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Protease Production by *Bacillus Subtilis* Using Fruit Peel Waste

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ABSTRACT

The production of protease enzymes using microorganisms has garnered significant attention in recent years due to their wide-ranging applications in industries such as food, pharmaceutical, and biotechnology. This study investigates the potential of Bacillus subtilis to produce protease enzymes using fruit peel extract as a substrate. Fruit peels, typically discarded as waste, offer a rich source of nutrients that can be harnessed to support microbial growth and enzyme production. In this project, we optimized the fermentation conditions for protease production by Bacillus subtilis using fruit peel extract. The effects of pH, temperature, and substrate concentration on enzyme production were evaluated. Our results showed that Bacillus subtilis produced significant amounts of protease enzymes when grown on fruit peel extract, with optimal production observed at pH 7.0, 37°C, and 2% substrate concentration. The crude enzyme extract exhibited promising proteolytic activity, with potential applications in various industrial processes. This study demonstrates the feasibility of utilizing fruit peel extract as a renewable substrate for protease production, contributing to a more sustainable and environmentally friendly approach to enzyme production.

Keywords: Bacillus subtilis, protease enzyme, fruit peel extract, fermentation optimization, sustainable production.

INTRODUCTION

Fruits are vital for human health, offering a rich source of vitamins, minerals, fiber, and bioactive compounds like antioxidants that help combat major diseases such as cancer, cardiovascular disorders, and diabetes. However, the food industry generates significant fruit waste—such as banana peels, citrus rinds, grape pomace, and dragon fruit and orange peels—which poses serious environmental challenges when discarded in landfills (Bharathiraja *et al.*, 2016). These wastes are rich in cellulose, hemicellulose, and reducing sugars, and they also contain valuable bioactive compounds and enzymes (Mohammad Jahid *et al.*, 2018).

Polyphenols extracted from fruit wastes exhibit antioxidative, anti-inflammatory, and antibacterial properties, making them valuable for food preservation, nutraceuticals, and pharmaceuticals. Integrating biotechnological approaches enables the conversion of these agro-wastes into high-value industrial products, particularly enzymes. Enzymes are biocatalysts widely used in industries such as food processing, detergents, pharmaceuticals, textiles, and leather. Among various microbial sources, *Bacillus subtilis*—a spore-forming, gram-positive soil bacterium—is recognized for its rapid growth and production of industrially significant enzymes including proteases, amylases, and lipases (Piggot, 2011).

Dragon fruit (*Hylocereus spp.*) and oranges (*Citrus sinensis*) are examples of fruits with beneficial peels rich in antioxidants, flavonoids, and phytochemicals. Dragon fruit peels have applications in the cosmetic and medical fields due to their vitamin C content and skin-repairing properties (Indah Purnamasari *et al*., 2021). Similarly, orange peels are rich in flavonoids like polymethoxy flavones (PMFs) and hesperidin, with known antimicrobial, anti-inflammatory, and skin-benefiting properties. These peels also serve as sources of protease and other functional biomolecules (Anwar Ali *et al*., 2014; Ayon, 2021).

This study aims to optimize the production of protease by *Bacillus subtilis* using fruit peel waste as a sustainable substrate. The objectives include molecular characterization of the isolate through 16S rRNA sequencing, optimization of protease production using dragon fruit and orange peels, and evaluation of enzyme yield through both qualitative and quantitative assays. Additionally, the study investigates potential applications of the produced protease in milk clotting activity and stain removal, highlighting its industrial relevance.

METHODOLOGY

Isolation and Screening of Protease-Producing Bacteria

Soil samples were collected from garden soil in Chennai and serially diluted for isolation of protease-producing bacteria. Pure cultures were obtained from isolated colonies (Seshan, 2018).

Culture Growth and Maintenance

The pure culture was grown on nutrient agar at 37 °C for 24 hours. For long-term storage, cultures were preserved in glycerol stocks at -20 °C, and for short-term storage at 4 °C. Biochemical and Morphological Characterization were carried out using the standard protocol.

