OriginalResearch Article Microbiology

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

CHARACTERIZATION OF PROTEASE FROM ALKALI-TOLERANT *BACILLUS* SP. DS2 ISOLATED FROM LONAR SODA LAKE, INDIA

VIJAYSHREE M. HEMKE[1] AND VISHAL R. DHUNDALE[2]

[1]*Jijamata Mahavidyalay Buldhana, Maharashtra,India.*
[2]*New Arts Commerce and Science College Ahmednagar, Maharashtra.India.*

ABSTRACT

The among several strains of moderately alkalophilic bacteria that were isolated from Lonar lake, *Bacillus* sp. DS2 was selected as the best producer of extracellular protease and was used for further studies. Phenotypic and biochemical classification analysis placed DS2 in the genus *Bacillus*. The bacterium was Gram positive, aerobic, motile, sporulating and rod-shaped bacterium and optimal growth temperature was found to be 37°C. The cells were able to grow well at a pH range of 8-10 which indicate that *Bacillus* sp DS2 was alkalophilic. The protease exhibited its optimal activity at 90°C and optimum pH 10.5. The enzyme activity was enhanced by Ethanol, Methanol, Chloroform, Benzene, Hexane, Propanol, Acetone, Xylene and Acetic acid. However, the protease activity was inhibited by Ethyl acetate. The optimum protease activity was enhanced in presence Chloroform (208.07%). The enzyme activity has enhanced by $FeCl_3$, $BaCl_2$ and $CaCl_2$ and optimum enzyme activity enhanced by $MgCl_2$ indicating it was a metalloenzyme with a potential to be a candidate for the biotechnological potential.

KEY WORDS: *Lonar Lake, Alkaliphiles, Halophiles, Bacillus, Protease, Metalloenzyme,*

VISHAL R. DHUNDALE
New Arts Commerce and Science College Ahmednagar, Maharashtra.India.

Received on : 19-04-2017
Revised and Accepted on : 05-06-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.b465-470

INTRODUCTION

Generally the most concentrated and widespread occurrence of the microorganism is observed in moderate environments. It has been believed that there are extreme environment on the earth which was thought to be the controversial about the existence of life. In this habitat, environmental condition such as pH, temperature and salinity concentration are extremely high or low. Extreme environments are a populated groups of organisms that are specifically adapted to these extreme conditions and these types of microorganism are usually referred as alkaliphiles, halophiles and thermophiles which are reflected the extreme environment.[1] The high alkaline condition which accesses naturally or artificially on the basis of extreme condition is referred to as alkaline environments and bacteria survive in this environment is called as alkaliphiles. In the naturally occurring alkaline environments, soda Lake are the most stable with pH values generally higher than 10 and occasionally reaching at 12[2]. These alkaline environments are naturally made by a combination of geological, geographical and climatic conditions[3]. At the time of alkalinity formation, other salts also accumulate, giving rise to haloalkaliphilic environment in which the native microflora is subjected to a number of extreme ecological pressure[4-5]. Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salt. Among halophiles microorganisms involves a variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic and heterotrophic bacteria, photosynthetic and heterotrophic eukaryotes. Halophiles can be loosely classified as slightly, moderately or extremely halophilic microorganism depending on their requirement for NaCl[6]. Extermophilic microorganisms, particularly *Bacillus* species, are attracted much attention in the past few decades because of their ability to produce extracellular enzymes that are active and stable at high alkaline and thermo stable condition. The unusual properties of these enzymes offer a potential opportunity for their utilization in processes which demanding such extreme conditions[7]. It can be assumed safely that the extremophilic *Bacillus* derived from the soda Lake environment will have a great quantity and quality, yet to be explored for biotechnological application. The awareness of existing microbe is ever increasing and the range of microbial diversity has proven to be remarkable. Microbial communities in natural alkaline condition such as soda Lake and hot spring have attracted the attention existing biotechnological useful in production of enzyme and metabolites from these organism. In a sense, the extremophiles, are commonly a specialist because of they have to be able to thrive under such harsh conditions. Several enzymes are largely used in industrial fields such as detergent, food, and feed production, leather and textile processing, pharmaceutical production, diagnostics, waste management and molecular biology.[8-9]The microbial diversity of saline lakes has been studied primararily acting on the isolation and characterization of organisms with biotechnological potential[2,9]. Martin *et al.*[10]were found both alkali tolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as the microbial species found in Ethiopian soda lake microbial population and known for being good enzyme producers. As far as Indian soda lakes are concerned, a culture-dependent approach has not been yet applied to analyze bacterial diversity and alkaliphilic protease producing microorganisms isolated from Lonar Lake[11]. In view of the above facts, the present study focused on the optimization of alkaline protease production from a haloalkaliphilic bacterial strain isolated from Indian Soda Lake.

