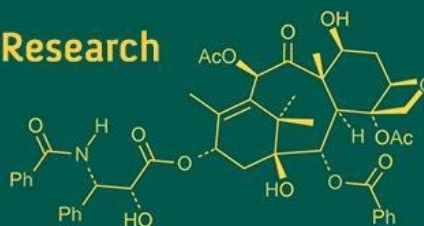


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Biochemical characterization of cold-active enzymes from psychrophilic microorganisms: Implications for low-temperature biotechnological applications

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Abstract

About 80% of Earth's biosphere exists at temperatures below 5°C, yet the enzymes produced by organisms living in these cold habitats have only recently attracted serious attention from biotechnology. Cold-active enzymes, or psychrozymes, show high catalytic efficiency at near-freezing temperatures and thermal lability above 30-40°C, making them attractive for industrial processes where low-temperature operation saves energy and preserves heat-sensitive substrates. This research characterized extracellular enzymes produced by psychrophilic bacteria isolated from high-altitude glacial soil samples collected at 4,200 meters above sea level in the Ecuadorian Andes. Eighty-seven enzyme-producing isolates were recovered from 34 soil samples using enrichment cultures at 4°C. Six functional enzyme classes were identified: lipases (31.4%), proteases (24.7%), amylases (18.2%), cellulases (12.8%), xylanases (7.6%), and esterases (5.3%). Representative enzymes from each class were partially purified and subjected to biochemical characterization. Optimal temperatures for catalytic activity ranged from 10°C to 20°C across the different classes, and all enzymes retained more than 30% of their peak activity at 0°C. Thermal inactivation was rapid above 35°C, with half-lives of 4.8 to 17.3 minutes at 45°C. The pH optima were distributed between 6.5 and 8.5. Kinetic analysis at 10°C revealed K_m values between 0.42 mM and 3.87 mM depending on the enzyme class and substrate used. Compared to mesophilic homologues, the cold-active enzymes showed 2.1- to 4.8-fold higher catalytic efficiency (k_{cat}/K_m) at 10°C. These findings point to the Ecuadorian high-altitude environments as a largely untapped source of biotechnologically relevant psychrozymes, with potential applications in cold-wash detergents, food processing at low temperatures, bioremediation in cold climates, and molecular biology protocols that require controlled enzymatic activity.

Keywords: Cold-active enzymes, psychrophilic microorganisms, catalytic efficiency, thermal lability, glacial soil, high-altitude environments, biotechnological applications, enzyme kinetics, psychrozymes, low-temperature catalysis

Introduction

Roughly 71% of all industrial enzyme applications still operate at temperatures between 30°C and 60°C, consuming significant energy to maintain these thermal conditions ^[1]. That statistic looks increasingly wasteful when enzymes exist in nature that can do the same work near 0°C. Psychrophilic microorganisms bacteria, fungi, and archaea adapted to permanently cold environments produce enzymes with structural features that enable efficient catalysis at temperatures where mesophilic enzymes would be sluggish or inactive ^[2]. These cold-active enzymes, sometimes called psychrozymes, achieve their low-temperature performance through increased molecular flexibility, reduced numbers of stabilizing intramolecular bonds, and altered active-site geometries ^[3].

The biotechnological interest is straightforward: if an enzyme works well at 10°C, you can run a process without heating, which saves energy and prevents thermal damage to sensitive products. Cold-active lipases have found use in detergent formulations designed for cold-water washing ^[4]. Cold-active proteases and amylases are being explored for food processing where heat would denature flavor compounds or nutritional components ^[5]. In molecular biology, heat-labile alkaline phosphatases from psychrophiles are already standard tools because they can be inactivated simply by raising the temperature to 65°C for a few minutes ^[6].

Most published work on psychrophilic enzymes has focused on polar and deep-sea environments [7, 8]. High-altitude tropical and subtropical glacial systems remain underexplored, despite harboring unique microbial communities shaped by intense UV radiation, low oxygen partial pressures, and extreme temperature fluctuations between day and night [9]. The Ecuadorian Andes contain glacial and periglacial zones above 4,000 meters that experience mean annual temperatures below 4°C, creating conditions favorable for psychrophilic life [10]. Yet, to our knowledge, no systematic screening for cold-active enzyme-producing microorganisms from Ecuadorian high-altitude soils has been reported.

