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Crude protease of *Bacillus smithii* AP67 isolated from the El Chichón volcano crater lake as a potential eco-friendly alternative for enzymatic dehairing in the leather industry

Proteasa cruda de *Bacillus smithii* AP67 aislada del lago cráter del volcán El Chichón como alternativa ecológica potencial para el depilado enzimático en la industria del cuero

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Abstract

Microbial proteases from thermophilic organisms are of great biotechnological interest due to their stability and activity under extreme conditions. Among their applications, enzymatic skin depilation is becoming a more environmentally friendly and specific alternative compared to conventional chemical methods. In this study, thermophilic bacteria were isolated from the geothermal water of the crater lake of the El Chichón volcano. Of the 50 strains isolated, 92% showed extracellular protease activity. The strain with the highest protease production showed high phylogenetic similarity to *Bacillus smithii*, reaching an activity of 102.75 ± 6.39 U/mL. The enzyme showed optimal conditions at pH 8.0 and 60°C. The presence of Ca^{2+} increased its activity and remained stable with Mg^{2+} and Mn^{2+}, while Co^{2+}, Fe^{2+}, and Cu^{2+} partially inhibited it. It also remained stable in ethanol and methanol but decreased in hexane, acetone, and chloroform. The activity remained stable in the presence of nonionic surfactants, although it was reduced with SDS. The application of the protease to goat skin allowed complete hair removal after 8 h at 60 °C without damaging the dermis, demonstrating its potential as an ecological biocatalyst in sustainable leather tanning.

Keywords: Thermophile, Protease, *Bacillus smithii*, Volcanic Crater Lake, Enzymatic Dehairing.

Resumen

Las proteasas microbianas de organismos termófilos son de gran interés biotecnológico debido a su estabilidad y actividad en condiciones extremas. Entre sus aplicaciones, la depilación enzimática de la piel se está convirtiendo en una alternativa más ecológica y específica en comparación con los métodos químicos convencionales. En este estudio, se aislaron bacterias termófilas del agua geotérmica del lago del cráter del volcán El Chichón. De las 50 cepas aisladas, el 92 % mostró actividad proteasa extracelular. La cepa con mayor producción de proteasas mostró una alta similitud filogenética con *Bacillus smithii*, alcanzando una actividad de 102.75 ± 6.39 U/mL. La enzima mostró condiciones óptimas a pH 8.0 y 60 °C. La presencia de Ca^{2+} aumentó su actividad y se mantuvo estable con Mg^{2+} y Mn^{2+}, mientras que Co^{2+}, Fe^{2+} y Cu^{2+} la inhibieron parcialmente. También se mantuvo estable en etanol y metanol, pero disminuyó en hexano, acetona y cloroformo. La actividad se mantuvo estable en presencia de tensioactivos no iónicos, aunque se redujo con SDS. La aplicación de la proteasa a la piel de cabra permitió la eliminación completa del vello después de 8 h a 60 °C sin dañar la dermis, demostrando su potencial como biocatalizador ecológico en el curtido sostenible del cuero.

Palabras clave: Termófilo, Proteasa, *Bacillus smithii*, Lago del cráter volcánico, Depilación enzimática.

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1 Introduction

The leather tanning industry has historically relied on the intensive use of chemicals that utilize lime and sodium sulfide to remove hair and soften hides. Although these practices produce effective results, they generate highly alkaline effluents with high levels of sulfides and suspended solids, which account for approximately 80% to 90% of the polluting waste generated in this sector (Hasan *et al*., 2022; George *et al*., 2014; Aruchalam & Saritha, 2009). This environmental issue, together with the risks posed by these compounds to workers' health, has intensified the need to develop safer and more sustainable alternatives aimed at reducing the use of aggressive chemicals, minimizing waste generation, and promoting more efficient tanning processes (China *et al.,* 2020). In this regard, enzymes from microorganisms have established themselves as industrial biocatalysts of great importance because they possess high efficiency, specificity, and robustness, which gives them the ability to function in a wide range of environments (Nachimuthu *et al.,* 2022). Studies based on crude enzyme extracts have demonstrated their efficiency in hydrolysis processes and their potential applicability in the development of sustainable bioprocesses (Hernández-Teyssier *et al.*, 2024). In particular, proteases stand out as key biotechnological alternatives for partially or totally replacing chemical agents in tanning, particularly during the unhairing stage (Biškauskaitė *et al.,* 2021). These enzymes hydrolyze non-collagenous proteins and components associated with hair follicles, thereby enabling hair removal without damaging the collagen matrix (Gao *et al.,* 2023; Shakilanishi & Shanthi, 2017). Their implementation has been shown to significantly reduce pollutant load, water consumption, and risks associated with handling hazardous compounds, making them valuable tools for advancing toward cleaner and safer tanning processes (Ismail *et al.,* 2025; Hasan *et al*., 2022).

Proteases account for about 60% of global demand for industrial biocatalysts used in leather processing, detergent formulation, pharmaceuticals, food, protein hydrolysate production, and waste treatment (Song *et al.,* 2023; Solanki *et al.,* 2021; Razzaq *et al*., 2019). However, industrial processes often involve extreme pH and temperature conditions that require more robust biocatalysts. In this regard, thermophilic proteases present substantial advantages, as they retain activity and stability under severe conditions and exhibit resistance to denaturing agents and organic solvents (Singh *et al.,* 2024; Kochhar *et al.,* 2022). These properties have encouraged the search for microbial sources that produce enzymes, with particular interest in bacteria of the genus *Bacillus*, recognized as one of the main sources of bacterial proteases due to their high yields in the production of enzymes with robust characteristics and outstanding stability in extreme conditions (Contesini *et al.,* 2018; Iqbal *et al*., 2015).

Extreme environments are considered underexplored reservoirs of thermophilic microorganisms producing enzymes with unique characteristics. These microorganisms are mainly found in hot springs in various parts of the world, as well as in areas of active or dormant volcanoes (Avila-Andrade *et al.,* 2025; Schultz *et al.,* 2023; Márquez & Blamey, 2019). These environments include shallow terrestrial thermal lakes, hydrothermal vents on the ocean floor, and coastal regions with geothermal activity, which have been identified as habitats for bacteria capable of producing enzymes of biotechnological interest (Ortega-Villar *et al.,* 2024; Guta *et al.,* 2024; Schultz *et al.,* 2023).

