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Whey as a screening and fermentation medium for protease-producing *Bacillus* strains

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ABSTRACT

Background: Microbial proteases provide sustainable alternatives to chemical processes and have wide applications in food and biotechnology. Among various microbial sources, *Bacillus* species are particularly recognized for their ability to produce extracellular proteases with high stability and broad substrate specificity.

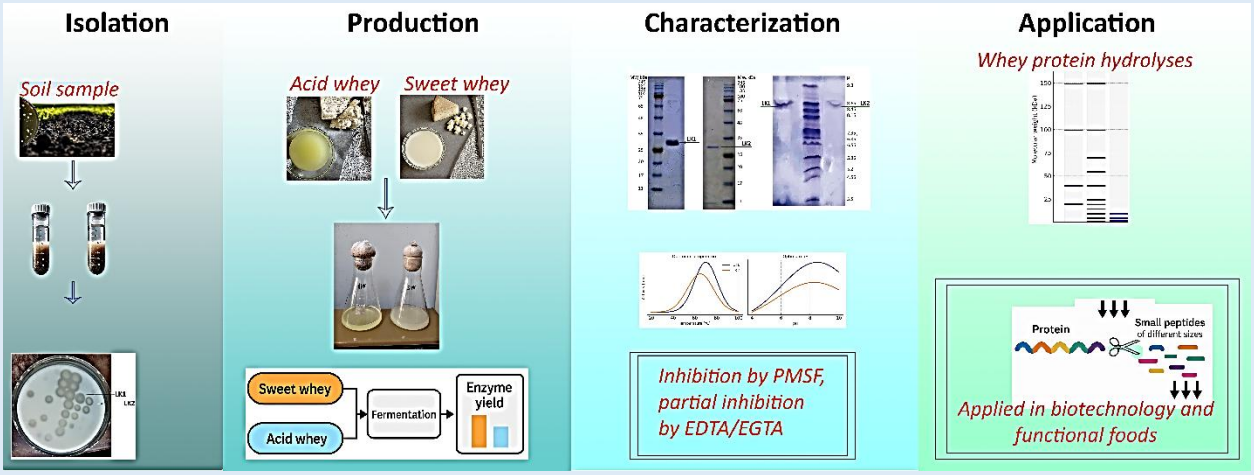
Objective: In this study, two protease-producing strains, *Bacillus amyloliquefaciens* LK1 (LK1) and *Bacillus subtilis* LK2 (LK2), were isolated from soil and cultivated in whey, an abundant and cost-effective dairy by-product. Both strains secreted extracellular serine proteases with molecular weights of about 30 kDa and isoelectric points around 8.5.

Methods: Whey was used as a screening and cultivation medium for protease-producing *Bacillus* strains. Strains were selected based on the size of clear zones. Proteases were purified by batch-column cation-exchange chromatography.

Results: The enzymes were active over a broad pH range (6–10), showing maximal activity at pH 8–9 and at temperatures between 60 and 65°C. The proteases remained stable at 55°C for several hours but were strongly inhibited by PMSF and partially by EDTA/EGTA, confirming their classification as serine proteases. Among the two proteases, LK1 exhibited superior tolerance to salts and detergents and efficiently hydrolyzed whey proteins into peptides and free amino acids. These hydrolysates were enriched in bioactive compounds, potentially exhibiting antioxidant, antimicrobial, and immunomodulatory activities.

Conclusion: The results highlight the suitability of whey-based fermentation for producing functional peptides and demonstrate the potential of LK1 protease to enhance protein digestibility, nutritional quality, and clean-label food processing. These findings underscore the enzyme's applicability in dairy science and its promise for developing functional food ingredients derived from whey proteins.

Keywords: Microbial proteases, *Bacillus amyloliquefaciens* LK1, *Bacillus subtilis* LK2, Fermentation optimization, Whey protein hydrolysis, Enzyme characterization, Functional foods



Graphical Abstract: Whey as a screening and fermentation medium for protease producing bacillus strains

INTRODUCTION

The excessive use of chemicals across industries has significantly impacted the environment, ecosystems, and human health. In response, environmentally friendly alternatives such as enzymes are increasingly being adopted. Various enzymes, including proteases, are widely applied in textile, detergent, leather, feed, waste, and other industries [1].

Among protease producers, *Bacillus* species are most prominent [2–6]. These extracellular proteases produced by *Bacillus* can be rapidly recovered from fermentation media and exhibit high catalytic activity [7-8]. There is growing interest in novel microbial proteases that can withstand harsh processing conditions and maintain high activity and stability. Whey, a significant by-product of cheese production, accounts for approximately 85–90% of milk volume. Whey proteins (WPs)-mainly α-lactalbumin, β-lactoglobulin, and immunoglobulins remain soluble after acid or rennet coagulation [9]. Enzymatic hydrolysis of WPs produces bioactive peptides with antioxidant, antimicrobial, antihypertensive, anticancer, and immunomodulatory effects [10–12]. Whey's rich composition, coupled with its low cost, makes it a suitable medium for microbial cultivation [13-14], although not all microorganisms can directly utilize lactose and proteins without enzymatic treatment [9].

