Salt Tolerant Protease Produced by an Aerobic Species Belonging to the Bacillus Genus Isolated from Saline Soil

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Abstract: Microbial proteases important hydrolytic enzymes represents largest group of industrial enzymes and have applications in various industries. Proteases capable to in harsh condition of salinity, alkalinity and temperature etc are thus of great concern. Extracellular protease producing bacterium isolated from saline soil of Akola district was related to *Bacillus licheniformis* on the basis of biochemical and16S rDNA gene sequence similarity. The enzyme was active in the range of pH 6–10 with the optimum at 9.0. Different carbon and nitrogen sources used in the study found increased protease production in Lactose and Yeast extract. The enzyme was highly stable at various NaCl concentrations retained 50% activity after 4hrs. Also, the enzyme was quite stable at higher temperature in the presence of KCl, NaCl and $CaCl_2$. Activity of this was affected by mono and divalent metal ions to varying extent. The study on this enzyme assumes significance with dual extremities of pH and salt also with moderate temperature stability.

Index Terms: Protease, *Bacillus licheniformis*, protease, salt and saline soil

I. INTRODUCTION

Enzymes are known biocatalysts and are used for various commercial purposes in industries. Microbial proteases are among the most important hydrolytic enzymes (Gupta *et al* 2002) representing largest group of industrial enzymes and account approximately 60% of total enzyme sale in the world (Rao *et al* 1998). Of the total enzymes used in various commercial and industrial processes, majority are obtained from mesophilic microorganisms. These have applications in various industries like detergents, foods, pharmaceuticals, leathers, diagnostic reagents and also for waste management and silver recovery (Gupta *et al* 2002). These enzymes function in a restricted range of pH, temperature, and ionic strength. Moreover, the use and application of these under harsh industrial conditions makes them unsuitable, inefficient or unrecommendable. This suggests need for search of new microbial sources. The microorganisms from diverse and exotic environments, Extremophiles, are considered an important source of enzymes, and their specific properties are expected to result in novel process applications (Govardhan and Margolin, 1995, Robertson *et al*, 1996).

In recent considerable focus has been given to the enzymes produced by moderately halophilic microorganisms and their biotechnological potentials (Ventosa, 2004; Ventosa et al., 1998) due to a advantage that these gives optimal activity at high salinities making them useful in many harsh industrial processes where the concentrated salt solutions used where many enzymatic conversions inhibited (Mohaparta et al., 1998). Protease production has been shown in some species of halophiles such as Pseudoalteromonas sp. Strain CP76 (Sa´nchez-Porro et al., 2003), Natrialba magadii (Gime´nez et al., 2000), Halobacterium mediterranei (Stepanov et al., 1992), Bacillus clausii (Kumar et al., 2004) and other halophilic isolates (Norberg and Hofsten, 1969; Kamekura and Onishi, 1974; Kamekura and Seno, 1990; Kamekura et al., 1992; Ryu et al., 1994; Lama et al., 2005). In all the cases, the proteases were shown to be secreted in the medium containing salts, particularly NaCl or KCl. The growth and protease production and its activity were exclusively observed in the presence of salts.

Halotolerant bacteria form a versatile group; can survive or adapted to life at the lower range of salinities and also have possibility of rapid adjustment to changes in the external salt concentration. In contrast, the halophilic archaea strictly require constant presence of high salt concentrations (3–4 M) for survival (Litchfield, 2002). This property of halotolerant bacteria makes them better candidates for bio-prospecting than their halophilic counterparts. Most of the Gram-positive, endospore forming rods with halotolerant properties, have been assigned to the genus Bacillus (Yoon *et al*, 2003). Bacillus sp. grows in a pH range of 7.0– 11.0 and produces extracellular protease. Currently, a large proportion of commercially available alkaline proteases are derived from different strains of Bacillus (Romero *et al*, 2007, Gupta *et al*, 2002).

Present investigation focused on the protease production and activity for a newly isolated, halotolerant bacterium of genus Bacillus obtained from saline soil of Akola, MS India.