El Chichón volcano, located in Chiapas, Mexico (17°21'N, 93°41'W; 1,100 masl), is the most active volcano in the region and dates to the Pleistocene epoch. Its last Plinian eruption took place in March 1982, leading to the development of a crater lake whose size, level, and chemical composition have since undergone continuous variation (Legrand *et al.,* 2024; Casas *et al.,* 2016; Armienta *et al.,* 2008). This lake is distinguished by its temperature variability at different points, ranging from 35 °C to 92 °C, and pH values between 1.9 and 6, making it a favorable environment for the proliferation of thermophilic bacteria with enzymatic potential (Peña-Ocaña *et al.,* 2022; Rincón-Molina *et al.,* 2020; Ovando-Chacón *et al.,* 2020). Several studies have addressed aspects of its hydrology, hydrochemistry, geology, evolution, and microbial diversity (Velázquez-Ríos *et al.,* 2022; Rincón-Molina *et al.,* 2019; Casas *et al.,* 2016; Jácome *et al*., 2016; Armienta *et al*., 2014; Cuoco *et al.,* 2013; Contreras & Salgado, 2012; Taran & Peiffer, 2009; Rouwet *et al*., 2008); however, knowledge remains limited regarding the bacterial community producing extremozymes with potential biotechnological applications. To date, no study has specifically focused on thermophilic protease-producing bacteria in this environment.

Consequently, in this study, thermophilic bacteria producing extracellular proteases were isolated, selected, and identified from geothermal water samples from El Chichón crater lake to explore their biotechnological relevance. As part of this approach, basic physicochemical parameters of the extracellular proteolytic activity of *Bacillus smithii* AP67 were evaluated, and its functional application in goat skin depilation was examined. Although functional genetic evidence for protease-related pathways in *Bacillus smithii* has been previously reported (Milano *et al.,* 1994), to our knowledge, no study has evaluated

its activity or considered its potential application in tanning processes, which positions this work as one of the first to propose its use as a sustainable alternative in the leather industry.

2 Materials and methods

2.1 Sampling site and sample collection

Geothermal water was sampled in October 2023 on the eastern side of the crater lake of El Chichón volcano, located at site 17°21'46.2' 'N, 93°13'41.7' 'W. The water was collected in triplicate using sterile 50 mL polypropylene conical tubes at a depth of 20 cm below the surface and kept refrigerated at 4 °C. The samples were then transferred to the microbiology laboratory located at the Tecnológico Nacional de México -Instituto Tecnológico de Tuxtla Gutiérrez for subsequent physicochemical and microbiological analysis. All bacterial isolates used in this work belong to the posgrado ITTG collection.

2.2 Physicochemical analysis of geothermal water samples

Temperature, pH, and total dissolved solids were evaluated *in situ* using a standard HANNA portable pH and temperature meter, model HI-98128 (Merk, Kenilworth, USA). The evaluation of the elemental components of the geothermal water was carried out using a PerkinElmer Optima 7000 inductively coupled plasma optical emission spectrometer (ICP-OES), using the methodology described by González-Terreros *et al*. (2018). All analyses were performed in triplicate.

2.3 Isolation of thermophilic bacteria

Geothermal water (500 μL) was inoculated on YT pH 6 agar plates composed of: casein peptone (MCD LAB, Mexico), 10 g/L; yeast extract (DIBICO, Mexico), 1 g/L; NaCl (MEYER, Mexico), 3 g/L; K_2HPO_4 (MEYER, Mexico), 1 g/L; $MgSO_4{\cdot}7H_2O$ (JALMEK, Mexico), 1 g/L; $MnSO_4{\cdot}2H_2$ O, 0.1 g/L; $CaCl_2{\cdot}2H_2O$ (MEYER, Mexico), 0.3 g/L; glucose (MEYER, Mexico), 2 g/L and agar (MCD LAB, Mexico), 20 g/L (Ellouz *et al.,* 2001) and incubated at 60 °C for 24 h. Bacterial colonies showing morphological variations were selected and re-streaked on a solid culture medium with the same composition until pure bacterial strains were obtained.

2.4 Detection of protease-producing bacterial isolates

The bacterial strains obtained were evaluated to identify the five strains with the highest capacity to produce extracellular proteases in vitro using a selective culture medium following the methodology described by Zhang *et al.* (2021). Briefly, the culture medium consisted of the following components: casein (LABESSA, Mexico), 10 g/L; glucose (MEYER, Mexico), 1 g/L; yeast extract (DIBICO, Mexico), 1 g/L; K_2HPO_4 (MEYER, Mexico),1 g/L; KH_2PO_4 (MEYER, Mexico), 0.5 g/L; $MgSO_4{\cdot}7H_2O$ (JALMEK, Mexico), 0.1 g/L, and agar (MCD LAB, Mexico), 20 g/L. Plates were inoculated at the center with 10 μl of inoculum previously grown on Luria Bertani culture medium (Bertani, 1951). After 10 min, the casein plates were incubated at 60 °C for 24 h. After the incubation, the diameter of bacterial growth was observed and measured, as well as the hydrolysis halo corresponding to the extracellular proteolytic activity using a TRUPER® digital vernier calibrator. The proteolytic activity index (PI) was estimated based on equation 1 (Abdollahi *et al.,* 2021):

$$PI: \frac{Diameter\ of\ the\ proteolysis\ zone\ \ (mm)}{Diameter\ of\ the\ bacterial\ colony\ (mm)} \quad (1)$$

PI: Proteolytic Index.

2.5 Molecular identification of isolates

Genomic DNA extraction from the selected strains was carried out using the phenol-chloroform technique described by Köchl et al. (2005). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using genomic DNA and the universal bacterial primers 8F (5'-AGAGAGTTTGATCCTGGCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Reaction conditions and thermocycler programming were adjusted as described by Weisburg et al. (1991). The integrity of the PCR products was verified on a 1% agarose gel, then purified and sequenced using the same primers (Macrogen, Seoul, South Korea). The 16S rRNA gene sequences were manually edited and assembled into consensus sequences using BioEdit software version 7.2.5 (Hall, 1999). The sequences were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) using the nucleotide BLAST tool. Genomes of type strains were compared to establish evolutionary relationships among the isolates by constructing a phylogenetic tree using the neighbor-joining method and Kimura's two-parameter model, incorporating a Bootstrap analysis with 1000 replicates (Saitou and Nei, 1987). Data processing was performed using the MEGA X software package version 10.1.8 (Kumar et al., 2018). The 16S rRNA gene sequences

corresponding to the strains of interest were registered in the GenBank database (PQ375433, PQ326436, PQ326437, PQ375434, and PQ375435).

