INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Volume: 2: Issue-2: April-June -2011 IJABPT *ISSN 0976-4550*

DETERGENT STABLE, HALOTOLERANT α-AMYLASE FROM *BACILLUS AQUIMARIS* VITP4 EXHIBITS REVERSIBLE UNFOLDING

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ABSTRACT: Halotolerant *Bacillus aquimaris* VITP4, isolated from Kumta coast (India), was used to produce extracellular α-amylase. The production was found to be maximal after 24 h of growth at pH 8.0 and 40 ºC. Optimal activity of the purified enzyme was in the pH range of 7.5 – 9.5 at 40 ºC. The enzyme was found to retain more than 60% of the initial activity even at NaCl concentration of 3.5 M, indicating that it is halotolerant. Calcium ion (0.01 mM) and CTAB (10 mM) enhanced the activity whereas EDTA decreased the enzymatic activity. Thermal inactivation kinetics suggested that the enzyme exhibits reversible unfolding even at high temperature (till 90 ºC) and the $t_{1/2}$ at 90 ºC was found to be 43.5 min. These results suggests that the α-amylase from *Bacillus aquimaris* strain VITP4 is halotolerant, metal ion dependent enzyme which is stable in the presence of cationic detergent and moderate temperature conditions.

Keywords: *Bacillus aquimaris* VITP4, halotolerant, α-amylase, calcium ion, reversible unfolding, CTAB.

INTRODUCTION

Amylases are extracellular enzymes which can hydrolyze starch to yield dextrins and ultimately to glucose units. 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 (A.T.George and L.F.J.Woods, 1995), they occupy 25% of the world market of enzymes (Niehaus, et al., 1999; Asgher, et al., 2007). Among the various starch hydrolyzing enzymes, α-amylases are on high demand. α-amylases (endo-1,4-α-D-glucan glucanohydrolase [E.C.3.2.1.1]) act on α-1,4 glycosidic bonds of starch to form maltoses, maltotrioses, maltotetrose, glucoses and a mixture of malto-oligosaccharides (H.Uhlig, 1998). Also α-amylases of microbial origin have replaced the chemical hydrolysis of starch (Pandey, et al., 2000). Of the micro-organisms, many fungal strains have been explored for the production of α-amylase, mostly owing to low cost in production. However, in certain applications like detergent and bakery industries the properties of α-amylase needed is quite challenging (Burhan, et al., 2003; Gupta, et al., 2003) and therefore continued efforts are being undertaken for obtaining α-amylases that are tolerant under extreme environmental conditions, especially salt, pH and temperature. Enzymes that are capable of surviving under these adverse conditions are termed either extremophilic or extremotolerant. Several extremophilic enzymes have been isolated and well characterized. However these differ from the extremotolerant enzymes as they are functional only under extreme conditions whereas the extremotolerant enzymes are functional even under mesophilic conditions. Recently we have isolated several salt tolerant bacterial strains from saltern of Kumta, coastal Karnataka, India. A few of these strains have been shown to produce proteases that are tolerant to salt and alkaline pH conditions (S.Pooja and G.Jayaraman, 2009). In continuation of our efforts to unravel the potential application of the strain, we explored the possibility of α-amylase production by the bacterial strain, *Bacillus aquimaris* VITP4 (GenBank ID: FJ687490). In this article we report the production and characterization of α-amylase from this halotolerant strain and the results show that the α-amylase from this strain is metal ion dependent and exhibits enhanced activity in the presence of cationic detergent cetyltrimethylammonium bromide (CTAB).

MATERIALS AND METHODS

Chemicals and experimental statistics

All chemicals used in the present study were of high quality analytical grade and media components of highest purity grade were purchased from Hi-Media, Mumbai. Production studies were carried out as batch cultures in 250 ml Erlenmeyer flasks, containing 100 ml of culture media. All the experiments were carried out independently in triplicates and repeated twice.

