

## Effect of Organic Solvents on the Activity and Stability of an Extracellular Protease Secreted by the Haloalkaliphilic *Bacillus firmus* HS4

Vijayshree M. Hemke<sup>1</sup> and Vishal R. Dhundale<sup>2\*</sup>

<sup>1</sup>Jijamata Mahavidyalay Buldhana, Maharashtra, India

<sup>2</sup>New Arts Commerce and Science College Ahmednagar, Maharashtra, India

\*Corresponding Author E-mail: [vrdvishal@gmail.com](mailto:vrdvishal@gmail.com)

Received: 23.07.2017 | Revised: 4.08.2017 | Accepted: 5.08.2017

### ABSTRACT

*Extremozymes are now-a-day replacing chemical catalyst in manufacturing of chemicals, textiles, pharmaceuticals, paper, food, leather processing and agricultural chemicals since the enzymes prepared with suitable properties with the advent of new knowledge in biotechnology. Partial characterization of protease from Bacillus was carried out by effect of pH, temperature, substrate, enzyme, metal ions and organic solvents on alkaline protease activity was then measured as per assay procedure. Total one hundred and fourteen isolates were obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied and fifty five isolates were selected on the basis of their pH salt and temperature tolerance bacteria were isolated from hypersaline alkaline lake. Out of them, a bacterium HS4 with an ability to produce extracellular halophilic, alkali-tolerant, organic solvent stable, and moderately thermostable protease producing strain was detected. Identification of the bacterium HS4 was done based upon biochemical tests and 16S rDNA sequence. The result of gene sequence analysis indicated that bacterium strain HS4 shows highest degree of similarities with Bacillus firmus. The optimum activity were revealed at 80°C, pH 10.5. Enzyme activity strongly exhibited enzyme activity by heavy metals such as BaCl<sub>2</sub>, CaCl<sub>2</sub>, MgCl<sub>2</sub>, FeCl<sub>3</sub>. Protease was found to be stable and enhance by organic solvents such as Methanol, Acetone, Benzene, ethanol, Hexane, propanol, ethyl acetate, xylene, acetic acid and Chloroform which has showed demonstrating the unique properties of protease enzymes. The enzyme is a haloalkaline, thermostable, metal ion and organic solvent tolerant in nature. The determination in the present study was an enzyme production analysis conducted to address this Bacillus firmus HS4 as a potential for biotechnology application.*

**Key words:** *Bacillus, Protease, Organic solvents, Metal ions.*

### INTRODUCTION

With increasing importance on the environmental protection with decreasing pollution, the use of enzymes particularly from extremophiles, gained considerable attention from the last several years. Extremozymes are now-a-day replacing chemical catalyst in

manufacturing of chemicals, textiles, pharmaceuticals, paper, food, leather processing and agricultural chemicals since the enzymes prepared with suitable properties with the advent of new knowledge in biotechnology<sup>1-3</sup>.

**Cite this article:** Hemke, V.M. and Dhundale, V.R., Effect of Organic Solvents on the Activity and Stability of an Extracellular Protease Secreted by the Haloalkaliphilic *Bacillus firmus* HS4, *Int. J. Pure App. Biosci.* 5(4): 1980-1988 (2017). doi: <http://dx.doi.org/10.18782/2320-7051.5293>

In leather industry, for removal of hairs from animal skin they used specific chemicals such as the Lime and sodium sulphide but these chemical are the remarkable source of water pollution and harmful to the tannery workers. Lime produces a poisonous sludge while sodium sulphide is highly toxic and has obnoxious odor<sup>4-6</sup>. There was a biological agent instead of chemicals such as enzymes which can be used in tannery for the removal hair from the animal skin and reduce the water pollution has yet to be explored<sup>7</sup>. As there is large demand of protease, isolation and production of extremozyme is most important to fulfill this demand. But such type of extremozyme are produced from microorganism which survive in extremophilic environment such as soda lake and alkaline hot springs<sup>8</sup>.