2.6 Morphological characterization

Morphological characterization of each isolate allowed the observation of characteristics such as shape, border, elevation, color, and surface of the bacterial colony, using the methodology described by Smith (2002). Microscopic observations were made using an AxioLab microscope (Carl Zeiss® brand) to assess bacterial cell shape, and Gram staining was performed using a differential staining kit (HYCEL, Mexico), following the procedure described by Mohan (2009).

2.7 Biochemical characterization and assessment of enzymatic production

The bacteria were subjected to biochemical characterization through a series of specific assays, including catalase (Ullah *et al*., 2021), indole (Harley *et al.,* 2002a), citrate (Masi *et al.,* 2021) triple sugar iron (Harley *et al.,* 2002b), methyl red (Masi *et al.,* 2021), Vogues-Proskauer (Masi *et al.,* 2021), amylase (Temsaah *et al.,* 2018), cellulase (Temsaah *et al.,* 2018), and lipase activity tests (Abdollahi *et al.,* 2021). The tests were performed to determine the metabolic properties and production of extracellular enzymes of the isolates.

2.8 Quantification of extracellular proteolytic activity

The extracellular proteolytic activity of the five strains that showed the highest protease production in the *in vitro* tests was quantified using the method described by Kuberan *et al*. (2010). The bacterial strains were incubated in YT medium at pH 6 and a temperature of 60 °C for 24 h. After incubation, the biomass was separated by centrifugation at 8000 rpm for 3 min, and the cell-free extract was established as a crude extract and used for the enzyme assay. The reaction mixture consisted of 1.25 mL of 100 mM Tris-HCl buffer pH 6.0, 0.5 mL of 1% (w/v) casein solution, and 0.25 mL of crude extract. The reaction mixture was incubated at 60 °C for 30 min and stopped with 3 mL of 5% (w/v) trichloroacetic acid. The mixture was then incubated at 4 °C for 10 min to promote protein precipitation, followed by centrifugation at 3500 rpm for 5 min. Next, 0.5 mL of the liquid fraction of the precipitate was taken to which 2.5 mL of 500 mM Na_2CO_3 was added, and the mixture was incubated for 20 min at room temperature. Finally, 0.5 mL of Folin-Ciocalteu reagent (Sigma, Aldrich) was added to measure the absorbance at 660 nm with a Velab UV/Vis spectrophotometer (model VE-5600 UV PC). Protease activity was determined in triplicate and recorded in units per mL (U/mL) by comparing the amount of tyrosine released from each sample with a standard tyrosine curve.

2.9 Biochemical properties of the crude protease

The biochemical properties of the crude extract were evaluated exclusively using the strain *Bacillus smithii* AP67, which was selected for exhibiting the highest proteolytic activity in the preliminary screening assays. The extract was obtained from the cell-free supernatant, and enzymatic activity was determined following the previously described protocol.

2.9.1 Effect of pH and temperature on protease activity

The influence of pH on the proteolytic activity of the crude extract was evaluated using 100 mM buffers according to the pH range: glycine-HCl (pH 4.0 and 5.0), Tris-HCl (pH 6.0-8.0), and glycine-NaOH (pH 9.0-10.0). The temperature profile was determined by performing the standard assay in a range of 30 to 90 °C, in increments of 10 °C, at a pH of 8.0. Enzymatic activity was reported as relative activity (%), considering the maximum activity obtained under control conditions as 100%. All assays were conducted in triplicate.

2.9.2 Effect of metal ions, organic solvents, and surfactants on protease activity

The effect of different chemical agents on protease activity was assessed under standard assay conditions (pH 8.0 and 60 °C). To evaluate the effect of metal ions, the crude protease extract was pre-incubated for 30 min with 10 mM solutions of $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $CuCl_2$, and the chelating agent EDTA before the enzymatic reaction.

The effect of organic solvents was analyzed by adding 10% (v/v) ethanol, methanol, hexane, acetone, and chloroform to the reaction mixture. The effect of surfactants was evaluated by pre-incubating the crude protease extract with 1% (w/v) SDS, Tween-20, or Tween-80 under the same conditions. Enzyme activity was expressed as relative activity (%) by considering the untreated crude protease extract as a control (100%). All assays were conducted in triplicate.

2.10 Application of crude protease extract in goat skin dehairing

The potential application of the crude protease extract in leather processing was evaluated using fresh goat skin, following a modified methodology described by

Pravin *et al.* (2014). Skin samples were thoroughly washed with distilled water, cut into uniform pieces of approximately 5 × 5 cm, and immersed in 100 mL of the crude protease extract, followed by incubation at 60 °C for 8 h. Control samples were incubated with distilled water under the same conditions. After incubation, the dehairing performance was assessed by manually scraping the skin surface with the fingers. The outcome was visually examined based on the cleared area of the skin, and the integrity of the dermal layer was evaluated to verify the absence of structural damage.

3 Results and discussion

3.1 Physicochemical characterization of geothermal water samples

The results of the *in situ* physicochemical characterization of the geothermal water from the El Chichón volcano lake are shown in Table 1. The thermal source represents a thermophilic environment (> 60 °C) with a slightly acidic pH (6), while the concentrations of elements and metals are very low.

According to the physicochemical characterization of the geothermal water collected from the crater lake of the El Chichón volcano, temperature and pH regimes conducive to the growth of thermoacidophilic microorganisms were identified. Previous studies have reported similar physicochemical conditions, highlighting the variability in the parameters at different points in the crater lake (Velázquez-Ríos *et al.,* 2022; Peña-Ocaña *et al*., 2022; Ortiz-Cortés *et al.,* 2021; Ovando-Chacón *et al.*, 2020). These characteristics can also affect the chemical form of the elements found in greater proportions and promote association with certain toxic elements, leading to the formation of primary or secondary minerals that reduce bioavailability to organisms (Ovando-Ovando *et al.,* 2023). This study reports the presence of macronutrients such as K and Mg at concentrations of 56.63 and 3.74 mg/L like as previously reported in water samples from the El Chichón volcanic lake by Rouwet *et al.* (2008), Armienta *et al.* (2008), Armienta *et al.* (2014), and Velázquez-Ríos *et al.* (2022). In addition, concentrations of 0.12, 0.02, and 1.88 mg/L of Mn, Cu, and Zn ions, respectively, were detected, which is consistent with the findings reported by Ovando-Ovando *et al*. (2023), who reported concentration ranges of 2.2-8.5, 0.005-0.02, and 0.08-0.12 mg/L, respectively. Likewise, the presence of Fe ions was determined with a concentration of 3.01 mg/L, in accordance with concentrations reported by Rouwet *et al.* (2008), who reported values ranging from 0.1 to 314 mg/L. Finally, the presence of the toxic element arsenic was detected at a concentration of 0.01 mg/L, which is comparable to the concentration of 0.05 mg/L reported by Ovando-Ovando *et al*. (2023). The chemical composition of crater lakes depends on factors related to the geology of the region in which they are located, climatic conditions, and the geothermal activity associated with the volcanic system., which is mainly evidenced in the form of magmatic heat and gas input, temporal variations mainly driven by dilution/concentration processes associated with rainfall and evaporation (Armienta *et al.,* 2008; Armienta *et al.,* 2014). The study of the chemical composition of these lakes is of particular interest, as it allows a more accurate assessment of precursor chemical changes that reflect volcanic activity, enabling timely warnings of changes in the state of a volcano (Armienta *et al.,* 2014). Microorganisms that reside in this type of extreme environment, despite the abundance of metals, display a remarkable variety of metabolic strategies that favor their survival (Ovando- Chacón *et al.,* 2020). Their diverse metabolisms contribute to the cycle of essential elements such as carbon, sulfur, and iron. These organisms can use both organic and inorganic molecules as substrates to obtain energy and carbon sources, allowing them to be classified as chemoorganotrophs or chemolithotrophs (Peña-Ocaña *et al*., 2022). However, most are considered chemoorganotrophic species that can survive in microaerophilic or anaerobic conditions, using ferric iron as a terminal electron acceptor (Velázquez-Ríos *et al.,* 2022).

