

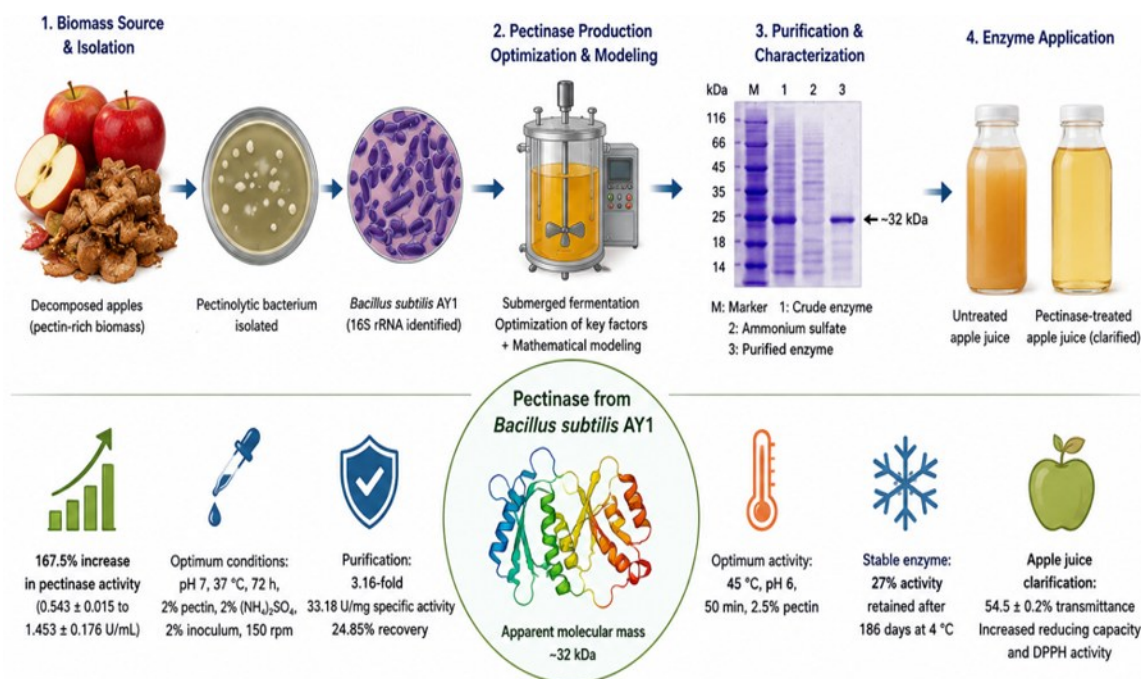
Isolation of *Bacillus subtilis* AY1 and Production, Characterization, Modeling, and Application of Pectinase for Apple Juice Clarification

Emad Abada ^{a,b,*}, Amjad Alzhrani,^a Norah S. Alothman ^a, Rasha M. Alzayed ^c, Sondos A. Alhajouj ^c, Shifaa O. Alshammari ^d, Khatib Sayeed Ismail ^a, Hanadi A. Alasiri ^a, Rania A Henawi ^a, and Ibrahim Y. Y. Sumaily ^{a,*}

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GRAPHICAL ABSTRACT



Isolation of *Bacillus subtilis* AY1 and Production, Characterization, Modeling, and Application of Pectinase for Apple Juice Clarification

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This study isolated a pectinase-producing bacterium from decomposed apple, optimized enzyme production, characterized the preparation, modelled responses, and tested its effect on apple juice clarification. 16S rRNA sequencing identified the isolate as *Bacillus subtilis* AY1. Optimization raised pectinase activity from 0.543 ± 0.015 to 1.453 ± 0.176 U/mL (167.5%). Ammonium sulfate precipitation and Sephadex G-100 chromatography yielded a preparation with specific activity of 33.18 U/mg, 3.16-fold purification, and 24.9% recovery. SDS-PAGE showed a prominent protein band at an apparent molecular mass of approximately 32 kDa. Maximum activity occurred at 45 °C and pH 6, with a 50-min reaction and 2.5% pectin. Quadratic models described responses in the one-factor-at-a-time production experiments but could not assess interactions among production variables. The temperature–time model showed a significant interaction for thermal stability. The enzyme retained 27% activity after 186 days at 4 °C. In apple juice, transmittance rose from $20.0 \pm 0.2\%$ to $54.5 \pm 0.2\%$. Reducing capacity increased by 30.5%, and DPPH scavenging activity by 15.9%. These laboratory-scale results indicate that AY1 pectinase improved juice clarification under tested conditions. Further validation, process development, and scale-up are needed before industrial application can be assessed.

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Keywords: *Bacillus subtilis*; Pectinase; Enzyme characterization; Mathematical modeling; Apple juice clarification

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INTRODUCTION

Apple-processing residues contain pectin and can provide inexpensive material for microbial pectinase production (Suryoprabowo *et al.* 2026; Nargotra *et al.* 2024). Pectin is a major component of fruit cell walls, and pectinases break down its complex structure. These enzymes are particularly useful in fruit-juice extraction and clarification (Haile and Ayele 2022). Microorganisms can produce pectinases on pectin-containing materials such as apple pomace and other fruit residues (Sharma *et al.* 2026).

Among bacterial producers, *Bacillus* species are promising sources of pectinases because many secrete extracellular enzymes, grow rapidly, and can be cultivated in

submerged fermentation (Kaissar *et al.* 2025). Pectinase production and characterization have been reported for *Bacillus* strains isolated from fruit wastes, decayed fruits, agricultural environments, and rhizospheric soils (Kh *et al.* 2022; Ja'afaru *et al.* 2025). Perveen *et al.* (2024), for example, reported extracellular pectinase production by *Bacillus licheniformis* using fruit-derived materials. Dhvani and Hardik (2024) also isolated pectinolytic bacteria from environmental and fruit-waste sources and examined production conditions and potential applications of a *Bacillus inaquosorum* pectinase.

Pectinase yields nevertheless depend on the strain and cultivation conditions, including temperature, pH, fermentation period, aeration, inoculum density, substrate concentration, nitrogen supply, and mineral composition (Zhang *et al.* 2026). Changing one factor at a time can identify conditions associated with higher production while the other factors are held constant. It cannot, however, measure interactions among variables or establish a comprehensive multivariable optimum. Empirical models fitted with such individual-factor experiments describe the observed trends within the tested ranges, but their predictions require further validation under combined conditions. Enzyme stability and translation to industrial use also remain important challenges for *Bacillus* pectinases (Sangeetha and Jagtap 2026).

Previous studies have examined different parts of the pectinase development pathway, including microbial isolation, production, purification, enzyme characterization, and juice clarification (Haile *et al.* 2022). Although *Bacillus* pectinases are well documented, the production behavior, biochemical properties, stability, and performance in apple juice clarification of the newly investigated *Bacillus subtilis* AY1 isolate have not yet been examined together. Assessing these features may show where this enzyme could be useful without assuming that it is inherently different from previously reported *Bacillus* pectinases. The joint effects of temperature and exposure time on residual activity can also be examined separately from the individual-factor experiments used to assess enzyme production.

As apples decompose, their pectin-rich tissues become accessible to resident microorganisms. In this work, decomposed apples were selected as an isolation source because bacteria growing on this material may include strains able to degrade pectin. Fruit and vegetable waste can also serve as low-cost fermentation substrates in other settings (Bhatt *et al.* 2024). Here, however, the decomposed apple material supplied the microorganism for isolation, whereas enzyme production was examined subsequently in the fermentation medium. Accordingly, this study isolated the pectinase-producing *Bacillus subtilis* AY1 from decomposed apple material and examined its extracellular enzyme production under selected cultivation conditions. The study combined individual-factor production experiments and empirical modeling with purification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), biochemical characterization, assessment of refrigerated stability, and apple juice clarification. A separate temperature-time response-surface analysis was used to examine residual enzyme activity after heat exposure. Taken together, these experiments characterize pectinase from the AY1 isolate and assess its potential usefulness in apple juice clarification.

EXPERIMENTAL

Collection of Apple Samples

Damaged and decomposed apples were collected from supermarkets, fruit markets, and restaurants in the Jazan region, Saudi Arabia. Samples were transferred aseptically into sterile bags, transported under cooled conditions, and processed within 3 h of collection for isolation of pectinolytic microorganisms (Vásquez and Millones 2023).

Isolation of Pectinolytic Bacteria

One gram of decomposed apple tissue was suspended in 10 mL of sterile distilled water and serially diluted. Appropriate dilutions were cultured on nutrient agar supplemented with 1% citrus pectin at 30 °C for 72 h, and morphologically distinct colonies were repeatedly subcultured to obtain pure isolates (Haile *et al.* 2022).

Screening for Pectinase Activity

Pure isolates were inoculated onto pectin-screening agar and incubated at 30 °C for 48 h. Plates were flooded with iodine solution, and isolates producing clear hydrolysis zones were considered pectinolytic; the isolate showing the greatest activity was selected for subsequent experiments (Ouedraogo *et al.* 2026).

