International Journal of Biotech Trends and Technology
ISSN: 2249–0183 / https://doi.org/10.14445/22490183/IJBTT-V15I3P101

Volume 15 Issue 3, 1-7, Sep-Dec 2025

Original Article

Characterization and Identification of Alkali Stable Protease Produced by *Citrococcous Paritis* using Skimmed Milk as a Substrate

Nargis Khan[1,2], Pratima Khare[1], Hemant Kumar Rawat[3]

[1]*Department of Botany, Govt Auto Girls PG College of Excellence, Sagar, MP, India.*
[2]*Maharaja Chhatrasal Bundelkhand University, Chhatarpur, MP, India.*
[3]*Department of Microbiology, Dr Harisingh Gour University, Sagar, MP, India.*

[3]*Corresponding Author : hemantrawat11@gmail.com*

Received: 02 October 2025 Revised: 26 November 2025 Accepted: 12 December 2025 Published: 27 December 2025

Abstract *- Citrococcous paritis was isolated and identified as an alkali-stable protease using 16S rRNA sequencing analysis. After 48 h of incubation in the CPYA medium with 1% casein and 1% yeast extract (pH 10.0), an isolate of the detergent factory (DF-10) showed maximum protease activity of 18.10 U/ml when haemoglobin was used as substrate. The alkaline protease showed optimal activity at pH 10.0 and a temperature of 50°C. The newly isolated C. paritis strain was efficiently characterized for alkaline protease production and blood stain removal, and therefore can be used as a suitable candidate in the detergent and leather industry.*

Keywords *- Citrococcous paritis, Alkaline protease, Casein, Detergent, Alkali stable.*

1. Introduction

Alkaline proteases (EC 3.4.21-24 and 99) are hydrolytic enzymes capable of degrading proteins into short peptides and amino acids. They cover approximately 60% of the total enzyme employed in industry. Alkaline proteases are more effectively used in leather, detergent, waste management, food, and the pharmaceutical industry. Most alkaline proteases show high catalytic activity and stability in a high alkaline range (pH 9.0-12.0) and are useful for biotechnological and industrial applications [1-4].

Alkaline proteases are produced by a variety of microorganisms, *viz.* bacteria, fungi, and actinomycetes; however, bacterial spp are highly capable of producing extracellular alkaline protease in higher amounts. Proteases are classified into two categories: (1) Exopeptidases, which catalyse the C or N-terminal reaction of the peptide bonds; (2) Endopeptidases, which catalyse peptide bonds. Endopeptidases are further classified into different categories like serine proteases (E.C. 3.4.21), aspartate proteases (E.C. 3.4.23), cysteine proteases (E.C. 3.4.22), threonine proteases (E.C.3.4.25), and metallo proteases (E.C. 3.4.24) [5]. Different bacterial spp. are suitable for the production of extracellular alkaline protease, *viz*. *Bacillus licheniformis* [6], *Bacillus stearothermophilus* [7], *Bacillus cereus* [8], *Pseudomonas aeruginosa* [9], and *Geobacillus thermoglucosidasius* [10].

In particular, alkaline-stable proteases are the key component for the detergent and leather industry. These are effectively employed as a supplementary additive in commercial detergents to improve cleaning efficiency by removing blood and organic food waste stains. The main feature to use alkaline protease as an additive in detergent powder is its stability in various temperatures, pH, and effectiveness to remove stains, which is very significant in the detergent industry [11; 1].

In the present study, newly isolated bacterial strains from the soil of the detergent factory of the Sagar region are used; the bacterial strain is highly suitable for producing alkali-stable protease. Production and characterization of the alkaline protease was performed and evaluated for its ability in stain removal, and proved to be suitable in the detergent industry.

