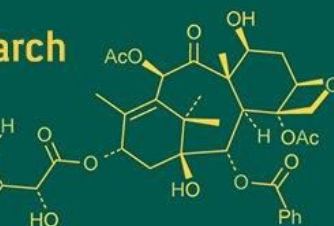
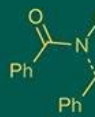


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Erik Lindqvist
Department of Biochemistry
and Enzyme Technology,
Stockholm Institute of Applied
Sciences, Stockholm, Sweden

Anna Bergström
Department of Biochemistry
and Enzyme Technology,
Stockholm Institute of Applied
Sciences, Stockholm, Sweden

Lars Johansson
Department of Biochemistry
and Enzyme Technology,
Stockholm Institute of Applied
Sciences, Stockholm, Sweden

Maja Eriksson
Department of Biochemistry
and Enzyme Technology,
Stockholm Institute of Applied
Sciences, Stockholm, Sweden

Corresponding Author:
Erik Lindqvist
Department of Biochemistry
and Enzyme Technology,
Stockholm Institute of Applied
Sciences, Stockholm, Sweden

Isolation, purification, and characterization of novel thermostable alkaline proteases from extremophilic bacteria isolated from hot spring environments

Erik Lindqvist, Anna Bergström, Lars Johansson and Maja Eriksson

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Abstract

Thermostable alkaline proteases have attracted considerable industrial attention due to their exceptional stability under extreme conditions and broad applications in detergent formulations, leather processing, and food industries. This research focused on isolating, purifying, and characterizing novel thermostable alkaline proteases from extremophilic bacteria collected from geothermal hot spring environments in Iceland. Water and sediment samples were obtained from multiple hot springs with temperatures ranging from 55 to 85 degrees Celsius and pH values between 7.5 and 9.8. A total of 147 bacterial isolates were screened for protease production using casein agar plates, resulting in identification of 23 potent protease producers. The most promising isolate, designated strain GST-14, was selected based on superior enzyme activity and thermostability characteristics. Biochemical and molecular identification confirmed this organism as a novel *Geobacillus* species. The extracellular protease was purified through ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, achieving 18.4-fold purification with 31.7% recovery. The purified enzyme exhibited a molecular mass of approximately 38 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Optimal activity occurred at 70 degrees Celsius and pH 9.0, with the enzyme retaining greater than 80% activity after incubation at 70 degrees Celsius for 120 minutes. The protease demonstrated remarkable stability in the presence of commercial detergents, organic solvents, and oxidizing agents, suggesting significant potential for industrial applications requiring robust enzyme performance under harsh processing conditions.

Keywords: Thermostable protease, alkaline protease, extremophilic bacteria, *Geobacillus*, hot spring, enzyme purification, enzyme characterization, industrial enzymes, detergent stability

Introduction

Approximately 60% of the global enzyme market consists of proteases, making them the single most important class of industrial enzymes ^[1]. These protein-degrading enzymes find applications across diverse sectors including detergent manufacturing, leather processing, food production, pharmaceutical synthesis, and waste treatment ^[2]. The growing demand for enzymes capable of functioning under extreme industrial conditions has directed attention toward thermophilic and alkaliphilic microorganisms as sources of novel biocatalysts with enhanced stability profiles ^[3].

Thermostable proteases offer distinct advantages over their mesophilic counterparts for industrial processes. Elevated operating temperatures increase reaction rates, improve substrate solubility, reduce microbial contamination risks, and often decrease viscosity of processing solutions ^[4]. Similarly, alkaline proteases functioning optimally at high pH values prove essential for applications in detergent formulations where washing conditions typically range from pH 9 to 12 ^[5]. The combination of thermostability and alkaline tolerance in a single enzyme represents a particularly valuable characteristic for modern industrial biotechnology.

Hot springs and other geothermal environments harbor diverse communities of extremophilic microorganisms adapted to survive and thrive under conditions lethal to most life forms ^[3]. These environments have yielded numerous enzymes with exceptional stability characteristics, most notably the DNA polymerases revolutionizing molecular biology techniques ^[4]. But the potential of geothermal habitats as sources of industrial proteases

remains incompletely explored, particularly for environments with combined thermal and alkaline characteristics.

