Indian Journal of Geo Marine Sciences
Vol. 47 (01), January 2018, pp. 240-248

Catalysis and stability of an extracellular α- amylase from a haloalkaliphilic bacterium as a function of the organic solvents at different pH, salt concentrations and temperatures

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Received 18 January 2016 ; revised 17 November 2016

The Ve1-10-8_2 amylase displayed significant tolerance against methanol, propanol and n-hexane. At the lower concentrations of the solvents, the enzyme catalysis was comparable to control. The enzyme had a broader range of alkaline pH for catalysis, the optimum being at pH 10-11. This pH range was higher than many reported amylases of the alkaliphilic *Bacillus* species. At the alkaline pH, the enzyme was stable for 3 hrs in the presence of 20 % (v/v) methanol. Optimal temperature at 60 ºC for the catalysis remained unaltered in organic solvents. However, the enzyme was active for up to 70 ºC with 5 and 10 % (v/v) methanol. The enzyme catalysis was optimum with 2 M Salt in most of the tested solvents and it retained significant activity at 4 M salt. To the best of our knowledge, this report is the first on the organic solvent tolerance of the extracellular amylase from the haloalkaliphilic bacteria.

[**Key Words:** Haloalkaliphilic bacteria; organic solvent tolerance; α-amylase; non-aqueous enzyme catalysis]

Introduction

Amylases hydrolyze natural polymeric substrate starch and are widely present among eucaryotes, bacteria, and archaea. There are varieties of enzymes implicated in the hydrolysis of starch, and many are widely used in various industrial processes, such as starch liquefaction and pulp process and in detergent [1-3]. Amylases have increasingly become one of the most valuable enzymes in biotechnology, particularly in the food and starch processing industries [4].

Haloalkaliphiles are a class of extremophilic organisms adapted to saline and high pH conditions [5, 6]. Ability to sustain under dual extremity makes them interesting system to study the biochemical and molecular basis of adaptation, besides biotechnological aspects.

Halophilic proteins are adapted to tolerate high salt concentrations, as reflected in their unique amino acid composition [7-11]. The surface of halophilic proteins is highly acidic leading to enhanced hydrophobicity compared to non-halophilic proteins. These features are important to prevent aggregation and, at the same time, retain structural flexibility with organic solvents [11]. Strikingly, the haloalkaliphilic enzymes require high salt for the activity and stability and at less than 1-2 M NaCl or KCl, most haloalkaliphilic proteins unfold and lose their activity [10]. High salt concentration appears to enhance hydrophobicity.

While many starch-degrading proteins are identified in various organisms, only limited attention has been paid to haloalkaliphilic bacteria in this context. The search for the organic solvent tolerant amylases would expand into new fields; such as clinical, medicinal and analytical chemistry. The present work aimed at the enzymatic potential of the haloalkaliphilic amylases in the organic solvents.

Materials and Methods

The amylase producing haloalkaliphilic bacteria were isolated from mud and seawater of Okha (22°28′0″N 69°4′0″E), Diu (20°43′N 70°59′E20.71°N 70.98°E) and Veraval (20°54′N 70°22′E20.9°N 70.37°E) along the Coastal Gujarat by Dr. Rupal Joshi and Dr. Mital Dodia in Prof. S.P. Singh's laboratory in the Department of Biosciences, Saurashtra University, Rajkot, India. Samples were subjected to

the enrichment culture techniques in Complex Medium Broth (CMB) consisting, (g/liter): Glucose, 10; Peptone, 5; Yeast extract, 5; KH_2PO_4, 5; with varying concentration of NaCl (10-20 %, w/v) at different pH 8-10. The pH of the medium was adjusted by adding separately autoclaved Na_2CO_3 (20%, w/v). After inoculation, flasks were incubated on an environmental shaker at 37 °C with regular monitoring on the turbidity. After 48-72 hour of growth, a loop full culture was streaked on the CMB agar (3%, w/v) plate and incubated at 37°C. After 48 hours of the incubation, on the basis of colony characteristics, various isolated colonies were selected and pure cultures were obtained by subsequent streaking on the CMB agar plate.

For the screening of the amylase producing bacteria, the actively growing cultures were prepared as mentioned above and inoculated on the starch agar plates (g/liter: Starch, 2; Yeast extract, 3; Peptone, 5; NaCl, 100, pH 8-10; Agar, 30) as spot. The plates were incubated for 24-48 hour at 37 °C and after sufficient growth, the amylase producing bacteria appeared on the starch agar plate were picked and preserved at 4 °C.