Micro-organism and the inocula preparation

Bacillus aquimaris VITP4 previously isolated from the Kumta coast of Karnataka, India (S.Pooja and G.Jayaraman, 2009), was initially cultured and maintained on Zobell marine agar plates (C.E.Zobell, 1941). To verify the capability of the strain to produce α-amylase, the micro-organism was grown on starch Zobell marine agar plate. After incubation at 37 ºC for 48 h, the plate was flooded with 0.5% (w/v) I_2 and 5% (w/v) KI solution and equal volume of 1 N HCl) (0.1 N KI_3 reagent). The 24 h old colony culture was used as inoculum in Zobell marine broth. Sub-culturing was performed for every two months and the colony forming unit (CFU) count was calculated by counting the number of colonies on the agar plate under the colony counter. Also, CFU was estimated from the measurement OD_{600} by using the equation $ln(CFU) = a + b\ ln(OD)$, where a and b were calculated using simple regression analysis (Eric, et al., 1997). Before inoculating for the α-amylase production the strain was further confirmed by certain biochemical identification methods like gram staining and morphological examination under light microscope (100x).

α-amylase production and assay

Overnight culture (1% v/v containing 1.5 x 10^{12}/ml CFU) was used to inoculate Zobell marine broth supplemented with 1% starch (w/v) for the enzyme production. Cultivation was performed on an incubator shaker (150 rpm) for 48 h at 37 ºC (pH 7.0) in 250-ml Erlenmeyer flasks with a working volume of 100 ml (C.E.Zobell, 1941). After defined time periods, the culture was centrifuged at 10,000 rpm for 20 min at 4 ºC, and the cell-free supernatants were used for the estimation of α-amylase activity and partial purification (McTigue, et al., 1995). Protein content was quantified by the method of Lowry (Lowry, et al., 1951) using Bovine Serum Albumin (BSA) as standard.

Determination of α-amylase activity

The liquefying activity of the enzyme was assayed using 0.1 mg of enzyme supernatant and 0.1 ml of 0.1% (w/v) soluble starch substrate at 40 ºC in the presence of 0.02 M sodium phosphate buffer (pH 6.9) in a final assay volume of 0.9 ml. After 15 min incubation, the reaction was stopped by the addition of 0.1 ml of KI_3 reagent. The starch-iodine complex formation was quantified using the absorbance at 550 nm by monitoring the presence of unhydrolysed/partially hydrolyzed starch (S.Srimathi and G.Jayaraman, 2005). Hydrolytic activity of enzyme was determined by measuring the decrease in iodine color, where the reaction showed dextrinizing of starch. One unit of enzyme activity is defined 0.0284 optical density reduction of blue color intensity of starch iodine solution at 40 ºC. Soluble starch (0.1 % w/v) was used as standard (P.K.Bajpai and N.P.Verma, 1990).

Optimum pH and temperature for production

Initial pH of the culture was adjusted to required pH (7.0 - 9.0) using either 1 N NaOH or 1 M HCl. The production of enzyme was examined at 37 ºC for a duration of 24 h taking the samples for every 6 h as described previously. Similarly, the temperature required for optimum production of enzyme was studied by culturing the strain at different temperatures, in the range 30 ºC to 60 ºC.

Optimum pH and temperature for activity

The effect of pH (4.5 - 10.5) on the activity of the enzyme was investigated by preparing 0.1 % (w/v) soluble starch substrate in various buffers of 20 mM concentration (pH 4.5 - 6.5, sodium acetate; pH 6.5 – 8.0, sodium phosphate; pH 7.5 - 9.0, Tris-HCl; pH 9.0-10.5, glycine-NaOH) at 45 ºC.

The effect of temperature on the activity of the enzyme was studied in the temperature range of 20 ºC to 90 ºC with 0.1 % soluble starch dissolved in 20 mM sodium phosphate buffer (pH 8.0). The residual activity of the partially purified enzyme was examined by incubating the enzyme preparation at various temperatures from 20 ºC to 90 º C after which the remaining activity was determined under the enzyme assay conditions (pH 8.0).