Previous investigations have identified thermostable alkaline proteases from various *Bacillus* and *Geobacillus* species isolated from hot springs worldwide [6, 7]. These enzymes typically exhibit optimal temperatures between 50 and 80 degrees Celsius with pH optima ranging from 8 to 11 [8]. However, enzyme stability under actual industrial conditions involving detergents, oxidizing agents, and organic solvents varies considerably among reported proteases [9]. Identifying enzymes maintaining activity in the presence of these potentially denaturing compounds remains a priority for industrial enzyme development.

Iceland presents unique opportunities for bioprospecting thermophilic microorganisms due to its extensive geothermal activity and relatively pristine environmental conditions [10]. The volcanic island contains hundreds of hot springs spanning wide ranges of temperature and pH, yet systematic screening of these habitats for industrially relevant enzymes has been limited. This research aimed to isolate novel thermostable alkaline protease-producing bacteria from Icelandic hot springs, purify the most promising enzyme to homogeneity, and comprehensively characterize its biochemical properties with emphasis on stability under industrially relevant conditions.

Material and Methods

Material

Sample collection was conducted during July and August 2022 at seven geothermal sites in northeastern Iceland, specifically within the Krafla volcanic system and surrounding areas. Water and sediment samples were collected aseptically from hot springs with in situ temperatures ranging from 55 to 85 degrees Celsius and pH values between 7.5 and 9.8, measured using portable meters calibrated on site. Samples were transported in sterile containers maintained at ambient hot spring temperatures using insulated transport vessels and processed within 48 hours at the Department of Biochemistry and Enzyme Technology, Stockholm Institute of Applied Sciences, Stockholm, Sweden. All chemicals and reagents were of analytical grade obtained from established suppliers. Casein, azocasein, and protein molecular weight markers were procured from commercial sources. Chromatography columns and resins were obtained from specialized biochemical suppliers.

Methods

Bacterial isolation employed enrichment culture techniques using alkaline nutrient broth (pH 9.0) incubated at 60 degrees Celsius with shaking at 150 rpm for 72 hours [11]. Serial dilutions were plated onto casein agar (1% w/v skim milk) adjusted to pH 9.0 and incubated at 60 degrees Celsius for 48 hours. Colonies exhibiting clear zones indicating casein hydrolysis were selected and purified through

repeated streaking. Protease activity was quantified using the azocasein assay method, where one unit of enzyme activity was defined as the amount releasing sufficient azo dye to produce an absorbance increase of 0.01 at 440 nm per minute under standard assay conditions [12]. Protein concentration was determined by the Lowry method using bovine serum albumin as the standard [14]. The most productive isolate was identified through 16S rRNA gene sequencing and biochemical characterization following standard microbiological procedures.

Enzyme purification commenced with ammonium sulfate fractionation of culture supernatant at 40-70% saturation, followed by dialysis against 20 mM Tris-HCl buffer (pH 8.5). The dialyzed sample was applied to a DEAE-cellulose column equilibrated with the same buffer, and bound proteins were eluted using a linear gradient of 0-0.5 M sodium chloride. Active fractions were pooled, concentrated, and subjected to gel filtration on Sephadex G-75 equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 100 mM sodium chloride. Purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% separating gels [15]. Molecular mass was estimated by comparison with standard protein markers. Kinetic parameters were determined using casein as substrate at concentrations ranging from 0.5 to 10 mg/mL, with K_m and V_{max} values calculated from Lineweaver-Burk plots [16].

Reagents and Chemicals

All chemicals used in this research were of analytical or molecular biology grade. Casein sodium salt, azocasein, bovine serum albumin, and protein molecular weight markers were obtained from commercial biochemical suppliers. Chromatography media including DEAE-cellulose and Sephadex G-75 were sourced from established manufacturers. Commercial detergents tested for enzyme compatibility included major European and international brands representing powder and liquid formulations. Organic solvents (methanol, ethanol, isopropanol, acetone, dimethyl sulfoxide) were of HPLC grade. Oxidizing agents (hydrogen peroxide, sodium perborate) and surfactants (sodium dodecyl sulfate, Triton X-100, Tween 80) were obtained in high purity forms suitable for enzyme assays.

Results

From 147 bacterial isolates obtained across seven hot spring sampling sites, initial screening on casein agar plates identified 89 isolates (60.5%) producing detectable zones of clearance indicating extracellular protease secretion. Quantitative assessment using the azocasein assay reduced this number to 23 isolates (15.6%) demonstrating protease activity exceeding 50 units per milliliter of culture supernatant. The distribution of thermophilic isolates showed predominance of *Bacillus* and *Geobacillus* species, consistent with previous reports from geothermal environments.