From the activated culture of the amylase producing isolate (A_{540}; 1.0), 5 % was inoculated into Starch medium (g/liter: Starch, 2; Yeast extract, 3; Peptone, 5; NaCl, 100, pH 9). The culture was harvested after 12 hours, which was pre standardized for the maximum amylase production. The culture was centrifuged at 8,000 rpm for 10 min at 4 °C and the cell free extract was used as the crude enzyme preparation. Amylase was measured by estimating reducing groups released from starch, by the reduction of 3, 5-dinitrosalicylic acid (DNS) with a slight modification of Bernfeld [12] method. The 0.5ml enzyme was added to 1ml (2%, w/v) starch prepared in NaOH-Borax buffer (20 mM, pH 10) and incubated at 37 °C for 20 min. One ml of DNS reagent (g/liter: DNS, 10; Sodium potassium tartrate, 300; Sodium hydroxide, 16) was added to the mixture and kept in boiling water bath for 10min. After cooling, the mixture was diluted with 8 ml distilled water and absorbance measured at 540_{nm}. One unit of the amylase activity was defined as the enzyme liberating 1µg of the maltose from starch per minute under the standard conditions of the enzyme assay. Methanol, butanol, propanol and n-hexane were selected for the non-aqueous enzyme catalysis by the amylase, based on their Log P_{ow} values (0.9, 0.25, 3.9 and 0.8).

Eight bacterial isolates; Kh-10-10_1, Dj-30-8_1, Ve1-10-8_1, Ve1-10-8_2, Ve1-10-8_3, Ve1-10-9_1, Ve1-10-9_2 and Ve1-10-9_3 were selected for the screening of their amylase tolerance against solvents. Kh-10-9_1, Dj-30-8_1 were isolated from Okha (22°28′0″N 69°4′0″E) and Diu (20°43′N 70°59′E20.71°N 70.98°E / 20.71; 70.98), respectively, while Ve1-10-8_1, Ve1-10-8_2, Ve1-10-8_3, Ve1-10-9_1 , Ve1-10-9_2 and Ve1-10-9_3 were from Veraval (20°54′N 70°22′E20.9°N 70.37°E). Amylase activity was measured in a reaction mixture of 0.5 ml enzyme and 1 ml starch solution (2 %, w/v) prepared in NaOH-Borax buffer (20 mM, pH 10) with 5, 10 and 20 % (v/v) of methanol, propanol, n-hexane and butanol. The enzyme estimation was as described above. Controls of each set were also included in the experiments.

Amylase catalysis in the organic solvents was monitored at different pH, using buffers (20 mM); Sodium Phosphate (pH 5.5 - 8), Tris-HCl (pH 8 - 9.5), NaOH-Borax (pH 9.5 - 10) and Glycine - NaOH (pH 8 - 12). The enzyme was incubated with 5, 10 and 20 % (v/v) of methanol and butanol along with the respective buffer. To investigate the effect of solvent on the stability of the enzyme at different pH, the pH was adjusted to 5-13 with the above buffers in the presence of 20 % methanol. After incubation for 30-180 minute, the residual activities were estimated.

The effect of NaCl and organic solvents in conjunction with enzyme activity was assessed by supplementing the reaction mixture with 0.5- 4M NaCl. Amylase assay was carried out at 37°C with 5, 10 and 20 % (v/v) of the tested solvents. For the enzyme stability in response to salt and solvent, the enzyme was incubated with NaCl in the range of 0-4M NaCl and the aliquots were withdrawn at regular time intervals for monitoring the residual activities. The amylase activity in the absence of NaCl was considered as a 100 %.

The temperature profile of the amylase catalysis was monitored by incubating the assay reaction mixture at different temperatures in the range of 37-80 °C. Amylase activity was determined as mentioned above. Temperature stability was studied by incubating the enzyme at different temperatures; 37-80 °C. Aliquots were withdrawn at 30, 60, 90, 120 and 180 min and the enzyme assay was performed at the optimum temperature. The residual enzyme activities were measured.