MATERIALS AND METHODS

Isolation of Alkaliphiles

The 1.0 g 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^{-7} dilutions. One ml of each diluted sample was spread by spread plate technique into petri plates containing Horikoshii medium and nutrient agar medium (A, B, C and D) and inoculated at 37°C for 24 h[12].

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^{o}C for 48 hrs and observed for zones of clearance, indicating proteolytic activities.

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^{o}C.

Preparation of crude enzyme extracts

The 100 ml 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.

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_2CO_3 and 1 ml of Folin-Ciacalteau 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* species 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_4) 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 the 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

Table 1
Morphological and biochemical characters of *Bacillus* sp. DS-2

Sr. No.	Test	Observation
1	Isolation Media	D
2	Color	White
3	Shape of Colony	Circular
4	Size of Colony	1 mm
5	Elevation	Convex
6	Edge	Entire
7	Gram Reaction	Positive
8	Shape	Long rod
9	Spore Reaction	+
10	Motility	+
11	Catalase	+
12	Oxidase	+
13	Glucose	+
14	Arabinose	+
15	Mannitol	-
16	Xylose	+
17	Sucrose	-
18	Maltose	-
19	Fructose	+
+ Positive and – Negative		

Screening of bacterial isolates, identifications and characterization of bacterial cultures

The bacterial isolates were isolated from water and sediment samples of hyperalkaline saline Lonar soda lake[13-17]. In the present study, totally one hundred and fourteen isolates obtained in the isolation exercise from different sediment samples and water samples of Lonar Lake. Out of one hundred and fourteen, thirty two bacterial cultures were found with proteolytic activity. All the bacterial strains were found alkaliphilic (7-12). Similar method was employed to observe the qualitative assay of extracellular protease production by *Bacillus altitudinis* GVC11[18]. Bacteria are the most prevailing alkaline protease producers with the genus *Bacillus* being the most prominent source[19-21].The selected isolates were studied morphologically and biochemically. According to Bergey's Manual of Systematic Bacteriology, the isolates were identified as *Bacillus* sp. All the bacterial cultures were found gram positive and spore forming bacilli. All of the alkaliphiles were identified as different species of the genus *Bacillus* on the basis of morphological and biochemical characterizations, which are known to produce a wide variety of enzymes with tolerance to thermal and alkaline conditions[22]. Out of these thirty two, one bacterial isolate was selected for the further production and characterizations on the basis of their maximum proteolytic activity of *Bacillus* sp. DS-2.

Enzyme Assay

As per the protocol as mentioned above, the assay was estimated and standard graph of tyrosine was prepared. The graph of absorbance versus concentration of maltose formed was plotted. One enzyme unit (unit/ml) is defined as the amount of enzyme which releases 1µ mole of maltose.

