

Biochemical Profiling and Functional Validation of Pectocellulosic Softening Enzymes Induced By Eight Banana (*Musa Spp.*) Cultivars

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Abstract

Utilizing agro-industrial byproducts such as banana peels for enzyme synthesis is vital for a circular bioeconomy, yet cultivar-specific inductive potential remains understudied. We evaluated eight *Musa* spp. cultivars as substrates for producing pectinase and cellulase using *Aspergillus japonicus*. Following proximate analysis and submerged fermentation, enzymes were concentrated via 50% ammonium sulfate precipitation and dialysis. Molecular profiling was conducted through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Industrial utility was tested via apple juice clarification and fruit peel softening assays. Cultivars 2 and 8 were identified as superior inducers, yielding enzymes with a 1.5–3.5-fold increase in specific activity post-purification. Gel electrophoresis revealed a secretome spanning 35–100 kDa. Mass spectrometry detected prominent signals at m/z 12,594.6, 76,096.2, and 86,244.2. Notably, the persistence of high-mass peaks despite tryptic digestion indicated a robust, proteolysis-resistant enzyme architecture likely stabilized by glycosylation. Applications resulted in a 40% increase in juice yield and accelerated maceration, evidenced by reduced firmness (6.2 ± 0.3 N) and pectin content (3.1 ± 0.2 mg/g). This study linked cultivar-specific biochemistry to fungal secretome complexity and identified a structurally resilient, dual-purpose biocatalyst uniquely suited for demanding industrial biotechnology and food engineering applications.

Keywords: *Aspergillus japonicus*, Banana peel valorization, Juice clarification, Mass spectrometry, Postharvest biotechnology

Introduction

The industrial management of banana peel waste—which typically represents 30% to 40% of the fruit's total mass—is no longer just an environmental necessity but a strategic priority for the circular bioeconomy (9). These peels are naturally rich in complex polysaccharides, specifically cellulose, hemicellulose, and pectin, making

them an ideal, low-cost "instructional" environment for microbes to synthesize high-value enzymes (25). In particular, fungal pectinases and cellulases are critical to the food industry for processes like juice clarification, and they play increasingly important roles in sustainable textile manufacturing and bioethanol production (4).

However, a significant gap exists in how we utilize these agricultural residues. Most industrial models treat banana waste as a uniform material, often ignoring the inherent biochemical differences between various cultivars (11). These varietal variations are not superficial; they fundamentally dictate how well an enzyme system is induced and how stable those enzymes remain during industrial catalysis (7,12). Furthermore, many studies focus only on the fermentation stage, leaving a technical "missing link" between the initial production and the rigorous molecular characterization required for real-world application (17).

The objective of our study was to address these challenges by exploring the potential of eight distinct banana (*Musa* spp.) cultivars as specialized feedstocks for *Aspergillus japonicus*. We present a comprehensive workflow that moves from proximate biochemical profiling to targeted protein purification via ammonium sulfate fractionation. By utilizing advanced molecular characterization—specifically sodium dodecyl sulfate–polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry—and validating the resulting secretome through apple juice processing and post-harvest peel softening assays, this research establishes a precise, varietal-specific framework for eco-efficient enzyme engineering.

Materials and Methods

Substrate Acquisition and Processing

Eight distinct banana (*Musa* spp.) cultivars were sourced from the Binni Mill Market in Bengaluru, India (Fig. 1 & Table 1). The peels were initially rinsed under running tap water to remove physical debris, followed by a secondary wash with distilled water to minimize surface microbial load. To preserve the biochemical integrity of the substrates, the samples were shade-dried at ambient temperature (28 °C) for a period of 5–7 days.

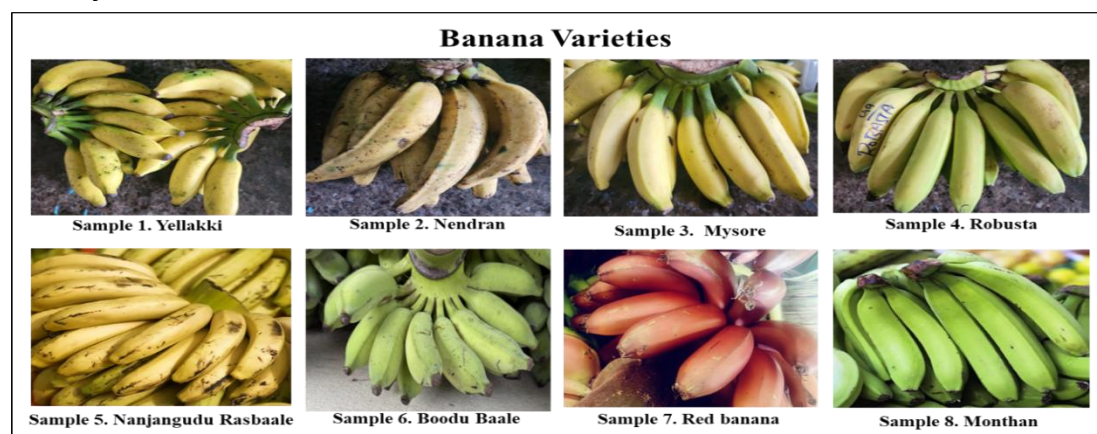


Fig. 1 Representative images of eight banana (*Musa* spp.) cultivars

Following the initial drying phase, the peels were oven-dried at 60 °C for 12 hours to achieve constant weight and ensure uniform dehydration. The desiccated material was subsequently pulverized into a fine powder using a sterile mechanical grinder under aseptic conditions. This varietal powder was stored in hermetically sealed containers at 4 °C for use in subsequent fermentation cycles. For analytical consistency, the cultivars were designated as Samples 1 through 8. This processing protocol was standardized based on the biorefinery framework established (25) to ensure the reproducibility of the agro-waste valorization process.

Table 1. Characteristics of Banana Peel Substrates Used for Enzyme Production

Sample ID	Common Name	Scientific Name	Genomic Group	ICAR–NRCB / NBPGR Accession Reference	Remarks
Sample 1	Elakkibale	<i>Musa acuminata Colla</i>	AB (Kunnan subgroup)	ICAR–NRCB Acc. No. 0113 (Elakkibale)	Indigenous Mysore-type variety; high sugar and volatile content; dessert banana (NRCB, 2020).
Sample 2	Nendran (Plantain)	<i>Musa paradisiaca L.</i>	AAB (Plantain subgroup)	ICAR–NRCB Acc. Nos. 266 & 268 (Nendran)	Kerala-origin dual-purpose banana; rich in starch and fiber; used for chips and cooking.
Sample 3	Mysore Bale (Poovan)	<i>Musa AAB (Mysore subgroup)</i>	AAB	ICAR–NRCB Acc. No. 0619 (Mysorebale)	Popular Indian cultivar; slightly acidic pulp; resistant to Sigatoka; used for both dessert and processing.
Sample 4	Robusta (Grand Naine)	<i>Musa acuminata Colla</i>	AAA (Cavendish subgroup)	ICAR–NRCB Acc. No. 0319 (Grand Naine)	Commercial clone of Cavendish group; high yield and shelf life; standard export cultivar.