Thermal stability and reversibility

Thermal stability of the sample was measured by incubating the crude extract at various temperatures in the range 20 ºC to 90 ºC for 6 h, collecting the sample for every 1 h, and the residual activity was measured as mentioned before. Half-life of inactivation ($t_{1/2}$) was calculated from the plot of log percentage residual activity versus time using the relationship $t_{1/2} = 0.693/k_i$ where k_i is the inactivation rate constant obtained from the slope of the curve (Srimathi, et al., 2007).

Effect of salt, metal ions and other additives

Effect of salt on enzyme activity was investigated in the NaCl concentration range of 0 M to 5 M (in 20 mM sodium phosphate buffer at pH 8.0). In order to investigate the role of cation, the hydrolytic activity of the enzyme was assayed in the presence of $CaCl_2$ dissolved in 20 mM sodium phosphate buffer at pH 8.0 (0.01 mM, 0.1 mM, 1 mM and 10 mM). In addition, effects of ethylenediaminetetraacetic acid (EDTA), tween 20, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB) and β-mercaptoethanol were also studied. In all these experiments, the enzyme was incubated for 15 min at optimum temperature and pH and the residual activity was determined. The activity of the enzyme in 20 mM sodium phosphate buffer, pH 8.0 (in the absence of additive) was taken as 100%.

RESULTS AND DISCUSSION:

Microbial growth and α-amylase production

The growth studies of *B. aquimaris* VITP4 were performed using inoculum of 24 h old culture (1.5 x 10^{12}/ml CFU) from the agar plates into 1% (w/v) starch Zobell marine agar medium (Figure 1). The ability of the organism to produce amylase(s) was initially confirmed by the observation of zone of clearance around the culture colonies grown in starch agar media when flooded with the KI_3 reagent. The growth pattern as determined by OD_{600} indicate that the culture is in 6 h of log phase but predominantly it is in stationary phase for 12 h followed by decline in the growth, as the viable cell density decreased upon observing the culture density. The growth profile shows that this strain has a fast growth rate as it entered log phase within 2 h of lag phase. The doubling time was calculated to be 15 min. In general, the bacterial growth is maximum till 24 h, excepting the cases of longer generation time (K. Toda, 2002).

Figure 1: Initial confirmation of enzyme activity using starch agar plate method

Maximum enzyme production was observed at 24 h of growth when the organism was in the stationary phase (Figure 2). This indicates that the enzyme production is growth associated and was induced by the presence of substrate. Similar results were shown by halophilic bacteria *Chromohalobacter* sp. TVSP 101 (Prakash, et al., 2009) but the time of incubation is comparatively high (96 h). The results were plotted (Figure 3) using specific activity (U/mg) as a function of time of incubation (h), wherein the maximum activity at 24 h was observed to be 450 U/mg. The decrease in the enzyme activity at longer growth time, could also be attributed to the co-production of protease. However, protease assay, confirmed that there was no proteolytic activity and thus ruling out the presence of protease.

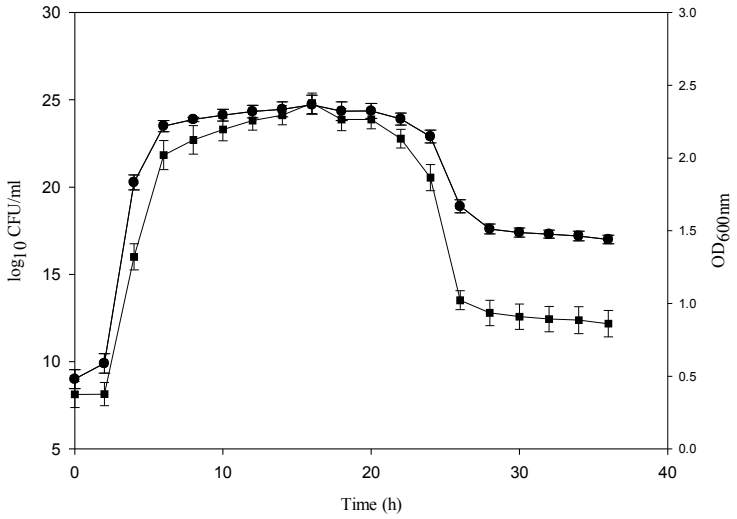

Figure 2: Growth curve of *B. aquimaris* VITP4 in 1% starch and Zobell marine broth media

