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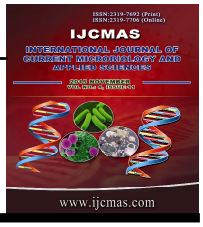

Original Research Article

Optimization, Partial Purification and Characterization of Halo-thermophilic Alkaline Protease from Moderately Halophilic Bacterium AH10 Isolated from Alexandria (Egypt)

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Keywords

Halo-thermophilic alkaline protease, Halophilic bacteria, Activity and stability of protease enzyme

A B S T R A C T

Among fifteen halophilic bacterial isolate were isolated from five regions of Alexandria (Egypt), the highest proteolytic activity was determined in the bacterial isolate AH10. Maximum protease production was determined in a broth medium inoculated with 6% of AH10, incubation period – 72 h, pH 8 and 40°C with galactose and tryptone as carbon and nitrogen source, respectively. Calcium chloride was used as supplement element for enhancement of production. About 22 and 48 purification fold was achieved by ammonium sulphate precipitation and column chromatography by sephadex G200 and then DEAE cellulose leading to partial purification of the protease enzyme. The partially purified enzyme exhibited its optimal activity at 60°C, pH 9 and 0.1M NaCl with a high tolerance to salt concentrations up to 5M. The enzyme maintained about 45.7 and 12.82 % of its activity at pH values 5 and 13 respectively. The protease was identified as a non-metalloprotease, which did not inhibited by EDTA-Na. The enzyme was resistant to Tween 80, Urea, H_2O_2, Na_2CO_3 and PMSF and some of them were shown to enhance the activity.

Introduction

Halophilic microorganisms or "salt loving" microorganisms live in environments with high salt concentration that would kill most other microbes. Halophilic and halotolerant microorganisms are found in all three domains of life, Archaea, Bacteria, and Eucarya (Oren, 1999). Halophiles, inhabiting saline environments, are considered as good source of useful salt stable enzymes (Oren, 2008). Their enzymes possess unique structural features to catalyze the reactions under high salt conditions. Proteases represent a group of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990; Fox *et al.*, 1991). Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. These are degradative enzymes that catalyze the

cleavage of peptide bonds leading to total hydrolysis of proteins via the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content (Sookkheo *et al.,* 2000; Beg *et al.,* 2003). Microbial proteases, especially from *Bacillus* species have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergents formulations (Gupta, 2003).

Materials and Methods

Screening for protease producers halophilic bacteria

Fifteen halophilic bacterial isolate were cultured on skimmed milk agar medium. Bacterial isolates were streaked on agar plates and incubated at 37 ^{o}C for 24–48 hours. Presence of clear zone around the bacterial growth is a positive result. The highest proteolytic activity was determined in the bacterial isolate AH10, which is a Gram positive bacillus bacterium.

Protease enzyme assay

Protease assay was estimated by the method described by (Beg *et al*, 2003) with reference to tyrosine standard. The enzyme (1ml) was added to 1 ml casein (0.65%, w/v in 50 mM potassium phosphate buffer, pH 7.5) and the reaction mixture was incubated at 37 °C for 40 min before the addition of 10% trichloroacetic acid.

The precipitates were removed by centrifuging the mixture at 5000 rpm for 15 min. 1 ml of the filtrate was mixed with 5 ml of 500 mM sodium carbonate solution and after incubation for 30 minutes, one ml of folin reagent (diluted 5 folds) was added, absorbance was measured at 660 nm using a UV visible spectrophotometer (spectrum SP 2000 UV l Taiwan). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute using tyrosine standard curve under the defined assay conditions.

Protein determination

Protein concentration was measured by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Optimization of nutritional and environmental factors for protease production

The effect of various physical parameters on protease production was assessed by growing bacterial strain in a broth medium containing gL^{-1}: casein, 10; bactopeptone, 10; NaCl, 3; $MgSO_4$, 1; K_2HPO_4, 1; $MnCl_2$, 0.008; $ZnSO_4$, 0.002; $CaCl_2$. $2H_2O$, 0.3; and pH 7.0.