Table 1. *In Situ* physicochemical characterization of the geothermal water from the crater Lake of El Chichón volcano.

Parameter	Average result
Temperature (°C)	65 ± 2.00
pH	6 ± 0.22
Total dissolved solids (ppm)	129 ± 3.01
As (mg/L)	0.01 ± 0.01
Cu (mg/L)	0.02 ± 0.16
Fe (mg/L)	3.01 ± 0.60
K (mg/L)	56.63 ± 3.05
Mg (mg/L)	3.74 ± 0.27
Mn (mg/L)	0.12 ± 0.01
Zn (mg/L)	1.88 ± 0.08

3.2 Isolation of thermophilic bacteria

Geothermal water samples from the crater lake of El Chichón volcano were inoculated onto plates containing YT solid culture medium, on which bacterial growth was observed after the incubation period. Based on colony morphology, each distinctive morphological characteristic of shape, edge, elevation, pigment, and surface was considered a different

bacterial species. A total of 50 different strains were subcultured using the cross-stripping technique and preserved in YT medium for future testing.

3.3 Screening and detection of thermophilic bacteria producing proteases

The *in vitro* proteolytic capacity of the isolated strains was assessed through specific activity tests. Among the 50 isolates obtained, 46 (92%) produced extracellular thermophilic proteases. Extracellular proteolytic activity was observed by the formation of clear zones around the colonies, resulting from the hydrolysis of casein molecules by the action of the enzyme secreted into the medium and the subsequent release of low molecular weight peptides and amino acids that act as a source of nutrients. (Zhang *et al*., 2021; Veloorvalappi *et al.,* 2013). The proteolytic index values of the positive isolates ranged from 1.0 to 2.11, as presented in Table 2.

Based on these results, five isolates AP19, AP25, AP31, AP34, and AP67 were prioritized for further analyses due to their high extracellular proteolytic activity, with strain AP67 exhibiting the highest proteolytic index value of 2.11 ± 0.12. The frequency of positive isolates and the range of IP observed in this study is similar to that reported in previous reports of from geothermal environments, where high positivity rates and PI values typically ranging from 0.31 to 2.50 (Muqarramah *et al.,* 2023), and occasionally reaching up to 4.65 (Sabaria *et al*., 2014), have been described depending on the substrate and assay conditions.

3.4 Molecular identification

Genomic DNA was extracted from strains considered potential protease producers (AP19, AP25, AP31, AP37, and AP67), and its integrity was confirmed by agarose gel electrophoresis. Subsequently, the universal 16S rRNA gene was amplified by polymerase chain reaction (PCR).

Table 2. Proteolytic activity index (PI) of thermophilic bacteria isolated from the crater lake of El Chichón Volcano.

Strain	Proteolytic Index (PI)	Strain	Proteolytic Index (PI)
AP10	1.15 ± 0.03 ^{fghijkl}	AP134	1.08 ± 0.04 ^{hijklm}
AP13	1 ± 0 ^{m}	AP135	1 ± 0 ^{m}
AP14	1.22 ± 0.04 ^{defgh}	AP136	ND
AP16	1.23 ± 0.12 ^{defgh}	AP141	1.23 ± 0.11^{defgh}
AP17	ND	AP143	1.24 ± 0.02^{defg}
AP18	1.16 ± 0.03^{efghijk}	AP144	1.01 ± 0.02^{klm}
AP19	1.42 ± 0.08 ^{c}	AP145	1.100 ± 0.02^{ghijklm}
AP25	1.59 ± 0.07 ^{b}	AP146	1.15 ± 0.02^{fghijklm}
AP31	1.65 ± 0.04 ^{b}	AP147	1.00 ± 0.02 ^{m}
AP33	1.22 ± 0.06^{defgh}	AP148	ND
AP34	1.35 ± 0.04 ^{cd}	AP149	ND
AP35	1.09 ± 0.01 ^{ghijklm}	AP150	1.24 ± 0.01^{defg}
AP37	1 ± 0 ^{m}	AP154	1.21 ± 0.00 ^{defgh}
AP39	1.20 ± 0.04^{defghi}	AP156	1.24 ± 0.00 ^{defg}
AP41	1.05 ± 0.04^{jklm}	AP157	1.15 ± 0.06^{ghijklm}
AP64	1.15 ± 0.08^{fghijklm}	AP159	1.14 ± 0.03 ^{fghijklm}
AP67	2.11 ± 0.12 ^{a}	AP161	1.17 ± 0.01 ^{efghij}
AP69	1.14 ± 0.05^{fghijklm}	AP165	1.21 ± 0.02 ^{defgh}
AP87	1.18 ± 0.04 ^{efghi}	AP166	1.17 ± 0.01 ^{efghijk}
AP119	1.22 ± 0.02 ^{defgh}	AP167	1.15 ± 0.08 ^{fghijklm}
AP129	1.16 ± 0.03 ^{efghijk}	AP169	1.16 ± 0.01 ^{efghijk}
AP130	1.17 ± 0.05^{efghij}	AP170	1.20 ± 0.01 ^{defghi}
AP131	1.26 ± 0.03 ^{def}	AP173	1.22 ± 0.06 ^{defgh}
AP132	1.31 ± 0.01 ^{cde}	AP175	1.22 ± 0.03 ^{defgh}
AP133	1.02 ± 0.01 ^{jklm}	AP176	1.17 ± 0.03 ^{efghijk}
		HSD	0.15

ND: Not detectable, HSD: honestly significant difference.

