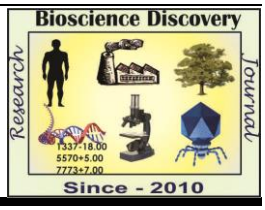
Bioscience Discovery, 8(2): 255-260, April - 2017

Print & Online, Open Access, Research Journal Available on http://jbsd.in
ISSN: 2229-3469 (Print); ISSN: 2231-024X (Online)
Research Article



Extracellular thermo-alkalophilic proteolytic activities of the *Bacillus aerophilus*: Detection and preliminary characterization

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Article Info

Received: 03-03-2017,
Revised: 26-03-2017,
Accepted: 29-03-2017

Keywords:
Bacillus, Haloalkaliphiles, Hypersaline environment, Lonar Lake, Protease.

Abstract

The Lonar Crater popularly called as the Lonar Soda Lake is situated in the Buldhana District of the Maharashtra State, India which is occupied by saline water and harbors various unidentified, unique haloalkaliphilic bacterial species which produces thermo-alkaliphilic enzyme. The attempt was made to isolate, protease producing bacterial strain from this hyper saline environment. Total six bacterial cultures were isolated by using Horikoshi (A, B, C and D) enrichment medium. Out of six, one bacterial strain was selected on the basis of its proteolytic activity and identified based on culturally, morphologically, biochemically and 16S rDNA sequencing as *Bacillus aerophilus.* This isolated bacillus spp was found to produce alkaline protease optimally at pH 10 and optimum activity at 80°C. The best substrate concentration 1.9 µg/ mL and enzyme concentration 1.72 µg/ mL were recorded. The present studies showed that the *Bacillus aerophilus* have ability to produced protease which can be exploited for biotechnological potential and medical use.

INTRODUCTION

In India the Lonar Crater, popularly called as the Lonar Soda Lake is situated in the Buldhana District of the Maharashtra State, which is occupied by saline water and harbors various unidentified, unique haloalkaliphilic bacterial species which produces thermo-alkaliphilic enzyme such as protease, amylase and lipase (Tambekar and Tambekar, 2012). Based on geological studies, it is postulated that the lake originated as a meteorite impact crater around 50-60 thousand years ago (Fredriksson *et al*., 1973). Lonar Lake occupied by saline water which was formed by meteoritic impact on basaltic rock and it has been well known as an inland saline lake with a considerable amount of sodium carbonate and chloride (Kanekar *et al.*, 2000). Extracellular enzyme like amylase, lipase, protease and celluloses producing bacterial strains were isolated from water and sediment samples of alkaline Lonar Lake (Joshi *et al*., 2007).

Thermostable protease which tolerates high temperature and salinity are useful for bioengineering and biotechnological application. Among the various type of proteases, alkaline proteases, have extensive applications in industries like detergent, pharmaceutical, food and leather industry where temperature and alkalinity are high (Tambekar and Dhundale, 2012). Protease is also been used in many other fields, such as clinical, medicinal, and analytical chemistries, as well as their widespread relevance in starch saccharification (Shanmughapriya, 2009).

The enzyme has better resistance to alkali and some other denaturing chemicals in the reaction mixture and has a higher affinity towards proteinaceous substrates. It is also thermostable organism growing in naturally alkaline habitats may have proteases with special characteristics (Shafee *et al.,* 2005). Very less study has been done on protease from *Bacilli* of Lonar Lake which can withstand at high temperature as well as high pH. Alkaline protease producing bacteria are of great importance in detergent and textile industry due to their high thermostability and pH stability and most important industrial enzymes, accounting for about 60% of total enzyme market (Borsodi *et al.,* 2005). As there is large demand of protease, isolation and production of protease enzyme is most important to fulfill this demand. Therefore, attempt was made to isolate new species of *bacillus* which can produce good quality of protease (Tambekar and Tambekar, 2013: Sawant and Nagendran, 2014).