Alkaline saline environment were occurred naturally in the world, these lakes are characterized by their highly basic pH values ranging from 10-12. These environments generated due to the combination of geological, geographical and climatic condition giving rise to the accumulation of sodium carbonate and sodium chloride which leads to alkaline saline environment. Haloalkaliphiles are vigorously grown in both saline and alkaline environments. Soda lakes are widely distributed however, as a result of their inaccessibility; few such lakes have been explored from the microbiological point of view. The microbial population of these lakes, which is considerably diverse phylogenetically, includes *Archaea*, *Cyanobacteria*, *Proteobacteria* and Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much interest because of their ability to produce extracellular metabolites that are active and stable at high pH. The unusual properties of these metabolites offer a potential opportunity for their utilization in processes demanding such extreme conditions<sup>9-11</sup>.

The bacterial diversity of an East African soda lake was studied and extensively reviewed for their biotechnological potential. The microbial diversity of saline lakes has

been studied primarily by focusing on the isolation and characterization of individual organisms with biotechnological potential. Lipolytic and Amylolytic microorganisms studied from lake Bogoria and Kenyan alkaline soda lake as well as from Ethiopian soda lakes respectively<sup>12-13</sup>. As far as Indian alkaline saline lakes are concerned, a culture-dependent approach has still not been used to analyze bacterial diversity. Haloalkaliphiles is focused on microbiological classification and genetic characterization, with limited work to discover their industrial application. We have applied this strategy to explore the proteolytic strain of aerobic bacteria from Lonar lake<sup>8</sup>.

## MATERIALS AND METHODS

**Sampling Site:-** Lonar Lake an Indian soda lake situated in Lonar, District Buldhana, Maharashtra, India (latitude 19° 58, longitude 76° 36), The alkaline Lonar crater is a unique basaltic rock meteorite impact crater, ranking third in the world and is filled with saline water having an average pH of 9.5- 10. The bacterial diversity particularly in concern to protease producing *Bacilli* prevailing in the Lonar Lake has not been studied in detail so far. So it is necessary to apply this strategy to study the protease producing bacteria from Lonar Lake.

### Isolation of Alkaliphiles:

One gram of soil sample was transferred to 100 ml sterilized distilled water in 250 ml conical flask and agitated (200 rpm) at 37°C for 15 min in shaker. The suspension was then diluted to 10<sup>-7</sup> dilutions. One ml of each diluted sample was spread by spread plate technique into petri plates containing Horikoshii medium and nutrient agar medium and inoculated at 37°C for 24 h<sup>14</sup>.

### Screening of bacterial alkaliphiles:

Individual bacterial colonies were screened for proteolytic activities on Skim milk agar medium. The pH of the medium was adjusted to pH 10 with 1N NaOH before and after sterilization. The inoculated plates were incubated at 37°C for 48 hrs and observed for zones of clearance, indicating proteolytic activities<sup>15</sup>.

**Identification of the proteolytic isolates:**

The bacterial isolates with prominent zones of clearance on casein agar medium were processed for identifications based on morphology, Gram characteristics, motility, oxidase, catalase tests and acid production from dextrose, fructose, sucrose, xylose, arabinose, maltose and mannitol. The isolates were also tested for their growth at different temperatures and pH. These isolates were identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology and Diagnostic Microbiology. The identified strains were maintained on nutrient agar slants having pH 10 at 4°C.

**16S rDNA sequences and Phylogenetic analysis:**

DNA was extracted from bacilli culture using standard phenol chloroform protocol<sup>16</sup>. The partial sequence of the 16S rRNA gene was amplified by using polymerase chain reaction and universal primer Eubacteria specific primers, 16F 27 (5' CCAGAATTGATCMTGGCTCAG- 3') and 16R 1525 (5' TTCTGCAGTCTAGAAGGAGGTGWTCCA GCC – 3'). The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl procedure<sup>16</sup>. The 16S rRNA sequence were analysed using BLAST program Multiple Sequence Alignment of approximately 900 bp sequences were performed using CLUSTAL W, version 1.8. A phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of MEGA 4 program package<sup>18</sup>.