Molecular Characterization

a) DNA Isolation

Genomic DNA was extracted from cultures using phenol-chloroform method. DNA was precipitated with ethanol, washed, and suspended in TE buffer.

b) Quantification of DNA

DNA concentration and purity were determined spectrophotometrically at 260/280 nm.

c) Polymerase Chain Reaction (PCR)

16S rRNA gene was amplified using specific primers. PCR products were visualized on 1.5% agarose gel stained with dye.

Submerged Fermentation for Protease Production

Fruit peels (dragon fruit and orange) were washed, dried at 70 °C, ground, and used as carbon sources. Three media types were prepared:

Sample I: Dragon fruit peel

Sample II: Orange peel

Sample III: No fruit peel (control)

Fermentation was carried out in protease production media (dextrose, peptone, yeast extract, salts, and fruit peels) inoculated with 2 ml of bacterial culture and incubated at 37 °C for 72 hours at 120 rpm.

Crude Enzyme Extraction

Post-incubation, the media were filtered and centrifuged. The supernatant was used as crude enzyme extract.

Protease Assays

Qualitative Assay – Ninhydrin Test

Crude enzyme was incubated with BSA, followed by TCA precipitation and ninhydrin reaction. Purple coloration indicated protease activity.

Quantitative Assay – Lowry's Method

Protein concentration was estimated using Lowry's method. Absorbance at 660 nm was measured and a standard curve was plotted using BSA.

Protease Activity Assay

BSA was used as a substrate. Reaction mixtures were incubated and terminated with TCA. The resulting peptides were quantified using Folin-Ciocalteu reagent and absorbance was read at 660 nm.

Application Studies

Milk Clotting Activity

1 ml of protease enzyme was added to milk and incubated at room temperature for 30 minutes. Curd formation indicated milk clotting.

Anti-Browning Activity

Apple slices were dipped in enzyme solutions for 15 minutes. Browning reduction was compared to control.

Stain Removal Efficiency

Blood-stained cotton pieces were treated with different combinations of enzyme, water, and detergent. Stain removal was visually assessed.

Digestive Supplement Potential

Mixtures of egg and protease were incubated, followed by Biuret reagent addition. Color intensity was used to evaluate proteolytic efficiency.

RESULTS

1. Isolation of *Bacillus subtilis*

Pure Culture of Isolate

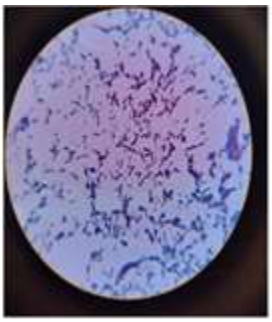
Figure 1 **shows** the Pure culture of *Bacillus subtilis* on nutrient agar. *Bacillus subtilis* was streaked onto nutrient agar plates and incubated at 37 °C for 24 hours. Post-incubation, discrete colonies were observed, characterized by uniform size, circular morphology, and a milky white appearance, indicative of pure culture growth.

Figure 1: *Bacillus subtilis* pure culture on nutrient agar plate for 24 hours at 37℃

Biochemical Tests for preliminary identification:

Table 1: Biochemical characterization test

S. No.	Tests	Results
1.	Gram Staining	Positive, Rod-shaped bacteria
2.	Oxidase Test	Positive
3.	Gelatin Liquefaction Test	Positive
4.	Citrate Test	Positive
5.	Methyl Red Test	Negative
6.	Voges Proskauer Test	Negative
7.	Nitrate Reduction Test	Negative
8.	Urease Test	Negative



a)

b)

c)

d) e) f) g) h) i)

Figure 2: Biochemical test

The biochemical characterization of the isolate confirmed it as a Gram-positive, rod-shaped bacterium. It tested positive for oxidase activity, gelatin liquefaction, citrate utilization, and nitrate reduction, indicating the presence of cytochrome c oxidase, gelatinase, and the ability to utilize citrate and reduce nitrate. However, it tested negative for the Methyl Red, Voges–Proskauer, and urease tests, suggesting the absence of stable acid production, acetoin formation, and urease activity.