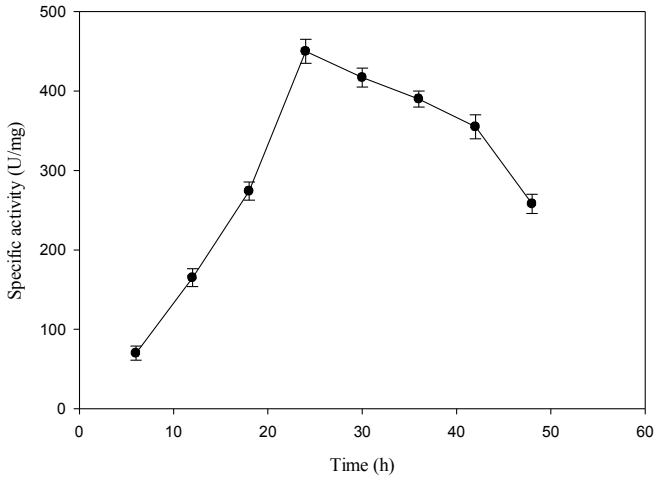

Figure 3: Optimum time of incubation for maximum enzyme production

Effect of pH and temperature on enzyme production

Growth of the strain occurred over a pH range of 7.0 to 9.0. However, the optimum pH for the maximum production of enzyme was observed at pH 8.0 (specific activity 525 U/mg) and also the growth was maximum at the same pH (Figure 4). Similar results were shown by a halophilic α-amylase from a moderately halophilic bacterium *Nesterenkonia* sp. Strain F, which showed substantial growth in the pH range 6.0 to 10.0 (Shafiei, et al., 2010). In general the production of halophilic and halotolerant enzymes carried out at neutral pH results in a slight raise in the pH probably due to the release of various metabolites as the growth proceeds that favors the production of enzyme (Carvalho, et al., 2008). These bacterial strains were (Carvalho, et al., 2008; Gupta, et al., 2003) allowed to grow on a wide range of temperatures ranging from 30 ºC to 60 ºC and majority of them with an optimal temperature of 40 ºC (Gupta, et al., 2003).

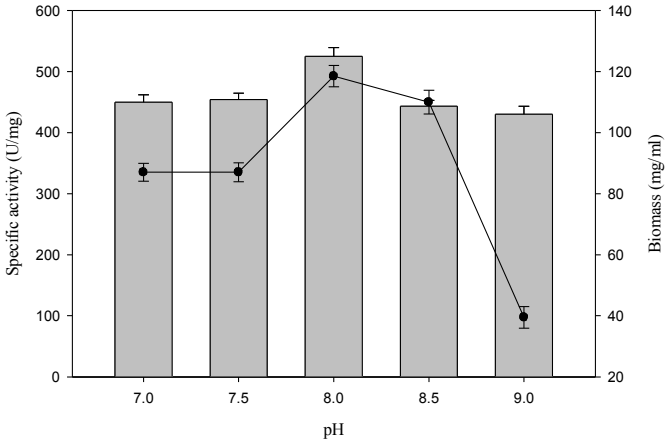

Figure 4: Optimum pH for enzyme production

Growth of a soil bacteria ANT-6 *Bacillus* sp. was observed at 20 ºC (Burhan, et al., 2003) whereas a hyperthermophile *Thermococcus profundus* (Chung, et al., 1995) was found to exhibit maximal growth at 80 ºC. (Figure 5) reveals that the strain *B. aquimaris* VITP4 exhibited maximum enzyme production at 40 ºC (650 U/mg). Although growth was observed in the temperature range 30 ºC to 60 ºC, it was found to be maximum at 40 ºC indicating one-to-one correlation between enzyme production and biomass, which clarifies that the enzyme production is growth dependent. These results are in agreement with the reports for *Bacillus* sp. YX-1 (X.D.Liu and Y.Xu, 2008) and a soil bacterium *Bacillus* sp. I-3 which exhibited optimal temperature of 45 ºC and 37 ºC respectively (Goyal, et al., 2005).