Sample 5	Nanjangu du Rasabale	<i>Musa sp. var. Nrb (Local landrace)</i>	AAB (Silk subgroup)	ICAR–NRCB Acc. No. 0618 (Nanjagud rasabale); GI Reg. No. 28 (Govt. of India, 2005)	GI-tagged variety unique to Nanjangud, Karnataka; aromatic, delicate texture; confirmed in NRCB gene bank.
Sample 6	Boodu Bale	<i>Musa ABB (Monthan type)</i>	ABB (Cooking banana group)	Corresponds to NRCB “Boothibale / Ash Monthan,” Acc. Nos. 0584 & 2254	Regional ABB cultivar; high starch and fiber; firm pulp; suitable substrate for enzyme fermentation.
Sample 7	Red Banana (Chenkadali / Lal Velchi)	<i>Musa acuminata Colla</i>	AAA (Red Dacca subgroup)	ICAR–NRCB Acc. Nos. 0575 (Chenkadali) & 0161 (Red Dacca)	Distinct red-skinned AAA type; high carotenoid and antioxidant content; nutrient-rich dessert variety.
Sample 8	Monthan (Cooking Banana)	<i>Musa ABB group</i>	ABB (Monthan subgroup)	ICAR–NRCB Acc. No. 0046 (Monthan)	Traditional South Indian cooking banana; high lignin and starch; used as industrial substrate in bioprocessing.

Quantitative Estimation of Pectin

The pectin concentration across the eight banana peel varieties was determined gravimetrically using the calcium pectate method as described by Ranganna (27), with minor refinements. A 5 g aliquot of homogenized banana peel powder was subjected to sequential extraction using 30 mL of 0.01 N and 0.05 N HCl at 85–90 °C for 30 minutes to facilitate the solubilization of protopectin.

The resulting extract was filtered and hydrolyzed using 0.3 N HCl, followed by neutralization with 1 N NaOH. The mixture was subsequently acidified with 1 N acetic acid and treated with an equal volume of 1 N CaCl₂ to precipitate pectin as calcium pectate. After a 30-minute stabilization period, the precipitate was recovered via

filtration, washed extensively with distilled water to remove residual chlorides, and dried at 60 °C until a constant weight was achieved. The pectin content was quantified using the following expression:

$$\text{Pectin content (\%)} = \frac{(W_2 - W_1)}{W_3} \times 100$$

Where:

W_1 = Weight of empty filter paper (g)

W_2 = Weight of filter paper with dried calcium pectate (g)

W_3 = Weight of the fresh sample taken (g)

Quantitative Estimation of Cellulose

The cellulose content of the banana peel samples was quantified using the gravimetric–colourimetric method established by Updegraff (32), with specific modifications for agro-industrial residues. A 1 g sample of dried banana peel powder was treated with 10 mL of acetic–nitric reagent (prepared as 80% acetic acid and concentrated HNO₃ in a 10:1 ratio). The mixture was boiled at 100 °C for 30 minutes to facilitate the oxidative removal of lignin, hemicellulose, and other non-cellulosic constituents.

The remaining cellulosic residue was recovered via filtration through Whatman No. 1 filter paper and washed with distilled water until reaching neutrality. The purified cellulose was then subjected to acid hydrolysis using 67% H₂SO₄. The concentration of glucose in the resulting hydrolysate was determined via the Anthrone method, using commercial-grade cellulose to establish a standard calibration curve. Absorbance was recorded at 620 nm. The cellulose concentration was initially calculated in mg/g of dry weight (Eq. 1) and subsequently expressed as a percentage for comparative analysis (Eq. 2):

$$\text{Cellulose (mg/g)} = \frac{C \times V \times D}{W} \quad \text{Eq.1}$$

Where:

C = concentration from standard curve (mg/mL),

V = total extract volume (mL),

D = dilution factor,

W = weight of the dry sample (mg).

For comparative analysis, cellulose values were converted to percentage cellulose content using:

$$\text{Cellulose (\%)} = \frac{\text{Cellulose (mg/g dry wt)}}{10} \quad \text{Eq.2}$$

All analyses were conducted in triplicate, and data are reported as the mean ± standard deviation (SD).

Submerged Fermentation (smf) and Enzyme Production

Submerged fermentation (SmF) was employed to assess the cellulase and pectinase inductive potential of *Aspergillus japonicus* and *Aspergillus carbonarius*. Fermentation parameters—including pH, temperature, incubation duration, and substrate loading—were established through preliminary optimization and maintained rigorously across all trials to isolate the specific effects of banana cultivars.

The pre-inoculum was generated by transferring a 5 mm mycelial plug from an actively growing fungal colony into 25 mL of a specialized production medium. This medium utilized 2% (w/v) varietal banana peel powder as the primary carbon source, supplemented with a mineral salt foundation consisting of 0.2% 0.2% KH_2PO_4 , 0.6% K_2HPO_4 , and 0.01% MgSO_4 . The cultures were maintained at 30 °C on an orbital rotary shaker at 120 rpm for a 72-hour incubation period.

To evaluate varietal influence on the enzyme secretome, banana peel powders from the eight distinct cultivars (designated Samples 1–8) were utilized as independent substrates. This methodology for submerged enzyme induction and nutrient supplementation was adapted from the standardized protocols described by Kashyap *et al.* (14) and the foundational work on cellulase systems edited by Hajny and Reese (10). All experiments were executed in triplicate. Following incubation, the crude fermented broth was centrifuged at 10,000 rpm for 15 minutes, and the clear supernatant was harvested as the extracellular enzyme source for subsequent activity assays.

Protein Estimation and Specific Activity Determination

The protein concentration across the crude, ammonium sulfate-precipitated, and dialyzed enzyme fractions was quantified using the colorimetric method of Lowry *et al.*, (20). Bovine Serum Albumin (BSA) was utilized to construct a standard calibration curve (10–100 $\mu\text{g/mL}$). For the assay, a 1 mL aliquot of the diluted enzyme fraction was treated with 5 mL of alkaline copper reagent and allowed to react for 10 minutes. This was followed by the addition of 0.6 mL of Folin–Ciocalteu reagent. After a 30-minute incubation at room temperature to allow for full colour development, the absorbance was recorded at 660 nm.

