International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 11(1): 187-194, February 2018
DOI: 10.30954/0974-1712.2018.00178.24

MICROBIOLOGY

Optimization of Extracellular Alkaline Protease Production From *Pseudomonas aeruginosa* Isolated from Soil Samples

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Paper No. 667 Received: 27-09-2017 Accepted: 09-01-2018

ABSTRACT

Proteolytic enzymes are ubiquitous in nature present in all forms of life from viruses to animals. Among them microbial proteases secure most significant position due to their vast industrial applications. In this study 148 bacterial strains were isolated from 50 different soil samples of slaughter house, fish market and sewage wastes of Lucknow, Uttar Pradesh, India. Out of which fifty six strains competent of secreting extracellular alkaline protease. In preliminary screening the isolate SSB1 showed highest ability to hydrolyzed casein and skimmed milk which was done on skim milk agar media. The isolates showed positive for casein, gelatin, oxidase, starch, and catalase test and negative for gram staining, indole, methyl red, voges proskauer, urea, hemolysis and triple sugar iron (TSI) test and found to be motile. The culture conditions were optimized for utmost enzyme production. Many physical parameters were deliberated to optimize the maximum yield of alkaline protease. The optimum conditions for the maximum protease production were when cultivated for 36 h at 37°C in a medium containing 1% glucose, 1% yeast extract, 1M NaCl, and 1mM Zn^{2+} at pH 9.0. Strain SSB1 with the maximum yield alkaline protease was identified as *Pseudomonas aeruginosa* based on nucleotide homology and phylogenetic analysis. The study revealed that the bacterial strain of *P. aeruginosa* is a potent source of extracellular alkaline protease and optimization of culture conditions for the maximum yield and their applications in different industries.

Highlights

- SSB1 strain identified as *Pseudomonas aeruginosa*, a protease producing bacterium from soils, a potential producer of protease which could find applications in industry & biotechnology.

Keywords: *Pseudomonas aeruginosa*, Skim milk agar, Enzyme-producing conditions, Extracellular alkaline protease, 16S rDNA, soil samples

Alkaline proteases (EC 3.4.21–24, 99) constitute one of the most important group of industrial enzymes and they catalyze the peptide bond cleavage in proteins. (Subba *et al*. 2009). In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts which are active in a neutral to alkaline pH range (Jisha *et al.* 2013). They also have a serine center or are of metal- type and the alkaline serine proteases are the most considerable group of enzymes so far exploited (Gupta *et al.* 2002). Microbial alkaline proteases have power over on the worldwide enzyme market, accounting for two-third of the share of the detergent industry. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate novel enzymes with altered properties that are desirable for their various applications in laundry detergent formulation, leather finishing, silk industry, feeds modification, food processing,

brewing, pharmaceuticals, diagnostic reagents, peptide synthesis, silver recovery from X ray/ photographic films, preparation of organic fertilizers and several processes of waste treatment (Kuddus and Ramteke 2008, 2009, 2011; Rawlings *et al*. 2011; Furhan and Sharma 2014; Sharma *et al*. 2017). The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry (Gupta *et al*. 2002; Kuddus *et al.* 2013. Sawant and Nagendrans 2014; Abdelnasser *et al.* 2015. Shine *et al.* 2016; Patil and Jadhav 2017). Therefore in commercial practice, the optimization of production parameters for cost effective yield of enzymes, has gained parameteric importance of alkaline protease from different microbial sources. In the present study, we report the isolation and selection of *Pseudomonas aeruginosa* (SSB1), which is the potent producer of extracellular alkaline protease, and the effects of culture conditions on the production of alkaline protease by the isolate.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strain

Soil samples from slaughter house (30), fish markets (15), sewage wastes (05) were collected at Lucknow, Uttar Pradesh, India and brought to the laboratory in sterile containers. The collected samples were serially diluted in sterile physiological saline (0.85% NaCl) and the diluted sample were plated on nutrient agar plates (pH 8) and incubated at 37°C for 48h. Colonies from the agar plates were selected based on divergence in morphology, size and color and were stored at -20°C for further processing.

Screening of protease producing bacteria

Primary screening was done on nutrient agar plates supplemented with 1% casein by spot inoculation. The plates were incubated at 37°C for 48 h. The protease producing strains were selected based on the zone of clearance and after that individual colonies were further screened onto skim milk agar plates (Chu 2007). A clear zone of skim milk hydrolysis was considered as an indication of the protease producing organism.