**Preparation of crude enzyme extracts:**

Hundred milliliters Casein nutrient broth (Casein 1gm, Peptone 0.5 gm, Yeast Extract 0.15 gm, Beef extract 0.15 gm, Sodium Chloride 0.5 gm, pH 10) was prepared. The sterile broth was inoculated with culture and incubated for 48 hrs at 37°C. After 48 hrs incubation, centrifugation of the broth at 5000 rpm for 15 min was carried out. The supernatant served as crude enzyme source<sup>19</sup>.

**Enzyme assay**

The standard graph of tyrosine was prepared by adding different concentration of standard tyrosine (1 mg/ml) into a series of test tubes and made the final volume in each test tube to 1 ml with distilled water. Estimation of proteases was carried out with 2.5 ml of casein in a test tube; 1 ml of enzyme source was added and incubated for 10min at room temperature. After incubation 2 ml of TCA was added to stop the reaction and centrifuged the reaction mixture at 5000-8000 rpm for 15 min. Supernatant was separated and 2 ml of Na<sub>2</sub>CO<sub>3</sub> and 1 ml of Folin-Ciocalteu reagent were added in 1 ml of supernatant. The reaction mixture was boiled for 1 min in a boiling water bath and 6 ml of distilled water was added to make a final solution to 10 ml. In control tube, the reaction was terminated the reaction at zero time and the absorbance was read at 650 nm.

**Partial characterization of protease:**

Partial characterization of protease from *Bacillus* was carried out by effect of pH, temperature, substrate, enzyme, metal ions and organic solvents on alkaline protease activity was then measured as per assay procedure.

**Effect of pH on alkaline protease activity:**

The effect of pH on alkaline protease was determined by assaying the enzyme activity at different pH values ranging from 7.0 to 12.0 using the phosphate (PO<sub>4</sub>) buffer systems with concentration of buffer as 0.2 M. The activity of the protease was then measured as per assay procedure.

**Effect of temperature on alkaline protease activity:**

The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (pH 10) for 10 min at different temperature ranging from 35°C to 100°C. The activity of the protease was then measured.

**Effect of substrate concentration on alkaline protease activity:**

The effect of substrate concentration on alkaline protease activity was determined by incubating the reaction mixture for 10 minutes with different substrate concentration, ranging

from 0.5 mg/ml to 5 mg/ml. The activity of the protease was then measured.

#### Effect of enzyme concentration on alkaline protease activity:

The effect of enzyme concentration on alkaline protease activity was determined by incubating the reaction mixture (pH 10) for 10 minutes at different enzyme concentration ranging from 0.5 ml to 5 ml. The activity of the protease was then measured.

#### Effect of different metal ions on protease activity:

The effect of different metal ions on alkaline protease activity was determined. The enzyme assay was performed in the reaction mixture as described above in the presence of various metal ions at a final concentration of 1 mM. The activity of the enzyme without any additives was taken as 100 %. The influence of various metal ions such as Potassium chloride, Sodium chloride, Ferric chloride, Barium chloride, Cupric chloride and Magnesium chloride (1 mM each) on protease activity was studied by pre-incubating the enzyme with the compounds for 15 min at 37°C. Then, the remaining activity was measured under the enzyme assay conditions.

#### Enzyme stability in presence of solvents

Stability of the protease enzyme was determined in the presence of various organic solvents such as Ethanol, Methanol,

Chloroform, Benzene, Hexane, Propanol, Acetone, Ethyl acetate, Xylene and Acetic acid (10 mM each). 0.25 ml of organic solvent was added to 1 ml of the protease solution in a 1.5 ml micro centrifuge tube with a screw cap. The mixture was shaken at 150 rpm for 100 min at 37 °C. Then, the remaining activity was measured under the enzyme assay conditions.

### RESULTS AND DISCUSSION:

During a previous study carried out from 2008 to 2017, total one hundred and fourteen isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied their tolerance of pH, salt and temperature<sup>8-9</sup>. Out of one hundred and fourteen, thirty two bacterial culture which were found proteolytic activity. All the bacterial strains were found both alkaliphilic and the optimum pH was revealed 10 for all the bacterial culture. Gessesse and Gashe<sup>20</sup> found both alkalitolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as microbial species found in soda lake microbial population and known for being good protease producers. In the present studies, out of thirty two, one bacterium HS4 strain was selected for further studies on production and optimization of protease on the basis of optimum zone of proteolytic activity<sup>21</sup>.