II. MATERIALS AND METHODS

1. Collection of soil sample and Screening of the Isolates for Extracellular Protease:

Soil Samples were collected from different parts from saline belt of Akola district, soil sample inoculated on same day or stored for no longer than 7 days at 6^0C. Bacterial isolation was done by the serial dilution plating technique on nutrient and halophilic agar containing excess NaCl (pH 7.5). Based on colony characteristics different organisms were isolated, cultured aerobically in similar broth (pH 7.5) at 35^0C and maintained as pure cultures. Protease-producing strain was selected by using a plate assay on medium Skim Milk Agar containing skim milk-10.0%, NaCl-50.0g/lit, and pH-7.0 and second medium containing Gelatin-300g/lit, Peptone-100g/lit, NaCl- 50.0g/lit and pH-7.0, incubated at 35^0C for 24 hrs. After incubation plates were observed for a clear zone of hydrolysis surrounding to bacterial colony indicative of hydrolysis due to proteolytic activity.

2. Characterization of Selected Isolates:

Identification of the protease positive isolates was done by microbiological, Gram's character, Motility, and ability to ferment various sugars was studied using Vitek. Selected isolate were subjected to 16S rDNA sequencing for molecular identification. The obtained 16S rDNA sequence was compared with data base sequences using BLAST program available at NCBI server. Hits obtained were analyzed and used for obtaining identification of the isolates. Query coverage, alignment score and E-value was considered for finding closest relative sequence for our sequence in the database. This basis was used for accepting nomenclature for the isolates.

3. Preparation of crude enzyme and determination of Protease Activity:

Protease positive isolates were inoculated in modified broth containing gelatin and peptone. Sterilized medium inoculated with 5% of culture (A_{600} = 0.1) and incubated on rotary shaker rotating with 120 rpm at 35^0C for 48 to 72 hrs. After dense growth in the medium the broth content were centrifuged in sterile centrifuge tubes at 15000 g for 20 min at 4^0C. Supernatant was collected in separate sterile bottles and used as crude protease enzyme. The proteolytic activity of the enzyme, with casein as the substrate, was determined by the modified method of Kunitz (1947). One-milliliter of crude enzyme solution was added to 3ml of 0.5% casein solution (pH 9), and incubated for 20 min at 60^0C. All Protein present was precipitated by adding 3ml of 10% trichloroacetic acid (TCA). After 20 min, the supernatant was separated by centrifugation at 10000 rpm for 20 min and free tyrosine produced due to activity of crude enzyme measured by Folin and Lowry method. Blanks were prepared in which 3ml TCA was added before incubation. All assays were done in triplicate. One unit of protease activity was defined as the amount of the enzyme yielding the equivalent of 1 mmol of tyrosine per minute under the assay conditions.

4. Effect of pH and Temperature on Protease Activity:

Optimization of pH for the activity of protease enzyme is determined by using buffered substrate of different pH, from pH 5.0 to 10.0. For acidic sodium acetate buffer is used and basic was tris HCl or phosphate. The enzyme substrate reaction in the same manner is carried out and incubated at varying temperature conditions ranging from 30^0C to 80^0C.

5. Effect of Carbon, nitrogen sources and metal ions on the production of protease:

Effect of various carbon, nitrogen and metal ions on protease production was studied. Carbon sources using glucose, sucrose, starch, fructose, maltose and lactose. Nitrogen using beef extract, yeast extract, peptone, ammonium nitrate, ammonium carbonate, urea, lysine, L-aspartic acid, glutamic acid and glycine. Metal ions used were K, Hg, Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}.

6. Salt Stability of Enzyme:

Crude enzyme was incubated in 5% NaCl and pH 9.0 at 60^0C after interval of 1 hr enzyme is withdrawn and protease activity was determined. Enzyme activity was also determined at various NaCl concentrations from 0 to 5%.

III. RESULT AND DISCUSSION

Screening of the isolates for extracellular protease:

Total of 120 bacterial isolates were obtained capable to withstand and grow at excess salt concentration. Out these 29% isolates further identified as *Bacillus* (Jadhav and Musaddiq, 2011). From these 21 isolates were efficient in protease production at 5% NaCl and mostly of Bacillus species (Bacillus AS-3, 47, 48, 54, 58, 72, 111, 46, 66, 102, 107, 68, 77, 103, 83 and Halomonas AS-11, 14, 38, 56, 98).

Identification of isolates:

Biochemical characterization of protease positive 21 isolates were Gm+ Ve and belongs to 5 different species of bacillus, these are as mentioned in table no.1.

Table no. 1. Biochemical characterization of various Bacillus sp isolates from saline soil.

