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Isolation, Screening and Characterization of Protease Producing Bacteria: Comparative Analysis of Solid-State and Submerged Fermentation Methods

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ABSTRACT

Proteases are among the most widely used industrial enzymes, with significant applications across food, detergent, leather, and pharmaceutical industries. This study reports the isolation of protease-producing bacteria from a protein-rich environment, with *Bacillus subtilis* I19 (GenBank Accession: PV942511) showing the highest activity. The culture was optimized under submerged fermentation (SmF) conditions with shaking at 35 °C and pH 7–9. The enzyme retained activity for up to three days, indicating good stability for industrial applications. The enzyme was purified using ammonium sulphate precipitation, dialysis, ion exchange, and gel filtration chromatography, resulting in a 4.5-fold purification with a final specific activity of 23.49 U/µg. SDS-PAGE revealed a prominent band at ~20 kDa, typical of *Bacillus* proteases. In SSF, finger millet (ragi) stem supplemented with skimmed milk powder yielded maximum protease levels (5673.2 U/g), indicating a novel substrate for protease production through *Bacillus* strains. These findings highlight *B. subtilis* I19 as a promising candidate for cost-effective protease production using agro-residues, contributing to sustainable enzyme biotechnology.

INTRODUCTION

Proteases are a diverse group of enzymes that catalyze the hydrolysis of peptide bonds in proteins, thereby releasing individual amino acids or smaller peptide fragments. These enzymes are vital to numerous physiological processes, including protein catabolism, cell signaling, immune responses, and tissue remodeling [1]. Due to their catalytic specificity and efficiency, proteases have garnered immense attention in both biological and industrial domains [2]. In fact, as of 2020, they constitute a substantial portion-nearly 60%-of the global industrial enzyme market [3].

Industrial applications of proteases are extensive and diverse. In the detergent industry, proteases are incorporated into formulations to enhance stain removal, particularly proteinaceous stains such as blood, sweat, and food residues [4, 5]. In food processing, they are employed for tenderizing meat, enhancing flavor development in cheese and soy products, and improving dough handling in baking [6, 7]. The

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leather industry utilizes proteases for dehairing and bating processes, reducing reliance on harsh chemicals and making the process eco-friendlier [8, 9]. In pharmaceuticals, proteases are exploited for the development of therapeutic agents, including enzyme replacement therapies, and as targets for antiviral drugs [1, 10].

Peptides also have important and emerging applications through the production of bioactive peptides. These bioactive peptides exhibit a wide range of functional properties, such as antioxidant, antimicrobial, antihypertensive, antidiabetic, and immunomodulatory activities, which are used to develop functional foods, nutraceuticals, and cosmeceuticals aimed at promoting health and preventing chronic diseases [11, 12, 13, 14]. Therefore, it is imperative to develop an industrially scalable, robust, and environmentally sustainable bioprocess that ensures cost-effective production of these proteases to address the diverse biotechnological applications.

Two popular fermentation strategies used to produce proteases are submerged fermentation (SmF) and solid-state fermentation (SSF). SmF is characterized by high reproducibility and precise control, while SSF leverages low moisture and agro-waste substrates-resulting in cost-efficient production with high enzyme titers [15]. SSF is more widely used to produce microbial proteases, majorly fungal proteases, due to their economic scalability and microbial diversity [16]. However, bacterial proteases, especially Bacillus sp., are also widely studied for protease production [17, 18, 19].

In the Indian context, common agro-residues used for the production of proteases from bacteria are wheat bran, rice bran, fruit and vegetable peels, and cattle feed [20]. One study even reported the use of cow dung as a substrate to produce alkaline proteases from Bacillus cereus with a yield of $4813 \pm 62 U/g$ [21]. However, substrates such as Napier grass, finger millet (ragi) stem, banana stem, and coconut husk, although relevant in the Indian agricultural space, are understudied for bacterial protease production.

Napier grass (Pennisetum purpureum) is widely cultivated in India and valued for its nutritional composition [22]. Finger millet stem (Eleusine coracana), commonly called "ragi" in India, is rich in cellulose and hemicellulose, serving as a low-cost carbon source for microbial growth [23]. Banana stem (Musa sp.) provides high moisture and degradable fibers that facilitate microbial colonization and protease secretion [24]. Coconut husk (Cocos nucifera), with its lignin-rich and porous structure, improves aeration and supports microbial activity [25]. These properties make all four substrates suitable for SSF-based protease production.

This study aims to isolate and characterize novel protease-producing bacteria from protein-rich waste sites and cultivate them under SSF using unexplored low-cost agro-residues. It also focuses on optimizing cultivation parameters under SmF , where the enzyme is further purified to evaluate specific activity and yield. The ultimate objective is to identify bacterial proteases with potential for industrialscale applications, thereby advancing sustainable enzyme biotechnology.