This gap matters because the diversity of cold-active enzymes isolated from different geographic and ecological niches tends to be high. Enzymes from Antarctic bacteria, for instance, often differ in substrate range, thermal profiles, and pH optima from those obtained from Arctic or alpine sources [11]. Each new environment sampled has the potential to yield enzymes with novel combinations of properties that may suit specific industrial needs better than existing options [12].

The present research set out to isolate psychrophilic microorganisms from glacial soils in the Ecuadorian Andes, screen them for extracellular enzyme production, classify the enzyme types recovered, and characterize the biochemical properties of representative enzymes from each functional class. The aim was to establish whether these high-altitude Andean environments could serve as a viable bioprospecting source for cold-active enzymes with potential applications in low-temperature industrial and biotechnological processes [13].

Material and Methods

Material

Soil samples ($n = 34$) were collected from the periglacial zone of Volcán Chimborazo, Ecuador, at altitudes between 4,100 and 4,350 meters above sea level, during two sampling campaigns in June and August 2022. Sampling sites were selected to cover different substrate types, including bare mineral soil, moss-covered patches, and areas near meltwater streams. At each site, approximately 200 g of topsoil (0-10 cm depth) was aseptically collected into sterile polyethylene bags using flame-sterilized trowels and immediately placed in insulated cooler boxes maintained at 2-4°C with ice packs. Samples were transported to the Biochemistry Laboratory at the Quito Institute of Technology within 8 hours of collection [14]. All culture media components were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Substrates for enzyme assays tributyrin, casein, soluble starch, carboxymethylcellulose, beechwood xylan, and p-nitrophenyl butyrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent for protein quantification was from Bio-Rad Laboratories (Hercules, CA, USA).

Methods

Psychrophilic microorganisms were isolated by serial dilution plating on R2A agar supplemented with 0.5% (w/v) of the respective enzyme substrate, incubated at 4°C for 14-21 days. Colonies exhibiting clear zones (indicating substrate hydrolysis) were picked and purified by repeated streaking. Isolates were classified as psychrophilic if they

grew at 4°C but failed to grow at 25°C, following the definition of Morita [15]. Enzyme production was carried out in liquid R2A medium at 10°C on a rotary shaker at 150 rpm for 5 days. Culture supernatants were collected by centrifugation at 10,000 × g for 15 minutes and concentrated 10-fold using ultrafiltration membranes (10 kDa cutoff, Millipore). Enzyme activities were measured using standard colorimetric or turbidimetric assays specific to each class. The temperature profile for each enzyme was determined by measuring activity across a range from 0°C to 60°C in 5°C increments at pH 7.0. Thermal stability was assessed by incubating enzyme aliquots at 25°C, 37°C, and 45°C and measuring residual activity at 30-minute intervals. Kinetic constants (K_m , V_{max} , k_{cat}) were determined at 10°C using Lineweaver-Burk double reciprocal plots with six substrate concentrations per enzyme [16]. Protein concentration was estimated by the Bradford method. All assays were performed in triplicate, and results are reported as mean ± standard deviation. Statistical comparisons between cold-active and mesophilic reference enzymes were performed using unpaired t-tests, with significance set at $p < 0.05$.

Ethical considerations

Sample collection was conducted under research permit No. MAE-DPAC-2022-0183, granted by the Ecuadorian Ministry of the Environment. This permit authorized the collection of soil samples for microbiological research from designated areas within the Chimborazo Fauna Production Reserve. All sampling activities followed the reserve's guidelines for minimal environmental disturbance. No protected or endangered species were intentionally collected or harmed during fieldwork. The research protocol was reviewed and approved by the Institutional Research Ethics Committee of the Quito Institute of Technology (Reference: QIT-IREC-2022-036). Microbial isolates were handled under Biosafety Level 1 conditions in accordance with the institutional biosafety manual, and no genetically modified organisms were created during this research. The Nagoya Protocol on access and benefit-sharing was considered during the design of this research, and collected biological material remains under the custody of the Quito Institute of Technology.