While many studies focus on lactose hydrolysis [15–17], protein hydrolysis is equally essential. Enzymes such as pepsin, trypsin, and Alcalase® have been used to generate WP hydrolysates with functional properties [18], though complete conversion into free amino acids remains less explored. Pintado et al. demonstrated that

6-hour hydrolysis with Protease 2A significantly increased the free amino acid content [19].

This study aims to isolate protease-producing bacteria and use whey as a low-cost medium for extracellular protease production. The resulting proteases will then be characterized and applied to the enzymatic treatment of sweet and acid whey (SW and AW) proteins, converting them into low-molecular-weight peptides and free amino acids. These hydrolysates have potential applications in functional foods, enhancing nutritional quality, digestibility, and bioactive compound content [20–22].

METHODS

To isolate strains with protease activity, sweet whey agar (SW_EA) plates were prepared. The lactose in sweet whey was hydrolyzed using β-glucosidase enzyme [16]. Before enzymatic treatment, the pH was adjusted to 7.0. The enzymatically treated SW was then used as a base for preparing agar plates.

Milk agar plates were prepared by diluting milk threefold, adding 1.5% agar, and pasteurizing at 70°C for 30 min.

Isolation of bacterial strain and screening for protease activity: Soil samples were collected aseptically in sterile containers from Vanadzor, Armenia, in sterile containers, serially diluted in 0.9% NaCl, and plated on SW_EA. Plates were incubated at 30°C for 48 h, and colonies were selected based on morphology, size, and color. Proteolytic activity was assessed on milk agar plates incubated at 30°C for 24 h. Two strains, *Bacillus amyloliquefaciens* LK1 and *Bacillus subtilis* LK2, showing the largest clear zones, were selected for further analysis.

Molecular identification and phylogenetic analysis: 16S rRNA gene sequences were compared with those in the NCBI database using the BLAST tool [23]. Multiple sequence alignments and phylogenetic tree construction were performed in MEGA 11 using the Neighbor-Joining method [24]. Alignments were done using ClustalW integrated in MEGA [25]. Sequences of LK1 and LK2 were submitted to GenBank (OQ727115 and OQ727116).

Optimization of incubation period and aeration: Inocula were prepared by cultivating strains in nutrient broth at 30°C for 24. SW_E and AW_E media were prepared as previously described by Poladyan et al [16].LK1 and LK2 bacterial cultures were inoculated at a 1:500 dilution into 100 mL of SW_E and AW_E, respectively, in 500 mL Erlenmeyer flasks. Cultivation was performed at 30°C for 72 h with shaking at 150 rpm (Senova ST-206R, China). To assess aeration, LK1 was grown at 100, 150, 200, and 250 rpm for 24 and 48 h. Experiments were conducted in triplicate (n=3 biological replicates, each with three technical replicates).

Protease activity assay and protein concentration: A modified version of the method by Braun and Schmitz (1980) was used for protease activity assay [26-27]. A 0.2 mL reaction mixture containing 1.5% azocasein (Megazyme, Bray, Ireland) in 50 mM phosphate buffer (pH 8.0) was incubated for 10 min at 60°C for LK1 and 65°C for LK2. The enzyme solution was added, and the reaction was stopped with 0.03 mL of 30% (w/v) trichloroacetic acid (TCA). After adding 1.2 mL of water, the samples were centrifuged (8000 rpm, 5 min), and the absorbance was measured at 440 nm. One unit of activity was defined as the amount of enzyme hydrolyzing one µg of azocasein per minute under optimal conditions (pH 8.0; 60°C for LK1, 65°C for LK2). An extinction coefficient of 35 was used to calculate activity in U/mL [27,28]. Protein concentration was determined using the Bradford method [29].

Purification of protease: Isolation and purification of proteases were performed at 4°C. Several purification

methods were tested to optimize the purification schema.

Ammonium sulfate precipitation: Crude supernatant was subjected to ammonium sulfate precipitation at 80% saturation. Pellets were dissolved in 5 mL 50mM phosphate buffer (pH 7.0).

Anion exchange chromatography: For further purification, the sample was applied to a DEAE-Toyoperl SuperQ-650M anion-exchange column (2.5 × 10 cm, Tosoh Bioscience, Japan) pre-equilibrated with 50 mM phosphate buffer (pH 7.0), and proteins were eluted with a 0–0.4 M NaCl linear gradient. Fractions of 5 mL were collected.

