OPTIMIZATION OF THE PRODUCTION AND PARTIAL CHARACTERIZATION OF AN EXTRACELLULAR ALKALINE PROTEASE FROM THERMO-HALO-ALKALOPHILIC LONAR LAKE BACTERIA

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ABSTRACT

LONAR Lake, an impact crater located in the Buldhana district of Maharashtra State, India is occupied by saline water and harbors various unidentified, unique haloalkaliphilic bacterial *bacillus* species which produces thermo-halo-alkaliphilic proteases. The present study deals with the isolation, production dynamics, purification, characterization and optimization of a protease from *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* isolated and identified by 16S rRNA ribotyping from the Alkaline Lonar Lake. The *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* produced protease at maximum rate after 72 h of incubation at 37^0C with agitation speed of 120 rpm and 5% of starter culture. The best carbon sources for this *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* were fructose, maltose, starch and lactose respectively where as the best nitrogen sources were yeast extract, soy tone and soyabean cake respectively. While the most effective inorganic nitrogen sources was ammonium carbonate for *Bacillus pseudofirmus, Cohnella thermotolerans* and urea for *Bacillus odysseyi*. Supplementation of the culture medium with amino acid L-glutamic acid for *Bacillus pseudofirmus* and L-glycine for *Cohnella thermotolerans and Bacillus odysseyi* and metal ion Mg^{2+} for all the three *bacillus* species improved the protease production substantially. Under these conditions, newly isolated *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* strain were found to produce alkaline proteases at a maximum rate of optimum pH 10 and temperature at 75^0C.

Key words: Alkaline Protease, *Bacillus pseudofirmus, Cohnella thermotolerans, Bacillus odysseyi*, Environmental factors, Nutritional conditions)

INTRODUCTION

LONAR Lake, an impact crater located in the Buldhana district of Maharashtra State, India is a circular 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.,* 2002). Extracellular enzymes like amylase, lipase, protease and cellulases produced by *Bacillus cereus, Bacillus firmus, Enterococcus caseliflavus, Bacillus fusiformis, Bacillus cohnii, Bacillus horikoshii* were isolated from water and sediment of alkaline Lonar Lake (Joshi *et al.,* 2008). Proteases are industrially important enzymes used in the detergent, food, pharmaceutical, leather industries, in peptide synthesis and also have application in silver recovery from photographic plates which account for about 60% of total industrial enzyme sales (Horikoshii, 1999). The detergent industry is the largest single market for this enzyme. 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). Therefore, attempt was made to isolate new species of *bacillus* which can produce good quality of proteases useful in the detergent and leather industry. A 16S rRNA gene sequence analysis was made for identification of the isolated species of *bacillus*.

Alkaline proteases produced by *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* are of great importance in detergent industry due to their high thermo-stability and pH stability which is the most important industrial enzymes accounting for about 60% of total enzyme market (Borsosi *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 and has wide applications in different industries. As there is large demand of proteases, isolation and production of protease enzyme is most important to fulfill this demand (Srinivasan *et al.,* 2009). Therefore, the present study deals with the isolation, characterization, production, purification and optimization of a protease from *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* isolated from the alkaline Lonar Lake.

MATERIALS AND METHODS

Collection of Lonar lake water and sediment sample: Total four sediment and eight water samples were collected in year 2009 - 2010 from alkaline Lonar Lake. Water samples collected in sterilized plastic cans and sediments in sterilized plastic bags were transferred to laboratory for isolation and identification of bacteria followed by their screening for proteolytic activity. During sample collection, the date, time and places were noted.

Isolation of Alkaliphiles: About 1.0 g of soil sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37^0C for 15 minutes in water bath shaker. The sample was then heated at 80^0C for 15 minutes to destroy all the vegetative microbial cells. The suspension was then diluted to 10^{-7} dilutions. One ml of each diluted sample was lawn into petri plates containing nutrient agar medium (pH 10) and incubated at 37^0C for 24 hours.