MATERIALS AND METHODS

Collection of samples: Total 12 (sediment, matt and water) samples were collected in sterilized plastic ziplock bags and plastic bottles respectively in August 2016 from alkaline Lonar Lake and were transferred to laboratory for isolation and identification of bacteria followed by their screening for proteolytic activity.

Enrichment of bacterial isolate: About 1g of each sediment and matt sample was transferred to 100mL sterilized distilled water in 250 mL conical flask and agitated (100 rpm) at 37^0C for 15 min on rotary shaker. The sample was then heated at 80^0C for 10 min to destroy all the vegetative microbial cells. Then the suspension was diluted to 10^7 dilutions and 1mL of each diluted sample was inoculated in Horikoshi medium (A, B, C and D) and incubated at 37^0C for 72h and four time repeated sub culturing was made in the same medium. After enrichment, it was inoculated on Nutrient agar plates (pH 10) and incubated at 37^0C for 24h for isolation of pure bacterial culture.

Screening and Identification of bacterial isolate: Individual and well isolated bacterial colonies were screened for proteolytic activity on skimmed milk agar medium plates. The inoculated plates were incubated at 37°C for 48h, after incubation, the zone of casein hydrolysis was observed for proteolytic activity of isolates. Then bacterial identifications was performed by morphological, cultural and biochemical characteristics. The selected strain was then analyzed by 16S rRNA gene sequencing.

Preparation of crude enzyme extracts: The 100mL Skimmed milk broth medium was inoculated with culture and incubated for 48h at 37°C. After 48h incubation, the broth was centrifuged at 5000 rpm for 15 min. The supernatant served as crude enzyme source.

Optimization of crude protease enzyme: The standard graph of tyrosine was prepared by using different concentration of standard tyrosine (20 µg/mL) into a series of test tubes and add 1mL FC reagent and 2mL of Na_2CO_3 in all tubes and then incubate in boiling water bath for 5 min after incubation, add 6mL distilled water and observe the OD at 650 nm.

Characterization of Protease:

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 to 12.

Effect of temperature on alkaline protease activity: The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture at different temperature ranging from 40°C to 90°C.

Effect of substrate on alkaline protease activity: The effect of substrate concentration on alkaline protease activity was determined by incubating the reaction mixture with different substrate concentration, ranging from 0.5 mg/mL to 4 mg/mL.

Effect of enzyme on alkaline protease activity: The effect of enzyme concentration on alkaline protease activity was determined by incubating the reaction mixture at different enzyme concentration ranging from 0.5mL to 4mL.

RESULTS AND DISCUSSION

In the present study a total of 6 bacterial cultures were isolated from water, matt and sediment sample of Lonar Lake. Then these cultures were inoculated on alkaline skimmed milk agar at pH 10 for studying their proteolytic activity. Out of six, three showed proteolytic activity. Out of three, one bacterial culture (DHT23) was found good protease producer. This isolate was further characterized on basis of morphological, cultural, biochemical (Table: 1) and 16S rDNA gene sequencing Table 2. Morphologically DHT 23 is single cell arranged with rod shape, gram positive in nature and is highly motile. Biochemically it is citrate and malonate utilizer and produce acid from glucose, dextrose, sucrose, mannose and arginine.

It is analyzed based on 16S rDNA sequencing identified as *Bacillus aerophilus*. According to Singh *et al.*, (2006) bacterial strains by using enrichment techniques at 20% (w/v) NaCl and pH 10 can be isolated and this isolates exhibited diversity towards gram's reaction, colony and cell morphology. Optimum protease activity and stability was recorded at 10% salt and pH 9-9.5. The activity and stability of the alkaline protease in a broader range of pH and salt would definitely make this enzyme and important candidate for various industrial applications.