Proteolytic activity assay

Protease assay was performed by a modification of the casein digestion method of Kunitz (1947). The assay was performed at alkaline range of 8-10 pH. Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Proteolytic activity with azocasein used as substrate was assayed by the modification method of Secades and Guijarro (1999). In briefly, 120 µl of the enzyme solution or cell-free supernatant was added to 480 µl azocasein (1% wt/vol) in reaction buffer (0.1 M Tris buffer, pH 9) and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 600 µl of 10% (wt/vol) trichloroacetic acid and resulting precipitate was allowed to settle for 30 min on ice, followed by centrifugation at 15000 × g at 4 °C for 10 min. A suitable control was run simultaneously, in which trichloroacetic acid was added into the incubation mixture at zero time used as a blank. For the neutralization 200 µl of 1.8 N NaOH was added to 800 µl of supernatant solutions. The final absorbance was measured at 420nm (A_{420}) using the 20 UV-Visible spectrophotometers. The protease activity is expressed in the unit as one unit of enzyme activity was defined as the yielded an increase in A_{420} of 0.01 in 30 min at 30 °C. Protein was determined by the method of Folin Lowry method (Lowry *et al*. 1951).

Identification of the bacterial strain

For identification of SSB1 isolate, both physiological and biochemical tests were performed as per Holt *et al*. (1994). The potential bacterial strain was identified using 16S rDNA gene sequence analysis as per the standard protocols. The bacterial isolate was grown overnight in 5 ml alkaline broth medium. Total DNA was extracted using D Neasy Kits (Qiagen, USA) according to the manufacturer's instructions. Gene-specific forward primer: 16s27F (5'-AGA GTT TGA TCC TGG CTC AG-3'), and reverse primer: 16s1115R (5'-AAG GAG GTG ATC CAG CCG CA-3') were used to amplify 16S rDNA gene. The purified 16S-rDNA was sequenced using an ABI 3730xl Genetic Analyzer (Eurofins Genomics India Pvt Ltd. Bangalore), and the obtained sequence was aligned with reference 16S rDNA sequences available in NCBI homepage (National Center for Biotechnology Information) using the BLAST algorithm.

Enzyme production

The basal culture medium for the protease production broth medium contained (g^{-1}): glucose 0.5, yeast extract 0.5, peptone 0.5, KH_2pO_4 0.1, $MgSO_47H_2O$ 0.01, pH 9. Desired pH was obtained by adding sterile 10% Na_2CO_3 solution after autoclaving. The medium (100ml) was inoculated with 1 ml of a 24 h old culture in 500 ml conical flask, and incubated at 37^0C with shaking at 250 rpm for 48h. The cell free supernatant was recovered by centrifugation at 10,000Xg for 15 min at 4 °C and used for determining the protease activity.

Optimization of incubation time, temperature, pH for protease production

To determine the various physical parameters for maximum production of protease, the 24h grown culture were subcultures (@1:100) in 250 ml flask contain 100 ml protease producing broth. For optimizing pH, the medium was prepared by varying the pH from 4.0 to 13.0 at 1.0 unit interval incubate in shaker (150 rpm), For optimizing temperature, the medium was prepared by varying the temperature at 30 to 50°C for 24-120 hours. Then 10 ml of the culture samples withdrawn from the medium were collected every 24 hours after incubation. The sample cultures were filtered, followed by centrifugation at 7000 rpm for 30 min. The culture filtrate solutions obtained were used to determine protease activity .All the experiments were conducted in triplicates.

Optimization of carbon, nitrogen sources, NaCl and metallic ions for protease production

The effect of various chemical parameters on protease production was studied by varying the carbon sources (glucose, fructose, maltose, sucrose, and lactose (1% w/v)) and nitrogen sources (peptone, yeast extract, beef extract, ammonium sulphate and sodium nitrate (1% w/v)). The NaCl salt concentrations various from 0 to 2 M was supplemented. Divalent metal ions such as Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+} ,Mg^{2+} and Ca^{2+} each 1mM were used to evaluate the impact on alkaline protease production. After 48 hours incubation at 37°C, 150 rpm, the cell-free supernatants were quantified for protease production.

Statistical analysis

All the experiments, enzyme assay, and cells growth measurement were carried out in triplicate, and the standard deviation for each test was calculated using SPSS 20. The standard deviations are indicated as error bars.