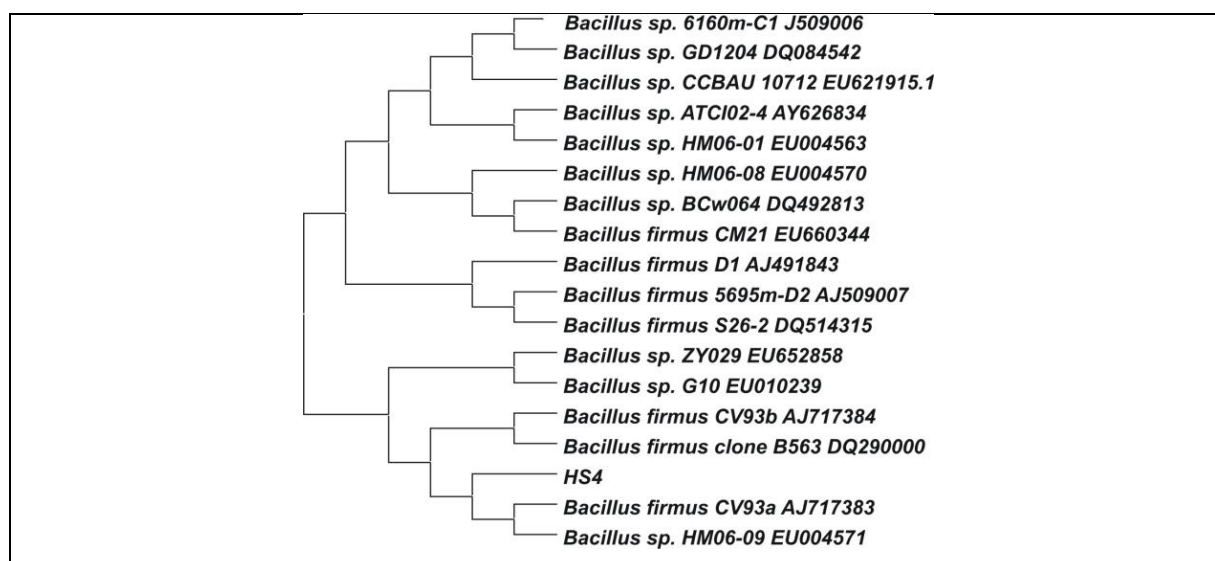


Fig 1: Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates and some of their closest phylogenetic relatives. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1,000 replications

Identification of the Strain on traditional and molecular-genetic analysis:

On the basis of the observed traditional morphological and phenotypic characteristics the HS4 was belong to genus *Bacillus*. Biochemical and physiological tests, growth properties and 16S rDNA sequencing indicated that the bacterial isolate obtained from the Lonar Lake was *Bacillus firmus* (Fig. 1).

#### **Effect of pH on activity of enzyme Protease:**

The effect of pH on protease activity of *Bacillus firmus* HS4 was determined by incubating the enzyme in different pH buffers ranging from 7-12 for 10 minutes at 37°C. The enzyme was incubated at 37°C for 10 min at the indicated pH in various buffers, and then the residual proteolytic activity was measured under the standard conditions of enzyme assay. The enzyme was stable over the pH range of 7-12. The protease was active in a wide pH range with optimum activity at pH 10.5 and was stable over a broad pH range 7-12 with retention of more than 60-80% of initial activity. The optimum activities of this enzyme were at pH 10.5 with 26 units/mL. The enzyme activity decreased dramatically at pH 11 (23 Units/mL) and 12 (20 Units/mL). The optimum pH range of alkaline proteases is between pH 9 and 11 (Kumar and Takagi, 1999). Alkaline protease from *Bacillus firmus* HS4 novel feature in the extremozyme which can produce the protease at alkaline pH which was beneficial for industrial status. The similar studies were performed by Kumar *et al.*,<sup>22</sup> protease produced by *Bacillus altitudinis* GVC11 was active between pH 7 and 12.5 but optimum activity was found at 9.5. Further the enzyme was stable up to pH 11- 11.5 and 12 with significant residual activity of 88.44% and 77% respectively. Hamas *et al.*,<sup>23</sup> investigated alkaline protease production by an *Actinomycete* MA1-1 isolated from marine sediments and optimum pH 10.5 was found. *Bacillus* sp UFLA 817CF presented greater enzymatic activity at pH 9.0. In the results obtained from the NB culture medium, with greater activity, this strain maintained about 80% activity at pH 7.0 and over 60% at pH 11.0. This optimum activity at values close to 9.0. *Bacillus* sp. UFLA 817CF isolated from coffee bean grown on cheese whey by Dias *et al.*,<sup>24</sup>. Similar results were reported by Giongo