Mean values with the same letter in the column are not statistically different ($p \leq 0.05$)

Table 3. Molecular identification of thermophilic bacteria from the crater lake of the El Chichón volcano.

Strain	Closest partial 16S rRNA gene sequence	Accession No.	16S rRNA seq. (bp)	Closest NCBI match/species identity	Reference
AP19	*Bacillus licheniformis*	PQ375433	1343	*Bacillus licheniformis* ATCC 14580^T (ON597434)/100%	Unpublished
AP25	*Bacillus licheniformis*	PQ326436	1210	*Bacillus licheniformis* DSM13^T (AE017333)/100%	(Veith *et al.,* 2004)
AP31	*Bacillus licheniformis*	PQ326437	1215	*Bacillus licheniformis* BCRC 11702 ^{T} (NR116023)/99.92%	(Wang et al., 2007)
AP34	*Alicyclobacillus mali*	PQ375434	1217	*Alicyclobacillus mali* FSL-W10-0018^T (MW694475)/100.00%	(Roth *et al.,* 2021)
AP67	*Bacillus smithii*	PQ375435	1423	*Bacillus smithii* DSM4216^T (CP012024)/99.09%	(Bosma *et al.,* 2016)

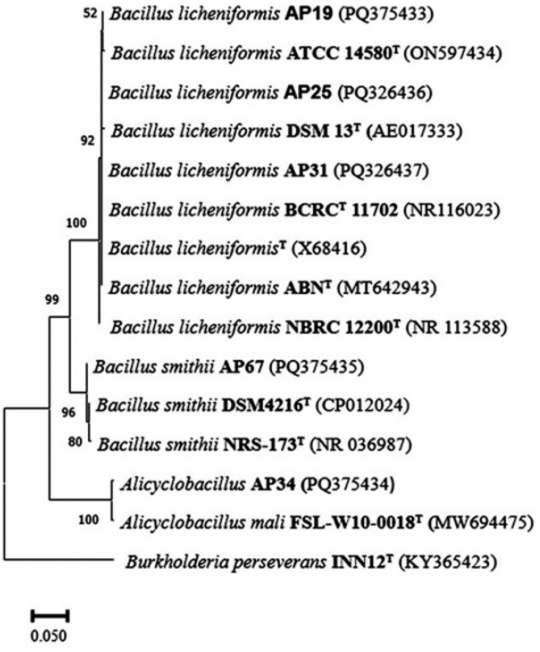

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the thermophilic bacteria AP19, AP25, AP31, AP34, and AP67 isolated from the crater lake of the El Chichón volcano. The tree contains the closest type of strain for each isolate.

The amplicons were approximately 1400 bp in size, and the resulting sequences were analyzed and compared with existing sequences in the NCBI database. Based on 16S rRNA gene sequence similarity analysis and the construction of a phylogenetic tree using type strains, strains AP19, AP25, and AP31 exhibited high phylogenetic similarity to *Bacillus licheniformis*, strain AP37 clustered within the genus *Alicyclobacillus*, and strain AP67 showed a strong phylogenetic relationship with *Bacillus smithii*, as summarized in Table 3. These results are highly relevant, as there are currently no specific reports on the isolation and identification of thermophilic microorganisms from the Chiapas Volcanic Arc capable of producing extracellular proteases with potential applications in the biotechnology industry.

Figure 1 shows the phylogenetic tree obtained using the neighborhood linkage method, based on 16S rRNA gene sequences grouped into clusters corresponding to type strains of bacterial species isolated from the geothermal water of the crater lake of the El Chichón volcano.

The bacterial genera identified in this study are consistent with those described in previous studies related to the isolation of thermophilic microorganisms in the crater lake of the El Chichón volcano, in which the bacterial genera *Geobacillus* and *Alicyclobacillus* have been described (Ovando-Chacón *et al.,* 2020; Ortiz-Cortés *et al.,* 2021). Similarly, Rincón-Molina *et al.* (2020) reported on a metagenomic study of the diversity of bacterial populations present in the sediments of the crater lake of the El Chichón volcano, in which the presence of 15 phyla was reported in sediments at 50 °C, with a predominance of Actinobacteria (33.1%), Proteobacteria (29.1%), and Acidobacteria (20.1%). The presence of nine phyla was also evidenced, with a predominance of Bacillota (52.7%; mainly *Alicyclobacillus* and *Sulfobacillus*) and Proteobacteria (44.8%; particularly *Bradyrhizobium*) in sediments at 92 °C. On the other hand, Velázquez-Ríos *et al.* (2022) reported on prokaryotic diversity along a pH gradient in the crater lake of the El Chichón volcano by sequencing the 16S rRNA gene amplicon,

using sediment and water samples collected within a pH/temperature gradient (pH 1.9-5.1, 38-89 °C). The results obtained showed that the microbial composition differed significantly between sediment and water. Sediment communities were different at the site with the highest temperature and lowest pH value, while water communities were similar at all sites. The identified genera were related to *Alicyclobacillus, Bacillus,* and *Paenibacillus*. Taken together, these results indicate that the crater lake of El Chichón volcano is a reservoir of thermophilic microorganisms that produce industrially important enzymes and that it harbors a microbial community capable of adapting to the different conditions of a highly active crater lake.

3.5 Morphological and biochemical characterization of thermophilic isolates

The bacterial strains with the highest proteolytic index were cultured in YT agar medium to evaluate the morphology of their colonies and cells. The colonies had wavy, complete edges, with circular or irregular shapes, flat convex elevations, and white to cream-colored pigmentation. Their surfaces were membranous and smooth. Gram staining showed that the strains were Gram-variable and bacillus shaped. The results of the morphological and biochemical characterization of the strains are presented in Table 4.