2. Materials and Methods

2.1. Isolation of Alkaline Protease-Producing Bacteria

Isolation of alkaline protease producers was done by the serial dilution method. Various soil samples were collected from the Detergent Factory (DF), the Chicken Market (CM), and the Paper Factory (PF). Isolation of bacterial species was done using Casein Peptone Yeast extract Agar (CPYA) medium. The CPYA medium (g/l) (pH 10.0; Casein 5.0, Peptone 5.0, Yeast extract 2.0, NaCl 5.0, $MgSO_4.7H_2O$ 0.2,

$CaCl_2$ 0.1, K_2HPO_4 1.0, Na_2CO_3 6.0) was employed for bacterial isolation. Isolated bacterial colonies were screened for hydrolysis of casein and further used in the experiment.

2.2. Protease Assay

The alkaline protease assay was performed by using casein as the substrate using the Folin–Ciocalteu method. An alkaline protease assay was performed according to the previously described method [11].

2.3. Assay of Proteolytic Activity in Culture Filtrate

Protease production by alkalophilic isolates was examined by the previously described radial diffusion assay method; for this purpose, casein agar medium was used. These plates were then incubated at 37°C for 24 h. Plates were observed for the transparent zone of hydrolysis around the agar well, and the area was calculated. One unit of alkaline protease activity was defined as the amount of enzyme that catalyses the liberation of 1 µg of tyrosine/ml/min from substrate used (haemoglobin and casein) under optimized assay conditions.

3. Method of Molecular Identification of Selected Bacterial Strains

3.1. Isolation of DNA

Bacterial genomic DNA was isolated from the overnight-grown selected culture. Furthermore, the bacterial DNA was purified using Hi-purA (Hi-media, USA) DNA purification kit. The purity of extracted genomic DNA was determined by measuring absorbance with a spectrophotometer (260-280 nm). The quality of the DNA was evaluated on a 1.0% agarose gel, where a single band and high-molecular-weight DNA were observed.

3.2. Molecular Identification of the Strain

The 16S rRNA gene fragment was amplified by using 16S rRNA-forward and 16S rRNA-reverse primers. A single self-contained PCR amplicon band (1500 bp) was observed when visualized on agarose gel electrophoresis. The PCR amplicon was purified to remove various contaminants. Forward and reverse DNA sequencing reaction of the PCR amplicon was performed using the ABI 3730xl Genetic Analyzer. Aligner software was used for the consensus sequence of the 16S rRNA gene from forward and reverse sequence data. The 16S rRNA gene sequence was used to perform a BLAST search against the NCBI GenBank database. The multiple alignment software program Clustal W was used for the maximum identity score. A phylogenetic tree and distance matrix curve were constructed using MEGA 10. The evolutionary history was done by using the Tamura-Nei model and the Maximum Likelihood method [12-13].

3.3. Effect of Different Carbon Sources

The effect of various carbon sources for the production of alkaline protease was studied with the casein-containing culture medium by incorporating different C-sources (1% w/v), including casein, glucose, lactose, galactose, and skimmed milk. For this purpose, 1.0 g of each C-source was added with various nitrogen sources (yeast extract, peptone, urea, and beef extract), and a One-Variable-At-a-Time approach (OVAT) was employed to see the effect of suitable culture medium for the higher alkaline protease production. The media were autoclaved and inoculated with an overnight-grown bacterial inoculum (1% v/v; OD 600 nm ~0.5), and then incubated for 48 h at 200 rpm. After that, the fermentation broth of the Erlenmeyer flask was centrifuged at 10000 rpm and analyzed for alkaline protease activity.

3.4. Effect of N-Source on Protease Production

The effect of various nitrogen sources (yeast extract, peptone, beef extract, and urea) on the production of bacterial alkaline protease by newly isolated bacterial strains was studied. Erlenmeyer flasks (150 ml) containing 50 ml of medium containing different N-sources (2% w/v organic and 1% w/v inorganic N-source) were autoclaved, and an overnight-grown bacterial inoculum was added (1% v/v; OD600 ~0.5) and incubated for 48 h at 200 rpm. After that, the fermentation broth was centrifuged at 10000 rpm, and the supernatant was analyzed for alkaline protease activity.