For optimizing pH, the medium was prepared with different pH in the range of 5.0–13.0 at 1.0 unit interval. Incubation temperature was determined by incubating the bacterial culture at a range of 20–60^{o}C. Effect of varying inoculum percentage from 1% to 12% on protease production was determined. Different incubation periods were studied from 12 to 96h. Various simple and complex carbon sources including glucose, fructose, galactose, xylose, lactose, ribose, mannose, cellulose and starch were used as a sole source of carbon (0.5% w/v) for studying the best carbon source for enzyme production. Ten nitrogen sources which used in this experiment were beef extract, peptone, yeast extract, gelatin, tryptone, ammonium hydrogen citrate, urea, ammonium nitrate, ammonium sulphate, and sodium nitrate (0.5% w/v).

The protease production medium was

supplemented with different sodium chloride concentrations from 0.2 to 3M. The enzyme activity was measured in each concentration of sodium chloride. Also, nine different amino acids were used to investigate their effects on protease production. These amino acids were lysine, isoleucine, glycine, tryptophan, proline, glutamic acid, cysteine, serine and methionine.

Partial purification of protease enzyme

The protease enzyme was partially purified with gradually steps, stared with ammonium sulphate precipitation and passing through dialysis, sephadex G-200 gel filtration and DEAE cellulose chromatography.

Characterization of partially purified enzyme

The activity of partially purified protease enzyme was measured at different incubation temperatures 20, 30, 40, 50, 60, 70, 80 and 90°C.

The enzyme stability was measured by incubating the partially purified enzyme at different temperatures 20, 30, 40, 50, 60, 70, 80 and 90 for 1 hour and then incubated again with casien for 30 min and followed with all activity measurement procedures to determine the residual activity.

The effect of different pH values on partially purified protease was determined with casein as substrate dissolved in potassium phosphate buffer (pH 5, 6, 7, and 8) and glycine–NaOH buffer (pH 9, 10, 11, 12 and 13).

The enzyme activity was determined at different NaCl concentrations (0.1M, 0.2M, 0.6M, 1M, 1.4M, 1.8M, 2.2M, 2.6M, 3M, 3.4M, 3.8M, 4.2M, 4.6M, and 5M).

The enzyme activity was determined also in the presence of different detergents (Tween 80, Urea, H_2O_2, Na_2CO_3) and inhibitors included (Ethylene diamine tetraacetic acid disodium salt (EDTA-Na_2), phenylmethyl sulfonyl fluoride (PMSF).

Results and Discussion

Screening for protease producers hlophilic bacteria

All the 15 halophilic isolates were examined for protease production on skim milk agar medium and the isolate AH10 showed the highest proteolytic activity (Fig. 1).

Effect of environmental factors on protease production

Protease enzyme produced after 12 h and increased continuously to maximum yield (176.5 Uml^{-1}) after 72 h of incubation. It was also observed that prolonged incubation decreased the enzyme activity to reach (142 Uml^{-1}) after 108 hours (Fig. 2). The productivity of protease is high at the slightly acidic, neutral, and slightly alkaline pH values with highest productivity at pH 8 (200 Uml^{-1}) (Fig. 3).

Data illustrated in figure 4 shows that, the best inoculum size was 6% (205 Uml^{-1}). There was a gradual increase in protease synthesis at 20-30^oC (Fig. 5) and a maximum production of enzyme was observed at 40^oC (202 Uml^{-1}) then, the productivity decreased again to (88 Uml^{-1}) at 60^oC.

Effect of nutritional factors on protease production

The most suitable carbon sources that achieve high productivity for protease enzyme was galactose (214 Uml^{-1}) followed

by ribose, xylose, lactose, cellulose, starch, fructose, glucose and mannose with activities of 212, 209, 202, 199, 192, 184, 169, and 167 Uml^{-1}, respectively (Fig. 6). Ammonium nitrate was the best inorganic nitrogen source with 162 Uml^{-1}. On the other hand, tryptone showed the maximum protease activity among all used organic and inorganic nitrogen sources with 167 Uml^{-1} (Fig. 7). Protease productivity and activity decreased gradually when the concentration of sodium chloride is increased so that the optimum concentration for maximum production was at 0.5 M (Fig. 8), while the best NaCl concentration for bacterial growth is 1.8 M. As compared to control, enzyme productivity not only did not increase with any of the studied amino acids but also, these amino acids cause inhibition in biosynthesis of enzyme (Fig. 9).