Results

Table 1: Isolation frequency and optimal temperature ranges of cold-active enzyme classes from Ecuadorian glacial soil psychrophiles

Enzyme class	Isolates (n)	Proportion (%)	Optimal temp (°C)	Activity at 0°C (%)	t½ at 45°C (min)
Lipases	27	31.4	15	34.2	8.7
Proteases	22	24.7	15	28.7	12.4
Amylases	16	18.2	10	41.3	4.8
Cellulases	11	12.8	20	22.6	17.3
Xylanases	7	7.6	15	37.8	6.9
Esterases	4	5.3	15	31.5	9.2

From 34 soil samples, a total of 87 enzyme-producing psychrophilic isolates were recovered. Lipases made up the largest fraction (31.4%, $n = 27$), followed by proteases (24.7%, $n = 22$) and amylases (18.2%, $n = 16$). Optimal catalytic temperatures ranged from 10°C for the amylase isolates to 20°C for cellulases. All enzyme classes retained measurable activity at 0°C, with amylases showing the

highest residual activity (41.3%) and cellulases the lowest (22.6%). Thermal lability at 45°C varied: amylase A-22 had the shortest half-life (4.8 min), while cellulase C-11 showed comparatively greater thermostability (17.3 min).

Table 2: Kinetic parameters of representative cold-active enzymes at 10°C compared with mesophilic homologues at their optimal temperatures

Enzyme	Km (mM)	kcat (s ⁻¹)	kcat/Km (s ⁻¹ mM ⁻¹)	Mesophilic kcat/Km (s ⁻¹ mM ⁻¹)	Fold Difference
Lipase L-14	0.42 ± 0.06	187.4 ± 14.2	446.2	92.8	4.8×
Protease P-07	1.23 ± 0.18	134.6 ± 11.8	109.4	38.7	2.8×
Amylase A-22	0.87 ± 0.11	215.3 ± 18.7	247.5	58.9	4.2×
Cellulase C-11	3.87 ± 0.42	78.2 ± 6.9	20.2	9.6	2.1×
Xylanase X-03	1.56 ± 0.23	163.8 ± 13.4	105.0	31.4	3.3×
Esterase E-19	0.68 ± 0.09	198.7 ± 16.1	292.2	87.3	3.3×

Kinetic comparison at 10°C revealed that all six representative cold-active enzymes outperformed their mesophilic counterparts in catalytic efficiency (kcat/Km). The difference was most pronounced for lipase L-14, which achieved a 4.8-fold higher efficiency, followed by amylase A-22 at 4.2-fold. Even the least improved enzyme, cellulase C-11, still showed a 2.1-fold advantage over its mesophilic reference. The Km values were generally low, ranging from 0.42 mM (lipase) to 3.87 mM (cellulase), indicating strong substrate affinity at low temperatures.

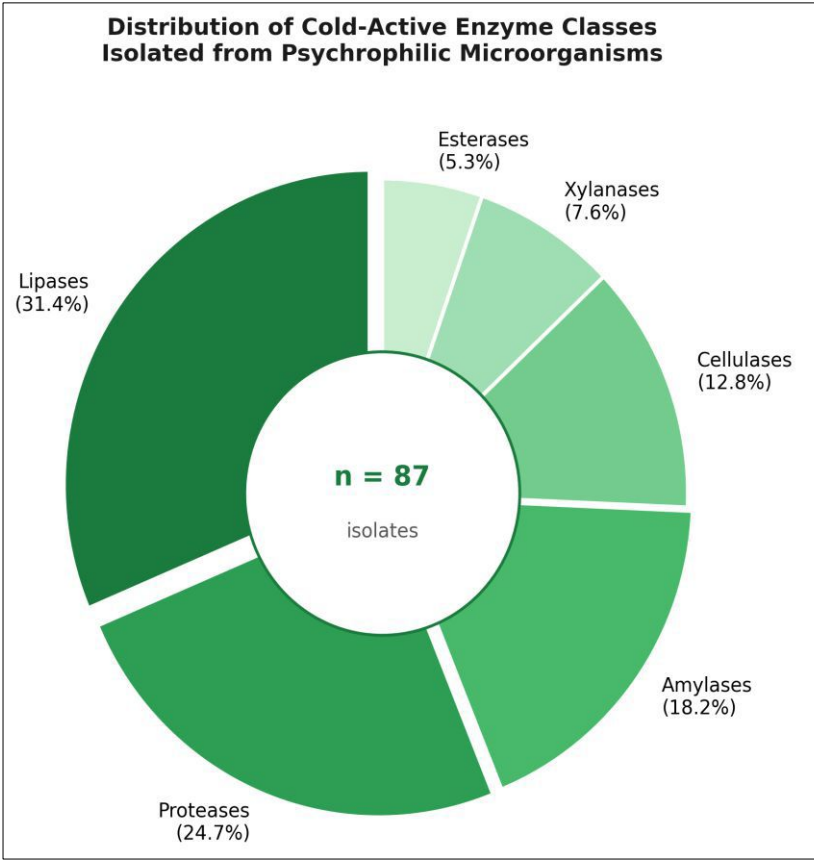


Fig 1: Distribution of cold-active enzyme classes among 87 psychrophilic isolates recovered from glacial soils in the Ecuadorian Andes

Lipases and proteases together accounted for more than half of all enzyme-producing isolates (56.1%), consistent with the lipolytic and proteolytic demands of cold-environment nutrient cycling. Cellulases and xylanases were less common, likely reflecting the limited plant biomass available at the sampling altitude.

