Annals of Plant and Soil Research 26(3): 498-502 (2024)
https://doi.org/10.47815/apsr.2024.10390

Exploration and characterisation of protease-producing bacterial strain *klebsiella pneumonia* isolated from soil

SONA A., EMI SARA SABU PHILIP AND PRIYA SENAN V.*

Post Graduate Department of Biotechnology, SAS SNDP Yogam College, Konni, Pathanamthitta, Kerala, India-689691

Received, March, 2024; Revised accepted, August, 2024

ABSTRACT

Microbial enzymes are recognized for their significance in today's world. They convert more intricate or complicated molecules into simpler components. The study aims to isolate, screen, and identify soil bacterial strains that exhibit the potential to produce industrially important enzymes, particularly protease. Bacteria capable of producing protease were extracted from polluted soil, assayed for protease production using skim milk agar plates, and the protease production was confirmed by protease assay. Four bacterial strains were collected, and among them, one strain exhibited protease activity. Protease producing bacterial strain PRS3A was selected on the basis of their protease test. Morphological and biochemical assessments were conducted on protease-producing organisms, and their identification through 16S RNA sequencing revealed that the bacterial cultures belong to Klebsiella genus and pneumonia species.

Keywords: Protease, Screening, contaminated soil, *Klebseilla sp.*

INTRODUCTION

Soil ecosystems, teeming with chemical and biological diversity, serve as an extensive reservoir of micronutrients and novel enzymes (Amritanshu *et al.*, 2023; Pachauri *et al.*, 2023). Microbial enzymes, known for their ability to function in extreme environmental conditions, find a particularly conducive habitat in soil an environment considered highly suitable for microbial growth among various unexplored habitat (Karlen and Stott, 1994). These enzymes from microorganisms play a crucial role globally due to their diverse applications in physiology, pharmaceuticals, and industries. More than 300 enzymes have been recognized, and among them protease and lipase have significant applications in industrial processes (Kumar *et al.*, 1994).Proteases, comprising a significant portion of the top three categories of industrial enzymes, hold a substantial share of approximately 60% in total global enzyme sales (Zhu *et al.*, 1994). Functioning as peptidyl-peptide hydrolases, proteases catalyze the hydrolysis of peptide bonds within protein molecules (Palsaniya *et al.*, 2012). Proteases are sorted based on their cleavage sites, classifying them into exoproteases and endo-proteases, while their acid-base behaviour categorizes them into acid, neutral, or alkaline proteases (Rao *et al.*, 1998, Sayaniya and Patel 2021, Ramkumar *et al.*, 2018) Their widespread commercial applications span food, leather, detergent, and pharmaceutical industries (de Castro Bizerra *et al.*, 2024, Gupta *et al.*, 2024, Khatoon *et al.*, 2023).Proteases play pivotal roles in diverse industries. In the food sector, they enhance quality through processes like meat tenderization and cheese production (Ismail *et al.*, 2019). In pharmaceuticals, proteases aid in drug formulation, delivery, and protein purification (Varanko *et al.,* 2020). Within textiles, they enable enzymatic stone washing and bio-polishing (Islam, 2021). In leather, proteases are crucial for cleaner products (Wanyonyi and Mulaa, 2020). Proteases also contribute to enzymatic laundry detergents, bioremediation, and optimizing brewing processes, showcasing their versatility and impact on innovation, efficiency, and sustainability across industries.

This study aims to isolate protease-producing bacteria from contaminated soil samples, contributing not only to the understanding of microbial proteases but also uncovering novel sources with potential applications across diverse fields.

MATERIALS AND METHODS

Soil samples were collected from Vytilla in Ernakulam, Kerala, India. The samples were transported to the laboratory in sterile bottles and stored at 37 ^{0} C for further analysis.

*Corresponding author- Dr. Priya Senan V, Associate Professor, Post Graduate Department of Biotechnology, SAS SNDP Yogam College, Konni, Kerala, India, Email-priyabiotech2021@gmail.com

Isolation of Bacteria and Screening for Protease Enzyme

The soil sample was serially diluted with sterile diluted water until reaching a 10^{-7}dilution, and isolation was performed using pour plate method on nutrient agar medium(Tanimu *et al.*, 2022, Abdel-Moniem *et al.*, 2021). Proteases producing bacterial strains were isolated on skim milk agar plates (Mohamed *et al.*, 2023).

Identification of the Protease-producing Bacteria

Morphological identification, including Gram staining; biochemical tests such as motility, indole production, MRVP, citrate utilization, catalase, oxidase, nitrate reduction, urease, starch hydrolysis and casein hydrolysis were employed for bacterial identification following Bergey's Manual of Determinative Bacteriology(Shinde *et al.*, 2023).