Molecular Characterization of the Isolate

Genomic DNA was successfully extracted from the isolate and confirmed through agarose gel electrophoresis (Figure 3). A distinct DNA band was observed in Lane 4, with a 100 bp molecular weight marker loaded in Lane 1 to determine fragment size. Subsequent PCR amplification of the 16S rRNA gene yielded a specific amplicon, which was visualized using agarose gel electrophoresis (Figure 4). The PCR product was observed in Lane 5, with a 100 bp DNA ladder used as a size reference in Lane 1.

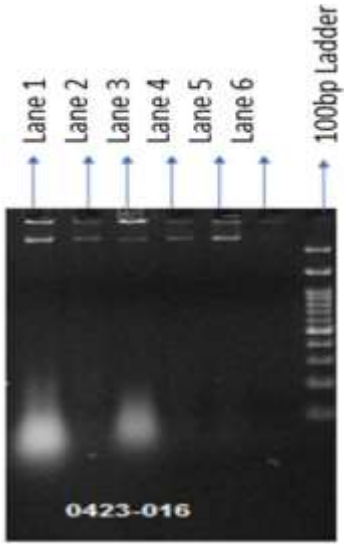


Fig 3 DNA Isolation

Lane 4: Genomic DNA

Lane ladder: 100bp ladder

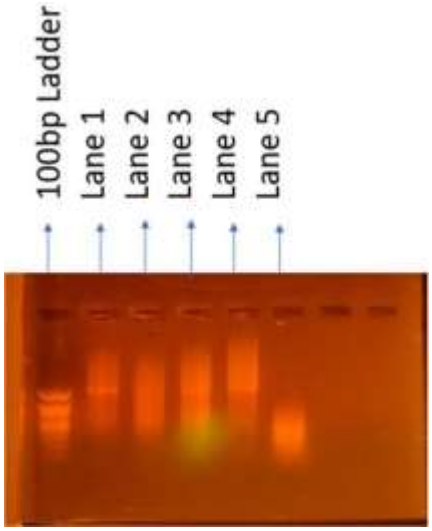


Fig 4 PCR Amplification of 16s rRNA

Lane 1: 100bp ladder

Lane 5: PCR amplicon of 16sr RNA

Table 2: Purity of the Isolated Genomic DNA

Sample	OD at 260nm	OD at 280nm	Concentration (ng/µl)	Purity
Blank	0.000	0.000	--	--
1	0.151	0.087	1241.3	1.73

Molecular Identification of the Isolate

The 16S ribosomal RNA (rRNA) gene of the bacterial isolate was amplified via PCR using the previously extracted genomic DNA. The resulting PCR amplicons were sequenced, and the obtained sequences were compared against the GenBank database using the BLAST algorithm. The sequence analysis revealed that the isolate showed 100% identity with Bacillus subtilis, confirming its taxonomic classification.

2. Submerged Fermentation for Protease Production

Submerged fermentation was carried out to evaluate protease production by the identified Bacillus subtilis isolate. The production medium was composed of dextrose, peptone, yeast extract, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), sodium carbonate (Na_2CO_3), fruit peel waste, and distilled water.