Gram Staining and Morphological Characterization

The selected bacterial isolate was characterized using Gram staining and microscopic examination. Heat-fixed cells were sequentially treated with crystal violet, iodine, decolorizer, and safranin, and cellular morphology and Gram reaction were recorded (Al-Talib *et al.* 2026).

Molecular Identification

Genomic DNA from the selected isolate was used to amplify the nearly full-length 16S rRNA gene with primers 27F and 1492R. PCR was performed at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 90 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The purified amplicon was sequenced and searched against the NCBI nucleotide database using (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignment can be viewed with the (<https://www.ncbi.nlm.nih.gov/projects/msaviewer/>) (Ghazouani *et al.* 2020).

Extracellular Pectinase Production

Bacillus subtilis AY1 was first grown in nutrient broth. A 2% (v/v) inoculum, adjusted to OD₆₀₀ = 0.5, was transferred to 50 mL of pectin-containing production medium in 100 ml flask. The medium contained 2% (w/v) pectin, 2% (w/v) ammonium sulfate, and was adjusted to pH 7. Cultures were incubated under submerged conditions at 30 °C and 150 rpm for 72 h. After fermentation, cultures were centrifuged at 8000 r.p.m at 4 °C. The cell-free supernatant was collected and used as the crude extracellular pectinase preparation (Ogbebor and Oshoma 2026).

Pectinase Activity Assay

Pectinase activity was measured by the DNS method. The reaction contained 500 µL of enzyme, 500 µL of 1% citrus pectin, and 500 µL of 0.1 M acetate buffer (pH 5). After incubation at 40 °C for 15 min, 1 mL of DNS reagent was added. The mixture was

heated at 100 °C for 10 min, cooled, and its absorbance measured at 540 nm. Reducing sugars were quantified against a glucose standard curve. One unit (U) was defined as the amount of enzyme releasing 1 μ mol of glucose equivalents per minute under these assay conditions; activity was expressed as U/mL (Sukmawati *et al.* 2025).

Protein Content Estimation

Protein concentration was determined colorimetrically using bovine serum albumin as the calibration standard, and the resulting protein values were used to calculate pectinase specific activity as U/mg protein (Kanzhigitova *et al.* 2025).

Optimization of Pectinase Production

Pectinase production was optimized using a one-factor-at-a-time approach (OFAT). Temperature (20 to 50 °C), pH (4 to 10), incubation time (24 to 144 h), agitation speed (0 to 300 rpm), inoculum size (0.5 to 4%), pectin concentration (0.5 to 4%), and ammonium sulfate concentration (0.2 to 3%) were tested, along with different carbon and nitrogen sources and metal ions. Each condition was evaluated in triplicate (Zhu *et al.* 2026).

Validation of Optimized Production Conditions

The selected conditions were combined using 2% pectin, 2% ammonium sulfate, selected K and Mg salts, 2% inoculum, pH 7, 37 °C, 72 h, and 150 rpm. Pectinase production under these combined conditions was experimentally compared with production under the initial cultivation conditions (Revathy *et al.* 2025).

Purification of Pectinase

The crude enzyme was subjected to 40 to 60% ammonium sulfate precipitation, followed by dialysis and Sephadex G-100 gel-filtration chromatography. Total activity, total protein, specific activity, purification fold, and activity yield were determined after each purification step (Nair and Devi 2025).

SDS-PAGE Analysis

Crude supernatant, ammonium sulfate fraction, and Sephadex G-100-purified enzyme were analyzed by SDS-PAGE together with molecular-weight protein standards. The apparent molecular mass of the enriched pectinase band was estimated from its electrophoretic migration relative to the marker proteins (Tikadar *et al.* 2026).

Biochemical Characterization of Purified Pectinase

Purified pectinase was evaluated at reaction temperatures of 20 to 100 °C, pH 4 to 10, reaction times of 10 to 60 min, and pectin concentrations of 0.5 to 4%. The effects of 0.5 mM AlCl_3 , CaCl_2 , FeCl_3 , KCl, MgCl_2 , MnCl_2 , NaCl, and NH_4Cl were also determined relative to the control (Oliveira *et al.* 2026).

Thermal Stability

The thermal stability of purified pectinase was evaluated at 0, 15, 30, 45, 60, 75, 90, 105, and 120 °C for 40 to 80 min. Treatments up to 90 °C were carried out in a thermostatically controlled water bath, while 105 and 120 °C treatments were performed in tightly sealed, heat-resistant vials to minimize evaporation. After heating, the samples

were cooled to room temperature, and residual enzyme activity was measured using the standard assay. Activity before heat treat (Mohammed *et al.* 2025).

Shelf Stability

Purified pectinase was maintained in phosphate buffer (pH 7) at 4 °C for up to 186 days. Residual activity at the experimentally assessed storage points was expressed as a percentage of the initial enzyme activity, following the general approach used for stability evaluation of microbial pectinases (Dhayalan *et al.* 2026).

Apple Juice Clarification

Purified pectinase was applied to apple juice, while untreated juice served as the control. Clarification was assessed spectrophotometrically (UV-1800, Shimadzu Corporation, Kyoto, Japan). and expressed as percentage transmittance (%T), with three measurements obtained for the enzyme-treated juice (Kereszturi *et al.* 2025).

Antioxidant Properties of Pectinase-treated Apple Juice

Reducing capacity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity were determined in untreated and pectinase-treated apple juice in triplicate. Reducing capacity was expressed as mg/L and DPPH scavenging as percentage inhibition to evaluate changes in antioxidant properties after enzymatic treatment (Li *et al.* 2025).

Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft Corporation, Version 2021, Redmond, WA, USA) and Python (SciPy). Experimental results were expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Multi-level datasets were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test, thermal-stability data by two-way ANOVA, and two-group comparisons by Welch's t-test; statistical significance was accepted at $p < 0.05$ (Pankaj *et al.* 2025).

Empirical Mathematical Modeling of Pectinase Production

To quantitatively describe the relationship between individual cultivation variables and pectinase production, the experimental data obtained from the one-factor-at-a-time optimization experiments were subjected to empirical regression analysis. Only continuous variables were included in the mathematical modeling, namely incubation temperature, initial pH, incubation period, agitation speed, inoculum size, pectin concentration, and ammonium sulfate concentration. Categorical variables, including carbon source, nitrogen source, and metal ion type, were excluded from polynomial regression analysis.

For each continuous factor, pectinase activity was modeled independently using a second-order polynomial equation, Eq. 1,

$$Y = \beta_0 + \beta_1 X + \beta_2 X^2 + \varepsilon \quad (1)$$

where Y represents the predicted pectinase activity (U/mL), X represents the investigated production variable, β_0 is the intercept, β_1 and β_2 are the linear and quadratic regression coefficients, respectively, and ε represents the residual error.

The regression coefficients were estimated by the least-squares method using the experimental measurements obtained from three independent replicates at each investigated level. The adequacy of each fitted model was evaluated using the coefficient

of determination (R^2), adjusted coefficient of determination (R^2_{adj}), root mean square error (RMSE), and examination of model residuals. The coefficient of determination was calculated according to Eq. 2,

$$R^2 = 1 - \frac{\sum_{i=1}^n (Y_i - \hat{Y}_i)^2}{\sum_{i=1}^n (Y_i - \bar{Y})^2} \quad (2)$$

where Y_i is the experimentally observed pectinase activity, $\hat{Y}_i$ is the corresponding model-predicted value, $\bar{Y}$ is the overall mean of the observed responses, and n is the number of observations.

The adjusted coefficient of determination was calculated as:

$$R^2_{adj} = 1 - (1 - R^2) \left(\frac{n-1}{n-p-1} \right) \quad (3)$$

where p represents the number of predictor terms included in the regression model.

The prediction error was further assessed using RMSE:

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum_{i=1}^n (Y_i - \hat{Y}_i)^2} \quad (4)$$

For quadratic models showing a concave response ($\beta_2 < 0$), the mathematically predicted optimum of each factor was calculated from the stationary point of the fitted equation:

$$X_{opt} = -\frac{\beta_1}{2\beta_2} \quad (5)$$

The mathematically predicted optimum was subsequently compared with the experimentally observed optimum within the tested range. The regression models were used exclusively to characterize the individual response of each production variable and were not used to estimate interactions among cultivation factors, because the production optimization experiments were performed independently using a one-factor-at-a-time design (Satpathy *et al.* 2025).

Mathematical response-surface modeling of pectinase thermal stability

Because thermal stability was experimentally evaluated using combinations of temperature and incubation time, the resulting two-factor dataset was additionally subjected to response-surface regression analysis. Residual pectinase activity was modeled as a function of temperature (T) and incubation time (t) using a second-order polynomial model,

$$Y = \beta_0 + \beta_1 T + \beta_2 t + \beta_{11} T^2 + \beta_{22} t^2 + \beta_{12} Tt + \varepsilon \quad (6)$$

where Y represents predicted residual pectinase activity (%), β_0 is the intercept, β_1 and β_2 represent the linear effects of temperature and incubation time, β_{11} and β_{22} represent their quadratic effects, β_{12} represents the temperature $\times$ incubation-time interaction, and ε represents residual error. Model adequacy was evaluated using R^2 , adjusted R^2 , RMSE, regression ANOVA, and residual diagnostics. The statistical significance of individual regression coefficients was assessed at a confidence level of 95% ($p < 0.05$). Three-dimensional response-surface and two-dimensional contour plots were generated from the fitted model to visualize the combined influence of temperature and incubation time on residual pectinase activity (Amin *et al.* 2026).