Results

Amylases of all the isolates were able to catalyze the reaction in all the tested solvents. Amylases from Dj-30-8_1, Ve1-10-8_2, Ve1-10-8_3 and Ve1-10-9_3 were quiet active with 20 % (v/v) of methanol, propanol, n-hexane and butanol.

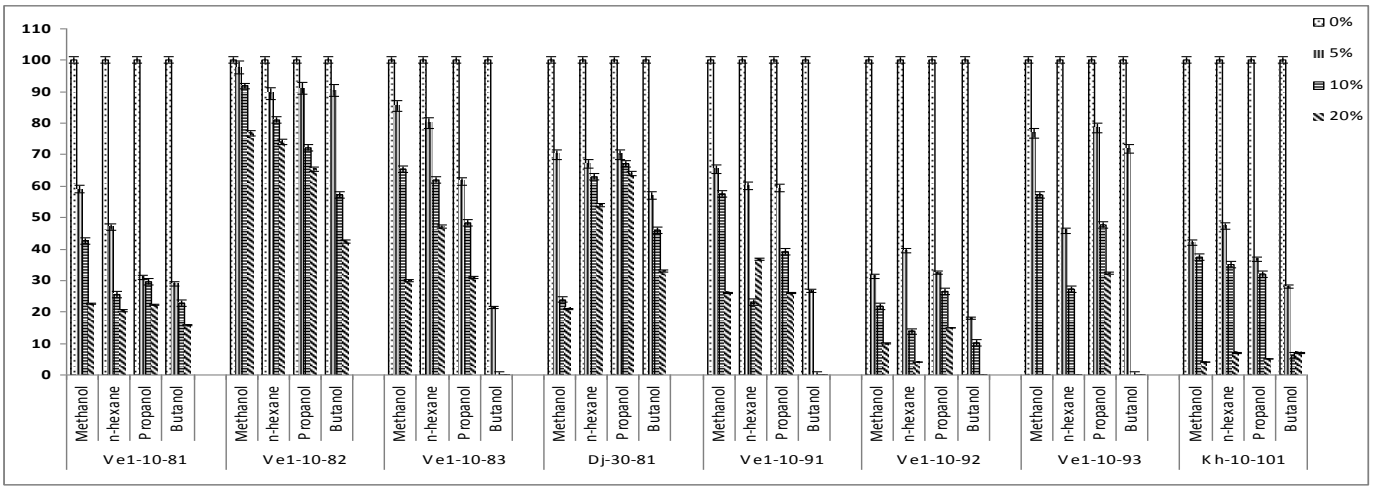

Fig. 1- Catalysis of Amylases in the presence of organic solvents

Figure 1captions- Effect of 0 % (light shaded square) 5 % (square with vertical lines) 10 % (square with horizontal lines) and 20 % (square with diagonal lines), V/V, solvents on amylases catalysis.

Table 1- Residual activities of Ve1-10-8_2 amylase in the presence of Methanol at various temperatures (°C)

Temp °C	Residual activity at different Time interval (Minute)						
	% Methanol (V/V)	0	30	60	90	120	180
50 °C	0	100	78.20	71.43	68.70	66.24	52.14
	5	53.70	58.14	50.94	50.61	42.38	26.80
	10	50.92	71.19	43.20	41.03	34.75	18.32
	20	40.74	33.77	30.86	29.27	18.63	13.93
60 °C	0	100	98.22	95.63	92.50	92.24	89.95
	5	83.59	79.46	68.13	64.27	56.58	44.46
	10	79.50	76.83	67.70	64.87	61.88	38.50
	20	59.51	56.39	47.58	46.77	42.08	25.42
70 °C	0	100	82.33	76.69	37.59	0	0
	5	73.30	37.59	38.34	34.15	0	0
	10	35.71	36.84	32.36	22.48	0	0
	20	30.82	21.66	19.81	14.39	0	0

The enzyme catalysis in 5 % (v/v) solvents was quite comparable to that in the control. However, at higher concentrations of the solvents, varying patterns emerged (Fig.1).

On the basis of the relative production of the amylases, the Ve1-10-8_2 was selected for further study. Based on 16S rRNA gene sequencing, Ve1-10-8_2 was phylogenetically nearest to *Oceanobacillus oncorhynchi* and hence the isolate was designated as *Oceanobacillus oncorhynchi* Ve1-10-8_2 (the accession number of 16S rRNA gene sequence-GQ121034).