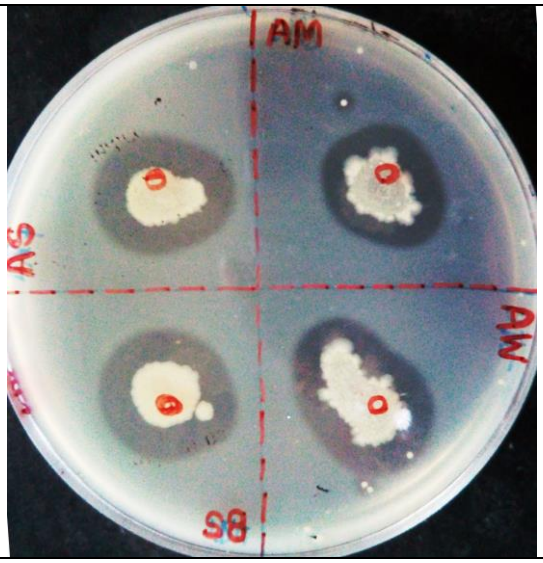
Table: 1. Cultural, morphological and biochemical characteristics of protease producing *Bacillus aerophilus*

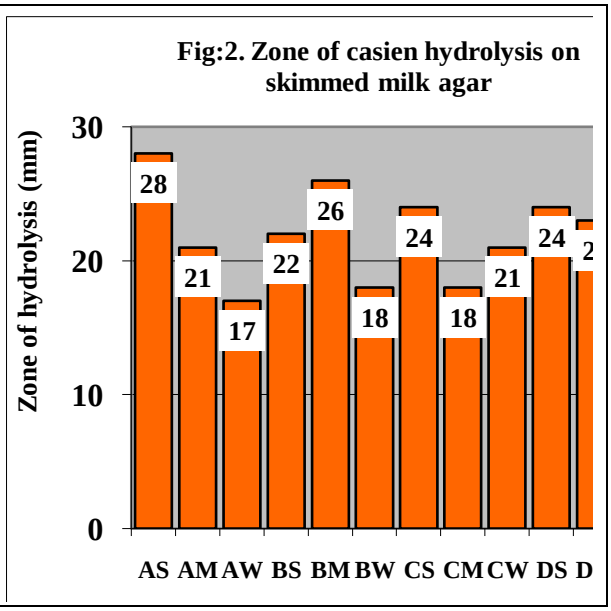
Gram character	+	Glucose	+	α-Methyl-D-glucoside	-
Shape of Bacteria	LR	Dextrose	+	Rhamnose	-
Arrangement of Cell	Single	Galactose	-	Cellobiose	-
Spore	+	Raffinose	-	Melezitose	-
Motility	+	Trehalose	-	α-Methyl-D-mannoside	-
Catalase	-	Melibiose	-	Xylitol	-
Oxidase	-	Sucrose	+	ONPG	-
Voges Proskauer	-	L- Arabinose	-	Esculin hydrolysis	-
Citrate utilization	+	Mannose	+	D-Arabinose	-
Nitrate reduction	-	Inositol	-	Malonate Utilization	+
Arginine	+	Sorbitol	-	Sorbose	-
Lactose	-	Mannitol	-	Inulin	-
Xylose	-	Adonitol	-	Sodium gluconate	-
Maltose	-	Arabitol	-	Glycerol	-
Fructose	-	Erythritol	-	Salicin	-

