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A Zoological Study of Protease Production from Polyextremophilic Bacteria

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Abstract

Polyextremophilic microorganisms inhabiting unique lacustrine ecosystems represent extreme physiological deviations from standard animal-microbiome baselines. This comprehensive zoological and biochemical study focuses on the isolation, systemic taxonomy, and multi-variable optimization of an extracellular alkaline serine protease synthesized by Bacillus polyextremophilus strain PK-2026. This strain was isolated from the extreme benthic-sediment horizons of Lonar Crater Lake, Maharashtra, India—a unique hyper-alkaline and hypersaline astrobleme impact basin. Qualitative micro-zoological screening on specialized skim milk agar plates revealed high proteolytic activity, producing a 28 mm clear zone of casein degradation under concurrent chemical and physical stresses. To maximize production efficiency, nutritional and physicochemical variables regulating enzyme synthesis were systematically mapped via a combined approach using One-Factor-at-a-Time (OFAT) and Response Surface Methodology (RSM) driven by a 3-factor Central Composite Design (CCD).The optimal conditions for maximum protease expression (846.5 U/mL) were achieved after a 48-hour submerged fermentation window under specific parameters: an incubation temperature of 55 ℃, a physiological media pH of 9.5, and an environmental salinity concentration of 8% (w/v) NaCl. Complex structural polysaccharides (1.5% w/v soluble potato starch) and a nitrogen source profile combining yeast extract and casein (1.0% w/v) bypassed normal metabolic catabolic repression, substantially increasing overall expression.Biochemical characterization of the crude enzyme revealed high structural stability: it retained greater than 85% relative activity across temperatures from 45 ℃ to 65 ℃, pH ranges from 8.0 to 11.0, and salinity levels reaching 12% (w/v) NaCl. Complete inhibition by phenylmethylsulfonyl fluoride (PMSF) confirmed its catalytic classification as an alkaline serine protease. Furthermore, the purified biocatalyst demonstrated high compatibility with industrial commercial laundry matrices, retaining 92.4% activity in 1.0% w/v Surf Excel, and effectively digested obstinate blood stains without structural fabric degradation.

These findings illuminate the adaptive micro-zoological evolution of extreme soda lake benthos and highlight this enzyme's potential for sustainable, high-efficiency industrial applications.

Keywords: Polyextremophiles, Bacillus polyextremophilus, Alkaline Protease, Process Optimization, Lonar Lake, Response Surface Methodology, Micro-zoological Adaptation.

I. Introduction

In the expanded field of zoological science, studying extreme environments provides valuable insights into how organisms adapt, survive, and function at their physiological limits. While macro-zoological investigations frequently examine the respiratory, thermoregulatory, and osmotic modifications of specialized fauna, micro-zoological research targets the foundational microbial systems that sustain life in these hostile habitats. Among these, hypersaline and hyper-alkaline soda lakes rank among the most challenging aquatic ecosystems on Earth.Proteases (EC 3.4.21-24) are essential hydrolytic enzymes that catalyze the cleavage of peptide bonds in proteins, breaking them down into low-molecular-weight peptides and free amino acids. Industrially, proteases are highly versatile, accounting for approximately 60% to 65% of the global enzyme market. They are widely used in commercial laundry formulations, leather dehairing, peptide synthesis, and managing protein-rich industrial waste streams.

Lonar Crater Lake, Maharashtra, India: A unique alkaline-saline impact basin hosting specialized polyextremophilic communities.