The specific activity of the recovered pectinases and cellulases was determined to evaluate the efficiency of the purification process. It was calculated as the ratio of total enzyme activity to the total protein content using the following expression:

Specific Activity (U/mg) = Enzyme activity (U/mL) / Protein concentration (mg/mL)

In this study, one unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar (galacturonic acid for pectinase and glucose for cellulase) per minute under the specified assay conditions (22). All determinations were performed in triplicate, and results are expressed as the Mean $\pm$ SD ($N = 3$, $p < 0.05$).

Determination of Cellulase Activity

Cellulase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method (22). Briefly, 0.1 mL of the enzyme source (representing crude, ammonium sulfate-precipitated, and dialyzed fractions) was incubated with 5 mL of 0.1% (w/v)

carboxymethyl cellulose (CMC) at 50 °C for 30 minutes. The reaction was terminated, and colour development was initiated by adding 1 mL of DNS reagent, followed by immersion in a boiling water bath for 10 minutes. Upon cooling to ambient temperature, the resulting optical density was measured at 540 nm utilizing a spectrophotometer. All readings were corrected against appropriate substrate and enzyme blanks to account for background interference. The calculation of activity units and specific activity followed the parameters established in the section titled "Protein Estimation and Specific Activity Determination."

Determination of Pectinase Activity

Pectinase activity was quantified following the same colorimetric procedure described for cellulase, with 0.1% (w/v) citrus pectin utilized as the substrate in place of CMC.

Selection of Fungal Isolates for Purification

Aspergillus japonicus was selected for downstream purification and characterization based on its superior enzyme yields and specific activities across all banana peel varieties. Conversely, *Aspergillus carbonarius* was excluded from further processing due to its comparatively lower enzyme production and the reported risks of ochratoxin synthesis. This selection strategy ensures the safety and suitability of the purified enzymes for industrial applications and aligns with the safety standards required for the "production of fungi-derived lignocellulolytic enzymes using agricultural wastes" as discussed by Huang *et al.*, (13) in *Mycology*.

Enzyme Concentration and Recovery

Extracellular enzymes were concentrated and partially purified using ammonium sulfate precipitation, followed by dialysis to ensure catalytic stability and purity. Based on internal laboratory optimization trials and established research protocols for fungal hydrolases, a 50% saturation level (314 g L⁻¹) was applied directly to 200 mL of chilled culture filtrate. This uniform threshold ensured methodological consistency for an objective comparative analysis across the eight banana peel varieties.

Salt was added incrementally with continuous stirring at 4 °C, followed by a 12-hour equilibration period to facilitate maximal protein aggregation. The resulting precipitates were harvested via centrifugation (10,000 rpm, 15 min, 4 °C) and reconstituted in 0.1 M sodium acetate buffer (pH 5.0). To eliminate residual salts and low-molecular-weight contaminants, the fractions were dialyzed at 4 °C against the same buffer for 12–16 hours, with buffer changes every 4 hours. The final dialyzed extract was clarified (5,000 rpm, 10 min, 4 °C) and used immediately for biochemical assays.

This systematic recovery and laboratory-scale purification of active proteins follows the principles described by Scopes (29) in *Protein Purification*.

Electrophoretic Analysis and Silver Staining

The molecular profile and purity of *Aspergillus japonicus* enzyme fractions were analyzed via SDS-PAGE using a discontinuous buffer system (5% stacking and 10% resolving gels) according to Laemmli (18). Samples were diluted 1:10 in standard

reducing buffer (containing 2% SDS and 5% β -mercaptoethanol), denatured at 95 °C for 5 min, and loaded (25 μ L/well) alongside a 11–245 kDa prestained marker. Electrophoresis was performed at 80 V through the stacking gel and 120 V through the resolving gel.

Protein bands were visualized using silver staining: gels were fixed (50% methanol, 12% acetic acid), sensitised (0.02% $\text{Na}_2\text{S}_2\text{O}_3$), and impregnated with 0.1% AgNO_3 .

Development was carried out in 2% Na_2CO_3 with 0.04% formaldehyde, then quenched with 5% acetic acid. Molecular weights were estimated by calculating relative mobility (R_f) against the standard markers.

Mass Spectrometric Analysis (MALDI–TOF–MS)

To resolve the molecular architecture of the most efficient secretome, Sample 8 was selected as the representative candidate for MALDI–TOF–MS analysis. This targeted selection was justified by its superior enzymatic activity in quantitative assays and the high protein enrichment observed via SDS-PAGE.

The partially purified enzyme fraction was characterized at Dextrose Technologies Pvt. Ltd. (Bengaluru, India) following SOP: DTPL/MALDI/21-22/002a (34). The analysis utilized a Bruker Ultraflex extreme system in reflector mode, employing an alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix for optimal ionization of low-molecular-weight peptides (<20 kDa), as established in standard sample preparation protocols (31). A low-mass gate of 700 Da was implemented to eliminate matrix noise and ensure precise peak detection.

Evaluation of Industrial Applicability in Fruit Juice Extraction

The potential industrial utility of the partially purified *Aspergillus japonicus* enzyme preparations was assessed through a juice extraction and clarification study using fresh apple mash, following established protocols (24, 26). For each treatment, 15 g of homogenized apple mash was incubated with 0.5 mL of dialyzed enzyme extract (standardized to 5 U/mL) at 50 °C for 30 min under gentle agitation. Control groups were treated under identical conditions but without the addition of the enzyme extract.

Following incubation, the mash was filtered through a double-layered muslin cloth to recover the juice. The efficiency of the enzymatic treatment was evaluated based on the juice yield and clarification degree (2). The juice yield (%) was determined using the following equation:

$$\text{Juice Yield (\%)} = \frac{\text{Volume of filtrate (mL)}}{\text{Weight of mash (g)}} \times 100$$

Juice clarification was assessed by measuring turbidity spectrophotometrically at 660 nm, with a reduction in absorbance values signifying enhanced clarity. All trials were conducted in triplicate, and results are reported as the mean $\pm$ (SD).

Enzymatic Maceration Analysis of Banana Peels

The maceration-inducing potential of the partially purified enzyme extract from Sample 8 was evaluated, following its optimal performance in juice clarification trials. Slices of unripe banana peel (10 g) were treated with 0.5 mL of the enzyme extract and incubated at 30 °C for 72 hours. Control samples were processed under identical conditions using an equivalent volume of buffer in place of the enzyme. The progression of peel softening was monitored through a multi-parametric analysis of colour index, textural firmness, reducing sugar concentration, pectin content, and moisture levels.

