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Article

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A Cold-Active GH42 β -Galactosidase from *Klebsiella variicola* for Aroma-Preserving, Energy-Efficient Processing of Reconstituted Tobacco

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Abstract

Cold-active enzymes are increasingly valued for energy-efficient biocatalysis at low temperatures. Here, a cold-active β -galactosidase (β -Gal 1527) from *Klebsiella variicola* GB3 was heterologously expressed in *Escherichia coli* BL21(DE3) and purified for reconstituted-tobacco processing. The 685-amino-acid protein encoded by GenBank PZ320841 has a theoretical molecular mass of 77.5 kDa, shares 89% identity with *K. variicola* β -galactosidases, and belongs to glycoside hydrolase family 42 (GH42), with conserved catalytic residues E157 and E314. Ni-NTA affinity and size-exclusion chromatography yielded 17.15 $\pm$ 0.32 mg/mL of purified enzyme ($\approx$ 214 mg/L culture), which migrated as a single $\approx$ 77 kDa band. The enzyme showed maximal activity at 10 °C and pH 6.5 ($>85\%$ activity at pH 6.0–7.0), retained $>90\%$ activity after 30 min at 0–20 °C, and lost $>80\%$ activity above 30 °C, confirming its cold-active, thermolabile nature. At 2 mmol/L, Fe²⁺ increased activity to 144.6 $\pm$ 3.2%, whereas Ca²⁺ ($\approx 19\%$) and Mn²⁺/Mg²⁺/Cu²⁺ (≈ 29 –36%) strongly inhibited activity. Michaelis–Menten analysis gave $K_m = 3.05 \pm 0.20$ mM, $V_{max} = 222.2 \pm 1.3$ $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $k_{cat} \approx 287$ s⁻¹, and $k_{cat}/K_m \approx 9.4 \times 10^4$ M⁻¹·s⁻¹. Treatment of Zhongyan-100 tobacco strands and reconstituted-tobacco concentrate at 10 °C improved sensory aroma quality, intensity, and smoothness compared with the water control. HS-SPME-GC-MS showed increases in all 16 monitored aroma compounds (16.6–91.0%, $p < 0.05$), including benzyl alcohol (+90.9%; 4.36 to 8.33 $\mu\text{g}/100$ mL), 9-hydroxy-megastigma-dien-3-one (+84.8%), theaspiron (+73.1%), β -damascenone (+30.7%), 4,7,9-megastigmatrien-3-one (tabanone; +16.6%), 2-phenylethanol (+28.5%), and solanone (+33.0%; 48.98 to 65.12 $\mu\text{g}/100$ mL).

These findings demonstrate that β -Gal 1527 efficiently releases glycosidically bound aroma compounds during processing at 10 °C, providing an aroma-preserving low-temperature environment that minimizes the volatilization and loss of valuable volatile and thermolabile aroma compounds. The enzyme,

therefore, represents a promising cold-active GH42 biocatalyst for energy-efficient applications in tobacco, flavor, fragrance, and food biotechnology.

Keywords: Cold-active β -galactosidase; *Klebsiella variicola*; GH42; low-temperature biocatalysis; aroma compounds; reconstituted tobacco

1. Introduction

β -Galactosidase (β -Gal), a glycoside hydrolase widely distributed across plants, microorganisms, and animal tissues, catalyzes the hydrolysis of β -galactosidic bonds, thereby releasing galactose units and modifying the physicochemical and biological properties of polysaccharides^[1-3]. In cell-wall polysaccharide metabolism, β -Gal targets galactan and arabinogalactan components of hemicellulose, as well as the rhamnogalacturonan side chains of pectic polysaccharides. Its hydrolytic activity alters the matrix polysaccharide structure, influencing cross-linking density, hydration capacity, and, ultimately, the mechanical strength and permeability of cell walls^[4]. These modifications are central to physiological processes such as fruit ripening, organ abscission, and tissue softening, where increased β -Gal activity facilitates cell-wall relaxation and structural remodeling^[5-6].

Advances in genetic engineering have enabled the heterologous production of β -Gal in high-efficiency microbial systems, with *Escherichia coli* emerging as a preferred host owing to its well-characterized genetics, cost-effectiveness, and rapid growth^[7-8]. Several studies have successfully cloned and expressed β -Gal genes from diverse microbial sources, yielding recombinant enzymes with enhanced activity relative to their native counterparts^[9-10]. To contextualize the present enzyme, we compared it with previously reported cold-active β -galactosidases. Most such enzymes have been isolated from psychrophilic or marine bacteria, with temperature optima of 15–25 °C; Fe^{2+} generally does not enhance their activity, and their applications have been largely confined to the food, dairy, and textile industries. Against this background, an enzyme with an optimum of 10 °C, marked Fe^{2+} activation, and demonstrated applicability to low-temperature tobacco processing is of distinct industrial interest: it further lowers energy consumption, limits the thermal degradation and volatilization of heat-sensitive aroma precursors, and helps preserve the intrinsic flavor of tobacco. However, significant differences in enzymatic properties and regulatory mechanisms persist among β -Gal enzymes from different species, underscoring the need for source-specific characterization^[11]. Notably, β -Gal from *Klebsiella variicola* remains largely unexplored, and its potential in industrial processes, particularly for tobacco reconstitution, has not been reported previously.

Tobacco reconstitution is an important area of applied biotechnology in which enzymatic treatments can improve product quality, optimize aroma profiles, and support harm-reduction strategies^[12]. Tobacco cell walls are rich in galactan, arabinogalactan, and pectic polysaccharides, many of which are covalently

linked to glycosidic aroma precursors. By hydrolyzing β -galactosidic bonds in these polysaccharides, β -galactosidase helps loosen cell-wall structures and release bound aroma compounds. Conventional high-temperature processing causes severe volatilization and thermal degradation of aroma components, resulting in poor smoke flavor and reduced smoking quality. In contrast, a cold-active β -galactosidase that functions efficiently at 0–30 °C minimizes aroma loss, preserves thermolabile compounds, and promotes the release of aglycone aroma compounds. In addition, the released galactose and modified polysaccharides may participate in Maillard reactions during subsequent processing, further enriching the tobacco aroma profile^[13]. A cold-active β -galactosidase is therefore a well-matched biocatalyst for improving the sensory quality and aroma richness of reconstituted tobacco under low-energy, environmentally friendly conditions.

Beyond their fundamental role in polysaccharide metabolism, β -galactosidases are among the most important industrial enzymes. Their best-known application is the hydrolysis of lactose to produce low-lactose and lactose-free dairy products for the large proportion of the world's adult population affected by lactose intolerance^[14-15]. Their transglycosylation activity is also exploited to synthesize prebiotic galacto-oligosaccharides (GOS) that promote a balanced gut microbiota^[16]. Cold-active (psychrophilic) enzymes are especially attractive here: high catalytic activity at low temperature combined with thermolability enables energy-saving processing, protects heat-labile products, and allows gentle heat-inactivation, which has driven their uptake across the dairy, detergent, food, and molecular-biology sectors^[17-19]. Accordingly, cold-active β -galactosidases have been characterized from diverse psychrophilic and cold-tolerant bacteria, including *Arthrobacter*^[20], *Pseudoalteromonas haloplanktis*^[21], *Paracoccus*^[22], *Alteromonas*^[23], and *Halomonas*^[24], yet reports from the genus *Klebsiella* remain scarce. A second, complementary application exploits the same catalytic chemistry to generate aroma: many plant aroma compounds accumulate as non-volatile glycosidic precursors, and glycoside hydrolases liberate the free, odor-active aglycones. This enzymatic release of bound volatiles is well established for enhancing varietal aroma in wine, fruit juices, and tea^[25-27], and provides the mechanistic rationale for applying a cold-active β -galactosidase to release bound aroma compounds from tobacco under mild, low-temperature conditions.