Partial purification of protease enzyme produced by halophilic AH10

The culture supernatant of halophilic AH10 containing an initial protease activity (2200 U/ml) was concentrated with ammonium sulfate (60 % saturation) and showed 21.2 or more fold increasing in specific activity (288 U/mg) compared to the un concentrated supernatant (Table 1). The protease was subjected to sephadex G200 and DEAE cellulose ion exchange chromatography resulted in specific activity 645.5 and 659.5 U/mg, purification yield 39.3 and 28.2% and purification fold 47.5 and 48.5 respectively.

Characterization of partially purified enzyme

Effect of temperature, pH and NaCl concentrations on the activity and stability of protease

The optimum temperature for partially purified protease activity was measured at various temperatures, ranging between 20 and 90°C. As shown in figure 10, the maximum protease activity was observed at 60°C. The increase of temperature above 60°C reduced the protease activity from 183.75 Uml^{-1} to 152.50, 127.75 and 97.50 Uml^{-1} at 70, 80 and 90°C respectively. Protease retained over 86 % of its hydrolytic activity between 20–50°C. The retained activity was 65.5, 57.2 and 40.5% of its activity at 60, 70 and 80°C, respectively. The percentage of relative stability was calculated by considering the activity at 60°C as 100% (350 Uml^{-1}).

The effect of pH on protease activity was estimated in three different buffer systems, 50 mM sodium citrate (pH 3.0–6.6), 50 mM potassium phosphate buffer (pH 6.9–8.0), and 50 mM $Na_2CO_3/NaHCO_3$ (pH9.1–13.0). Data presented in figure 11 show that, the optimum pH value of protease was 9. The stability of protease enzyme incubated for 1 hour in different pH values was estimated. The relative activity of hydrolytic protease was 53.9, 55.5, 57.2, 65.4, 67.1, 57.2 and 35.8 at pH values 6, 7, 8, 9, 10, 11 and 12, respectively. The protease enzyme maintained its activity with 45.7 and 12.82 % at extreme values 5 and 13.The percent relative activity was calculated by considering the activity at pH 9 as 100% (370 Uml^{-1}).

Data presented in figure 12 show the activity of protease with different concentrations of sodium chloride. The relative activity of protease produced by AH10 was increased with the addition of 0.1M sodium chloride to 123.5% and above this concentration, the activity of enzyme was decreased. The results of halostability showed that the enzyme retained 94.7, 80, and 36.8% of its activity after incubation at 0.6, 3, and 5M NaCl, respectively. Percentage relative

activity was calculated by considering the activity without sodium chloride as 100% (370 Uml^{-1}).

Effects of different metal ions on the activity of the protease

Effects of Co^{2+}, Ni^{2+},Li^{2+}, Ba^{2+}, Ca^{2+}, and Mn^{2+}, with final concentration of 10 mM of each cation on partially purified protease activity are presented in figure 13. Compared to control, most of the tested metal ions had an inhibitory effect on enzyme activity. The inhibition ratios were approximately 20, 12, 14, 19, and 30 % with cobalt chloride, nickel chloride, lithium chloride, barium chloride, calcium chloride, and manganese chloride, respectively. The enzyme stability is presented in figure 13 shows that the enzyme retained 52.5, 39.2, 54.5, 44.7, 52.7 and 48.6% with cobalt chloride, nickel chloride, lithium chloride, barium chloride, calcium chloride, and manganese chloride, respectively. The percentage of relative activity was calculated by considering the activity without any treatments as 100% (370 Uml^{-1}).

Effect of some detergent and inhibitors

All of the tested detergents did not inhibit the partially purified protease enzyme. The oxidizing agent, Tween 80, achieved an increasing in enzyme activity with about 20.9, 16.6 and 9% at concentrations of 1, 5 and 10%, respectively when compared to control. On the other hand, the enzyme retained about 93.2 % of its initial activity with 10 % of the detergent. Urea has also stimulatory effect on partially purified enzyme increasing 6.1, 3.1, and 0.16% of its activity at 1, 10, and 15% concentrations, respectively. The protease enzyme from AH10 showed a good stability against all examined concentrations of urea with about 19, 8 and 5 % activity increase at concentrations of 1, 10, and 15% respectively compared with control.