Colour Index Normalization

Fruit colouration was assessed using the seven-point visual scale established by Von Loesecke (13) and further detailed for post-harvest screening by Dadzie and Orchard (3). In this system, Stage 1 represents "all green" and Stage 7 represents "mostly brown." To maintain consistency with established maceration benchmarks, visual observations initially recorded on a 0–10 scale were normalized to the standard 1–7 scale using a linear proportional transformation, where a multiplier of 6 was employed to correspond with the six-unit interval defining the target peel softening stages:

$$\text{Normalized value (1 – 7)} = 1 + \left(\frac{\text{Original value (0–10)}}{10} \times 6 \right)$$

By applying this transformation, an experimental score of 8.7 (0–10 scale) was converted to 6.2, aligning with the "Yellow with brown flecks" stage (Stage 6) of the standardized index.

Physicochemical Characterization

Peel firmness was quantified using a penetrometer to assess structural softening. Biochemical changes were monitored by measuring reducing sugars via the dinitrosalicylic acid (DNS) method (22) and total pectin content through the calcium pectate gravimetric technique (27). Moisture content was determined gravimetrically by drying the samples to a constant weight. All measurements were conducted in triplicate, and data are presented as the mean ± standard deviation (SD).

Results and Discussion

Proximate Composition of Banana Peel Substrates

The efficiency of fungal enzyme synthesis is largely governed by the specific carbohydrate architecture of the biomass used. In this study, the biochemical profiles of eight banana peel varieties were evaluated to determine their potential as inductive substrates (Fig. 2 & Fig. 3).

Cellulose Content

Cellulose levels varied significantly across the eight banana peel varieties ($p < 0.05$; Fig. 2). Sample 8 yielded the highest concentration at 22.5% (Group a), followed by Sample 7 at 19.07% (Group b), providing the necessary carbohydrate framework to

trigger the fungal cellulolytic machinery (21). Intermediate levels were recorded in Samples 2 and 6 (~18.1–18.3%; Group c), while Samples 3 and 4 formed a distinct moderate cluster (~16.3–16.8%; Group d). The lowest concentrations were found in Samples 1 and 5 (~16.1%; Group e).

Tukey's post-hoc analysis categorized the cultivars into five distinct statistical tiers (a–e), highlighting the significant impact of genotypic diversity on cell wall architecture. This chemical variability confirms that banana peel composition is strictly dependent on the specific variety utilized (11). From a biotechnological standpoint, these proximate values provide the foundational baseline necessary to interpret the performance of the resulting multi-enzyme secretome.

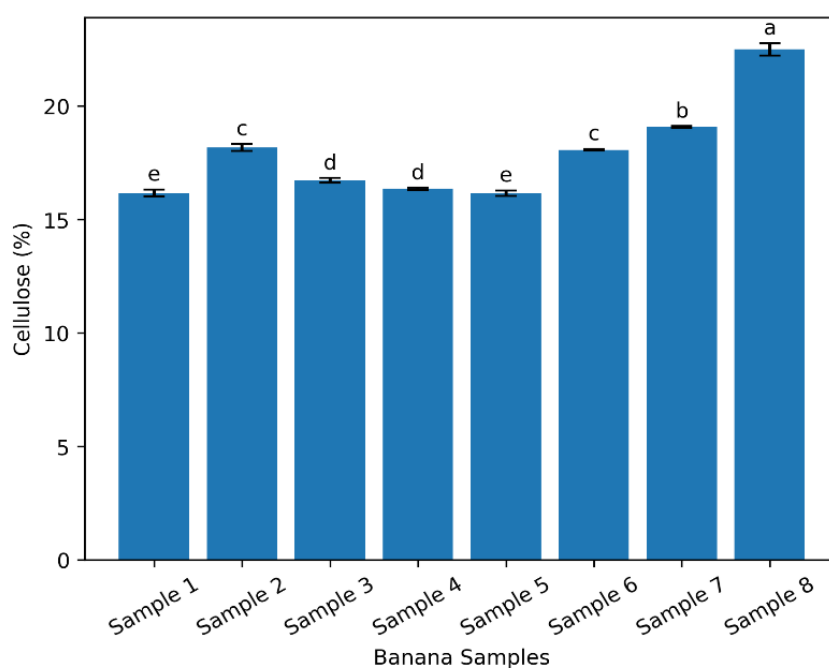


Fig. 2. Comparative assessment of cellulose levels (%) across eight banana peel cultivars. Values are expressed as mean $\pm$ standard deviation ($n=3$). Distinct lowercase letters (a–e) indicate statistical significance between varieties as determined by one-way ANOVA and Tukey's HSD post-hoc analysis ($p < 0.05$).

Pectin Content

The concentration of pectin exhibited a highly diverse profile, with nearly every variety falling into a unique statistical category ($p < 0.05$; Fig. 3) and Tukey groups (a–h), highlighting the complex relationship between substrate architecture and fungal secretome expression (11). Sample 2 emerged as the most pectin-rich substrate at 9.4% (Group a), significantly outpacing Sample 6 (6.7%, Group b) and Sample 1 (6.3%, Group c). Moderate levels were detected in Sample 4 (~5.1%, Group d) and Sample 3 (~4.7%, Group e). Conversely, pectin content diminished in Sample 8 (4.3%, Group f) and Sample 7 (2.8%, Group g), with Sample 5 yielding the minimum at 1.4% (Group h). Since the substrate used is a complex natural matrix rather than a refined commercial

standard, these results establish the intrinsic inductive potential of each variety. While future industrial benchmarking is planned to standardise these native isolates against commercial preparations (e.g., Pectinex), the current data validate the effectiveness of heterogeneous agro-waste as a baseline for enzyme induction.