RESULTS AND DISCUSSION

Isolation, Screening and identification of the bacterial strain

One hundred forty eight bacterial strains were isolated from 50 different soil samples of slaughter house, fish market and sewage wastes of Lucknow, Uttar Pradesh, India. Out of which fifty six strains competent of secreting extracellular alkaline protease. The isolates were then purified, preserved and tested for their proteolytic ability. In preliminary screening the isolate SSB1 showed highest ability to hydrolyzed casein and skimmed milk (Fig. 1) and was finally selected for further optimization of the extracellular alkaline proteases production.

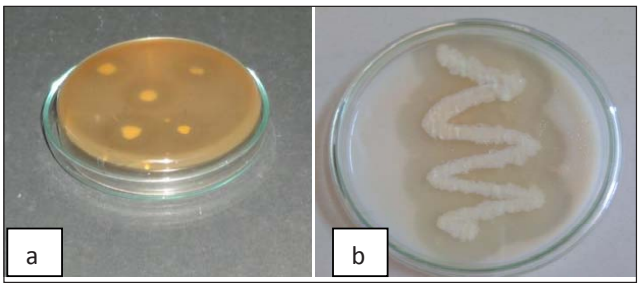

Fig. 1: Proteolytic activity of SSB1 **(a)** Spot inoculation, **(b)** Spread inoculation

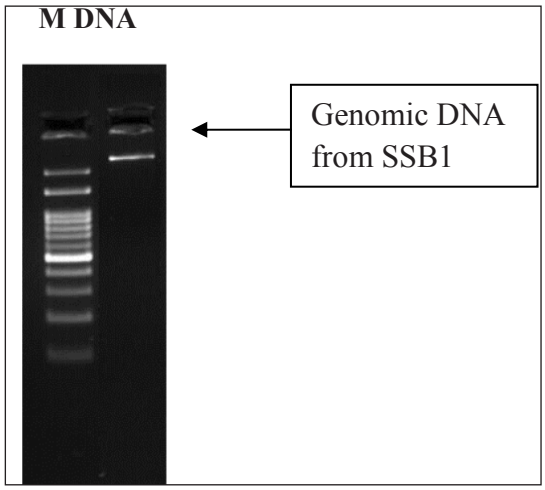

Fig. 2: Agarose gel electrophoresis of P.aeruginosa (SSB1) genomic DNA. Lane M: 1 kb DNA ladder. Lane DNA: bacterial genomic DNA marked by an arrow

The isolate showed positive for casein, gelatin, oxidase, starch, and catalase test and negative for gram staining, indole, methyl red, voges proskauer, urea, hemolysis and triple sugar iron (TSI) test and found to be motile. Based on nucleotide homology and phylogenetic analysis, SSB1 was identified as *Pseudomonas aeruginosa*. (Fig. 2-5).

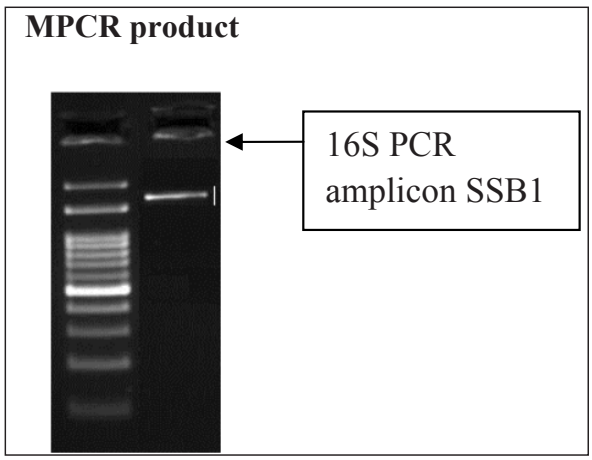

Fig. 3: Agarose gel electrophoresis of PCR amplicon P.aeruginosa (SSB1) Lane M: 1 kb DNA ladder. Lane DNA: PCR amplicon marked by an arrow

Optimization of parameters for maximum enzyme production

Incubation time

Maximum enzyme production was obtained after 36 h of incubation (Fig. 6). These results are in accordance with observations made by other workers, where maximum enzyme production was observed during continuous growth of the culture at the late exponential phase and early stationary phase of the growth (Durhams *et al.* 1987; Gashaw and Gessesse 1997 and Qadar *et al.* 2009). The reduction in protease yield after the optimum period was probably due to depletion of nutrients available to microorganisms. (Akcan 2011; Sandhya *et al*. 2005).