4. Results and Discussion

4.1. Isolation of DNA

DNA of the DF-10 strain was isolated and evaluated on agarose gel, and a single band of high-molecular-weight genomic DNA was observed (Figure 1). A fragment of the 16S rRNA gene was amplified by 16S rRNA-Forward and 16S rRNA-Reverse primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on a gel. The PCR amplicon was purified. Forward and reverse DNA sequencing reaction of the PCR amplicon was carried out with 16S rRNA-F and 16S rRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

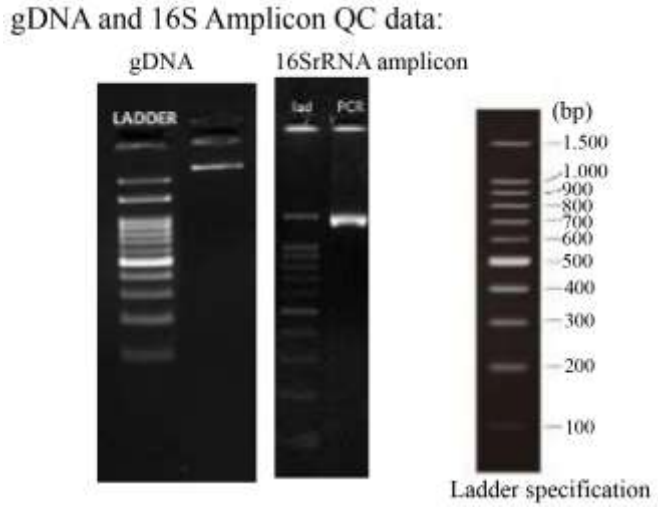

Fig. 1 Genomic DNA (gDNA) and 16S Amplicon data and specification of ladder used in this study

4.2. Molecular Identification of Alkaline Protease-Producing Bacteria

The 16S rRNA gene sequence of DF-10 was 1500 bp long and used in GenBank BLAST search analyses, revealing a high homology of up to 99% with *Micrococcus* sp. BLAST analysis identified the nearest species as *Citrococcus parietis*, which was further confirmed by phylogenetic tree construction using MEGA X software (Figure 2). Additionally, analysis of the 16S rRNA of isolate number DF-10 was found to be *Citricoccus parietis*, showing high similarity based on phylogenetic analysis, nucleotide homology, and distance matrix study revealed that the sequence was 1500 bp long and indicated its classification within the genus *Citrococcus*, with the highest homology observed with *Citrococcus* (99.48% homology) (Tables 1 and 2).

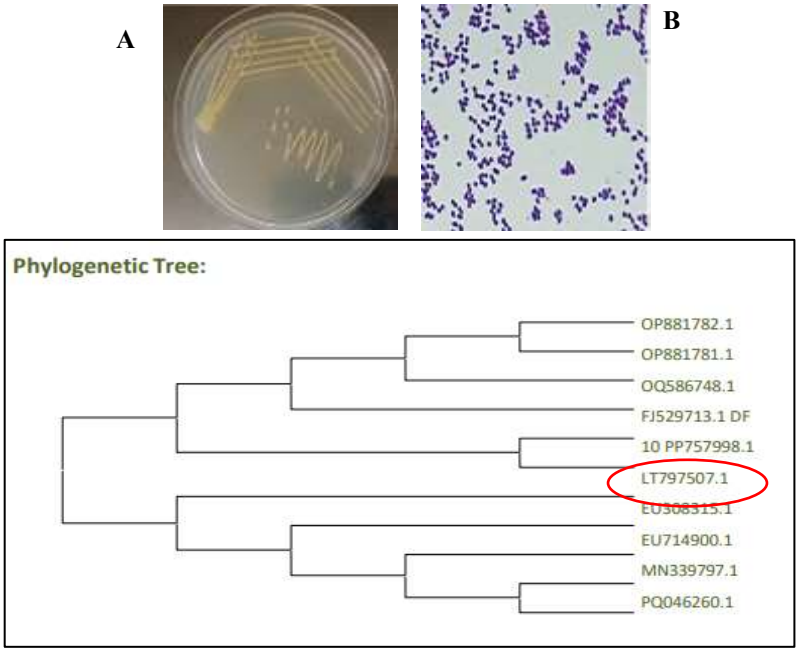

Fig. 2 (A) Colonial characteristics of *Citrococcous parietis* (DF-10), (B) Gram staining showed Gram-positive character of *Citrococcous parietis,* (C) Molecular phylogenetic analysis based on 16S rRNA sequence of the isolate DF-10. Evolutionary analysis by the Maximum Likelihood method