RESULTS

Collection of Apple Samples

All of the decomposed apple samples obtained from the Jazan region were examined for the presence of pectinolytic bacteria. The samples exhibited varying degrees of visible deterioration, indicating active microbial degradation of the fruit tissues. These samples were subsequently used as the source material for bacterial isolation, and the isolation results are presented in Fig. 1.

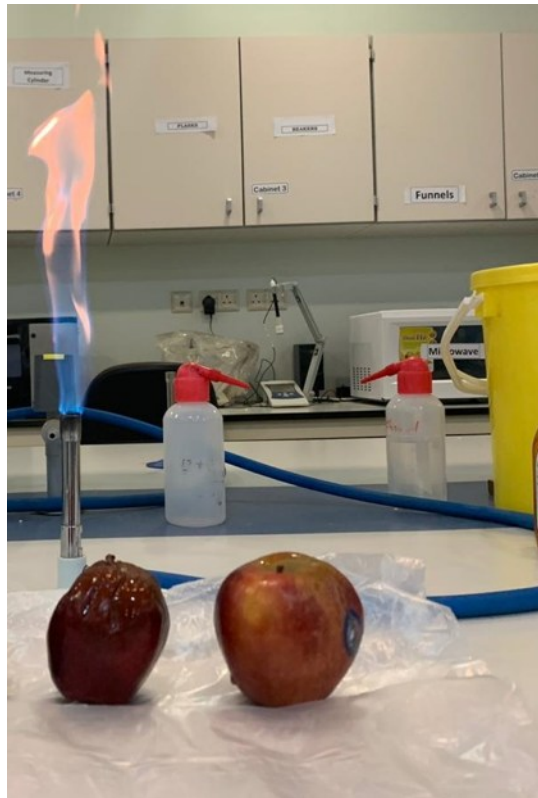


Fig. 1. Apple samples: the left is the spoiled apple, and the control apple is on the right

Isolation of Pectinolytic Bacteria

Pectinolytic bacteria were successfully recovered from the damaged apple samples following serial dilution and cultivation on nutrient agar supplemented with 1% pectin. After incubation at 30 °C for 72 h, morphologically distinct colonies were observed and repeatedly subcultured until pure bacterial isolates were obtained. The viable bacterial count reached approximately 5×10^3 CFU/mL in the analyzed samples (Fig. 2).

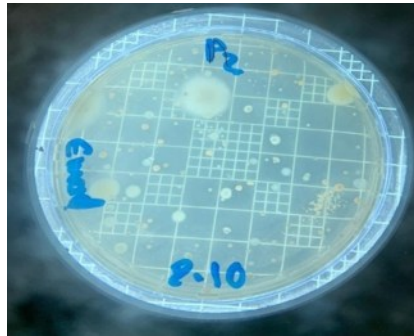


Fig. 2. Pectinolytic bacteria recovered from damaged apples were cultured on nutrient agar supplemented with 1% pectin for 72 h at 30 °C

Screening for Pectinase Activity and Gram Characterization

The pectinase-producing potential of the bacterial isolates was evaluated on pectin-containing agar. Following incubation at 30 °C for 48 h and flooding with iodine solution, isolate AY1 produced a distinct clear hydrolysis zone surrounding the bacterial growth, confirming extracellular pectinolytic activity (Fig. 3). Gram staining further showed that isolated AY1 was Gram-positive, based on the characteristic blue-purple staining of the cells under oil-immersion microscopy.



Fig. 3. Iodine-stained pectin agar showing a hydrolysis zone around isolate AY1, indicating extracellular pectinase activity

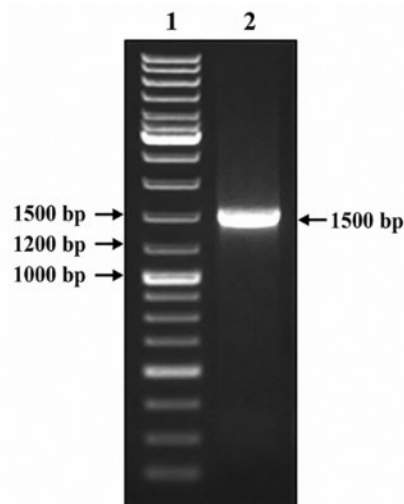


Fig. 4. Agarose gel of the 16S rRNA gene PCR product from isolate AY1. Lane 1, DNA size marker; lane 2, approximately 1,500-bp amplicon

Molecular Identification of Pectinase-producing Bacteria

The highest pectinase-producing isolate, AY1, was subjected to molecular identification based on 16S rRNA gene sequencing. The polymerase chain reaction (PCR) amplification generated a single DNA fragment of approximately 1500 bp (Fig. 4). Sequence comparison using NCBI BLAST showed 99% sequence identity with *Bacillus subtilis*, and phylogenetic analysis placed isolate AY1 within the *Bacillus* clade (Fig. 5). Accordingly, the isolate was designated *Bacillus subtilis* AY1.

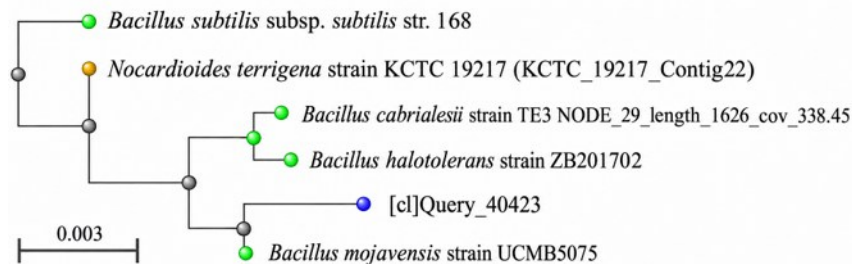


Fig. 5. Phylogenetic relationship of isolate AY1 based on 16S rRNA gene sequence analysis: The AY1 sequence clustered within the *Bacillus* group, supporting its identification as *Bacillus subtilis*

Optimization of Growth Parameters for Maximum Pectinase Production

Effect of temperature

Temperature significantly affected pectinase activity ($F(6,14) = 224.06$, $p < 0.001$). Enzyme activity increased progressively from $0.163 \pm 0.021 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 20°C to a maximum of $0.543 \pm 0.015 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 37°C , corresponding to 100% relative activity. Activity remained relatively high at 40°C ($0.497 \pm 0.012 \mu\text{mol min}^{-1} \text{mL}^{-1}$; 91.98% relative activity) before declining markedly at higher temperatures, reaching $0.093 \pm 0.025 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 50°C . Tukey's HSD analysis showed that the activity at 37°C was significantly higher than that observed at 30°C and lower temperatures and at 45 to 50°C , whereas the difference between 37 and 40°C was not significant. These results identified 37°C as the experimental optimum for pectinase production/activity under the tested conditions (Fig. 6).

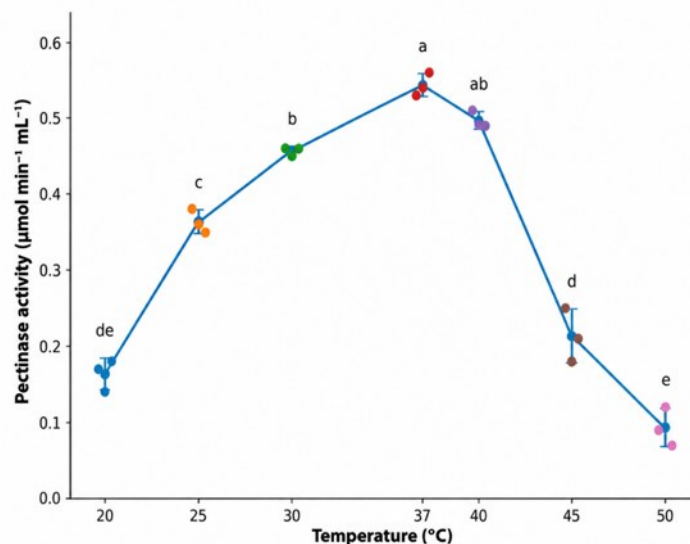


Fig. 6. Pectinase activity of *B. subtilis* AY1 at different temperatures. Data are mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of pH

Pectinase activity varied significantly across the tested pH values ($F(10,22) = 100.65$, $p < 0.001$). The activity increased from $0.193 \pm 0.032 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at pH 4 to a maximum of $0.543 \pm 0.015 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at pH 7. Activity remained high at pH 7.5 ($0.523 \pm 0.015 \mu\text{mol min}^{-1} \text{mL}^{-1}$) and declined progressively at higher pH values, reaching

$0.053 \pm 0.021 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at pH 10. Tukey's HSD test showed no significant difference between pH 7 and 7.5 (Fig. 7).