In this study, the β -Gal gene from *Klebsiella variicola* GB3 was cloned into an *E. coli* expression system, and the recombinant enzyme was purified and systematically characterized. Beyond biochemical profiling, the enzyme was applied to tobacco strands and reconstituted-tobacco concentrate, and sensory evaluation and HS-SPME-GC-MS were used to assess improvements in aroma quality and composition. The work thus links enzyme characterization with an industrial application and provides a preliminary basis for developing low-temperature enzymatic strategies in reconstituted-tobacco production.

2. Materials and Methods

2.1 Materials and Equipment

2.1.1 Strains and plasmids

The target gene encoding β -galactosidase (GenBank accession PZ320841.1) was derived from the pectinase-producing strain *K. variicola* GB3, maintained in our laboratory culture collection. The β -galactosidase open reading frame (685 codons) was retrieved from the genome of this strain; BLASTp of the deduced protein against the NCBI non-redundant database returned *K. variicola* β -galactosidases as the closest homologs (89% identity, full-length coverage). Recombinant *E. coli* BL21(DE3) competent cells and the prokaryotic expression vector pET-28a(+) were obtained from Beijing Bomeide Biotechnology Development Co., Ltd. Primer synthesis and sequencing services were provided by the same company.

2.1.2 Reagents

Protein markers (15–180 kDa) were purchased from Thermo Fisher Scientific. IPTG, o-nitrophenol (ONP), o-nitrophenyl- β -D-galactopyranoside (ONPG), kanamycin sulfate, and Tris were obtained from Sangon Biotech Co., Ltd. (Shanghai). A 5 mL Ni-NTA His-affinity chromatography column was purchased from Qiagen (Germany).

2.1.3 Media

LB liquid medium contained (per liter): 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride. LB solid medium was supplemented with 20 g agar per liter. Media were sterilized at 121 °C for 20 min, and kanamycin sulfate was added to a final concentration of 50 μ g/mL.

2.1.4 Instruments

Major instruments included an SW-CJ-1F ultra-clean workbench (Suzhou Antai), a J6-MI refrigerated high-speed centrifuge (Zhengzhou Liyan), a JY92-IIDN ultrasonic cell disruptor (Ningbo Xinzhi), a DYY-6C electrophoresis apparatus (Beijing Liuyi), an Agilent 8890 GC-MS system (Agilent, USA), and an ÄKTA protein purification system (GE Healthcare, USA).

2.2 Experimental Methods

2.2.1 Construction of the recombinant β -Gal expression vector

Specific primers were designed from the β -Gal gene sequence: forward 5'-AGAATGCGGCCGCATGAATAAATTTGCACCTTTACATCC-3' and reverse 5'-CCGGAATTCTTAGGCGTGACGACGAAGAAT-3', incorporating restriction sites for cloning. The gene was amplified by PCR and cloned into pET-28a(+) (kanamycin resistance). The PCR product and vector were digested with NotI and EcoRI, respectively, and ligated with T4 DNA ligase. (The restriction enzyme

was NotI, at position 166 of the vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3) and plated on LB agar containing 50 µg/mL kanamycin, followed by overnight incubation at 37 °C.

2.2.2 Phylogenetic and structural analysis

The deduced amino acid sequence of β-Gal was analyzed using BLASTp against the GenBank database. Homologous full-length β-galactosidase sequences were aligned in MEGA, and a neighbor-joining phylogenetic tree was constructed to determine evolutionary relationships (Fig. 1). For structural analysis, the sequence was used to build a template-based homology model on the thermostable GH42 β-galactosidase Gan42B (PDB 4OIF), and, independently, a de novo model was generated with AlphaFold. Model confidence was assessed using the predicted local-distance difference test (pLDDT), and structures were rendered in PyMOL. To distinguish the two approaches, the homology model built on 4OIF is shown in Fig. 3, whereas the AlphaFold prediction is shown in Fig. 4. For the multiple sequence alignment, the query sequence together with the GH42 templates 5DFA and Gan42B (PDB 4OIF) was aligned and rendered with ESPript 3.0; fully conserved residues are boxed in red, and the catalytic residues E157 and E314 are marked (Fig. 2).

2.2.3 Expression of recombinant β-Gal

Recombinant colonies were inoculated into 20 mL of LB with kanamycin and cultured at 37 °C, 150 rpm for 12 h. The culture (3% inoculum) was transferred into 1 L of LB containing kanamycin and grown to an OD₆₀₀ of approximately 0.8. Expression was induced with 1 mM IPTG at 16 °C, 120 rpm for 16 h. Cells were harvested (10,000 rpm, 10 min, 4 °C), washed twice with sterile water, and stored at −20 °C.

2.2.4 Purification and assay of recombinant β-Gal

Cells were resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl) and disrupted by ultrasonication (300 W, 5 s on/5 s off, 20 min, ice bath). Lysates were centrifuged (10,000 rpm, 15 min, 4 °C), and the supernatant was loaded onto a Ni-NTA column on the ÄKTA system. Proteins were washed with buffer A (20 mM Tris-HCl, pH 8.0) and eluted with a gradient of buffer B (20 mM Tris-HCl, pH 7.5, 1 M imidazole). The affinity eluate was further purified by size-exclusion chromatography on a Superdex 200 10/300 GL column and stored at 4 °C. Purity was assessed by SDS-PAGE (10% gel)^[28], and concentration was determined by the BCA assay using bovine serum albumin as the standard^[29].

2.2.5 Enzymatic characterization

Enzyme activity was assayed using ONPG as the substrate. After the reaction, 2 mol/L Na₂CO₃ was added to raise the pH to 9.0 and terminate the reaction. One unit (U) was defined as the amount of enzyme that releases 1 µmol of ONP per minute under optimal conditions^[30]. Specific activity was calculated as

U/mg = $(\Delta A \times V \times 103) / (\epsilon \times t \times m)$, where ΔA is the blank-corrected absorbance, V is the reaction volume (L), ϵ is the molar extinction coefficient of ONP at 420 nm and pH 9 ($4500 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$), t is the reaction time (min), and m is the enzyme mass (mg). The temperature optimum was determined between 0 and 70 °C; thermostability was measured after incubation at 0–50 °C for up to 50 min. The pH optimum was determined in buffers of pH 4–9, and pH stability was assessed after 18 h of incubation at pH 4–9. Metal-ion effects were tested by pre-incubating the enzyme with 2 mmol/L chloride salts of K^+ , Mg^{2+} , Cu^{2+} , Mn^{2+} , Ca^{2+} , Na^+ and Fe^{2+} before activity measurement. Kinetic parameters were determined with ONPG at 2, 8, 12, 16, and 20 mM (a range spanning K_m) and were obtained by nonlinear regression of the Michaelis–Menten equation in OriginPro 2026; the Lineweaver–Burk linearization is provided only for reference (Fig. 14). All assays were performed in triplicate (mean $\pm$ SD). Statistical analysis used one-way ANOVA followed by Tukey’s HSD test in Origin 2026; groups not sharing a letter differ at $p < 0.05$.

2.2.6 Preliminary application in tobacco processing

2.2.6.1 Standard curve

A standard curve was established by plotting OD_{420} (y-axis) against ONP concentration (x-axis, 0.2–1.0 mM). The calibration was linear ($R^2 = 0.998$; Fig. 12), providing a reliable basis for activity determination. (The axes are arranged in the conventional calibration orientation, with the measured signal on the y-axis.)

2.2.6.2 Sensory evaluation of tobacco strands

Fifty grams of air-cured tobacco strands (upper leaves, Zhongyan 100) were treated with recombinant β -Gal at 0.01% (w/w), sprayed evenly onto the strands. Sterile water served as the blank control, and, to distinguish catalytic from non-catalytic (protein/handling) effects, a heat-inactivated enzyme control (enzyme boiled for 10 min before application) was included in parallel. Samples were sealed and incubated at the enzyme optimum (10 °C, pH 6.0) for 6 h, then dried, rolled into cigarettes, and equilibrated for 48 h before evaluation. Sensory evaluation followed the industrial standards YC/T 529-2015^[31] and YC/T 138-2014^[32], with a trained nine-member panel scoring aroma quality, aroma volume, smoke concentration, offensive odor, irritation, aftertaste, and combustibility on blind-coded samples.