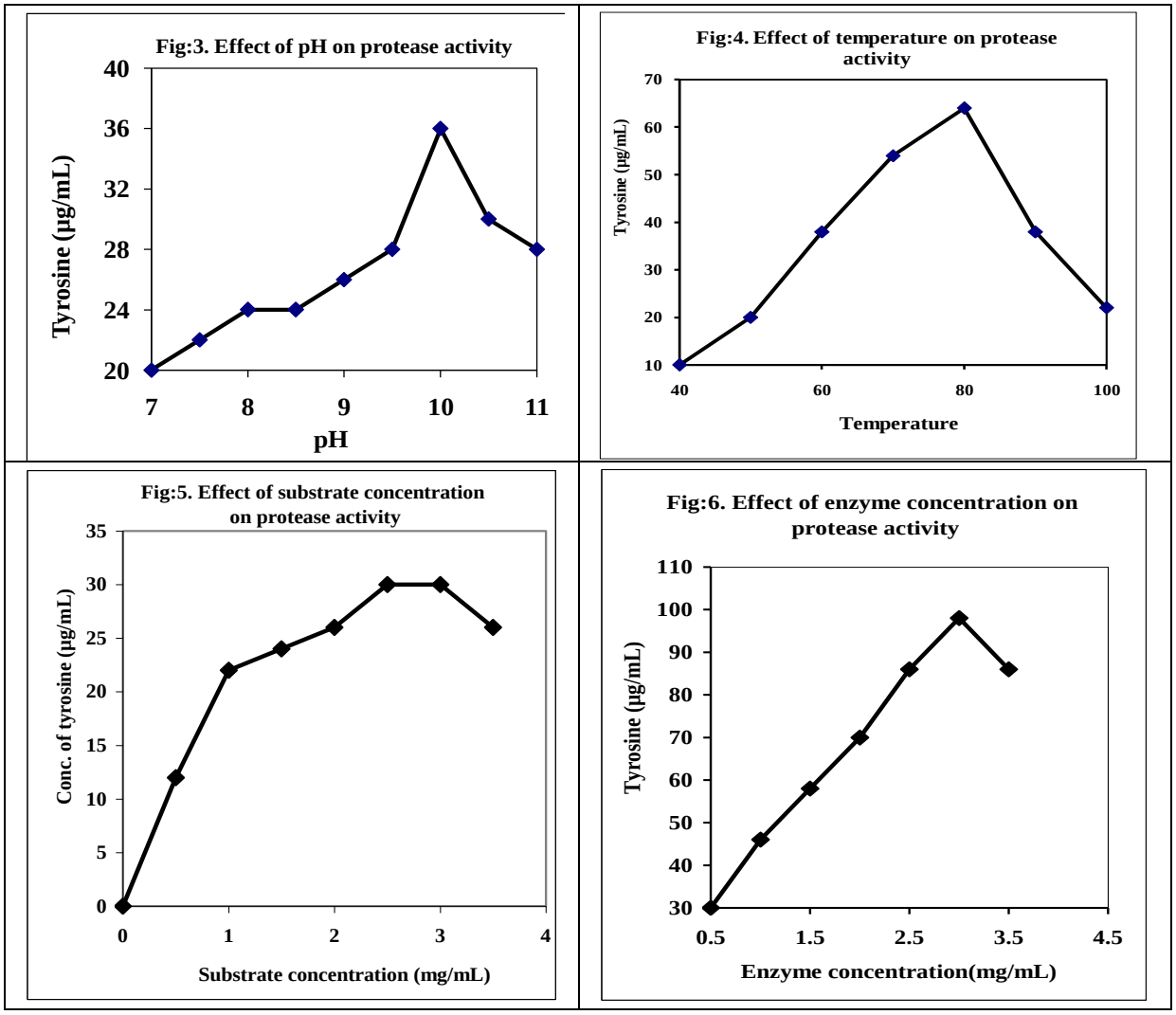
Note: LR- Long Rod, (+) = Positive, (-) = Negative

The growth of *Bacillus aerophilus* (DHT 23) was found to be optimum at 80^0C. From the data it showed that, the maximum proteolytic activity was observed at pH 10 (Fig.3) and at 80^0C (Fig.4). Optimum pH for proteolytic activity of protease producing bacteria was observed between pH 8-12. The optimum substrate concentration required for maximum activity of protease 1.9 µg/mL (Fig.5) and enzyme concentration was found 1.72 µg/mL (Fig.6).