Temperature

Temperature is one of the most important factors that affect the enzyme production. The incubation temperature of a fermentation process has a profound role to play in the growth and in turn on

>SSB1 Consensus data

TGATACGTGGAGCGGACGAAGGGAGCTACGCACCCGGATTCCGCGGCGGTAGGGTGAGTAATGCCTAGGAATCTGCCT
GGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACC
TCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG
TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAG
GAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCG
CGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGT
GAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTG
TGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTTGACTGATACTGACACTGAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGAT
CCTTGAGATTTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGAC
ATGCTGAGAACTTTCCAGAGATGTAGTGGTGCCTTCCGGGGAACCTCGTAGTACCTTAGCTGCGCTGCGTGCG

Description	Max score	Total score	Query coverage	E value	Max ident	Accession
Pseudomonas aeruginosa strain HMR16 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KU174205.1
Pseudomonas aeruginosa strain SNCK1 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KT588297.1
Pseudomonas aeruginosa strain Zn 7 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KT444586.1
Pseudomonas aeruginosa strain Zn 2 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KT444583.1
Pseudomonas aeruginosa strain KASK 1142 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KT456549.1
Pseudomonas aeruginosa strain KASK 1061 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KT456548.1
Pseudomonas aeruginosa strain PA018 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KP728995.1
Pseudomonas aeruginosa strain PA017 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KP728994.1
Pseudomonas aeruginosa strain PA016 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KP728993.1
Pseudomonas aeruginosa strain PA015 16S ribosomal RNA gene, partial sequence	928	928	100%	0	99%	KP728992.1

Fig. 4: Sequences producing significant alignments *P.aeruginosa* (SSB1) carry out BLAST with the nrdatabase of NCBI GenBank database

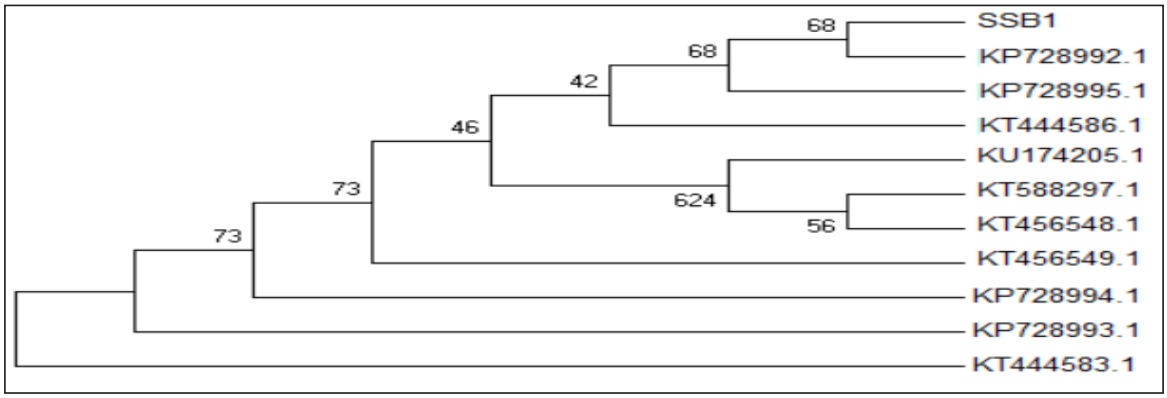

Fig. 5: Phylogenetic tree *P. aeruginosa* (SSB1) was constructed using MEGA 4

the metabolic activities of the microbial cells. When the optimum temperature for the production of the protease was investigated from 30°C to 50°C, 37°C was found to be the optimum temperature for protease production. (Fig. 7). Several reports indicate that maximum protease production was achieved at 37- 45°C for *Bacillus subtilis* (Mukherjee *et al*. 2008). Zambare *et al*. (2011) reported that optimum temperature for maximum production of protease by *P. aeruginosa* MCM B-327 was 35°C.