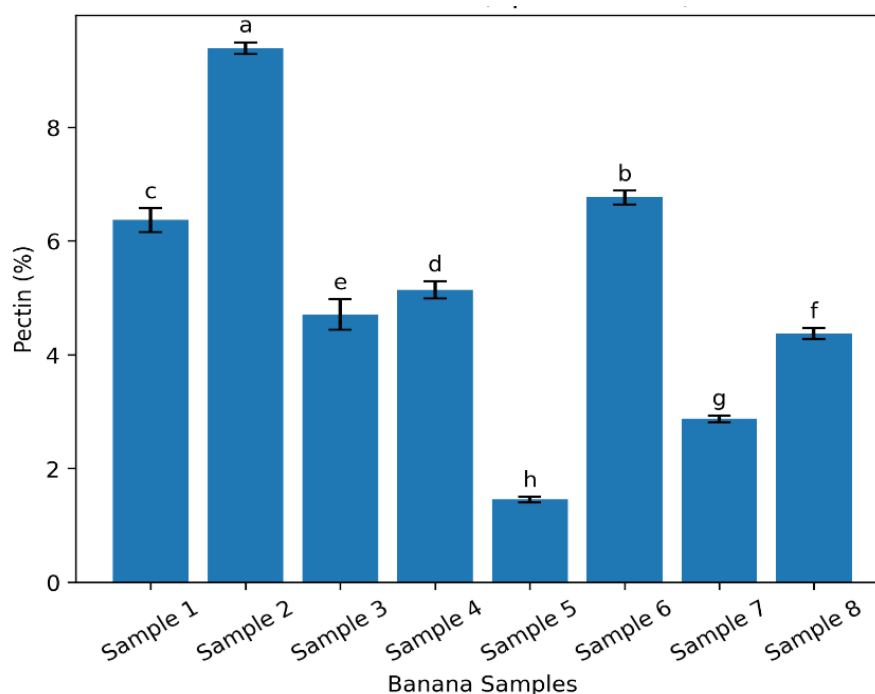


Fig 3: Comparative assessment of pectin levels (%) across eight banana peel cultivars. Results are presented as mean $\pm$ standard deviation ($n=3$). Unique lowercase letters (a–h) denote significant statistical variance between varieties (one-way ANOVA; Tukey’s HSD; $p < 0.05$).

Enzyme Production by *Aspergillus japonicus*

Submerged fermentation (SmF) utilizing banana peel as the primary carbon source facilitated robust enzyme synthesis by *Aspergillus japonicus*, though significant performance fluctuations were noted across the eight varieties (Fig. 4). Enhanced mycelial proliferation and accelerated substrate degradation were prominently observed in Samples 1, 2, 3, 7, and 8, suggesting more potent enzyme induction and elevated metabolic activity within these specific lignocellulosic environments.

Conversely, Samples 4, 5, and 6 demonstrated relatively diminished biomass accumulation and protracted substrate utilization rates. These variations emphasize the critical role of specific substrate constituents—such as the cellulose and pectin profiles established in the preceding analysis—in modulating fungal metabolic pathways and the subsequent secretion of hydrolases. The successful bioconversion observed across these samples reinforces the viability of banana peel as a sustainable and economically efficient substrate for industrial enzyme biosynthesis (5, 8)

Fig 4: Visual assessment of *Aspergillus japonicus* growth and substrate bioconversion in submerged fermentation. Illustrates the comparative mycelial density and degradation of banana peel particles across eight varieties (Samples 1–8) compared to a control flask. Varied levels of turbidity and biomass accumulation indicate the differential inductive potential of the peel substrates.



Comparative Enzyme Production Analysis

The comparative evaluation of pectinase and cellulase secretion by *Aspergillus japonicus* and *Aspergillus carbonarius* under submerged fermentation (SmF) revealed distinct variations across both banana peel substrates and fungal species. In all instances, *A. japonicus* demonstrated a significantly higher enzymatic output compared to *A. carbonarius*, underscoring its superior hydrolytic efficiency and stronger regulation of cell-wall-degrading enzyme systems. A two-way ANOVA confirmed that both the banana peel variety and the fungal species, as well as their interaction, significantly influenced enzyme titers ($p < 0.05$; (Fig. 5A–B).

The production of both enzymes was highly variety-dependent, reaching its peak in Sample 8, which yielded approximately 0.20 U/mL for pectinase and 0.38 U/mL for cellulase, followed closely by Sample 2. The use of fungi as the primary drivers of "lignocellulose degradation" (1) highlights the specific enzymatic mechanisms required to break down complex agro-waste. Such waste valorization is a critical component of a "multiproduct biorefinery" as discussed by Saini & Sharma (28), where fungal lignocellulolytic enzymes play a central role in converting biomass into value-added products. By repurposing these materials as a "functional ingredient" for microbial fermentation, this approach offers a "green sustainable insight for waste management" (6) that aligns with circular economy goals.

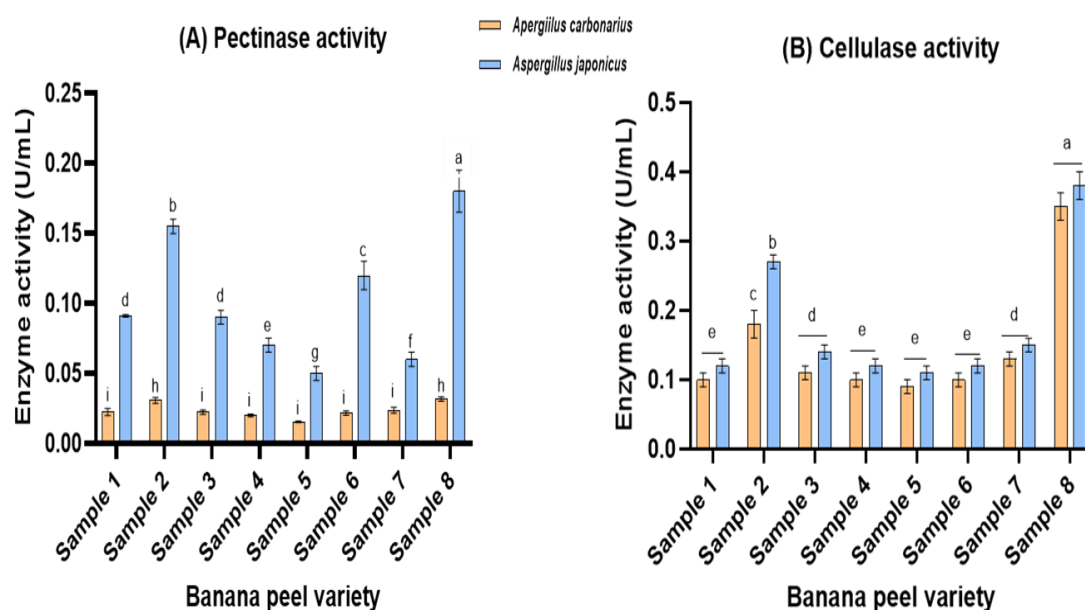


Fig. 5. Comparative (A) Pectinase and (B) Cellulase activity of *Aspergillus japonicus* and *Aspergillus carbonarius* using eight banana peel varieties. Values represent the mean $\pm$ SD (n=3). Different lowercase letters denote statistically significant differences between samples and strains determined by two-way ANOVA followed by Tukey's HSD post-hoc test ($p < 0.05$).