Isolate	Biochemical property								
	Catalase	V-P reaction	Growth at 50°C	Growth in 7% NaCl	NO3 reduced to NO2	Starch hydrolyzed	Acid in glucose	Acid and gas in glucose	Hydrolysis of casein
B. cereus	+	+	+	-	+	-	+	+	+
B. licheniformis	+	+	+	+	+	+	+	-	+
B. pumilus	+	+	+	+	-	-	+	-	+
B. firmus	+	-	-	+	+	+	+	-	+

Bacillus licheniformis AS-3, 47, 48, 54, 58, 72, 111, *B. cereus* AS-46, 66, 102, 107, *B. firmus* AS-68, 77, 103, *B. pumilus* AS-12, 83, *Halomonas stevensii* AS-11, 14, 38, 56, 98

Few isolates also identified by 16S rDNA comparison result obtained summarized in table no.2.

Table no. 2. Comparison of 16S rDNA sequences of selected isolates.

Query	Hit Closest BLAST relative	Accession	Similarity
AS-2	Bacillus firmus strain BVC76 16S ribosomal RNA gene, partial sequence	JQ407798.1	87%
AS-3	Bacillus licheniformis strain CICC10266 16S ribosomal RNA gene, partial sequence	DQ112221.1	94%
AS-4	*Bacillus sonorensis* NRRL B-23154(T)	AF302118	99.286
AS-5	*Bacillus subtilis* subsp. spizizenii NRRL B-23049(T)	AF074970	47.731
AS-12	Bacillus pumilus strain ATCC 7061 16S ribosomal RNA, partial sequence	NR_043242.1	98%
AS-14	*Halomonas stevensii* S18214(T)	AM941388	99.713

Determination of Protease enzyme activity:

Among these only four representative isolates were selected showing higher protease production at 5% NaCl and comparison of these four revealed that isolate no AS- 3 followed by AS-8 showed highest protease production in basal medium containing 5% NaCl. It was found to have maximum enzyme activity at pH 9.0 and 60^{0}C, average value of three different experiments was as shown in fig. 1.

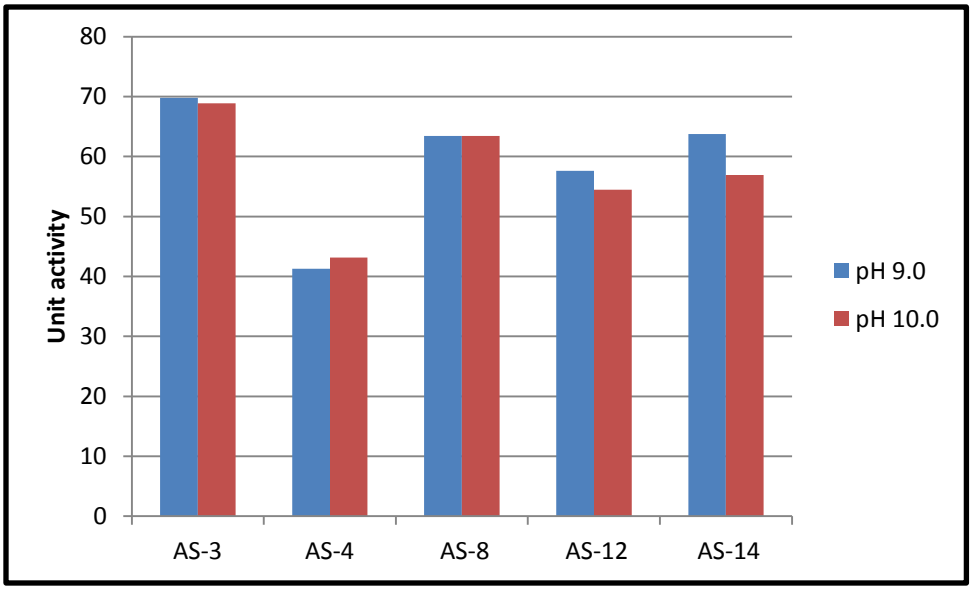

Fig 1: Protease activity of selected protease positive isolates at 5% NaCl.

The isolate AS-3 *B. licheniformis* being the highest protease producer in assay condition further used for enzyme production, the influence of various parameters on protease activity was determined. Enzyme was active over a range of pH from 5.0 to 11.0, having maximum activity at pH 9.0 – 10 (Fig. 2).

