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# HALOSTABLE ALKALINE AMYLASE AND PROTEASE FROM AN EXTREMELY HALOALKALIPHILIC ARCHAEON, *NATRINEMA SP*. SSBJUP-1 ISOLATED FROM LONAR LAKE.

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## ABSTRACT

An extremely haloalkaliphilic archaeon *Natrinema* sp. SSBJUP-1 showing potent amylolytic and proteolytic activities was isolated from the saline alkaline Lonar Lake, India. Amylase and protease were active over the neutral to alkaline pH range from 6 to 9. Amylase was more stable over a narrower temperature range of 40-60$^{o}$C than the protease but had the higher optimum (55$^{o}$C) for activity. By contrast, protease was active over a broader range 10-75$^{o}$C with an optimum of 40$^{o}$C. The amylase was stable over a broader salinity range, but with an optimum salt concentration of 10-12%, whereas the protease was with a higher optimum concentration of 18% salt in the reaction mixture. Calcium and Magnesium behaved as activators for both enzymes while Copper was an activator for protease while being an inactivator for amylase. Amylase was highly tolerant to five commercial detergents tested while the protease was tolerant only to one.

**KEYWORDS:** Haloalkaliphiles, Archaea, *Natrinema sp,* Amylase, Protease.

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# INTRODUCTION

Amylases and proteases constitute two of the most extensively studied enzymes on account of their application in several biotechnology based industries and constituting two of the largest selling in the world accounting for approximately 25% and 18% respectively[1].Most industrial processes employ harsh physicochemical conditions that may not be definitively adjusted to the optimal points required for the activity of the available enzymes that are today mostly of mesophilic or neutrophilic origin. Thus enzymes that exhibit optimal activities at various ranges of salt concentrations, pH and temperature would be of great significance[2]. This is where the extreme haloalkaliphiles could be of tremendous help as pointed out by Horikoshi [3].Haloalkaliphilic archaea constitute a group of microorganisms that grow in extreme conditions of alkaline pH from 8.0 to 11.0 and at least 1.5M NaCl with most strains growing best at 3.5-4.5M NaCl[4]. They have been isolated from different habitats, including alkaline salt lakes, marine salterns, the Dead Sea and saline soils[5, 6,7]. In contrast to halophilic bacteria, the archaea adopt a high salt strategy to survive osmotic challenges associated with life in hypersaline environments[8, 9]. Thus, their enzymes are active in up to 5M or higher concentration of NaCl or 4M KCl [10]. These enzymes also show catalytic functions in low water activity, a situation common with organic solvents [11]. Enzymes of such extremophilic archaea would therefore prove very useful in several biotechnological applications [12,13,14] . Despite advances in understanding the diversity and systematics of haloarchaea, their hydrolytic enzymes and their characterisation has received less attention [15]. *Natrinema* is a genus, whose members are known to be potent producers of several hydrolytic enzymes [14]. Only one though, *Natrinema ejinorense*[7] is a confirmed amylase producer and *Natrinema versiforme* shows doubtful results[17]. However, studies on the characterisation of either are lacking. A *Natrinema sp* SSBJUP-1 was isolated from the alkaline (pH 9.5-10.0) soils of Lonar Lake, a well known closed basin lake formed in a meteorite impact crater – the only one in the world formed in basaltic rock, situated in the district of Buldhana in Central Maharashtra, India (Lat 19$^o$58'; Long76$^o$34').The following study reports the production and characterisation of halo- and alkalistable amylase and protease from this isolate.

# MATERIALS AND METHODS

### *Bacterial Isolation, Media and Cultural Conditions*

*Natrinema sp.* SSBJUP-1 was isolated in Specific Haloalkaliphilic (SH) medium of composition (g/L): Casamino acids, 7.5; Yeast extract, 10; Tri-sodium citrate, 3; $MgSO_4.7H_2O$, 1; KCl, 2; $FeSO_4.7H_2O$, 0.05; NaCl, 200; $Na_2CO_3$, 18.5, agar agar 25. Sodium chloride (NaCl) and $Na_2CO_3$ were sterilised separately and the pH was self adjusted to 8.5 after the ingredients were mixed. Incubation was at 40$^o$C for up to 21 days. Screening for amylase and protease production was done on SH-starch (0.5% w/v soluble starch) agar and SH-casein (1% w/v casein) agar. Standard schemes of morphological and biochemical characterisation were adopted for the identification of the isolate [19] and confirmed by 16S rRNA gene sequencing (courtesy Microbial Culture Collection, National Centre for Cell Science, Pune). Homology with sequences in GenBank database of NCBI was analysed with BLAST software. Phylogenetic analysis was performed using the software package MEGA 4.0 version[20].