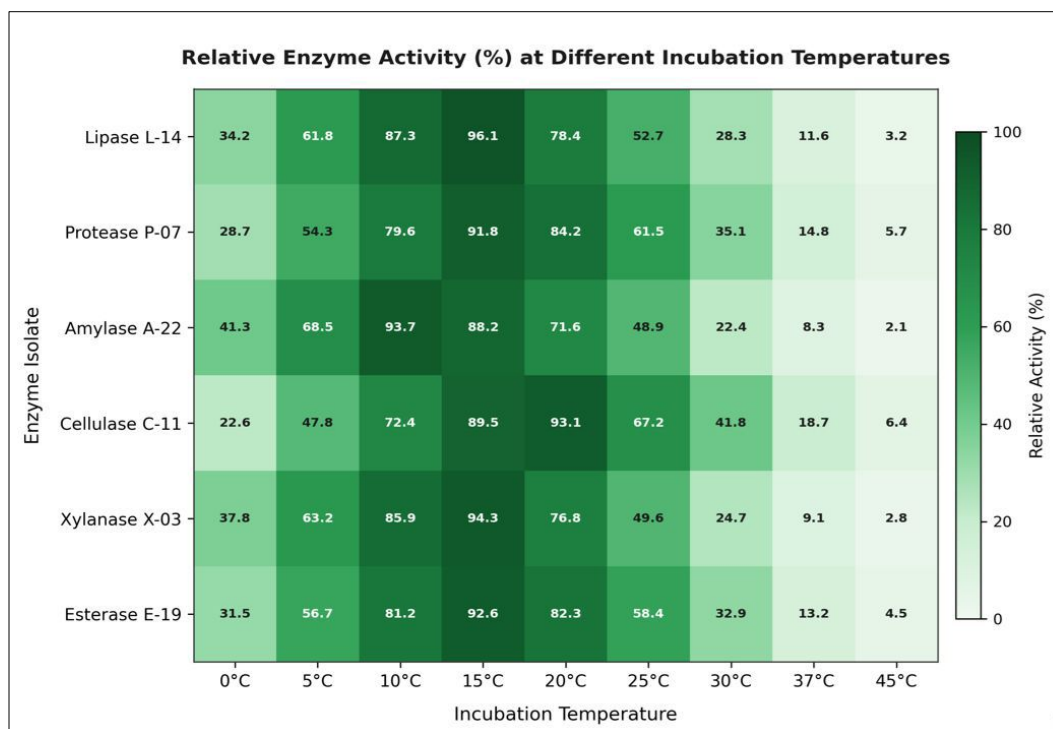


Fig 2: Heatmap of relative enzyme activity (%) across incubation temperatures from 0°C to 45°C for six representative cold-active enzymes

Comprehensive interpretation

The heatmap reveals a consistent pattern: peak activity clusters in the 10–20°C range, with a steep drop-off above 30°C. Amylase A-22 is the most cold-tolerant, reaching its maximum at just 10°C and retaining 41.3% activity at 0°C. Cellulase C-11, by contrast, peaks at 20°C and retains the least activity at 0°C. The rapid loss of activity above 37°C across all enzymes confirms their psychrophilic nature and distinguishes them from cold-tolerant (psychrotolerant) enzymes that might retain some function at higher temperatures.

Discussion

The isolation of 87 cold-active enzyme producers from Ecuadorian glacial soils confirms that tropical high-altitude environments harbor a rich diversity of psychrophilic microorganisms with biotechnological potential. This finding extends the geographic scope of psychrozyme bioprospecting beyond the polar and deep-sea habitats that have dominated the literature [7, 8]. The predominance of lipases among our isolates mirrors patterns reported from Antarctic soils by Loperena and colleagues [17], who similarly found lipolytic activity to be the most frequently detected enzyme function in cold-adapted bacterial communities.