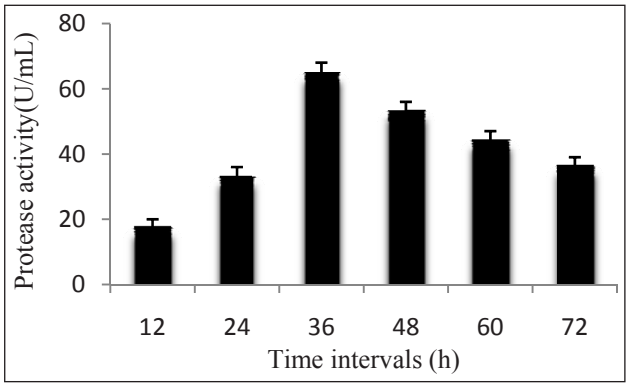

Fig. 6: Incubation period of protease production in *P. aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3).

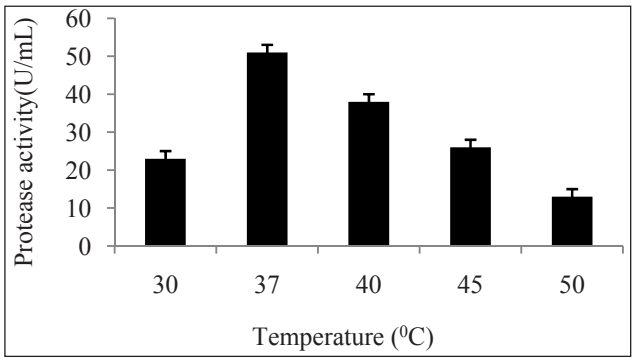

Fig. 7: Effect of incubation temperature on protease production in *P. aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

pH

The pH of culture strongly affects many enzymatic processes. The pH change observed during the growth of microbes also affects product stability in the medium. The isolate *P.aeruginosa* was studied at pH ranging from 4.0 to 13.0. Best suited pH for *P. aeruginosa* was 9.0 (Fig. 8). Comparable values of pH optima were reported for other proteolytic strains. The optimum pH for protease production in a *Bacillus subtilis* SAL1 was 9.0 (Almas *et al*. 2009). Protease production was best at pH 9.0-12.0 in *B. patagoniensis* PAT05 (Olivera *et al*. 2006). Studies related with optimum pH 9.0 for protease production in *P. aeruginosa* PseA (Gupta and Khare 2007).

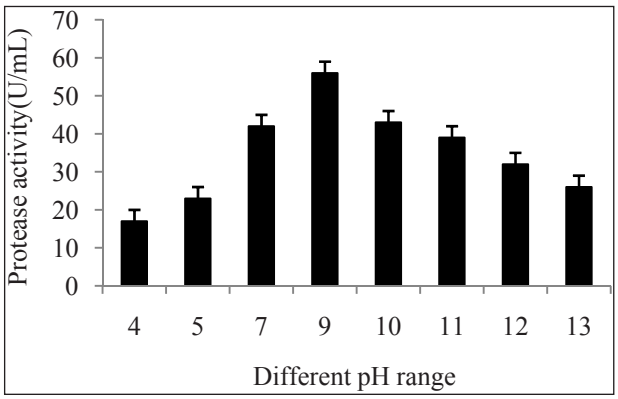

Fig. 8: Effect of pH on protease production in *P. aeruginosa.* The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Carbon

Different carbon supplements were added in the protease production broth. The results indicate that the maximum enzyme production was enhanced by the addition of carbon enriched supplements like glucose, fructose, maltose, sucrose, and lactose.

Among these supplements, maximum enzyme production were obtained when glucose was used as carbon supplement (Fig. 9). These results are related with previous studies showing highest protease production when glucose is used as a carbon source in the protease production broth (Pastor *et al*. 2001; Santhi 2014).

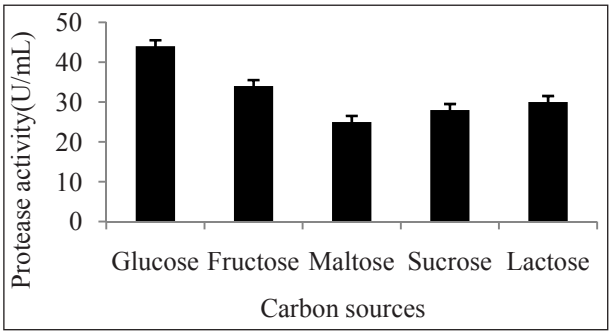

Fig. 9: Effect of carbon sources on protease production in *P.aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Nitrogen sources

Peptone, yeast extract, beef extract, ammonium sulphate and sodium nitrate were added in the production medium as a nitrogen supplements. Among these supplements, maximum enzyme production was obtained when yeast extract and peptone were used after 48 hours of incubation. (Fig. 10). Several studies also suggest that yeast extract and peptone is a highly suitable nitrogen source, which stimulates protease production by using various microbial species (Yum *et al.* 1994; Ashour *et al*. 1996). The production of protease probably was enhanced due to the high protein and amino acid components in the yeast extract and peptone.