Table 1. Isolate number DF-10 was found to be *Citricoccus parietis*, showing high similarity based on nucleotide homology and phylogenetic analysis

Sequences producing significant alignments:						
Description	**Max Score**	**Total Score**	**Query Cover**	**E value**	**Per. Ident**	**Accession**
Citricoccus parietis strain 02-Je-010	2453	2453	100%	0.0	99.63%	PP757998.1
Micrococcus xinjiangensis strain SRDD42	2444	2444	100%	0.0	99.48%	EU714900.1
Citricoccus sp. strain Qhu-S38	2444	2444	100%	0.0	99.48%	OQ586748.1
Actinomycetia bacterium strain Qhu-S38	2444	2444	100%	0.0	99.48%	OP881782.1
Micrococcus sp. CPCC100074	2444	2444	100%	0.0	99.48%	FJ529713.1
Micrococcus sp. strain MGB 2690	2444	2444	100%	0.0	99.48%	MN339797.1
Citricoccus sp. strain SW10	2444	2444	100%	0.0	99.48%	PQ046260.1
Actinomycetia bacterium strain Qhu-S35	2444	2444	100%	0.0	99.48%	OP881781.1
Citrococcus sp. FIB146_2	2438	2438	100%	0.0	99.40%	EU308315.1
Citricoccus alkalitolerans isolate AT1RP19	2438	2438	100%	0.0	99.48%	LT797507.1

Table 2. Estimates of Evolutionary Divergence between Sequences

Distance Matrix:											
DF_10		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
PP757998.1	0.003		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.02	0.002
EU714900.1	0.005	0.005		0.002	0.002	0.002	0.000	0.000	0.002	0.001	0.001
OQ586748.1	0.005	0.004	0.006		0.000	0.001	0.002	0.002	0.000	0.002	0.002
OP881782.1	0.005	0.004	0.006	0.000		0.001	0.002	0.002	0.000	0.002	0.002
FJ529713.1	0.005	0.005	0.006	0.002	0.002		0.002	0.002	0.001	0.002	0.002
MN339797.1	0.005	0.005	0.000	0.006	0.006	0.006		0.000	0.002	0.001	0.001
PQ046260.1	0.005	0.005	0.000	0.006	0.006	0.006	0.000		0.002	0.001	0.001
OP881781.1	0.005	0.004	0.006	0.000	0.000	0.002	0.006	0.006		0.002	0.002
EU308315.1	0.006	0.006	0.002	0.008	0.008	0.008	0.002	0.002	0.008		0.001
LT797507.1	0.005	0.004	0.001	0.006	0.006	0.006	0.001	0.001	0.006	0.004	

4.2. Effect of Carbon and Nitrogen Source on Enzyme Production

Bacterial colonies grown on CPYA medium plates showed the usability of casein as a substrate (18.10 U/ml). The effect of various carbon sources (casein, skimmed milk, glucose, etc.) on the alkaline protease production is represented in Figure 3. The highest enzyme activity was reported in the incorporation of casein, showing 9.47 U/ml.

Furthermore, the effect of various nitrogen sources (beef extract, yeast extract, peptone, and urea) was used (1% w/v). Among different N-sources used, yeast extract showed the maximum protease activity with 7.216 U/ml. The effect of a prominent nitrogen source is shown in Figure 4. Similarly, Al-Dhabi et al [14] reported yeast extract as a suitable nitrogen source for the growth of *Streptomyces* sp. Fu et al [15] demonstrated that the alkaline protease activity of *Alkalihalobacillus clausii* FYX showed higher protease activity in the presence of casein as substrate.