Different concentrations of the bleaching agent H_2O_2 (1, 5 and 10 %) increased the activity with 26, 20 and 8 % (Table 2). Data in table 2 indicate that the partially purified enzyme was stable in the presence of 1, 5 and 10% of H_2O_2 for 1 hour at standard measurement conditions with–activities of 113.2, 106.8 and 101.1 %, respectively. In addition, an enhancement in the enzymatic activity and stability was observed also in the presence of concentrations of Na_2CO_3 (50 and 75 mM). The enzyme stability against the same concentrations of Na_2CO_3 (50 and 75 mM) was 104.6, 103.6 and 90.8 % residual activity compared with control. EDTA-Na_2 has also stimulatory effect on protease activity. Enzyme activity or stability was not affected by PMSF. The percentage of relative activity was calculated by considering the activity without any treatments as 100% (370 Uml^{-1}).

From industrial point, it is necessary to monitor and control parameters that affect the production process starting from the selection of optimum carbon and nitrogen sources, inocula volume, salt concentration, pH, temperature, incubation period, etc. (Ray *et al.*, 2007). Maximum protease production with AH10 (176.5 U/ml) was observed in the end of stationary phase. The enzyme activity decreased considerably after this time. These results are in accordance with observations made by Kumar and Parrack (2003) with *Bacillus sp*. and Okafor and Anosike (2012) with *Bacillus sp*. SW2. Shorter optimum incubation period for protease production (48h) was reported by Reddy *et al.*, (2011) with *Bacillus* sp., while longer optimum incubation period (96h) was reported by Anand *et al.*, (2010) with *Halobacterium* sp. Different recorded optimum incubation periods may be due to different inoculation sizes used or due to different culturing conditions used.

pH is an important physical parameter affecting microbial protease production (Puri *et al.,* 2002). Various enzymatic processes and transportation of components through cell membrane is dependent on pH of the media (Moon and Parulekar, 1991). AH10 could grow and produce protease over a wide range of pH values (5–12). Maximum protease production was observed at pH of 8 (200 U/ml). These results are in agreement with those obtained by Bose *et al.* (2014). Suganthi *et al.* (2013) reported that the pH of 8 was the optimum pH for protease production from *Bacillus licheniformis* (TD4) after 24 hours of incubation. Also, these data showed the ability of AH10 to grow at different pH values and predict stability of purified enzyme. Most of the reported *Bacillius* sp. has pH optima from 7.0 to 11.0 (Joo and Chang, 2005; Shivanand and Jayaraman, 2009).

The inoculum quantity normally used between 3 and 10 % of the medium volume (Hunt and Stieber, 1986). In the present study, maximum protease production (205 U/ml) was observed at 6% inoculum. There was a reduction in protease production when inoculum size was reduced (1, 2, 3, 4 and 5 %), these may be due to insufficient bacterial cells, which would lead to reduced amount of enzyme production. Dutta *et al.,* (2004) has been reported that 1.5% of inoculums showed maximum enzyme production from *Pseudomonas sp*. Also, Ray *et al.,* 2012 reported that the optimum inoculum volume was 3 % for maximum production of protease from both two bacterial strains, *Bacillus licheniformis* BF2 and *Bacillus subtilis* BH4 isolated from the digestive tract of bata, *Labeobata* (Hamilton). The differences in results may be due to using of different bacterial strains and/or using inocula with different physiological conditions.

Microorganisms grow slowly at a temperature below or above the normal growth temperature because of a reduced rate of cellular production (Ray *et al.,* 2007). The optimum temperature for enzyme production was varied from 30 to 50^{o}C and reported as 36, 50, 37, 40, 37, 37, 30^{o}C (Chu, 2007; Amoozegara *et al.,* 2007; Sinsuwan *et al.,* 2008; Abusham *et al.,* 2009; Hezayen *et al.,* 2009; Vijayaraghavan *et al.,* 2012b; Anbu *et al.,* 2013) from *Salinivibrio* sp. strain AF-2004, *Bacillus* sp., *Virgibacillus* sp. SK33, *Bacillus subtilis* strain Rand, *Bacillus subtilis* strain KO, *Halobacterium* sp. and *Exiguobacterium profundum* BK-P23, respectively. There was a gradual increase in protease synthesis by AH10 at 20–30^{o}C and the maximum production was observed at 40^{o}C (202 U/ml) then, the productivity decreased again to (88 U/ml) at 60^{o}C.