16S rRNA Sequence Analysis

The bacterial strain PRS3A is selected for 16S rRNA sequence analysis. The isolated bacterial gene's 16S rRNA underwent PCR amplification with 27F & 1492R forward and reverses primers. The gel-purified amplicon was then processed using NucleoSpin® Tissue Kit (Macherey-Nagel) and sequenced on an ABI 3730 XL cycle sequencer.

Phylogenetic Analysis

Sequence analysis utilized the NCBI database's BLAST tool for comprehensive analysis. Sequences of maximum identity were aligned using ClustalW and a phylogenetic tree was constructed using Neighbor Joining method in MEGA11.

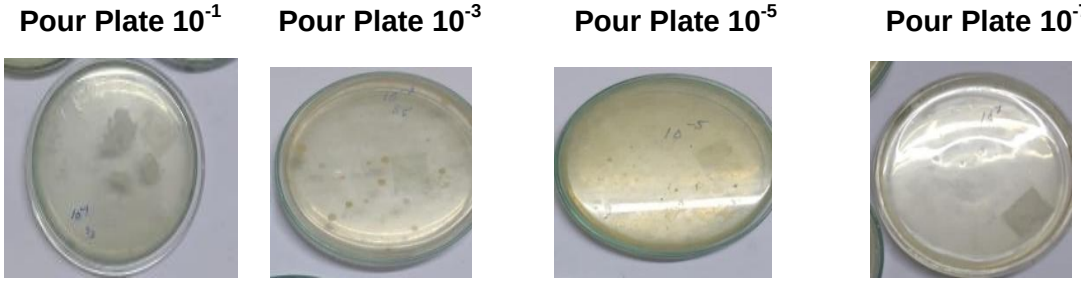


Figure 1: Isolation of protease producing microorganisms by pour plate method

RESULTS AND DISCUSSION

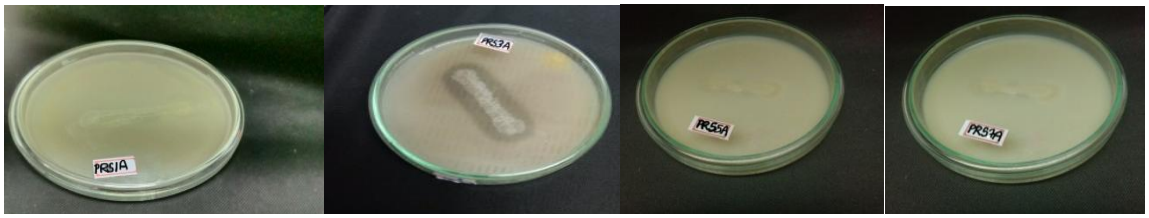
Isolation and Screening of Protease Producing Microorganisms

In this work, soil samples subjected to serial dilution, pour-plated, and then incubated at 37^0C for 24 hours on nutrient agar. Four predominant morphologically distinct colonies, designated as PRS1A, PRS3A, PRS5A, and PRS7A, were isolated from the soil sample. (Figure 1).

After isolation the bacterial strains were screened for protease activity on skim milk agar. Only one isolate displayed significant proteolytic activity with a distinct zone of clearance, while the other three isolates exhibited poor proteolytic activity, as shown in Figure:2 and Table:1, respectively. Therefore, the efficient protease producing isolate, PRS3A was chosen for furtherinvestigations and biochemical test. In accordance with Bergey's Manual of Determinative Bacteriology, PRS3A underwent comprehensive morphological and biochemical characterization, with the strain's morphology identified through Gram staining. Table 2 presents PRS3A's identification based on its biochemical characteristics. The table indicates that the bacterial strain PR3SA exhibits a rod-shaped morphology and is non-motile. However, upon staining, the isolate displayed pink colour, indicating it is gram negative. The isolate is negative for indole, methyl red, and oxidase tests, while being positive for the remaining tests.

Table 1: Protease activity of various bacterial strains

SL No.	Bacterial strains	Protease activity (Qualitative)
1	PR S1A	Negative
2	PR S3A	Positive
3	PR S5A	Negative
4	PR S7A	Negative



PR S1A PR S3A PR S5A PR S7A

Figure 2: Screening of microorganisms for proteolytic activity in skim milk agar

Table 2: Morphological and Biochemical characteristics of protease producing isolates

Biochemical Test	Bacterial strain PR S3A
Grams Staining	Negative
Motility	Negative
Indole (I)	Negative
Methyl red (MR)	Negative
Vogues Proskauer's(VP)	Positive
Citrate utilization	Positive
Nitrate reduction	Positive
Urea hydrolysis	Positive
Catalase	Positive
Oxidase	Negative
Casein hydrolysis	Positive