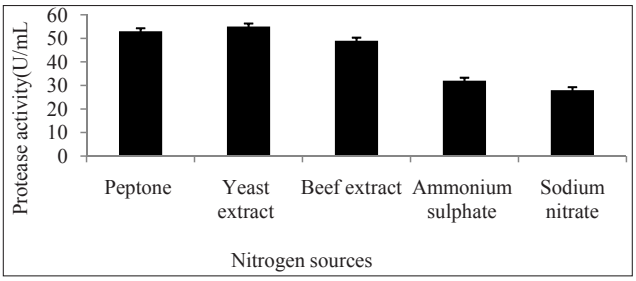

Fig. 10: Effect of nitrogen sources on protease production in *P. aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

NaCl

The *Pseudomonas aeruginosa* was showed to NaCl tolerance up to 2 M concentration. It was observed that the growth medium containing 1.0 M NaCl yielded the maximum activity in *P.aeruginosa* (Fig. 11). The production of the protease was gradually reduced when salt concentration was increased above 1.5 M NaCl. Shivanand and Jayaraman (2009) reported a similar finding for the maximum protease production.

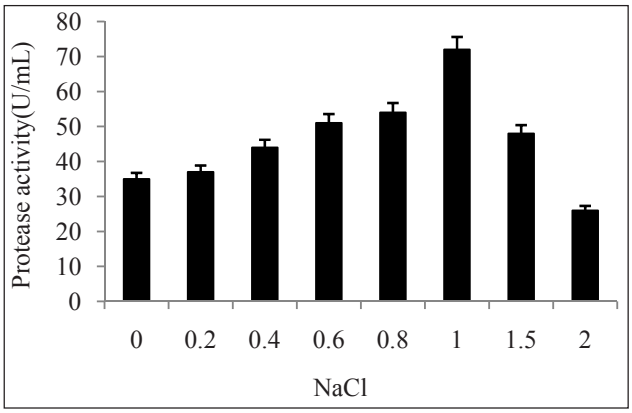

Fig. 11: Effect of NaCl concentration on the production of protease in *P. aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

Metallic ions

The impact of various metal ions on alkaline protease production was evaluated, and the results are demonstrated in Fig. 12. The protease production significantly enhanced by adding cations such as Cu and Zn compared to the control. However, most of the other tested ions caused a significant reduction of the enzyme yield, particularly at high concentration. These results corroborate the earlier findings of metal ions such as Zn^{2+} enhancing the activity of protease in *Bacillus subtilis*. Mcconn *et al*. (1964) and *Bacillus licheniformis* Vigneshwaran *et al.* (2010).

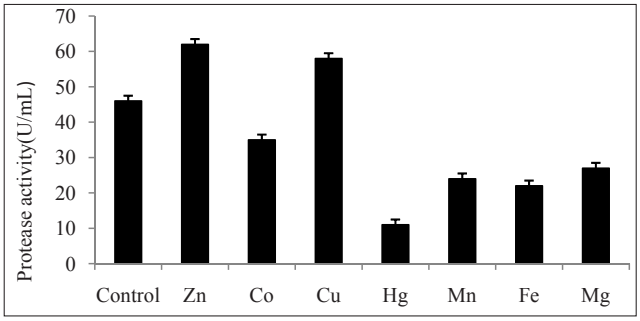

Fig. 12: Effect of metallic ions on the production of protease in *P. aeruginosa*. The bars indicate the standard deviation (± S.D) of three replicates (n = 3)

CONCLUSION

In conclusions, the present study suggests that strain SSB1 is a novel alkaline protease producing bacterium isolated from the soil sample of slaughter house from Lucknow, Uttar Pradesh, India. The organism was identified as *Pseudomonas aeruginosa* based on biochemical tests and 16S rDNA sequence analysis. The optimum conditions for the maximum protease production were when cultivated for 36 h at 37°C in a medium containing 1% glucose, 1% yeast extract, 1M NaCl, and 1mM Zn^{2+} at pH 9.0. This suggests that *P. aeruginosa* SSB1 can be a potential producer of extracellular protease which could find applications in the industry and biotechnology. Further studies will be carried on purification, characterization and industrial applications of alkaline protease enzyme are in progress.