Effect of pH on activity of enzyme protease

The effect of pH on protease activity of *Bacillus* sp. DS-2 was determined by incubating the enzyme in different pH buffers ranging from 7.0-12.0 for 10 minutes at 37° C. The optimum pH of *Bacillus* sp. DS-2 protease was found to be 10.5. The enzyme was active between pH ranging from 10-12. Protease activity was relatively low at pH 7, 8 and 9; the enzyme has relative activities found to be 58.82%, 64.71% and 76.47% respectively. The optimum activity of this enzyme was at pH 10.5 with 2.83 Units/ml, which considered as 100%. Earlier studies have shown that proteases were active up to the pH of 5-10. It is comparatively less and the enzyme of *Bacillus* sp. DS-2 strain has still wider ranges of tolerances than the previously reported for proteases. Previous studies states that, *Bacillus* strains were able to produce high level of enzyme at pH 10 and pH 10.5.[23].Alkaline protease from *Bacillus* sp. isolated from coffee bean and grown on cheese whey was employed by Dias *et al.,*[24]. *Bacillus* sp. UFLA 817CF and *Bacillus patagoniensis* showed highest enzymatic activity at pH 9.0[25]. Similar results were obtained alkaline protease production from *Actinomycetes* MA1-1 isolated from marine sediments and optimum pH 9[26].

Effect of temperature on activity of enzyme protease

Influence of temperature on *Bacillus* sp. DS-2 protease activity was observed by incubating the enzyme at different temperature ranging from 35-100°C and residual activity were determined under enzyme assay condition. The optimum activity of enzyme was taken as 100%. The temperature profile of protease activity of *Bacillus* sp. DS-2 were showed maximal enzymatic activity of 1 Unit/ml (100%) at 90°C, which indicated that the enzyme was thermo stable at high temperature. The protease retained more than 75% of the highest activity between 70-80°C. Subsequently, the enzyme activity progressively decreased at 100°C. While the proteolytic activity of the crude supernatant was thermo sensitive at 80°C and high activity was detected at 90°C. Similar characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis* and *Bacillus licheniformis* strain BA17 studied by Olivera *et al.,* and Ates *et al*. respectively[25,27].

Effect of substrate concentration on activity of enzyme protease

The influence of different concentrations of substrate was assayed ranging from 0.5-9 ml under constant assay conditions. The activity at 7.5 ml of substrate concentration was 2.33 Units/ml and it was considered as 100%. However, the activity was retained more than 75% with the substrate concentration from 4.5-7 ml. There was very less activity at 0.5 ml of substrate concentration (0.25 Units/ml).

Effect of enzyme concentration on activity of enzyme protease

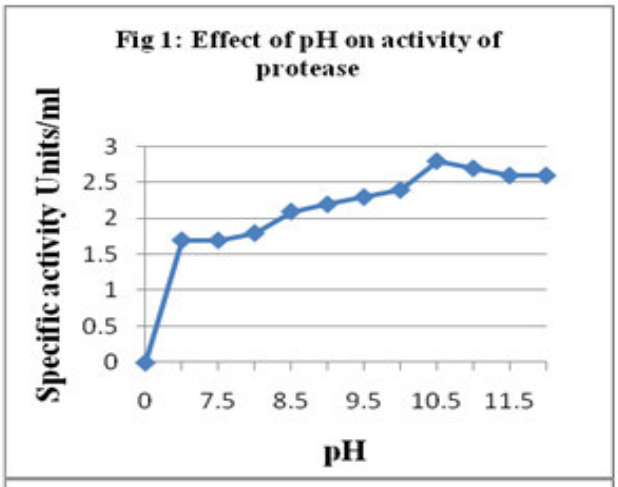
The effects of different enzyme concentrations ranging from 0.5-9 ml was carried out under assay conditions. The enzyme shows maximum enzymatic activity (6.25 Units/ml) at 7.5 ml of enzyme concentration and it was considered as 100%. The activity of protease decreases as the enzyme concentration increases more than 8 ml. There was very less activity at 0.5 ml of enzyme concentration (0.17 Units/ml).

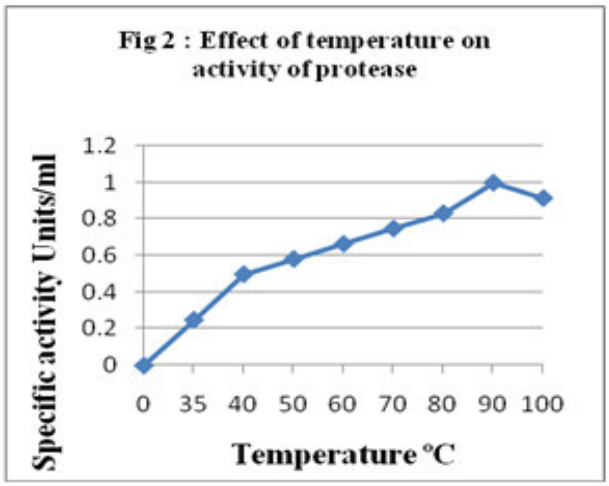
Influence of different metal ions on activity of enzyme protease