Among the carbon sources tested, galactose achieved maximum production of protease (214 U/ml). There was slight variation in protease productivity among the carbon sources used. Different carbon sources are recorded as optimum carbon sources by different authors. For example, Vijayaraghavan *et al.* (2014) reported that maltose was the optimum carbon source with 1% concentration for production of protease from *Bacillus cereus* strain AT. Also, an increased yield of enzyme production using various carbon sources such as lactose (Kumar *et al.,* 2014), starch (Padmapriya and Williams, 2012), fructose (Sevinc and Demirkan, 2011) and glucose (Sankaralingam *et al.,* 2011) have been reported.

Effects of a specific nitrogen supplement on protease production differ from organism to another organism although complex nitrogen sources are usually used for alkaline protease production (Kurmar and Tagaki,

1999). Among all nitrogen sources tested, tryptone resulted in the highest activity of protease by AH10 (167 U/ml). Various nitrogen sources were recorded as optimum by different authors, potassium nitrate 3% (Muthulakshmi *et al.*, 2011), Skim milk (Sevinc and Demirkan, 2011), Peptone (Das and Prasad, 2010), peptone and yeast extract (Shivanand and Gurunathan, 2009) and yeast extract (Gouda, 2006), these differences may be due to using different strains and different culturing conditions.

Table.1 Summary of the purification steps of protease by halophilic AH10

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	1620	22000	13.58	100	1
$(NH_4)2SO_4$ precipitation	51.7	14900	288.20	67.72	21.22
Sephadex G-200	13.4	8650	645.52	39.3	47.53
DEAE	9.4	6200	659.57	28.2	48.56

Table.2 Effect of different concentrations of some detergents and inhibitors on partially purified protease activity and stability

Detergent/ Inhibitor	**Concentration**	**Protease relative activity (%)**	**Protease residual activity (%)**
Non	---	100	100
Tween 80	1% (v/v)	120.9812	105.5485
	5%	116.6981	100.3217
	10%	109.5589	93.2486
Urea	1% (w/v)	106.177	119.3656
	10%	103.172	108.8481
	15%	100.1669	105.0083
H_2O_2	1% (v/v)	126.2523	113.2522
	5%	120.3489	106.8297
	10%	108.2241	101.1177
Na_2CO_3	50 mM	125.8765	104.6745
	75 mM	103.6728	103.672
	100 mM	92.8364	90.8330
Non	---	100	100
EDTA-Na_2	1 mM	102.8381	119.6995
	5 mM	108.6811	110.1836
	10 mM	107.0117	107.1786
PMSF phenylmethylsulfonyl fluoride	5 mM	100.2543	100.0247
	10 mM	100.0941	100.1001