### *Production and Partial Purification of the Enzymes*

The isolate was cultivated in liquid SH-starch and SH-casein medium aerobically at 40$^o$C on a rotary shaker-incubator (LabHosp) at 125rpm. Cell-free supernatants were collected by centrifugation at 10000xg for 20' at 4$^o$C and the enzymes precipitated with 60% saturation (for amylase) and 45% (for protease) ammonium sulphate, stirring for 30' and refrigeration at 4$^o$C overnight. The precipitates were collected through centrifugation at 5000xg for 15' at 4$^o$C,

dissolved in 50ml 50mM Tris-HCl buffer (pH 8.5) and dialysed against the same buffer at 4$^{o}$C overnight.

### *Assay of Amylase*

Amylase activity was assayed by mixing 500µl of 0.5% soluble starch solution (prepared in 50mM Tris-HCl buffer, pH 8.5 and 10% NaCl) with 500µl dialysed enzyme and incubated at 40$^{o}$C for 15'. The further assay was performed as previously described [21]. One unit of enzyme was defined as the amount that released 1 µmol of reducing sugar (glucose) min$^{-1}$ under the assay conditions.

### *Assay of Protease*

This was performed as described in Jayaraman [22]. One unit of protease was defined as the amount that released 1µg of tyrosine min$^{-1}$ under the assay conditions [13].

### *Effect of pH, Temperature and Salt (NaCl) Concentration on the activity of the Enzymes*

The enzyme substrate reaction mixtures were incubated at different pH from 3.0 to 11.0, different temperature from 10-100$^{o}$C and various NaCl concentrations from 0-26% w/v. Buffers used for maintaining the pH values were 50mM solutions of acetate (pH 3.0–5.0); sodium phosphate (pH 6.0–7.0); Tris–HCl (pH 8.0–9.0) and carbonate bicarbonate (pH 10.0–11.0).

### *Effect of Substrate Concentration on Enzyme Activity and Determination of $V_{max}$ And $K_M$*

Different substrate concentrations from 0.5- to 15mg/ml were taken in the reaction mixtures that were incubated at the pH, temperature and salinity optima of the enzymes. The two kinetic constants were determined using the Lineweaver-Burk plot.

### *Effect of Metals and Detergents on Enzyme Activity*

The effect of metal ions $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Na^{2+}$, $Hg^{2+}$, and $Zn^{2+}$ and commercial detergents like Nirma, Active Wheel, Surf Excel, Ariel, Oxyblue and Tide on the enzymes was tested by incubating the enzymes with the metals and detergents for 30 minutes and determining the relative activity by standard assay thereafter.

## RESULTS

### *Isolation and Identification of Natrinema sp. SSBJUP-1*

Based on morphological, physiological and biochemical characteristics, the isolate was tentatively identified as a member of genus *Natrinema* and confirmed by 16S rRNA sequence analysis. It was code named *Natrinema sp.* SSBJUP-1 and its sequence deposited in the Gene Bank of NCBI (www.ncbi.nlm.nih.gov/Genebank/submit.ht ml) with the accession number JX478270.1. The phylogenetic tree based on neighbour joining method was constructed using MEGA 4.0 and is presented in Fig. 1.