4.3. Effect of pH and Temperature on Alkaline Protease Production

The optimum pH and temperature conditions for *Citrococcous parietis* (DF-10) alkaline protease were identified as pH 10.0 (Figure 5) and 50°C (Figure 6), respectively. The newly isolated bacterial species exhibited activity at pH 10.0, drawing keen attention to its potential uses in the detergent industry, as similar results for bacterial alkaline proteases have been reported earlier [14, 16-17]. A similar type of result was obtained by using *Bacillus patagoniensis* alkaline protease, which showed a relationship with the present study [11]. Shah et al [1] reported protease activity of *Bacillus amyloliquefaciens* at pH 9.0 and 40 °C after 24 h of incubation. The present study showed higher pH and temperature stability when compared with others. Furthermore, the *Citrococcous parietis* (DF-10) revealed a long-lasting high alkaline protease activity and effective pH stability at high alkaline conditions, showing its effectiveness in the detergent industry is comparable to previous reports [18-19].

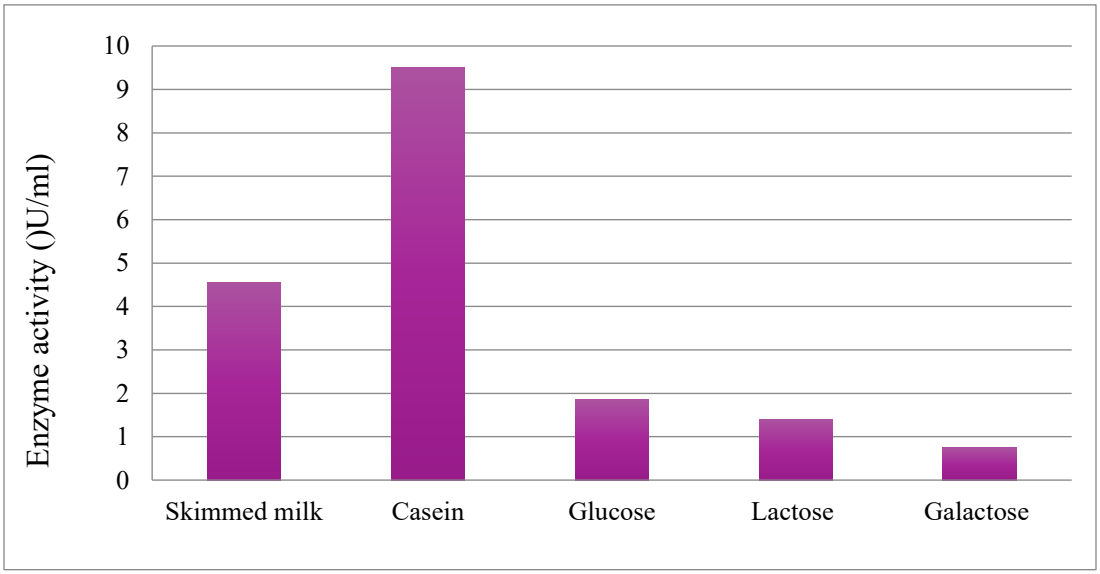

Fig. 3 Effect of various carbon sources (One variable at a time) on production of alkaline protease (Experiments were performed in duplicates)