Screening of bacterial alkaliphiles: Individual bacterial colonies were screened for proteolytic activities on Skim milk agar medium (skim milk 1%, Peptone 1%, sodium chloride 0.5%, Agar-Agar 2%, pH 10). The pH of the medium was adjusted to 10 with 1N NaOH before sterilization at 121^oC for 15 minute. The inoculated plates were incubated at 37^oC for 72 hrs and observed for zones of clearance, indicating proteolytic activities.

Identisfication 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, citrate utilization, oxidase, urease, gelatin liquification, catalase, Vogous-proskaur, Indol tests and acid production from glucose, arabinose, lactose, mannitol, galactose and maltose. 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 4.0 ^{o}C. The isolated strains were then analyzed by 16S rRNA at NCCS, Pune and BLAST identification was made.

Preparation of crude enzyme extracts: The 100 ml Yeast extract casein medium (Glucose 1%, Casein 0.5%, yeast extract 0.5%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $MgSO_4$ 0.1%, pH.10.5) was dispensed (50 ml each) into two 250 ml capacity conical flasks, after adjusting the pH to 10.5 and sterilized it by autoclaving. After cooling, the broth was inoculated with *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* cultures and incubated for 72 h at 37^0C in shaking incubator. After 72h incubation, centrifuged the broth at 5000-8000 rpm for 15 min. The supernatant served as crude enzyme source.

Optimization of crude enzyme protease: 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 1 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 adds 1ml of Folin-Ciacalteau reagent and 2 ml of Na_2CO_3 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 at zero time and the absorbance was read at 650 nm (Lalitha *et al.,* 2010).

Determination of proteolytic activity: Proteases activity was determined by a slightly modified method of Yang *et al.,* 2001. The reaction mixture containing 1 ml of 1.0 % casein solution in 0.2 M Glycine-NaOH buffer having pH 10.5 and 1 ml of a given enzyme solution was incubated at 40^{o}C for 10 minutes and the reaction was then stopped with 2 ml of 10 % tri-chloroacetic acid (TCA). The amount of tyrosine liberated was determined as per tyrosine assay procedure at 650 nm. The proteolytic unit was defined as the amount of the enzyme that released 1µg of tyrosine per minute under the assay conditions.

Partial characterization of protease: Partial characterization of protease was carried out (Joo *et al.,* 2002).

Effect of pH on alkaline protease activity: The effect of pH on alkaline protease from *Bacillus* species was determined by assaying the enzyme activity at different pH values ranging from 7.0 to 10.5 using the different concentration of each buffer were 0.2 M: phosphate (pH 6-7), tris–HCl (pH 8-9) and Glycine-NaOH (pH 10-12).

Effect of temperature on alkaline protease activity: The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (pH 10.5) for 20 minutes at different temperature ranging from 55^{o}C to 90^{o}C.

Effect of substrate on alkaline protease activity: The effect of substrate concentration on alkaline protease activity was determined by incubating the reaction mixture (pH 10.5) for 20 minutes with different substrate concentration, ranging from 5 mg/ml to 40 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 (pH 10.5) for 20 minutes at different enzyme concentration ranging from 0.5ml to 4ml. The activity of the protease was then measured as per assay procedure (Shafee *et al.,* 2005).

Purification of enzymes: Purification of enzyme was made by chilled acetone, isopropyl alcohol and Ammonium sulphate precipitation method.

Optimization of environmental and nutritional conditions for the production of alkaline protease: Optimization of proteases production was studied with help of fermentor by optimization of medium composition (variation in carbon, nitrogen sources and metal ions) and environmental conditions such as pH, temperature, incubation period etc (Kumar *et al.,* 2002). The present investigation deals with the optimization of medium components which were predicted to play a significant role in enhancing the production of alkaline proteases. Carbon sources chosen for the study were glucose, sucrose, starch, fructose, maltose and lactose. These carbons occur which led to cell lysis and increased cell sources were used to replace the carbon source available permeability due to abrasion by shear forces (Gupta *et al.,* 2002). Sources of nitrogen include organic nitrogen, inorganic nitrogen and amino acid in which sources throughout the study were soy tone, Soya bean cake, beef extract, yeast extract, peptone, ammonium nitrate, ammonium carbonate, urea, L-lysine, L-aspartic acid, L-glutamic acid and L-glycine. Metal cations tested to replace metal ion source in the Media were Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+} (Adinarayana *et al.,* 2003).