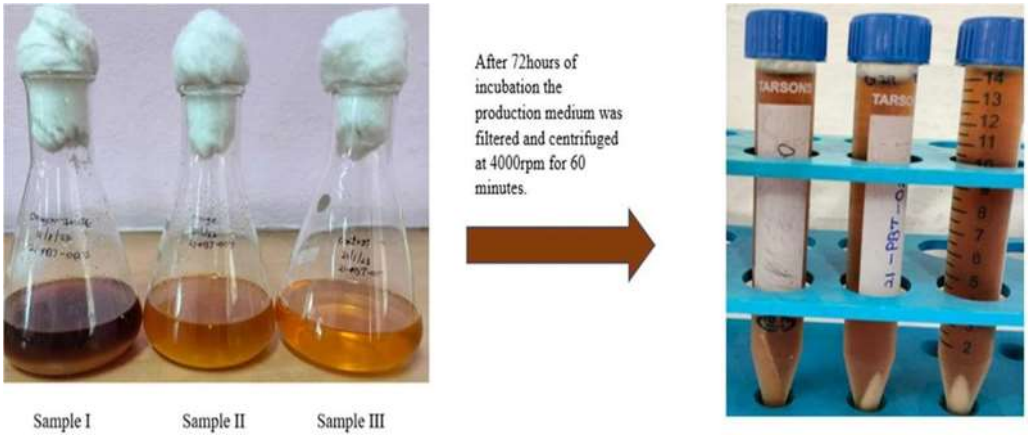
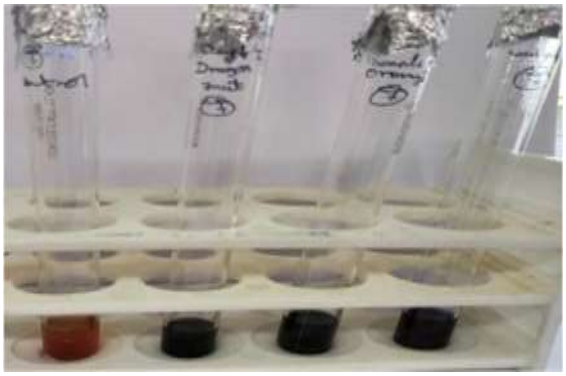


Figure 5 illustrates the composition of the fermentation media: Sample I: Production medium supplemented with dragon fruit peel powder, Sample II: Production medium supplemented with orange peel powder, Sample III: Control medium containing only the production medium without any fruit peel supplement.

i) Qualitative Assay

Ninhydrin Test

The Ninhydrin test was performed to qualitatively detect the presence of protease activity. Figure 6 illustrates a positive result for the Ninhydrin test, where the appearance of a purple color indicates the presence of protease enzymes. The color change occurs due to the breakdown of the substrate by the protease, confirming the enzymatic activity.



Control Sample I Sample II Sample III

Fig. 6 Ninhydrin test to detect presence of protease

ii) Quantitative Assay

Protein Lowry's Assay

The Lowry's protein assay was performed using Bovine Serum Albumin (BSA) standards at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, with OD measured at 660 nm. The corresponding OD values were 0.51, 0.86, 1.26, 1.35, and 1.52. For the samples, the absorbance was 1.34 for Sample I, 0.94 for Sample II, and 0.70 for Sample III, with protein concentrations of 168 µg/mL, 110 µg/mL, and 78 µg/mL, respectively. Sample I showed the highest protein content.

Table 3: Protein Lowry's assay

Volume of BSA (ml)	Volume of Distilled water (ml)	Concentration of protein (µg/ml)	Volume of Reagent C (ml)	Volume of Reagent D (ml)	Absorbance at 660nm
Blank	1	-	5	0.5	-
0.2	0.8	40	5	0.5	0.51
0.4	0.6	80	5	0.5	0.86
0.6	0.4	120	5	0.5	1.26
0.8	0.2	160	5	0.5	1.35
1.0	-	200	5	0.5	1.52
Test 1 (1ml)	-	168	5	0.5	1.34
Test 2 (1ml)	-	110	5	0.5	0.94
Test 3 (1ml)	-	78	5	0.5	0.70