Fig.1 Isolate (AH10) on skim milk agar medium

Fig.2 Effect of different incubation periods on protease production by AH10

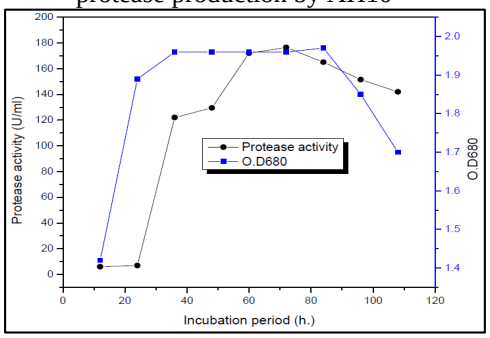


Fig.3 Effect of different pH values on protease production by AH10

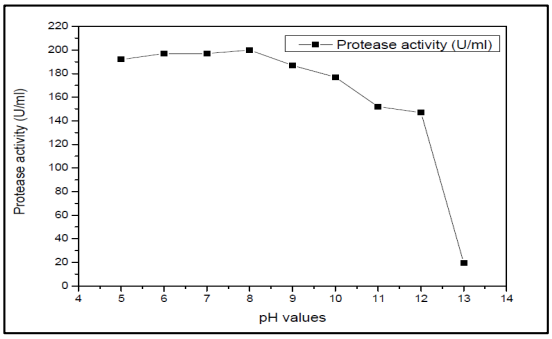


Fig.4 Effect of different inoculum sizes on protease production by AH10

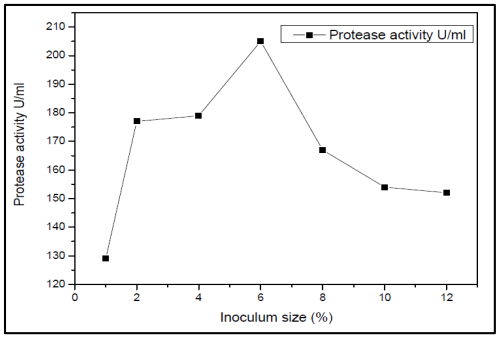


Fig.5 The effect of different incubation temperature on protease production by AH10

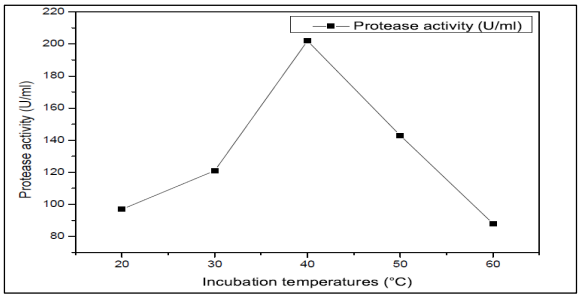


Fig.6 Effect of different carbon sources on protease production by AH10

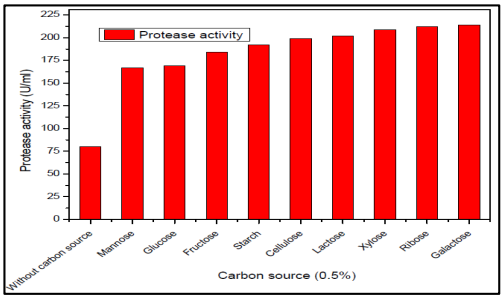


Fig.7 Effect of different nitrogen sources on protease production by isolateAH10

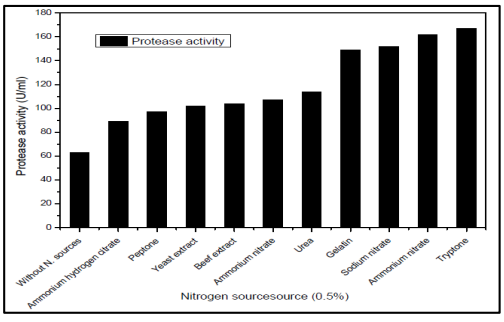


Fig.8 Effect of different NaCl concentrations on protease production

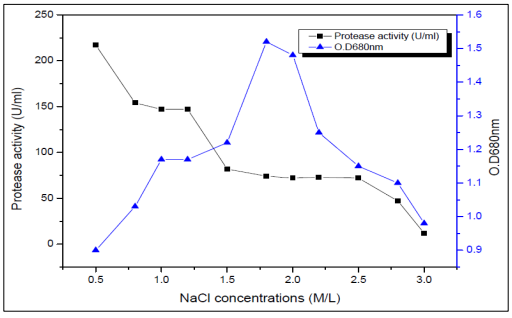


Fig.9 Effect of different amino acids on protease production

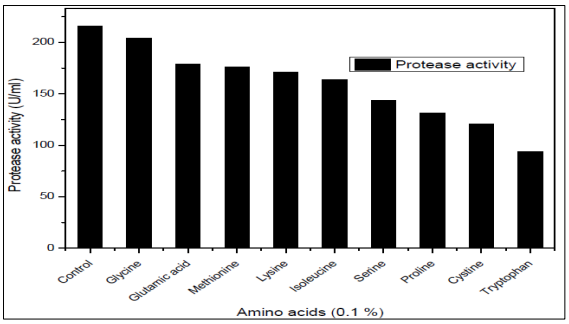


Fig.10 Effect of different temperatures on partially purified protease activity and stability