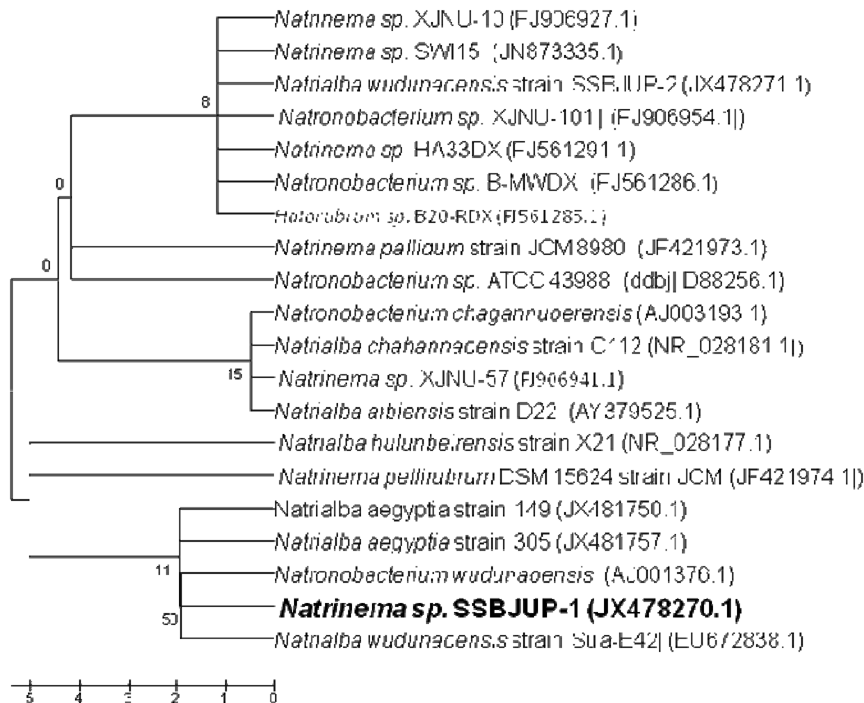


**Figure 1**

***Phylogenetic tree based on partial 16S rRNA sequences, showing the relationship of the isolate Natrinema sp. SSBJUP-1 to other members. Accession numbers of the sequences are shown in parentheses. The evolutionary history was inferred using the Neighbour-Joining method [23]. The optimal tree with the sum of branch length = 30.48292977 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.***

### *Production and characterisation of the enzymes*

The isolate was used for production of the enzymes in the respective liquid media. The enzymes were partially purified and used for further characterisation as follows.

### *Effect of pH, Temperature and Salt (NaCl) Concentration on activity*

The results of these studies are shown in Figures 2, 3 and 4 respectively. It is evident that both the enzymes are alkalistable, thermostable as well as halostable. The protease is more alkali stable than amylase, with higher activity in a narrower alkaline range from pH 8 to 10 and an optimum of 9. Where temperature is considered however, amylase is superior in its optimum at 55$^{o}$C but within a narrower range. Halostability for both the enzymes is expectedly very good with both enzymes showing stability up to 20% NaCl concentration. Here again, the protease steals a march over amylase showing its maximum activity at 20% NaCl with significant activity levels from 14% upward. Amylase shows maximum activity at 10 -12% concentration retaining 80% activity at 20%.

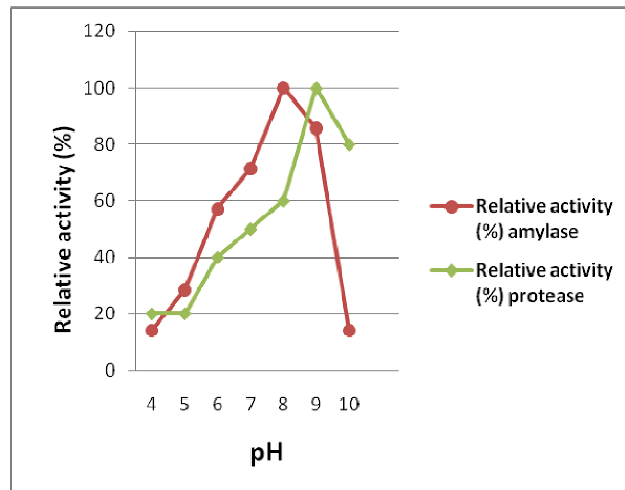


**Figure 2**
***Effect of pH on the activity of amylase and protease from Natrinema sp. SSBJUP-1. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments.***