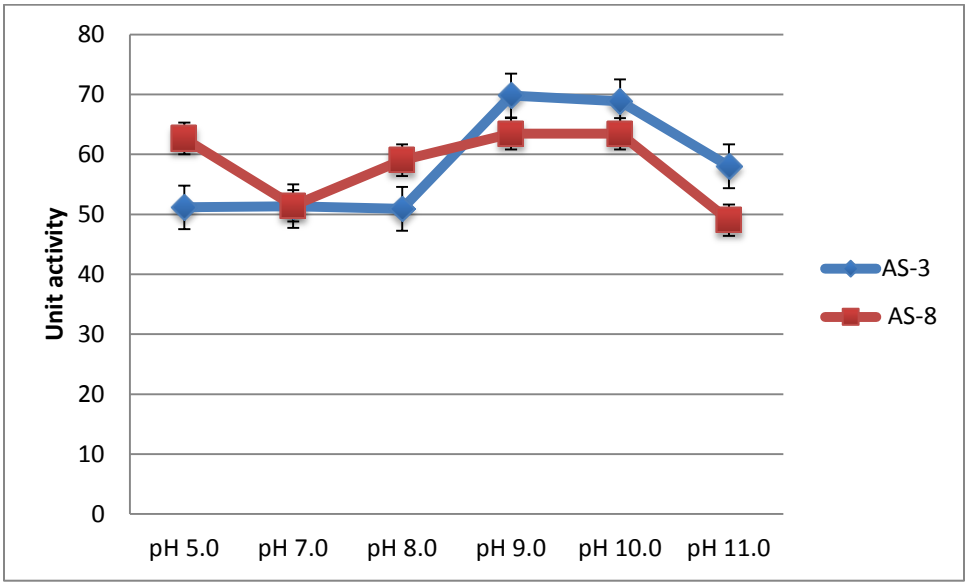

Fig. 2: Influence of pH on the activity of protease enzyme from isolate AS-3.

Temperature found as one of influencing factor, maximum being 60^0C where as below and above temperature showed substantial fall in activity (Fig.3).

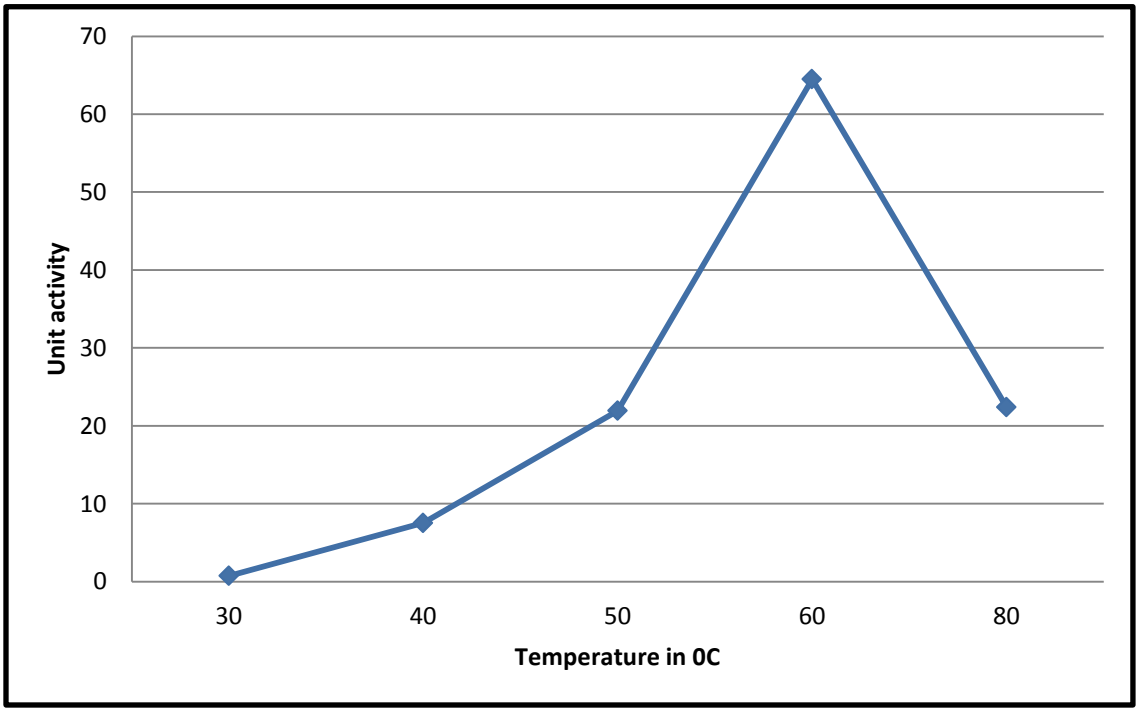

Fig. 3: Influence of temperature on the activity of protease enzyme from isolate AS-3.