Fig 1: Zones of casein hydrolysis







These indicate that for the growth of protease producing bacteria, alkaline environment is more suitable. Similarly, Tambekar *et al.*, (2016), reported that the bacterial isolates were characterized and identified as *Bacillus halodurans* and alkaline protease production was maximum at pH 8 and the activity was 1.5 unit/mL. The isolated *Bacillus halodurans* strain produces the protease enzymes which were thermostable, alkaliphilic and has potential to produce good quality protease which can be used in food, pharmaceutical and the detergent industries (Tambekar and Tambekar, 2013; Sawant and Nagendran, 2014).

CONCLUSION

In the present study, a different bacterial species were isolated from water, sediment and matt sample of Lonar Lake. The isolate DHT 23 was screened for production and partial characterization of protease on the basis of their optimum casein hydrolysis and was found to be 25 mm. On the basis of 16S rDNA sequencing indicated that strain DHT 23 was affiliated genera *Bacillus* and showed species *Bacillus aerophilus*. We have determined the optimum production of alkaline protease by the newly isolated thermophilic bacterium. The isolate *Bacillus aerophilus* produce alkaline protease at optimum temperature 80°C at pH 10 and showed maximum activity at substrate concentration 1.9 µg/ mL and enzyme concentration 1.72 µg/ mL. The isolated *Bacillus aerophilus* strain produces the protease enzyme which was thermostable, alkaliphilic and has potential to produce good quality protease which can be used in food, pharmaceutical and the detergent industries.

Sequence DHT 23

TGGGGACGTACTCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGA
AACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGC
CACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCT
CTTCTGCACTCAAGTTTCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCA
CATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTT
GCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGT
ACCGTCAAGGTGCGAGCAGTTACTCTCGCACTTGTTCTTCCCTAACAACAGAGCTTTAC
GATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGG
AAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGC
CGATCACCCTCTCACGTCGGCTACGCATCGTCGCCTTGGTGAGCCATTACCCCACCAAC
TAGCTATGCGCCGCGGGTCCATCTGTAAGTGACAGCCGAAACCGTCTTTCATCCTTGAA
CCATGCGGTTCAAGGAACTATCCCGGTATTAGCTCCGGTTTCCCGGAGTTATCCCAGTC
TTACAGGCAGTTACCCACGTGTTACTCACCCGTCGCGCTGACATCCGGAGCAGCTCCCT
TCTGTCGCTCGACTTGCATGTATAGCACGCGCAGCGTCGTCATGATCATGATCAATCTT
CTCTCGCGGCCCCGTTGACTCGTTTAGCCGTATTTTATTTTAGAACACGCGGGCACACTA
TTTAAGCGTGGTCGAGCAGCACATAAACTCTTATGTTTTTTTGTCTCCGCACCGAGAAG
GACGAGAGTCTCCGCTTTGTTTTTTCTCTCCGCAAGAGAGTAGTATGTAGTCAAACCCT
GCACGACTGCAGCTTGTTGTATCTCACTTACATCTTCGTAGTTGAGACTGATACTCGCTA
CGCCGAAAGAAGGGGGGGTTTATATCTCGAGTCTCAACCTCAGGGCCTAATCGCTGAT
AGATATGAACGCACGCGACG

Root rank Root (0/10/12749) (selected/match/total RDP sequences)

- domain Bacteria (0/10/12239)
- phylum Firmicutes (0/10/2443)
- class Bacilli (0/10/1571)
- order Bacillales (0/10/1067)
- family Bacillaceae 1 (0/10/314)
- genus Bacillus (0/10/244)