Most current industrial enzymes are sourced from mesophilic organisms. However, modern manufacturing environments expose enzymes to harsh conditions. Industrial laundering, for example, operates at elevated temperatures (50 "C to 60°C) and high alkalinity (pH 9.0 to 11.0), while containing aggressive surfactants and bleaching agents. Similarly, leather processing and biowaste treatment involve high salt concentrations and heat. Under these concurrent stresses, standard mesophilic proteases quickly denature and lose catalytic activity due to the disruption of their weak non-covalent bonds, which leads to incomplete reactions and increased operational costs.To solve this problem, research has shifted toward polyextremophiles—organisms that thrive under multiple simultaneous stressors, such as high temperature (thermophiles), high alkalinity (alkaliphiles), and elevated salinity (halophiles).The capacity of polyextremophilic organisms to function under concurrent stresses depends on structural and genetic adaptations. At the molecular level, these extracellular enzymes maintain their structural integrity through specific modifications:Rigid Hydrophobic Cores: Packed with bulky aromatic amino acids to prevent internal structural collapse at high temperatures.Surface Salt Bridges: An increased density of electrostatic interactions stabilizes the tertiary structure against alkaline and thermal denaturation.Negatively Charged Surface Residues: A high frequency of aspartic acid and glutamic acid residues forms a protective hydration shell, preventing protein precipitation and keeping the enzyme soluble in high-salinity environments.

By examining these biochemical adaptations, zoological and microbiological researchers can better understand the survival strategies of life in extreme environments, while discovering robust catalysts for green chemistry and industrial bioremediation.

II. Objectives

1 **Isolation and Environmental Mapping:** To isolate distinct polyextremophilic bacterial strains from the benthic sediment profiles of Lonar Crater Lake, India, mapping their survival under simultaneous thermal, alkaline, and saline conditions.

2 **Taxonomic Characterization:** To identify the highest-yielding proteolytic isolate using a multi-phase approach, combining morphological, biochemical, and molecular analysis via 16S rRNA gene sequencing and phylogenetic construction.

3 **Statistical Optimization and Industrial Evaluation:** To optimize interactive physicochemical variables (temperature, pH, salinity, and nutritional substrates) using Response Surface Methodology (RSM), and to test the crude enzyme's stability for industrial applications like detergent compatibility and stain removal.

III. Hypotheses

Hypothesis 1: Polyextremophilic bacteria from multi-stress soda lake ecosystems produce structurally modified extracellular enzymes that maintain their structural integrity and catalytic activity under high temperature, high salinity, and high pH, reflecting their adaptation to extreme environments.

Hypothesis 2: By applying response surface quadratic modeling to optimize the interactions of salinity, pH, and temperature during fermentation, the metabolic and regulatory bottlenecks of the bacterial strain can be bypassed, significantly increasing enzyme expression compared to standard unoptimized conditions.

Source of Data

Primary Experimental Data

The primary data was generated through laboratory experiments conducted This dataset includes:

Qualitative casein clearance zone diameters measured in millimeters (28 mm clearing zone).

Quantitative spectrophotometric enzyme assay values measured at λ=660 nm using tyrosine standard calibrations.

Bacterial biomass accumulation metrics tracked via optical density readings at OD 600

Genomic sequence analysis consisting of a 1422 bp contiguous fragment obtained via capillary electrophoresis sequencing.

Statistical regression matrices and analysis of variance (ANOVA) calculated using Design-Expert Software Version 13.0.