Effect of pH on the enzyme catalysis was judged in the presence of Methanol and butanol. Amylase was quite active over the broader range of the acidic and alkaline pH. Activity at pH 10, without any solvent was assumed as 100 %. With many combinations of the pH and concentrations (v/v) of the solvents, the enhanced activities were recorded. At pH 5, relative to control; 92, 108 and 57 % activities with 5, 10 and 20 % (v/v) of methanol and 40, 30 and 20 % activities at the same concentrations of butanol, respectively, were evident. The amylase activity was quite

significant at pH 6. The activities were 106, 100 and 82% of control at 5, 10 and 20 % (v/v) of methanol and 100, 80 and 96 % with the same concentrations of butanol. At the neutral pH, however, the activities declined in the presence of methanol, while it remained unaltered with butanol. At pH 7, the residual activities were 50, 75 and 50 % at 5, 10 and 20% of methanol, respectively. At the same pH with butanol, the residual activities were 92, 108 and 74 % at the tested concentrations of the solvent.

In the alkaline pH range, better activities were recorded. At pH 8, the enzyme exhibited 61, 73 and 87% residual activities at 5, 10 and 20 % (v/v) methanol. At pH 9; 70, 90 and 85 of the residual activities with 5, 10 and 20 % (v/v) methanol, while 92, 87 and 82 % activities with the corresponding concentrations of butanol were observed. However, at pH 10, the residual activities were 125, 115 and 90 % of control with 5, 10 and 20% of methanol. With the same concentrations of butanol, at pH 10; the residual activities were 90, 68 and 52 %. pH 11 was quite favorable for the enzyme activity with methanol , although butanol at the same pH was not as favored. At pH 11, the activities were 116, 92 and 85 % with methanol and 60, 52 and 50% with 5, 10 and 20 % v/v of butanol. The residual activities were significantly reduced with both solvents at pH 12. The enzyme exhibited 70, 67 and 57% residual activities with methanol and 36, 18 and 13 % with butanol. At pH 13, with 5, 10 and 20 % v/v methanol; highly reduced residual activities at 35, 30 and 20 % were recorded. While with butanol at pH 13, the activities were totally lost at higher solvent concentrations. At pH 13, with 5% (v/v) butanol, 30% residual activity was detected (Fig. 2 a and Fig. 2 b).

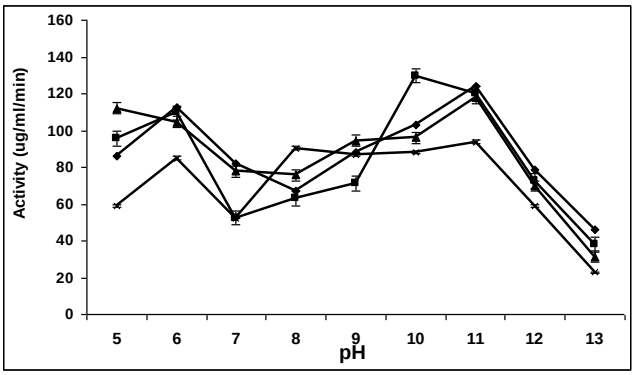

Fig. 2- (a) Effect of pH on Ve1-10-8_2 Amylases) in the presence of methanol

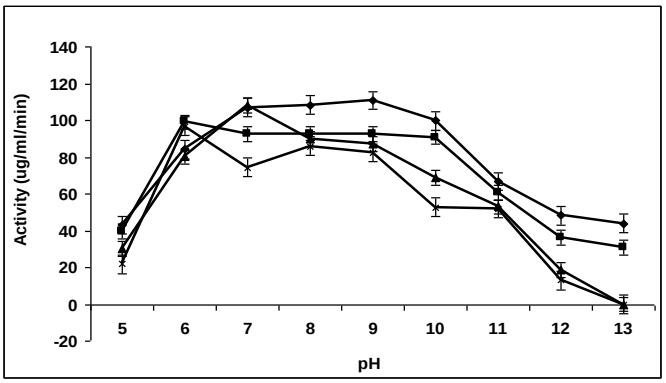

Fig. 2- (b) Effect of pH on Ve1-10-8_2 Amylases) in presence of butanol

Fig. 2 captions- Effect of pH on Ve1-10-82 Amylase activity with 0% (black closed diamond), 5% (black closed square), 10 %(black closed triangle) and 20% (cross) (v/v) Methanol (2a) and Butanol (2b).