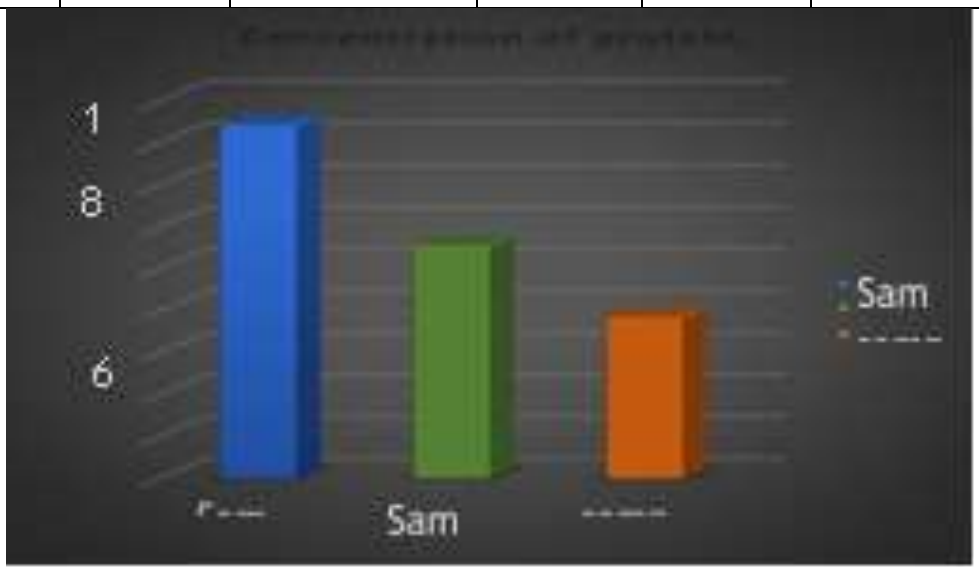


Figure 7: Protease Enzyme Activity

Table 4 shows the protease enzyme activity over five days. On Day 1, enzyme activity was 0.40 µg/mL for Sample I, 0.007 µg/mL for Sample II, and 0.006 µg/mL for Sample III. On Day 2, enzyme activity increased to 0.512 µg/mL (Sample I), 0.464 µg/mL (Sample II), and 0.159 µg/mL (Sample III). On Day 3, values were 0.760 µg/mL (Sample I), 0.587 µg/mL (Sample II), and 0.353 µg/mL (Sample III). Day 4 saw the highest enzyme activity: 1.3616 µg/mL for Sample I, 0.9872 µg/mL for Sample II, and 0.5202 µg/mL for Sample III. On Day 5, the enzyme activity decreased to 0.7562 µg/mL (Sample I), 0.6514 µg/mL (Sample II), and 0.3535 µg/mL (Sample III). Sample I showed the highest enzyme activity on Day 4.

Table 4: Protease Activity was assessed using UV Spectroscopy

FERMENTATION HOURS	**ABSORBANCE AT 660nm**		
	SAMPLE 1 (Dragon fruit peel)	**SAMPLE 2 (Orange fruit peel)**	**SAMPLE 3 (Control)**
24 hours	0.4065	0.0781	0.00631
48 hours	0.5127	0.4642	0.1594
72 hours	0.7605	0.5877	0.3535
96 hours	1.3616	0.9872	0.5202
120 hours	0.7562	0.6814	0.3535
144 hours	0.7186	0.5362	0.2324

Figure 8: shows the absorbance at 660 nm for all three samples during a 6-day fermentation period. On Day 4, the absorbance for all samples increased, indicating that 96 hours is the optimal fermentation time for maximizing protease enzyme yield. The absorbance values recorded were 1.3616 µg/mL for Sample I, 0.9872 µg/mL for Sample II, and 0.5205 µg/mL for Sample III. Sample I (Dragon fruit peel) demonstrated the highest enzyme activity and yield, likely due to its bioactive compounds, including betalains, phenolics, and its high protein content.