METHODOLOGY

Isolation and Screening of Protease Producing Bacteria

Chemicals, reagents, and culture mediums required for these experiments were sourced from SRL Chemicals, Himedia Laboratories Private Limited, and Sigma Aldrich Chemicals Private Limited. Six protein-rich waste samples (dairy effluents, spoiled food, and meat shop soil) were collected from six distinct protein-rich microenvironments in Bengaluru, Karnataka, India (dairy effluent drain, spoiled food waste bin, meat shop floor soil, paneer kitchen waste, and two chicken waste sites). Samples were collected aseptically using sterile 50 mL falcon tubes, transported on ice, and processed within 4 hours of collection and serially diluted for bacterial isolation on skimmed milk agar (SMA). Colonies showing proteolysis (clear zone of casein breakdown) were selected, and extracellular enzymes were extracted by centrifugation at 5200 RPM for 10 minutes. Uninoculated broth served as negative controls. All fermentation experiments were performed in biological triplicates ($n = 3$), and data are reported as mean ± SD. Proteolytic activity was assayed using casein as a substrate (here, "optimum" denotes the condition yielding peak EPA across the tested range). Enzyme extracts were incubated with $1\%(w/v)$ casein in 50 mM Tris- HCl buffer (pH 8.0) at $37^{\circ}C$ for 10 min ; the reaction was stopped with trichloroacetic acid (TCA) and centrifuged at 5000 RPM for 2 minutes. The resultant supernatants were treated with Folin-Ciocalteu (FC) reagent ($1:3$ dilution) and 0.5 M sodium carbonate and incubated at $37^{\circ}C$ for 30 min before measurement. Absorbance was measured at 660 nm to quantify activity through the amount of tyrosine released against a tyrosine standard curve ($0 - 100\mu\, mol/mL$). One unit of extracellular protease activity (EPA; U/mL, where 1 U liberates $1\mu\, mol$ tyrosine per minute) was defined as the amount of enzyme that liberates $1\mu\, mol$ of tyrosine per minute under the assay conditions. The following equation was used to calculate enzyme activity in U/mL:

$$EPA\left(\frac{U}{mL}\right) = \frac{(\mu\, mol\ of\ tyrosine\ released\ )\times(\ total\ volume\ of\ assay\ )}{(\ incubation\ time\ )\times(\ volume\ of\ enzyme\ used\ )}$$

Optimization of Submerged Fermentation Conditions

Isolates were cultured in skimmed milk broth (SMB) under varying conditions: agitation (shaking vs. static, $35\ ^{\circ}C, 24\ h), pH(4 - 10, 35\ ^{\circ}C, 24\ h$, shaking), and temperature ($30 - 45\ ^{\circ}C, 24\ h$, shaking). The initial temperature of $35\ ^{\circ}C$ was chosen because it mimics the warmth of the tropical environment where isolation took place and is within the recommended optimal temperature for mesophilic Bacillus proteases. The pH range of $4 - 10$ was used to screen for alkaline proteases that can be used in detergents. Salting-out was done using 80% saturation because pilot testing using 60% and 80% saturation showed that 80% had higher specific activity. Extracellular enzymes were extracted, and EPA was assayed by the abovementioned method.

Purification Of Enzyme Obtained Through Submerged Fermentation

Crude enzyme was precipitated with ammonium sulfate (80%), dialyzed, and freeze-dried. Dialysis was performed against 50 mM Tris-HCl buffer $(pH8.0)$ using a 10 kDa molecular weight cut-off membrane for 48 hours at $4\ ^{\circ}C$ with three buffer changes to remove small molecules and salt. Total protein was quantified using the Bradford assay (with Casein as standard), and specific activity ($U/\mu g$ protein) was calculated at each purification step to track enrichment. Purification was performed by DEAE cellulose ion-exchange and Sephadex G-75 gel filtration, and purity was confirmed by Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

Solid-State Fermentation Using Agro-Based Substrates

Napier grass, coconut husk, banana stem, and ragi stem wastes were dried, powdered, and then used as substrates for solid-state fermentation. Isolates were inoculated into substrates with and without skimmed milk powder (SMP) and incubated at room temperature for 7 days. Cultures were suspended in 100 mL buffer, homogenized, and centrifuged at 5200 rpm for 10 min . Supernatants were analyzed for EPA (Fig. 1).

Figure 1. Agro-based lignocellulosic substrates (From left to right: Napier grass, coconut husk, banana stem, and ragi stem) after drying and pulverization, prepared for use in SSF experiments.

Identification of Protease Producing Bacteria

The isolate was Gram-stained and identified by 16 S rRNA sequencing. The amplified gene was sequenced, BLAST-analyzed, and used for phylogenetic analysis in MEGA 11. The confirmed sequence was deposited in GenBank. All experiments were performed in triplicates, and the data are represented as mean ± SD ($n = 3$).

RESULTS

Isolation And Screening of Protease Producing Bacteria

From the six samples, a total of 19 isolates were identified, which indicated a clear zone of casein breakdown (Fig. 2). The sample collection sites were selected because they are rich in proteins or regularly exposed to protein waste, making them suitable for finding protease-producing bacteria. Soil and effluent samples from the dairy industry contain milk proteins, such as casein and whey, which support the growth of bacteria that break down proteins. Soil from a meat shop is often exposed to meat residues like blood and tissue, creating a protein-rich environment for proteolytic bacteria. Kitchen waste samples containing paneer and chicken come from home and restaurant sources, both rich in food proteins and ideal for isolating bacteria involved in protein degradation. These sources are known to harbor proteolytic microbes due to their high organic content (Fig. 3).