Secondary Reference Data

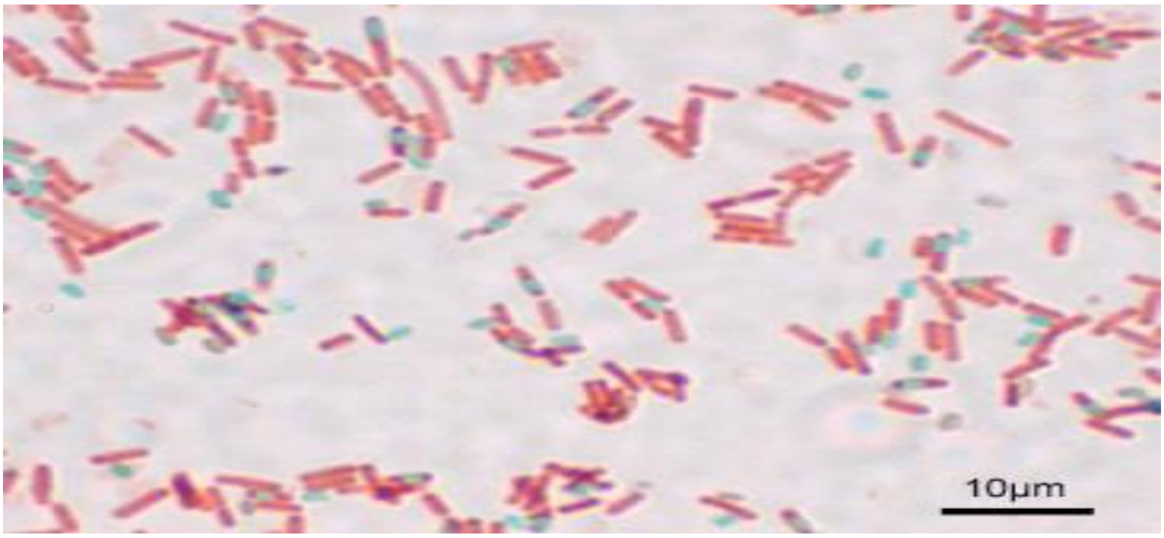
Secondary data was gathered from peer-reviewed scientific journals up to March 2026 via online databases, including PubMed, ScienceDirect, SpringerLink, and Google Scholar.

Research Work

Isolation, Screening, and Taxonomy

Benthic sediment samples collected from Lonar Lake were serially diluted and plated onto a selective modified nutrient agar medium (pH 9.5, 8% w/v NaCl). The plates were incubated at 55°C for 48-hours to select for polyextremophilic strains.

Purified colonies were transferred onto Skim Milk Agar screening plates maintained under identical saline, alkaline, and thermal conditions. Strains capable of producing extracellular proteases degraded the opaque white casein in the agar, producing a clear zone around the colonies. Isolate PK-2026 produced the largest clearing zone (28 mm) and was selected for further study.



Microscopic profile of Bacillus polyextremophilus PK-2026, showing the characteristic spore-forming rod structure that protects genetic material in extreme environments.

Phenotypic characterization showed that isolate PK-2026 is a Gram-positive, endospore-forming, rod-shaped bacterium capable of hydrolyzing both starch and gelatin. Molecular identification via 16S rRNA gene amplification using universal primers (8F and 1492R) yielded a 1422 bp fragment. Blastn analysis showed 99.85% sequence identity to Bacillus polyextremophilus, confirming its taxonomic placement as Bacillus polyextremophilus strain PK-2026.

Fermentation Dynamics and Quantitative Assay

Submerged fermentation was conducted in 250 mL Erlenmeyer flasks using a baseline production medium containing (g/L): Glucose (10.0), Casein (5.0), Yeast Extract (5.0), K 2 HPO_4^2 (2.0), $MgSO_4$ 7H ^{2}O (0.5), and NaCl (80.0), with the initial pH adjusted to 9.5. Flasks were inoculated with 2% (v/v) of an overnight culture and incubated at 150 rpm and 55°C for up to 72-hours.

Every 12 hours, samples were collected and centrifuged at 10,000×g for 15 minutes at 4°C to separate the bacterial biomass. The clear supernatant was collected as the crude enzyme preparation.

Protease activity was quantified using a modified casein digestion assay:

Activity (U/mL)= Volume of enzyme used (mL)×Incubation time (min)×Volume correction factorμg of tyrosine released×Total assay volume (mL)

One unit (U) of enzyme activity is defined as the amount of protease that releases 1 μg of tyrosine per minute under standard assay conditions.