Enzyme Purification Efficiency and Catalytic Enhancement

The transition from crude extract to dialyzed fraction resulted in a significant enhancement of catalytic performance, with pectinase and cellulase activities increasing 1.5–3.5-fold across all banana peel varieties (Fig. 6). This marked improvement is attributed to the systematic removal of low-molecular-weight inhibitory metabolites and residual salts during the dialysis process. Analysis confirmed that the 50% saturation level successfully targeted medium-molecular-weight fungal hydrolases while minimizing the risk of non-specific protein aggregation typically observed at higher salt concentrations.

This selective recovery approach is consistent with findings by Yadav and Shastri (35), who noted that precise ammonium sulfate fractionation is vital for maintaining the properties of extracellular pectinases during purification. Furthermore, the successful enrichment of these enzymes aligns with the innovations in fungal pectinase production and industrial application described by Dwivedi *et al.*, (6). Samples 2, 6, and 8 demonstrated the highest pectinase recovery, while Samples 2, 4, 7, and 8 exhibited the most robust cellulase activity.

Overall, the purification strategy substantially strengthened enzyme activity, demonstrating the suitability of ammonium precipitation and dialyzed extracts for downstream biochemical and industrial applications.

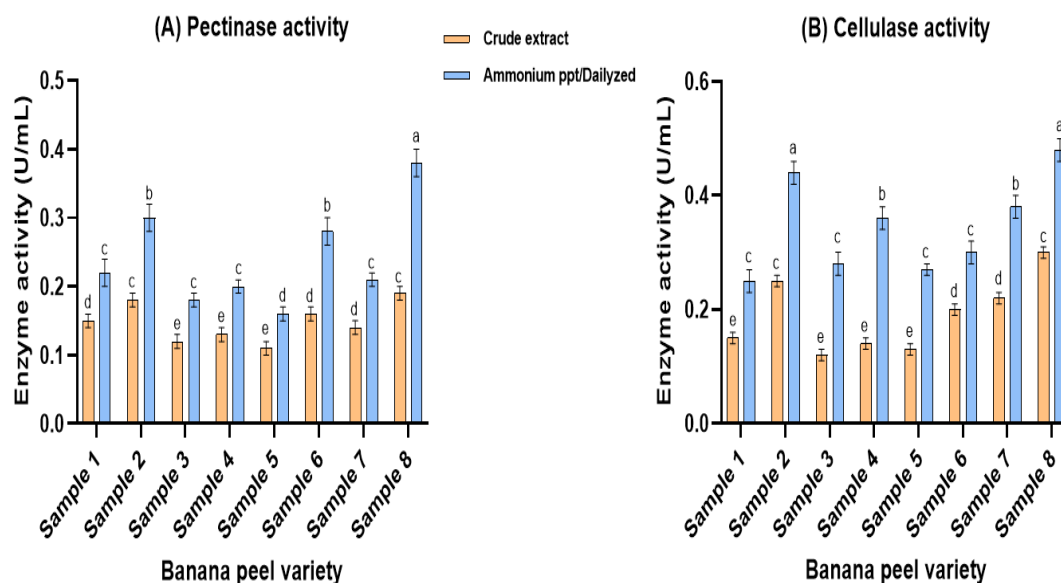


Fig 6. Comparative enzyme activity across banana peel varieties assessment of (A) Pectinase and (B) Cellulase activities in crude versus purified extracts from *Aspergillus japonicus*. Different lowercase letters (a–e) indicate significant differences ($p < 0.05$) between varieties.

Protein Profiling via SDS–PAGE and Silver Staining

The analysis revealed a multifaceted protein profile characterized by distinct bands spanning the 35–100 kDa range (Fig. 7). Considering the partial purification status of the extract, this distribution likely represents a consortium of co-secreted proteins rather than isolated isoenzymes. Banana peel is a rich, heterogeneous lignocellulosic substrate; its degradation typically requires a synergistic array of enzymes, potentially including cellulases, pectinases, and hemicellulases.

The observed molecular weight distribution is consistent with established literature regarding the secretomes of *Aspergillus* species when induced by agricultural residues. Research by De Vries and Visser (5) highlights that *Aspergillus* produces a vast repertoire of enzymes specifically tailored to dismantle plant cell wall polysaccharides, many of which fall within this kilodalton range. Furthermore, Matsuzawa (30) emphasizes that the expression of these degradation-related enzymes is highly adaptive, responding to the specific complexity of the carbohydrate matrix provided.

Notably, the increased band intensity in Sample 8 corresponds with the superior enzyme activities measured (Fig 5 & 6), suggesting a higher concentration of the targeted hydrolases in these specific samples. These results underscore the capacity of *A. japonicus* to express a diverse extracellular enzyme system tailored for the breakdown of complex polysaccharide matrices.

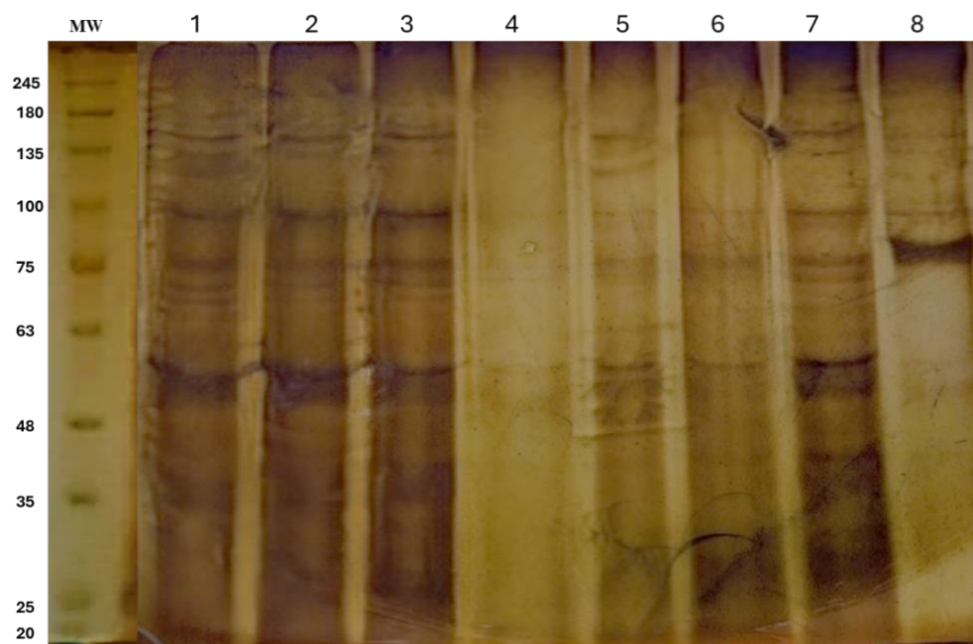


Fig 7: Protein profiling of *Aspergillus japonicus* extracts across eight banana peel varieties. **Lane M:** Molecular weight marker; **Lanes 1–8:** Dialyzed fractions from respective banana peel substrates.