Effect of NaCl (0.5 - 4M) on the enzyme catalysis was investigated in the presence of 5, 10 and 20% (v/v) methanol and butanol. With 0.5 M Salt, in 5, 10 and 20 % (v/v) methanol and butanol; 31, 25, 39 and 46, 44, 39 percent of the residual activities were recorded. With 1 M salt, comparatively better activities were observed at the tested concentrations of both solvents. In case of butanol, there were slightly enhanced activities, while for methanol; the enhancement of the activities was nearly threefold of the value at 0.5M salt (Fig.3).

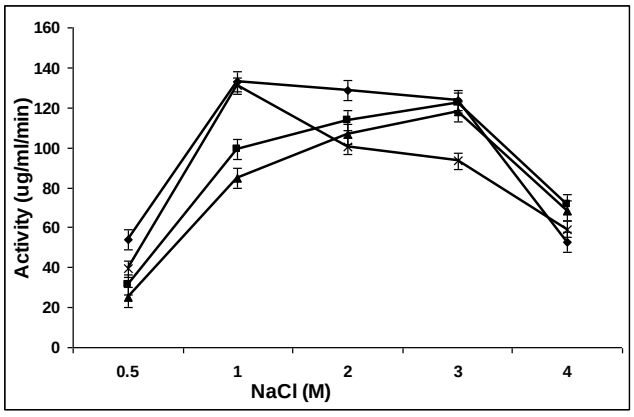

Fig. 3-(a) Effect salt on Ve1-10-8_2 Amylases in the presence of methanol

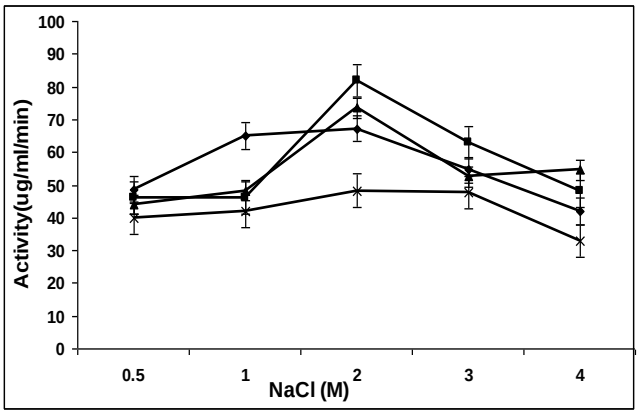

Fig. 3- (b) Effect salt on Ve1-10-8_2 Amylases in the presence of butanol

Fig. 3 captions- Effect of salt (NaCl) concentration on Ve1-10-8_2 amylase activity in the presence of 0% (Black closed diamond) , 5% (black closed square), 10% (black closed triangle) and 20% (cross) (v/v) Methanol (3a) and Butanol (3b).