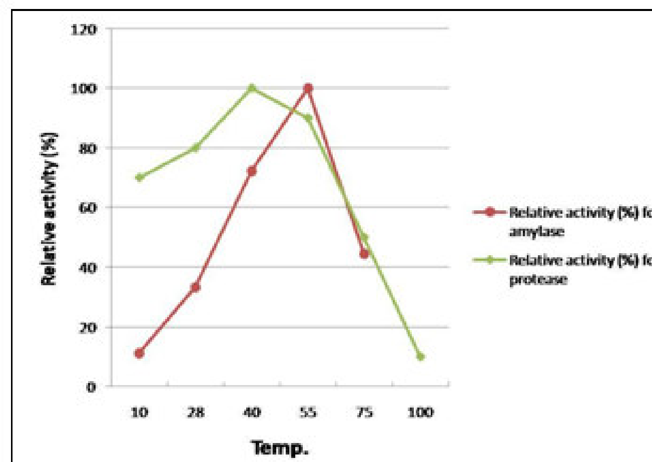


**Figure 3**
***Effect of temperature ($^{o}C$) on the activity of amylase and protease from Natrinema sp. SSBJUP-1. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments***

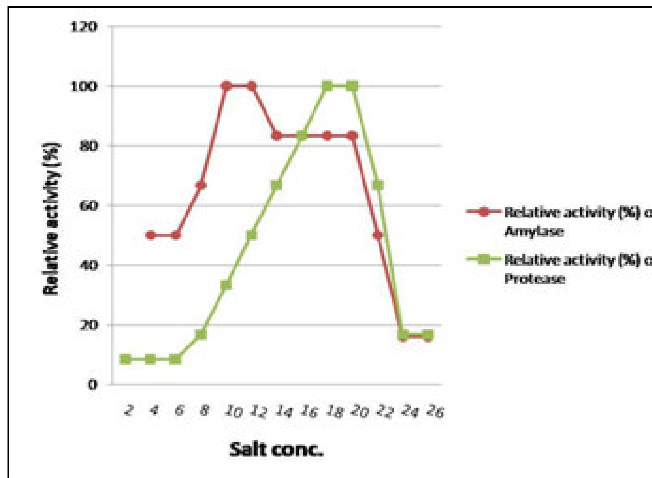


**Figure 4**

***Effect of salt (NaCl) concentration (%) on the activity of amylase and protease from Natrinema sp. SSBJUP-1. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments***

***Kinetic Constants Of The Enzymes***

The two kinetic constants $V_{max}$ and $K_M$ determined for the enzymes at their respective pH, temperature and salt optima can be observed in Figure 5. The $V_{max}$ and $K_M$ for amylase are 100 $Uml^{-1}$ and 0.88$mgml^{-1}$ respectively while for the protease they are 100 $Uml^{-1}$ and 1.51$\mu gml^{-1}$.

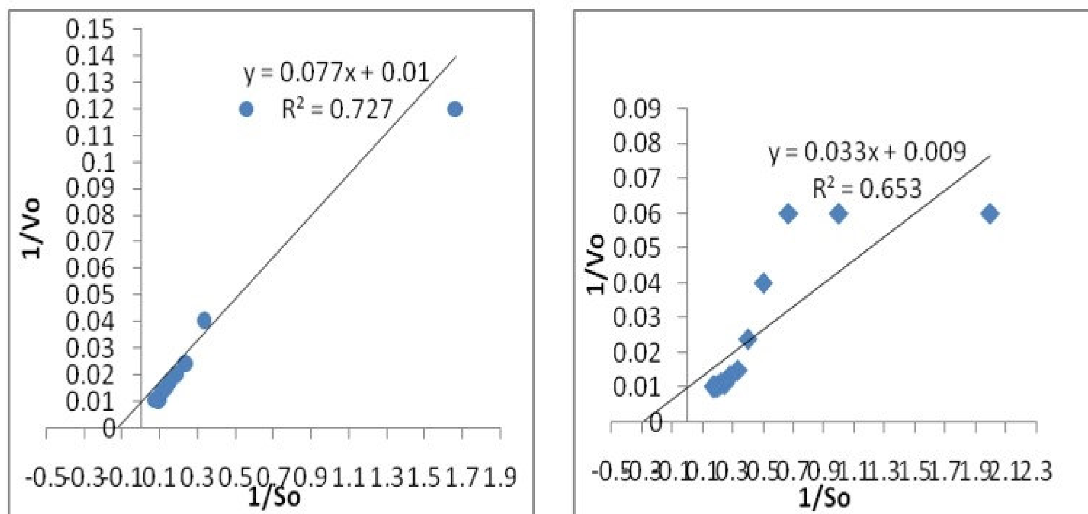


**Figure 5**

***Lineaweaver-Burk plot showing the $K_M$ and $V_{max}$ intercepts for Amylase (a) and Protease (b). Experiment performed in triplicates under optimum conditions of pH (8.0 and 9.0), temperature (55 and 40$^oC$) and salt concentration (12 and 20%) for each enzyme.***