Maldi–Tof–Ms Characterization

The MALDI–TOF–MS spectrum of Sample 8 exhibited dominant peaks at m/z 12,594.6, 76,096.2, and 86,244.2 (Fig. 8). Interestingly, the persistence of high-molecular-weight species in the 76–86 kDa range, despite the application of tryptic digestion protocols, indicates a high degree of structural stability within the *A. japonicus* enzyme complex.

This resistance to proteolysis likely stems from extensive post-translational modifications, such as glycosylation, which are common in fungal glycosyl hydrolases and can sterically hinder tryptic cleavage sites (19). The 12.6 kDa peak may represent a successfully cleaved auxiliary protein or a stable catalytic domain fragment. The presence of these intact-mass signals suggests that the enzymes induced by the banana peel substrate possess a robust molecular architecture, a characteristic that is highly advantageous for industrial applications involving lignocellulosic biomass degradation where enzyme longevity and stability are critical.

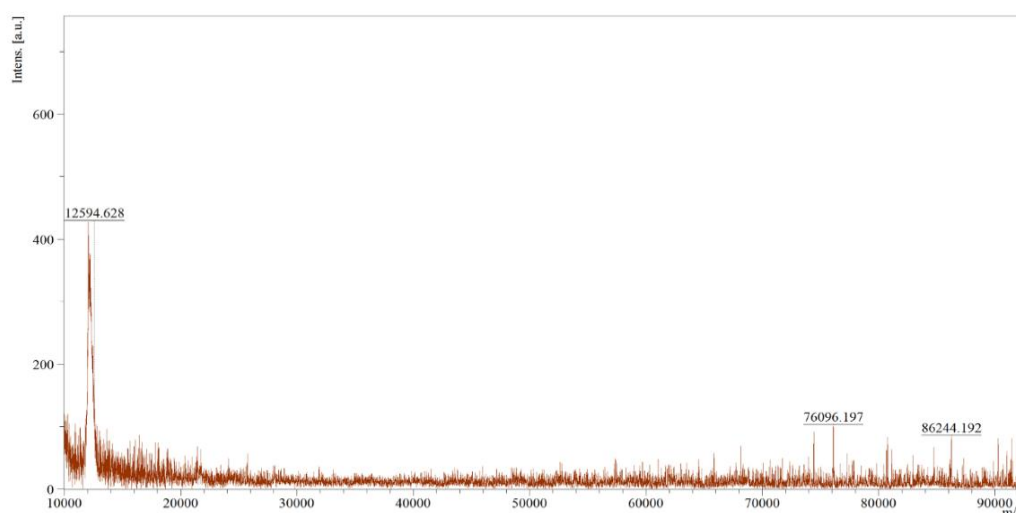


Fig 8. MALDI–TOF–MS spectrum of the partially purified enzyme extract from Sample 8 (Bruker Ultraflexxtreme MALDI–TOF/TOF MS, CHCA matrix).

Enzyme Application Studies in Apple Fruit Juice Clarification

The application of enzyme extracts derived from eight banana peel varieties significantly enhanced the clarification of apple mash, demonstrating a clear inverse relationship between juice recovery and residual turbidity. The cellulase-treated mash recorded the highest yield ($40 \pm 1.2\%$), confirming that cellulolytic activity is a primary driver in the degradation of the apple tissue matrix.

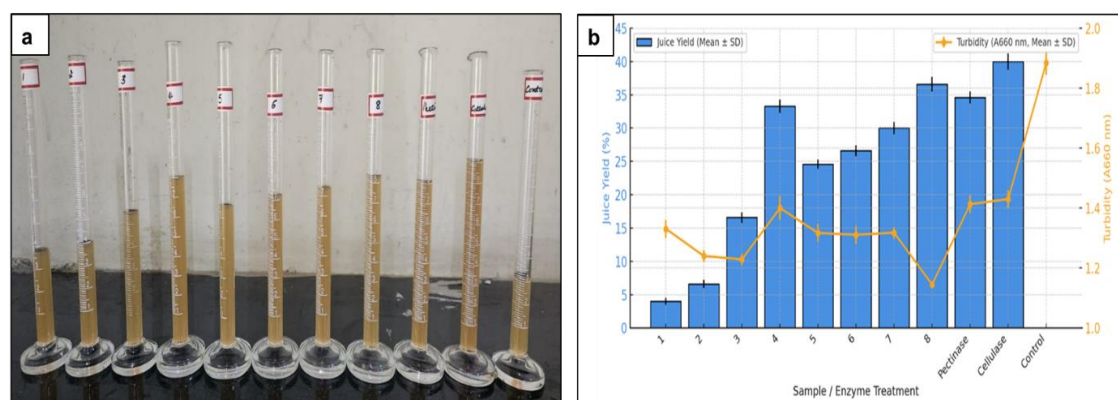
The control treatment produced minimal juice recovery and the highest recorded turbidity (1.885 ± 0.04), highlighting the necessity of enzymatic intervention for effective extraction. Among the tested varieties, Sample 8 achieved the lowest turbidity (1.145 ± 0.01) with a competitive yield of $36.6 \pm 1.1\%$.

This performance is attributed to the high cellulose-to-pectin ratio characteristic of apple fruit, where cellulase is essential for the hydrolysis of the primary cell wall. Because the enzymes were derived from complex banana peel substrates, the observed effects likely reflect the complementary interaction of a multi-enzyme system—including co-secreted hydrolases—rather than isolated activities.

While these results align with Kc *et al.*, (15) and Mwaheb *et al.*, (23) regarding the utility of fungal hydrolases for juice recovery, it is important to note that this study establishes baseline biocatalytic efficacy using a water control. Future investigations incorporating heat-inactivated controls and standardized commercial benchmarks (e.g., Pectinex) will further clarify the market competitiveness and non-enzymatic contributions within this sustainable, low-cost secretome.

Fig 9. Enzyme application studies in apple fruit juice clarification

(a) Visual comparison of clarified apple juice following enzyme treatment.



(b) Impact of enzyme treatment on juice yield (%) and turbidity (A₆₆₀ nm). Data points represent the mean ± SD (n=3), illustrating the inverse relationship between increased yield and reduced turbidity, particularly in Sample 8.