According to the morphological evaluation, strains AP19, AP25, and AP31 exhibit morphological characteristics like the reported thermophilic isolates. Mohammad *et al.* (2017) and Verma *et al*. (2018): irregular shape, wavy edges, flat elevation, white color, and smooth surface. On the other hand, strain AP34 presented a morphology like that reported by. Ding *et al*. (2008) and Ortiz Cortés *et al.* (2021), which indicates that the morphology of *Alicyclobacillus* isolates has characteristics of circular shape, wavy edges, convex elevation, and creamy color. Likewise, strain AP67 presented characteristics like those reported by Nakamura *et al.* (1998), who reported that the colonial morphology of the *Bacillus smithii* isolate presents a circular shape, entire edge, translucent color, smooth surface, and a flat elevation. As for the results of the microscopic observation, they indicated that all the strains presented the form of bacilli and a Gram-variable staining response, predominantly showing a coloration characteristic of Gram-negative bacteria. It is important to mention that these bacterial species have been reported as Gram-positive (Gallo & Aulitto, 2024; Ramírez-Olea *et al*., 2022; Gordon, 2017). However, the variability in Gram staining could be due to a structural change in the components of the cell membrane caused by mechanisms of adaptation to common phenomena of heat and matter transfer, such as changes in temperature, pressure, and phase change related to gas production at the edges of the crater lake of the El Chichón volcano (Ovando-Chacón *et al.,* 2020).

Table 4. Characterization of macroscopic, microscopic, and biochemical tests of thermophilic protease-producing bacteria.

Observation	Isolated strain code				
	AP19	AP25	AP31	AP34	AP67
Macroscopic feature					
Form	Irregular	Irregular	Irregular	Circular	Circular
Border	Undulate	Undulate	Undulate	Undulate	Integer
Elevation	Flat	Flat	Flat	Convex	Flat
Pigment	White	White	White	Cream	Translucent
Surface	Smooth	Smooth	Smooth	Membranous	Smooth
Microscopic feature					
Gram stain	-	-	-	-	-
Cellular form	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Biochemical tests					
Simmons Citrate	-	-	-	-	-
Voges Proskauer	-	-	-	-	-
Methyl red	+	+	+	+	+
Indol	-	-	-	-	-
Catalase	+	+	+	+	+
TSIA	A/A	A/A	A/A	A/A	A/A
Starch	+	+	+	+	+
Cellulose	+	+	+	-	-
Lipase	+	+	+	-	+

+ = positive, - = negative, A= Yellow.

The results of biochemical tests showed that the strains were unable to metabolize citrate as a carbon source or ammonia as a nitrogen source, nor could they degrade tryptophan to produce indole. Similarly, they did not produce 3-hydroxybutanone (acetoin) because of glucose metabolism. However, the methyl red test showed that they can ferment glucose and produce organic acids such as acetic, lactic, and formic acids. Similarly, the TSI test showed that all bacteria were capable of fermenting lactose and sucrose to produce acids. Likewise, the catalase test revealed that all bacteria possessed aerobic metabolism (Harley *et al.,* 2002b). On the other hand, the production of extracellular hydrolytic enzymes such as amylase, cellulase, and lipase was evaluated. The results showed that all strains can produce extracellular amylases, in contrast to cellulase production, as strains AP34 and AP67 did not reveal such activity. Similarly, strain AP34 did not produce the enzyme lipase. These findings suggest that the bacterial strains isolated in this study have a chemoheterotrophic metabolism, demonstrating their dependence on organic compounds for growth and energy generation. These results are consistent with the findings of Ovando-Chacón *et al*. (2020) and Ortiz-Cortés *et al.* (2021), who indicated that the strains isolated from the crater lake of the El Chichón volcano showed chemoheterotrophic traits, indicating their dependence on organic compounds for metabolism. However, unlike the results obtained in this study, our strains were unable to metabolize citrates, which could suggest differences in metabolic adaptations to the environmental conditions of the crater lake of El Chichón volcano.

3.6 Quantification of extracellular proteolytic activity

The extracellular proteolytic activity of strains AP19, AP25, AP31, AP34, and AP67 was evaluated using casein as a substrate. The enzymatic activities obtained ranged from 23.68 ± 5.25 to 102.75 ± 6.39 U/mL, with the *Bacillus smithii* strain AP67 showing the highest extracellular protease activity. The results are shown in Figure 2.

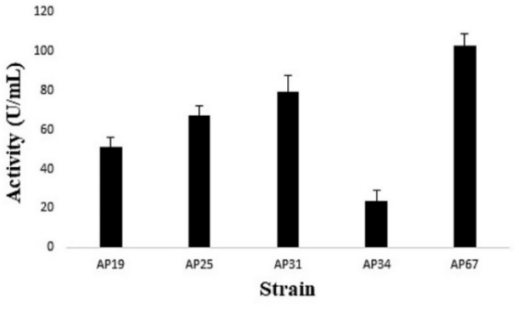

Figure 2. Proteolytic activity of selected thermophilic bacteria.

The differences obtained in proteolytic activities are because protease production depends on several physicochemical and nutritional factors, such as temperature, pH, carbon and nitrogen sources, as well as aeration and agitation, which play an important role in the expression of these enzymes (López-Trujillo *et al.,* 2023; Solanki *et al.,* 2021; Ahmad *et al.,* 2020). The results obtained in this study suggest that the strains isolated from the crater lake of the El Chichón volcano show moderate proteolytic activities compared to reports from other authors. For example, Suleiman *et al.* (2020) reported proteolytic activities in the range of 75 to 175 U/mL in bacteria isolated from hot springs in Malaysia, and Fachrial *et al.* (2021) reported activity ranges of 0.96 to 23.67 U/mL in preliminary tests on thermophilic bacteria isolated from hot springs in Indonesia. Due to the concordance between the high IP in plates and the maximum enzymatic activity in the crude extract, we decided to select the *Bacillus smithii* AP67 strain as the main strain of interest. In this regard, we evaluated the biochemical properties of the crude enzyme extract to assess its performance under relevant operating conditions and support its applied evaluation.

3.7 Biochemical properties of the crude protease

3.7.1 Effect of pH and temperature on protease activity

The crude proteolytic extract of *Bacillus smithii* AP67 showed maximum activity at a pH of 8.0, confirming its classification as an alkaline protease with an absolute activity of 136.64 ± 7.50 U/mL designated as 100% relative activity, as shown in Figure 3.