***Effect of Metal Ions***

**Table1**

***Effect of metal ions on the activity of amylase and protease from Natrinema sp.SSBJUP-1.Residual activity is activity of enzymes with individual metal concentration in relation to the activity in the control* taken as 100% activity. Values are averages of three independent experiments.***

| Metal ion (mM) | Residual activity (%) in the presence of metals | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ca | | Mg | | Hg | | Cu | |
| | Amylase | Protease | Amylase | Protease | Amylase | Protease | Amylase | Protease |
| 0.1 | 16.65 | 16.65 | 25 | 45 | 16 | 16 | 33 | 65 |
| 0.2 | 30.76 | 30.76 | 38.46 | 38.46 | 15 | 15 | 7.6 | 87 |
| 0.3 | 42.85 | 42.85 | 50 | 50 | 7.14 | 7.14 | 0 | 95 |
| 0.4 | 102 | 50 | 58.33 | 70.33 | 0 | 0 | 0 | 105.26 |
| 0.5 | 116.66 | 66.60 | **103** | **115** | 0 | 0 | 0 | 111 |
| 0.6 | 116.66 | 116.66 | 100 | 100 | 0 | 0 | 0 | 119.60 |
| 0.7 | **140** | **140** | 100 | 100 | 0 | 0 | 0 | 125 |
| 0.8 | 80 | 130 | 100 | 70 | 0 | 0 | 0 | **135** |
| 0.9 | 50 | 50 | 50 | 50 | 0 | 0 | 0 | 100 |
| 1.0 | 50 | 50 | 20 | 20 | 0 | 0 | 0 | 80 |

****Activity in presence of Na ions at corresponding concentration was taken as control for amylase and activity in absence of any metal was the control for protease.***

Data in Table 1 show amylase activity being markedly stimulated by $Ca^{2+}$ in the range of 0.4 to 0.7mM concentration but inhibited by $Cu^{2+}$ and $Hg^{2+}$ with marginal stimulation by $Mg^{2+}$. The protease activity was also markedly stimulated by $Ca^{2+}$ in the range of 0.6 to 0.8mM concentration but was inhibited by $Hg^{2+}$. Copper ($Cu^{2+}$) however, showed unexpected results enhancing the enzyme activity with increasing concentration. $Mg^{2+}$ had a marginally inhibitory effect on the enzyme.

***Effect of Detergents***

The stability of the two enzymes in the presence of five commercial detergents at different concentrations was tested and the response shown in Fig.6.

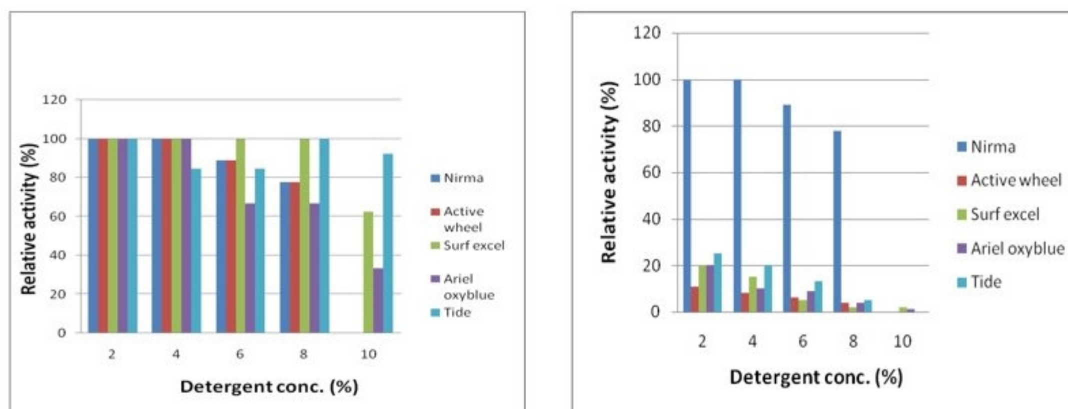


**Figure 6**

***Effect of detergent concentrations on the activity of amylase (a) and protease (b) from Natrinema sp. SSBJUP-1. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments.***

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Amylase shows maximum tolerance to 4% detergent concentration of Nirma and Active Wheel, and up to 8% to Surf Excel and Tide. Its maximum resistance to Ariel Oxyblue however is at 2% followed by a steady decline at increasing concentrations. By contrast unfortunately, the protease was inhibited by all detergents except Nirma which it tolerated up to a concentration of 8%.