2.2.6.3 Enzymatic treatment of reconstituted-tobacco concentrate and aroma analysis

Reconstituted-tobacco concentrate was diluted 1:1 (v/v) with distilled water, adjusted to the enzyme optimum, and treated with 0.05% (w/w) β -Gal at 10 °C for 12 h; a water blank and a heat-inactivated-enzyme control were processed identically. The reaction was stopped by heat inactivation, and 1 mL of the 0.2- μm -filtered solution was spiked with 5 μL of 18.3 mg/100 mL dioctanol (internal standard). Volatiles were analyzed by HS-SPME-GC-MS (Agilent 7890A-5977B; DB-5ms, 60 m $\times$ 250 μm $\times$ 0.25 μm).

Samples were incubated at 60 °C for 20 min and adsorbed for 20 min. The oven program was 40 °C (2 min), 3 °C/min to 60 °C (5 min), 3 °C/min to 220 °C, then 2 °C/min to 230 °C (25 min); inlet 250 °C, split 3:1; helium 1 mL/min; full-scan 35–700 amu. Compounds were identified by retention index (C-n alkane calibration) and library matching.

2.2.6.4 Gradient (time-course) analysis of aroma components

The concentrated material treated as in section 2.2.6.3 was sampled daily over five days, and the evolution of volatile-compound classes was profiled by GC-MS. All experiments were performed in triplicate (mean ± SD); significance was assessed by one-way ANOVA with Duncan's multiple-range test ($p < 0.05$).

3. Results and Discussion

3.1 Molecular identification of recombinant β -Gal

Sequencing showed that the recombinant strain encodes a β -galactosidase of 685 amino acids with a theoretical molecular mass of 77.5 kDa. BLASTp against the NCBI database returned the highest identity (89%) to members of *Klebsiella*. A phylogenetic tree built from closely related sequences placed the target with *K. variicola* β -galactosidases annotated as “1–685” (Fig. 1); the strain was designated 1527.

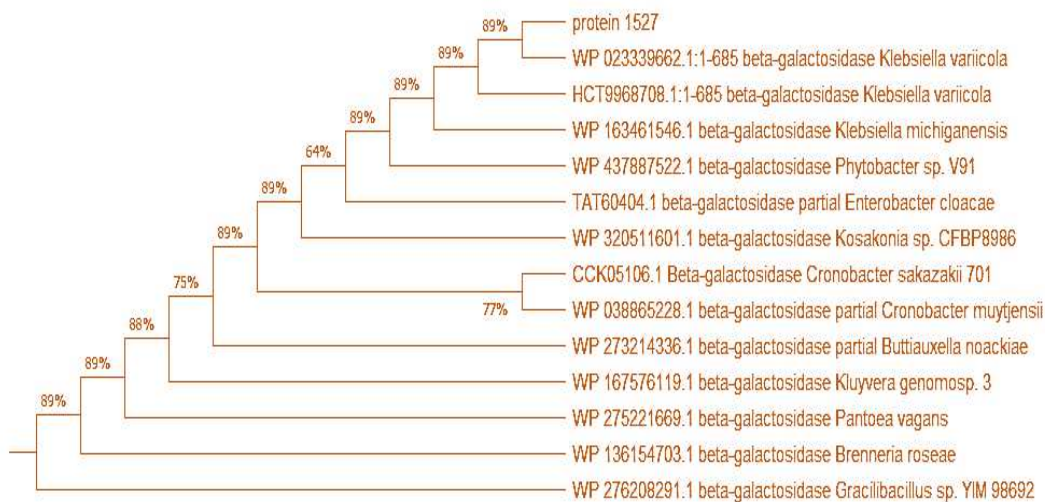
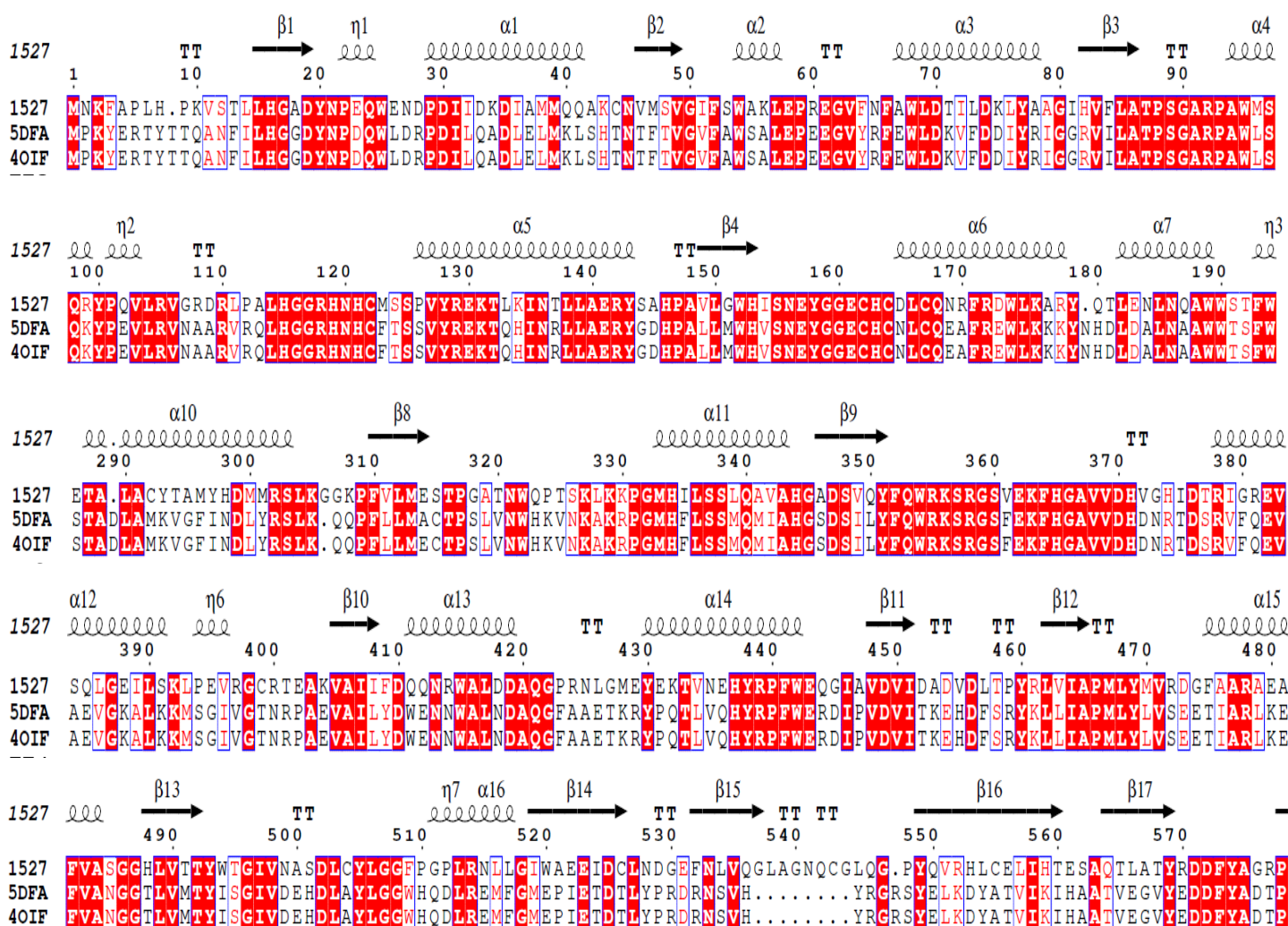


Figure 1. Neighbor-joining phylogenetic tree of recombinant β -galactosidase based on amino-acid sequence alignment. Protein 1527 clusters with *K. variicola* β -galactosidases annotated as 1–685.