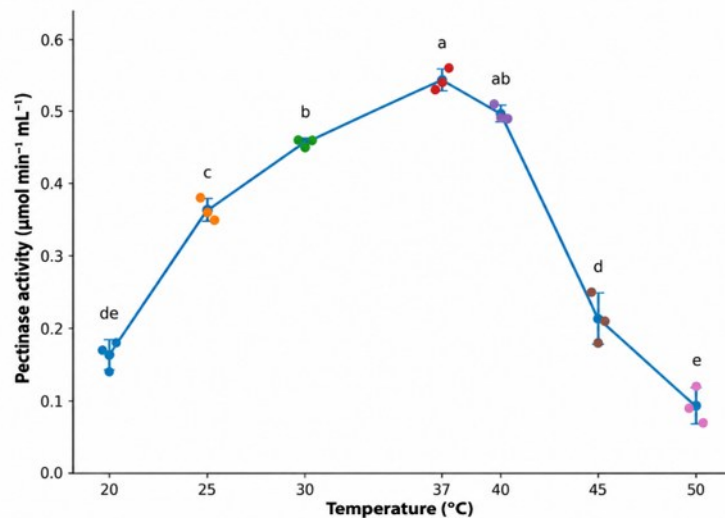


Fig. 7. Pectinase activity of *B. subtilis* AY1 at different pH values. Data are mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of incubation time

Pectinase activity differed significantly among incubation periods ($F(5,12) = 111.54$, $p < 0.001$). Activity increased from $0.173 \pm 0.032 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 24 h to a maximum of $0.820 \pm 0.036 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 72 h. Thereafter, activity decreased to $0.673 \pm 0.035 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 96 h and remained lower at 120 and 144 h. Tukey's HSD test confirmed that the activity at 72 h was significantly higher than at all other incubation periods (Fig. 8).

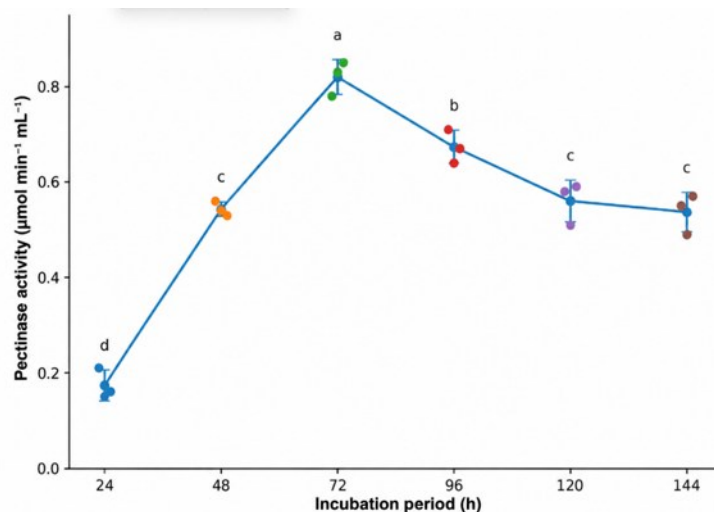


Fig. 8. Pectinase activity of *B. subtilis* AY1 at different incubation times. Data are mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of metal ions

Metal ions significantly affected pectinase activity ($F(9,20) = 44.01$, $p < 0.001$). The highest activity was obtained with Mg^{2+} ($0.783 \pm 0.032 \mu\text{mol min}^{-1} \text{mL}^{-1}$), followed by K^{+} (0.727 ± 0.021) and Ca^{2+} (0.700 ± 0.026). Mg^{2+} , K^{+} , and Ca^{2+} were statistically comparable, whereas Co^{2+} , Mn^{2+} , and Ni^{2+} showed the lowest activities (Fig. 9).

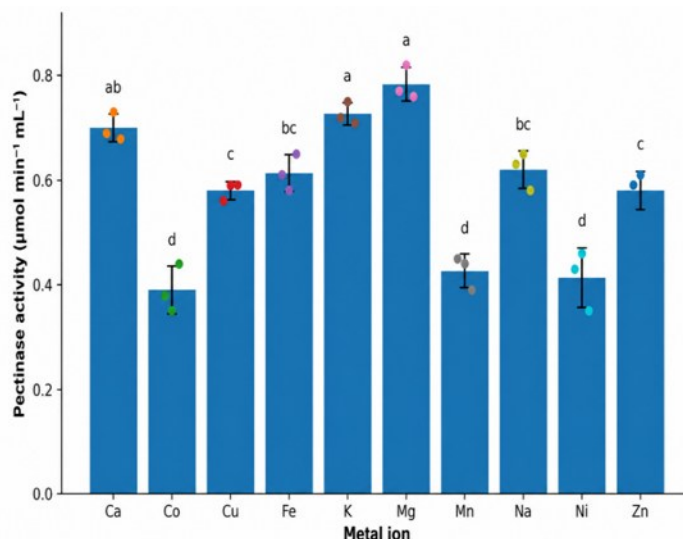


Fig. 9. Effect of metal ions on pectinase activity of *B. subtilis* AY1. Data are mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of Inoculum Size

Inoculum size significantly affected pectinase activity ($F(5,12) = 156.75$, $p < 0.001$). Activity increased from $0.203 \pm 0.023 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 0.5% inoculum to a maximum of $0.820 \pm 0.026 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 2%. Activity decreased at higher inoculum levels, reaching $0.520 \pm 0.036 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 4%. Tukey's HSD test showed that the activity at 2% was significantly higher than at all other inoculum levels (Fig. 10).

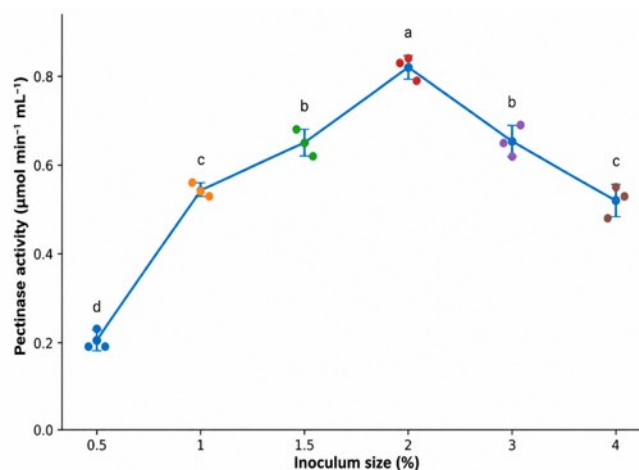


Fig. 10. Effect of inoculum size on pectinase activity of *B. subtilis* AY1. Data are mean $\pm$ SD ($n = 3$); different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of agitation speed

Agitation speed significantly affected pectinase activity ($F(5,12) = 52.18$, $p < 0.001$). Activity increased from $0.323 \pm 0.042 \mu\text{mol min}^{-1} \text{mL}^{-1}$ under static conditions to a maximum of $0.713 \pm 0.035 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 150 rpm. Activity remained statistically comparable at 200 and 250 rpm but declined to $0.540 \pm 0.026 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 300 rpm (Fig. 11).

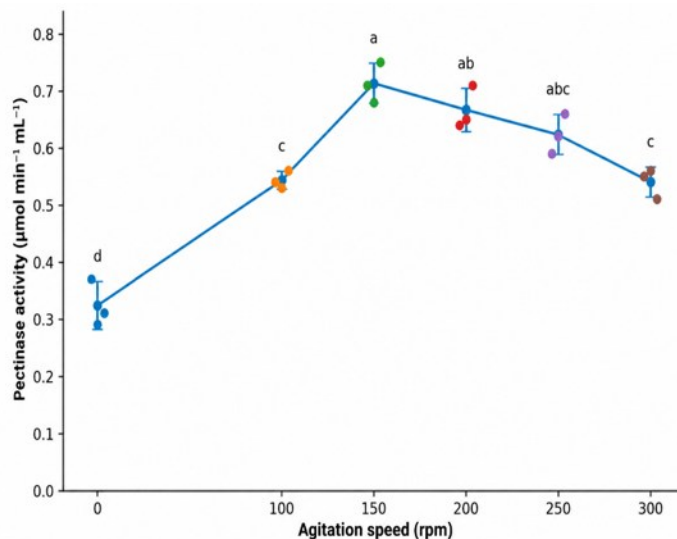


Fig. 11. Effect of agitation speed on *B. subtilis* AY1 pectinase activity; data are mean $\pm$ SD ($n = 3$), and different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of nitrogen sources

Nitrogen source significantly affected pectinase activity ($F(5,12) = 28.82$, $p < 0.001$). The highest activity was obtained with ammonium sulfate ($0.610 \pm 0.026 \mu\text{mol min}^{-1} \text{mL}^{-1}$), followed by peptone (0.543 ± 0.015) and sodium nitrate (0.527 ± 0.040). The lowest activity was recorded with yeast extract ($0.330 \pm 0.036 \mu\text{mol min}^{-1} \text{mL}^{-1}$). Tukey's HSD test showed that ammonium sulfate was statistically comparable to peptone and sodium nitrate but significantly higher than ammonium nitrate, tryptone, and yeast extract (Fig. 12).