Effects of carbon sources on protease production:

Lactose and maltose showed increases protease production (Fig.4) as compared to other carbon sources at 72 h of incubation. This is similar to reported in previous studies, larger amount of enzyme was synthesized when carbon sources used were poorly utilized for growth purposes (Ismail and Fattah, 1999).

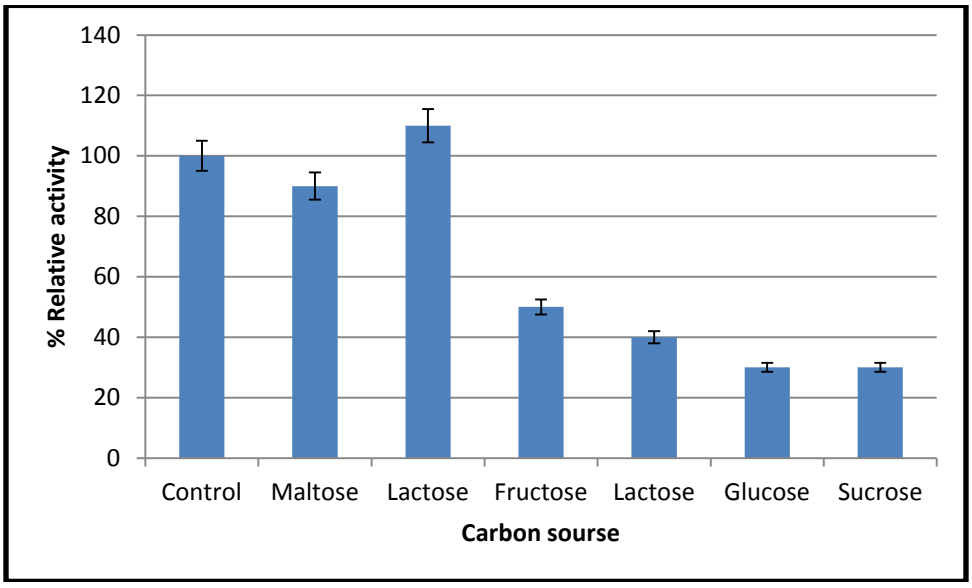

Fig. 4: Effect of carbon sources on the activity of protease enzyme from AS-3 isolate.

Effect of nitrogen sources on protease production:
It was showed that yeast extract (Fig.5) resulted in the highest level of protease activity compared to other sources of organic nitrogen. this was followed by beef extract and peptone. Other inorganic and amino acid showed increase in growth but in comparison lesser protease production.