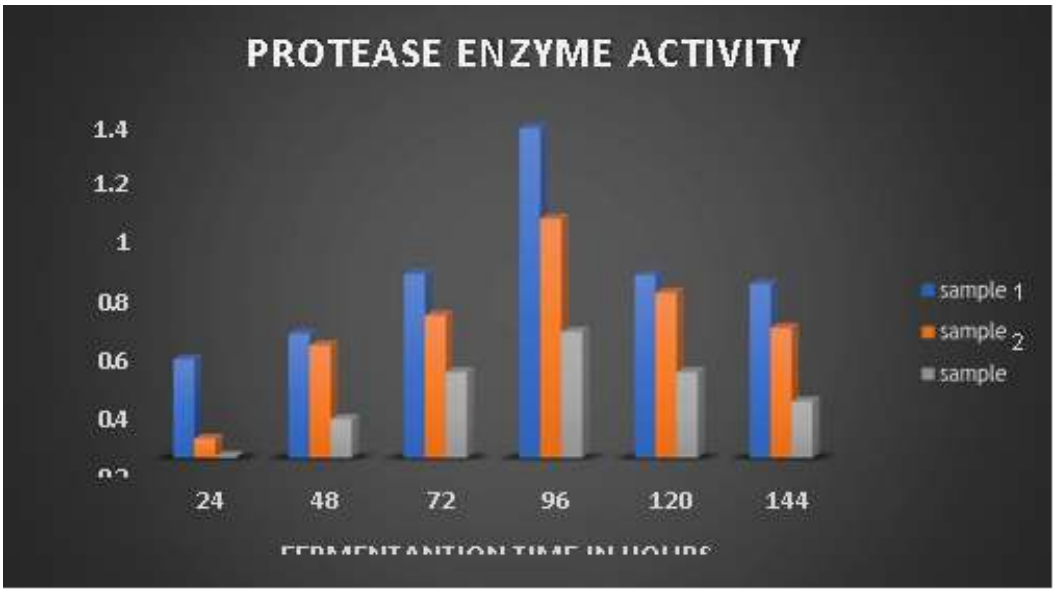


Figure 8: Protease enzyme activity measured using different substrates.

3. Application Studies

a) Milk Clotting Activity: The enzyme supernatant was added to 7 mL of milk, mixed thoroughly, and incubated at room temperature for 30 minutes. After incubation, the milk was observed for coagulation due to the activity of peel protease. The peel protease from Sample I demonstrated strong milk curdling activity, indicating its potential for use in cheese formation. In contrast, Sample II's peel protease showed weaker curdling activity and took longer to produce the same effect. Therefore, Sample I, with its higher concentration of peel protease, is more effective for cheese-making. (Figure 9)

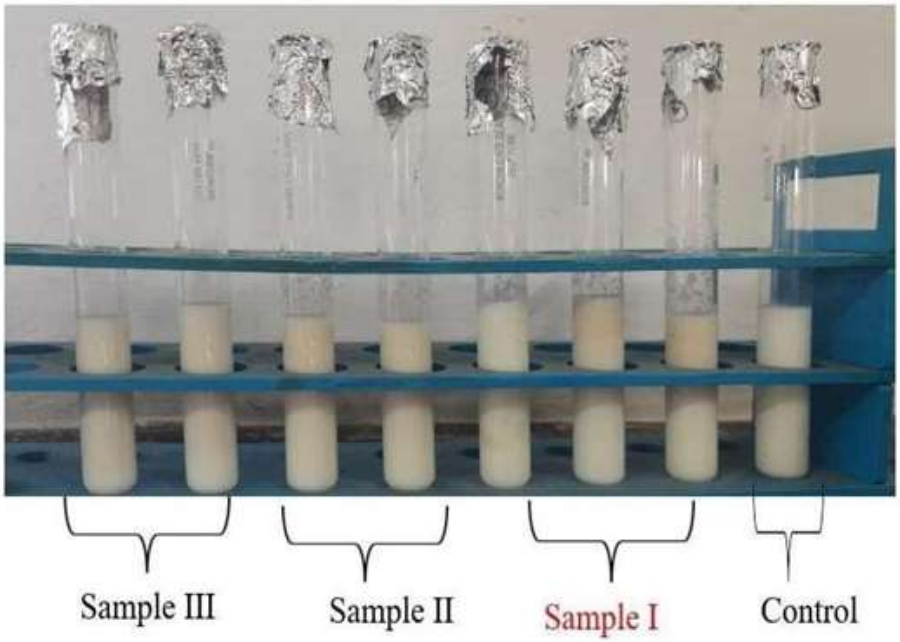


Fig 9: Milk clotting activity by peel protease enzyme

Table.5: Time of curdling & Volume of whey formed

Sample	Time of Curdling (minutes)	Volume of Whey (ml)
Sample I	30	5
Sample II	30	3
Sample III	30	-