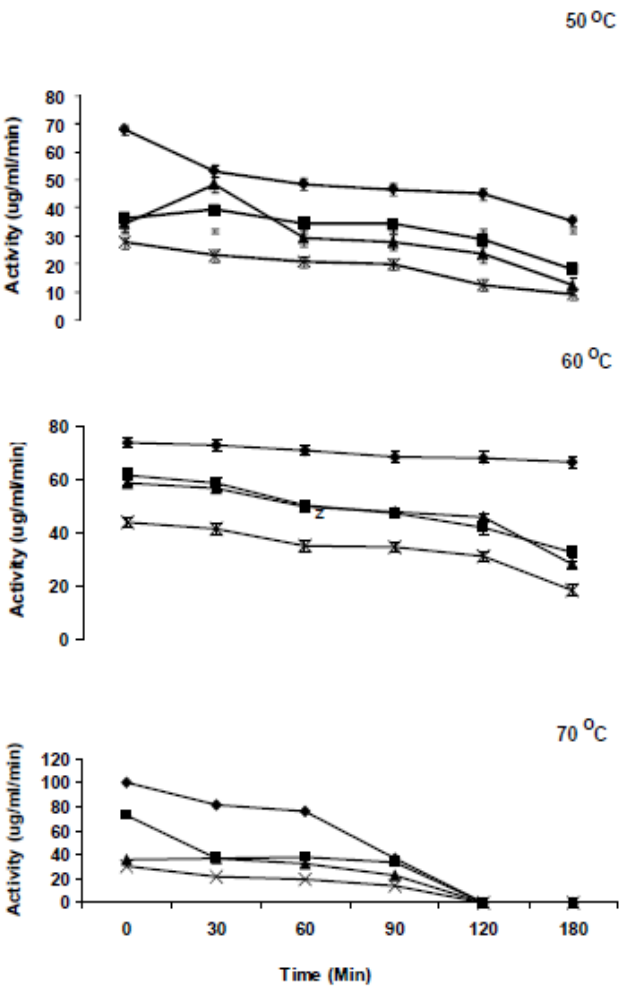

Fig.4- Thermostability of Ve1-10-8_2 Amylase

Fig. 4 captions- Effect of methanol on thermo stability of Ve1-10-8_2 amylase with 0% (black closed diamond), 5% (black closed square), 10% (black closed triangle) and 20% (cross) (v/v) methanol.

At 1 M salt, the activities were 100, 84 and 130 µg/ml/min, correspondingly. Salt at 2 M was optimum for the catalysis in both solvents. At this salt concentration; 80, 73 and 48 µg/ml/min activities with butanol and 113,105 and 100 µg/ml/min activities with methanol were observed. On further increase in salt concentrations, the activities were reduced for both tested solvents. At 3 M salt; 63, 52, 47 and 122, 113, 93 µg/ml/min activities were observed in the presence of 5, 10 and 20 % butanol and methanol, respectively. At 4 M salt; 48, 54, 33 and 70, 68, 60 µg/ml/min activities were evident with butanol and methanol, correspondingly (Fig.4).

Amylase of Ve1-10-8_2 was quite stable in methanol up to 180 minutes, at 50 and 60°C. While at 70°C, the enzyme was active for 90 minutes. The residual activities at various methanol concentrations are shown in (Table 1). Amylase was stable and active over the acidic and alkaline pH range. While, in the acidic range (pH 5-6), the enzyme was stable up to 90 minutes , at neutral pH with 5 and 10% (v/v) methanol, it was stable up to 180 minutes (Fig. 5). In alkaline range, at pH 9-11, the enzyme was stable up to 180 minutes retaining significant activity, the pH at 11 being optimum for the activity and stability. At pH 12, the activities and stability decreased significantly. The residual activities with different concentrations of methanol at varying pH are presented in (Table 2a, b).

Discussion

Despite the great scope for biotechnological applications, extremozymes have not been explored extensively [13-15]. Few enzymes from the extreme and moderate halophilic and haloalkaliphilic bacteria are cited in the literature which might have ample potential in food, chemical, pharmaceutical, leather, tanning, paper pulp and waste-treatment industries [16-19].

Studies on the enzymatic potential of amylases from halophilic and haloalkaliphilic bacteria in the non-aqueous medium are further restricted. Haloalkaliphilic bacteria in the present report were screened against 4 organic solvents; methanol, propanol, n-hexane and butanol. The isolates displayed varying diversity with respect to the catalysis in these solvents. With quiet less Log P_{ow} value, butanol is extremely toxic for the living cells and their macromolecules. Therefore, the catalytic of Ve1-10-8_2 amylase even in the presence of butanol reflected a unique feature of the enzyme.

Enzyme catalysis with 5 % (v/v) water miscible and immiscible alcohols and alkane, similar to control, signify its robust nature at lower solvent concentrations. At higher concentrations, varying results were evident.