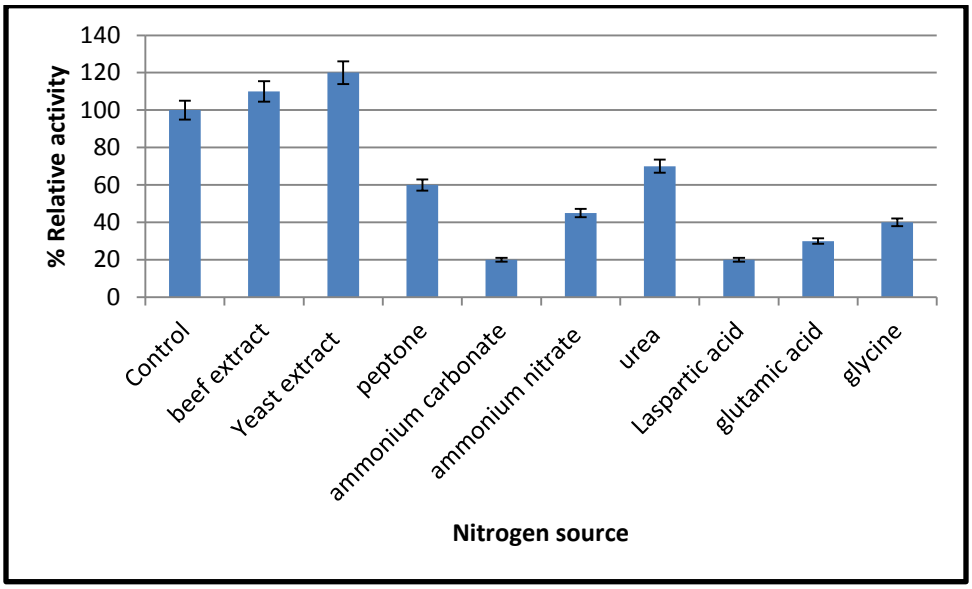

Fig. 5: Effect of Nitrogen sources on the activity of protease enzyme from AS-3 isolate.

Effects of metal ions on protease production:
The highest level of protease production was observed in the presence of KCl at 72 h incubation (Fig.6). Addition of Ca2+, Cu2+ and Mn2+ resulted in lesser protease production whereas $HgCl_2$ was found inhibitory effect. It was suggested that these metal ions increases stability of proteases, even though effects of the different metal on protease production vary, their presence in the medium improved activity.

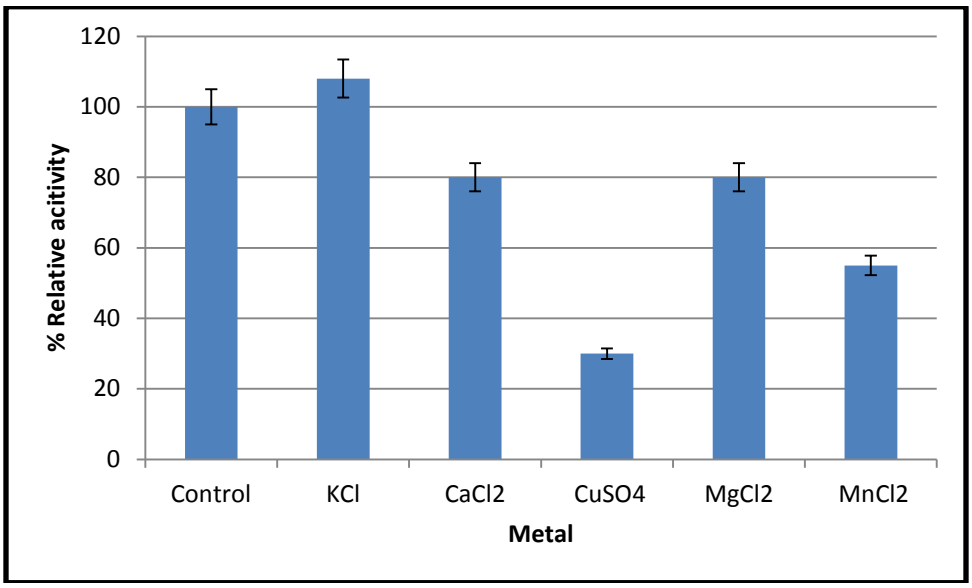

Fig. 6: Effect of Metal on the activity of protease enzyme from AS-3 isolate.

Stability and effect of NaCl

Protease activity was reported at presence and absence of NaCl, without NaCl the activity was highest and decreases with increase in NaCl concentration from 0 to 5% (Fig. 7). In this study only activity of this is considered in response to NaCl not the production it will be also worthwhile to check production at varying salt concentrations. Protease activity is found stable at 5% NaCl and 50% retained upto 4hrs (Fig. 8).