S000012241 - 0.609 1440 Bacillus vallismortis (T); DSM11031; AB021198
S000417318 - 0.653 1415 Bacillus altitudinis (T); type strain:41KF2b; AJ831842
S000458519 - 0.667 1354 Bacillus safensis (T); FO-036b; AF234854
S000481068 - 0.667 1352 Bacillus pumilus (T); ATCC 7061; AY876289
S000644546 - 0.613 1372 Bacillus idriensis (T); SMC 4352-2; AY904033
S001014161 - 0.655 1443 Bacillus stratosphericus (T); type strain:41KF2a; AJ831841
S001014162 - 0.6551443 Bacillus aerophilus (T); type strain:28K; AJ831844
S004007309 - 0.650 1426 Bacillus xiamenensis (T); MCCC 1A00008; JX680066
S004067892 - 0.609 1456 Bacillus amyloliquefaciens (T); DSM7; FN597644
S004071803 - 0.663 1338 Bacillus invictae (T); Bi.FFUP1; JX183147

REFERENCES

Borsodi AK, Micsinai A, Rusznyak A, Vladar P, Kovacs G, Toth EM, Marialigeti K, 2005. Diversity of alkaliphilic and alkali tolerant bacteria cultivated from decomposing reed rhizomes in Hungarian Soda Lake. *Microb ecol*; **50**: 9-18.

Fredriksson K, Dube A, Milton DJ and Balasundaram MS, 1973. Lonar Lake, India: An impact crater in basalt. Sci, 180 (4088): 862-864, doi: 10.1126/Science.**180**.4088.862.

Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Wani AA, Borgave SB and Sarnaik SS, 2008. Cultivable bacterial diversity of alkaline Lonar Lake, India. *Microb Ecol*; **55**(2): 163- 172.

Kanekar PP, Nilegaonkar SS, Sarnaik SS and Kelkar AS, 2000. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresource Technol*; **85**(1): 87- 93.

Sawant R and Nagendran S, 2014. Protease: An enzyme with multiple industrial applications. *World J Pharmacy and Pharmaceutical sciences*, **3**(6):568-579.

Shafee N, Norariatis AS, Zaliha ARN and Basri M, 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus Cereus* strain, *J Appl Sci Res*, **1**: 1-8.

Shanmughapriya S, Kiran GS, Selvin J, Gandhimathi R, Baskar TB, Manilal A and Sujith S, 2009. Optimization, Production, and Partial characterization of an alkaliphilic amylase produced by sponge associated marine bacterium *Halobacterium salinarum*. MMD047", *Biotechnology and Bioprocess Engineering*, 14: 67-75, DOI/ 10.1007/s12257-008-0060-1.

Singh SP, Dodia MS, Joshi RH and Patel RK, 2006. Characterization and stability of extracellular alkaline protease from halophilic and alkaliphilic bacteria isolated from saline habitat of coastal Gujarat, India, *Broz J Microbiol*, **37**(3): 1-12.

Tambekar DH and Dhundale VR, 2012. Studies on the physiological and cultural diversity of *bacilli* characterized from Lonar Lake (MS) India. *Biosci Disco*; **3**(1):34-39.

Tambekar DH and Tambekar SD, 2012. Partial characterization and optimization of alkaline protease production of *Bacillus pseudofirmus* from Lonar Lake. *Int J Adv Pharm Biol Sci*, **2**(1): 130-138.

Tambekar DH, Tambekar SD, Jadhav AS and Babhulkar BV, 2016. Isolation and partial characterization of protease from *Bacillus halodurans* (aj302709) from alkaline Lonar Lake, *Int J Pharma Sci Research*, **7**(11): 4546-4549.

Tambekar SD and Tambekar DH, 2013, Optimization of the production and partial characterrization of an extracelluar alkaline proteases from termo-halo-alpophillic Loanar lake bacteria *Bisciecnec Discovery*, **4**(1):30-38**.**

How to Cite this Article:

Tambekar DH, Tambekar SD, Jadhav AS and Kolhe SG, 2017. Extracellular thermo-alkalophilic proteolytic activities of the *Bacillus aerophilus*: Detection and preliminary characterization. *Bioscience Discovery*, **8**(2):255-260.