Phylogenetic Analysis

The phylogenetic analysis of the 16S rRNA sequences was conducted using neighbour-joining method, leading to the identification of the strain PRS3A as *Klebsiella pneumonia*, with reference toFigure:3.In this research, we isolated a bacterial strain capable of producing the industrially significant enzyme, protease. Proteases crucial for commercial applications can be derived from microbial, animal, and plant sources. However, microbial-derived proteases are preferred to their rapid growth, ease of manipulation, and shorter production and purification timelines (Yao*et al.*, 2023, YUAN*et al.*, 2021, Ashraf*et al.*, 2023, North *et al.*, 1982). The presently available proteases are not sufficient to meet industrial demands. Therefore, the on-going quest involves exploring diverse bacterial isolates to discover new proteases exhibiting unique characteristics suitable for industrial applications.

Fig 3: Phylogenetic tree of bacterial strain PRS3A

Klebsiella pneumoniae, known for its versatile capabilities, produces enzymes crucial for biotechnological applications, including industrial processes and biofuel production (Li *et al.*, 2023). Its genetic toolkit enables genetic engineering, and its adaptability makes it suitable for various environmental conditions. Despite its pathogenic nature, certain strains are explored for bioremediation, nitrogen fixation in agriculture, ethanol production, and potential

probiotic use (Bai *et al.*, 2023). Additionally, *Klebsiella pneumoniae* is a model organism in genetic studies and has applications in biomedical engineering for biosensor development (Tomulescu *et al.*, 2021).

Isolating this protease-producing bacterial strain from contaminated soil samples not only addresses current concerns but also opens up avenues for exploring its biotechnological potential in the future.

CONCLUSION

This research involved the identification, isolation, and genetic characterization of a novel protease-producing strain of *Klebsiella pneumoniae* from contaminated soil samples collected in Vytilla (Kochi), Ernakulam district, Kerala. The isolates were obtained through serial dilution and cultured on nutrient agar medium. Following 24 hours of incubation, four distinct bacterial strains were identified and from this, one isolate (PRS3A) showed protease activity on skim milk agar medium. The efficient protease producing isolate PRS3A was chosen for gene sequencing and identification. The morphological and biochemical features suggested that the isolate belonged to the category of Gram-negative bacteria possessing encapsulation and lacking motility.16S rRNA sequence homology was compared and phylogenetic tree was constructed and the result indicated that the isolate was *Klebsiella pneumoniae*. The present study disclosed that, this protease producing strain, PRS3A could be helpful for industrial application. The initial screening report on the diversity of *Klebsiella* species and their enzyme-producing potential indicates a high taxonomic diversity among the isolated *Klebsiella* strains, emphasizing the significant biotechnological potential of bacterial isolates from soil samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors concede Xcellogen Biotech Pvt. Ltd, Thiruvananthapuram for the facilities and technical support rendered on us.

REFERENCES

Abdel-Moniem, S. M., El-Liethy, M.A., Ibrahim, H.S., and Ali, M.E. (2021) Innovative green/non-toxic Bi2S3@ g-C3N4 nanosheets for dark antimicrobial activity and photocatalytic depollution: Turnover assessment. *Ecotoxicology and Environmental Safety*, ***226***, 112808.

Amritanshu, S. P., Tyagi, A., Srivastava, A. J. A. Y. A., and Pathak, A. N. A. N. D. (2023) Status of some extractable macro-and micro-nutrients in the soils of Dehradun district of Uttarakhand. *Annals of Plant and Soil Research*, ***25***(2), 221-229.

Ashraf, M., Hussain, N., Baqar, Z., Kumar, A., Ferreira, L. F. R., and Iqbal, H. M. (2023) Bioprospecting microbial proteases in various industries/sectors. In *Microbial Biomolecules* (pp. 301-324). Academic Press.

Bai, Y., Feng, H., Liu, N., and Zhao, X. (2023) Biomass-derived 2, 3-butanediol and its application in biofuels production. *Energies*, ***16***(15), 5802.

de Castro Bizerra, V., Sales, M.B., Melo, R.L. F., do Nascimento, J.G.A., Junior, J. B., Silva, M.P.F., and Dos Santos, J.C.S. (2024) Opportunities for cleaner leather processing based on protease enzyme: Current evidence from an advanced bibliometric analysis. *Renewable and Sustainable Energy Reviews*, ***191***, 114162.

Gupta, M., Choudhury, B., and Navani, N. K. (2024) Production and characterization of an organic solvent activated protease from haloalkaliphilic bacterium Halobiforma sp. strain BNMIITR. *Heliyon*.

Islam, M.T. (2021) Enzymatic Wet Processing. *Sustainable Practices in the Textile Industry*, **87.**

Ismail, B., Mohammed, H., and Nair, A. J. (2019) Influence of proteases on functional properties of food. *Green bio-processes: enzymes in industrial food processing*, 31-53.