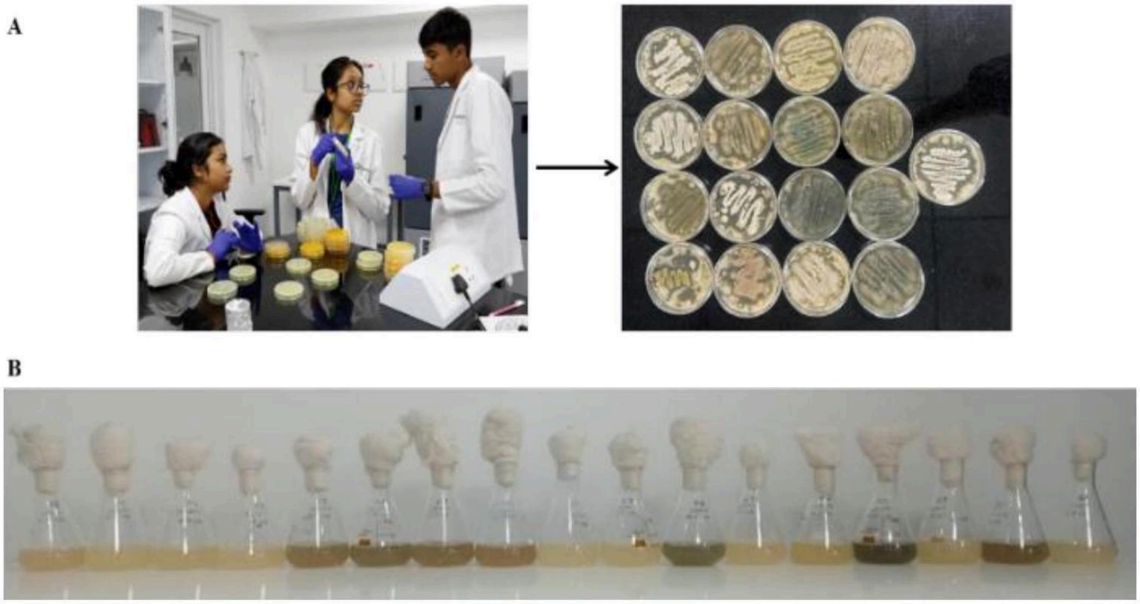


Figure 2. Screening of selected bacterial isolates (I1-I19) for extracellular protease production. (A) Casein hydrolysis zones visualized on SMA, and (B) growth and proteolytic activity in SMB cultures

On assessment of EPA, isolates I11, I17, and I19 were chosen to be the top three isolates for production of extracellular (EC) enzyme (Figure 3). I11 exhibited the maximum initial EPA, i.e., $304.67 U/mL$ at 24 h , which slightly declined over time. I17 exhibited similar activity, while I19 showed a gradual increase, reaching $297.29 U/mL$ at 72 h (Table 1). Despite slightly lower overall activity, I19 was selected for further study due to its unique flaky and uniform colony morphology and longer enzyme stability.