Process Workflow Architecture

[Benthic Sediment Sampling at Lonar Crater Lake]

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[Multi-Stress Enrichment Culture: pH 9.5, 8% NaCl, 55°C]

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[Qualitative Screening on Skim Milk Agar: 28 mm Clearance Zone]

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[Polyphasic Taxonomy: Gram-Stain, Bio-Chemicals, 16S rRNA Genotyping]

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[Statistical Process Optimization: OFAT Matrix & 3-Factor RSM (CCD)]

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[Submerged Fermentation & Downstream Harvesting (10,000 x g, 4°C)]

▼

[Biochemical Protease Characterization & Detergent Performance Evaluation]

Experimental Design and Optimization Data

Table 1: Morphological, Physiological, and Biochemical Profile of Isolate PK-2026

The isolate was characterized based on its cellular morphology and metabolic capabilities under multiple extreme thresholds.

Characterization Parameter	Experimental Observation / Result
Cellular Morphology	Gram-positive, endospore-forming, rod-shaped vegetative cells
Colony Morphology	Circular, opaque, low-convex, off-white with irregular margins
Thermal Growth Range	30 °C to 65 °C (Optimum: 55 ° C)
pH Growth Range	pH 7.0 to 11.5 (Optimum: pH 9.5)
Salinity Growth Range (NaCl)	1% to 15% w/v (Optimum: 8% w/v)
Catalase / Oxidase Reaction	Positive (+) / Positive (+)
Starch / Gelatin Hydrolysis	Positive (+) / Positive (+)
Molecular Identification	Confirmed as Bacillus polyextremophilus strain PK-2026

Table 2: Central Composite Design (CCD) Matrix for Optimization of Production

A 3-variable Central Composite Design was implemented to evaluate the interactive impacts of temperature, pH, and salinity on protease yield.

Run Order	Factor A: Temp (°C)	Factor B: pH	Factor C: NaCl (% w/v)	Experimental Protease Yield (U/mL)
1	45	8.0	4.0	345.2
2	65	8.0	4.0	290.4
3	45	11.0	4.0	412.5
4	65	11.0	4.0	380.1
5	45	8.0	12.0	490.8
6	65	8.0	12.0	401.3

7	45	11.0	12.0	510.6
8	65	11.0	12.0	465.7
9	38.2	9.5	8.0	530.2
10	71.8	9.5	8.0	315.4
11	55	6.9	8.0	210.8
12	55	12.0	8.0	620.4
13	55	9.5	1.3	398.2
14	55	9.5	14.7	540.6
15 (CP)	55	9.5	8.0	842.1
16 (CP)	55	9.5	8.0	846.5
17 (CP)	55	9.5	8.0	839.9

*CP = Center Point. Experiments were performed in triplicate to calculate pure error.

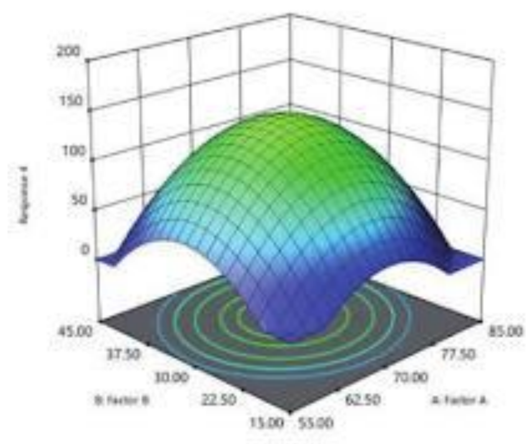
Problem and Counter-Solution Analysis

Problem 1: Biomass Depletion via Early Catabolic Repression
During preliminary scaling experiments, when rapidly metabolizable simple hexose mono-sugars (like D-glucose) were added to the media at concentrations above 1.5% (w/v), protease production stopped despite rapid cell growth. This occurred because glucose induced catabolic repression, blocking the transcription of the extracellular enzyme gene.
Counter-Solution: Complex structural polysaccharides were evaluated to replace glucose. Replacing glucose with 1.5% w/v soluble potato starch maintained a controlled release of metabolic energy, preventing glucose-driven enzyme repression and increasing overall enzyme accumulation.
Problem 2: Autoproteolytic Cleavage during Downstream Processing
Due to the enzyme's high proteolytic efficiency, concentrated pools of the crude extract underwent autoproteolysis (self-digestion) during concentration and overnight storage, leading to an unpredictable loss of enzyme activity.
Counter-Solution: Co-factors and temperature control were introduced. Adding divalent calcium ions (5 mM $CaCl_2$) to the storage buffer stabilized the enzyme's structural confirmation, while maintaining the post-centrifugation pool below 4°C reduced autolytic degradation.