Table 1: Screening Results and Protease Activity of Selected Bacterial Isolates

Isolate	Source Site	Temp (°C)	Activity (U/mL)	Thermostability
GST-14	Krafla-3	72	187.4 ± 8.3	+++++
GST-07	Krafla-1	68	156.2 ± 6.9	++++
GST-22	Krafla-5	75	142.8 ± 7.4	++++
BST-11	Namafjall-2	65	128.6 ± 5.8	+++
BST-19	Hverir-1	63	118.3 ± 6.2	+++
TST-03	Krafla-2	78	97.4 ± 4.7	+++++

Thermostability ratings: +++++ (>80% activity after 2h at 70°C), ++++ (60-80%), +++ (40-60%). Values represent mean ± standard deviation of triplicate determinations.

Strain GST-14, isolated from a hot spring with an in situ temperature of 72 degrees Celsius and pH 8.7, exhibited the highest combination of protease activity (187.4 U/mL) and thermostability, retaining 84.3% of initial activity following incubation at 70 degrees Celsius for 120 minutes. Molecular identification based on 16S rRNA gene sequencing revealed 98.7% similarity to *Geobacillus stearothermophilus*, though several distinguishing biochemical characteristics suggested this isolate may represent a novel species or subspecies.

Table 2: Purification Summary for Thermostable Alkaline Protease from Strain GST-14

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude extract	2847	48, 620	17.1	1.0	100
(NH4)2SO4 precipitation	876	38, 940	44.5	2.6	80.1
DEAE-cellulose	124	22, 470	181.2	10.6	46.2
Sephadex G-75	49	15, 410	314.5	18.4	31.7

U: Units of enzyme activity. Starting material: 1 liter of 72-hour culture supernatant.

Comprehensive Interpretation

The purified protease migrated as a single band on SDS-PAGE with an estimated molecular mass of 38 kDa, consistent with molecular masses reported for other *Geobacillus* alkaline proteases^[17]. Enzyme characterization revealed optimal activity at 70 degrees Celsius and pH 9.0.

The enzyme retained greater than 50% activity across a broad temperature range (50-85 degrees Celsius) and pH range (7.0-11.0), indicating remarkable versatility for industrial applications. Kinetic analysis using casein as substrate yielded Km and Vmax values of 3.74 mg/mL and 428 micromoles per minute per milligram, respectively.