Multiple-sequence and secondary-structure alignments among target protein 1527, 5DFA, and thermostable Gan42B (PDB 4OIF)^[33] showed that the core catalytic residues E157 and E314 are strictly conserved across all three sequences (Fig. 2), confirming that protein 1527 is a GH42-family β -

211 galactosidase with the canonical tri-domain architecture (a TIM-barrel catalytic domain, a middle domain,
 212 and a C-terminal β -domain). Although the overall arrangement of α -helices and β -strands aligns well with
 213 thermophilic Gan42B, protein 1527 shows extended loop regions at several sites ($\alpha 7$ – $\eta 3$, $\alpha 8$ – $\beta 5$, $\beta 9$ – $\alpha 11$,
 214 $\beta 11$ – $\beta 12$ and the C-terminal tail). Consistent with a cold-adaptation strategy, the cold-active character of
 215 protein 1527 appears to arise mainly from these extended, flexible surface loops rather than from a global
 216 compositional bias: the whole-sequence composition (36 Pro, 47 Gly; Gly/Pro ≈ 1.31) is close to that of the
 217 thermostable template Gan42B (28 Pro, 39 Gly; Gly/Pro ≈ 1.39), so the increased backbone flexibility that
 218 lowers the energy barrier for low-temperature catalysis is contributed chiefly by loop elongation and local
 219 substitutions^[34–36] (Supplementary Table S2). Together, these features indicate that protein 1527 is a cold-
 220 active β -galactosidase with high predicted activity at low temperature but relatively weak thermostability.



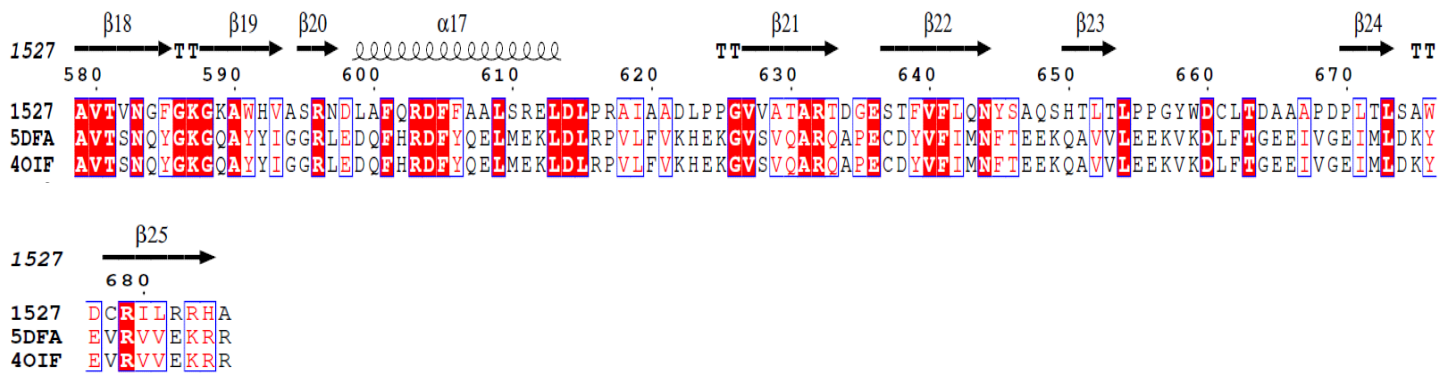


Figure 2. Multiple-sequence and secondary-structure alignment of the target β -galactosidase with GH42 templates 5DFA and Gan42B (PDB 4OIF), rendered with ESPrpt 3.0. Strictly conserved residues are boxed in red; the catalytic glutamates E157 and E314 are conserved across all three sequences.

The homology-modeled three-dimensional structure of protein 1527 shows the typical GH42 tri-domain organization: Domain A (a catalytic TIM-barrel), Domain B, and Domain C (Fig. 3A). The two catalytic glutamates E157 and E314 lie within the central cavity of the TIM-barrel, forming the active-site cleft, consistent with their strict conservation in the alignment. Superposition on the thermophilic template Gan42B (4OIF) shows a globally well-aligned backbone, with the main divergences confined to surface loops and inter-domain linkers rather than the catalytic core (Fig. 3B). An independent AlphaFold prediction reproduced the same tri-domain fold with high per-residue confidence, corroborating the homology model (Fig. 3C).

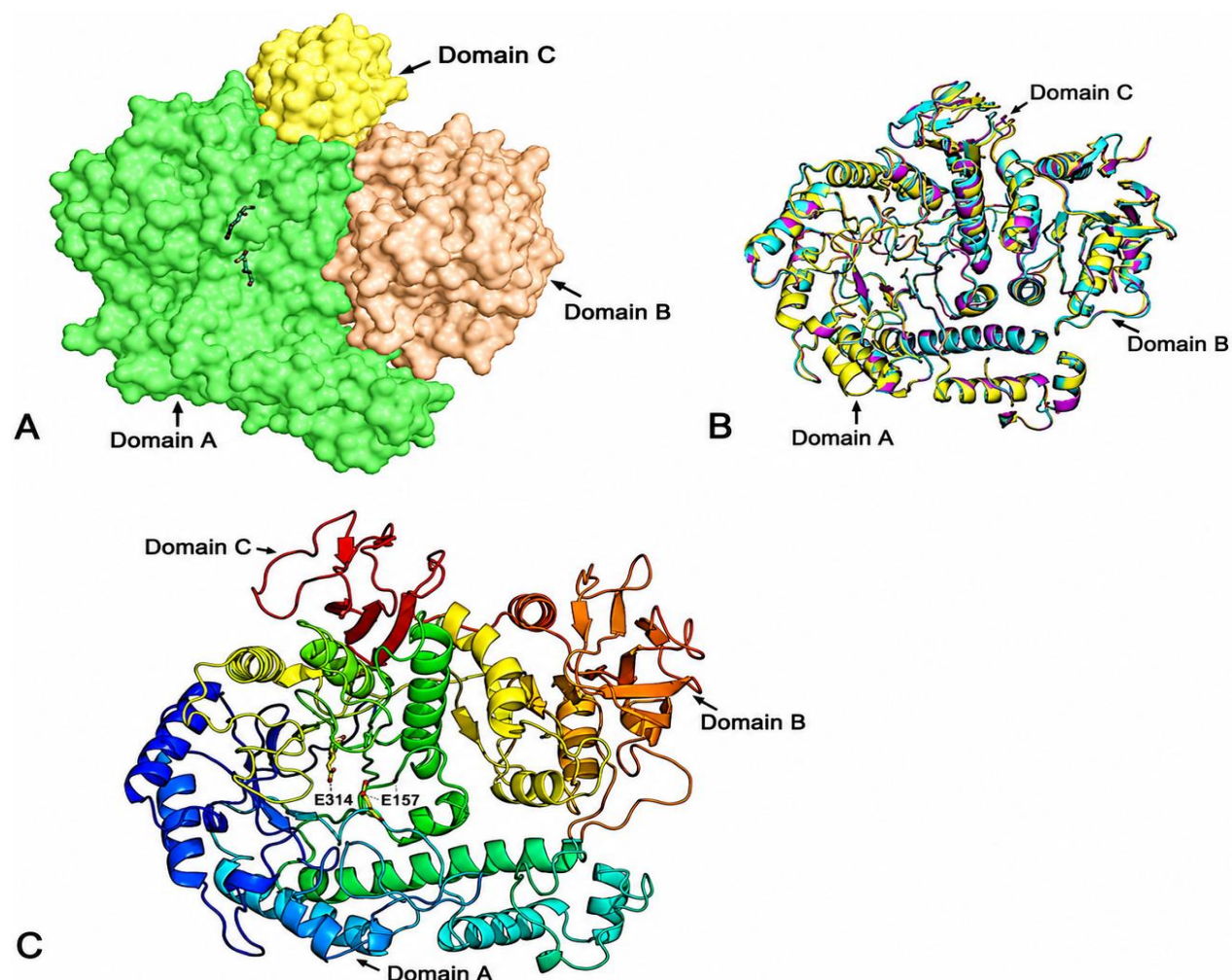


Figure 3A,B,C. Structural models of the target GH42 β -galactosidase. **A:** Homology-modeled structure of the target β -galactosidase, built using Gan42B (PDB 4OIF) as the template and rendered in PyMOL, showing the tri-domain architecture: Domain A (catalytic TIM-barrel, green), Domain B (beige), and Domain C (yellow), with the two catalytic glutamate residues shown as sticks within the active-site pocket. **B:** Independent de novo AlphaFold model of β -galactosidase 1527, colored according to domain and prediction confidence, reproducing the characteristic GH42 tri-domain fold. **C:** Superposition of the homology-modeled target structure (cyan/magenta) onto the Gan42B template (gold), demonstrating the structural similarity between the modeled target and template. The AlphaFold prediction provides an independent structural validation of the domain organization and overall fold observed in the template-based homology model.