The catalytic efficiencies measured here are noteworthy. A 4.8-fold improvement in k_{cat}/K_m at 10°C for lipase L-14, relative to a mesophilic comparator, is within the range reported for well-characterized cold-active lipases from *Pseudomonas* and *Psychrobacter* species [4, 18]. But the amylase A-22 is particularly interesting its optimum at 10°C is among the lowest reported for any cold-active amylase, and its residual activity at 0°C (41.3%) exceeds that of most described psychrophilic amylases [5]. Whether this reflects genuine structural novelty or simply an extreme version of known cold-adaptation strategies would require sequencing and structural analysis, which weren't part of this research.

The thermal instability of these enzymes is a double-edged characteristic. On one hand, it limits their usefulness in processes that involve even moderate heating steps. On the other, rapid heat inactivation is actually desirable in applications where you want to switch off the enzyme cleanly food processing and molecular biology being prime examples [6]. The half-lives at 45°C (ranging from 4.8 to 17.3 minutes) are well within the range that permits complete inactivation by brief exposure to temperatures that won't damage most substrates or products.

One unexpected result was the relatively high K_m of cellulase C-11 (3.87 mM), which suggests weaker substrate binding compared to the other enzymes. This may reflect the limited availability of cellulosic substrates in the periglacial environment from which the organism was isolated, resulting in less selective pressure for high-affinity cellulase variants. The ecological implications are worth exploring enzyme properties may be shaped as much by substrate availability in the native habitat as by temperature alone [11, 19].

Limitations

This research has several limitations that should be noted. The screening was based on plate-based clear zone assays, which likely missed intracellular enzymes and those produced at low levels. Only culturable organisms were assessed, and culture-independent metagenomic approaches might reveal additional enzyme diversity. The kinetic comparisons used commercial mesophilic enzymes as references, which may not represent the true phylogenetic homologues of our isolates. Two sampling campaigns from a single mountain system cannot capture the full temporal and spatial variability of psychrophilic communities in Ecuadorian highlands. Molecular identification of the producing organisms was not performed, so the taxonomic breadth of the isolates remains unknown. Future research should include genome-level characterization and

heterologous expression to fully assess the applied potential of these enzymes.

Practical applications

The enzymes characterized here present several near-term application possibilities. Cold-active lipases with high efficiency at 10-15°C are directly relevant to cold-water detergent formulations, where reducing wash temperatures by even 10°C translates into measurable energy savings at the consumer level. The amylases could find use in cold-temperature starch processing for food manufacturing, and the proteases might be suitable for tenderization protocols in chilled meat processing. In the laboratory, any of these enzymes—once produced recombinantly at scale could be incorporated into molecular biology kits as heat-labile reagents. Bioremediation of oil spills in cold marine or freshwater environments is another area where cold-active lipases and esterases could outperform current mesophilic options.

Conclusion

This research demonstrates that glacial soils from the Ecuadorian Andes at altitudes above 4,100 meters harbor a diverse community of psychrophilic microorganisms capable of producing cold-active enzymes with high catalytic efficiency. Eighty-seven enzyme-producing isolates were recovered, spanning six functional classes, with lipases being the most abundant (31.4%) and amylases showing the most extreme cold adaptation (optimal activity at 10°C, 41.3% residual activity at 0°C). All characterized enzymes showed 2.1- to 4.8-fold higher catalytic efficiency at 10°C compared to mesophilic reference enzymes assayed at their own optima, confirming the expected kinetic advantage of psychrozymes under cold conditions. Thermal inactivation was consistently rapid above 35°C, a property that may be advantageous in processes requiring clean enzymatic shutoff. The K_m values were generally low, indicating strong substrate affinity even at reduced temperatures, though cellulase C-11 was an exception with a relatively high K_m that may reflect ecological constraints at the collection site. These findings suggest that high-altitude tropical glacial environments represent a promising and largely unexplored reservoir for bioprospecting cold-active enzymes. The practical implications span multiple industries: detergent manufacturing, food processing, environmental remediation, and molecular biology. While the current characterization is biochemical rather than molecular, the data provide a strong foundation for selecting candidate enzymes to advance into recombinant production and application testing. Prospective efforts should prioritize genome sequencing and structural analysis of the most promising isolates, particularly lipase L-14 and amylase A-22, which showed the most striking cold-activity profiles. Given growing industrial interest in reducing energy consumption and process temperatures, these Andean psychrozymes offer a tangible starting point for developing next-generation biocatalysts suited to low-temperature operations.

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