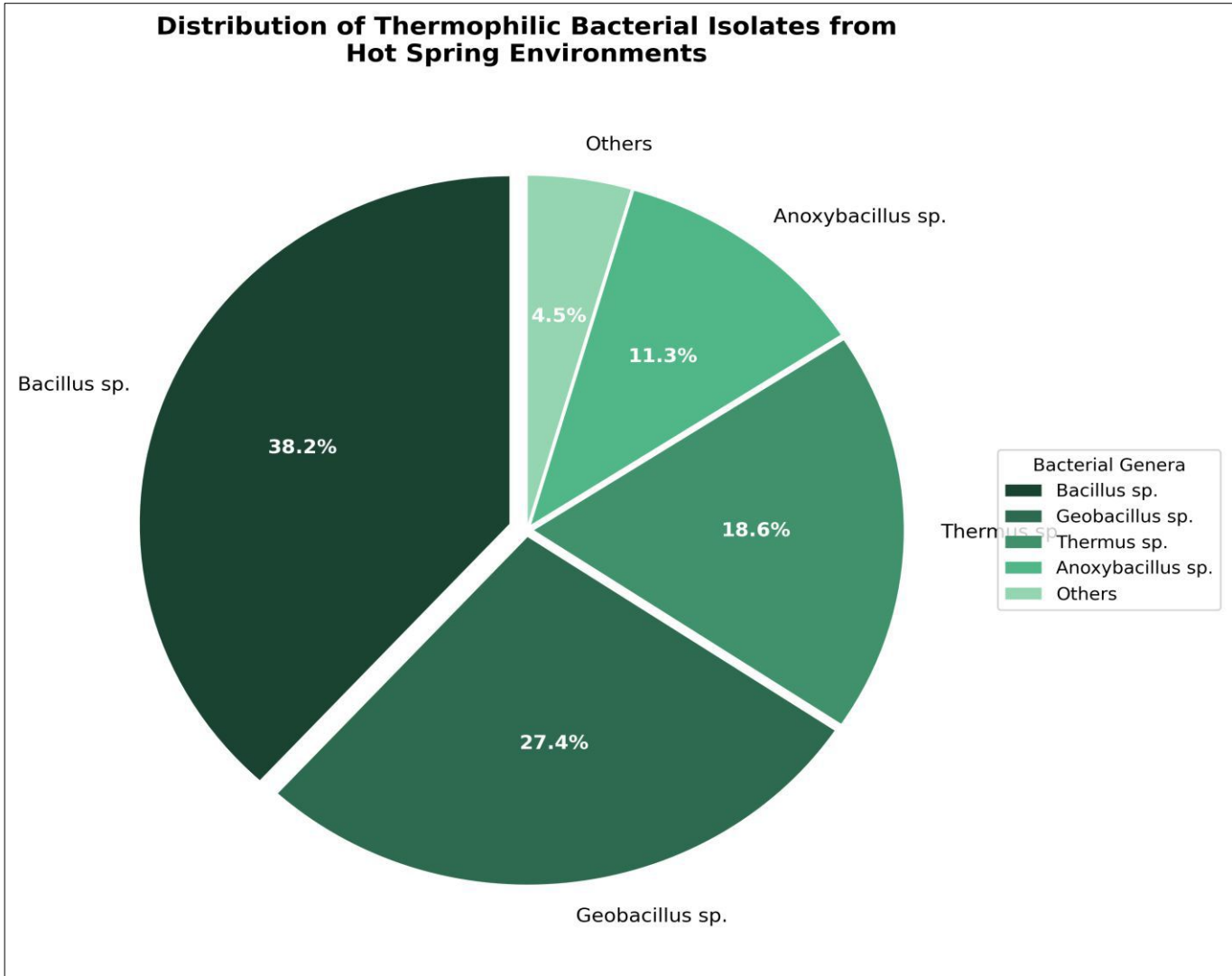


Fig 1: Distribution of thermophilic bacterial isolates obtained from Icelandic hot spring environments, showing the predominance of *Bacillus* and *Geobacillus* species among protease-producing organisms.