ACKNOWLEDGEMENTS

This paper is the part of PhD research work of the first author. The authors are thankful to the Director, MRD Life Sciences, Lucknow and Eurofins Genomics India Pvt. Ltd. Bangalore. Authors also express their gratitude to the Vice Chancellor, Sam Higginbottom University of Agriculture, Technology and Sciences, for his guidance and the necessary facilities provided.

REFERENCES

Abdelnasser, S.S., I. Ali, A. Al-Salamah, Y.B. Elbadawi, M.A. Tayeb, and S.S. Ibrahim. 2015. Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes. *Electronic Journal of Biotechnology,* **18**: 236–243.

Akcan, N. 2011. Production of extracellular protease in submerged fermentation by *Bacillus licheniformis* ATCC 12759. *African J. Biotech.,* **11(7):** 1729-1735.

Almas, S., A. Hameed, D. Shelly, and P. Mohan. 2009. Purification and characterization of alkaline protease from *Bacillus subtilis* SAL1. *African J. Biotechnol.,* **8**: 3603-3609.

Anwar, A. and Saleemuddin, M. 2000. Alkaline protease from *Spilosoma obliqua*: potential applications in bioformulation. *Biotechnol. Appl. Biochem.,* **31**: 85-89.

Ashour, S.A., Shore, E.L. Metwally, M. and Habib. S.A. 1996. Fungal fermentation of Whely incorporated with certain supplements for the production of protease. *Microbios,* **86**: 59-69.

Banerjee, R. and Battacharyya, B.C. 1992. Optimization of multiple inducers effect on protease biosynthesis by *Rhizopus oryzae. Bioprocess Engineering,* **7**: 225-229.

Banerjee, U.C., Sani, R.K, Azmi, W. and Soni, R. 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Proces Biochemistry* **35**:213-219.

Chu, W.H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. *J.Ind. Microbiol. Biot.,* **34**: 241-245.

Durham, D.R., Stewart, D.B. and Stellwag, E.J. 1987. Novel alkaline-and heat-stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. *Journal of Bacteriology*, **169(6):** 2762-2768.

Furhan, J. and Sharma, S. 2014. Microbial alkaline proteases: findings and applications. *Int. J. Inv. Pharm. Sci.,* **2(4)**: 823-834.

Gashaw, M. and Gessesse, A. 1997. Thermostable amylase production by immobilized thermophilc *Baccillus* sp. *Biotechnology Techniques,* **11(6):** 447-450.

Gupta, R., Beg. Q.K. and Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, **59**: 15-32.

Gupta, A. and Khare, S.K. 2007. Isolation and optimization of alkaline protease from *Pseudomonas aeruginosa* PseA *.Enzyme Microb.Technol.,* **42***:* 11-16.

Holt, J.G., Krieg, N.R., Sneth, P.H.A, Stately, J.T. and Williams, S.T. 1994. *Bergeys manual of Determinative Bacteriology,* 9th Ed., Williams and Wilkins, Baltimore.

Jisha, V.N., Smitha, R.B, Pradeep. S, Sreedevi. S, Unni. K. N, Sajith. S, Priji. P, Josh. M.S. and Benjamin, S. 2013. Versatility of microbial proteases. *Adv. Enz. Res.,* **1**:39-51.

Kuddus, M. and Ramteke, P.W. 2008. A cold active-extracellular metalloprotease from *Curtobacterium luteum* (MTCC 7529): enzyme production and characterization. *J. Gen. Appl. Microbiol*., **54:** 393-398.

Kuddus, M. and Ramteke, P.W. 2009. A cold active-extracellular alkaline protease from an alkaliphic *Stenotrophomonas maltophilla:* production of enzyme and its industrial applications. *Can. J. Microbiol*., **55**: 1-8.

Kuddus, M. and Ramteke, P. W. 2011. Production optimization of an extracellular cold active alkaline protease from *Stenotrophomonas maltophilia* MTCC 7528 and its application in detergent industry. *African Journal of Microbiology,* **5(7):** 809-816.