3.2 Construction of the recombinant β -Gal expression vector

The recombinant plasmid pET-28a(+)-1527 (β -Gal) was successfully constructed (Supplementary Fig. 1). Key elements include the T7 promoter, *lacI* operator, 6 $\times$ His tag, and kanamycin-resistance gene. The target gene was inserted between the NotI (position 166) and EcoRI (position 2231) sites, confirming correct cloning.

3.3 Expression and purification of recombinant β -Gal

SDS-PAGE of lysates before and after induction (Fig. 4A) showed no distinct band pre-induction but a clear band after induction; the apparent mass was ≈ 70 – 100 kDa, consistent with the theoretical mass of 77.5 kDa deduced from the 685 -residue sequence. Ni-NTA affinity chromatography (Fig. 4B) resolved a flow-through peak and a target-protein peak eluting at $\approx 20\%$ buffer B (≈ 200 mM imidazole), reaching ≈ 2050 mAU. Size-exclusion chromatography on Superdex 200 gave a single, symmetric peak, indicating a homogeneous, monodisperse preparation (Supplementary Fig. 2). Quantitative re-analysis of the raw chromatogram confirmed a single elution peak accounting for $\approx 95\%$ of the total A280 peak area, with no aggregate or secondary peaks exceeding 5% of the main peak and a low A254/A280 ratio (≈ 0.42), together indicating a highly pure, monodisperse, and largely nucleic-acid-free preparation. The purified enzyme reached 17.15 ± 0.32 mg/mL, corresponding to ≈ 214 mg per liter of culture.

The apparent mass of ≈ 77 kDa recovered by SEC matches the theoretical mass of a single subunit and the SDS-PAGE band. However, GH42 β -galactosidases are characteristically homotrimeric (≈ 200 – 230 kDa); the apparent ≈ 77 kDa obtained here therefore most likely reflects the subunit (monomer) mass under the calibration used rather than a genuinely monomeric native state. A definitive assignment of the native oligomeric state will require calibrated SEC coupled to multi-angle light scattering (SEC-MALS) or analytical ultracentrifugation.

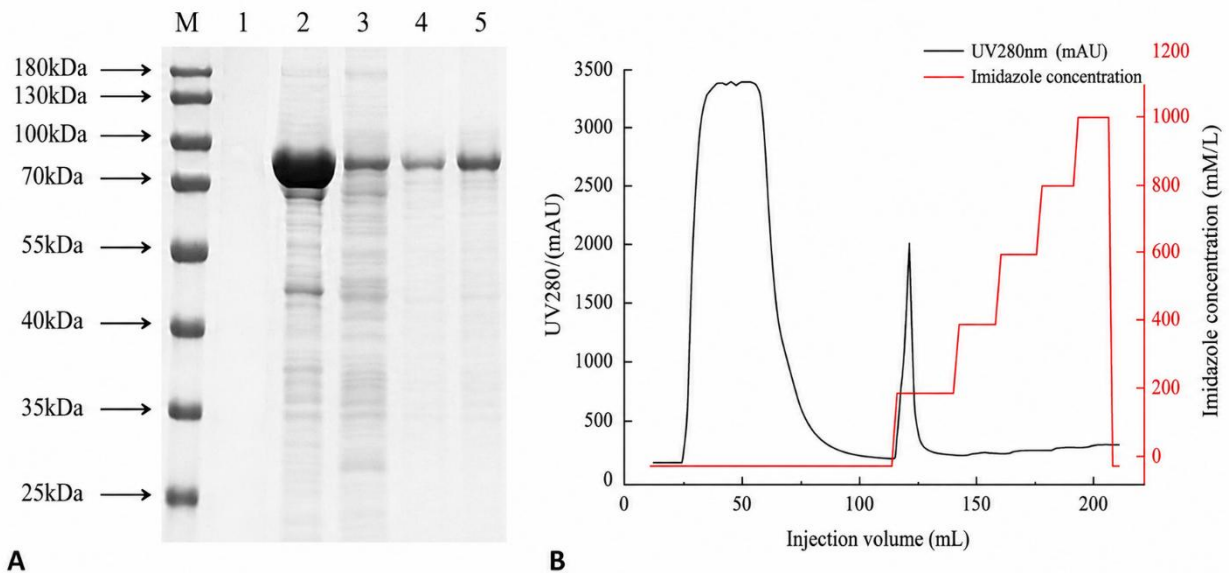


Figure 4A,B. Purification of recombinant β -galactosidase 1527. **A:** SDS-PAGE analysis of recombinant β -galactosidase 1527: lane M, protein marker; lane 1, pre-induction lysate; lane 2, post-induction lysate; lane 3, Ni-NTA flow-through; lane 4, Ni-NTA eluate; lane 5, size-exclusion-purified enzyme. A single band at ≈ 77 kDa is observed in the purified enzyme, consistent with the theoretical subunit molecular mass. **B:** Ni-NTA affinity chromatography

profile of recombinant β -galactosidase 1527, showing UV280 absorbance (black) and the stepwise imidazole gradient (red). The target protein eluted at approximately 20% buffer B (≈ 200 mM imidazole).

3.4 Functional characterization

3.4.1 Temperature dependence and thermostability

The enzyme was maximally active at 10 °C (defined as 100%); activity rose steeply between 0 and 10 °C, fell sharply between 10 and 30 °C, and dropped below 20% at ≥ 30 °C (Fig. 5A). The mesophilic organism *K. variicola* thus encodes an enzyme with an unusually low, sharp temperature optimum; such a mismatch between host physiology and enzyme optimum is not unprecedented, as psychrophilic-type, thermolabile enzymes can occur in mesophilic and even thermophilic genomes. The cold-active behavior here is supported by the sequence-level flexibility features noted above. To exclude assay artifacts due to temperature-dependent ONP color development and substrate/enzyme handling, activities were normalized to ONP standard curves determined at each assay temperature, and reactions were terminated with Na_2CO_3 before absorbance measurement. Thermostability (Fig. 5B) was high at 0–20 °C ($>90\%$ residual activity after 30 min) but decreased markedly at higher temperatures, falling to $\approx 20\%$ at 50 °C after 30–50 min, confirming a heat-labile, cold-active character. For comparison, the cold-adapted β -galactosidase from *Rahnella* sp. R3³⁵ has a higher optimum (35 °C) and retains only $\approx 30\%$ activity at 10 °C; the enzyme reported here is therefore comparatively more cold-active, although we avoid describing it as categorically “superior”.