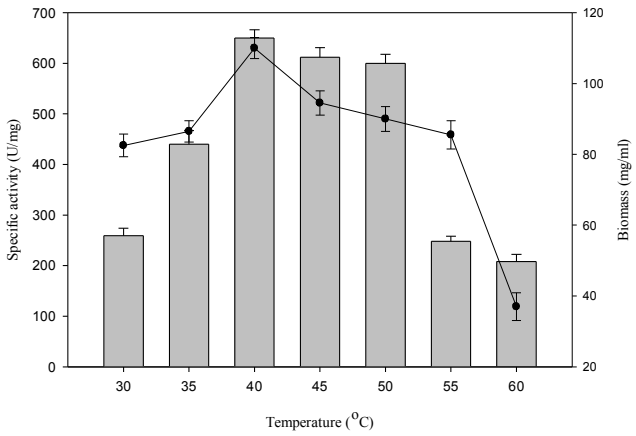

Figure 5: Optimum temperature for enzyme production

Effect of pH on the α-amylase activity

The effect of pH on the amylolytic activity of *B. aquimaris* VITP4 was investigated in the pH range 4.5 to 10.5 using appropriate buffers (see materials and methods for details). The enzyme showed maximal activity in the alkaline pH range 7.5 to 9.5 (Figure 6). Enzyme activity at pH 8.0 and 8.5 nearly same (751.5 U/ml and 747 U/ml respectively), as recently shown by *Bacillus* sp I-3 α-amylase (Goyal, et al., 2005). However, the activity decreased rapidly at pH 10.0 (213.3 U/ml). On the contrary, α-amylases produced by other *Bacillus* sp like *Bacillus* sp isolate L1711 (Bernhardsdotter, et al., 2005) and *Bacillus subtilis* have shown optimum activities at pH values as low as 3.5 or as high as 12.0 respectively (Carvalho, et al., 2008; Z.Konsula and M.Liakopoulou-Kyriakides, 2004).

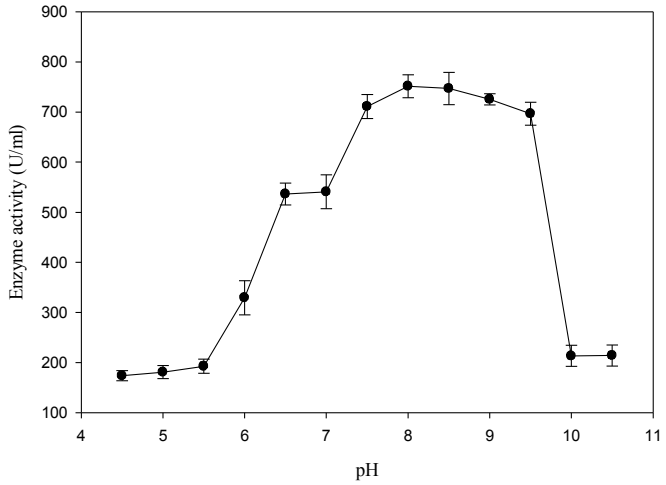

Fig. 6: Effect of pH on the activity of α-amylase

Effect of temperature on the α-amylase activity

The effect of temperature on the amylase activity was tested in the temperature range 20 ºC to 90 ºC. There was a substantial increase in the enzyme activity till 40ºC (Figure 7). Considerable enzyme activity was seen even at temperatures beyond the optimum temperature (40ºC). At 50 ºC and 60ºC, the enzyme activity was 676.8 U/ml and 459.9 U/ml, respectively, indicating that the enzyme was moderately thermostable. Halophilic α-amylases from *Micococcus* sp 4 (J.M.Khire, 1994), *Haloarcula hispanica* (Hutcheon, et al., 2005) and haloarchaeon *Haloarcula* sp strain S-1 (Fukushima, et al., 2005) have been reported to have optimal temperature of 50 ºC whereas the halotolerant bacterium, *Nesterenkonia* sp. strain F showed optimal activity at temperature 55 ºC (Shafiei, et al., 2010).