Cation exchange chromatography: Cation-exchange chromatography was performed using CM-23 resin (Servacel, Serva) in a 2.5 × 10 cm column pre-equilibrated with 50 mM acetate buffer (pH 5.5). The enzyme preparation from the previous step was applied, and proteins were eluted with a pH 5–10 gradient established using 100 mM acetate (pH 5.0) and 100 mM borate (pH 10.0). Fractions of 5 mL were collected.

Batch-column cation-exchange chromatography: For hybrid mode (batch-column) cation exchange chromatography, the supernatant was concentrated fivefold by ultrafiltration using a 10 kDa molecular weight cut-off membrane (transmembrane pressure of approximately 1-2 bar at 10°C) and the pH was adjusted to 5.5. CM-23 resin was then added at a ratio of 1 g resin per 4 mg of protein. The mixture was stirred continuously overnight at 4°C to allow protein binding.

After incubation, the supernatant was decanted, and the resin was transferred to a chromatography column (2.5 cm diameter) pre-packed with 1 cm of the same resin equilibrated in 50 mM acetate buffer, pH 5.5. Proteins were eluted using the pH gradient described above.

All collected fractions were analyzed for protein content by measuring absorbance at 280 nm and for enzyme activity using the azocasein assay. All buffers were filtered (0.22 µm) before use.

Characterization of purified proteases; Purity and molecular weights of the proteases were determined by SDS-PAGE on 15% acrylamide gels [30], using Triple color protein marker 10-245 kDa (Biofact, South Korea).

Isoelectric points (pI) were determined by isoelectric focusing on a 5% polyacrylamide gel per the Pharmacia method (Sweden) [31], using 1.6% ampholines at 4–6 (75%) and 3.5–9 (25%) pH. Electrode buffers were 0.1 M NaOH and 0.1 M H_2SO_4. Isoelectric points markers included a broad spectrum of proteins such as trypsinogen (pI-9.30), lentil lectin basic group (pI-8.65), lentil lectin neutral group (pI-8.45), lentil lectin acid group (pI-8.15), myoglobin basic group (pI-7.35), myoglobin acid group (pI-6.85), human anhydrase (pI-6.55), bull anhydrase (pI-5.85), β-lactoglobuline A (pI-5.20), soybean trypsin inhibitor (pI-4.55), amyloglucosidase (pI-3.50) was used.

Temperature and pH optima were determined across 20-95°C and pH 3-10, respectively, using Britton-Robinson buffer (40 mM) [32].

Temperature stability was tested by pre-incubating enzymes at 55, 60, 65, and 70°C for up to 6 h, followed by incubation on ice. Residual activities were then measured.

Effects of protease inhibitors (PMSF, EDTA, EGTA), surfactants (SDS, Triton X-100, Triton X-405, Tween 20/80), and metals (NaCl, Na_2SO_4, KCl, K_2SO_4, $FeSO_4$, $FeCl_3$, $CaCl_2$, $MgSO_4$, $MgCl_2$, $MnSO_4$, $BaCl_2$, $ZnSO_4$, $CuSO_4$, $CoSO_4$, $CdCl_2$, $NiCl_2$, $CuCl_2$) were studied. Enzymes were incubated with inhibitors (0.05-1 mM), 1% surfactants, and five mM metal ions at 4°C for one h. A control assay was conducted in the absence of these compounds. All assays were performed in triplicate (n=3).

Enzymatic Hydrolysis of AW and SW Proteins: Before hydrolysis, the pH of AW and SW was adjusted to 7.0 with dilute NaOH, and the samples were pasteurized at 70°C for 10 min. AW was previously concentrated twofold to 2.5 mg/mL protein, and SW was used at its native form (3.1 mg/mL).

Hydrolysis was performed with LK1, LK2, and commercial enzymes: Protease P "Amano" 6SD (semi-alkaline protease from *Aspergillus melleus*), ProteAXH (peptidase from *Aspergillus oryzae*), PROTIN SD-NY10 (neutral protease from *Bacillus amyloliquefaciens*), THERMOASE PC10F (neutral metalloprotease from *Geobacillus stearothermophilus*), and Proteinase S (thermostable serine protease from *Pyrococcus furiosus*). These enzymes differ in their optimal temperatures and activities. To standardize conditions, all assays (except ProteAXH at 55°C) were conducted at pH 7.0 and 40°C (Supplementary Table 1). Equal enzyme activity (0.04 U) was used in 0.5 mL reaction mixtures containing 0.3 mL substrate; the volume was adjusted with ultrapure water.

Reactions were incubated at 40°C for 4 h (ProteAXH at 55°C). Controls were run identically without the enzyme. Reactions were stopped by adding SDS-PAGE sample buffer and heating at 95°C for 10 min. Hydrolysates were analyzed via 15% SDS-PAGE.