## DISCUSSION

Characteristics of the two enzymes studied here are presented along with data of some similar enzymes reported by earlier workers in Table 2.

**Table 2**
***Characters of Amylase and protease of Natrinema sp SSBJUP-1 in comparison with previous reports of similar enzymes from other halophiles and haloalkaliphiles***

| Isolate | Reference | Optimum pH | Optimum temp. (°C) | Optimum salt requirement (%) | Response to metals: Activated | Response to metals: Inhibited |
|---|---|---|---|---|---|---|
| **Amylase** | | | | | | |
| *Halobacillus sp.* LY9 | Li and Yu (2011) | 8.0 (4 – 12) | 60 (50 – 70) | 10 – 12 (20) | Ca | Zn, Fe, Cu, Hg |
| *Salimicrobium halophilum* LY20 | Li and Yu (2012) | 10.0 (6 – 12) | 70 (30 – 80) | 10 (2.5 – 20) | ND | ND |
| ***Natrinema* sp SSBJUP-1** | Present study | 8.0 (6 – 9) | 55 (40 – 75) | 8 – 12 (7 – 22) | Ca, Mg | Hg, Cu |
| **Protease** | | | | | | |
| *Natrinema* sp. J7 | Shi et al (2004) | 8.0 | 50 | 14 | ND | ND |
| *Bacillus megaterium* RRM2 | Renganathan et al (2011) | 9.0 – 10.0 | 60 | ND | Ca, Mg, K, Na | Hg, Cu, Fe, Co, Zn |
| *Salimicrobium halophilum* LY20 | Li and Yu (2012) | 10.0 (6 – 12) | 80 (30 – 80) | 12.5 (2.5 - 20) | ND | ND |
| ***Natrinema* sp SSBJUP-1** | Present study | 9.0 (5 – 10) | 40 (10 -75) | 18– 20 (12 – 22) | Ca, Mg, Cu | Hg |

*ND – not determined; Figures in parentheses indicate the range within which at least 50% activity is retained*

The extracellular protease SptA from *Natrinema* sp. J7 retained 20% of its activity after removal from NaCl and 60% of its activity could be restored by reintroduction of 2.5 M NaCl [26]. Similarly, reports on a β–amylase from *Halobacillus sp*. LY9 with an optimum pH of 8.0 within a wide range from 4 to 12 was seen to retain >55% activity over a range of higher temperature from 50-70$^{o}$C with an optimum at 60$^{o}$C and showed an optimum salt (NaCl) requirement of 10-12% concentration with a >70% retention of activity at 20% salt [27].Calcium and Magnesium appear to be universal activators to all enzymes while mercury and copper are expectedly inhibitory to all. The only remarkable feature is the stimulation of protease from *Natrinema* sp SSBJUP-1 by copper.The amylase produced by *Salimicrobium halophilum* strain LY20 was completely inhibited by EDTA, diethyl pyrocarbonate (DEPC) and phenylarsine oxide (PAO) and the protease by phenylmethylsulfonyl fluoride, DEPC and PAO. They were however, resistant to the surfactants SDS, Tween 80, and Triton X-100 and showed remarkable stability in the presence of water-miscible organic solvents as well [24]. Similarly, the protease from *Bacillus megaterium* RRM2 was seen to exhibit a high degree of tolerance to SDS. The partially purified protease when used as an additive in the commercial detergents was found to be a suitable source for washing clothes especially those stained with blood [28]. Of the two

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enzymes produced by *Natrinema sp.* SSBJUP-1, amylase was found to be more resistant to the several commercial detergents tested and the partially purified enzymes were seen to be capable of removing food and blood stains from cloth when tested independently and as additives in detergents.The present study describing alkalistable, thermostable, halostable and most significantly detergent stable amylase and protease from *Natrinema sp.*SSBJUP-1 indicates a significant biotechnological potential for this isolate.

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