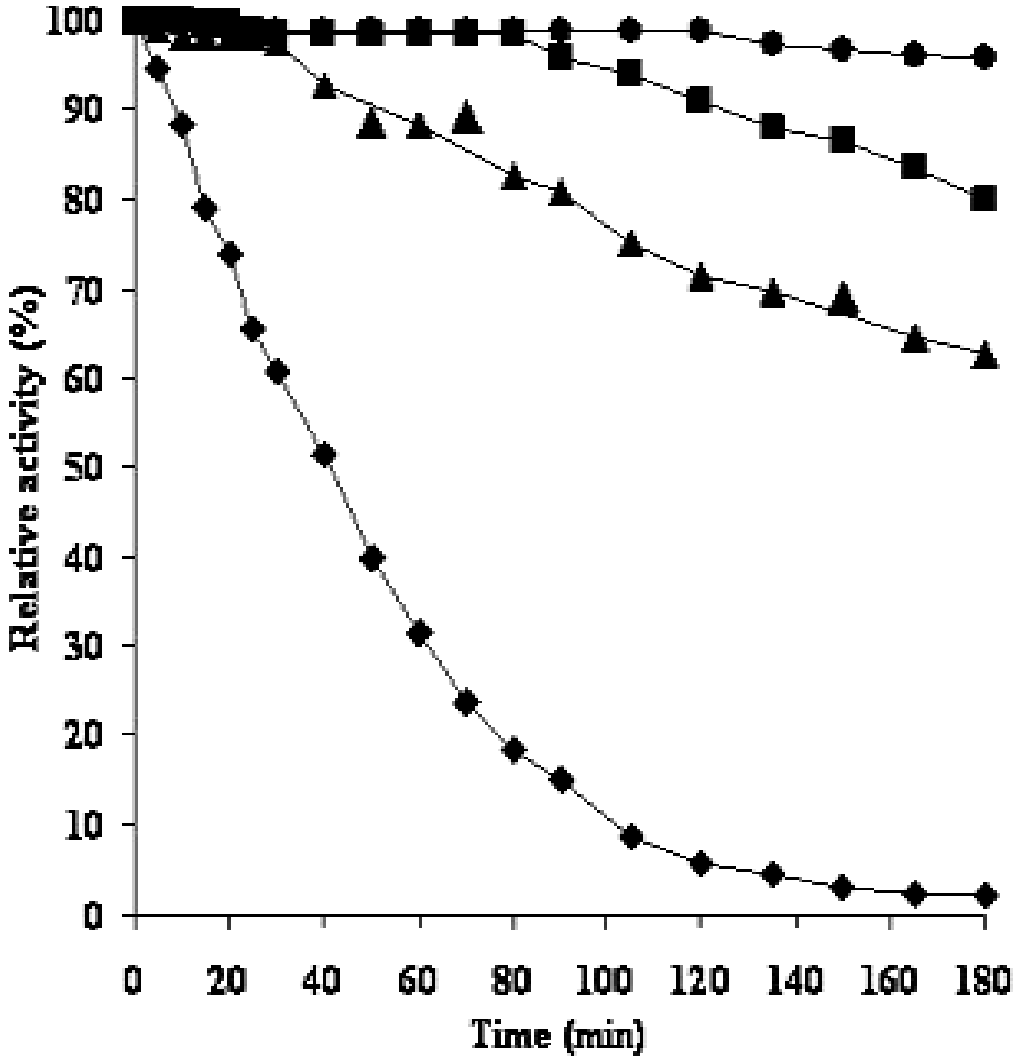

Figure 3. Stability of 0.1 M Na^+ containing α-amylase from *Bacillus licheniformis* ATCC 6346, at 60 (●); 70 (■); 80 (▲) and 85 °C (♦). α-amylase activity was measured using 20 g L^{-1} starch as substrate and incubating for 5 min at pH 7.0.

Table 3. Half-life of α-amylase in the presence of 0.1 M Na^+ at different temperatures at pH 7.0.