RESULTS

Isolation of bacterial strains with protease activity: The primary objective of this study was to isolate bacterial strains with proteolytic activity capable of hydrolyzing whey proteins into low-molecular-weight peptides and free amino acids. This strategy was employed to develop whey-based media suitable for cultivating a range of unique microorganisms. Enriching whey with small peptides and free amino acids derived from protease treatment adds value to dairy side streams and creates an efficient cultivation medium.

Agar plates prepared with SW_EA were used for bacterial screening. Soil samples were diluted in series and plated onto SW_EA to monitor colony growth. A total of 25 bacterial isolates were selected based on colony morphology, color, and size (Figure 1A). The formation of clear zones around the colonies on milk agar plates confirmed proteolytic activity. The diameters of these zones served as an index for selecting strains with higher protease production.

Two bacterial colonies, named LK1 and LK2, which exhibited relatively larger clear zones, were studied in more detail (Figure 1B). The isolates were sub-cultured and maintained in LB medium for future testing.

Interestingly, the orange pigment produced by some colonies on SW_EA disappeared when growing them on milk agar plates. This might be due to shorter cultivation time as secondary metabolites such as colors are being produced during a longer growth period.

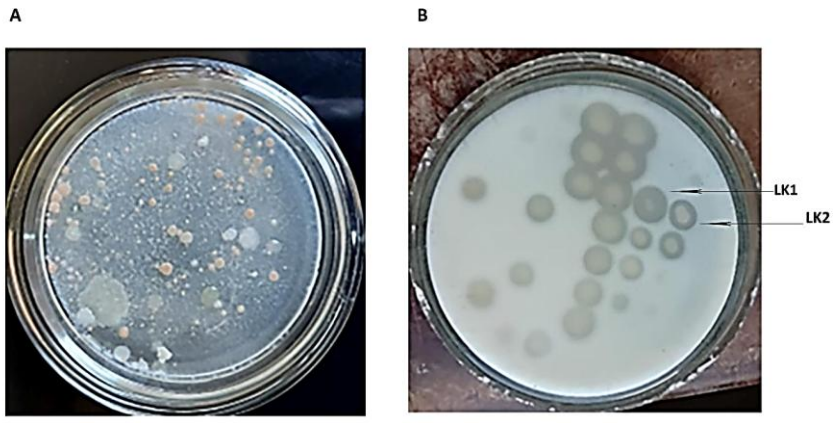


Figure 1. Diversity of soil microorganisms grown on SW_EA and assessment of their proteolytic activity on skim milk agar plates. (A) Serial dilutions (10^6) of soil samples were plated to obtain single, isolated colonies. The plates containing SW_EA were incubated at 30°C for 48 h. (B) Twenty-five colonies were selected and grown on milk agar plates at 30°C for 24 h

Molecular identification: BLAST analysis confirmed that both isolated strains belong to the genus *Bacillus*. The 16 rRNA gene sequence of LK1 exhibited 100% identity with *Bacillus amyloliquefaciens* EGE-B-2d.1, (JF926530.1), CICC 20604 (GQ375216.1) and HB03102 (KY417124.1) strains, while LK2 showed 100% identity with *Bacillus subtilis* JX-2 (KX708699.1) and Bs21(OQ423156.1) strains. Both strains also showed higher identity with other *Bacillus* strains.

The phylogenetic tree was constructed based on 16S rRNA gene sequences of isolate LK1 (*Bacillus amyloliquefaciens LA1*- OQ727115) and LK2 (*Bacillus subtilis* LA2 -OQ727116) (Supplementary Figure 1). Based on the data obtained, we confirmed that LK1 is *Bacillus amyloliquefaciens* and LK2 is *Bacillus subtilis*. *Bacillus amyloliquefaciens* LK1 is clustered with *Bacillus subtilis* Y4, *Bacillus velezensis* LT2, and *Bacillus amyloliquefaciens* P3, despite having relatively lower identity to those strains. As for *Bacillus subtilis* LK2, it is clustered with *Bacillus subtilis* JX-2 and *Bacillus subtilis* Bs21 strains and shows the highest identity with these strains (Supplementary Figure 1).

Effect of incubation time and aeration on protease production: Enzyme synthesis is linked to cell growth, with incubation time and aeration (shaking speed) directly affecting production. *Bacillus* species typically secrete extracellular proteases during the late exponential or early stationary phase. At the same time, prolonged incubation leads to declining activity, as proteases remain stable in the medium for up to 24 h. [33-34].