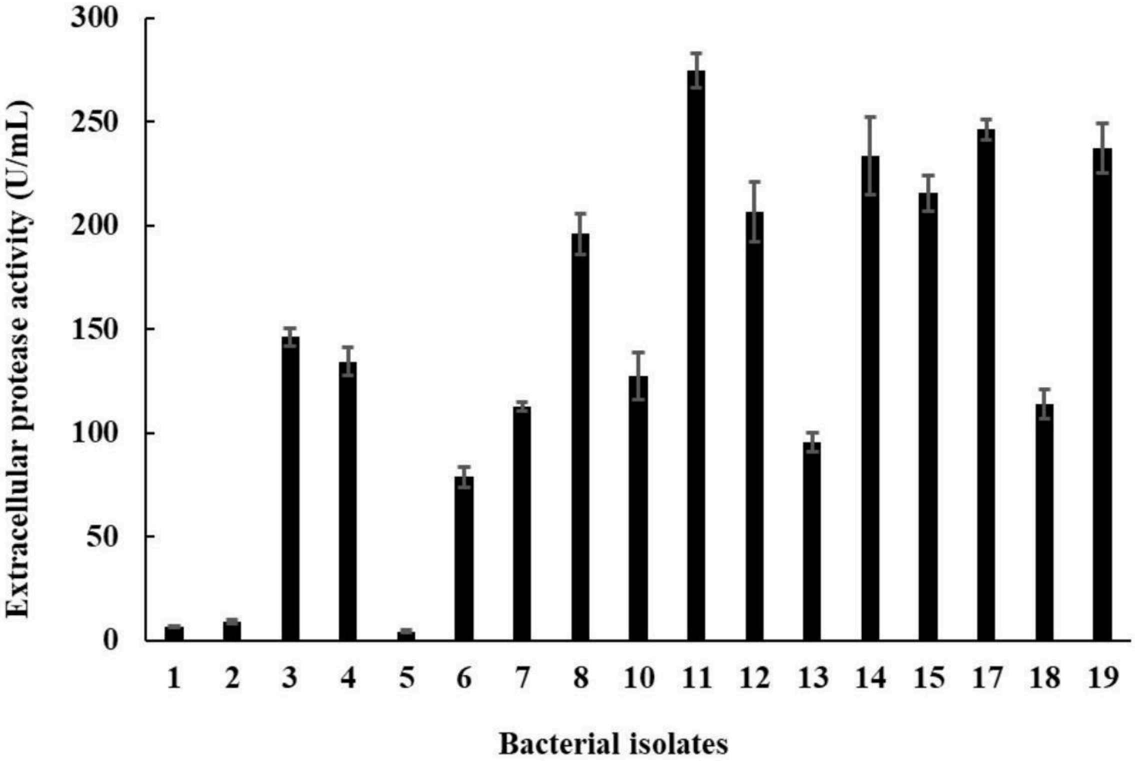


Figure 3. Comparative EPA exhibited by bacterial isolates in SmF cultures at 24, 48, and 72 h . Error bars represent the mean ± SD ($n = 3$).

Table 1. Time-course analysis of EPA of the top three bacterial isolates (I7, I12, and I19) cultured in SmF for 24, 48, and 72 h .

Isolates	Extracellular enzymes	
	Sampling interval	Extracellular protease activity (U/mL)
I11	24 hours	304.67 ± 9.14
	48 hours	289.91 ± 14.19
	72 hours	285.74 ± 8.57
I17	24 hours	296.16 ± 14.80
	48 hours	289.91 ± 8.69
	72 hours	285.41 ± 11.41
I19	24 hours	284.13 ± 6.68
	48 hours	290.23 ± 14.51
	72 hours	297.29 ± 8.91

Optimization Of Submerged Fermentation Conditions

Optimization experiments demonstrated that agitation enhanced protease yield, with shaking conditions outperforming static culture (Figure 4B). Temperature studies indicated optimum activity at $35\ ^{\circ}C$, while pH optimization revealed strong EPA between pH 7 and 9, with a peak at $pH8(360.95 \pm 12.3U/mL)$, suggesting that the extracted enzyme is tolerant to alkaline conditions (Figure 4A).

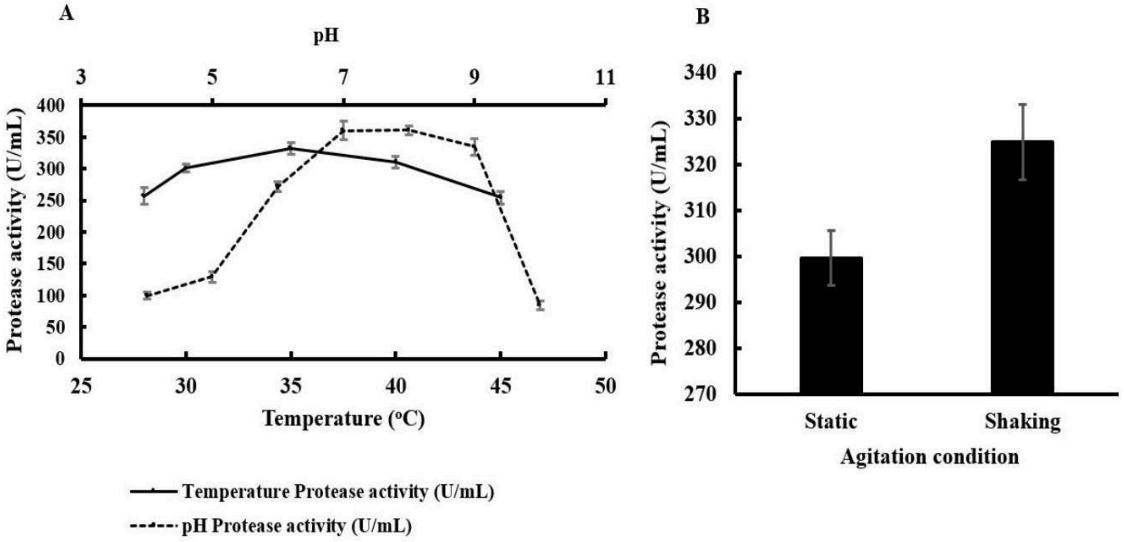


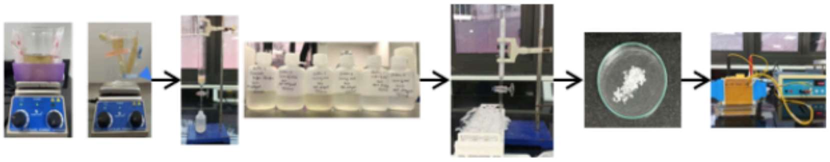
Figure 4. Optimization of SmF conditions: (A) Effect of initial pH (range 4-10) and incubation temperature ($30 - 45^{\circ}C$) on protease yield (U/mL); (B) Comparative protease production under static and shaking conditions at $35\ ^{\circ}C$, $24\ h$. Error bars represent the mean $\pm\ SD(n = 3)$.