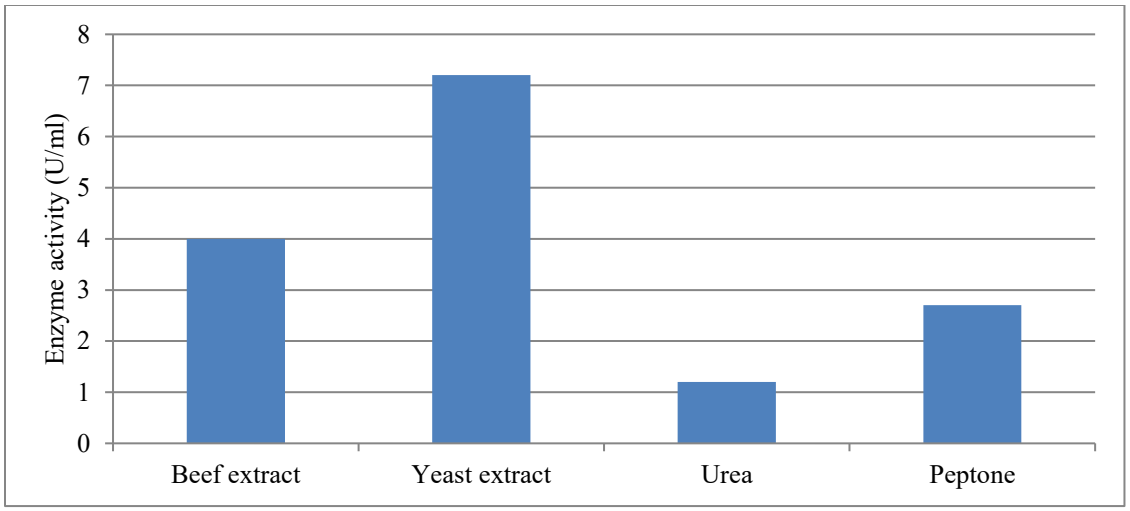

Fig. 4 Effect of various nitrogen sources (One variable at a time) on production of alkaline protease (Experiments were performed in duplicates)

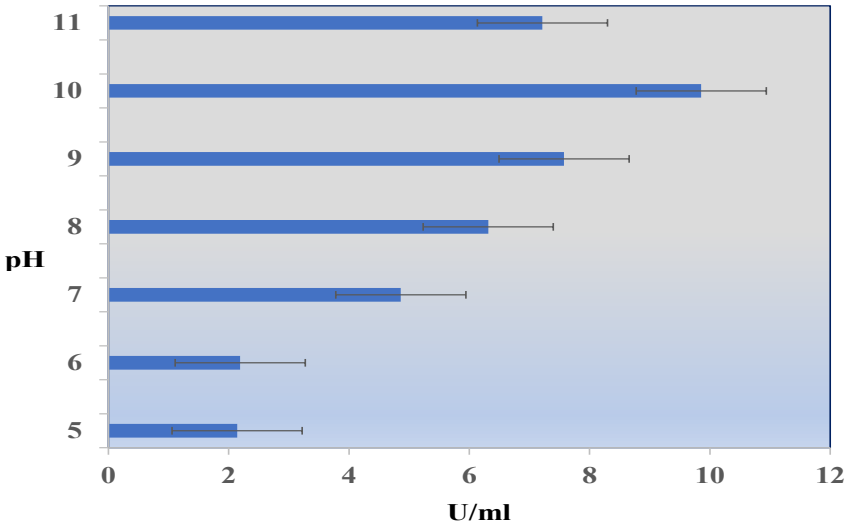

Fig. 5 Effect of pH on the activity of alkaline protease obtained from *Citrococcus parietis*. One unit of protease activity was defined as the amount of enzyme that released 1g tyrosine/ml/min under the standard conditions. Vertical bars are ±SE of the mean