B. amyloliquefaciens LK1 and *B. subtilis* LK2 were cultivated in shake flasks using SW_E and AW_E (Figure 2). Protease production proceeded gradually, reaching a maximum value at 48 hours for both isolates. Similar findings were reported for *B. subtilis* S1 [35], with maximum enzyme production observed after 48 h of growth. Both strains exhibited higher protease activity when cultivated in SW_E than in AW_E, with maximum activities of 748 U/L and 650 U/L, respectively. Of the two isolates studied, *Bacillus amyloliquefaciens* LK1 showed the highest protease production and was selected for further optimization studies.

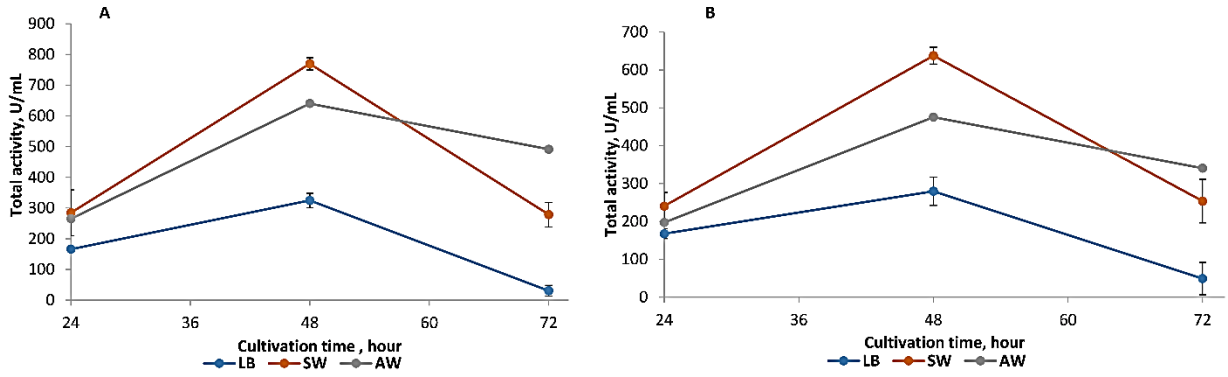


Figure 2. Effect of incubation time on protease production by *B. amyloliquefaciens* LK1 (A) and *B. subtilis* LK2 (B).

Protease production was monitored at 30°C, pH 7.0, under 150 rpm shaking for 72 h. Total activity (U/L) was measured every 24 hours. Three different media were used: Luria-Bertani (LB), sweet whey (SW), and acid whey (AW).

Excessive or limited aeration can cause shear stress or oxygen limitation, negatively affecting enzyme secretion and stability [36]. In the present study, *Bacillus amyloliquefaciens* LK1 was cultivated in SW_E at different shaking speeds for 72 hours. (Supplementary Figure 2).

Protease activity increased gradually over time, reaching a maximum at 48 h, with the highest activity observed at 200 rpm (645 ± 60 U/L).

Purification of proteases: Purification of proteases was optimized using *B. amyloliquefaciens* LK1 as a model enzyme source. The initial purification approach involved ammonium sulfate precipitation followed by anion-exchange chromatography. However, this purification step was ineffective, as protease activity was detected predominantly in the early elution fractions. This behavior is likely related to the protease's higher isoelectric point, as confirmed by isoelectric focusing analysis.

In the second step, cation-exchange chromatography was performed using a pH gradient elution system (Supplementary Figure 3). Although cation-exchange chromatography allowed effective purification, the use of large amounts of ammonium sulfate is not suitable for industrial applications [21]. To address this, the protein binding to the resin was performed in batch mode. The supernatant volume was concentrated 5-fold by ultrafiltration, after which the culture supernatant pH was optimized to 5.5. Under these optimized conditions, more than 80% of the protease activity successfully bound to the resin, enhancing process scalability and cost-effectiveness. After purification, the specific activities of *B. amyloliquefaciens* LK1 and *B. subtilis* LK2 proteases increased approximately 30-fold and 20-fold, respectively (Supplementary Tables 2 and 3). These results demonstrate the robustness of the optimized purification workflow and its potential for adaptation to industrial enzyme production, particularly for the valorization of dairy side-streams within functional food manufacturing systems [37].

Characterization of LK1 and LK2: The purity of the proteases was confirmed using SDS-PAGE and isoelectric focusing. Both LK1 and LK2 enzymes displayed an approximate molecular weight of 30 kDa (Figure 3A and 3B). The isoelectric points (pI) of both enzymes were estimated to be around 8.5 (Figure 3C), matching their basic nature. These findings are consistent with typical *Bacillus*-derived extracellular proteases and provide a basis for further biochemical characterization and industrial application.