Purification of Enzyme Obtained Through Submerged Fermentation

The crude extract contained $17,357.1\mu g$ of protein and $89,625.0U$ of activity (specific activity 5.16 $U/\mu g$), serving as the baseline. After 80% ammonium sulfate precipitation, protein decreased to $12,237.7\mu g$ and activity to $83,318.7U$, while specific activity rose to $6.80U/\mu g$ with 92.96% recovery. Dialysis and freeze-drying further reduced protein $(10,142.6\mu g)$ and activity $(73,881.0U)$, yielding $7.28U/\mu g$ and 82.43% recovery (Table. 2).

Ion exchange chromatography (DEAE-cellulose) enriched protease activity, with $0.4 - 0.8MNaCl$ eluates pooled ($4,435.8\mu g$ protein, $55,365.7U$ activity, $12.48U/\mu g$; 61.77% recovery). Gel filtration (F25-F30) yielded the maximum specific activity $(1,982.3\mu g$ protein, $46,576.4U$ activity, $23.49U/\mu g)$, with 51.96% recovery and a > 4.5-fold purification. SDS-PAGE confirmed a single prominent band at approximately 20 kDa (Figure 5), consistent with the molecular weight range reported for subtilisintype Bacillus proteases [17].

Table 2. Purification summary of protease enzyme from isolate I19 SmF culture supernatant

Steps of purification							
	Volume (mL)	*Protein content (µg/mL)*	*Total protein (µg)*	*Protease activity (U/mL)*	*Total activity (U)*	*Specific activity (U/µg)*	*% recovery of enzyme*
Crude extract	250	69.42	17357.1	358.5	89625.0	5.16	100
Ammonium sulphate precipitation (80%)	25.5	479.90	12237.7	3267.4	83318.7	6.80	92.96
Dialysis and Freeze drying	30	338.08	10142.6	2462.7	73881.0	7.28	82.43
Ion exchange chromatography (Pooled active fractions - 0.4, 0.6 and 0.8 M NaCl eluents)	120	36.96	4435.8	461.38	55365.7	12.48	61.77
Gel-exclusion chromatography (Pooled active fractions (F25 - F30)	9	220.25	1982.3	5175.15	46576.4	**23.49**	**51.96**

Solid-State Fermentation Using Agro-Based Substrates

Enzymes obtained using Ragi stem as substrates presented the highest EPA among all substrates, at $5120.8U/g$, which further increased to $5673.2U/g$ upon SMP supplementation. This suggests that the Ragi stem is the most suitable substrate for protease production by I19, and SMP enhances enzyme secretion. Napier grass as substrate demonstrated moderate EPA, which increased significantly to $3028.8U/g$ with SMP. This indicates SMP supplementation positively influences enzyme production even on moderately effective substrates. Banana stem and banana stem + SMP as substrates resulted in relatively low EPA, $872.4U/g$ and $994.4U/g$, respectively, showing only a marginal improvement with SMP. These substrates are less favorable for enzyme production. Coconut husk as a substrate presented minimal EPA, $539.3U/g$ and no change upon SMP addition, indicating it is the least effective substrate for I19 under these conditions (Fig. 6).

The extracted EC enzyme was quantified for protein content using the Bradford method. Enzymes obtained from Ragi and Ragi + SMP substrates exhibited relatively high protein levels, with SMP addition causing a slight reduction from $100.07\mu g/mL$ to $90.98\mu g/mL$. Napier grass-derived enzymes displayed a lower baseline protein content of $70.72\mu g/mL$, but SMP supplementation markedly increased it to $90.72\mu g/mL$, highlighting its protein-enhancing effect in this case. Banana stem as substrate yielded the highest protein levels overall, both with and without SMP, though supplementation appeared to slightly reduce the extractable protein, possibly due to interference or binding interactions or the presence of non-enzyme proteins. Coconut husk derived enzymes exhibited the lowest protein content at $40.33\mu g/mL$, with SMP raising it only marginally to $46.05\mu g/mL$, indicating limited improvement.