*et al.*,<sup>25</sup>, when culturing *Bacillus* isolates and by Tremacoldi *et al.*,<sup>26</sup> when culturing *Aspergillus clavatus* (Fig. 2).

#### **Effect of temperature on activity of enzyme protease:**

The casein solution and enzyme dilutions prepared in different buffers of pH ranging between 6 and 12 were used in the reaction mixtures and incubated at 40 °C. For assessing the effect of temperature on enzyme activity, the reaction mixtures were incubated at different temperatures 35°C-100°C and residual activity were determined under enzyme assay condition. The temperature profile of protease from *Bacillus firmus* showed maximal enzymatic activity of 83 Units/mL at 80°C. Subsequently, the enzyme activity progressively decreased from 45-70°C. About 15-49% of activity was retained at 78-90°C. Purified protease of GVC11 was active between 30 and 70 °C with an optimum of 45°C and maximum loss of activity was observed at 65 °C<sup>27</sup>. The protease isolated from *Bacillus Patagoniensis* and proteolytic activity of the crude supernatant was thermo sensitive with a half-life of 2.3 min at 70° C, while high activity was detected at moderate temperatures was studied by Olivera *et al.*,<sup>28</sup>. While in the present studies showed that the protease from *Bacillus firmus* was potential for thermotolerant up the 80°C as shown in Fig. 3.

#### **Effect of Enzyme concentration on activity of protease**

The effects of different enzyme concentrations ranging from 1-12 mL was carried out under assay conditions. The enzyme shows maximum enzymatic activity (40 Units/mL) at 8 mL of enzyme concentration. The activity of protease decreases as the enzyme concentration increase with 12ml. The enzyme retained about 27-95of its activity at enzyme concentration of 1 and 9 mL respectively. There was very less activity at 1 mL of enzyme concentration revealed 11 Units/mL (27%) (Fig. 4).