These findings are consistent with previous reports describing similar optimal pH values for proteases from *Bacillus infantis* (Saggu and Mishra, 2017), *Bacillus licheniformis* LBA 46 (dos Santos Aguilar *et al.,* 2019), *Bacillus subtilis* BSP (Majeed *et al.,* 2024), *Bacillus subtilis* S1 (Hashmi *et al.,* 2022), *Bacillus amyloliquefaciens* (Hashmi *et al.,* 2022), and *Bacillus cereus* PW3A (Tennalli *et al.,* 2022). Consistently, it has been reported that most microbial proteases used in industrial sectors exhibit their highest catalytic activity in an alkaline pH range between 8.0 and 12.0 (Veloorvalappi *et al.,* 2013). In this study, the enzyme retained more than 70% of its activity in a pH range of 6 and 7, suggesting greater operational compatibility compared to strictly alkaline proteases. In contrast, a marked loss of activity was observed under acidic conditions, a behavior consistent with reports of alkaline enzymes exhibiting low stability under acidic stress conditions (Solanki *et al.,* 2021). This phenomenon has been linked to alterations in the electrostatic balance and conformation of proteins, which compromise the geometry of the active

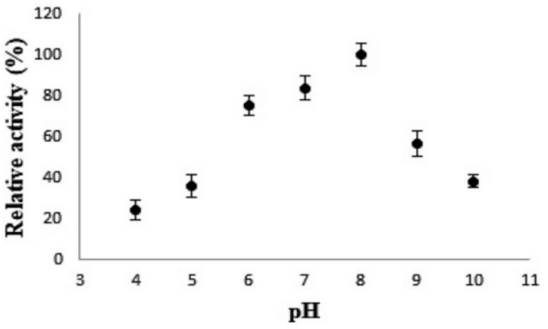

Figure 3. Effect of pH on enzyme activity.

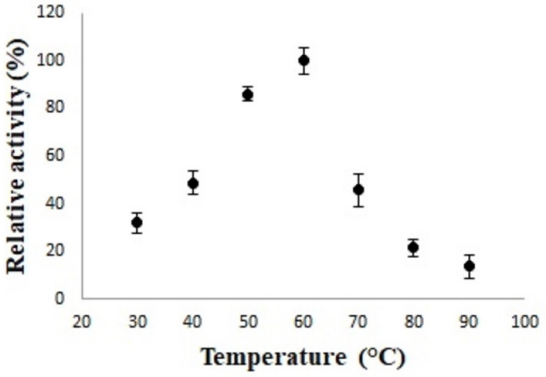

Figure 4. Effect of temperature on enzyme activity.

site and reduce its catalytic capacity (Meena *et al.,* 2024; Gemechu *et al.,* 2020).

The crude proteolytic extract of *Bacillus smithii* AP67 presented an activity profile as a function of temperature, which reached its maximum value at 60 °C, defined as 100% relative activity, as shown in Figure 4.

These findings are consistent with previous reports of proteases from *Bacillus licheniformis* LBA46 (dos Santos Aguilar *et al.,* 2019), *Bacillus stearothermophilus* (Karray *et al.,* 2021), *Bacillus cereus* PW3A (Tennalli *et al.,* 2022), *Bacillus subtilis* K-5 (Shad *et al*., 2024), and *Geobacillus* sp. PLS (Iqbalsyah *et al.,* 2019), which reported optimal activities in a range of 55 and 60 °C. In this study, the crude protease retained more than 80% of its activity at 50 °C, demonstrating thermal stability suitable for biotechnological applications in processes requiring high temperatures. On the other hand, proteolytic activity was significantly reduced at temperatures above 70 °C, probably reflecting partial denaturation of the enzyme. The reduction in activity observed at high temperatures is consistent with that described in microbial proteases, in which thermal stress disrupts the network of non-covalent interactions responsible for stabilizing the tertiary structure of the enzyme, affecting the catalytic capacity of the active site (Song *et al.,* 2023; Gupta *et al.,* 2002). Taken together, the pH and temperature profile results reflect characteristics typical of thermoalkaline enzymes. These results provide a solid basis for investigating their stability under other conditions relevant to industry.

3.7.2 Effect of metal ions, solvents, and surfactants on protease activity

The crude protease from *Bacillus smithii* AP67 was evaluated with different metal ions, organic solvents, and surfactants to determine its stability under diverse chemical conditions. The results showed that divalent cations such as $CaCl_2$, $MgCl_2$, and $MnCl_2$ enhanced or maintained enzyme activity, while transition metals such as $CoCl_2$, $FeCl_2$, and $CuCl_2$ produced a notable inhibition. In contrast, the chelating agent EDTA partially decreased activity, which could indicate that divalent ions are necessary to maintain structural stability. When evaluating activity with solvents, the enzyme maintained its activity in short-chain alcohols such as ethanol and methanol it showed a pronounced decrease in the presence of hexane, acetone, and chloroform. As for surfactants, the protease retained much of its activity in the presence of Tween-20 and Tween-80, while SDS induced a loss of activity. These findings are summarized in Table 5.

Analysis of the effects of metal ions revealed that proteolytic activity increases in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+}. This behavior coincides with that reported for alkaline proteases from bacteria such as *Bacillus licheniformis* A10 (Yilmaz *et al.,* 2016), *Bacillus infantis* SKS1 (Saggu and Mishra, 2017), *Bacillus stearothermophilus* (Karray *et al.,* 2021), *Bacillus subtilis* BSP (Majeed *et al.,* 2024), *Geobacillus toebii* LBT77 (Thebti *et al.,* 2016), and *Geobacillus stearothermophilus* (Iqbal *et al.,* 2020).

Table 5. Effect of metal ions (10 mM), organic solvents 10% (v/v), SDS 1% (w/v), and surfactants 1% (v/v) on the activity of the crude protease produced by *Bacillus smithii* AP67.

Chemical agent	Relative activity (%)
$MgCl_2$	91.68 ± 7.03
$MnCl_2$	89.33 ± 2.67
$CaCl_2$	116.34 ± 5.86
$CoCl_2$	55.95 ± 6.14
$FeCl_2$	41.26 ± 3.69
$CuCl_2$	21.72 ± 2.37
EDTA	94.99 ± 4.36
Ethanol	92.60 ± 7.74
Methanol	89.54 ± 2.32
Hexane	78.13 ± 6.82
Acetone	55.75 ± 6.14
Chloroform	50.63 ± 4.75
SDS	82.82 ± 7.27
Tween-20	97.50 ± 9.56
Tween-80	103.00 ± 3.98

All the results were presented as mean ± SD.

These reports describe how divalent cations, such as Ca^{2+} and Mn^{2+}, coordinate with the carboxylate residues of Asp and Glu located in flexible regions, such as surface loops, reducing electrostatic repulsions and providing greater rigidity to the protein conformation, which protects the active site from thermal denaturation. In contrast, the presence of transition ions such as Cu^{2+}, Fe^{2+}, and Co^{2+} caused a notable inhibition of enzymatic activity. This behavior has been described in alkaline proteases from *Bacillus infantis* SKS (Saggu and Mishra, 2017), *Bacillus subtilis* VBC7 (Ramalingam *et al.,* 2022), *Bacillus stearothermophilus* (Karray *et al.,* 2021), and *Bacillus subtilis* BSP (Majeed *et al.,* 2024).