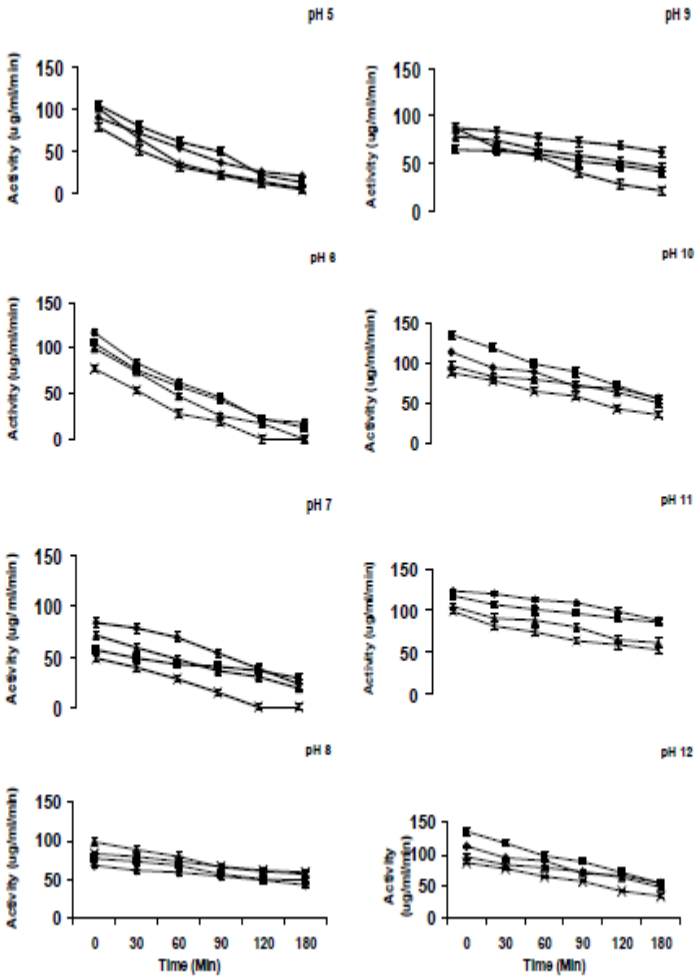

Fig. 5- Stability of Ve1-10-8_2 Amylase at different pH

Fig. 5 captions- Stability of Ve1-10-8_2 amylase at various pH in the presence of 0% (Black closed diamond), 5 %(black closed square), 10 %(black closed triangle) and 20 % (cross) (v/v) Methanol.

Amylase from Ve-10-8_2 was active at both acidic and alkaline pH, while at pH 7, it lost activity. The optimal pH for the catalysis was 10-11, which appeared to be higher than an amylase from alkaliphilic *Bacillus* sp. (pH 8.0-8.5) [20]. The Ve-10-8_2 enzyme retained stability over a wide range of pH; 6.0-10.0, which is quite higher than an amylase from *Halobacterium salinarum* [21]. Other prokaryotes, such as a thermophilic and halotolerant bacterium, *Halothermothrix orenii* was reported to have optimal amylase activity in the range of pH 6.0-9.5 [22].Regarding the stability at different pH; the enzyme was quite active over an acidic and alkaline range. At pH 5 and 6 with 5 and 10 % (v/v) methanol, the enzyme was stable up 90 min, while it was highly stable at pH 10-11. While the enzyme retained activity over a wide pH range of 5-11, enhanced activities were observed at alkaline pH range. Therefore, the alkaliphilic nature of the enzyme was highly pronounced.

Normally structure of the halophilic enzymes are not stable in low salt concentrations because of the ionic charge required for the stability of the enzyme[23-24].Therefore, decrease in the salts required by the halophilic enzymes may lead to the loss of their structure and function [25-27]. Amylase reported here appears to be adapted to the high NaCl concentrations. The increase in the enzyme activity with salt is a common feature of the halophilic enzymes [28]. Amylase of Ve1-10-8_2 highlights its halophilic character with upward shifting of the enzyme activity from 0.5-1M NaCl. Higher concentrations of salt affect the binding mechanism between the enzyme and substrate (starch). However, as described earlier, the activity increased at higher salt concentrations, indicating that salt had an overall positive effect on the reaction rate. Most of the halophilic and haloalkaliphilic enzymes studied are inactivated when the NaCl or KCl concentration decreases to less than 2 M [29].

In contrast to this general acceptance, the amylase in the present report was optimally active at 1M NaCl with methanol. While with butanol, the enzyme required 2M NaCl concentration for its maximal activity. Optimal performance at comparatively low salt concentrations and ability to retain activity with border range of the salt concentrations was evident in a moderately halophilic and mesophilic aerobic bacteria, *Halomonas meridiana* [30]. There were substantial changes in the salt profile of the enzyme. In the presence of butanol, the optimum activity shifted from 1M to 2M salt concentration.