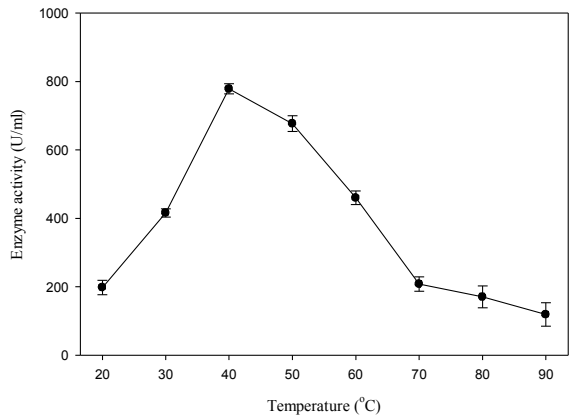

Figure 7: Effect of temperature on activity of α-amylase

Thermostability studies and half-life of the enzyme

The stability of the enzyme was studied at different temperatures for 6 h (Table 1). The half-life calculated at the optimum temperature (40 °C) was 156 ± 5 min. The enzyme stability profiles showed the biphasic nature of enzyme in the temperature range studied. For the first 4 h nearly 75% (562.5 U/ml) of the initial activity (758.8 U/ml) was retained. This nature was clarified from the plot between log (residual activity %) versus time in minute. The enzyme's half-life was found to be 120 min and 43.5 min at 50 °C and 90 °C, respectively. The half-life of the α-amylase from *Bacillus* sp. Ferdowsiceus had a half-life of 48 min at 80 °C (Asoodeh, et al., 2010). A psychrophilic α-amylase from the organism *Pseudoalternomonas haloplanktis* was reported to exhibit half-life of 83 min at 40 °C (Srimathi, et al., 2007). Prakash et al., 2009 reported the half-life of α-amylases from *Chromohalobacter* sp. TVSP 101 was 1 h at 80 °C. These studies indicate that the thermal stability of the α-amylase from the halotolerant *B. aquimaris* VITP4 is comparable to that shown in literature for true halophiles. However, it should be pointed out that comparison of the temperature dependent enzymatic activity and the thermal inactivation kinetics indicates that the enzyme has exhibited reversible unfolding. At high temperatures, greater than the optimal temperature, there is reduction in the activity owing to increased flexibility and probably denaturation at still higher temperatures. But even after heating for one hour the enzyme at 90 °C, it was found to exhibit 80% (606.4 U/ml) enzyme activity (Table 1), indicating that enzyme has successfully refolded to its native conformation. This is further confirmed by the fact that prolonged (6 h) heating of enzyme at 90 °C, the half-life of the enzyme has decreased only to 43.5 min. It should be pointed that there are not many reports in literature, depicting the halotolerant hydrolyzing enzymes that have been shown to successfully refold after heat denaturation.

Table 1: Thermal Inactivation Kinetics. The relative activity (%) was calculated by taking control (Enzyme activity 758.8 U/ml) as 100%. The activity given is for the enzyme incubated at the specified temperature for 1 hour. $t_{1/2}$ was calculated from the plot of log percentage residual activity versus time. Standard deviation showed was calculated from three experimental replicates.