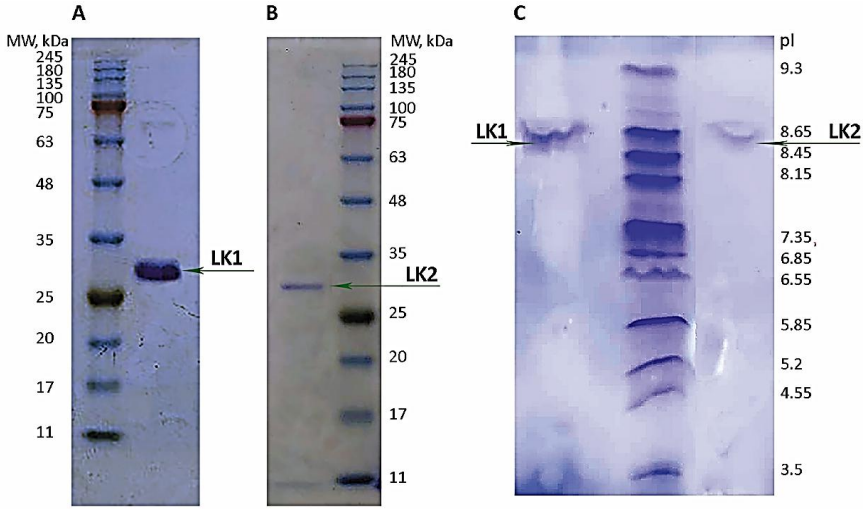


Figure 3. SDS-PAGE and isoelectric focusing analysis of LK1 and LK2 proteases. (A) SDS-PAGE of LK1 enzyme, (B) SDS-PAGE of LK2 enzyme, and (C) Isoelectric focusing of of both enzymes (~8.5). Molecular weight markers (kDa) are indicated. Experiments were performed in triplicate (n = 3 biological replicates × 3 technical replicates).

Effect of temperature and pH on protease activity: The temperature and pH dependence of LK1 and LK2 proteases was evaluated (Figure 4). Both enzymes were active across pH 6–10, with optima at pH 8–9, similar to other *Bacillus* proteases [38,39]. LK1 displayed maximal activity at 60°C, while LK2's reached its optimum at 65°C. At 40°C and 80°C, they retained approximately 35% and 40% of their maximal activity, respectively indicating moderate thermal stability.

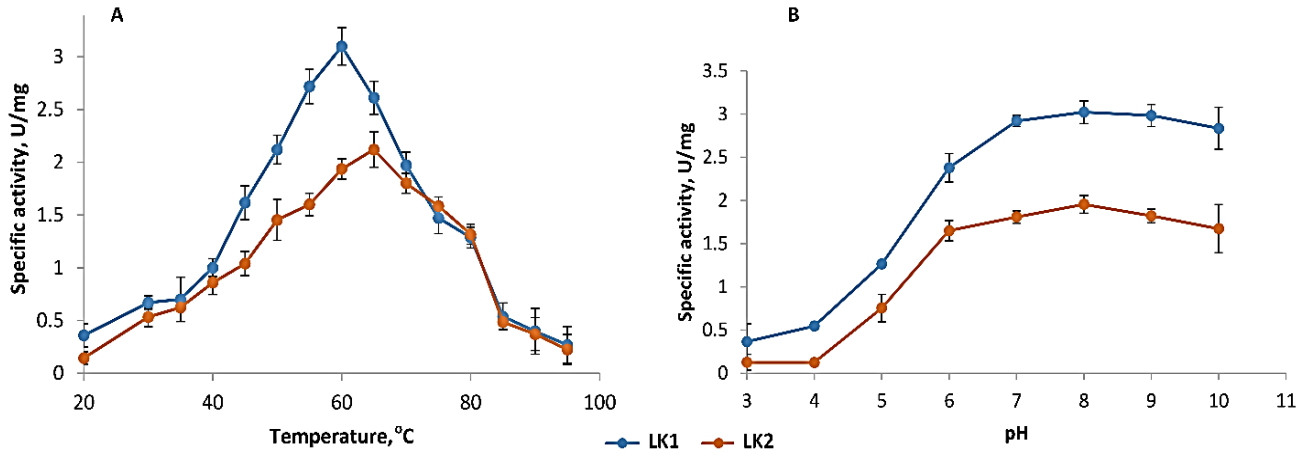


Figure 4. Temperature and pH dependence of LK1 and LK2 proteases. (A) Temperature profiles; (B) pH profiles. Blue and orange lines indicate LK1 and LK2 activity, respectively. Data represent mean ± SD of three biological replicates (n = 3).

The enzymes exhibited similar temperature stability, remaining stable at 55°C after 6 h of incubation. At 60°C, LK1 and LK2 retained approximately 70% and 60% of their initial activity, respectively (Supplementary Figure 4). Both proteases were partially inhibited by 1 mM EDTA, losing approximately 53% (LK1) and 43% (LK2) of activity, and were inactivated entirely by PMSF, indicating that they belong to the serine protease class (Table 1). Previous reports have shown that PMSF at M generally leads to complete inactivation of serine alkaline proteases [40-41]. Members of the subtilisin family are strongly inhibited by PMSF, which covalently binds to the active-site serine in subtilisin E [42]. Chelating agents such as EDTA and EGTA have also been reported to reduce the activity of serine proteases [43].