Table 2a- Residual activities of Ve1-10-8_2 amylase at different pH with % (v/v) Methanol

pH	% Methanol (v/v)	Residual Activity at different Time interval (Minute)					
		0	30	60	90	120	180
5	0	100	79.77	59.70	41.01	27.97	21.87
	5	116.9	89.82	68.81	54.06	24.37	13.79
	10	113.59	71.98	39.50	25.58	15.23	5.02
	20	87.61	56.89	34.59	23.34	11.91	3.57
6	0	100	71.81	52.88	39.30	18.08	15.70
	5	89.64	65.20	49.63	37.01	19.64	11.24
	10	85.62	63.11	40.74	22.07	14.73	0
	20	66.40	45.50	23.68	16.04	0	
7	0	100	93.54	83.37	63.87	45.21	27.27
	5	67.94	58.13	51.31	48.31	43.52	34.56
	10	85.59	70.21	57.24	43.31	35.35	22.24
	20	58.49	43.54	33.01	17.41	0	0
8	0	100	91.21	87.26	79.25	72.76	63.39
	5	113.47	107.40	100.29	83.74	73.79209	71.15
	10	143.19	129.98	116.54	95.08	88.05	83.16
	20	121.81	116.39	107.61	98.38	89.70	87.52

Table 2b- Residual activities of Ve1-10-8_2 amylase at different pH with % (v/v) Methanol

pH	% Methanol (v/v)	Residual Activity at different Time interval (Minute)					
		0	30	60	90	120	180
9	0	100	95.56	87.90	82.92	78.24	70.07
	5	73.75	71.33	67.78	58.75	53.84	46.29
	10	89.49	84.01	73.73	67.32	58.64	52.12
	20	99.96	76.10	65.84	45.26	31.55	23.37
10	0	100	83.59	79.26	62.45	60.21	49.27
	5	119.90	104.81	87.27	78.23	63.55	48.30
	10	85.50	73.81	70.24	64.50	56.30	43.87
	20	77.60	68.98	57.68	52.12	37.75	31.73
11	0	100	97.79	92.08	88.65	80.57	71.34
	5	96	86.77	82.04	78.66	73.92	69.87
	10	85.06	73.87	71.42	65.14	52.81	50.04
	20	80.48	66.13	60.57	51.83	47.73	42.77
12	0	100	78.48	64.79	39.89	14.64	4.69
	5	95.69	64.66	38.46	24.11995	6.25	0
	10	88.39	63.36	29.15	18.59	0	0
	20	64.66	47.58	24.11	0	0	0

The optimal temperature for the Ve1-10-8_2 amylase was in the same of 55-60 ºC, as also reported for the enzyme from Halobacterium salinarum. However, it retained activity at higher temperatures. Similar to Ve1-10-8_2 amylase, an enzyme from alkaliphilic Bacillus sp. had an optimal temperature at 55 ºC [31]. Other Halophilic enzymes such as NAD and NADP glutamate dehydrogenases from *Halobacterium salinarum* displayed maximal activity at 70 ºC, with high temperature stability [32], which are quite comparable to an amylase from *Thermus* sp. AMD33 [33]. Along the similar lines, a halophilic and thermophilic bacterium, *Halothermothrix orenii*, had temperature optima at 65 ºC [34].

Ve1-10-8_2 amylase displayed thermophilic character in its stability as well. The thermal stability, however, decreased sharply at 80 °C. In the literature, there are some reports on the thermostable amylases from mesophilic and halophilic organism's [35].The high optimal temperature may be considered as an adaptive response to the high temperatures that these enzymes have to endure in their natural salt environments, i.e., salterns exposed to intense sunlight. Thermophilic nature has also been reported for some other halophilic enzymes [35].

Recent studies have highlighted the enormous potential of haloalkaliphilic bacteria in context with production, biochemical characterization, structure to function aspect and solvent tolerance of highly alkali-thermo stable amylases and proteases [36-43]. Considering these properties, the haloalkaliphilic protease may find potential application in several industries.

Conclusion

In conclusion, the enzyme described in the present report reflected several unique features similar to those reported for halophilic enzymes. However, the effect of solvents on the amylase from moderately halophilic bacteria reflected new trends. The action of various solvents in combination with varying conditions of pH, salt and temperatures further highlighted the significance of the enzymatic studies under non-aqueous conditions. The catalysis of the haloalkaliphilic amylases in the organic solvents will, therefore, significantly add to the knowledge of the non-aqueous enzymology.

Acknowledgement

Authors are grateful to University Grants Commission (UGC), New Delhi for financial support. SP & AKS gratefully acknowledges the Research Fellowship in Sciences granted by UGC, New Delhi, India.

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