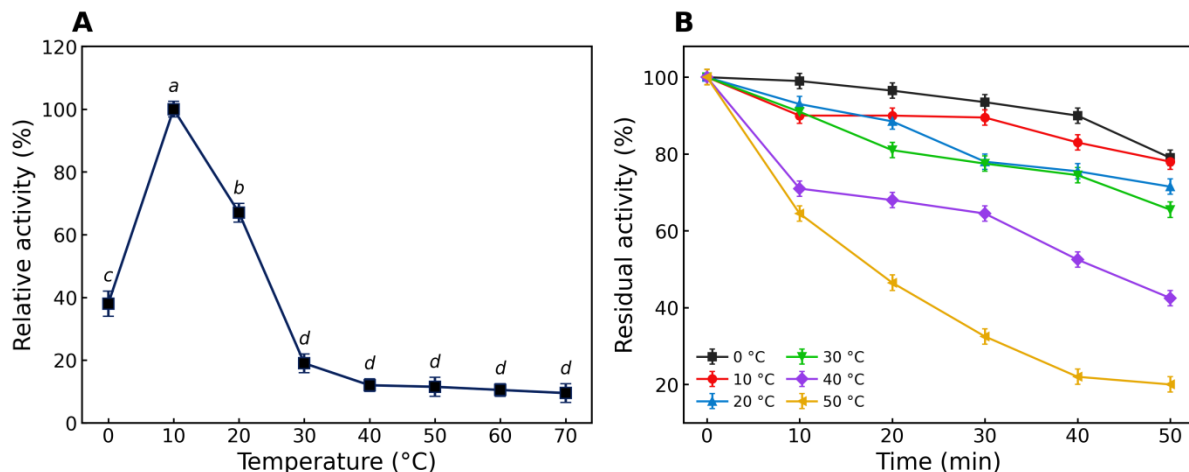


Figure 5A,B. Temperature characteristics of recombinant β -galactosidase 1527. **A:** Effect of temperature on enzyme activity (optimum at 10 °C). **B:** Thermostability: residual activity after incubation at 0–50 °C for 10–50 min. Values are mean $\pm$ SD ($n = 3$); different letters in (A) indicate significant differences ($p < 0.05$, one-way ANOVA with Tukey’s HSD).

3.4.2 Effect of pH on activity and stability

The enzyme was optimally active at pH 6.5, with a broad, weakly acidic plateau that retained > 85% activity between pH 6.0 and 7.0 (Fig. 6A). pH stability was greatest near pH 6.0, where > 85% of activity was retained after 18 h (Fig. 6B). (The application experiments were performed at pH 6.0, within this near-optimal plateau and close to the natural pH of tobacco extracts.) Across pH 4.5–9.5, the enzyme retained a substantial fraction of activity, indicating good operational stability under weakly acidic conditions. This broad pH tolerance aligns with the general biophysical signature of cold adaptation: compared with mesophilic and thermophilic homologs, psychrophilic enzymes typically have a lower density of ion pairs, hydrogen bonds, and other stabilizing interactions, along with a higher proportion of flexible surface loops. This looser, more mobile architecture lowers the activation energy for catalysis at low temperatures and tends to flatten the pH-activity profile by allowing catalytic and surface-ionizable residues to adopt favorable protonation states over a wider pH range^[37]. The extended, cold-adaptive surface loops identified in protein 1527 relative to the thermostable template Gan42B (Section 3.1) are therefore a plausible structural basis for both its low-temperature optimum and its comparatively broad pH plateau, a combination also noted in other cold-active hydrolases.

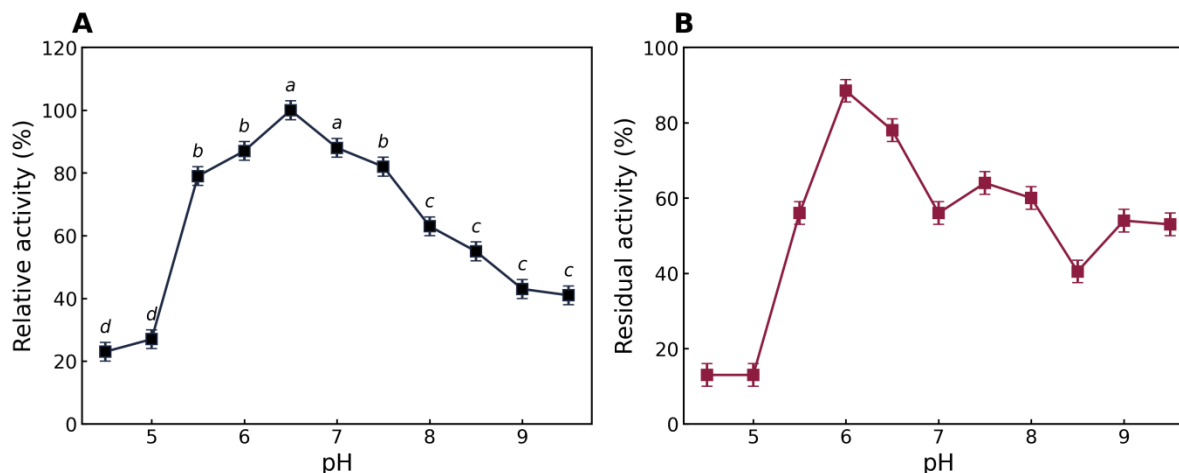


Figure 6A,B. pH characteristics of recombinant β-galactosidase 1527. **A:** Effect of pH on relative activity (optimum pH 6.5). **B:** pH stability after 18 h of incubation at each pH. Values are mean ± SD (n = 3); different letters in (fig. 6a) indicate significant differences (p < 0.05, one-way ANOVA with Tukey's HSD).

3.4.3 Effect of metal ions

Metal-ion effects were assessed at 2 mmol/L relative to a no-ion control (100%). Fe²⁺ strongly activated the enzyme, raising relative activity to 144.6 ± 3.2%, whereas Na⁺ had little effect (≈91%) and K⁺ was mildly inhibitory (≈75%). Mg²⁺, Cu²⁺, and Mn²⁺ were more strongly inhibitory (≈29–36%), and Ca²⁺ was the most inhibitory (≈19%) (Fig. 7). Fe²⁺ is thought to enhance β-galactosidase activity by stabilizing the active-site conformation³⁶. By contrast, the pronounced inhibition by Ca²⁺ (≈19%) and by Mg²⁺, Cu²⁺,

and Mn^{2+} ($\approx 29\text{--}36\%$) indicates that these ions do not simply occupy the same activating site as Fe^{2+} but instead perturb the enzyme through non-productive binding or conformational effects. In the structurally related GH42 enzyme Gal42 from a marine *Bacillus* sp., divalent cations bound at loops flanking the active-site cleft: several (e.g., Mg^{2+} , Mn^{2+}) stabilized the catalytic domain and enhanced activity, whereas Cu^{2+} produced pronounced inactivation, showing that closely related divalent cations can have opposite effects on the same GH42 fold depending on the local coordination environment^[38]. A plausible explanation for protein 1527 is that Ca^{2+} , Cu^{2+} , and Mn^{2+} compete with Fe^{2+} for a shared regulatory site near the catalytic TIM-barrel, or rigidify the extended cold-adaptive surface loops (Section 3.1) important for low-temperature turnover, thereby offsetting any structural stabilization these ions might otherwise provide. Because such ion-specific and species-specific divergence is common among β -galactosidases from different sources, direct structural or mutagenesis evidence (for example, alanine substitution of candidate coordinating residues near E157/E314) will be required to confirm the site(s) responsible for activation versus inhibition in protein 1527.