#### **Effect of substrate concentration on activity of protease**

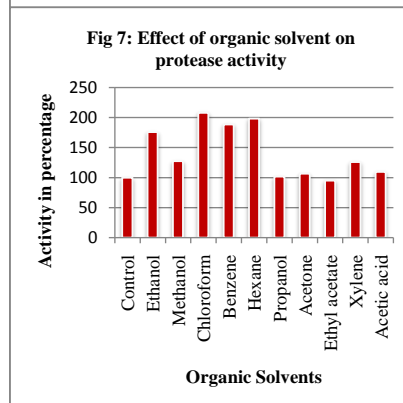
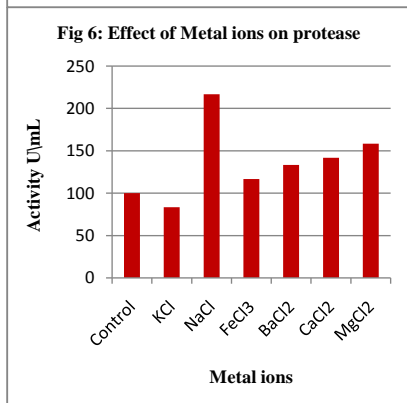
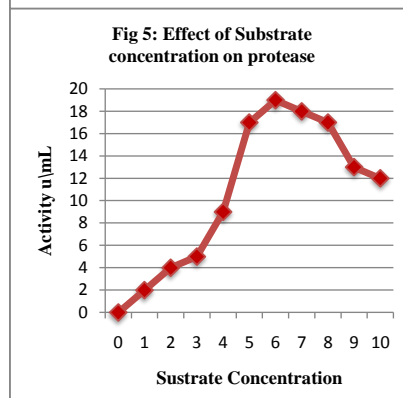
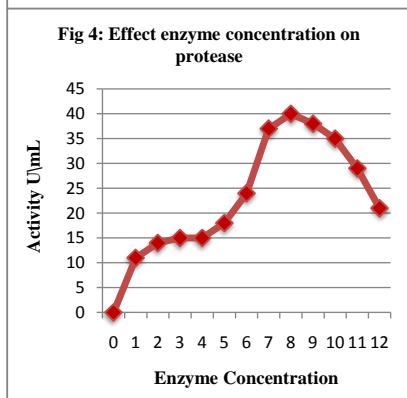
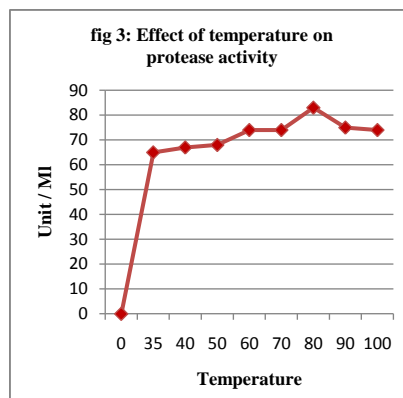
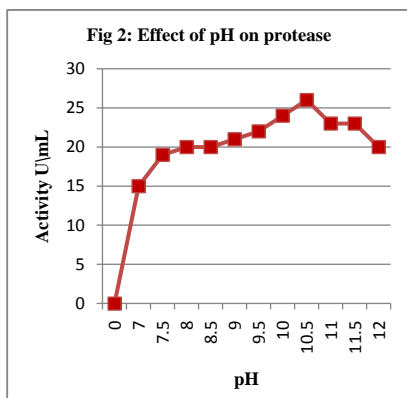
The influence of different concentrations of substrate was assayed ranging from 1-10 mL under constant assay conditions. Substrate

utilization revealed that 94% of substrate was utilized (18Units/mL) but maximum substrate utilization (19Units/mL) occurred at 6 mL of substrate concentration while the 89% activity was found at 5 and 8ml substrate concentration (Fig. 5).

**Influence of different metal ions on protease activity:**

The influence of different metal ions on protease activity of *Bacillus firmus* HS44 was carried out under the standard assay conditions. Metal ions have different effects on activity of protease. The enzyme activity without any additives was taken as 100% (Control). The enzyme activity was enhanced by BaCl<sub>2</sub> and CaCl<sub>2</sub>. However, the protease

activity was inhibited only by KCl. Similar results, inhibitory effect was observed in the presence of KCl, BaCl<sub>2</sub>, CuSO<sub>4</sub>, ZnCl<sub>2</sub> and HgCl<sub>2</sub> inhibited completely the enzyme at 1mM concentrations<sup>29</sup>. The optimum protease activity 19 and 17 Units/mL (158% and 141%) was enhanced in presence of MgCl<sub>2</sub> and CaCl<sub>2</sub> respectively. The enzyme activity was positively influenced by the presence of Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> ions and negatively affected by Fe<sup>2+</sup> and Ba<sup>+2</sup> ions was investigated by Kezia *et al.*,<sup>30</sup>. Metal ion and pH stable protease production using agro-industrial waste was studied by Saxena and Singh<sup>31</sup> (Fig. 6).



### Influence of various organic solvents on protease activity:

The use of organic solvents as reaction media for enzymatic reactions furnish various industrially attractive beneficial features compared to base on response. Despite the advantages, native enzymes almost widely revealed low reactive and stabilities in the presence of organic solvents. The effect of organic solvents on the activity of the protease was determined. The data elucidate that the enzyme was highly enhanced to when ethanol, methanol, chloroform, benzene, hexane used as organic solvent as compared to control which was considered as 100%. The loss of enzyme activity was found in presence of all tested organic solvents except ethyl acetate. Thus, all solvents, except ethyl acetate, have an inhibitory effect on the activity of protease produced by *Bacillus firmus* HS4 while there was no effect on protease in presence of propanol and acetone. The maximum activity was enhanced in presence of chloroform (208%) after that hexane (188%) were revealed. *Saccharophlyspora* species was also found solvent-stable enzyme producer and can be used as biocatalyst for peptide synthesis in the presence of organic solvents<sup>32</sup>. Ruiz *et al.*,<sup>33</sup> was studied on the effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii* (Fig. 7).