RESULTS AND DISCUSSION

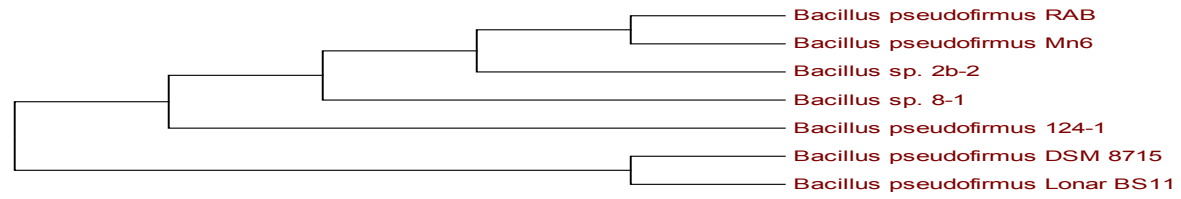
In the present study, a total of 104 bacterial species were isolated from water and sediment sample of Lonar Lake. Out of them, 67 were from water and 37 from sediments which were maintained on nutrient agar slant (pH 10.5) and various tests were performed for identification of these bacterial species. Then these bacterial cultures were inoculated on alkaline skim milk agar at pH 10.5 for studying their proteolytic activity using morphological and biochemical characteristics. Out of 104 cultures, 37 isolates were identified as *Bacillus*. Out of 67 isolates of water, only 22 isolates and out of 37 isolates of sediments, only 15 isolates were efficient in protease production and most efficient *bacillus* species were used to study different enzyme parameter.

The results of 16 S rRNA analyses at NCCS, Pune and BLAST identification were as follows.

On the basis of 16S rRNA gene sequence analysis, it was confirmed that the *Bacilli* species isolated from Lonar Lake having class "*Bacilli*" and order *Bacillales.* The 16S rRNA gene sequence analysis demonstrated that this isolate was moderately related to species of the genus *Bacillus*, with <98.5% sequence similarity to all other described *bacillus* species and represent novel species of a

Table No. 1: 16S rRNA analysis (Blast analysis), Dendrogram showing phylogenic relationship of *Bacillus pseudofirmus* isolated from Lonar Lake

CACCCCAATCATCTGTCCCACTTTAGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCAATTCCG
GCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTATGGGATTCGCTCAACCTCGCGGTTTTGC
AGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCCACC
TTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGT
TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGG
AAAGCTCTATCTCTAGAGTGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATG
CTCCACTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAAT
GTGTTAACTTCGGCACTAAGGGCATCGAAACCCCTAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTAT
CTAATCCTGTTTGCTTCCCCACGCTTTCGCGCCTTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTT
CCTCCACATATCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGTACTCAAGTCTCCCAGTTTCCAAT
GACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAGACCGCCTGCGCGCGCTTTACGCCCAATAATTCC
GGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAA
GGTGCCGCCTTATTCAAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCG
GCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCA
GTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTAAGCCGTTACCTTACCAACTAGCTA
ATGCGCCGCGGGCCCATCTGTAAGTGATAGCCAGAGGCCATCTTTTACCGCTCCACCATGAGGTGGAACGGGTTATC
CGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAA
CATCAGGAGCAAGCTCCCATCAGTCCGCTCGACTTGCATGTATTA



new genus of endospore-forming bacteria for which propose the name *Bacillus pseudofirmus* with <98.5% sequence similarity (Table No.1). Among them, a bacterial culture identified as *Cohnella thermotolerans* from sediment sample of Lonar Lake by 16S rRNA analysis were used for detail study of protease production and optimization.

On the basis of 16S rRNA gene sequence analysis, it was confirmed that the *Bacilli* species (S3) isolated from Lonar Lake having class "*Bacilli*" and order *Bacillales*. 16S rRNA gene sequence analysis demonstrated that this isolate was moderately related to species of the genus *Alicyclobacillus* with <98.9% sequence similarity to all other described *Alicyclobacillus* species and represent novel species of a new genus of a Gram positive, rod shaped, endospore forming organism *Cohnella thermotolerans* strain with <98.9% sequence similarity (Table No. 2).

On the basis of 16S rRNA gene sequence analysis, it was confirmed that the *Bacilli* species (S9) isolated from Lonar Lake having class "*Bacilli"* and order *Bacillales.* 16S rRNA gene sequence analysis demonstrated that this isolate was moderately related to species of the genus *Bacillus* with <94.6% sequence similarity to all other hitherto described *Bacillus* species and represent novel species of a new genus of endospore-forming bacteria for which propose the names *Bacillus odysseyi* with <94.6% sequence similarity (Table No. 3). **Partial characterization of protease**

Effect of substrate on alkaline protease activity: Alkaline protease production was maximum at pH 9-10.5 where as maximum protease production was recorded after 72 h of incubation at 37°C. In the effect of substrate concentration on enzyme activity of protease, the Michalies Menten constant (K_M) and Maximum velocity (V_{Max}) was found to be 7.69 µg/ml and 0.01 µg/ml by Line weaver-Burk plot in *Bacillus pseudofirmus* where as in *Cohnella thermotolerans,* it was found to be 9.09 µg/ml and 0.018 µg/ml respectively.

Table No. 2: 16S rRNA analysis (Blast analysis), Dendrogram showing phylogenic relationship of *Cohnella thermotolerans* isolated from Lonar Lake

TCGGTATTTCTTTTATTTTGGGTGTCAGCGCGCGGGGGTGTCCTTATGTAAGATGTGAGTCAAGGAAAAAAAAAAAAC
CTTTGTTATCTGACAGAGGTAGAATGAATATAAGAGAGGAGATTTACGTGTGGCGGGGAGAAGTGTAGAGATGTGG
AGGAGACCCCAGGCGAAAGGGAAACTTTTCCTCTTTAAATGTTGCTGAAAGGGAAAGCGTGGGGGGGGAGAAAAG
ATTATTTAATCCGGTGGTCGTCCCCCCCAAAATGAATTGCTAAGAGTTTGTGGGGGTCATGGCCCTCTGTGCCGAAAG
CAACACATGAATAAATCACCCCCGGCGGGGTGCGGGGGGGACAGGTGGAAAATCATAAAAAAAAATAATGGAGGGA
CCCCCCGCCCACCGGAGAAGATTGTGGTATTTTTTTTTTTTAAACAAGAAAAAATATCAAGACTTGTTGGGGATTTGTG
TAACACCTTAAGAAAATAAGTTTCCTCCTCCTTTTAACAATCTGAGAAAAAGGTGCATGGTGGGTGTCATCTCCCTCTT
GTGGATACGAGTGTGGGAGATAAAGCCCCAAATCCACCCCCCCCCTCGTTAATCTTTTGG
CATTAAATTTTACA

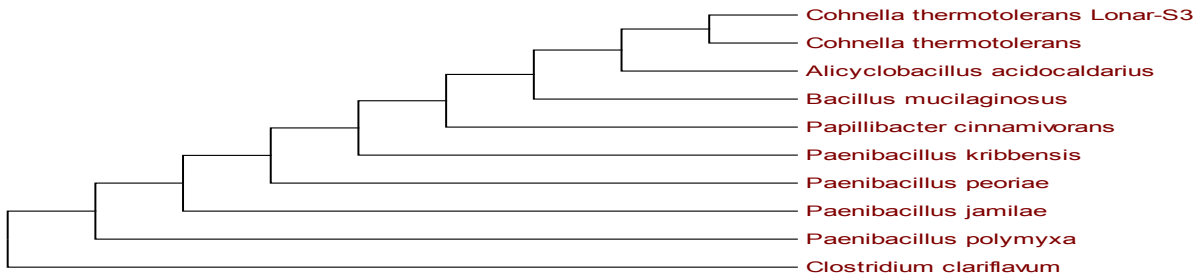
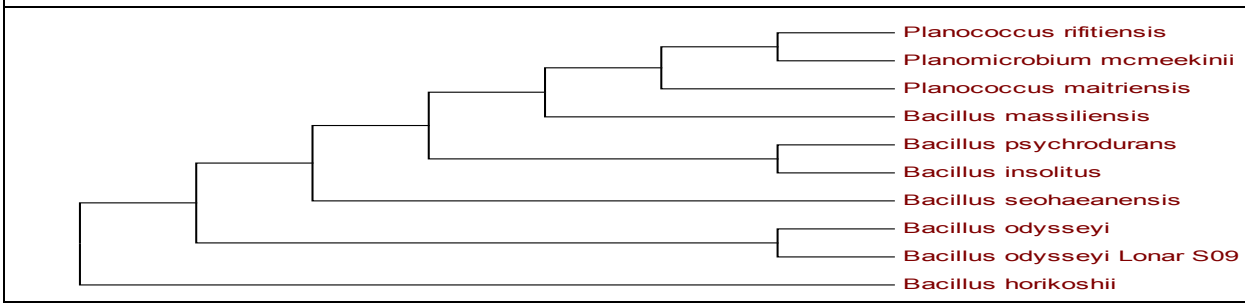


Table No. 3: 16S rRNA analysis (Blast analysis), Dendrogram showing phylogenic relationship of *Bacillus odysseyi* isolated from Lonar Lake

AGGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAG
GGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATT
TCACCGCTACACTTGGAATTCCACTTTCCTCTTCTGCACTCAAGTCCCCCAGTTTCCAATGACCTTCCACGGTTGAGCC
GTGGGCTTTCACATCAGACTTAAAAGACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTGAGGTACCGTCAAGGTACCAGCAT

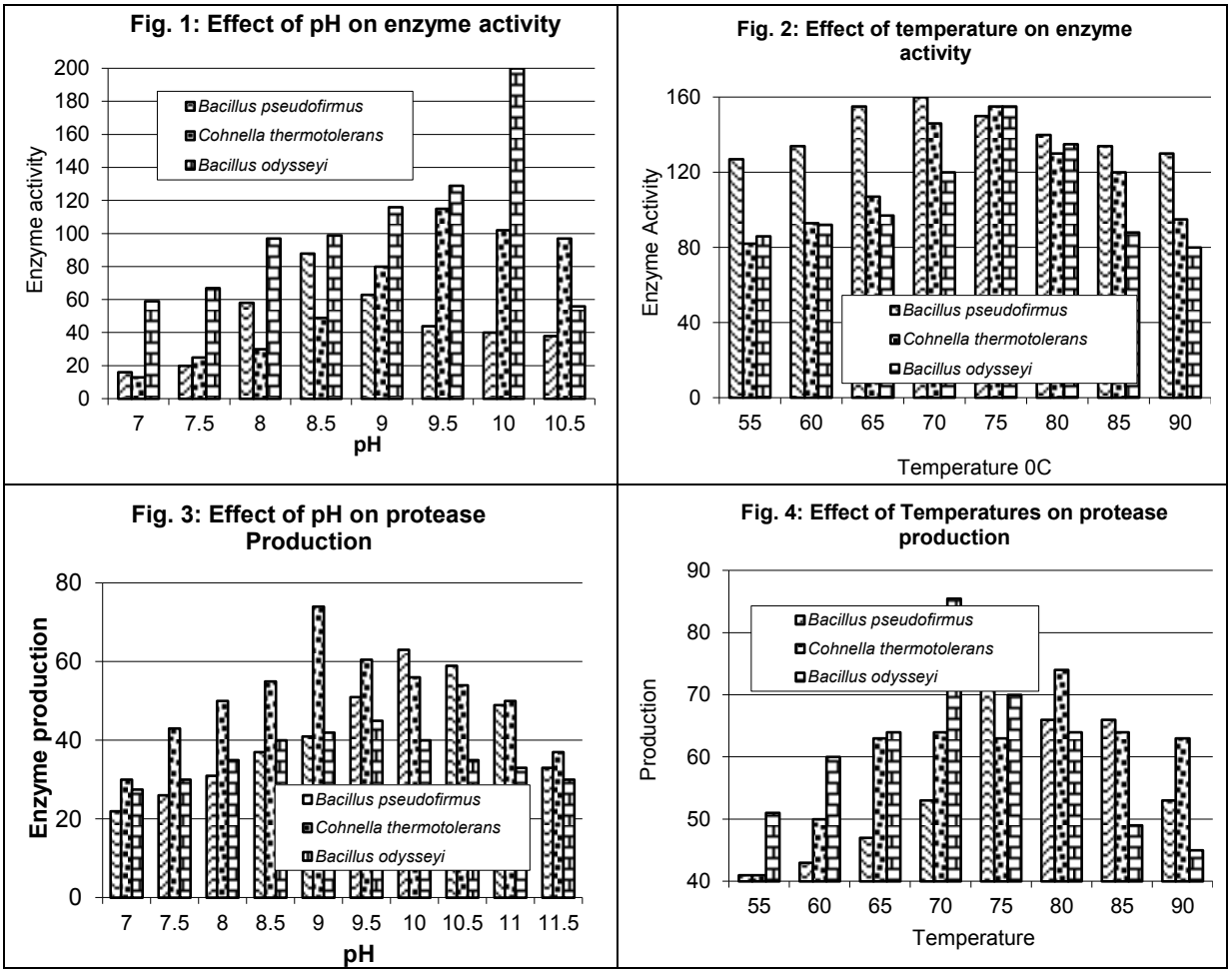


In the effect of substrate concentration on enzyme activity of protease, the Michalies Menten constant (K_M) and Maximum velocity (V_{Max}) was found to be 4.16 µg/ml and 0.007 µg/ml by Line weaver-Burk plot in *Bacillus odysseyi*.

Effect of enzyme on alkaline protease activity: The optimum enzyme concentration required for maximum activity of protease was 2.5 ml, 3.5 ml and 1.5 ml in *Bacillus pseudofirmus*, *Cohnella thermotolerans* and *Bacillus odysseyi* respectively.

Effect of pH and temperature on alkaline protease activity: The optimum pH and temperature required for maximum activity of protease was 8.5 (Fig.1) and 70^0C respectively (Fig.2) in *Bacillus pseudofirmus*, where as in *Cohnella thermotolerans,* the optimum pH and temperature required for maximum activity of protease was 9.5 (Fig.1) and 75^0C respectively (Fig.2).

The optimum pH and temperature required for maximum activity of protease was 10 (Fig.1) and 75^0C respectively (Fig.2) in *Bacillus odysseyi*.



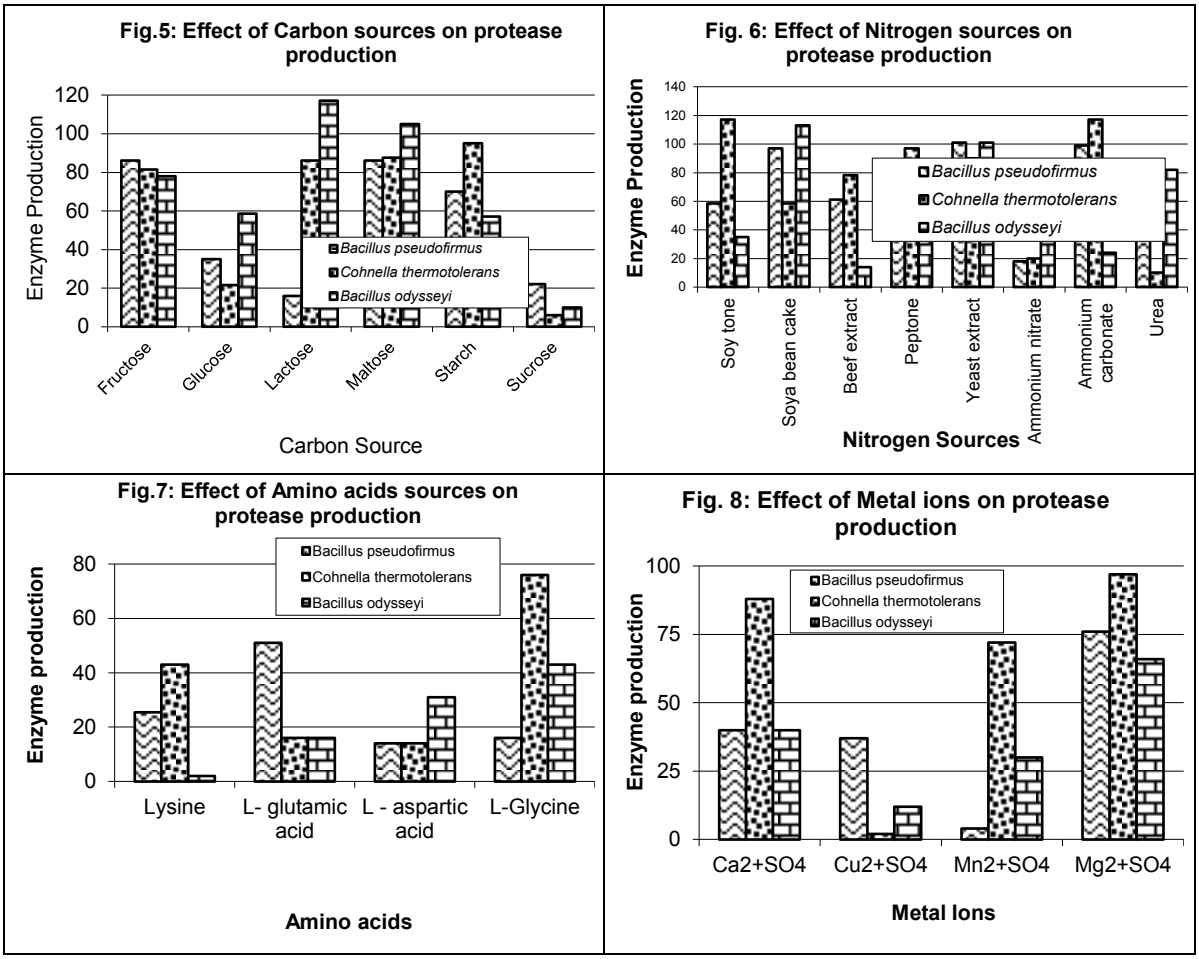
Effect of pH and temperature on protease production: This observation was corroborated by different pH and Temperature range. *Bacillus pseudofirmus* strain produced maximum alkaline protease at pH 10 (Fig.3) and temperature at 75^0C (Fig.4). *Cohnella thermotolerans* strain produced maximum alkaline protease at pH 9 (Fig.3) and temperature at 80^0C (Fig.4) where as *Bacillus odysseyi* strain produced maximum alkaline protease at pH 9.5 (Fig.3) and temperature at 70^0C (Fig.4).

Effects of carbon sources on protease production: It has been reported that pure sugars affected protease production considerably. Utilization of pure sugars as carbon and energy sources was also shown to result in good growth with increase protease production. This observation was in agreement with previous studies which suggested that larger amount of enzyme was synthesized when carbon sources were poorly utilized for growth purposes (Tambekar and Tambekar, 2012). Various sources of carbon such as glucose, fructose, sucrose, maltose, starch and lactose were used in enhancing the production of alkaline proteases. The best carbon sources for this *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* were fructose, maltose, starch and lactose (Fig.5) respectively, instigated highest protease production compared to other carbon sources at 72 h of incubation due to the prolonged incubation time perhaps led to auto digestion of proteases and proteolytic attack by other proteases (Horikoshii *et al.,* 2006).

Effect of nitrogen sources on protease production

Effect of organic nitrogen sources on protease production: In this study, sources of organic nitrogen like soy ton, Soya bean cake, beef extract, yeast extract and peptone were used. The best nitrogen sources were yeast extract, soy tone and soyabean cake for *Bacillus pseudofirmus, Cohnella thermotolerans* and *Bacillus odysseyi* (Fig.6) respectively which showed highest level of protease activity compared to other sources of organic nitrogen. Despite the luxurious bacterial growth, the presence of beef extract, peptone and tryptone resulted in low protease production.

This observation contradicted (Phadatare *et al.,* 1993) which reported that protease production in *Conidiobolus coronatus* was enhanced by organic nitrogen sources like yeast extract, peptone and tryptone.



Effects of inorganic nitrogen sources on protease production:

Inorganic nitrogen sources like ammonium carbonate, ammonium nitrate and urea were tested on the growth and protease production. While the most effective inorganic nitrogen sources was ammonium carbonate for *Bacillus pseudofirmus, Cohnella thermotolerans* and urea for *Bacillus odysseyi* (Fig.6) led to high protease activity at 48 and 72 h. Ammonium nitrate did not enhance protease production at early stages of incubation but at later stages (48 and 72 h) protease production increased. Even though growth was stimulated, only moderate levels of enzyme activities were obtained when ammonium nitrate was used as a nitrogen source. This was perhaps due to the inability of bacteria to utilize ammonia in the media (Ellaiah *et al.,* 2005).

Effects of amino acid on protease production:

This observation was corroborated by L-aspartic acid; glutamic acid and glycine which were also tested as sources of amino acids for protease production. Supplementation of the culture medium with amino acid L-glutamic acid for *Bacillus pseudofirmus* and L-glycine for *Cohnella thermotolerans and Bacillus odysseyi* (Fig.7) improved the protease production substantially.

Effects of metal ions on protease production:

The highest level of protease activity was observed in the presence of Mg^{2+} at 72 h incubation for all the three *bacillus* species which improved the protease production substantially (Fig.8). Addition of Ca^{2+}, Cu^{2+} and Mn^{2+} resulted in high protease production only at 48 h incubation. It was suggested that these metal ions increased stability of proteases, even though effects of the different

metal cations on protease production vary where Supplementation of culture medium with metal cations improved substantially the protease production and growth of these *bacillus* strains. This observation strongly suggested the requirement of some metal ions for protease production by this organism. These results were in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions and it was suggested that these metal ions increased stability of proteases (Banerjee *et al.,* 1999).

In summary, isolated *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* species from Lonar Lake produced alkaline protease and maximum growth at pH 8.5-10.5. The isolated *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* strain produces the proteases enzyme which was theomorphic, alkaliphilic and has potential to produced good quality of proteases which can be used in the industry. The *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* species were most efficient for protease producing at pH 10.5 incubated at 37^0C for 72h. The protease produced from this species was highly efficient at high temperature, high salt concentration and tolerate the other environmental conditions. This bacterial species is ubiquitous and non-pathogenic, not causing any diseases to human beings and most efficient for protease production among all isolated protease-producing bacteria. Protease enzymes have importance in various industries like detergent industry, leather industry etc. *Bacillus* species particularly *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi* were known for their ability to produce proteolytic enzymes with wide application in industries. In addition to the limited number of reports, protease production by these *bacillus* species was shown to be affected by various environmental and nutritional conditions. In the present investigation, it was determined the optimum parameters for maximum production of alkaline protease by the newly isolated thermophilic bacterium *Bacillus pseudofirmus, Cohnella thermotolerans and Bacillus odysseyi*. This information has enabled the ideal formulation of media composition for maximum protease production by these organisms.

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How to Cite this Article:

Sandhya D Tambekar and DH Tambekar, 2013. Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-Halo-Alkalophilic Lonar Lake Bacteria. *Biosci. Disc.,* **4**(1): 30-38.