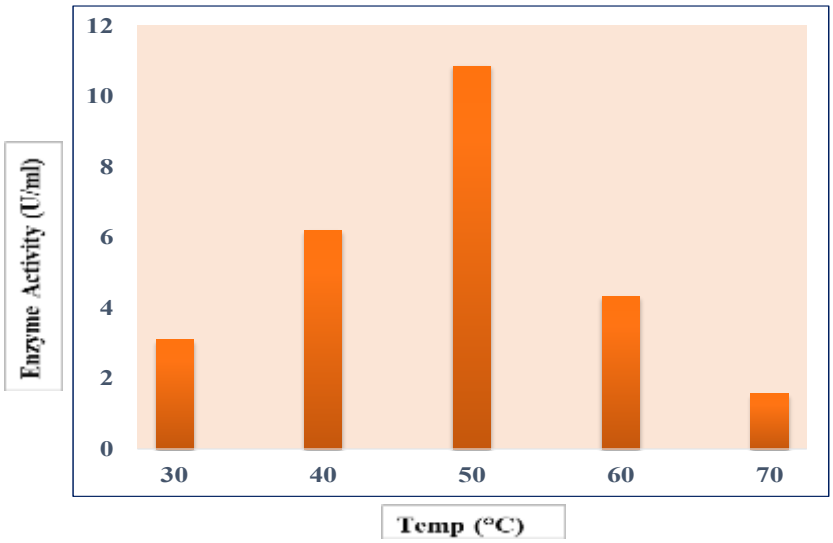

Fig. 6 Effect of temperature on the activity of alkaline protease obtained from *Citrococcus parietis*. One unit of protease activity was defined as the amount of enzyme that released 1g tyrosine/ml/min under the standard conditions. Experiments performed in duplicates.

5. Conclusion

The newly isolated bacterial species from detergent factory soil was screened and identified as *Citrococcus parietis*. In this study, various experiments were performed to optimize the fermentation medium and production process for *C. parietis* DF 10 alkaline protease. The results confirmed that yeast extract and casein are suitable nitrogen and carbon sources for the production of alkaline protease. With increasing demand for environmental protection, the role of biocatalysts has gained great attention in biotechnology. There is always a need to screen out new microbes for the production of efficient enzymes that have the ability to meet industrial demand. The novel alkaline protease producer *C. parietis* showed maximum activity at higher pH and temperature. The alkaline protease is promised to be used in extreme alkalinity conditions and also showed good activity when mixed with a detergent.

Acknowledgments

The authors are highly thankful to Prof. Naveen Gopal Kango, Head, Department of Microbiology, Dr. Harisingh Gour University, Sagar, MP, for providing the facility for research.

References

[1] Tawaf Ali Shah et al., "Production of Alkaline Protease from *Bacillus Amyloliquefaciens* TAS-2 and Optimizing Fermentation Conditions," *Biocatalysis and Biotransformation*, vol. 43, no. 5, pp. 496-509, 2025. [CrossRef] [Google Scholar] [Publisher Link]

[2] Polson Mahakhan et al., "Alkaline Protease Production from *Bacillus Gibsonii* 6BS15-4 Using Dairy Effluent and Its Characterization as a Laundry Detergent Additive," *Journal of Microbiology Biotechnology*, vol. 33, no. 2, pp. 195-202, 2023. [CrossRef] [Google Scholar] [Publisher Link]

[3] Cheng Zhou et al., "A Novel Alkaline Protease from Alkaliphilic *Idiomarina* Sp. C9-1 with Potential Application for Eco-Friendly Enzymatic Dehairing in the Leather Industry," *Scientific Reports*, vol. 8, no. 1, pp. 1-18, 2018. [CrossRef] [Google Scholar] [Publisher Link]

[4] Kanupriya Miglani Sharma et al., "Microbial Alkaline Proteases: Optimization of Production Parameters and their Properties," *Journal of Genetic Engineering and Biotechnology*, vol. 15, no. 1, pp. 115-126, 2017. [CrossRef] [Google Scholar] [Publisher Link]

[5] Ali Eatemadi et al., "Role of Protease and Protease Inhibitors in Cancer Pathogenesis and Treatment," *Biomedicine & Pharmacotherapy*, vol. 86, pp. 221-231, 2017. [CrossRef] [Google Scholar] [Publisher Link]

[6] Yuan Yuan et al., "Enhancing the Thermostability of *Bacillus Licheniformis* Alkaline Protease 2709 by Computation-Based Rational Design," *Molecules*, vol. 30, no. 5, pp. 1-16, 2025. [CrossRef] [Google Scholar] [Publisher Link]

[7] Aida Karray et al., "Novel Thermostable and Alkaline Protease Produced from *Bacillus Stearothermophilus* Isolated from Olive Oil Mill Sols Suitable to Industrial Biotechnology," *Molecules*, vol. 26, no. 4, 2021. [CrossRef] [Google Scholar] [Publisher Link]