Karlen D.L, Stott D.E. (1994) A framework for evaluating physical and chemical indicators of soil quality. In: Doran, J.W., Coleman, D.C., Bezdicek, D.F. Stewart, B.A. (Eds.), Defining Soil Quality for a Sustainable Environment, SSSA. Madison, WI. 53-72.

Khatoon, N., Ullah, N., Sarwar, A., Ur Rahman, S., Khan, A. A., Aziz, T., Alharbi M. and Alshammari, A. (2023) Isolation and identification of protease-producing bacillus strain from cold climate soil and optimization of its production by applying different fermentation conditions. *Applied Ecology & Environmental Research*, ***21***(4).

Kumar M, Mukherjee J, Sinha M, Kaur P, Sharma S, Gupta M.N, Singh T.P. (1994) Enhancement of stability of a lipase by subjecting to three-phase partitioning (TPP): structures of native and TPP-treated lipase from *Thermomyceslanuginosa*. Sustain Chem Process. **3**: 14.

Li, Y. Q., Wang, M. J., Gan, X. F., and Luo, C. B. (2023) Cleaner 2, 3-butanediol production from unpretreated lignocellulosic biomass by a newly isolated Klebsiella pneumoniae PX14. *Chemical Engineering Journal*, ***455*,** 140479.

Mohamed, A. M., Sayed, A., A Abd El-Aziz, F., and Abd-Elmonsef Mahmoud, G. (2023) Screening of bacterial isolates for protease production with special reference to molecular identification of highly producer strains. *Journal of Applied Molecular Biology*, ***1***(1), 12-30.

North MJ. (1982) Comparative biochemistry of the proteinases of eucaryotic microorganisms. Microbiological Rev. **46**(3): 308-340.

Pachauri, S. P., Pathak, A., Tyagi, A., Pant, C., Tiwari, S. N., and Pal, Y. (2023). Assessment of some extractable macro and micro-nutrients along with multivariate analysis of their spatial distribution in soils of Haridwar district of Uttarakhand. *Annals of Plant and Soil Research*, *25*(1), 70-78.

Palsaniya P, Mishra R, Beejawat N, Sethi S, Gupta BL. (2012) Optimization of alkaline protease production from bacteria isolated from soil. J Microbiol Biotech Res. **2**(6): 858-865.

Ramkumar A., Sivakumar N, Gujarathi AM, Victor R. (2018) Production of thermotolerant, detergent-stable alkaline protease using the gut waste of *Sardinella longiceps* as a substrate: Optimization and characterization. Sci Rep. **8**: 12442.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., and Deshpande, V. V. (1998) Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, ***62***(3), 597-635.

Sayaniya, A. V., & Patel, P. (2021) Isolation and Characterization of Detergent Compatible Alkaline Protease Producing Bacillus Subtilis APO-1. *Biomed. J. Sci. Tech. Res*, ***35***, 27949-27955.

Shinde, S. S., Patil, A. M., Wagh, S. G., and Janvale, G. B. (2023) Detection and identification of oxalate-degrading bacteria from high oxalate containing vegetables. *Int J Adv Biochem Res*, ***7***(2S), 105-113.

Tanimu, A. M., Yusuf, M., and N. daiji, A. (2022) Isolation of Hydrocarbon Utilizing Bacteria from Petro-Chemical Polluted Soil in Bauchi Metropolis, Nigeria.

Tomulescu, C., Moscovici, M., Lupescu, I., Stoica, R. M., and Vamanu, A. (2021) A Review: Klebsiella pneumoniae, Klebsiella oxytoca.

Varanko, A., Saha, S., and Chilkoti, A. (2020) Recent trends in protein and peptide-based biomaterials for advanced drug delivery. *Advanced drug delivery reviews*, ***156***, 133-187.

Wanyonyi, W. C., and Mulaa, F. J. (2020) Alkaliphilic enzymes and their application in novel leather processing technology for next-generation tanneries. *Alkaliphiles in Biotechnology*, 195-220.

Yao, H., Liu, S., Liu, T., Ren, D., Zhou, Z., Yang, Q., and Mao, J. (2023) Microbial-derived salt-tolerant proteases and their applications in high-salt traditional soybean fermented foods: a review. *Bioresources and Bioprocessing*, ***10***(1), 82.

YUAN, Y., WANG, L., and SHI, Y. W. (2021) Research advances in strategies for improving the activity of microbial-derived alkaline proteases. *Biotechnology Bulletin*, ***37*(**5), 231.

Zhu D, Wu Q, Hua L. Industrial Enzymes. In: Moo-Young, M. (Ed.) (1994) Comprehensive Biotechnology (Third Edition), Pergamon.; 1-13. ISBN 9780444640475.