Kuddus, M., Joseph, B. and Ramteke, P. W. 2013. Production of laccase from newly isolated *Pseudomonas putida* and its application in bioremediation of synthetic dyes and industrial effluents. *Biocatalysis and Agriculture Biotechnology,* **2:** 333-338.

Kunitz, M.J. 1947. Crystalline soybean trypsin inhibitor:II. General properties. *Gen. Physiol.,* **48**: 456-466.

Lowry, O.H., Rosebrough. N.J, Farr. A.L. and Randal, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193**: 265-275.

Mcconn, J.D., Tsuru. D. and Yasunobu, K.T. 1964. *Bacillus subtilis* Neutral proteinase A zinc enzyme of high specific activity. *The J. Biological. Chemistry,* **239(11):** 3706-3711.

Mukherjee, A.K., Adhikari. H. and Rai, S.K. 2008. Purification and characterization of alkaline protease from *Bacillus subtilis. Biochem. Eng. J.*, **39**: 353-361.

Olivera, N., Sequeiros. C , Sineriz. F, and Breccia J.D. 2006. Isolation and optimization of alkaline protease from *B. patagoniensis* PAT05. *World J. Microbiol. Biotechnol.*, **22**: 737-743.

Pastor, M.D., Lorda. G.S. and Balatti. A. 2001. Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration rates. *Brazilian Journal of Microbiology*, **32(1):** 6-9.

Patil, R.C. and Jadhav, B.L. 2017. Isolation and Characterization of Protease Producing *Bacillus* Species from Soil of Dairy Industry. *Int. J. Curr. Microbiol. App. Sci.*, **6(6):** 853-860.

Qadar, S.A.U., Shireen, E. Iqbal, S. and Anwar, A. 2009. Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. *Indian Journal of Biotech***. 8:** 286-290.

Rawlings, N.D., Barrett. A.J. and Bateman. A. 2011. Asparagine peptidase lyases: a seventh catalytic type of proteolytic enzymes. *J Biol Chem* **286**: 38321-38328.

Sandhya, C., Sumantha. A, Szakacs. G. and Pandey. A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process Biochem*., **40**: 2689-2694.

Santhi, R., 2014. Microbial production of protease by *Bacillus cereus* using cassava waste water. *Eur. J. Exp. Biol.*, **4(2):** 19-24.

Sawant, R. and Nagendrans. 2014. Proteaase: An Enzyme with multiple industrial applications. *World Journals of Pharmacy and Pharmaceutical Sciences,* **3(6):** 568-579.

Secades, P. and Guijarro. J.A. 1999. Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Appl. Environ. Microbiol.*, **65(9)**: 3969-3975.

Shankar, S., Rao, M. and Laxman, R.S. 2011. Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp. *Process Biochem*., **46**: 579–85.

Sharma, K.M., Kumar. R., Panwar, S. and Kumar, A. 2017. Microbial alkaline protases: Optimization of production parameters and their properties. *J. Genetic Engg. Biotechnol*., **15**: 115-126.

Shine, K., Kanimozhi, K, Panneerselvam. A, Muthukumar. C. and Thajuddin. N. 2016. Production and optimization of alkaline protease by *Bacillus cereus* RS3 isolated from desert soil. *Int. J. Adv. Res. Biol. Sci.* **3(7):** 193-202.

Shivanand, P. and Jayaraman. G. 2009. Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochemistry,* **44(10):** 1088-1094.

Subba, Rao., C. Sathish, T. Brahamaiah, P. Kumar T.P. and Prakasham, R.S. 2009. Development of a mathematical model for *Bacillus circulans* growth and alkaline protease production kinetics. *J. Chem. Technol. Biot*., **84**: 302-307.

Vigneshwaran, C., Shanmugam, S., Satish, T.K. 2010. Screening and characterization of keratinase from *Bacillus licheniformis* isolated from namakkal poultry farm. *Researcher*. **2(4)**: 89-96.

Yum, D.Y., Chung, H.C., Bai, D.H., Oh. D.H. and Yu. J.H. 1994. Purification and characterization of alkaline serine protease from an alkalophilic *Streptomyces* sp. *Biosci Biotechnol Biochem.,* **58**: 470-474.

Zambare, V., Nilegaonkar, S. and Anovel, P.K. 2011. *New Biotechnol. http://dx.* doi.org/10.1016/j.nbt.2010.10.002.