Temperature (°C)	Half-life of α-amylase (min)
60	838.9
70	428.4
80	249.4
85	42.8

Table 4. Half-life of α-amylase in the presence of different concentrations of Ca^{2+} at 85 °C and pH 7.0.

Ca^{2+} (mM)	Half-life (min)
0	26.9
0.05	275.5
0.1	433.2
0.5	1443.5
0.7	1750.3
1.0	1782.0

Effect of Ca^{2+} on the stability of α-amylase

In the presence of 0.7 and 0.05 mM Ca^{2+} at 85 ^{o}C, the enzyme retained 96.3 and 82.0% relative activity (Fig. 4) of its initial activity at 60 min. In the absence of Ca^{2+} the enzyme retained 30% of its initial activity at 60 min at 85 ^{o}C and pH 7.0. In the presence of 1.0 mM Ca^{2+} α-amylase showed 6.5 fold increase in half-life when compared to that in the presence of 0.05 mM Ca^{2+} at 85 ^{o}C and pH 7.0 (Table 4).

Effect of Stability of Na^{+} and Ca^{2+} containing α-amylase at different temperatures

In the previous experiment when Na^{+} and Ca^{2+} were added separately, the stability of α-amylase was improved. Therefore, the combined effect of the two ions on α-amylase stability at different temperatures was studied. α-amylase containing 0.1 M Na^{+} and 1mM Ca^{2+}, was pre-incubated at different temperatures (85, 90 and 95 ^{o}C). At 180 min in the presence of both 0.1 M Na^{+} and 1 mM Ca^{2+}, the enzyme retained 94 and 64% (Fig. 5) of the initial activity at 85 and 90 ^{o}C respectively. α-amylase retained 77 and 23 % of its initial activity at 180 min in the presence of 1 mM Ca^{2+} at 85 and 90 ^{o}C respectively. However, the enzyme lost all of its activity at 210 and 120 min (data not shown in the figure) in the presence of 0.1 M Na^{+} respectively at 85 and 90 ^{o}C. Half-life of α-amylase in the presence of either 0.1 M Na^{+} or 1 mM Ca^{2+} or both, decreased with increasing temperature (Table 5). When α-amylase was incubated at 90 and 95 ^{o}C in the presence of 0.1 M Na^{+} and 1 mM Ca^{2+}, the enzyme showed respectively 8.7 and 32 fold decrease in half-life when compared at 85 ^{o}C. In the presence of 0.1 M Na^{+} and 1 mM Ca^{2+}, at 180 min, α-amylase lost 83% of its initial activity at 95 ^{o}C (Fig. 5). However, in the presence of either 1 mMCa^{2+} or 0.1 M Na^{+}, the enzyme lost its total activity at 120 and 90 min respectively at pH 7.0.