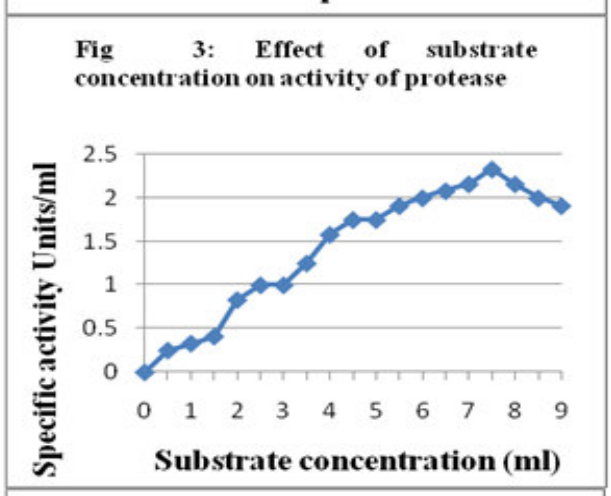
The influence of different metal ions on activity of *Bacillus*sp. DS-2 protease was carried out under the assay conditions. Metal ions have different effects on activity of protease. The enzyme activity without any additives was taken as 100%. The enzyme activity was enhanced by KCl, NaCl, $FeCl_3$, $BaCl_2$, $CaCl_2$ and $MgCl_2$. The optimum protease activity (341.67%) was enhanced in presence of $MgCl_2$.The enzyme activity has enhanced by KCL, NaCl, $FeCl_3$,$BaCl_2$and $CaCl_2$, these activity indicating it was a metalloenzyme. The dissimiller results obtained from bacterium L21, protease enzyme retained 93% of its activity after 1 h of incubation with EDTA and was completely inhibited by 0.01 M PMSF[23].

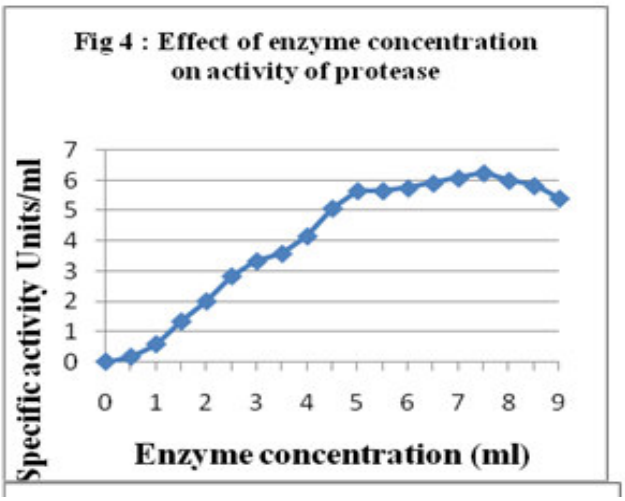
Influence of various organic solvents on activity of enzyme protease

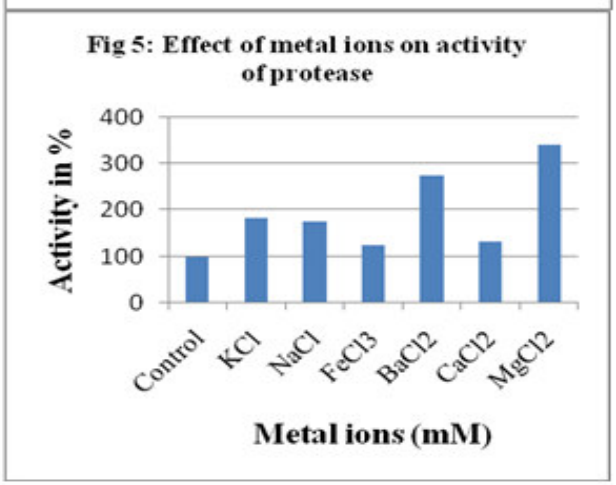
The effect of organic solvents on the activity of *Bacillus*sp. DS-2 protease was determined. Organic solvents have different effects on activity of protease. The enzyme activity without any additives was taken as 100%. The enzyme activity was enhanced by Ethanol, Methanol, Chloroform, Benzene, Hexane, Propanol, Acetone, Xylene and Acetic acid. However, the protease activity was inhibited by Ethyl acetate. The optimum protease activity (208.07%) was enhanced in presence Chloroform.