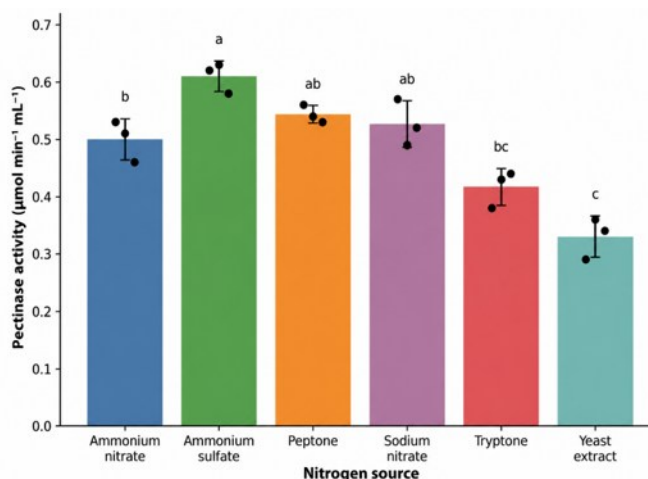


Fig. 12. Effect of nitrogen source on *B. subtilis* AY1 pectinase activity; data are mean $\pm$ SD ($n = 3$), and different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of ammonium sulfate concentration

Ammonium sulfate concentration significantly affected pectinase activity ($F(6,14) = 68.07$, $p < 0.001$). Activity increased from $0.233 \pm 0.055 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 0.2% to a maximum of $0.713 \pm 0.035 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 2.0%. Activity remained statistically comparable at 1.5 and 2.5%, but it decreased at 3.0% to $0.497 \pm 0.049 \mu\text{mol min}^{-1} \text{mL}^{-1}$ (Fig. 13).

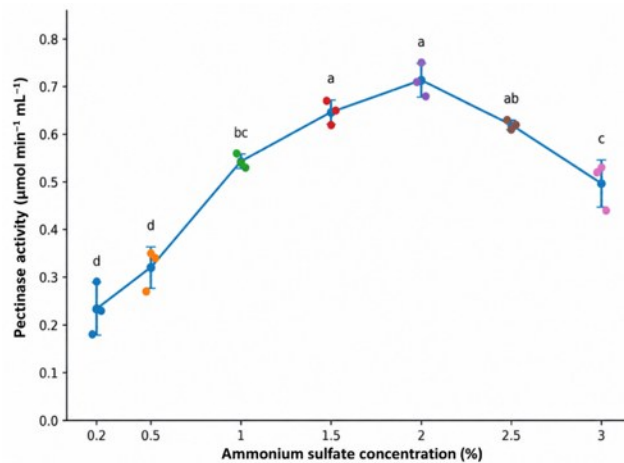


Fig. 13. Effect of ammonium sulfate concentration on *B. subtilis* AY1 pectinase activity; data are mean $\pm$ SD ($n = 3$), and different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of carbon sources

Carbon source significantly affected pectinase activity ($F(4,10) = 14.04$, $p < 0.001$). Citrus pectin produced the highest activity ($0.543 \pm 0.015 \mu\text{mol min}^{-1} \text{mL}^{-1}$), followed by glucose (0.413 ± 0.040). The lowest activity was recorded with sucrose ($0.267 \pm 0.080 \mu\text{mol min}^{-1} \text{mL}^{-1}$). Tukey's HSD test showed that citrus pectin was significantly higher than starch, wheat bran, and sucrose, but not significantly different from glucose (Fig. 14).

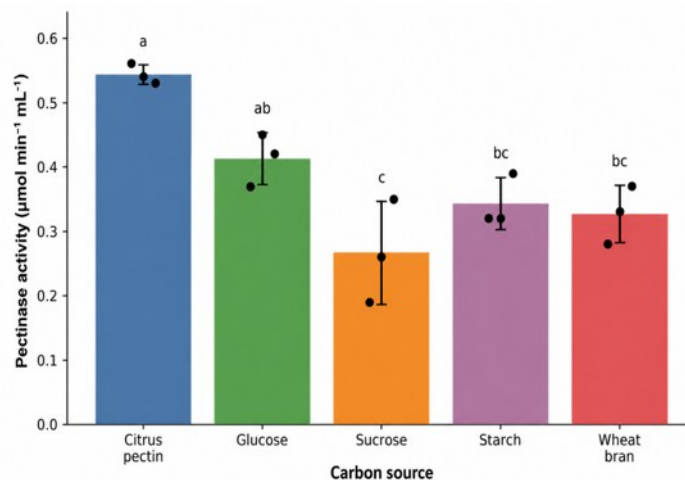


Fig. 14. Effect of carbon source on *B. subtilis* AY1 pectinase activity; data are mean $\pm$ SD ($n = 3$), and different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Effect of pectin concentration

Pectin concentration significantly affected pectinase activity ($F(6,14) = 87.86$, $p < 0.001$). Activity increased from $0.257 \pm 0.015 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 0.5% pectin to a maximum of $0.743 \pm 0.047 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 2%. Activity at 2.5% remained statistically comparable to 2%, whereas further increases reduced activity to $0.310 \pm 0.026 \mu\text{mol min}^{-1} \text{mL}^{-1}$ at 4% (Fig. 15).

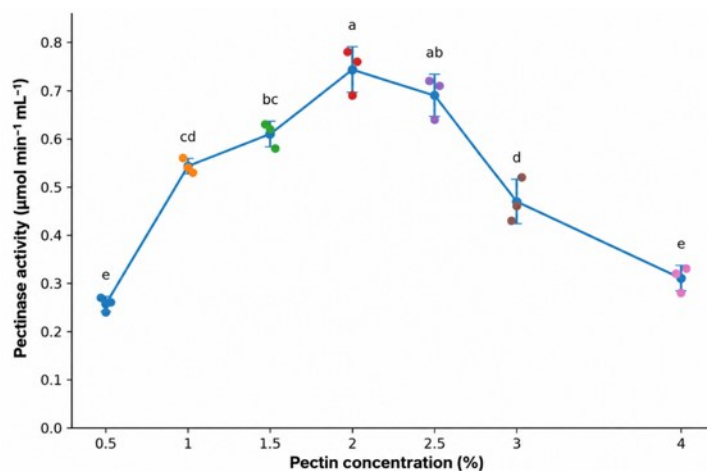


Fig. 15. Effect of pectin concentration on *B. subtilis* AY1 pectinase activity; data are mean $\pm$ SD ($n = 3$), and different letters indicate significant differences (ANOVA with Tukey's HSD, $p < 0.05$).

Validation of Optimized Conditions for Pectinase Production

Application of the optimized conditions significantly increased pectinase activity from 0.543 ± 0.015 to $1.453 \pm 0.176 \mu\text{mol min}^{-1} \text{mL}^{-1}$ ($p = 0.012$), representing an approximately 167.5% increase over the initial condition. The optimized culture contained 2% pectin, 2% ammonium sulfate, K^+ and Mg^{2+} salts, and 2% inoculum and was incubated at pH 7, 37°C , and 150 rpm for 72 h. The corresponding specific activity was 5.19 IU/mg (Fig. 16).

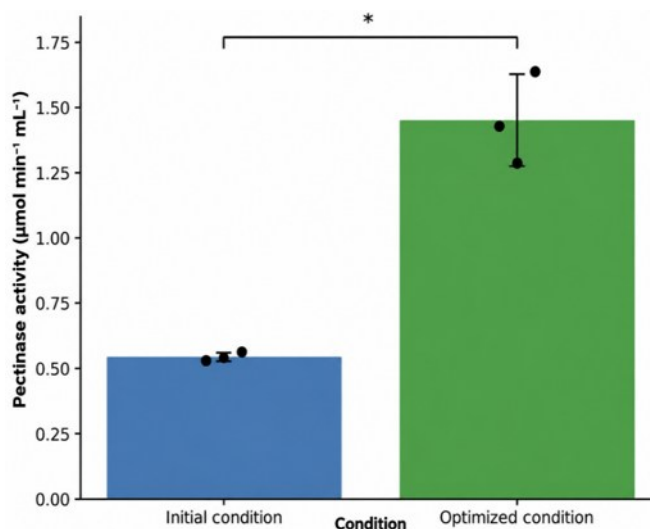


Fig. 16. Pectinase activity before and after application of the optimized production conditions; Values are mean $\pm$ SD ($n = 3$); * indicates a significant difference between conditions ($p < 0.05$)

Purification Profile of *Bacillus subtilis* AY1 Pectinase

Pectinase was purified from the cell-free supernatant by ammonium sulfate precipitation followed by Sephadex G-100 gel filtration. The crude enzyme showed a total activity of 261.72 ± 1.40 IU and a specific activity of 10.49 IU/mg. Following ammonium sulfate precipitation, the specific activity increased to 13.14 IU/mg, corresponding to a 1.25-fold purification with 36.55% activity recovery. Sephadex G-100 purification further increased the specific activity to 33.18 IU/mg, resulting in a 3.16-fold purification with 24.85% recovery of the initial activity. (Fig. 17).