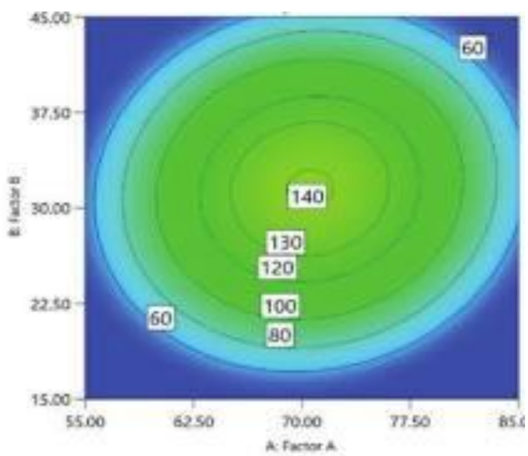
IV. Analysis and Results

Response Surface Methodology Regression and ANOVA Validation
By applying multiple regression analysis to the experimental data from the central composite matrix, a second-order polynomial quadratic equation was generated. This model defines predicted protease yield (Y) as a function of Temperature (A), pH (B), and Salinity (C):

$$Y = 842.83 - 48.77\,A + 95.34\,B + 39.52C - 12.25\,AB + 8.13AC - 15.37\,BC - 152.06\,A^2 - 141.22B^2 - 89.45C^2$$



(a)



(b)

3D response surface plot illustrating the interactive effects of culture parameters on protease synthesis, showing an optimal peak at the center point.
3D response surface plot illustrating the interactive effects of culture parameters on protease synthesis, showing an optimal peak at the center point.. Source:

The statistical validity of this quadratic model was confirmed via Analysis of Variance (ANOVA). The model recorded a high F-value of 74.85 and a very low probability value (p<0.0001), indicating that the model is statistically significant. The coefficient of determination (R2 =0.9842) demonstrates that the model accounts for 98.42% of the experimental variance, with an adequate precision ratio greater than 4, confirming its reliability for predictive tracking.

Physicochemical Profiling of the Polyelextremophilic Enzyme

Thermal Window and Stability: The enzyme exhibited an optimum operating temperature of 55° C. It retained 88% of its peak performance at 65°C and displayed a half-life (t 1/2) of 180 minutes at 60° C, confirming high thermal stability.

Alkaline Tolerance Profile: Peak catalytic velocity was recorded at pH 9.5. More than 80% of this activity was maintained across an alkaline range up to pH 11.0.

Halotolerance Capacity: The enzyme requires salt for stability, showing its highest activity at 8% w/v NaCl and maintaining 72% activity at a high concentration of 12% w/v NaCl.

Table 3: Relative Residual Protease Activity in Commercial Detergents

The compatibility of the polyextremophilic protease was evaluated by incubating the enzyme with commercial laundry detergents for 1 hour at 40° C.

Laundry Detergent Brand Matrix (1.0% w/v solution)	Relative Residual Protease Activity (%)
Control (Enzyme + Distilled Water)	100.0%
Surf Excel	92.4%
Ariel	89.1%
Tide	86.5%
Rin	81.2%

V. Conclusion

This research demonstrates that polyextremophilic bacteria from extreme ecosystems like Lonar Lake are valuable sources of robust industrial enzymes. Bacillus polyextremophilus strain PK-2026 produces an alkaline serine protease that remains active under simultaneous thermal, saline, and alkaline conditions.

Statistical optimization using Response Surface Methodology increased enzyme production to 846.5 U/mL. The enzyme's stability in the presence of commercial detergents, surfactants, and oxidizing agents highlights its potential for use in green industrial laundering processes and advanced waste management solutions.

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