Enzymatic Maceration and Peel Softening Analysis

The application of Sample 8 enzyme extract to banana peels resulted in significant structural modifications, characterized by accelerated softening and biochemical changes compared to the control (Table 2). This specific extract was selected for its superior performance in apple mash clarification, where it demonstrated high yield and low turbidity—a result of the complementary action of its pectinase and cellulase components. Peel softening was evaluated using the Von Loesecke (33) colour index (Fig. 10), where enzyme-treated peels reached stage 6–7 (yellow to mostly brown), while the control remained at stage 4–5 (greenish yellow). Treated peels showed reduced firmness (6.2 ± 0.3 N), and higher reducing sugars (29.1 ± 0.3 mg/g), and lower pectin content (3.1 ± 0.2 mg/g), confirming enzymatic degradation of cell wall polysaccharides. Increased moisture content ($78.4 \pm 0.6\%$) indicated enhanced cell permeability.

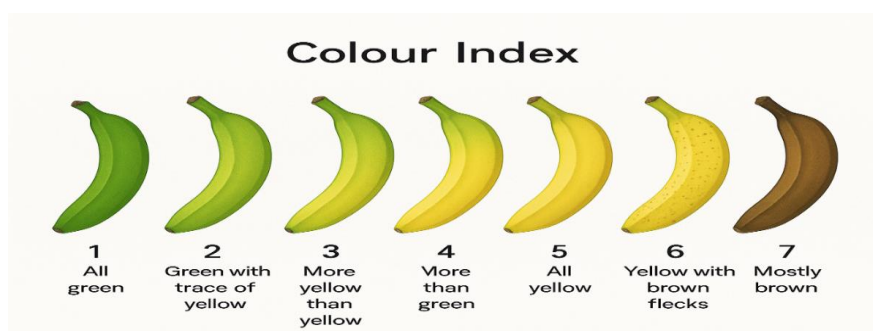


Fig 10. Comparative softening stages of banana peels correspond to Von Loesecke stages 1–7.

Table 2: Banana peel softening analysis using Sample 8 enzyme extract

Sample	Color Index (0–10)	Firmness (N)	Reducing Sugar (mg/g)	Pectin Content (mg/g)	Moisture Content (%)
Sample 1	8.7 ± 0.3	6.5 ± 0.4	28.4 ± 1.2	3.2 ± 0.2	78.1 ± 0.8
Sample 2	8.5 ± 0.2	6.8 ± 0.3	27.6 ± 1.1	3.4 ± 0.3	77.4 ± 0.7
Sample 3	7.9 ± 0.4	7.3 ± 0.5	24.8 ± 1.3	3.9 ± 0.2	76.8 ± 0.9
Sample 4	6.6 ± 0.3	8.5 ± 0.3	21.5 ± 1.3	4.6 ± 0.4	74.5 ± 1.0
Sample 5	6.4 ± 0.4	8.7 ± 0.4	20.8 ± 1.2	4.8 ± 0.3	74.2 ± 0.8
Sample 6	6.8 ± 0.5	8.1 ± 0.4	22.4 ± 1.0	4.5 ± 0.2	75.0 ± 0.9
Sample 7	8.3 ± 0.2	7.0 ± 0.3	26.7 ± 1.4	3.6 ± 0.2	77.9 ± 0.7
Sample 8	8.9 ± 0.3	6.2 ± 0.3	29.1 ± 0.3	3.1 ± 0.2	78.4 ± 0.6
Control (Buffer)	5.2 ± 0.3	10.2 ± 0.4	17.8 ± 1.2	5.1 ± 0.3	73.1 ± 1.0

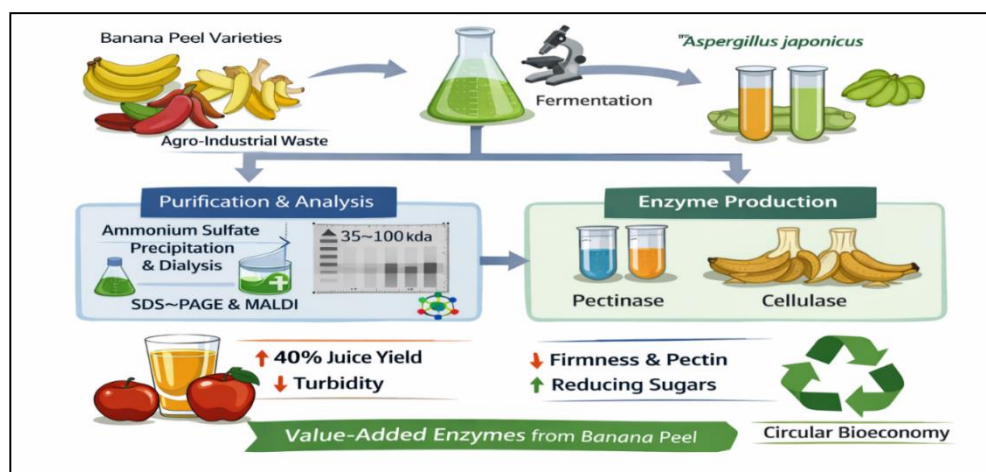
Conclusion

This study validates the potential of banana peel as a sustainable, cost-effective feedstock for inducing a robust extracellular enzyme system in *Aspergillus japonicus*. Our findings demonstrate that the specific biochemical architecture of different banana cultivars significantly dictates enzyme output, with Samples 2 and 8 emerging as the most effective inductive substrates.

The integration of ammonium sulfate precipitation and dialysis successfully enriched the catalytically active fraction, yielding a 1.5–3.5-fold increase in activity. While molecular profiling via SDS–PAGE and MALDI–TOF–MS revealed a heterogeneous suite of co-expressed hydrolases within the 35–100 kDa range, this diversity underscores the metabolic versatility of *A. japonicus* in deconstructing complex plant cell wall matrices. From a functional perspective, these preparations showed substantial industrial promise, significantly enhancing juice recovery in apple mash and accelerating the maceration of fruit tissues through targeted pectocellulosic breakdown.

As these results are derived from a complex, non-refined biomass, they establish a fundamental performance baseline for native fungal secretomes. While this research focuses on the intrinsic biocatalytic capacity of the isolates, future industrial standardization—incorporating heat-inactivated controls and commercial benchmarks—will further refine their market applicability. Ultimately, this work highlights a viable pathway for transforming agro-industrial waste into high-value biochemical tools, aligning with the core objectives of a circular bioeconomy.

Fig 12. Overview of the study illustrating enzyme production from banana peel waste by *Aspergillus* species, purification, characterization, and its successful application in apple juice clarification and banana peel softening.



Acknowledgment

All authors gratefully acknowledge Bangalore University for its continued encouragement of our research activities and for providing the necessary laboratory facilities and infrastructure support. The authors declare that no external financial grants or industrial affiliations supported this work.

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