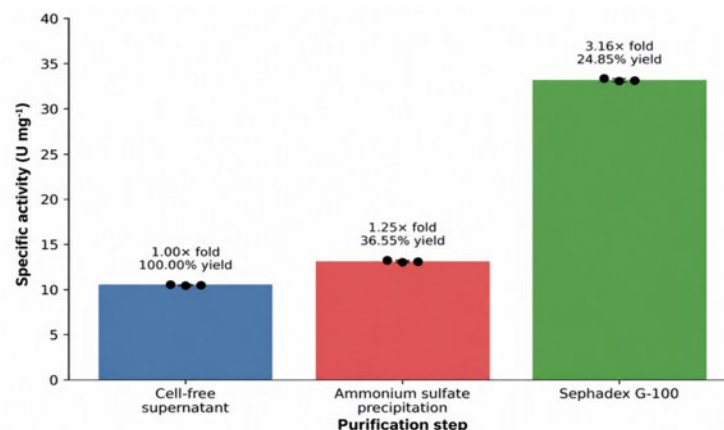


Fig. 17. Purification profile of pectinase from *Bacillus subtilis* AY1; Specific activity is presented as mean $\pm$ SD ($n = 3$); purification fold and activity yield are indicated above each purification step

SDS-PAGE Analysis and Molecular Weight Determination of Purified Pectinase

SDS-PAGE analysis showed progressive purification of the enzyme. The crude cell-free supernatant contained several protein bands, whereas fewer bands were detected after ammonium sulfate precipitation (Fig. 18). Following Sephadex G-100 gel filtration, a prominent single band of approximately 32 kDa was observed. Together with the pectinase activity detected in the purified fraction, this result suggests that the 32 kDa protein is associated with the purified pectinase preparation, although further enzyme-specific analysis would be required for definitive identification.

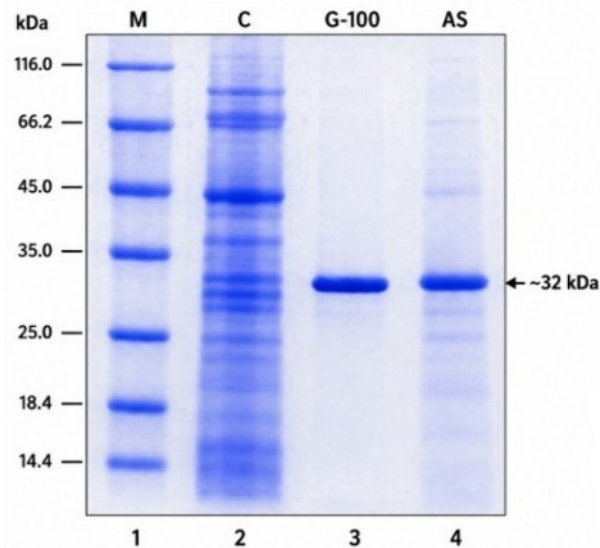


Fig. 18. SDS-PAGE profile of *Bacillus subtilis* AY1 pectinase during purification: Lane M, protein molecular-weight marker; lane C, cell-free supernatant (crude enzyme); lane G-100, Sephadex G-100-purified pectinase; lane AS, ammonium sulfate fraction

Biochemical Characterization of Purified Pectinase

The activity of purified pectinase was significantly affected by reaction temperature, pH, reaction time, substrate concentration, and salt composition (Fig. 19A through E). Maximum activity was observed at 45 °C ($1.833 \pm 0.085 \mu\text{mol min}^{-1} \text{mL}^{-1}$; Fig. 19A) and pH 6 ($2.697 \pm 0.181 \mu\text{mol min}^{-1} \text{mL}^{-1}$; Fig. 19B). Activity increased with reaction time up to 50 min, reaching $1.893 \pm 0.055 \mu\text{mol min}^{-1} \text{mL}^{-1}$ (Fig. 19C).

The highest activity at different substrate concentrations was obtained at 2.5% substrate ($1.803 \pm 0.023 \mu\text{mol min}^{-1} \text{mL}^{-1}$; Fig. 19D). Among the tested salts, NaCl produced the highest activity ($1.723 \pm 0.070 \mu\text{mol min}^{-1} \text{mL}^{-1}$), followed by KCl and NH_4Cl (Fig. 19E). All tested factors showed significant effects on pectinase activity by one-way ANOVA ($p < 0.001$).

Thermal Stability of Purified Pectinase

Thermal stability was significantly influenced by temperature, incubation time, and their interaction. Two-way ANOVA showed highly significant effects of temperature ($F(8,90) = 134097.27$, $p < 0.001$), incubation time ($F(4,90) = 353848.17$, $p < 0.001$), and the temperature $\times$ incubation-time interaction ($F(32,90) = 14534.81$, $p < 0.001$).

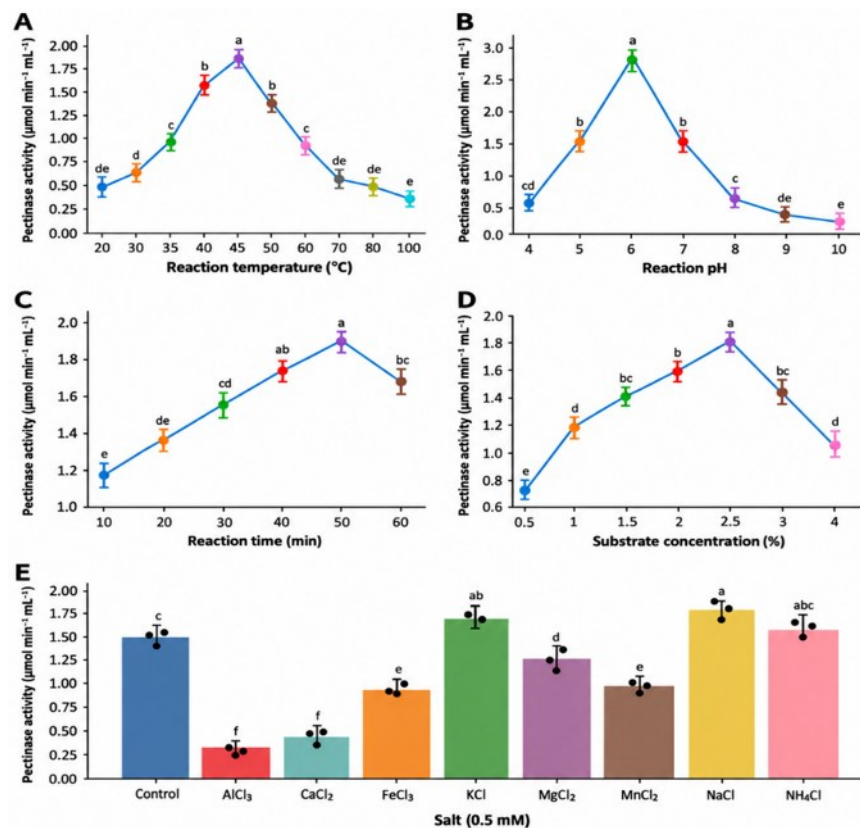


Fig. 19. Biochemical characterization of purified pectinase from *Bacillus subtilis* AY1: (A) Reaction temperature, (B) reaction pH, (C) reaction time, (D) substrate concentration, and (E) effect of salts; Values are mean $\pm$ SD ($n = 3$); Different lowercase letters indicate significant differences by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$)

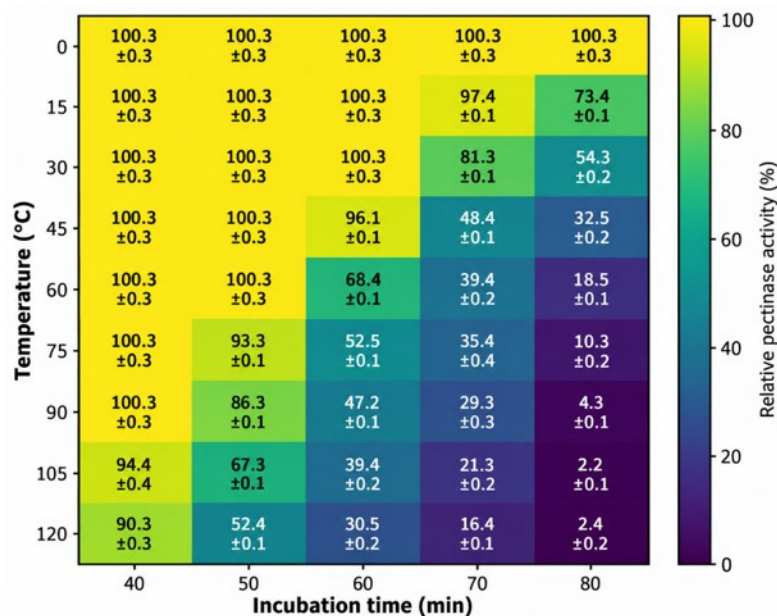


Fig. 20. Thermal stability of purified pectinase from *Bacillus subtilis* AY1 at different temperatures and incubation times; Values are mean relative activity $\pm$ SD ($n = 3$); Effects of temperature, incubation time, and their interaction were significant by two-way ANOVA ($p < 0.001$)

Relative activity remained close to 100% under shorter incubation periods and progressively decreased as both temperature and exposure time increased. At 120 °C, the enzyme retained $90.3 \pm 0.3\%$ activity after 40 min, decreasing to $52.4 \pm 0.1\%$, $30.5 \pm 0.2\%$, $16.4 \pm 0.1\%$, and $2.4 \pm 0.2\%$ after 50, 60, 70, and 80 min, respectively (Fig. 20).