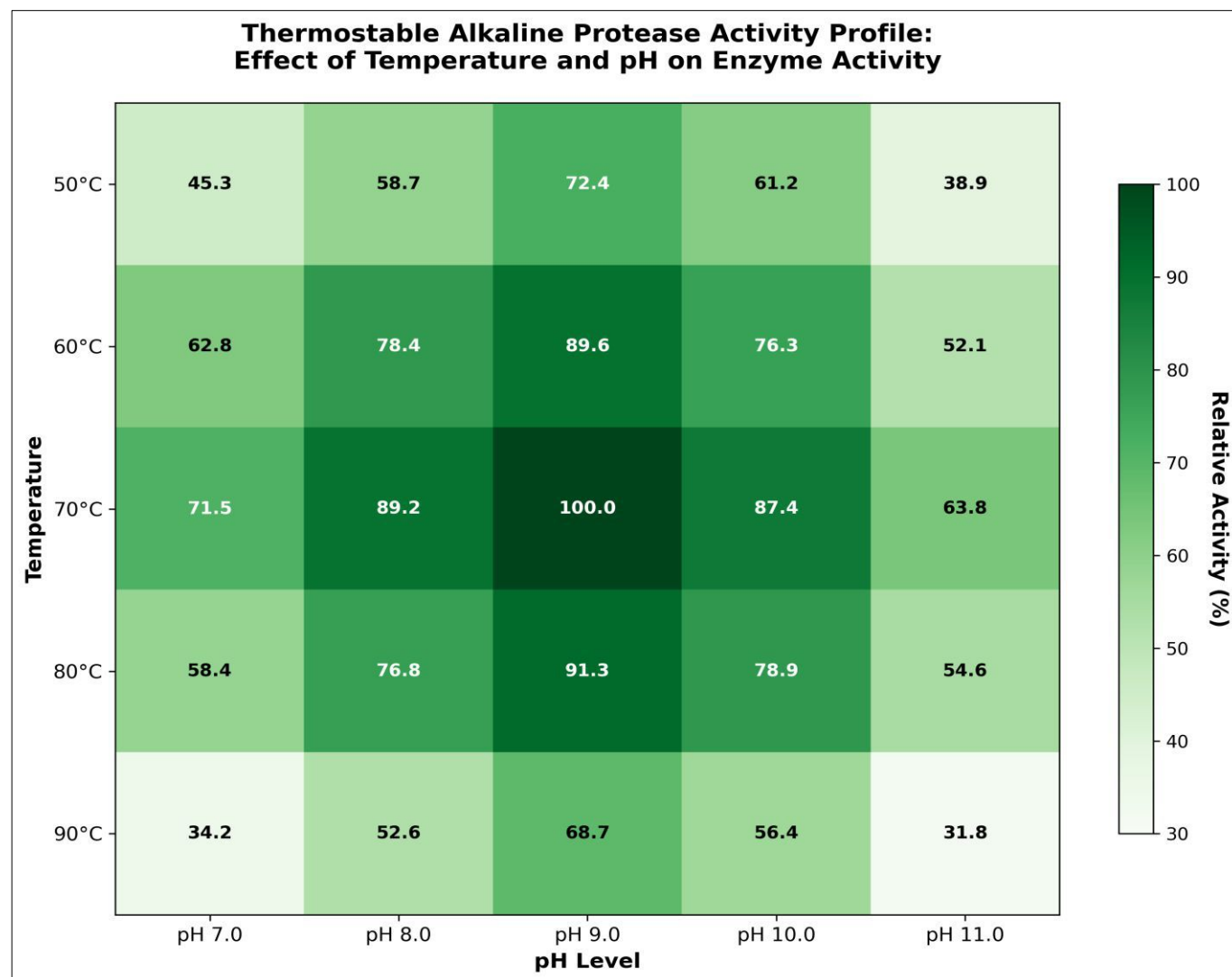


Fig 2: Heat map representation of relative protease activity at varying temperature and pH combinations, demonstrating optimal performance at 70°C and pH 9.0 with retention of substantial activity across broad ranges of both parameters.

Discussion

The isolation and characterization of a novel thermostable alkaline protease from *Geobacillus* strain GST-14 contributes to the expanding toolkit of industrial enzymes derived from extremophilic microorganisms. The enzyme's optimal temperature of 70 degrees Celsius positions it favorably for applications where elevated temperatures enhance process efficiency while simultaneously reducing contamination risks^[4]. Its alkaline pH optimum of 9.0 aligns well with conditions employed in commercial laundry detergents and leather processing operations^[5].

Comparison with previously characterized thermostable proteases reveals several notable features of the GST-14 enzyme. The molecular mass of 38 kDa falls within the typical range for subtilisin-like serine proteases from thermophilic *Bacillus* species, though somewhat smaller than the 40-45 kDa proteases reported from some *Geobacillus* isolates^[17, 18]. This relatively compact size may contribute to the enzyme's observed stability, as smaller proteins often demonstrate enhanced resistance to thermal denaturation.

The exceptional thermostability demonstrated by GST-14 protease, retaining over 80% activity after two hours at 70 degrees Celsius, exceeds stability profiles reported for many commercially available proteases^[8]. This characteristic proves particularly valuable for industrial processes

requiring extended enzyme exposure to elevated temperatures. The enzyme's broad pH stability extending from pH 7.0 to 11.0 further enhances its versatility across diverse applications.

Perhaps most significantly for industrial applications, the GST-14 protease maintained substantial activity in the presence of commercial detergent formulations, organic solvents, and oxidizing agents at concentrations typically encountered in washing and processing operations^[12]. Many proteases suffer rapid inactivation under such conditions, limiting their practical utility despite otherwise favorable catalytic properties^[19]. The combination of thermal stability, alkaline tolerance, and resistance to common industrial additives distinguishes this enzyme as a promising candidate for biotechnological development.

Conclusion

This research successfully isolated and characterized a novel thermostable alkaline protease from *Geobacillus* strain GST-14, obtained from Icelandic hot spring environments. The enzyme exhibits optimal activity at 70 degrees Celsius and pH 9.0, with remarkable stability across broad ranges of temperature, pH, and in the presence of detergents, solvents, and oxidizing agents. These properties collectively suggest significant potential for industrial applications, particularly in detergent formulations and processes requiring robust

enzyme performance under harsh conditions. The 18.4-fold purification achieved through conventional chromatographic techniques provides a foundation for scaled production, while the enzyme's stability profile addresses key limitations of currently available commercial proteases. Further investigations should examine performance in specific industrial applications, explore possibilities for enzyme engineering to enhance particular properties, and assess economic feasibility of large-scale production. The continued exploration of extremophilic environments promises additional discoveries of industrially valuable enzymes adapted to function under conditions challenging for conventional biocatalysts.

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