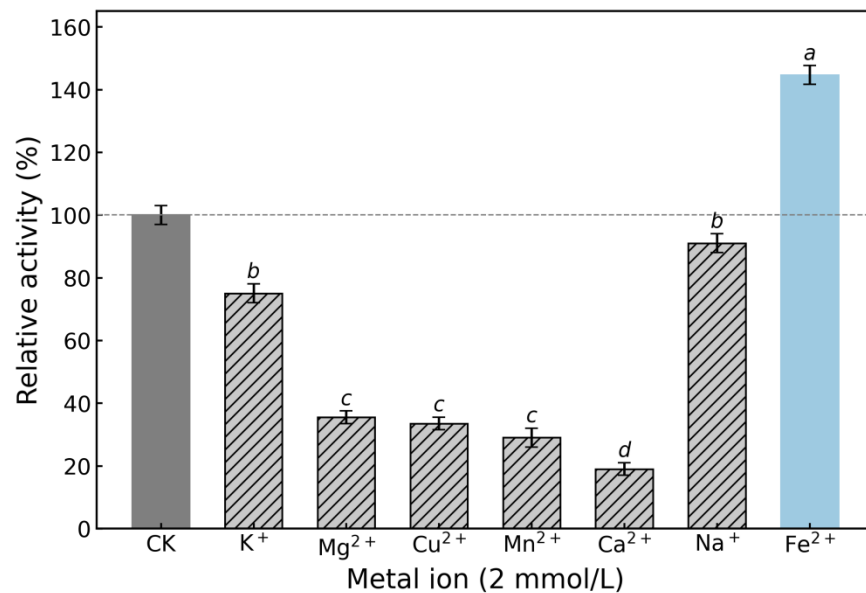


Figure 7. Effect of metal ions (2 mmol/L) on the activity of recombinant β -galactosidase 1527, relative to a no-ion control (CK). Values are mean $\pm$ SD ($n = 3$); different letters indicate significant differences ($p < 0.05$, one-way ANOVA with Tukey's HSD).

3.4.4 Purification summary

A summary of the purification is given in Table 1. Protein concentrations of the crude extract, affinity eluate, and SEC-purified enzyme were 30.1 ± 0.2 , 28.1 ± 0.2 , and 21.7 ± 0.1 mg/mL, and the corresponding specific activities were 3.05 ± 0.06 , 10.5 ± 0.15 , and 82.74 ± 1.23 U/mg, giving an overall ≈ 27 -fold increase in specific activity. This ≈ 27 -fold enrichment, achieved with a two-step Ni-NTA affinity/size-exclusion

protocol, compares favorably with other recombinant cold-active GH42 β -galactosidases purified from *E. coli*; for example, the deep-sea enzyme from *Alteromonas* sp. ML52 was reported to yield 55.2 mg of purified protein per liter of induced culture with a final specific activity of 467.2 U/mg on ONPG. The higher volumetric yield obtained here (≈ 214 mg/L) alongside a lower final specific activity (82.74 U/mg) most likely reflects the low assay temperature used throughout this study (10 °C, matched to the activity optimum of protein 1527) rather than incomplete purification, since SDS-PAGE and the quantitative SEC re-analysis (Section 3.3) both indicated a single, homogeneous species; a side-by-side comparison of specific activities determined at each enzyme's own temperature optimum would be needed to disentangle intrinsic catalytic differences from assay-condition effects.

Table 1. Purification summary of recombinant β -galactosidase 1527.

Purification step	Protein conc. (mg/mL)	Specific activity (U/mg)	Purification (fold)
Crude cell extract	30.1 ± 0.2	3.05 ± 0.06	1.0
Ni-NTA affinity eluate	28.1 ± 0.2	10.5 ± 0.15	3.4
SEC-purified enzyme	21.7 ± 0.1	82.74 ± 1.23	27.1

Note: values are mean $\pm$ SD ($n = 3$). A full mass balance (total activity and % yield) requires the individual fraction volumes, which were not archived with the present dataset; these columns should be completed by the authors from the purification records.

3.4.5 Enzyme kinetics

Enzyme activity was quantified using an ONP standard curve prepared in the conventional calibration orientation (OD₄₂₀ versus ONP concentration), which was linear over the range 0.2–1.0 mM and had $R^2 = 0.998$ (Fig. 8A).

Steady-state kinetics were determined using ONPG at 2–20 mM and fitted by nonlinear regression of the Michaelis–Menten equation (Fig. 8B). The fit, $v = 222.2 \cdot [S] / (3.05 + [S])$ ($R^2 = 0.998$), yielded $V_{max} = 222.2 \pm 1.3$ $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 3.05 \pm 0.20$ mM. Using the subunit mass of 77.5 kDa, these values correspond to a turnover number $k_{cat} \approx 287$ s^{-1} and a catalytic efficiency $k_{cat}/K_m \approx 9.4 \times 10^4$ $\text{M}^{-1} \cdot \text{s}^{-1}$, indicating efficient catalysis at low temperature (Table 2). Because the fitted K_m is ≈ 3 mM, and the substrate series (2–20 mM) brackets this value, the Lineweaver–Burk double-reciprocal plot (Fig. 8C), which gave the same V_{max} , is retained only for reference because linear transformation distorts the error structure.

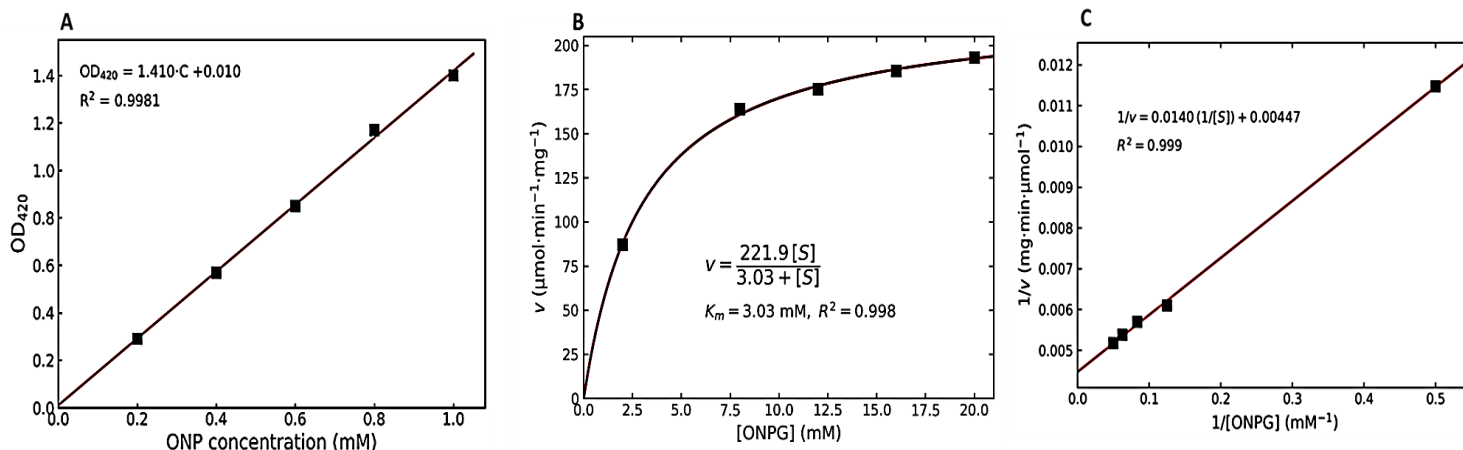


Figure 8A,B,C. ONP standard curve and kinetic characterization of recombinant β -galactosidase 1527. **A:** ONP standard curve used for quantification of β -galactosidase activity, plotted as OD₄₂₀ versus ONP concentration. Data are presented as mean $\pm$ SD ($n = 3$). **B:** Michaelis–Menten kinetics of recombinant β -galactosidase 1527 using ONPG as the substrate. Data points represent the mean of three independent measurements ($n = 3$), and the curve represents the nonlinear regression fit to the Michaelis–Menten equation. **C:** Lineweaver–Burk (double-reciprocal) plot of recombinant β -galactosidase 1527 shown for reference. Nonlinear regression was used as the primary method for estimating kinetic parameters because linear transformations can distort the experimental error structure.

Table 2. Steady-state kinetic parameters of recombinant β -galactosidase 1527 (ONPG substrate, 10 °C, pH 6.0).

Parameter	Value	Method
K_m	3.05 ± 0.20 mM	Nonlinear MM regression
V_{max}	222.2 ± 1.3 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$	Nonlinear MM regression
k_{cat}	≈ 287 s^{-1}	$V_{max} \times M$ (77.5 kDa)
k_{cat}/K_m	$\approx 9.4 \times 10^4$ $\text{M}^{-1}\cdot\text{s}^{-1}$	Derived

Note: all values highlighted as revised to a single internally consistent set (mean $\pm$ SD, $n = 3$).