### CONCLUSION

In this work, alkaline-adapted protease producing bacterium was isolated and identified as *Bacillus firmus* HS4 on the basis of its phenotypes, biochemical testing and 16S rDNA sequencing. The different characteristics toward the sensitivity to organic solvent, metal ions revealed the unique features of protease. The enzyme described in the present report emphasizing several features like to those found in other protease enzymes, including, the alkaline pH profile and thermal stability closely resembled to features reflected in alkalo-thermophilic organisms. In the case of enzyme characterization, the amino acid

sequence of an enzyme will be determined and kinetic studies done with a purified enzyme.

### Acknowledgements

The authors would like thanks to Dr. Yogesh souche, National Centre For Cell Science, Pune for helping us in 16S rDNA sequencing

### REFERENCES

1. Kanekar, P.P., Nilegaonkar S.S., Sarnaik S.S and Kelkar, Process for production of protease using deoiled soybean cake and alkaliphilic bacteria *Arthobacter ramosus* and *Bacillus alkalophilus*. *Indian Patent*. (188072). (1997).
2. Yang, J.K., Shih L., Tzeng Y.M., Wang S.L., Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enz Microbial Technol* **26(5)**: 406-13 (2000).
3. Sumantha, A., Sandhya, C., Szakacs, G., Soccol, C.R. Pandey, A., Production and partial purification of a neutral metalloprotease by fungal mixed substrate fermentation. *Food Technol. Biotechnol.*, **43(4)**: 313-319. (2005).
4. Malathi, S., Chakraborty R., Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent. *Appl environ microbial*, **57(3)**: 712-716 (1991).
5. Alessandro, R., Silvia O., Adriano B., Dehairing activity of extracellular proteases produced by keratinolytic bacteria. *J Chem Technol Biotechnol* **78**: 855–859 (2003)
6. Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T., Approach towards zero discharge tanning: role of concentration on the development of eco-friendly liming–reliming processes. *Journal of cleaner production*, **11(1)**: 79-90 (2003).
7. Purushotham, H., Malathi, S., Rao, P.V., Rai, C.L., Dehairing enzyme by solid state fermentation. *Journal of the Society of Leather Technologists and Chemists*, **80(2)**: 52- (1996).
8. Hemke V.M., Joshi S.S., Fule N.B.,