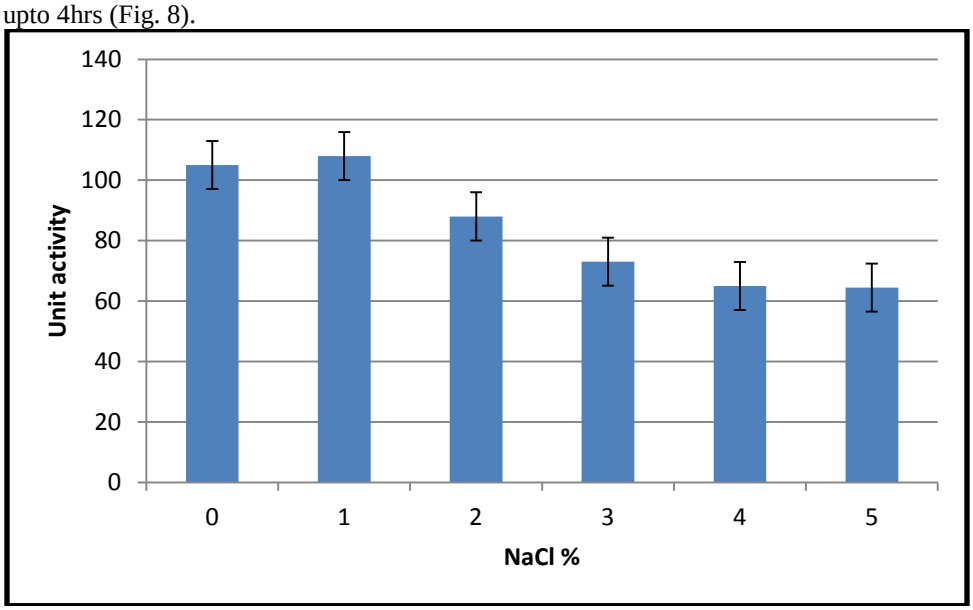

Fig. 7: Activity of crude protease enzyme from AS-3 isolate at various NaCl concentrations.

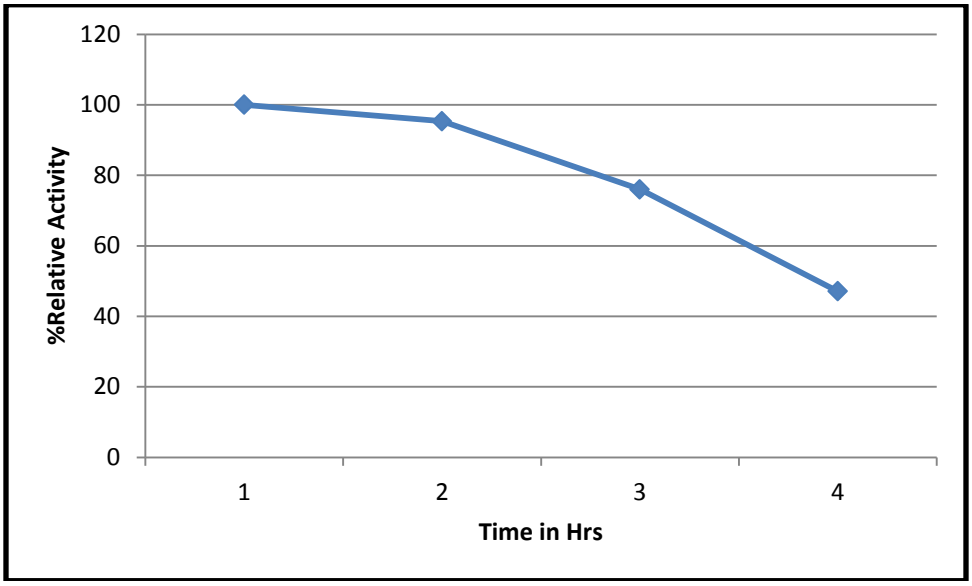

Fig. 8: Stability of protease enzyme activity at 5% NaCl of AS-3 isolate. 12

This result clearly indicated the potential of isolates in producing protease enzyme at 5% of salt, also stability of the enzyme. Similar results were obtained with the other enzymes produced by halophilic microorganisms (Prakash *et al* 2005, Amoozegar *et al.*, 2003, Sanchez-Porro *et al.*, 2003; Coronado *et al.*, 2000; Kamekura and Seno, 1990). The isolate showed protease production in basal medium containing 5% (w/v) NaCl. It will worthwhile to check capability of producing enzyme in the presence of $NaNO_3$, KCl and sodium citrate as well as in varying concentration of NaCl. The production of protease in the presence of Na_2SO_4 has not been reported by others so far (Lama *et al.*, 2005; Patel *et al*., 2005; 2003; Kumar *et al.*, 2004).

Protease activity was found in presence and absence of NaCl, this is in contrast to the activity of most proteases from extremely halophilic microorganisms that falls off dramatically and irreversibly when the enzyme is exposed to lower salt concentrations. These characteristics make the protease of this study an interesting candidate for application in biotechnological processes, such as the treatment of saline waters or waste solutions with proteinaceous materials.

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