Fruit Juice Clarification by Purified Pectinase

Treatment with purified pectinase clearly improved the clarity of the apple juice compared with untreated control. The untreated juice showed a mean transmittance of $20.0 \pm 0.2\%$, whereas the pectinase-treated juice reached $54.5 \pm 0.2\%$ based on three independent measurements ($n = 3$) (Fig. 21A-B), representing a significant improvement in juice clarification ($p < 0.001$).

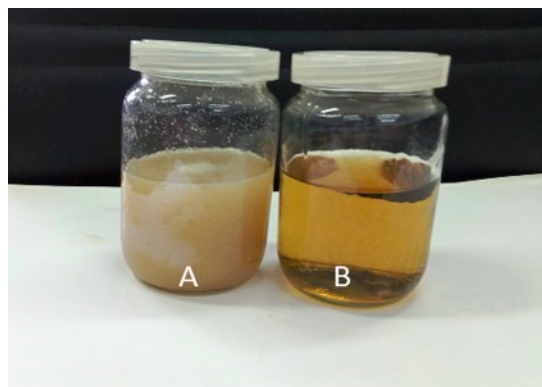


Fig. 21. Application of purified pectinase from *Bacillus subtilis* AY1 in apple juice clarification: (A) Untreated juice (control); (B) pectinase-treated juice

Antioxidant Capacity and DPPH Radical Scavenging Activity of Pectinase-Treated Apple Juice

Pectinase treatment significantly increased the reducing capacity of apple juice from 954.0 ± 18.7 mg/L in the untreated control to 1245.0 ± 43.2 mg/L in the clarified juice ($p = 0.0027$).

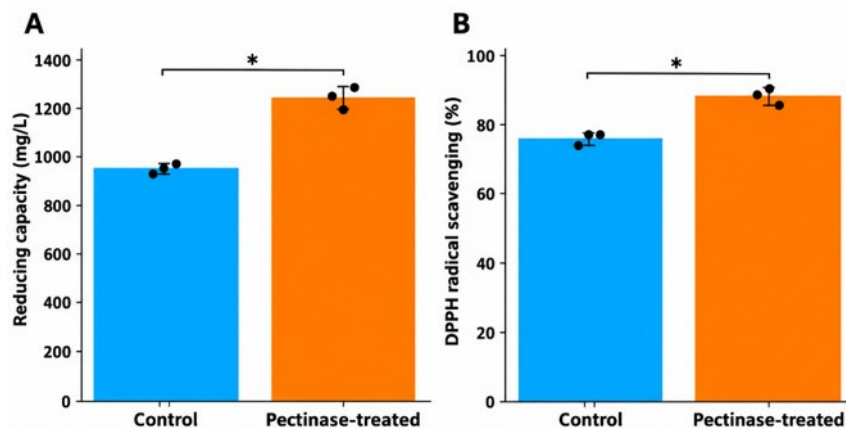


Fig. 22. Effect of pectinase treatment on the antioxidant properties of apple juice: (A) Reducing capacity and (B) DPPH radical scavenging activity; Values are mean $\pm$ SD ($n = 3$); * indicates a significant difference between untreated and pectinase-treated juice ($p < 0.05$)

The DPPH radical scavenging activity also increased significantly from $76.07 \pm 1.62\%$ to $88.20 \pm 2.39\%$ following pectinase treatment ($p = 0.0031$) (Fig. 22).

Shelf Stability of Purified Pectinase

The purified pectinase gradually lost activity during storage at 4 °C in phosphate buffer (pH 7). Residual activity decreased to approximately 50% after 60 days and further declined to 27% after 186 days of storage (Fig. 23).

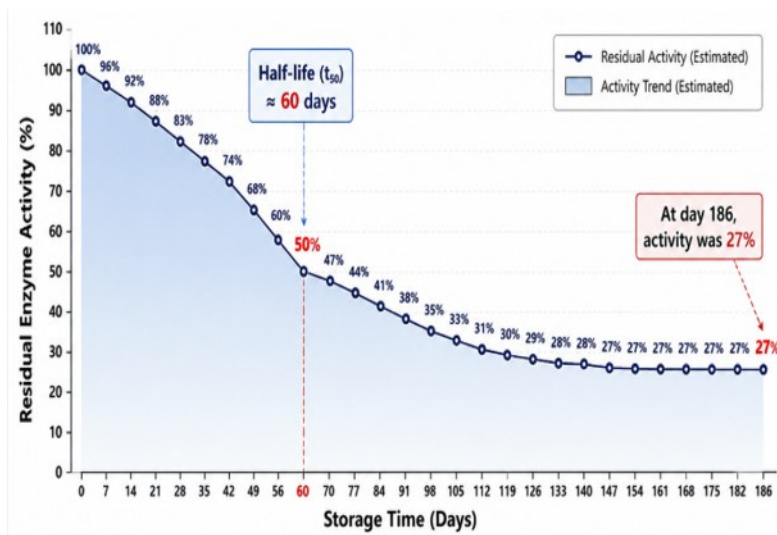


Fig. 23. Shelf stability of purified pectinase from *Bacillus subtilis* AY1 during storage at 4 °C; Residual activity was approximately 50% after 60 days and 27% after 186 days

Empirical Mathematical Modelling of Pectinase Production

Second-order polynomial regression models were fitted independently to the continuous production variables. All fitted models were statistically significant ($p < 0.001$). The coefficients of determination (R^2) adjusted coefficients of determination (adjusted R^2), RMSE, and model-predicted optima are summarized below.

Table 1. Regression Model Performance and Predicted Versus Experimental Optimum Conditions for Pectinase Production

Factor	R^2	Adjusted R^2	RMSE	Predicted Optimum	Experimental Optimum
Temperature	0.919	0.910	0.047 U/mL	34.1 °C	37 °C
Initial pH	0.832	0.821	0.065 U/mL	6.75	7.0
Incubation time	0.809	0.784	0.087 U/mL	93.4 h	72 h
Agitation speed	0.897	0.884	0.041 U/mL	186.7 rpm	150 rpm
Inoculum size	0.887	0.872	0.064 U/mL	2.49%	2.0%
Ammonium sulfate	0.950	0.944	0.037 U/mL	1.93%	2.0%
Pectin concentration	0.837	0.819	0.070 U/mL	2.20%	2.0%

R^2 , coefficient of determination; adjusted R^2 , adjusted coefficient of determination; RMSE, root mean square error

For temperature, the fitted quadratic equation was:

$$Y = -1.5866 + 0.12337T - 0.001809T^2$$

The model yielded $R^2 = 0.919$, adjusted $R^2 = 0.910$, and RMSE = 0.047 U/mL, with a predicted stationary point at 34.1 °C.

For initial pH, the fitted equation was:

$$Y = -1.5448 + 0.59859(\text{pH}) - 0.04435(\text{pH})^2$$

The model yielded $R^2 = 0.832$, adjusted $R^2 = 0.821$, and RMSE = 0.065 U/mL, with a predicted stationary point at pH 6.75.

For agitation speed, the fitted equation was:

$$Y = 0.3132 + 0.003852R - 0.0000103R^2$$

The model yielded $R^2 = 0.897$, adjusted $R^2 = 0.884$, and RMSE = 0.041 U/mL, with a predicted stationary point at 186.7 rpm.

For inoculum size, the fitted equation was:

$$Y = -0.0518 + 0.67603I - 0.13575I^2$$

The model yielded $R^2 = 0.887$, adjusted $R^2 = 0.872$, and RMSE = 0.064 U/mL, with a predicted stationary point at 2.49%.

For ammonium sulfate concentration, the fitted equation was:

$$Y = 0.0882 + 0.61650N - 0.15974N^2$$

The model yielded $R^2 = 0.950$, adjusted $R^2 = 0.944$, and RMSE = 0.037 U/mL, with a predicted stationary point at 1.93%.

For pectin concentration, the fitted equation was:

$$Y = 0.0525 + 0.57024P - 0.12936P^2$$

The model yielded $R^2 = 0.837$, adjusted $R^2 = 0.819$, and RMSE = 0.070 U/mL, with a predicted stationary point at 2.20%.

For incubation time, the fitted equation was:

$$Y = -0.2087 + 0.020416t - 0.000109t^2$$

The model yielded $R^2 = 0.809$, adjusted $R^2 = 0.784$, and RMSE = 0.087 U/mL, with a predicted stationary point at 93.4 h.

Response Surface Modelling of Pectinase Thermal Stability

Residual pectinase activity was fitted as a function of temperature and incubation time using a second-order polynomial response-surface model. The overall regression model was statistically significant ($p < 0.001$), with $R^2 = 0.929$, adjusted $R^2 = 0.926$, and an RMSE of 9.20 percentage points.