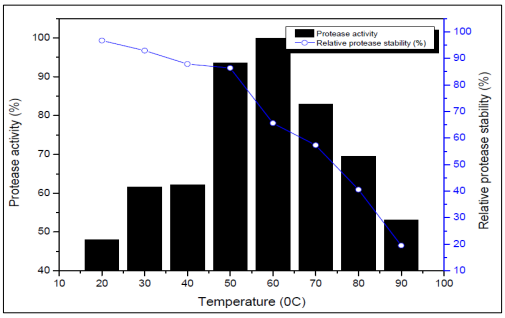


Fig.11 Effect of different pH values on partially purified protease activity

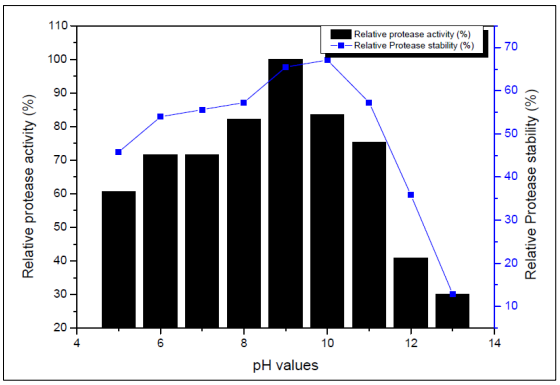


Fig.12 Effect of different sodium chloride concentrations on protease activity and stability

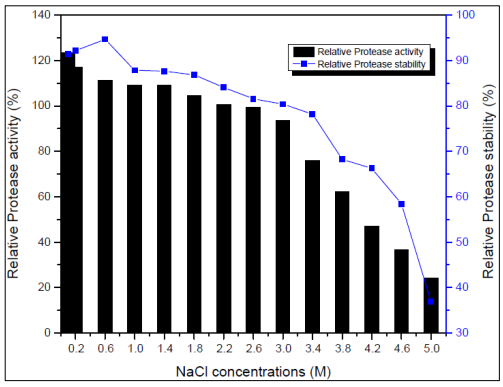
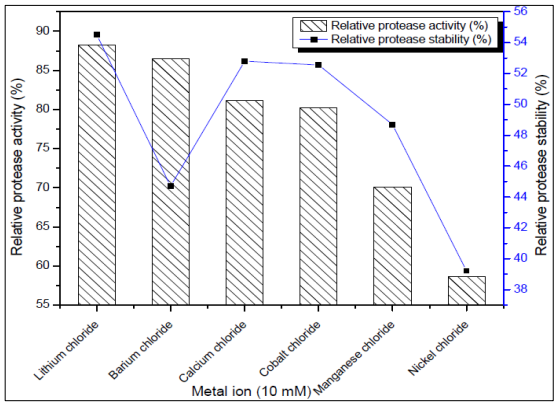


Fig.13 Effect of metal ions on partially purified protease activity and stability



The maximum protease production was observed in the medium containing 0.5 M NaCl (217 U/ml) after 72 h of incubation. The growth and production of protease was gradually reduced when salt concentration increases above 0.5 M NaCl. Mostly, reduction of enzyme production by Gram positive moderate halophiles is often reported at high salt concentration (Ventosa *et al.*, 1998). Concentration of 1 M NaCl was found to be optimum for the production of protease from *Bacillus aquimaris* strain VITP4 (Shivanand and Jayaraman, 2009) and Sinsuwan *et al.*, (2008) reported in their

paper that 5 % NaCl was the optimum concentration for production of protease from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. In our study, the used amino acids inhibited protease production from AH10 compared to control. Various nitrogen sources including amino acids are known to repress enzyme production (McDonald and Chambers, 1966; O'reilly and Day, 1983). It may be subjected to feedback inhibition by some of the amino acids, which are end products of proteolytic degradation. Amino acids, even at low concentrations, are known to repress protease synthesis (Moon and Parulekar, 1991).