- Dhundale V.R., Tambekar D.H., Phenetic diversity of alkaliphilic protease producing bacteria from alkaliphilic environment. *Indian J Sci Res* **10(1)**: 47-52 (2015).
9. Dhundale VR and Hemke VM. Phylogenetic analysis of Bacilli from haloalkaline Lonar Soda Crater. *Int J Pharm Bio Sci* **6(4)**: (B) 279 – 290, (2015).
  10. Joshi, A.A., Kanekar, P.P., Kelkar, A.S., Shouche, Y.S., Vani, A.A., Borgave, S.B. and Sarnaik, S.S., Cultivable bacterial diversity of alkaline Lonar Lake, India. *Microbial Ecology*, **55(2)**: 163-172. (2008).
  11. Tambekar D.H., Dhundale V. R., Studies on the physiological and cultural diversity of bacilli characterized from Lonar lake (MS) India. *Biosci Discov* **3(1)**: 34-39 (2012)
  12. Hashim, S.O., Delgado, O., Hatti-Kaul, R., Mulaa, F.J. Mattiasson, B., Starch hydrolysing *Bacillus halodurans* isolates from a Kenyan soda lake. *Biotechnology letters*, **26(10)**: 823-828 (2004).
  13. Martins, R.F., Davids, W., Al-Soud, W., Levander, F., Rådström, P. and Hatti-Kaul, R., Starch-hydrolyzing bacteria from Ethiopian soda lakes. *Extremophiles*, **5(2)**: 135-144, (2001).
  14. Dhundale, V.R., More V.R., Nagarkar R.D., Hemke V.M., Tambekar D.H., Isolation and characterization of a novel amylase from *Bacillus pseudofirmus* DW4(1). *Indian J life science*. **4(1)**: 69-76 (2014).
  15. Tambekar D.H. Dhundale V.R., Multienzyme producing haloalkaliphilic bacteria from Lonar Lake. *Int J Pharm Bio Sci*. **4(3)**: (B) 1271 – 1276(2013).
  16. Sambrook J, Fritsch EF, Maniatis T., Molecular Cloning: a Laboratory Manual, 2<sup>nd</sup> Edn. Cold Spring Harbor, New York (1989)
  17. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Short protocols in molecular biology. 5th Edn., Vol. II, Wiley, New York (1992).
  18. Kumar, S., Tamura, K., Nei M., MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in bioinformatics*, **5(2)**: 150-163 (2004).
  19. Dhundale, V.R., Nagarkar R.D., More V.R., Hemke V.M., Partial characterization and optimization of alkaline amylase production from *Bacillus lehensis* isolated from alkaline saline Lonar Lake BW32. *Int J Advanced Biotech Res* **53**: 1531–1536 (2014).
  20. Gessesse, A., Gashe, B.A., Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake. *Biotechnology letters*, **19(5)**: 479(1997).
  21. Hemke, V.M, Dhundale V.R., Characterization of Protease From Alkali-tolerant *Bacillus sp.* DS2 Isolated From Lonar Soda Lake, India. *Int J Pharma Biosci* **8(3)**: (B) 465 -470 (2017).
  22. Kumar, E.V., Srijana, M., Kumar, K.K., Harikrishna, N., Reddy, G., A novel serine alkaline protease from *Bacillus altitudinis* GVC11 and its application as a dehairing agent. *Bioprocess Biosystems Engineering*, **34(4)**: 403-409(2011).
  23. Hameş-Kocabaş, EE., Uzel A., Alkaline protease production by an actinomycete MA1-1 isolated from marine sediments. *Annals Microbiology*. **1;57(1)**: 71-75 (2007).
  24. Dias, D.R., Vilela, D.M., Silvestre, M.P.C., Schwan, R.F., Alkaline protease from *Bacillus sp.* isolated from coffee bean grown on cheese whey. *World J.Microbiol. Biotechnol*, **24(10)**: 2027-2034 (2008).
  25. Giongo, J.L., Lucas, F.S., Casarin, F., Heeb, P. Brandelli, A., Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable dehairing activity. *World J. Microbiol. Biotechnol*, **23(3)**: 375-382 (2007).
  26. Tremacoldi, C.R., Monti, R., Selistre-De-Araújo, H.S., Carmona E.C., Purification and properties of an alkaline protease of *Aspergillus clavatus*. *World J. Microbiol.*



- Biotechnol*, **23(2)**: 295-299 (2007).
27. Kumar, C.G. Takagi H., Microbial alkaline protease; from a bio industrial view point. *Biotechnol Adv* **17**: 561–594 (1999).
  28. Olivera, N., Sequeiros, C., Siñeriz, F. Breccia, J.D., Characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis*. *World J. Microbiol Biotechnol*, **22(7)**: 737-743. (2006).
  29. Usharani, B., Muthuraj, M., Production and characterization of protease enzyme from *Bacillus laterosporus*. *African journal of microbiology research*, **4(11)**: 1057-1063 (2010).
  30. Kezia, D., Chandrakala G., Prasanthi V., Naidu S. Rao M.N., Influence of different factors on production of purified protease by *Bacillus subtilis* DKMNR. *Int J Pharma Bio Sci* **1;2**: 73-85 (2011).
  31. Saxena, R., Singh, R.. Metal ion and pH stable protease production using Agro-industrial waste. *Journal of Ecobiotechnology*, **2(4)**: (2010).
  32. Raut, G.R., Chakraborty S., Chopade B.A., Kokare C.R., Isolation and characterization of organic solvent stable protease from alkaliphilic marine Saccharopolyspora species. **42(1)**: 131-138 (2013).
  33. Ruiz, D.M., De Castro, R.E., Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii*. *J Industrial Microbiol Biotechnol.*, **34(2)**: 111-115 (2007).