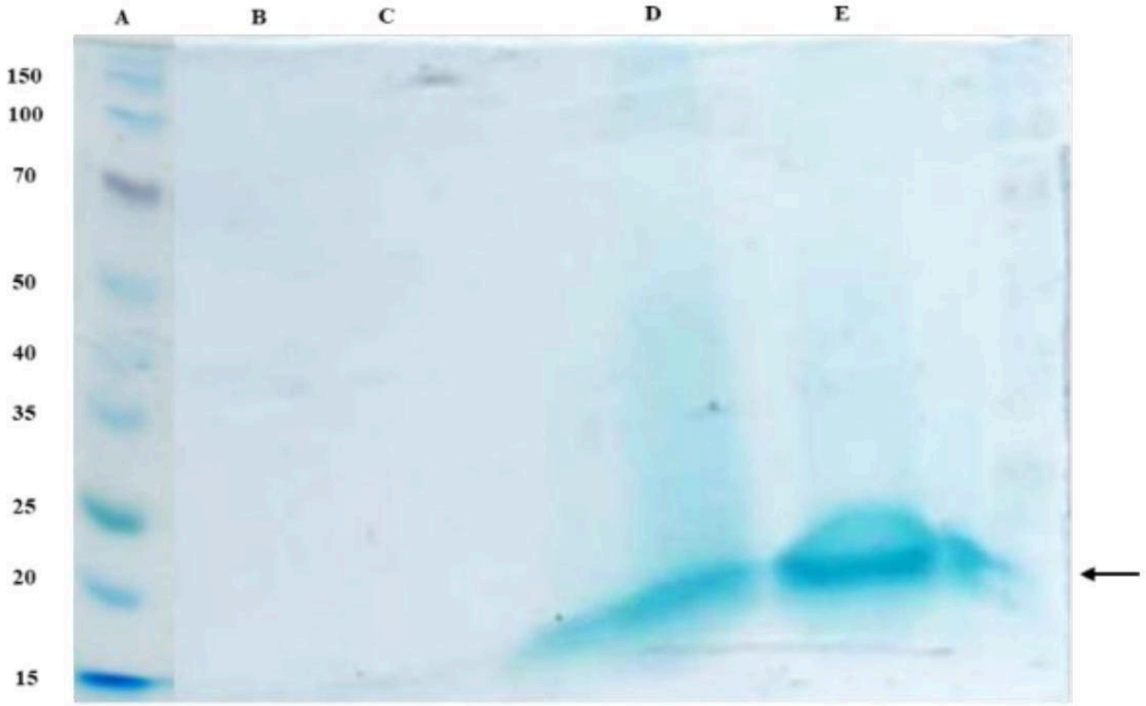


Figure 5. SDS-PAGE analysis of purified protease. Lane A: molecular weight standards; Lanes B and C: buffer controls; Lane D: ion-exchange chromatography-purified protease; Lane E: gel-filtrationpurified protease (indicated by arrow) on a 10% polyacrylamide gel.