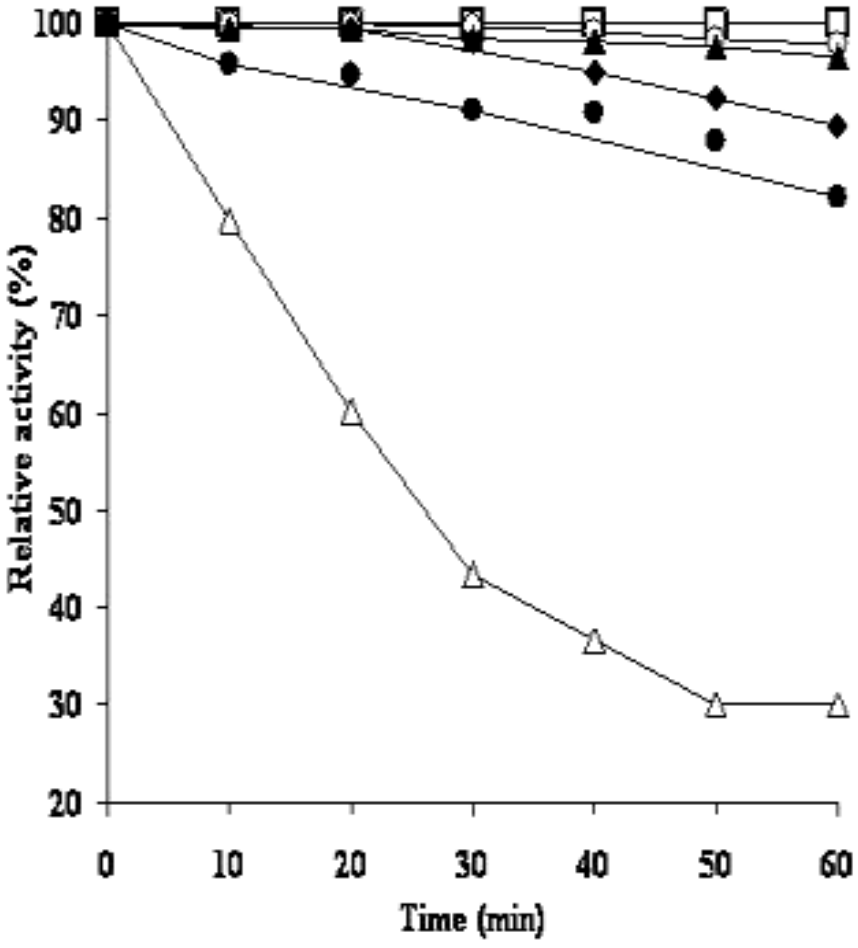

Figure 4. Effect of different concentration of Ca^{++}: 0 (Δ), 0.05 (●), 0.1 (♦), 0.5 (▲), 0.7 (O) and 1 mM (□), on the stability of α-amylase from *Bacillus licheniformis* ATCC 6346. α-amylase activity was measured at 85 ^{o}C using 20 g L^{-1} starch as substrate and incubating for 5 min at pH 7.0.

Table 5. Half-life of α-amylase in the presence of either 0.1 M Na^+ or 1 mM Ca^{2+} or both cations at different temperatures at pH 7.0.

Temperature (°C)	Half-life of α-amylase (min)		
	0.1 M Na^+	1 mM Ca^{2+}	0.1 M Na^+ and 1 mM Ca^{2+}
85	42.8	1782.0	2421.2
90	17.7	93.0	279.5
95	10.6	23.4	75.5