b) Anti-browning Activity: Figure 10 shows that peel protease treatment effectively prevented browning in apple pieces compared to the control. Apple pieces treated with Sample I, Sample II, and Sample III peel protease for 15 minutes were observed. Sample I showed no browning, while apple pieces treated with Sample II and Sample III turned brown. Among all the samples, Sample I exhibited the best anti-browning activity. Since commercial anti-browning agents may cause adverse reactions, Sample I peel protease could serve as a natural, safe alternative for the food industry.

Sample I Sample II Sample III

Figure 10: Apple pieces treated with peel protease enzyme for anti-browning effect

Table 6: Anti-browning Activity of peel protease

S. No.	Sample	Colour
1.	Control	Dark Brown
2.	Sample I	Colourless
3.	Sample II	Light Brown
4.	Sample III	Light Brown

c) Enzyme in Stain Removal: Figure 11 illustrates the destaining activity of protease on blood stains. Peel protease was applied to white cotton cloth and incubated for 15 minutes, effectively removing the blood stain. The detergent solution supplemented with peel protease also successfully removed the stain. Sample I demonstrated complete removal of the blood stain, indicating its superior potential for use in detergent industries compared to Sample II and Sample III peel proteases.

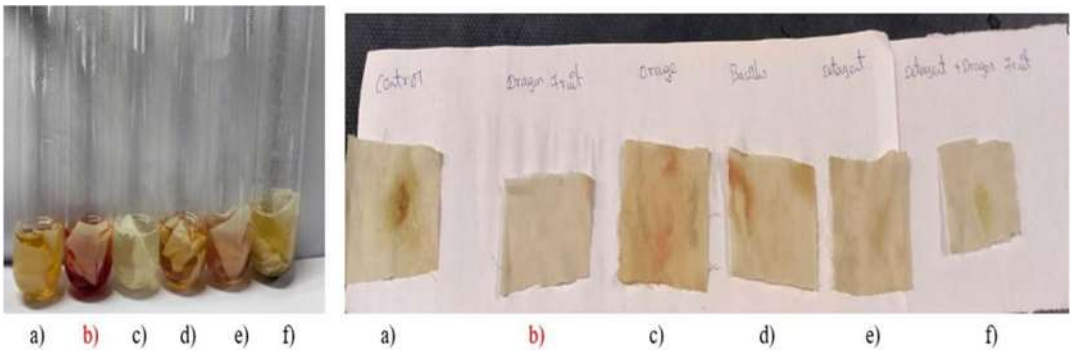
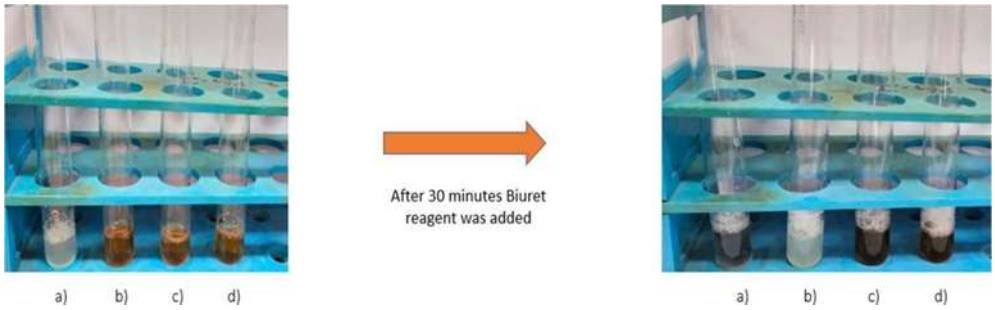


Fig 11. Destaining activity of protease on blood.

a) Control; b) Sample I; c) Sample II; d) Sample III; e) Detergent; f) Detergent + Sample

d) **Digestive Supplement:** Results showed that Sample I peel protease effectively digested proteins. Egg white was supplemented with all the samples and incubated for 30 minutes at room temperature. Protein digestibility was determined using the biuret test, with Sample I exhibiting the highest protein digestibility compared to Sample II and Sample III. This could be attributed to the higher protein content in Sample I. The purple or blue color indicated a high affinity for the substrate, and Sample I demonstrated the highest substrate affinity compared to the other samples (Figure 12).



a) Control; b) Sample I; c) Sample III; d) Sample I

Fig 12: Substrate affinity of peel protease.