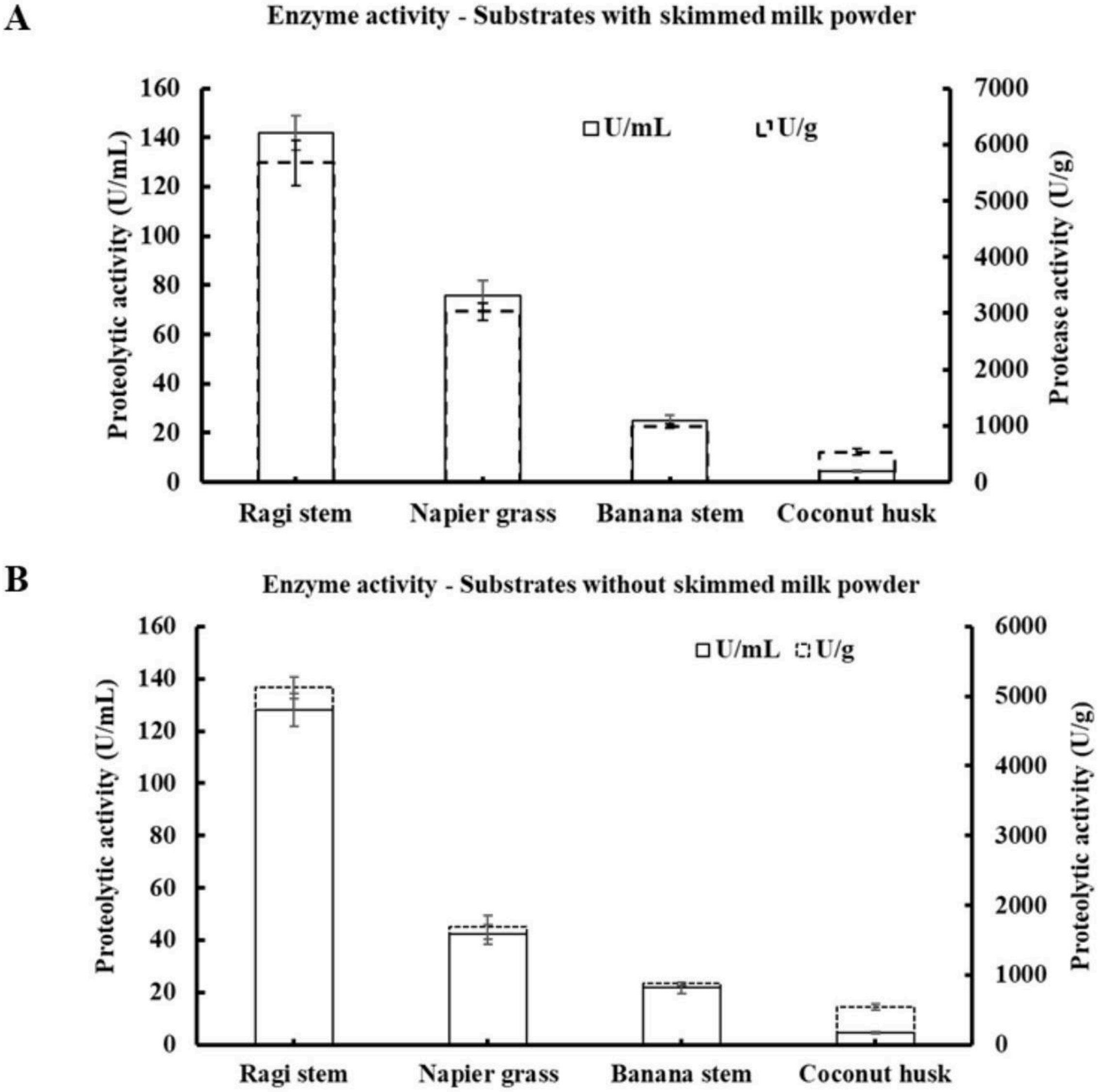


Figure 6. Substrate-specific protease production by I19 in SSF: Comparative analysis of protease yields in media supplemented with (A) and without (B) SMP. Activity is expressed in both U/mL and U/g of dry substrate. Error bars represent mean ± SD ($n = 3$). Substrates tested: Napier grass, coconut husk, banana stem, and ragi stem; incubation at room temperature for 7 days.

Identification of Protease Producing Bacteria

Microscopic examination revealed that the cells retained the crystal violet stain, appearing purple, which confirms that Isolate 19 is a Gram-positive rod-shaped bacterium. The 16S rRNA gene sequence analysis confirmed that the isolate belonged to Bacillus subtilis, showing high similarity (>99%) with reference sequences available in the NCBI database. The sequence was successfully submitted and accepted by GenBank (accession number PV942511) with sequence ID PRGDNRSVkb_16S_Bacillus_subtilis (Fig. 7).

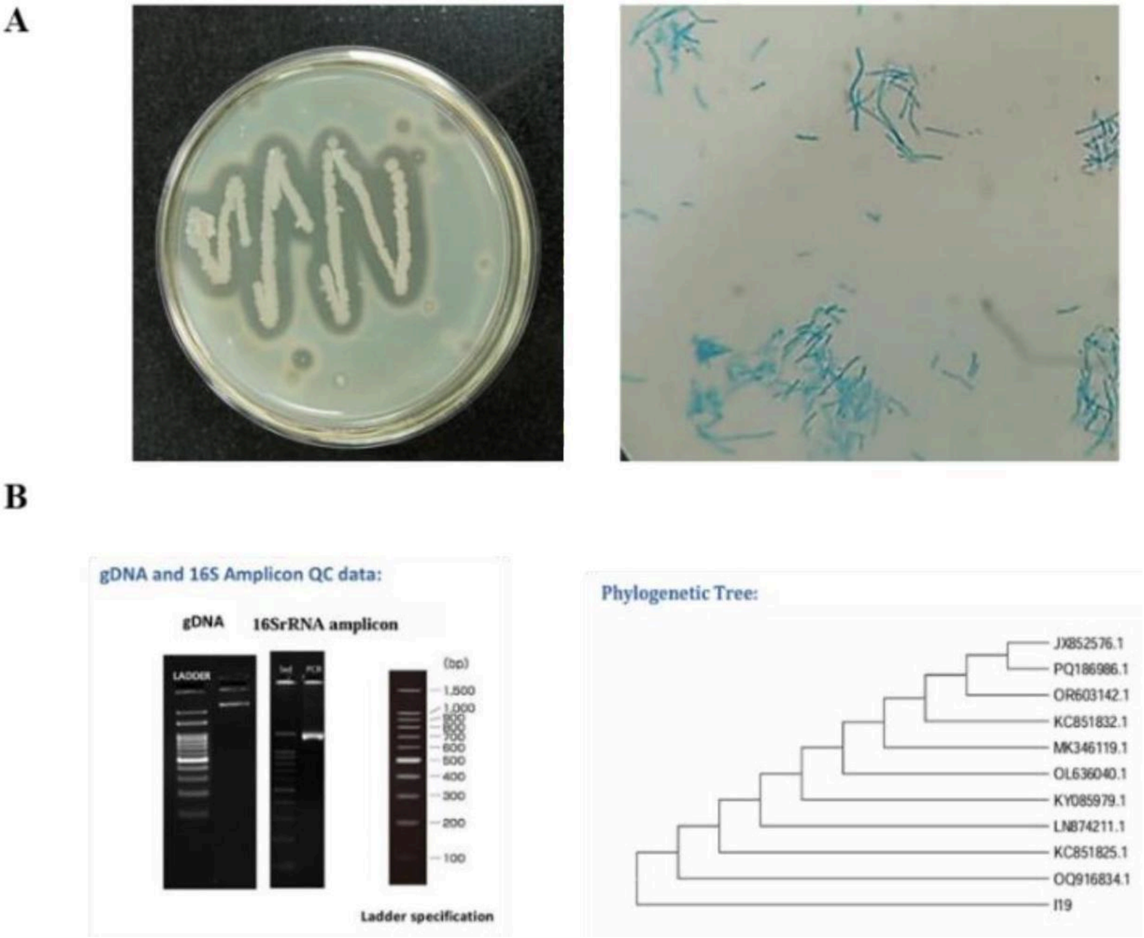


Figure 7. Morphological and molecular characterization of isolate I19. (A) Growth of I19 on SMA medium and Gram staining, revealing Gram-positive, rod-shaped bacterial cells. (B) 16S rRNA gene amplification confirming closest homology of isolate I19 to Bacillus subtilis.