[8] Liangliang Li et al., "An Alkaline Protease from *Bacillus Cereus* NJSZ-13 Can Act as a Pathogenicity Factor in Infection of Pinewood Nematode," *BMC Microbiology*, vol. 23, no. 10, 2023. [CrossRef] [Google Scholar] [Publisher Link]

[9] Chendi Jing et al., "Antibodies Against *Pseudomonas Aeruginosa* Alkaline Protease Directly Enhance Disruption of Neutrophil Extracellular Traps Mediated by this Enzyme," *Frontiers in Immunology*, vol. 12, pp. 1-16, 2021. [CrossRef] [Google Scholar] [Publisher Link]

[10] Suleiman D. Allison et al., "Molecular Cloning, Characterization, and Application of Organic Solvent-Stable and Detergent-Compatible Thermostable Alkaline Protease from *Geobacillus Thermoglucosidasius* SKF4," *Journal of Microbiology Biotechnology*, vol. 34, no. 2, pp. 436-456, 2023. [CrossRef] [Google Scholar] [Publisher Link]

[11] Zhimin Li et al., "Redox and Solvent-Stable Alkaline Serine Protease from *Bacillus Patagoniensis* DB-5: Heterologous Expression, Properties, and Biotechnological Applications," *Frontiers in Microbiology*, vol. 16, pp. 1-16, 2025. [CrossRef] [Google Scholar] [Publisher Link]

[12] S. Kimura et al., "Human Thyroid Peroxidase: Complete cDNA and Protein Sequence, Chromosome Mapping, and Identification of Two Alternately Spliced mRNAs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 16, pp. 5555-5559, 1987. [CrossRef] [Google Scholar] [Publisher Link]

[13] Sudhir Kumar et al., "MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms," *Molecular Biology and Evolution*, vol. 35, no. 6, pp. 1547-1549, 2018. [CrossRef] [Google Scholar] [Publisher Link]

[14] Naif Abdullah Al-Dhabi et al., "Characterization and Fermentation Optimization of Novel Thermo Stable Alkaline Protease from *Streptomyces* Sp. Al-Dhabi-82 from the Saudi Arabian Environment for Eco-Friendly and Industrial Applications," *Journal of King Saud University - Science*, vol. 32, no. 1, pp. 1258-1264, 2020. [CrossRef] [Google Scholar] [Publisher Link]

[15] Yuxi Fu et al., "Protein Engineering, Expression Optimization, and Application of Alkaline Protease from *Alkalihalobacillus Clausii* FYX," *International Journal of Biological Macromolecules*, vol. 307, 2025. [CrossRef] [Google Scholar] [Publisher Link]

[16] Essam Kotb et al., "Isolation, Screening, and Identification of Alkaline Protease-Producing Bacteria and Application of the Most Potent Enzyme from *Bacillus* sp. Mar64," *Fermentation*, vol. 9, no. 7, pp. 1-17, 2023. [CrossRef] [Google Scholar] [Publisher Link]

[17] Ying Zhang et al., "Enhancement of Alkaline Protease Production in Recombinant *Bacillus Licheniformis* by Response Surface Methodology," *Bioresources and Bioprocessing*, vol. 10, pp. 1-11, 2023. [CrossRef] [Google Scholar] [Publisher Link]

[18] Aishwarya Ramkumar et al., "Production of Thermotolerant, Detergent Stable Alkaline Protease Using the Gut Waste of *Sardinella Longiceps* as a Substrate: Optimization and Characterization," *Scientific Report*, vol. 8, pp. 1-15, 2018. [CrossRef] [Google Scholar] [Publisher Link]

[19] Haichuan Yang et al., "Enhancing Alkaline Protease Stability through Enzyme-Catalyzed Crosslinking and Its Application in Detergents," *Processes*, vol. 12, no. 3, pp. 1-14, 2024. [CrossRef] [Google Scholar] [Publisher Link]