Table 1. Residual activity of LK1 and LK2 proteases in the presence of inhibitors

Inhibitor	Concentration, mM	Relative activity, %	
		LK1	LK2
PMSF	0.05	17	18
	0.1	10	9
	1	0	0
EDTA	0.05	94	77
	0.1	66	57
	1	47	38
EGTA	0.05	101	97
	0.1	77	76
	1	53	43

The effects of various metals on the specific activities of LK1 and LK2 were evaluated (Supplementary Figure 5A). The addition of NaCl and Na_2SO_4 significantly enhanced LK1 activity (~4.0 U/mg), whereas LK2 activity was unaffected. Conversely, heavy metal ions such as Cu^{2+}, Cd^{2+}, Ni^{2+}, and Fe^{3+}, strongly inhibited both enzymes, with LK2 showing higher susceptibility. Ca^{2+} activated both LK1 and LK2, while Mg^{2+} and Mn^{2+} selectively increased LK1 activity.

Non-ionic detergents notably stimulated LK1, with Triton X-405 causing the greatest activation, whereas LK2 displayed only moderate enhancement in the presence of Tween 80 and Triton X-100 (Supplementary Figure 5B). Both enzymes were inhibited by thiol-based reducing agents (DTT, ME, and DTNB) compared to the control.

Application possibilities of LK1 and LK2; The applicability of LK1 protease was evaluated using whey substrates to explore its role in the development of functional foods. Enzymatic hydrolysis of whey markedly increases the concentration of free amino acids and small peptides, thereby enhancing its nutritional and bio-functional value [44]. Whey protein hydrolysates are further enriched with amino acids and bioactive peptides, offering applications in functional foods and pharmaceuticals [45]. Moreover, enzymatic hydrolysis improves the functionality and techno-functional properties of whey proteins, such as solubility, emulsifying, and foaming capacities, thereby broadening their industrial applications [46].

The enzymatic hydrolysis profiles of SW and AW proteins by LK1 and several commercial proteases are shown in Figure 5**.** SW proteins were efficiently degraded by multiple proteases, with LK1 demonstrating high hydrolytic activity comparable to Proteinase S (5), but slightly lower than Protease P (1) and Prote AXH (2) (Figure 5A). In contrast, AW proteins were completely hydrolyzed by Protease P (1) and LK1 (6). Prote AXH (2), Thermoase PC10F (4), and Proteinase S (5) showed lower activity, particularly against high-molecular-weight proteins, whereas Protin SD-NY10 (3) displayed reduced activity only toward proteins below 25 kDa (Figure 5B).

Collectively, these findings indicate that LK1 is effective for the hydrolysis of AW and SW proteins, underscoring its potential utility in diverse biotechnological applications and as a functional food ingredient.

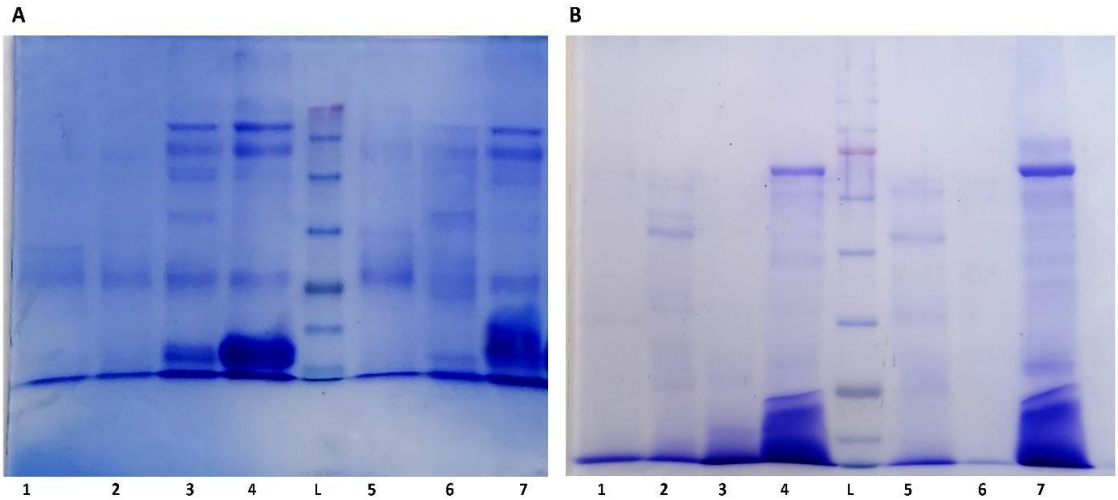


Figure 5. SDS–PAGE profiles of SW (A) and AW (B) after hydrolysis with different proteases. Lanes: (1) Protease P, (2) Prote AXH, (3) Protin SD-NY10, (4) Thermoase PC10F, (5) Proteinase S, (6) LK1, (7) control (thermal incubation without enzyme), (8) control (non-incubated sample). Incubations were performed at 40°C for 4 h, except for Prote AXH, which was incubated at 55°C.