DISCUSSION

In this study, B. subtilis I19 was identified as a potent protease producer from protein-rich environmental sample (soil from a meat shop). The isolate exhibited robust extracellular protease activity across all conditions tested, with a maximum yield achieved under SSF using ragi stem supplemented with skimmed milk powder ($5673.2U/g$). This performance is well within the range of high-yielding Bacillus strains previously reported. For instance, Bacillus sp. JB-99 has been shown to produce high yields of protease on wheat bran and sugarcane residues ($7000U/g$), while B. pumilus SG2 reached even higher levels using seed cake substrates ($9840U/g$), and B. cereus produced moderate activity with red gram husk $(258U/g)[26, 27, 28]$. Compared to these, the yield of I19 using ragi stem + SMP highlights the suitability of this substrate as an inexpensive and effective raw material for enzyme production and constitutes the first report of ragi stem as a substrate for bacterial protease production via SSF. Among the other agro-residues tested, Napier grass supplemented with SMP supported moderate protease activity ($3028.8U/g$), whereas banana stem and coconut husk were relatively poor substrates, with activities below $1000 U/g$. Such variations are likely due to differences in lignocellulosic composition and nutrient availability, which influence microbial growth and enzyme secretion.

Under SmF, isolate I19 initially produced $304.67 U/mL$, which improved to $360.95 U/mL$ after optimization at $35\ ^{\circ}C$, with shaking and $pH 7-9$. This yield is higher than some Bacillus strains, such as B. alkalitelluris ($322.8 U/mL$), though lower than B. pumilus ($776.5 U/mL$) [29, 30]. Importantly, I19 protease displayed optimal activity at pH 8 and stability across $pH 7-9$, confirming its tolerance to alkaline conditions. The alkaline resistance characteristic of I19 is mechanistically feasible since subtilisin-type serine proteases have an active site catalytic triad (Ser-His-Asp) that is maximally protonated in alkaline media and is the predominant extracellular protease synthesized by B . subtilis [35]. The enhancement of protease production through shaking may be explained by better oxygen and nutrient transport, which promotes aerobic synthesis of the enzyme in Bacillus species. The 20 kDa band observed on SDS-PAGE is within the range of reported molecular weights for subtilisin-type proteases of Bacillus species [17]. Considering its yield characteristics and other properties, the industrial relevance of I19 could be explored for its use in detergents, food, and leather industries, provided it meets certain requirements. Future directions should focus on scaling up SSF using ragi stem-based substrates, assessing the enzyme's performance in detergent or food industry applications, and exploring strategies such as strain improvement or immobilization to further enhance yield and stability. Ragi stem's superior SSF performance is attributable to its abundant arabinoxylan hemicelluloses (arabinose and xylose as predominant sugars) that serve as readily fermentable carbon sources [31], combined with moderate lignin that does not significantly impede microbial growth. Skimmed milk powder supplementation provides casein, which is well established as an inducer of Bacillus extracellular protease gene expression, particularly aprE and nprE acting via nutrient-sensitive regulatory proteins (CodY, ScoC) that derepress protease synthesis under nitrogen-limiting conditions [32]; this mechanism accounts for the protease activity increase from 5120.8 to $5673.2 U/g$. The SSF advantage over SmF on a per-gram basis further reflects the resistance of SSF systems to catabolite repression, which allows enzyme induction even at high sugar concentrations [33, 34], alongside the high substrate surface-area-to-volume ratio that promotes microbial colonization and oxygen transfer.

LIMITATIONS AND FUTURE PERSPECTIVES

There are a number of limitations to the present study that need to be taken into account. First, all the experiments conducted during this study were carried out on a lab scale ($50-100\,mL$); whether Bacillus subtilis I19 works at an industrial scale remains unknown. Second, enzyme stability under different application conditions, for example, when the enzyme is added to commercial detergents and in situations involving high temperatures associated with food processing, needs to be studied further. Third, the comparative study of the four agro-residues used for SSF of the bacteria mentioned here is one of the limitations of the present study; future studies will undoubtedly find more agro-residues suitable for biotechnological purposes.

CONCLUSION

This study demonstrates that Bacillus subtilis I19 is a strong producer of extracellular protease, with stability across broad pH and temperature ranges. Beyond submerged fermentation, SSF using agrowaste substrates proved effective for cost-efficient enzyme production. Ragi stem supported the maximum yields, which further improved with SMP supplementation, emphasizing the importance of nitrogen-rich additives. Napier grass also exhibited moderate potential, particularly when supplemented, while banana and coconut husk were less effective, likely due to unfavorable nutrient composition or structural limitations. These findings align with reports highlighting the role of substrate composition in protease yields and underscore the promise of SSF as a sustainable strategy for enzyme production, with further scale-up and application-specific validation needed to confirm industrial suitability.

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ANNEXURE I

Project group

From left to right

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