Temperature (°C)	Relative activity (%)	$t_{1/2}$ (min)
40	94 ± 1	156 ± 5
50	93 ± 1	120 ± 4
60	89 ± 1.5	108 ± 3
70	83 ± 1.5	103 ± 1
80	84 ± 1.7	73.2 ± 0.7
90	80 ± 1.9	43.5 ± 0.7

Effect of salt on enzyme activity

In order to investigate the salt tolerance, amylase activity was assayed in the presence of different NaCl concentrations (0 - 5 M). At 0 M salt concentration, 758.8 U/ml of hydrolytic activity was observed. (Figure 8) depicts that the enzyme activity is not significantly changed till 1 M NaCl concentration. However, when NaCl concentration was increased beyond 1 M, the activity decreased, but was above 75% (562.5 U/ml) till 3 M salt concentration. The activity was close to zero only when the salt concentration was as high as 5 M. This clearly indicates that the enzyme is halotolerant and is comparable with a few halophilic amylases reported in literature. Halotolerant bacterium *Chromohalobacter* sp.

TVSP 101 (Prakash, et al., 2009) α-amylase was shown to exhibit 90% of the relative activity till 2.5 M. *Bacillus* sp. strain TSCVKK retained 100% activity at 1.7 M NaCl (K.K.Kiran and T.S.Chandra, 2008). Broad range of salt tolerable concentrations was reported in halophilic amylases from *M. halobia* (H. Onishi and K.Sonada, 1979) moderate halophile *Halomonas meridian* (Coronado, et al., 2000), *Bacillus dipsosauri* (C.E.Deutch, 2002) and *Halobacterium halobium* (W.M.Good and P.A. Hartman, 1970). A thermophilic *Bacillus* sp strain SMIA-2 retained 63.4% enzyme activity in 2.0 M NaCl (Carvalho, et al., 2008). Highest halotolerance was reported only for a recombinant bacterium, *Bacillus* sp. MD 124 which retained 100% amylase activity even in 5 M NaCl solution (Jana, et al., 1997). This indicates that the salt tolerance of the α-amylase produced from this *Bacillus* strain is maximum, reported thus far in literature.

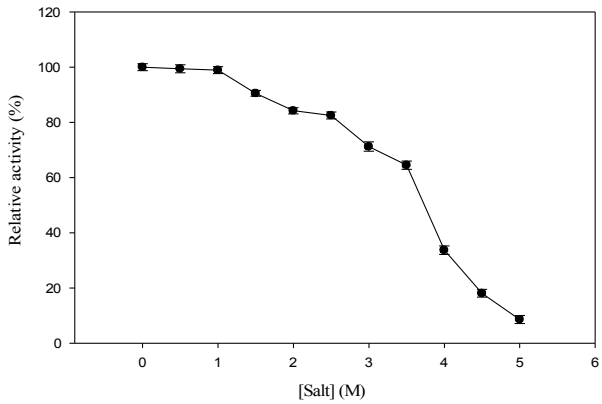

Figure 8: Effect of salt on enzyme activity

Effect of co-factor and other additives on the enzyme activity

The effect of Ca^{2+} ion on the amylolytic activity (0.01 mM, 0.1 mM, 1 mM and 10 mM) was investigated under optimum temperature and pH conditions (Figure 9). Most of the α-amylases are activated in the presence of Ca^{2+}. In the present study the enzyme activity was enhanced in the presence of lower Ca^{2+} ion concentrations by 4.4% (33.35 U/ml). Most of the bacterial α-amylases are calcium dependent. Recent reports on α-amylase from *Bacillus licheniformis* ATCC 9945a (Natasa, et al., 2011), halotolerant bacteria like *Chromohalobacter* sp TVSP 101 (Prakash, et al., 2009) and *Nesterenkonia* sp strain F (Shafiei, et al., 2010) have shown similar results where the enzyme activity enhanced in the presence of Ca^{2+} metal ion. On the contrary there are calcium-independent α-amylases were also reported from *Bacillus* sp. KR-8104 (Sajedi, et al., 2005), *Bacillus thermooleovorans* NP54 (Malhotra, et al., 2003), *Bacillus* sp WN11 (Mamo, et al., 1999).