The purification of proteolytic enzymes presents the normal challenges associated with the purification of all proteins (Jugran *et al.,* 2014). In the present study the protease from moderately halophilic AH10 was partially purified from the culture supernatant of AH10 through ammonium sulphate precipitation at 60 % of saturation and (21.2) % fold purification was achieved at this step, this data was closely similar with that reported by Meng *et al.,* (2013) who obtained 21.6 fold of purification after salt precipitation for protease from *Lactobacillus brevis* 1.12. In a similar report, 15.2 purification fold was reported by Sinha and Khare (2013) for protease of *Bacillus* sp. EMB9 isolated from sea water samples of Goa, India, however much lower purification fold (1.78) of bacterial alkaline proteases through salt precipitation has been reported by Maruthiah *et al.,* (2013).

In our investigation, the ammonium sulfate precipitation step was followed by Sephadex G-200 gel filtration and DEAE ion exchange chromatography that led to a total of 47.5 and 48.5 fold enzyme purification, respectively (Table 1). Much similar to this, Sephadex G-100 and DEAE ion exchange chromatography have been used earlier by various workers for bacterial protease purification (Kumar and Bhalla, 2004; Rajkumar *et al.,* 2011; Maruthiah *et al.,* 2013). Meng *et al.* (2013) achieved 34, 47.1% fold of purification of protease from *Lactobacillus brevis* 1.12 after DEAE and sephadex G-200 chromatography, respectively.

The optimum temperature for AH10 partially purified protease was 60°C (Figure 10) with maximum activity of 350 Uml-1. These results are in agreement with that obtained by other researchers (Lama *et al.,* 2005; Deng *et al.,* 2010; Jayakumar *et al.,* 2012). Protease was produced from a moderately halophilic bacterium *Halobacillus karajensis* with an optimum temperature 50°C and pH 9.0 (Heidari *et al.,* 2009).

The AH10 protease exhibited activity over a broad range of pH values, (5.0 -13.0) with optimum activity at pH 9.0 (370) Uml-1. This result is similar to that reported by Annamalai *et al.,* (2013); Sinha and Khare, (2013) for *Bacillus alveayuensis* CAS and *Bacillus* sp., respectively, with using casein as a substrate. Therefore, this enzyme could be alkaline protease and can be used in many industrial processes especially in detergent industries. Other pH values varied from 8-11 were reported by many researchers (Vijayaraghavan *et al.,* 2012a; Heidari *et al.,* 2013; Bose *et al.,* 2014).

In our study, partially purified protease from moderately halophilic AH10 showed an increase in activity in the presence of sodium chloride. Protease activity was increased with addition of 0.1 M NaCl to 123.5 %. The results of halostability studies depicted that the enzyme was 100% stable up to 15% NaCl and it retained 80% activity even at 35%. Maruthiah *et al.,* (2013) reported that protease from *Bacillus subtilis* AP-MSU6 showed maximum activity at 0.5

M NaCl. Annamalai *et al.,* (2013) produced protease from *Bacillus alveayuensis* CAS5 with optimum NaCl at 30 % and an increased activity with increasing concentrations of NaCl.

Studying the influence of metal ions on alkaline protease activity of moderately halophilic AH10 showed that all the tested cations, (Co^{2+}, Ni^{2+},Li^{2+}, Ba^{2+}, Ca^{2+} and Mn^{2+}) inhibited the enzyme activity to various extents (Fig. 13). Bose *et al.,* (2014) found that Mn2+ (5mM) inhibited the enzyme activity, but Ca^{2+} (5mM) enhanced the activity of protease from a novel marine isolate (*Bacillus tequilensis* P15) up to about 126%. Li *et al.,* (2011) found that the activity of a novel nonionic surfactant and hydrophilic solvent-stable alkaline serine protease was enhanced by Mn^{2+}.

Data presented in our study showed that, the partially purified AH10 protease was not only stable in the presence of all examined detergents (Tween 80, Urea, H_2O_2 and Na_2CO_3) but was also enhanced. These results are in accordance with the result obtained for protease from *Bacillus pumilus* CBS, *Serratia proteamaculans* AP-CMST, *Salinivibrio* sp. strain MS-7 (Jaouadi *et al.,* 2008; Esakkiraj *et al.,* 2011 and Heidari *et al.,* 2013).

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