The inhibition of activity is attributed to the interaction of these metals with amino acids such as histidine or sulfhydryl, which alter the conformation of the active site and reduce hydrolytic capacity. In some cases, the affinity of transition ions for carboxylate groups can interfere with the binding of stabilizing cations, causing structural alterations that decrease enzyme stability (Eijsink *et al.,* 2011; Gupta *et al.,* 2002). Additionally, the partial inhibition observed with EDTA suggests that divalent cations play an important role in the structural stabilization of the enzyme. However, since much of the enzymatic activity is retained, there is evidence of a certain degree of functional independence from these metal cofactors.

The crude protease showed variable stability with organic solvents, demonstrating high tolerance to short-chain alcohols such as ethanol and methanol, while a reduction was observed with hexane, acetone, and chloroform. This behavior is consistent with that described for proteases from *Bacillus caseinilyticus* (Mothe and Sultanpuram, 2016), *Bacillus licheniformis* A10 (Yilmaz *et al.,* 2016), *Geobacillus toebii* LBT77 (Thebti *et al.,* 2016), *Bacillus stearothermophilus* (Karray *et al.,* 2021), and *Bacillus subtilis* BSP (Majeed *et al.,* 2024), in which proteolytic activity has been reported to be preserved in the presence of ethanol and methanol but inhibited by less polar solvents. However, exceptions exist, such as the protease from *Bacillus subtilis* VBC7 (Ramalingam *et al.,* 2022), which retains its activity with acetone, chloroform, and hexane, reflecting that stability with solvents depends on the structure of each enzyme. In general, tolerance to water-miscible alcohols has been attributed to their ability to preserve the enzyme's hydration layer, unlike non-polar solvents, which interfere with hydrophobic interactions, leading to structural destabilization of the enzyme (Ramalingam *et al.,* 2022; Gupta *et al.,* 2002; Ogino and Ishikawa, 2001).

Regarding the effect of surfactants, crude protease retained its activity in the presence of Tween-20 and Tween-80, reflecting high compatibility with nonionic surfactants. In contrast, the anionic detergent SDS reduced activity to 82.82%, suggesting a reduction in the structural stability of the enzyme. These findings are consistent with reports on proteases from *Bacillus caseinilyticus* (Mothe and Sultanpuram, 2016), *Bacillus stearothermophilus* (Iqbal *et al.,* 2020), *Bacillus stearothermophilus* (Karray *et al.,* 2021), and *Bacillus subtilis* BSP (Majeed *et al.,* 2024), in which Tween has been reported to have a protective effect, while SDS interferes with critical hydrophobic regions, altering the structural conformation of the enzyme (Razzaq *et al.,* 2019; Gupta *et al.,* 2002).

The results obtained in this study show that crude protease from *Bacillus smithii* is stable under moderate conditions, as it retained its activity in the presence of divalent cations, short-chain alcohols, and nonionic surfactants, but is more sensitive in the presence of strongly disruptive agents, such as transition metal ions, nonpolar solvents, and anionic detergents. This pattern suggests that crude protease has sufficient stability for use in industrial applications. Specifically, its compatibility with water-miscible alcohols and mild surfactants reinforces its potential for applications in leather tanning, where these conditions are relevant and highly disruptive agents are not usually present in significant concentrations.

3.8 Dehairing capacity of crude protease from Bacillus smithii AP67

The crude protease extract from *Bacillus smithii* AP67 was tested for its dehairing potential on fresh goat skin. After 8 h of incubation at 60 °C, the enzyme preparation completely removed the hair, while the dermal layer remained intact. The absence of apparent damage to the dermal layer after enzymatic treatment is consistent with the selective hair removal mechanism described for proteases, which supports the preservation of dermal collagen under controlled conditions (Gao *et al.,* 2023; Sivasubramanian *et al.,* 2008). In contrast, the control samples treated with distilled water showed no visible dehairing effect. The outcome of the enzymatic treatment is presented in Figure 5. The time required for complete hair removal was relatively short compared with previous studies on alkaline proteases that reported unhairing periods of 12 to 48 h (Singh *et al.,* 2024; Ullah *et al.,* 2022; Briki *et al.,* 2016; Nadeem *et al.,* 2010; Mukhtar, 2008). Even in recent reports, such as Uddin *et al.* (2025), the process required 9 h.

Overall, the results obtained in this study correspond to an initial stage of research and demonstrate the potential of crude protease from *Bacillus smithii* AP67 for possible application in enzymatic hair removal from goat skin. However, further studies are needed to complement the visual

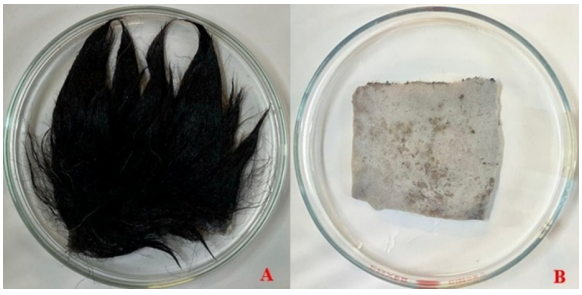

Figure 5. Dehairing of goat skin using the crude protease from *Bacillus smithii* AP67. (A) Untreated control showing intact hair, and (B) enzyme-treated sample exhibiting complete hair removal with preservation of the dermal structure.

assessment of the depilation effect observed in this work, through more detailed analyses aimed at defining its feasibility and reproducibility in industrial processes.

Conclusions

This study identifies *Bacillus smithii* AP67, isolated from the crater lake of the El Chichón volcano, as a new source of extracellular proteases with relevance in the enzymatic depilation of goat skin. The crude enzyme extract exhibited a thermoalkaline profile, moderate chemical stress tolerance, and achieved complete depilation of goat skin without apparent dermal damage, suggesting its potential applicability as an ecological alternative to conventional chemical processes. To our knowledge, no previous reports have been found that describe the functional activity of *Bacillus smithii* protease experimentally validated in leather processing.

By integrating microbial ecology and applied enzymology, this study highlights the crater lake of the El Chichón volcano as a unique natural reservoir of thermophilic microorganisms capable of producing enzymes of biotechnological importance. Rather than closing a line of research, these findings constitute one of the first contributions that pave the way for new research on the biotechnological potential of the microbial communities that inhabit this extraordinary geothermal environment.

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