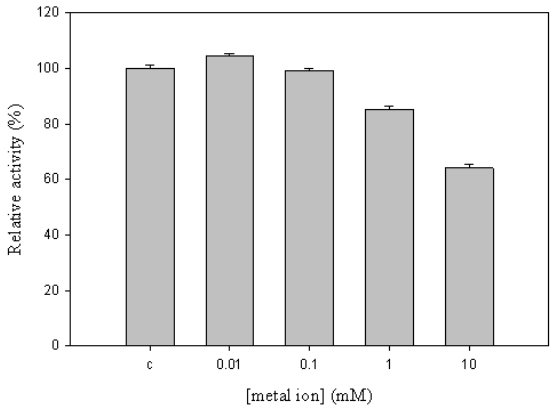

Figure 9: Effect of Ca^{+2} metal ion on the activity of crude α-amylase

The effect of metals can vary widely. For instance, there are reports in which no effect of Ca^{2+} and Fe^{2+} on the α-amylase from *B. licheniformis* NH1 (Hmidet, et al., 2008) and also Ca^{2+} was found to strongly enhance the enzyme activity of *Geobacillus* sp LH 8 (Mollania, et al., 2010).

Various additives viz., EDTA, SDS, CTAB, Tween 20, β-mercaptoethanol were used to investigate their effect on enzyme activity (Table 2). Enhanced enzyme activity (160%, 1212.8 U/ml) was recorded when 10 mM CTAB was used in the assay buffer. However when the additives such as SDS, EDTA and Tween20 were added to the enzyme assay mixture, drastic decrease in enzyme activity was observed. Presence of higher concentration of these additives resulted in complete inhibition. Thus cationic detergent CTAB appears to be only stimulant of *B. aquimaris* VITP4 α-amylase and first ever reported, although lower concentrations of it showed partial inhibition. In general EDTA shows non-competitive inhibition (Asgher, et al., 2007), that is irreversible. The inhibitory effect showed by EDTA confirms that binding to metal ions is required for enzyme activity. This behavior can be compared with *B. licheniformis* NH1 α-amylase (Hmidet, et al., 2008). It was observed in the present study that the enzyme activity decreased as the concentration of β-mercaptoethanol was increased. Tolerable concentration of β-mercaptoethanol thus was 2 mM with a relative enzyme activity of 48% (363.8 U/ml). Increase in β-mercaptoethanol brings change in the conformation of the enzyme in the active site by breaking disulfide bonds, results in loss of enzyme activity. This indicates that the disulfide bonds play an important role in maintaining the structure of α-amylase from *Bacillus aquimaris* VITP4. β-mercaptoethanol is equivalent to dithiotritol (DTT) whose action has non-significant effect on the enzyme activity in case of recombinant maltogenic amylase from *Bacillus* sp. WPD616 (Liu, et al., 2006).

Table 2: Effect of additives. The relative activity (%) was calculated by taking control (Enzyme activity 758.8 U/ml) as 100%. Standard deviation showed was calculated from three experimental replicates.

Additive	Relative Activity (%)		
	2 mM	5 mM	10 mM
Tween	42 ± 1	6 ± 0.3	0
CTAB	4 ± 0.2	28 ± 0.5	160 ± 1.5
SDS	2 ± 0.1	0	0
EDTA	20 ± 0.4	0	0
β-mercaptoethanol	48 ± 1.2	36 ± 0.7	18 ± 0.4

CONCLUSION

The present study on the α-amylase from halotolerant bacteria *B. aquimaris* VITP4 from the saltern of Kumta coast has shown optimal activity at pH range of 7.5 – 9.5 at 40 °C. The activity is maintained even in the presence of moderate concentration of NaCl and further enhanced in the presence of calcium ion and cationic detergent CTAB. Substantial activity, even after incubation at higher temperatures indicates that the enzyme could be refolded efficiently unlike many of its counterparts that are reported in literature. Its salt tolerance and moderate thermotolerant property makes this enzyme a potential candidate to be employed in applications requiring high salt concentration and stress containing process like clarification of juices.

ACKNOWLEDGMENT

The authors thank the management of VIT University for providing the facility and support.

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