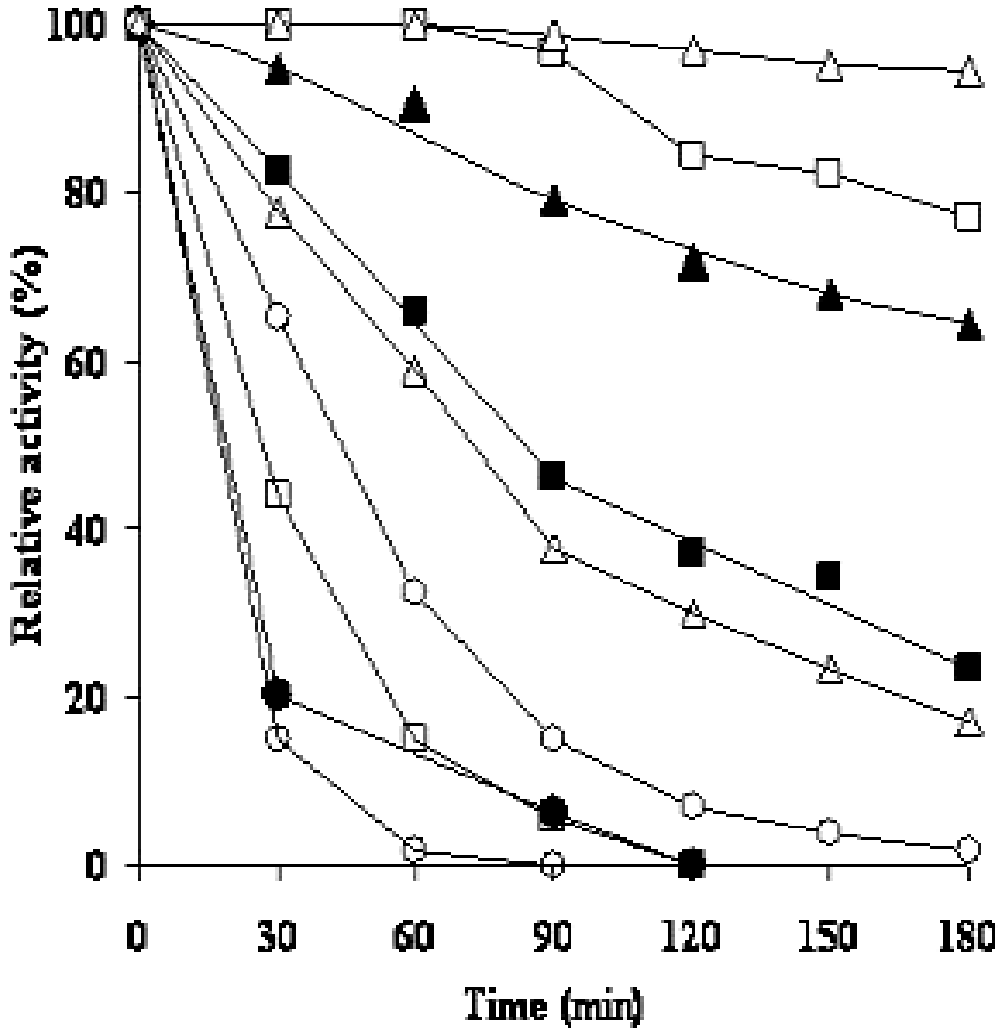

Figure 5. Stability of α-amylase from *Bacillus licheniformis* ATCC 6346; in the presence of 0.1 M NaCl (○), 1 mM Ca^{2+} (□) and both 0.1 M NaCl and 1 mM Ca^{2+} (Δ) at 85 °C; in presence of 0.1 M NaCl (●), 1 mM Ca^{2+} (■) and both 0.1 M NaCl and 1 mM Ca^{2+} (▲) at 90 °C; and in presence of 0.1M NaCl (⊖), 1mM Ca^{2+} (⊟) and both 0.1M NaCl and 1mM Ca^{2+} (⍋) at 95 °C.

DISCUSSION

In the presence of 2 mM Ca^{2+} and Na^{+} ions separately, α-amylase from *B. licheniformis* ATCC 6346 showed higher activity than in the control (which contained no ions) and in the presence of other ions. Therefore, according to our study, α-amylase requires Ca^{2+} and Na^{+} for catalytic activity. A slight inhibition was observed with Mg^{2+} and Ba^{2+} and a stronger inhibitory effect was observed with Cu^{2+}, Mn^{2+} and Hg^{2+}. The effect of metal ions on the activity of α-amylase in *Bacillus* sp strain KSM-1378, a relative of *Bacillus firmus* was investigated by Lgarashi *et al.* (1998) who revealed that Ni^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} strongly inhibited the enzyme activity by 82, 100, 91 and 100%, respectively. Of the cations, Na^{+}, Ca^{2+}, and Mg^{2+}, showed stimulatory effect on α-amylase of *Bacillus licheniformis* CUMC 305, whereas Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Al^{3+} and Mn^{2+} showed inhibitory effects (Krishnan *et al.,* 1983). Cu^{2+} has been reported to inhibit the activity of amylases from *B. circulans* (Takasaki, 1982), *B. coagulans* (Babu *et al*., 1993) and *B. licheniformis* (Krishnan *et al.,* 1983). Based on our initial findings on cations, Ca^{2+} and Na^{+} ions were selected for further investigation to find their effects on the stability of α-amylase from *B. licheniformis* ATCC 6346.