The fitted response-surface equation was:

$$Y = 88.957 + 0.5108T + 1.0766t + 0.000714T^2 - 0.01352t^2 - 0.01884Tt$$

The temperature $\times$ incubation-time interaction term was statistically significant ($p < 0.001$). The quadratic incubation-time term was also significant ($p = 0.006$), whereas the quadratic temperature term was not significant ($p = 0.248$). The linear temperature term was significant ($p < 0.001$), while the linear incubation-time term was not significant ($p = 0.070$).

DISCUSSION

The recovery of AY1 from decomposed apple material is consistent with the conditions in pectin-rich fruit residues, where plant cell-wall polymers can provide substrates for pectin-degrading microorganisms. The iodine-clearance zone supported extracellular pectin hydrolysis, and 16S rRNA sequencing placed the isolate within *Bacillus subtilis* at 99% sequence identity. Other fruit-waste studies have also recovered bacteria capable of producing pectinase on citrus pectin and peel-based substrates. These findings support decomposed fruit as a reasonable source for screening pectinolytic bacteria (Liew *et al.* 2025).

Pectinase production by *B. subtilis* AY1 responded to cultivation conditions, likely because these conditions affect both bacterial growth and enzyme synthesis or secretion. Temperature and pH can influence cellular metabolism and protein production, while pectin can serve as both a carbon source and an inducer of pectinolytic enzymes. Fermentation time determines how long cells have to grow and produce enzyme; longer incubation may eventually become less favorable as nutrients are depleted or enzyme activity declines. Agitation and inoculum size can alter mixing, oxygen transfer, and the balance between growth and enzyme production. Ammonium sulfate may provide an accessible nitrogen source, although its effect is strain-dependent. These are plausible explanations for the observed responses; the underlying regulatory mechanisms were not examined directly. The near-neutral pH and mesophilic temperature profile is consistent with the range reported for microbial pectinase production, while the nitrogen-source response highlights the need to optimize conditions for each strain (Ametefe and Orji 2024).

The production maximum at 37 °C is consistent with a mesophilic growth range that can support cellular metabolism and enzyme secretion. A pH of 7 may favor cellular homeostasis and pectinase production, whereas more acidic or alkaline conditions can disrupt transport and protein function. The 72-h maximum may represent a balance between growth and pectin-induced enzyme synthesis before nutrient depletion, pH changes, by-product accumulation, or extracellular proteolysis reduces activity. These explanations are biologically plausible but were not tested directly.

The 2% pectin optimum likely provided enough substrate and induction for pectinase production. At higher concentrations, increased medium viscosity may have limited mixing and mass transfer, reducing access to substrate and oxygen. Because viscosity and substrate inhibition were not measured, this explanation should be considered a possibility rather than a demonstrated mechanism.

Combining the selected production conditions increased pectinase activity by 167.5% relative to the initial condition. The single-factor quadratic equations described several responses reasonably well and predicted values close to the experimental optima for pH, pectin, and ammonium sulfate. However, these equations were fitted to one-factor-at-a-time (OFAT) experiments. Because only one variable was changed at a time, the models cannot estimate interactions among temperature, pH, incubation time, agitation, and nutrient concentrations or establish a global optimum. The predicted values should therefore be interpreted as estimates within the tested ranges, not as a fully optimized multivariable condition. This differs from the response-surface approach used for *Bacillus pumilus*, in which pH, temperature, and fermentation time were evaluated together and the predicted production condition was experimentally validated (Zafar *et al.* 2024).

The largest differences between predicted and observed optima occurred for incubation time, agitation, and inoculum size: 93.4 h versus 72 h, 186.7 versus 150 rpm, and 2.49% versus 2.0%, respectively. The incubation-time model may smooth discrete measurements, while actual enzyme accumulation also depends on changing biomass, substrate depletion, pH shifts, and enzyme decay. Agitation responses depend on oxygen transfer and shear, which are also

affected by flask geometry and fill volume; inoculum size can alter growth phase and competition for nutrients and oxygen. These factors were not modeled jointly. The predicted values should therefore be treated as estimates, with the experimentally observed conditions providing the practical reference for this study (Patel and Shah, 2024).

Purification increased the specific activity of the AY1 preparation, while the final recovery reflected a trade-off between enrichment and enzyme loss during the selected steps. SDS-PAGE showed fewer detectable protein bands after purification, including a prominent band with an apparent molecular mass of approximately 32 kDa in the Sephadex G-100 fraction. Since SDS-PAGE alone does not establish protein identity, this band should not be assigned conclusively to pectinase without enzyme-specific evidence. The purification gain was lower than that reported for a cold-active *Cladosporium* pectinase purified using a different sequence of steps, but fold purification depends on the starting material, purification protocol, and balance between purity and recovery. The comparison therefore provides context rather than a direct measure of enzyme quality (Moharram *et al.* 2022).

The purified AY1 preparation showed its highest activity under moderately warm, mildly acidic conditions. Temperature and pH affect enzyme flexibility, substrate binding, and catalytic-residue ionization, while pectin concentration determines substrate availability. This profile broadly agrees with a recent pectinase study reporting an approximately 35-kDa protein with maximal activity near 50 °C and activity across pH 5 to 7 (Mwaheb *et al.* 2024). Salt effects may reflect changes in ionic strength or enzyme–pectin interactions; because these mechanisms were not tested, the observed effects should be interpreted as specific to this assay.

Thermal-stability measurements showed that residual activity depended on both temperature and exposure time, and the response-surface analysis detected a significant temperature-by-time interaction. This is consistent with heat inactivation being cumulative: the effect of a given temperature can depend on how long the enzyme is exposed. The model described the measured thermal-stability data well ($R^2 = 0.929$), but the unusually high activity reported after exposure at 105 and 120 °C requires cautious interpretation and independent confirmation. A recent study of another pectinase reported declining stability across 50 to 65 °C and a longer thermal half-life after immobilization, illustrating the expected importance of temperature and exposure duration (El-Shora *et al.* 2025). Unlike the OFAT production equations, the thermal model included experimentally measured temperature–time combinations and could therefore describe their joint response within the tested range. Even so, the extreme-temperature observations should not be taken as evidence of routine operational stability at those temperatures. Quadratic response models can capture such interactions when the experimental design includes combined factor levels, as also demonstrated in pectinase studies using response-surface methods (Kottilingal *et al.* 2026; Zhao *et al.* 2025).

The residual activity reported after exposure at 105 and 120 °C is particularly unusual and should be regarded as provisional until confirmed under independently verified, pressure-rated heating conditions with effective control of evaporation and sample temperature. These observations do not establish routine process stability at temperatures above 100 °C.

During refrigerated storage, the enzyme retained measurable activity over the study period, although activity declined with time. In apple juice, treatment increased transmittance from $20.0 \pm 0.2\%$ in the untreated control to $54.5 \pm 0.2\%$ in the treated sample and was accompanied by higher reducing capacity and DPPH scavenging activity. These results indicate improved clarification and changes in the measured antioxidant-assay responses under the conditions tested. They do not, by themselves, establish improved nutritional value or health benefits; changes in phenolic availability and sample turbidity could affect assay readings and would require further investigation. A continuous apple-juice clarification study reported a larger increase in clarity, from 12.6% to 89.3%, alongside changes in phenolic and

antioxidant measurements. Direct comparison is limited because enzyme dose, juice composition, and processing conditions differ between studies (Mahmood-Fashandi *et al.* 2026).

Relative to untreated control, the treated juice showed a 34.5-percentage-point increase in transmittance. This direct comparison supports improved clarification under the tested conditions, while the absolute transmittance should not be interpreted as evidence of performance across other juice batches or processing systems.

Overall, the results identify *B. subtilis* AY1 as a laboratory-scale source of extracellular pectinase and show that its production and measured activity depend on cultivation and assay conditions. The OFAT models are useful for describing individual-factor responses, but they cannot identify interactions or replace a multivariable optimization design. Likewise, the thermal-stability and apple juice results support further evaluation rather than immediate claims of industrial readiness. Confirmation of the protein identity, validation of the high-temperature observations, multivariable optimization, and testing at larger processing volumes would help determine the enzyme's suitability for juice processing.

The present application was evaluated at laboratory scale. Scale-up, enzyme immobilization, production economics, and food-safety assessment were not examined; therefore, industrial readiness cannot yet be concluded. These questions, together with process validation in larger volumes and sensory evaluation of the treated juice, should be addressed in future work.

CONCLUSIONS

1. *Bacillus subtilis* AY1 was isolated from decomposed apples and produced extracellular pectinase. Under the selected conditions, enzyme activity increased from 0.543 to 1.453 U/mL.
2. Purification yielded preparation with a specific activity of 33.2 U/mg. Pectinase treatment increased apple-juice transmittance from 20.0% to 54.5% under the conditions tested.
3. These findings are from laboratory-scale experiments. The OFAT models do not assess factor interactions, the identity of the approximately 32 kDa band remains unconfirmed, and the high-temperature results require verification.
4. Future work should test apple pomace and other apple-processing residues as fermentation substrates and assess scale-up, safety, and production costs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Use of Generative AI

The authors would like to disclose that AI-assisted language editing tools (e.g., Grammarly) were used only to improve the language and clarity of the manuscript. All scientific content, data generation, simulations, analyses, and figures were produced entirely by the authors.

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