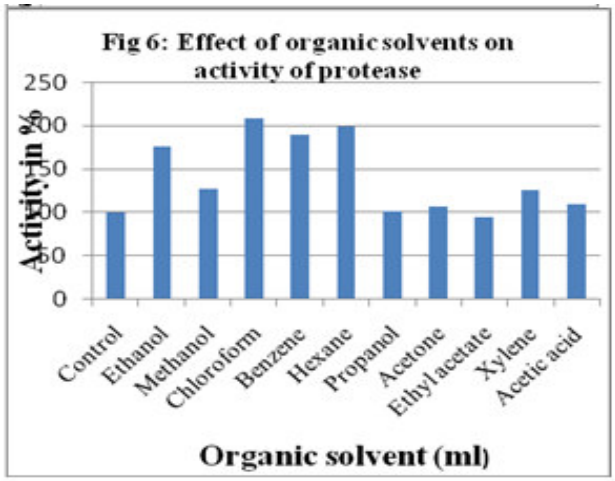












CONCLUSION

In the present studies, protease isolated from the *Bacillus sp DS2* was tolerant and stable in the presence of extreme conditions such as pH (optimum pH 10.5), temperature (90°C) and high salt concentrations and organic solvent tolerant compared to other bacterial protease. The enzyme activity has enhanced by $FeCl_3$, $BaCl_2$ and $CaCl_2$ and optimum enhanced by $MgCl_2$ indicating it was a metalloenzyme with a potential to be a candidate for the biotechnological potential.

CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Horikoshi K, Akiba T. Alkalophilic microorganisms: a new microbial world. Springer, New York. 1982 10: 3540109242 ISBN 13: 9783540109242
2. Jones BE, Grant WD, Duckworth AW, Owenson GG. Microbial diversity of soda lakes. Extremophiles. 1998 2:191–200.
3. Grant WD, Tindall BJ. The isolation of alkalophilic bacteria. In: Gould GW, Corry ICL (Eds.) Microbial Growth and Survival in Extremes of Environment. Academic Press, London, 1980 27–36.
4. Joshi AA, Kanckar PP, Kelkar AS, Shouche YS, Wani AA, Bogave SB, Sarnaik SS. Cultivable bacterial diversity of an alkaline Lonar Lake, India. Microb Ecol. 2007 55:163–172, DOI 10.1007/s00248-007-9264-8.
5. Wani AA, Surakashi VP, Siddharth J, Raghwan RW, Patole MS, Ranade D., Shouche YS, Molecular analyses of Microbial diversity associated with the Lonar soda Lake in India: An impact crater in a basalt area. Res in Microbiol. 2006 157(10):928-937.
6. DasSarma S, Arora P. (2001). Halophiles Encylopediaof life science, 2001 Nature Publishing Group / http://www.openwetware.org/images/2/2d/Halophiles.pdf
7. Takami H, Kobata K, Nagahama T, Kobayashi H, Inoue A, Horikoshi K. Biodiversity in deep sea sites located near the south part of Japan. Extremophiles. 1999 3:97–102.
8. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y. Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures. Extremophiles. 1998 2:185–190.
9. Horikoshi K. Alkaliphiles: some applications of their products for biotechnology. MicrobiolMolBiol Rev. 1999 63:735–750.
10. Martins RF, Davids W, Abu Al-Soud W, Levander F, Radstrom P, Hatti-Kaul R. Starch hydrolyzing bacteria from Ethiopian soda lakes. Extremophiles. 20015: 135–144.
11. Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Wani AA, Borgave SB, Sarnaik. Cultivable Bacterial Diversity of Alkaline Lonar Lake, India. Microb Ecol. 2007 55:163–172.
12. Tambekar DH, Dhundale VR.Studies on the physiological and cultural diversity of *Bacilli* characterized from Lonar Lake. Biosci Discovery. 2012 3(1): 34-39.
13. Hemke VM, Joshi SS, Fule NB, Dhundale VR, Tambekar DH.Phenetic diversity of alkaliphilic protease producing bacteria from alkaliphilic environment. 20145 10(1): 47-52.
14. Dhundale VR. Characterization of organic solvent tolerant halo-alkali- thermostable amylase from *Bacillus cereus* OCW3(1). Int J PharmaBiosci . 2015 6(4): (B) 457 – 69.
15. Dhundale VR, Hemke VM.Phylogenetic analysis of Bacilli from haloalkalineLonar Soda Crater. Int J Pharm Bio Sci. 2015 6(4): (B) 279 – 90.
16. Dhundale VR, More VR, Nagarkar RD, Hemke VM. HaloalkaliphilicBacillus *flexus*AW3(2): potential for biotechnological applications. Int J Pharm Bio Sci. 2014 141: 1745–61.
17. Dhundale VR, More VR, Nagarkar RD, Hemke VM, Tambekar DH. Isolation and characterization of a novel amylase from *Bacillus pseudofirmus* DW4(1). Indian J Life sci 2014 4(1): 69-76.
18. Kumar EV, Srijana M, Kumar KK, Harikrishna N, Reddy G. A novel serine alkaline protease from *Bacillus altitudinis* GVC11 and its application as a dehairing agent. Bioprocess Biosyst Eng. DOI 10.1007/s00449-010-0483-x
19. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 2002 59:15-32.
20. Ban OH, Han SS, Lee YN. Identification of a potent protease-producing bacterial isolate, *Bacillusamyloliquefaciens*CMB01. Ann. Microbiol. 2003 53: 95-103.
21. Rahman RNZR, Basrı M, Salleh AB. Thermostable alkaline protease from *Bacillusstearothermophilus* F1; nutitional factors affecting protease production. Ann. Microbiol. 2003 53: 199-221.
22. Nthangeni M, Patterton, van Tonder A, Vergeer W, Litthauer D. Over-expression and properties of a purified recombinant *Bacillus licheniformis*lipase: a comparative report on *Bacillus* lipases. Enzyme Microb. Technol. 2001 28: 705–712.
23. Genckal H, Tari C. Alkaline protease production from alkalophilic*Bacillus* sp. isolated from natural habitats. Enzyme and Microbial Technology 2006 39:703–710.
24. Dias DR, Vilela DM, Silvestre MPC, Schwan RF. Alkaline protease from *Bacillus* sp. isolated from coffee beangrown on cheese whey. World J MicrobiolBiotechnol2008 24:2027–2034. DOI 10.1007/s11274-008-9706-6
25. Olivera N, Sequeiros C, Sineriz F, Breccia JD. Characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis.* World J MicrobiolBiotechnol2006 22:737–743. DOI 10.1007/s11274-005-9099-8
26. Hames- Kocabas EE, Uzel A. Alkaline protease production by an *actinomycete* MA1-1 isolated from marine sediments. AnnalsMicrobiol, 2007 57 (1) 71-75.
27. Ates O, Oner TE, Arikan B, Denizcl AA, Kazani D. Isolation and identification of alkaline protease producer halotolerant. *Bacillus licheniformis* strain BA17. Annals of Microbiology 2007 57 (3) 369-375.

Reviewers of this article

Dr.Ashok pawarr

Assistant professor
Department of microbiology

Asso.Prof.Dr. R. Usha, MSc, M.Phil, Ph.D.

Associate Professor, Department of Microbiology,
Karpagam University, Eachanari (PO),
Coimbatore - 641 021, Tamil Nadu, India.

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript