INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 5, p. 130-140, 2019

RESEARCH PAPER OPEN ACCESS

Characterization and optimization of alkaline protease producing thermostable bacteria

Beena Kanwar Shekhawat[1], Shabori Bhattacharya[2], Mohd Tanvir Qureshi[3], P.J. John[2*]

[1]*Centre for Converging Technology, University of Rajasthan, Jaipur, Rajasthan, India*

[2]*Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur, Rajasthan, India*

[3]*University Innovation Cluster-Biotechnology, Centre for Converging Technologies, University of Rajasthan, Jaipur, Rajasthan, India*

Key words: Alkaline proteases, Extremophiles, *Bacillus sp.,* Semi-arid soil, Rajasthan.

http://dx.doi.org/10.12692/ijb/14.5.130-140 Article published on May 02, 2019

Abstract

Thermophilic bacteria that secrete alkaline proteases have been attracting attention in industrial applications such as tanneries and laundry industry due to their ability to act as a source of cheap but efficient enzymes that reduce dependence on environmentally harmful chemicals. The aim of this study was to isolate the therrmophilic bacteria that produce alkaline protease enzymes from soil of Rajasthan (Jodhpur, Nagaur, Barmer), India by using specific isolation media defined by Horikoshi. The isolates were identified as gram positive bacteria on the basis of gram staining and by various biochemical methods. 16S rDNA gene sequencing suggested these strain *Bacillus* sp. Which have been submitted to NCBI (National Center for Biotechnology Information). These isolates were tested for their ability to produce extracellular proteases and their proteolytic activity was checked on the basis of casein hydrolysis on skim milk agar plate by halo formation surrounding the isolate. Thermostability study revealed that all the isolates are found to survive up to 90°C. Optimization of culture conditions was also done to enhance productivity and activity of the protease enzymes.

* **Corresponding Author:** P. J. John ✉ placheriljohn@gmail.com

Introduction

Proteases (protein hydrolysing enzyme) are produced by plants, animals and microorganisms. Out of all the proteases produced, the microbial forms constitute approximately 40% of the total world-wide production of enzymes gaining highest commercial value over enzymes obtained from animals and plants (Godfrey *et al.*, 1996; Beg *et al.*, 2003). Among various microbes *Bacillus* is highest protease producer. As per literature protease is isolated from various bacillus species such as *Bacillus pumilus, Bacillus subtilis, Bacillus mycoides* and *Bacillus megaterium* (Baweja *et al.*, 2016; Mukhtar and Haq 2008; Abdel-Naby *et al.*, 1998; Askar *et al.*, 2013). Proteases have diverse biological roles for example in signal transduction, post-translational modification, proliferation, apoptosis and pathogenicity through specific processing or non-specific degradation (Kumar, 2007; Huston, 2010; Soh *et al.*, 2010). On the basis of their optimum activity in a particular pH range, these are classified into acid, neutral and alkaline proteases (Sandhya *et al.*, 2005).

Alkaline proteases are a kind of endopeptidases that show enhanced activity in neutral to alkaline pH range. These either have a serine center (serine proteases) or are of metallo-type (metalloprotease) (Ward, 1985). They are also active in the presence of surfactants and oxidizing agents (Gupta *et al.*, 1999; Kalisz, 1988) and are primarily used as detergent additives (Sandhya *et al.*, 2005; Krik *et al.*, 2002).

This study was aimed to isolate and identify alkaline protease producing bacteria from semi-arid soil of Rajasthan.

This area has extreme climatic conditions, so the extremophiles present here are expected to produce an alkaline protease enzyme that can work at high alkaline pH of 10 while also being stable at high temperature (approx. 60°C). Due to this reason soil of this area was screened for isolation of such species.

Materials and methods

Isolation of alkaline protease producing bacteria

The isolation of alkaline protease producing bacteria from the soil of semi-arid regions of Rajasthan (i.e. Jodhpur, Bikaner and Nagaur) India (Fig. 1) has been done by the method given by Horikoshi (Horikoshi *et al.*, 1999).

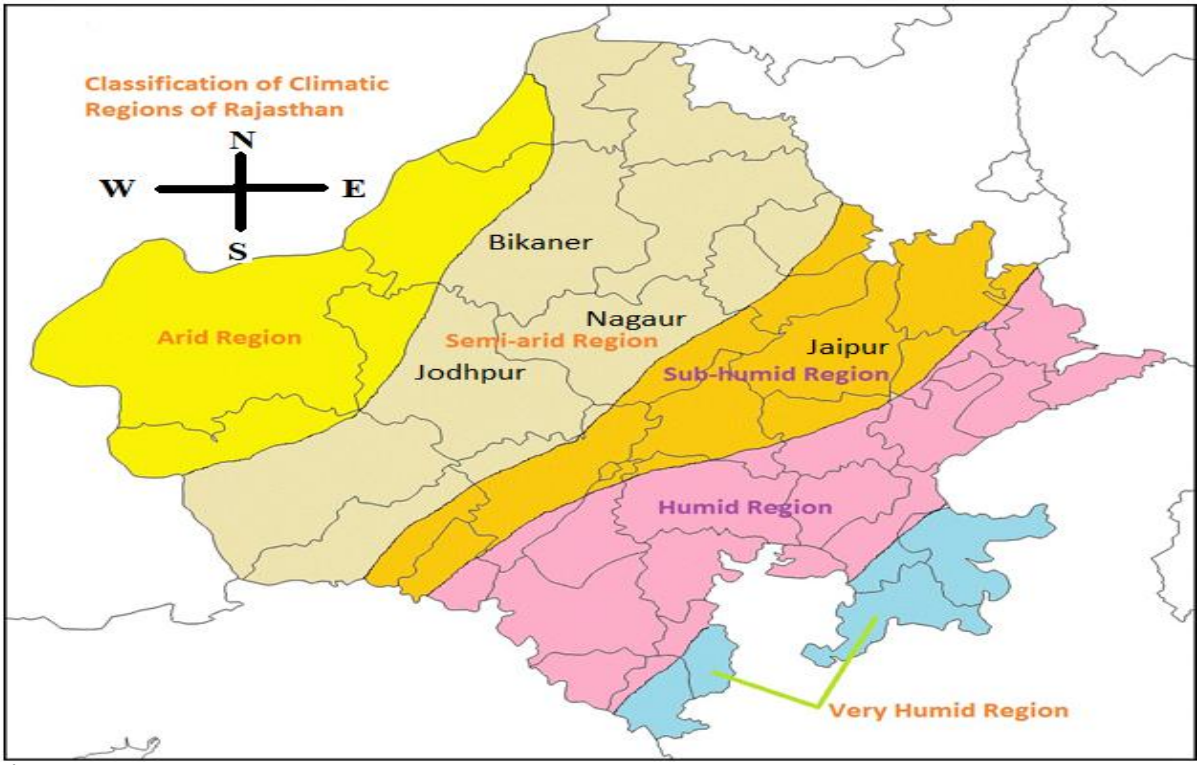

Fig. 1. Semi-arid region of Rajasthan.

For Serial dilution 1.0 g soil was suspended in 10ml of sterilized distilled water and 1 ml of resulting suspension was transferred to 9 ml of sterilized distilled water. This process of serial dilution was repeated till 10 fold dilutions. 1ml of the resulting suspension from each dilution was transferred to screening agar medium plate which contained: 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% potassium dihydrogen phosphate, 0.02% magnesium sulphate, 1.5% Agar. Sterile 1% sodium carbonate was aseptically added after autoclaving. pH of the medium was adjusted at 10.5. The culture was incubated at 37°C for 72 hours. The best results obtained from 10^3 and 10^5 dilution tubes. Three replicate plates were prepared for each sample.

The pure isolates were obtained purified by repeated streaking. A positive (inoculated with MCC 2211 strain, *Bacillus licheniformis* strain was purchased from National Centre for Microbial Resource and gene bank (NCMR), National Centre for Cell Science (NCCS, Pune, India) and negative flask (no bacterial inoculation) were also incubated on same standard conditions.

The isolates obtained were named as N, B from Nagaur and Bikaner respectively whereas J1, J2 from Jodhpur. All the isolates were stored in 30% glycerol stocks and Cultures were regenerated in every 3-4 weeks on a fresh plate from frozen stock culture.

Protease assay

The protease producers among pure strains were detected by plating on skim milk agar plate. The strain showing maximum halo formation (clear zone diameter) was selected as potent producer of the alkaline protease enzyme (Fig. 2) and was maintained in glycerol stock at -20°C for further studies and protein purification.

Total protein content

Total protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. The concentration of protein was estimated spectrophotometrically at 660 nm.

Assay for proteolytic activity

Alkaline protease activity was determined by the method given by Takami (Takami *et al.,* 1989) with slight modification. According to the protocol, 0.25 ml of glycine:NaCl:NaOH (50 mM, pH 10.5) buffer was incubated with 2.5 ml of 0.65% casein (Merck) dissolved in the same buffer at 30°C to 40°C until equilibrium was achieved. An aliquot of 0.25 ml of enzyme solution was added to this mixture and incubated for 20 min.

The reaction was stopped by adding 2.5 ml TCA solution (0.6 N). After 10 min the entire mixture was centrifuged at 8000 rpm for 10 min. The supernatant (0.5 ml) was mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature.

The optical density of the solutions was determined with respect to the sample blank at 660 nm using spectrophotometer. For these studies, one alkaline protease unit was defined as the enzyme amount that could produce 1µg of tyrosine/ min under the defined assay conditions.

Identification of isolates

The bacterial isolates showing maximum protease activity and maximum clear zone were further subjected to biochemical and molecular characterization.

Biochemical characterization

Gram staining was done by standard protocol. Various biochemical test (Bergey's manual, 1974) including indole test, methyl red test, Voges-Proskauer test, citrate test, lactose fermentation test, starch hydrolysis test, gelatin test, urease test nitrate reduction test, mannitol test, citrate utilization test, hydrogen sulphide production test, catalase production test, casein hydrolysis test, oxidase test and H_2S production tests were performed.

All the isolates were screened on Hi-chrome media in triplicates. All the isolates were checked for their thermostabilty also at various temperatures ranging

from 60°C to 90°C, i.e. 60°C, 70°C, 80°C, 90°C.

Molecular characterization

Molecular Characterization was done by using 16s rRNA sequencing. The 16S rDNA sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA5.

Optimization of culture conditions for protease producing isolates

The selected protease producing isolates were further subjected to culture condition optimization experiments so as to increase their enzyme producing capacity. Six parameters were studied for optimization. The broth culture medium with isolated strain inoculation were subjected to various incubation periods (12, 24, 36, 48, 60, 72, 84, 96 hours), agitation speed (90 rpm, 120 rpm, 150 rpm, 180 rpm, 210 rpm, 240 rpm), carbon source (glucose, lactose, fructose, sucrose, maltose, xylose), temperature (25°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C), pH range (7, 8, 9, 10, 11, 12, 13), nitrogen source (yeast extract, peptone, casein, tryptone, beef extract and gelatin), inoculum percent (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40), nitrogen source in combination at a time (yeast extract-casein, yeast extract-peptone, casein-tryptone, beef extract-yeast extract, tryptone-peptone, gelatin-beef extract and yeast extract-tryptone) and divalent ion (Mg^{+2}, Mn^{+2}, Ca^{+2}, Fe^{+2}, Zn^{+2} and Ba^{+2}). All the optimization experiments were done in triplicates.

Results

Morphological and physiological characterization of isolates

The isolates from semi-arid soil of Rajasthan were found to be Gram positive, long, rod-shaped bacilli (Fig. 3) after Gram staining and negative staining.

They showed green colonies on Hi-chrome media (Fig. 4) and were also found to be spore formers. All the four isolates were observed to grow in the temperature range of 30°C to 90°C. All were thermostable upto 90°C. Growth of the isolates was found to occur in the pH range of 8.0 to 13.0 and in the presence of 1% glucose.

Biochemical characterization

The isolates were tested and found to be positive for casein hydrolysis, starch hydrolysis, gelatin hydrolysis, and catalase test. Negative results were obtained for growth on MacConkey agar, indole test, methyl red test, Voges-Proskauer tests, oxidase test, and nitrate reduction test (Table 1).

Table 1. Biochemical tests for bacterial identification.

S. No.	Name of Test	Control	Sample N	Sample J_1	Sample J_2	Sample B	MCC
1	Indole Test	(–)	(–)	(–)	(–)	(–)	(–)
2	Methyl Red Test	(–)	(–)	(–)	(–)	(–)	(+)
3	VogesProskauer Test	(–)	(–)	(–)	(–)	(–)	(+)
4	Citrate Utilization Test	(–)	(+)	(+)	(+)	(+)	(+)
5	Fermentation Test	(–)	(–)	(–)	(–)	(–)	(–)
6	Starch Hydrolysis Test	(–)	(+)	(+)	(+)	(+)	(+)
7	Gelatin Test	(–)	(+)	(+)	(+)	(+)	(+)
8	Urease Test	(–)	(–)	(–)	(–)	(–)	(–)
9	Citrate Utilization Test	(–)	(+)	(+)	(+)	(+)	(+)
10	Casein Hydrolysis Test	(–)	(+)	(+)	(+)	(+)	(+)
11	Catalase Test	(–)	(+)	(+)	(+)	(+)	(+)
12	Oxidase Test	(–)	(–)	(–)	(–)	(–)	(+)
13	Nitrate Reduction Test	(–)	(–)	(–)	(–)	(–)	(–)
14	H_2S production Test	(–)	(+)	(+)	(+)	(+)	(+)

Foot note: - Sample N-Bacterial strain from soil of Nagaur (Rajasthan), Sample J_1 and Sample J_2- Bacterial strain from soil of Jodhpur (Rajasthan), Sample B- Bacterial strain from soil of Bikaner (Rajasthan).

Molecular characterization

The partial nucleotide base sequencing of 16S rDNA gene of all isolates were done to identify the bacteria. Sequence retrieval subjected to pairwise local alignment using National Center for Biotechnology Information – Basic Local Alignment Search Tool (NCBI-BLAST) against 16s rRNA gene sequence. The top 10 similar sequences with 94-95% query coverage and around 99% identity were selected for further studies.

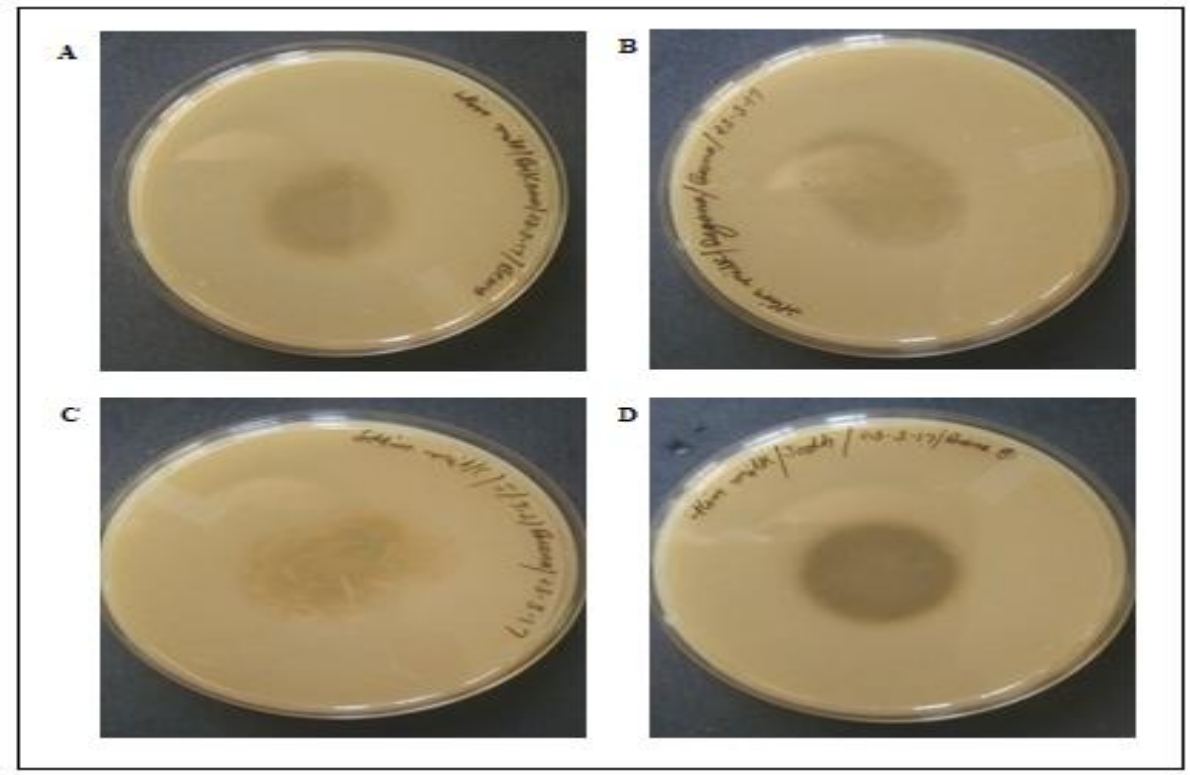

Fig. 2. Skim-milk agar plate showing protease activity of various Bacillus strains isolated from semi-arid soils of: A (Bikaner), B (Nagaur), C and D (Jodhpur).

The selected sequences were the input to Multiple Sequence Alignment (MSA) using Clustal Omega (web) and .aln file was retrieved. The .aln file was used as an input for phylogenetic analysis using MEGA 5 software.

The phylogenetic tree of NJ-method was retrieved. The phylogenetic trees for all isolates are shown in Fig 5. On the basis of above described biochemical and molecular characterization, the isolates were identified as a new strain of *Bacillus licheniformis*.

The 16S rDNA gene sequence of the isolates from B, N, J_1 and J_2 was deposited at Genebank with the accession numbers MG461611 (Bacillus BJ2), MF276798 (Bacillus BKS), MF276898 (Bacillus BSJ) and MG461608 (Bacillus BB1) respectively.

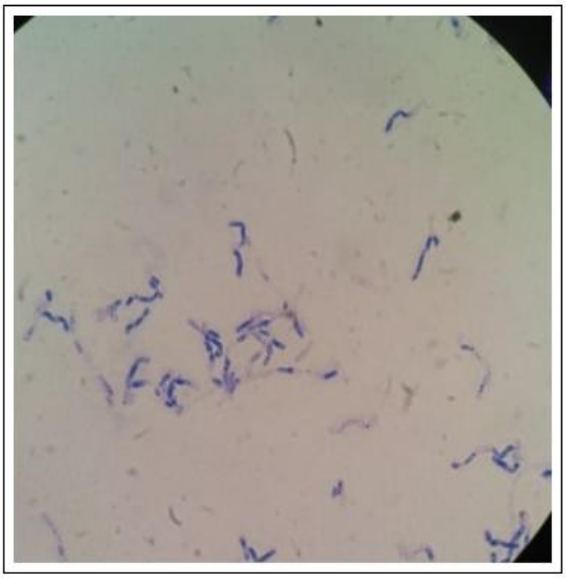

Fig. 3. Gram staining of the isolates (here Jodhpur isolate) identified them as Bacillus species (Gram positive).

Optimization of protease enzyme

After optimization of various culture conditions, the protease activity was calculated to identify the best alkaline protease producer, as mentioned in material and methods. Temperature optimization was done using a range of 25°C to 80°C. The maximum activity was shown by all the strains at 40°C on an average except one specific strain (MG461608) from Jodhpur was 50°C (Fig. 6A). Optimum agitation for protease production was 180 rpm for MG461608 Jodhpur strain giving 230U/ml protease activity.

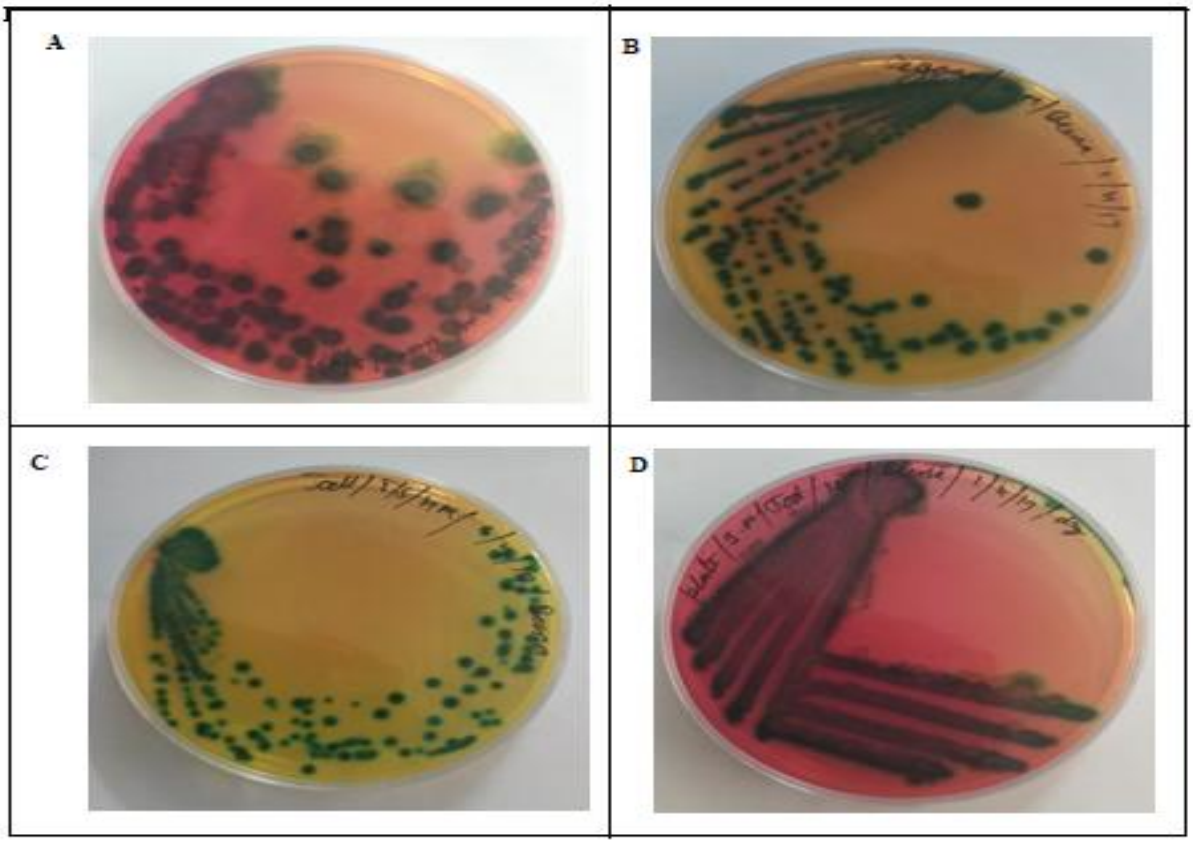

Fig. 4. Hi-Chrome agar plates showing various Bacillus strains isolated from semi-arid soils of: A (Bikaner), B (Nagaur), C and D (Jodhpur).

The other bacterial strains MF276798 produced 170 U/ml whereas MG461611 and MF276898 produced 210U/ml of alkaline protease at 150 rpm (Fig. 6B). Protease production at various pH range from 7 to 13 when studied, exhibited highest activity at pH 10, which was between 200U/ml to 250U/ml (for all 4 strains) (Fig. 6C). All the strains MF276798, MG461608 and MG461611 showed similar activity for any given incubation time while, the Jodhpur strain MF276898 gave an enhanced activity at an incubation period of 72 hours. The activity measured was 270U/ml which was much higher than any other strain studied by us (Fig. 6D). The bacterial strain obtained from Bikaner soil (MG461611) showed maximum production at 60 hours which was 210U/ml, while for the rest three isolates 72 hours was the optimised incubation time. Protease production using varied inoculum concentration was assessed for all strains under study.

With the increasing inoculum concentration, the growth rate along with the protease activity was also found to be enhanced. 0.3% v/v was identified as the optimal inoculum concentration for all our bacterial strains. An inoculum concentration greater than 0.3% decreased the protease activity despite an increased growth rate (Fig. 6E). Glucose proved to be the best carbon source for the maximum protease production, among all the other carbon sources used for all the four strains (Fig. 7A).

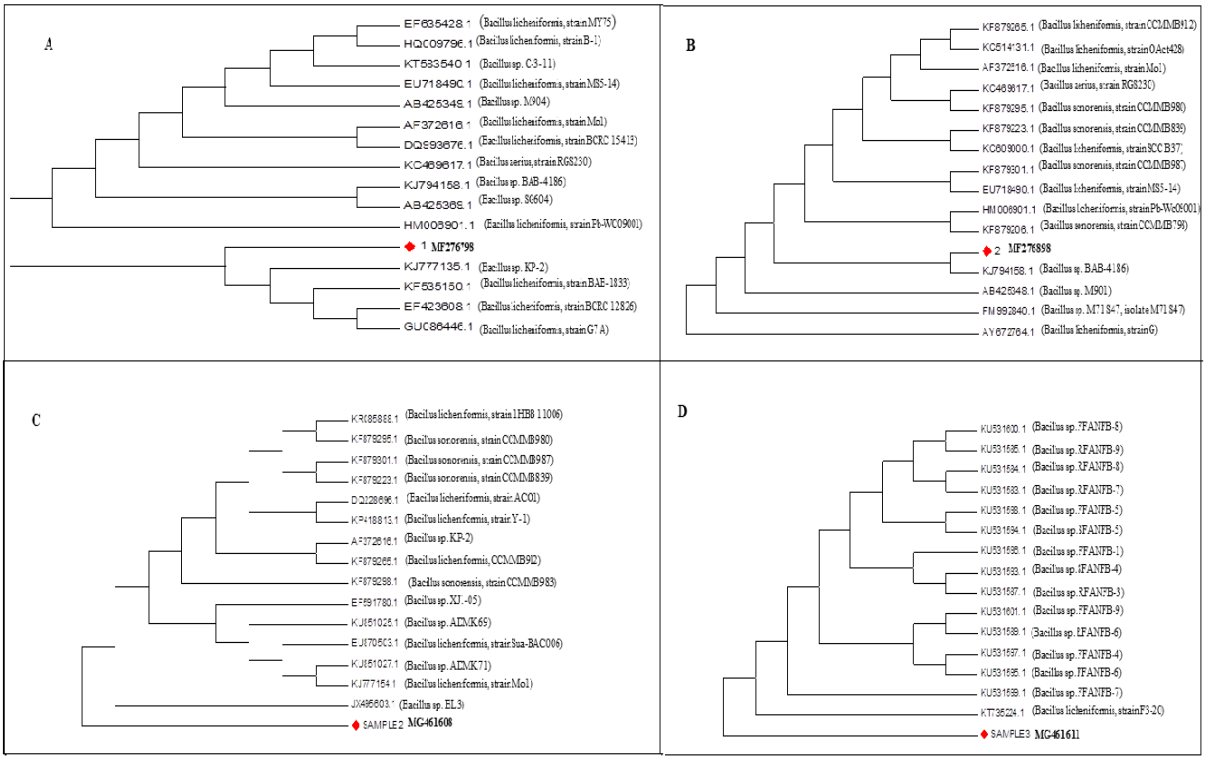

Fig. 5. Phylogenetic analysis of isolated strains from semi-arid soil of Rajasthan A(MF276798), B(MF276898), C(MG461608) and D(MG461611).

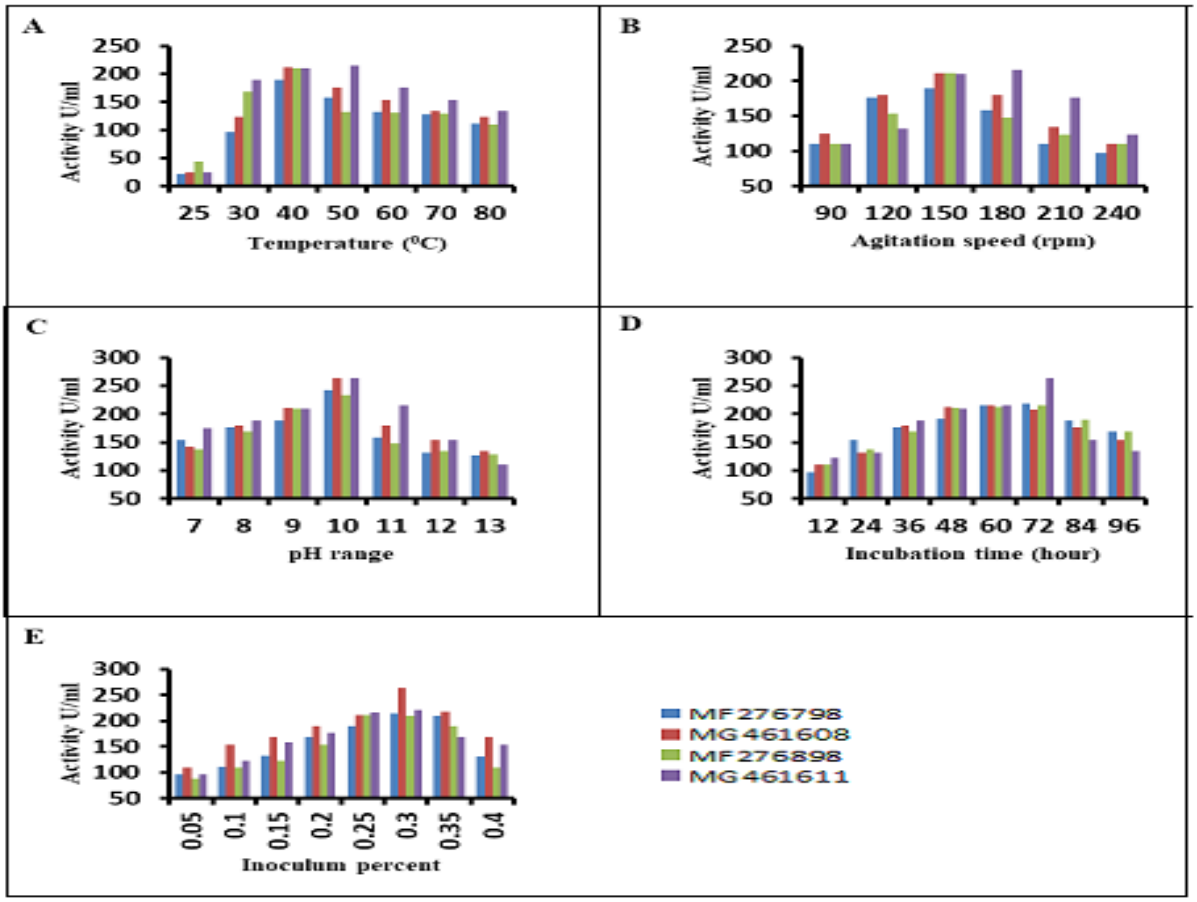

Fig. 6. Alkaline protease activity by isolated Bacillus strains with various culture conditions A- Temperature, B- Agitation Speed, C-pH range, D- Incubation time, E-Inoculum percent.

The protease activity measured was in between 230U/ml to 270U/ml. Protease production using nitrogen sources such as yeast extract, casein, beef extract, peptone, tryptone, gelatin was studied. Casein was found to be most suited for maximum protease production in all four strains (Fig.7B). Various nitrogen source combinations were examined for maximum protease production. Yeast extract with casein and peptone with yeast extract showed maximum protease activity (Fig. 7C). Mn+2 were the optimized divalent ion on which all the studied bacterial strains exhibited maximum protease activity (Fig. 7D).

Discussion

In the present study we were able to identify four *Bacilli* strains *viz*. MF276898, MG461608, MG461611 and MF276798 from the semi-arid soil of Rajasthan with an inherent ability to produce alkaline protease enzyme.

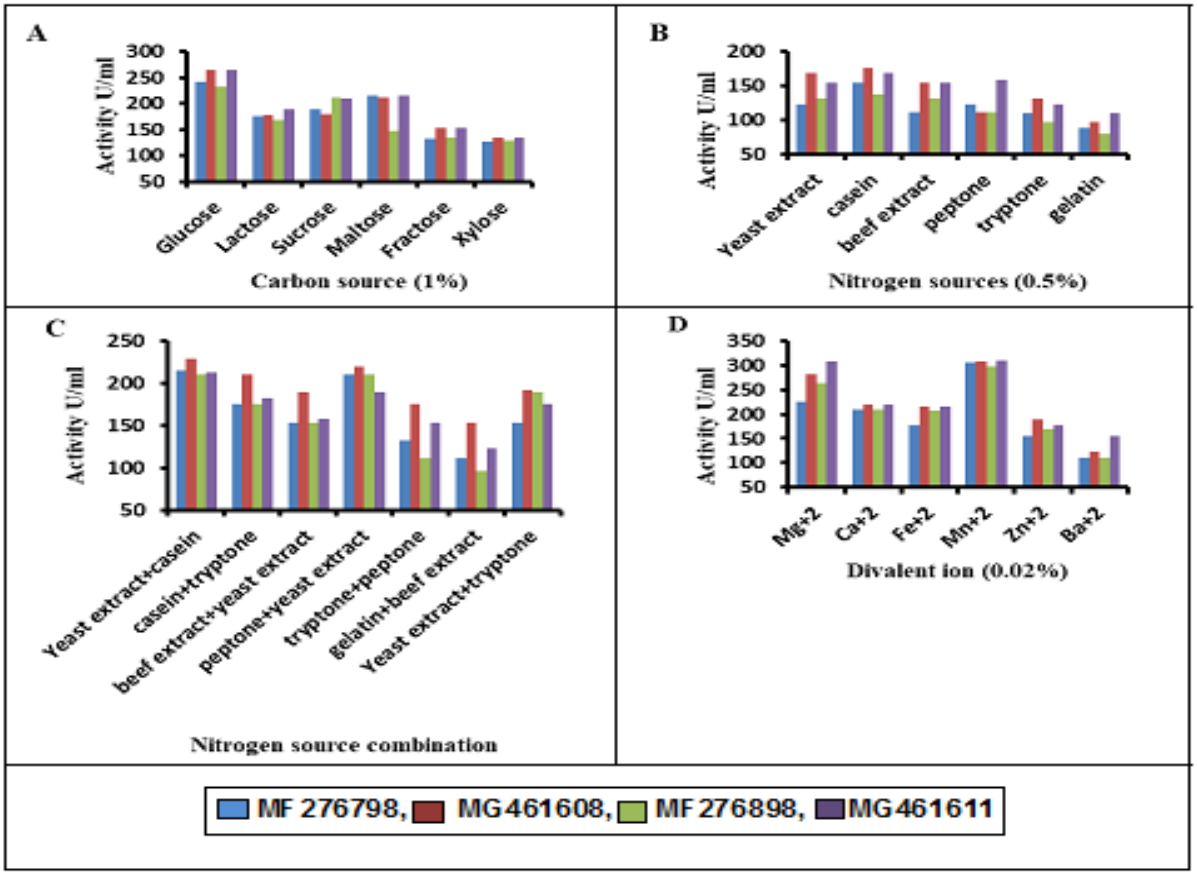

Fig . 7. Alkaline protease activity by isolated *Bacillus* strains with various culture conditions A- Carbon source, B- Nitrogen source, C-Nitrogen source combination, D- Divalent ion.

Various extrinsic parameters optimized for protease production showed the optimum temperature conditions were between 40°C and 50°C. Frankena *et al.*, 1986 reported that the enzyme synthesis and its mechanism in bacterial culture are controlled by temperature as seen in our study as well. Ahamed *et al.,* 2016, Dorcas and Pindi, 2016 and Kalpana *et al.,* 2016 also optimized the temperature for the production of alkaline protease by different *Bacillus* strains from environmental samples. Our findings also showed similar results. Moon and Parulekar, 1991 reported that enzymatic processes of alkaline protease enzyme were strongly affected by the culture pH.

This was also seen for the present investigation with pH 10 giving much enhanced activity in all the four strains. Palsaniya *et al.*, 2012 also optimized pH 10 for the production of enzyme from bacteria (*Bacillus subtillis* and *Serratia marscens*) isolated from soil of

Rajasthan. The optimum incubation period was 72 hours which is similar to that stated by Dorcas and Pindi, 2016 for *Bacillus cereus* isolated from poultry farm. The best carbon source for our study was glucose in comparison to lactose, sucrose, maltose, fructose and xylose. Badhe *et al.,* 2016 and Dorcas and Pindi, 2016 also reported that among various carbon sources, glucose showed maximum enzyme production by bacillus strains. Sonnleitner, 1983 and Gajju *et al.,* 1996 reported that lack of glucose in media resulted in dramatic decrease in enzyme production. The optimized metal ion was Mn^{+2} at 0.02% concentration when highest protease production was observed. Similar findings were reported by Asha and Palaniswamy, 2018 by bacterial strain isolated from organic waste containing soil.

Optimized nitrogen source was casein for the present study. Ward, 1995 and Mehta *et al.,* 2006 reported that enzyme production was influenced by nitrogen source and Gupta and Khare, 2007 explained the different nitrogen source requirements by various bacterial strains. Our results are in concomitance with those of El Zawahry *et al.* 2007; Jayasree *et al.,* 2010 and Asha and Palaniswamy, 2018 who also reported casein to be the best nitrogen source. In several other studies, as a sole nitrogen source, yeast extract resulted in higher protease production than casein (Vanitha *et al.*, 2014; Badhe *et al.,* 2016). In our study, a combination of two nitrogen sources gave better results than any single nitrogen source. The optimized combination was yeast extract and casein for one *Bacillus* strain MG461608 while it was a combination of peptone and yeast extract for other three. As reported in literature as well as in the present study, *Bacillus* strains obtained from soils have been the most potent alkaline protease producers.

Conclusion

The present study reports four new strains of *Bacillus licheniformis* from the semi-arid soil of Rajasthan. These isolates can survive on high pH and temperature and produce significant amount of alkaline protease enzyme. Optimized culture conditions of these bacteria prove that they are suitable for various industrial processes. These isolates are being further subjected to isolation and purification of alkaline protease enzyme.

Acknowledgement

The authors thank the Director, Centre for Converging Technologies (CCT) for providing laboratory facilities. Author BKS acknowledges DST PURSE program for providing financial assistance (NET JRF) for undertaking the studies. We are also thankful to Dr Ajit Kumar, DBT-Bioinformatics Infrastructure Facility, and Centre for Bioinformatics, Maharshi Dayanand University, Rohtak, Haryana (MDU) for Bioinformatics studies.

Conflict of Interest

The authors declare no conflict of interest

References

Abdel-Naby M, Ismail AMS, Ahmed SA, Fattah AFA. 1998. Production and Immobilization of alkaline protease from Bacillus Mycoides. Bioresource Technology **64,** 205-210. https://doi.org/10.1016/S0960-8524(97)00160-0

Ahamed M, Rehman R, Siddique A, Hasan F, Ali N, Hameed A. 2016. Production, purification and characterization of detergent-stable, halotolerant alkaline protease for eco-friendly application in detergents industry. International Journal of Biosciences **8,** 47-65. http://dx.doi.org/10.12692/ijb/8.2.47-65

Asha B, Palaniswamy M. 2018. Optimization of alkaline protease production by Bacillus cereus FT1 isolated from soil. Journal of Applied Pharmaceutical Science **8(02),** 119-127. http://dx.doi.org/10.7324/JAPS.2018.8219

Asker MMS, Mahmoud MG, Shebwy KE, Aziz MSA. 2013. Purification and Characterization of two thermostable protease functions from *Bacillus* megaterium. Journal of Genetic Engineering and Biotechnology **11(2),** 103-109.

https://doi.org/10.1016/j.jgeb.2013.08.001

Badhe P, Joshi M, Adivarekar R. 2016. Optimized production of extra cellular proteases by Bacillus subtilis from degraded abattoir waste. Journal of Bioscience and Biotechnology **5,** 29-3.

Baweja M, Tiwari R, Singh PK, Nain L, Shukla P. 2016. An Alkaline Protease from Bacillus pumilus MP 27: Functional Analysis of Its Binding Model toward Its Applications as Detergent Additive. Frontiers in microbiology **7,** 1195. https://dx.doi.org/10.3389%2Ffmicb.2016.01195

Beg KB, Sahai V, Gupta R. 2003. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochemistry **39,** 203-209. https://doi.org/10.1016/S0032-9592(03)00064-5

Bergey DH, Buchanan RE, Gibbons NE. 1974. Bergey's Manual of Determinative Bacteriology, 8th edition. The Williams and Wilkins Co., Baltimore, USA 15-36.

Dorcas K, Pindi PK. 2016. Optimization of protease production from Bacillus cereus. International Journal of Current Microbiology and Applied Sciences **5,** 470-478. http://dx.doi.org/10.20546/ijcmas.2016.506.054

El Zawahry YA, Awny M, Tohamy EY, AbouZeid AAM, Reda FM. 2007. Optimization, characterization and purification of protease production by some Actinomycetes isolated under stress conditions. Proceeding of The Second Scientific Environmental Conference Zagazig University, 153-175.

Frankena J, Koningstein GM, Van Verseveld HW, Stouthamer AH. 1986. Effect of different limitations in chemostat cultures on growth and production of exocellular protease by Bacillus licheniformis. Applied Microbiology Biotechnology **24,** 106-12.

Gajju H, Bhalla TC, Agarwal OH. 1996. Thermostable alkaline protease from thermophilic Bacillus coagulans PB-77, Indian Journal of Microbiology **36,** 153-155.

Godfrey T, West S. 1996. Industrial enzymology. 2nd ed., New york: McMillan Publishers Inc. app.

Gupta A, Khare SK. 2007. Enhanced production and characterization of a solvent stable protease from solvent tolerant Pseudomonas aeruginosa PseA. Enzyme and Microbial Technology **42,** 11-16. http://dx.doi.org/10.1016/j.enzmictec.2007.07.019

Gupta R, Gupta K, Saxena RK, Khan S. 1999. Bleach stable alkaline protease from Bacillus sp. Biotechnology letters **21**, 135-138.

Horikoshi K. 1999. Extracellular Enzymes. Isolation procedures for alkaline enzymes. In: Alkaliphiles. Tokyo: Kodenshs Ltd 147.

Huston WM. 2010. Bacterial proteases from the intracellular vacuole niche; protease conservation and adaptation for pathogenic advantage. FEMS Immunology and Medical Microbiology **59(1),** 1-10. https://doi.org/10.1111/j.1574-695X.2010.00672.x

Jayasree D, Sandhya Kumari TD, Kavi Kishor PB, Vijayalakshmi M, LakshmNarasu M. 2010. Optimization of production protocol of alkaline protease by Streptomyces pulvereceus. Inter JRI Science Technology **1(2),** 79- 82.

Kalisz HM. 1988. Microbial Proteinases. Advances in Biochemical Engineering/Biotechnology **36,** 1-65.

Kalpana VN, Sravani N, Vigneshwari T, Devi Rajeshwari V. 2016. An inexpensive substrate for the production of alkaline protease by Bacillus sp and its application studies of Manihotesculenta. Der Pharmacia Lettre **8,** 220-232.

Krik O, Borchert TV, Fuglsang CC. 2002. Industrial enzyme applications. Current Opinion in

Biotechnology **13,** 345–435. https://doi.org/10.1016/S0958-1669(02)00328-2

Kumar S. 2007. Caspase functions in programmed cell death. Cell Death and Differentiation **14(1),** 2-43. https://doi.org/10.1038/sj.cdd.4402060

Lowry OH, Rosebrough N, Farr AL, Rondall RL. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193,** 265–73.

Mehta VJ, Thumar JT, Singh SP. 2006. Production of alkaline protease from alkaliphilic actinomycete. Bioresource Technology **97,** 1650-1654. https://doi.org/10.1016/j.biortech.2005.07.023

Moon SH, Parulekar SJ. 1991. A parametric study of protease production in batch and fed-batch cultures of Bacillus firmus. Biotechnolology and Bioengineering **37,** 467-83. https://doi.org/10.1002/bit.260370509

Mukhtar H, Haq I. 2008.Production of alkaline protease by Bacillus subtilis and its application as a depilating agent in leather processing. Pakistan Journal of Botany **40(4),** 1673-1679**.**

Palsaniya P, Mishra R, Beejawat N, Sethi S, Gupta BL. 2012. Optimization of alkaline protease production from Bacteria isolated from soil. Journal of Microbiology and Biotechnology Research **2(6),** 858-865.

Sandhya C, Sumantha A, Szakacs G, Pandey A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae*in submerged and solid-state fermentation. Process Biochemistry **40,** 2689–2694. https://doi.org/10.1016/j.procbio.2004.12.001

Soh UJ, Dores MR, Chen B, Trejo J. 2010. Signal transduction by protease-activated receptors. British Journal of pharmacology **160(2),** 191-203. https://dx.doi.org/10.1111%2Fj.14765381.2010.00705.x

Sonnleitner B. 1983. Biotechnology of thermophilic bacteria growth, products and application. Advances in Biochemical Engineering/Biotechnology **28,** 70-138.

Takami H, Akiba T, Horikoshi K. 1989. Production of extremely thermostable alkaline protease from Bacillus sp. no. AH-101. Applied Microbiology and Biotechnology **30(2),** 120-124.

Vanitha N, Rajan S, Murugesan AG. 2014. Optimization and production of alkaline protease enzyme from Bacillus subtilis 168 isolated from food industry waste. International Journal of Current Microbiology and Applied Sciences **3,** 36-44.

Ward OP, Blanch HW, Drew S, Wang DI. 1985. Proteolytic enzymes In: Comprehensive Biotechnology. Vol **3.** Oxford, UK: pergamon press, 789-818.

Ward OP. 1995. Proteolytic enzymes. In M Moo-Young edition comprehensive Biotechnology **3,** 789-818.