DISCUSSION

In this work, a targeted screening approach was employed to isolate and characterize protease-producing bacteria capable of efficiently hydrolyzing whey proteins. SW was selected as the primary substrate owing to its higher protein content compared with AW [16]. Proteolytic activity was confirmed on skim milk agar, and two isolates were selected for detailed characterization (Figure 1). Based on phenotypic traits and 16S rRNA gene sequencing, the strains were identified as *Bacillus amyloliquefaciens* and *Bacillus subtilis*. The protease activities obtained in this study (748 U/L for LK1 and 650 U/L for LK2) are comparable to or exceed values reported for related *Bacillus* strains [38], supporting the feasibility of utilizing dairy by-products as low-cost fermentation substrates. This aligns with current sustainable bioprocessing strategies aimed at whey valorization into high-value bioactive compounds and functional ingredients [20-21].

Bacillus species are well-recognized industrial producers of extracellular proteases due to their high secretion capacity, stability, and Generally Recognized as Safe (GRAS) status [1]. Our results are consistent with previous findings, confirming that *Bacillus* isolates are highly effective protease producers [47]. Under optimized cultivation conditions, protease activities reached 748 U/L for LK1 and 650 U/L for LK2, with SW providing a superior medium compared to AW (Figure 2). This aligns with current sustainable bioprocessing strategies aimed at whey valorization into high-value bioactive compounds and functional ingredients [20,21]. Biochemical characterization revealed that both LK1 and LK2 proteases share several features typical of alkaline serine proteases. They exhibited similar molecular weights (~30 kDa) and isoelectric points (pI ≈ 8.5) (Figure 3) with optimal activity at pH 8–9 (Figure 4B). Temperature optima were 60°C and 65°C for LK1 and LK2, respectively (Figure 4A). Both enzymes retained over 60% of activity after six hours of incubation at 55°C (Supplementary Figure 4). Inhibition studies further confirmed their subtilisin-like nature, showing complete inactivation by PMSF and partial inhibition by EDTA and EGTA (Table 1). Such traits are consistent with industrially relevant subtilisins, which dominate nearly two-thirds of the global protease market owing to their high stability, broad specificity, abundant secretion, and ease of downstream processing [48-49].

We have shown that using 0.04 U of LK1 enzyme, it is possible to hydrolyze whole whey proteins into small peptides and amino acids <15 kDa (Figure 5). These findings highlight the suitability of whey not only as an inexpensive fermentation medium but also as a substrate for the production of bioactive peptide-enriched hydrolysates. Recent research has shown that enzymatic hydrolysis of whey can enhance amino acid bioavailability and generate peptides with antioxidant, antihypertensive, and prebiotic properties [21,50]. The novel protease from *B. amyloliquefaciens* LK1 demonstrated high catalytic efficiency and substrate specificity on both SW and AW, comparable to or surpassing several commercial enzymes (Figure 5). Such characteristics, combined with robust thermal and pH stability, suggest strong potential for applications in functional food manufacturing, particularly in improving protein digestibility, generating functional peptides, and supporting clean-label formulations [51-52].

Overall, this study contributes to the ongoing valorization of dairy side-streams by demonstrating that whey can serve both as a nutrient-rich fermentation substrate and as well as a precursor for functional peptide production. The protease from *B. amyloliquefaciens* LK1, in particular, is a promising candidate for industrial-scale bioconversion processes aimed at developing sustainable and health-promoting food systems.

Supplementary Information: All data used to prepare this manuscript are available as supplementary materials.

Abbreviations: TCA: trichloroacetic acid; LK1: *Bacillus amyloliquefaciens* LK1; LK2: *Bacillus subtilis* LK2; LB: Luria-Bertani, SW: sweet whey, and AW: acid whey.

Competing Interests: The authors declare that they have no competing interests

Author Contributions: Conceptualization: AP**;** Isolation and identification of strains: LK, MK, TD; Sequencing, identification, and phylogenetic analysis: AH, AP, LK, GH**;** Preparation of whey-based cultivation media: AP, AM, LK, GH**;** Purification and characterization: AP, AM, LK, KD, MI; Biochemical analyses and assays: AH, AP, AM**;** Writing—original draft: AP; Writing—review and editing: AP, AH, AM.; All authors have read and approved the final version of the manuscript.

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