DISCUSSION

Proteases are specialized enzymes with proteolytic functions, capable of breaking down peptide bonds. They are found ubiquitously in all living organisms, including animals, plants, and microbes. Proteases play a critical role in cell growth and differentiation. Among microbial sources, *Bacillus subtilis* is widely used for protease production due to its rapid growth and high yield. This Gram-positive, rod-shaped bacterium is non-pathogenic and forms heat-resistant spores. *Bacillus subtilis* is commonly found in soil and produces various commercially important products, including proteases and amylases.

Biochemical tests were performed for morphological characterization. The Gram stain revealed *Bacillus subtilis* as a Gram-positive, rod-shaped bacterium. The citrate test showed positive results, indicating the bacterium's ability to utilize citrate as a sole energy source. Methyl Red and Voges Proskauer tests were negative, suggesting the absence of stable acid and neutral end products from glucose fermentation. The urease test was also negative, indicating that the organism does not hydrolyze urea. The nitrate reduction test, however, was positive, showing the organism's ability to reduce nitrate to nitrite.

The protease enzyme production in this study utilized submerged fermentation, a widely adopted method due to its low contamination risk and high yield. Fruit peels were used as substrates, providing a sustainable source of enzymes and bioactive compounds for industries such as food, medicine, and cosmetics. Fruit wastes like banana peel, citrus waste, and apple pomace are rich in cellulose, hemicellulose, and reducing sugars, making them suitable for protease production.

In the study, the protease enzyme activity was confirmed through qualitative and quantitative analysis. The Ninhydrin test, which detects free amino acids, yielded a positive result, indicating the presence of protease activity (Marathe *et al.*, 2018). Protein concentration was measured using the Lowry's method, revealing that Sample I (Dragon fruit peel) had the highest protein content (168 µg/ml), followed by Sample II (Orange peel) and Sample III (Control) at 110 µg/ml and 70 µg/ml, respectively.

Enzyme activity was monitored over five days, with peak activity observed on Day 4. Sample I exhibited the highest protease activity (1.3616 µg/ml), followed by Samples II (0.9872 µg/ml) and III (0.5202 µg/ml). This suggests that the Dragon fruit peel was the most effective substrate for protease production.

Several applications of peel protease were investigated. In milk clotting activity, Sample I demonstrated the highest potential for curdling milk, indicating its suitability for cheese production. Sample II and Sample III showed weaker curdling activity. In anti-browning assays, Sample I effectively prevented browning in apple pieces, outperforming Samples II and III. Sample I also demonstrated superior stain removal activity in blood destaining tests, making it a promising candidate for detergent applications. Finally, Sample I exhibited the best protein digestibility when tested on egg white, likely due to its higher protease content.

This study highlights the significant potential of *Bacillus subtilis*-derived protease, particularly from Dragon fruit peel, in various industrial applications, including cheese making, food preservation, detergent formulation, and as a digestive supplement.

CONCLUSION

This study identified *Bacillus subtilis* through morphological, biochemical tests, and 16S rRNA sequence analysis. Protease enzyme production was assessed using Dragon fruit peel and orange peel as substrates. The enzyme showed maximum activity (1.34 µg tyrosine/minute/ml) at an initial pH of 7.2 and 37°C after 72 hours of fermentation. Dragon fruit peel produced the highest protease activity compared to orange peel. The protease demonstrated potential applications in milk clotting, stain removal, and as an anti-browning agent. Future work could optimize fermentation conditions, use diverse substrates, and explore new bacterial and fungal strains to enhance enzyme production.

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