Thermal stability of α-amylase from *B. licheniformis* is suggested to be due mainly to the additional salt bridges involved with lysine residues (Tomazic *et al*., 1988). Similarly, the α-amylase from *B. sterothermophilus* and mutant α-amylase from *B. amyloliquefaciens* have been suggested to be stabilized against thermal denaturation through ionic interactions (Janecek *et al*., 1992). In our study when the concentration of Na^{+} was increased above 0.1 M, the stability of α-amylase was decreased. This could be due to the osmotic effect exhibited by high concentrations of NaCl. Accordingly, 0.1 M Na^{+} was selected to stabilize α-amylase from *B. licheniformis* ATCC 6346. In the presence of 0.1 M Na^{+}, α-amylase showed 1.9 fold increase in half-life than in the presence of 0.4 M Na^{+} at 85^{o} C. When the pre-incubating temperature was decreased from 85 to 60 ^{o}C, the half-life of α-amylase containing 0.1 M Na^{+} was increased by 19.6 fold at pH 7.0. Therefore, Na^{+} improved the stability of α-amylase. However, 0.1 M Na^{+} could not continue to keep the α-amylase in the active state at higher temperatures, above 70 ^{o}C. This could be due to the breakdown of the Na^{+}-α-amylase bond with the increase in temperature.

It can be concluded that Ca^{2+} ions improved the stability of α-amylase from *B. licheniformis* ATCC 6346. At liquefying temperatures (90-100 ^{o}C), commercially used amylases require Ca^{2+} as a stabilizer (Kumar *et al.,* 1990). Since 1.0 mM Ca^{2+} improved the stability of α-amylase it was selected for further investigation of the stability of α-amylase with temperature. Since Ca^{2+} increased the stability of α-amylase it could be concluded that this enzyme is a calcium dependant α-amylase. α-amylase from *B. licheniformis* is far more thermostable than the α-amylases produced by other *Bacillus* species (Declerck *et al*., 2002). Presence of Ca^{2+} ions showed positive effect on the thermostability of amylolytic enzymes, including the amylases from *B. licheniformis* (Violet *et al.,* 1989), *Pyrococcus furiosus* (Dong *et al.,* 1997), *Thermococcus litoralis* (Brown *et al.,* 1993) and α-amylase from *B. amyloliquefaciens*, leading to increased stability (Farez-Vidal *et al.,* 1995).

It is evident from this study that the metal ions such as Ca^{2+} and Na^{+} increase the stability of the α-amylase from *B. licheniformis* ATCC 6346. The half-life of α-amylase from *B. licheniformis* ATCC 6346 at 75 and 85 ^{o}C was 203.2 and 13.8 min respectively at pH 7.0. α-amylase from *Bacillus subtilis* AX20 showed 60 and 35% of maximum activity at 40 and 70 ^{o}C, respectively, and stability at 50^{o} C for 45 min (Mohsen *et al*., 2005). Temperature stable α-amylase from *Bacillus licheniformis* 584 rapidly lost its activity at temperatures above 76 ^{o}C (Saito and Yamamoto, 1975).

Since Ca^{2+} and Na^{+} ions when used separately improved the stability of α-amylase from *B. licheniformis* ATCC 6346, combined effect of these two ions on α-amylase stability at different temperature were studied. Presence of $Ca2^{+}$ and Na^{+} ions together increased the stability of α-amylase more than when they were present alone. Therefore, this supported the previous hypothesis that these two ions form linkages with α-amylase. Of these two ions, Ca^{2+} ions stabilized the enzyme more than Na^{+} ions. Calcium has a direct role in increasing the stability of α-amylases from *B. licheniformis* ATCC 6346. When the temperature was increased from 85 to 95 ^{o}C, the stability of 0.1 M Na^{+} and 1 mM Ca^{2+} containing α-amylase was reduced. Hence, it is inferred that high temperatures disturb the bonds formed between Ca^{2+} and Na^{+} ions and the enzyme. In addition to protein stabilization (Feller *et al.,* 1999), Ca^{2+} has been reported to exhibit roles in allosteric activation (Feller *et al.,* 1994).

The findings indicate that the α-amylase produced by *B. licheniformis* ATCC 6346 possesses properties of an industrial enzyme. Presence of Ca^{2+} increases the activity and stability of the enzyme. Na^{+} also enhances the activity and stability of the enzyme, but the influence of Na^{+} was less than Ca^{2+}. Combined addition of Na^{+} and Ca^{2+} increased the enzyme stability at high temperatures. Its moderate salt tolerance and thermostable property makes this enzyme a potential candidate to be employed in applications requiring salt concentrations and stress containing processes.

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