3.4.6 Comparison with previously reported cold-active β -galactosidases

To place the enzyme in context, its properties were compared with those of the marine cold-active β -galactosidase reported by Cui et al.^[39](Table 3). Protein 1527 has a lower temperature optimum (10 vs 15 °C) and strong Fe^{2+} activation, which the reference enzyme lacks.

Compared with the broader panel of cold-active β -galactosidases, protein 1527 is competitive rather than exceptional. Its turnover number ($k_{cat} \approx 287$ s^{-1}) exceeds that of the GH42 enzyme from an Antarctic *Arthrobacter* isolate (≈ 102 s^{-1}) and is comparable to those of enzymes from *Halomonas* sp. S62 (≈ 390 s^{-1}) and *Pseudoalteromonas haloplanktis* (≈ 200 s^{-1}), while remaining below that of the highly active deep-sea enzyme from *Alteromonas* sp. ML52 ($\approx 3.7 \times 10^3$ s^{-1}). Its catalytic efficiency on ONPG ($k_{cat}/K_m \approx 9.4 \times 10^4$ $\text{M}^{-1}\cdot\text{s}^{-1}$) is comparable to that of the cold-active *Paracoccus* sp. 32d enzyme ($\approx 6 \times 10^4$ $\text{M}^{-1}\cdot\text{s}^{-1}$) and typical of the family (usually 10^4 – 10^6 $\text{M}^{-1}\cdot\text{s}^{-1}$ on ONPG). The principal distinguishing feature of protein

1527 is therefore not a record catalytic efficiency but its combination of an unusually low temperature optimum (10 °C), good specific activity, and pronounced Fe²⁺ activation, which together suit it for low-temperature bioprocessing.

Table 3. Comparison of recombinant β -galactosidase 1527 with a reported marine cold-active β -galactosidase.

Property	This study: β -Gal 1527 (<i>K. variicola</i> GB3)	Reference: marine cold-active β -Gal (Cui et al., 2013)
Source	<i>K. variicola</i> GB3; first cold-active β -Gal from this species	Undefined wild marine psychrotolerant strain
Temperature optimum/stability	10 °C; >90% after 30 min at 0–20 °C; <20% above 30 °C	15 °C; $\approx$ 70% of maximum at 10 °C; drops above 25 °C
pH optimum/stability	pH 6.5; broad plateau pH 6.0–7.0	pH 6.5; stable pH 6.0–7.5
K_m	3.05 mM	28 μ M (0.028 mM)
V_{max}	222.2 μ mol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹	$\approx$ 112 μ mol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹
k_{cat}/K_m	$\approx 9.4 \times 10^4$ M ⁻¹ $\cdot$ s ⁻¹	not reported
Metal-ion response (2 mmol/L)	Strong Fe ²⁺ activation (144.6%); strong Ca ²⁺ inhibition; Na ⁺ negligible	No Fe ²⁺ activation; slight Na ⁺ /K ⁺ stabilisation; inhibited by heavy metals

3.5 Application to tobacco

3.5.1 Sensory evaluation of Zhongyan-100 tobacco strands

Sensory evaluation (Fig. 9) showed that β -Gal-treated strands scored higher than the water control for aroma quality, intensity, smoothness, and sweetness^[40]. A plausible mechanism is the hydrolysis of β -galactosidic bonds in cell-wall polysaccharides, loosening the matrix and releasing or generating flavor-related compounds; released oligosaccharides could also feed Maillard reactions during subsequent thermal processing.

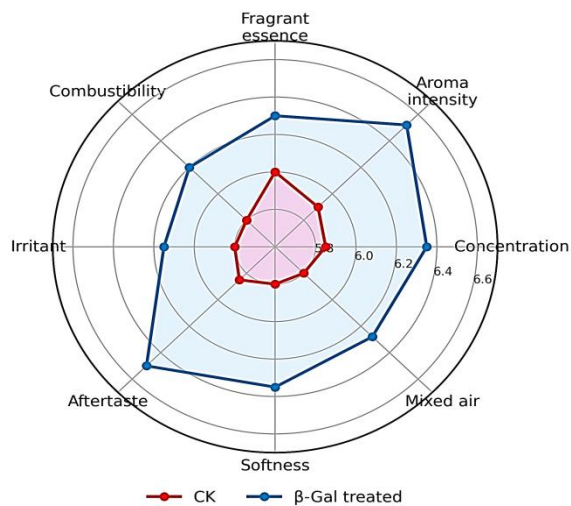


Figure 9. Sensory radar comparing the water control (CK) with recombinant β -galactosidase-treated Zhongyan-100 tobacco strands across eight attributes. Values are the mean of the panel scores (n = 3).

3.5.2 Effect on aroma components of reconstituted-tobacco concentrate

HS-SPME-GC-MS analysis showed that all 16 monitored aroma compounds increased after β -Gal treatment, by 16.6–91.0% (Fig. 10; Table 4). Benzyl alcohol showed the largest relative increase, from 4.36 ± 0.08 to 8.33 ± 0.16 $\mu\text{g}/100$ mL (+90.9%); benzyl alcohol contributes floral–fruit notes^[41]. Other notable increases included 9-hydroxy-megastigma-dien-3-one (+84.8%), theaspirone (+73.1%), 1-octen-3-ol (+68.1%), and 2-acetylpyrrole (+61.6%), which contribute herbal, sweet-floral, mushroom, and roasted notes^[42–44]. In absolute terms, solanone increased most, from 48.98 ± 0.95 to 65.12 ± 1.28 $\mu\text{g}/100$ mL (+16.1 $\mu\text{g}/100$ mL, +33.0%).

Mechanistically, these increases are consistent with the enzymatic release of glycosidically bound aroma precursors: in tobacco, as in grape, tea, and fruit matrices, many odor-active compounds, particularly C_{13} -norisoprenoids such as β -damascenone and the megastigmatrienones, accumulate as non-volatile glycoconjugates that glycoside hydrolases can convert into their free, volatile aglycones^[45]. Because β -damascenone and related norisoprenoids have extremely low odor-detection thresholds (β -damascenone is perceptible at nanogram-per-liter levels), even moderate percentage gains observed here are expected to translate into disproportionately large sensory effects^[46]. This positions the cold-active β -galactosidase not merely as a processing aid but as a tool for the controlled, low-temperature generation of high-value aroma compounds relevant to the flavor and fragrance industries. Beyond glycoside hydrolases, endogenous tobacco enzymes, such as those in the lipoxygenase pathway, also shape the leaf volatile pool, underscoring that enzyme-based interventions are central to tailoring tobacco aroma^[47].

The remaining compounds in Table 4 further support this glycoside-hydrolysis mechanism, and each carries a distinct sensory role in tobacco. Linalool (+19.1%) and 2-phenylethanol (+28.5%) are floral, rose-like monoterpene and phenylpropanoid volatiles, respectively, long recognized as principal aroma-active markers of flue-cured and cigar tobacco^[48]. Dihydro- β -ionone (+49.3%) and 4,7,9-megastigmatrien-3-one (tabanone; +16.6%) are carotenoid degradation products, together with β -damascenone (+30.7%, above), that impart floral-fruity and characteristic sweet ‘tobacco’ notes. They are considered among the most sensorially important norisoprenoids in cured tobacco because of their very low odor thresholds; their concerted increase after β -Gal treatment is consistent with the release of shared or structurally related glycosylated carotenoid-derived precursors. Dihydroactinidiolide (+32.1%) and 6-methyl-5-hepten-2-one (+25.4%), described respectively as oily-fruity-sweet and fruity in cigar tobacco, are further oxidative degradation products of the same carotenoid pool. 2-Phenylacetaldehyde (+20.3%), a Strecker-type aldehyde with a honey-floral odor commonly reported among the principal volatiles of cured tobacco extracts, and 2,3-dihydro-2,2,6-trimethylbenzaldehyde (+36.4%), a related cyclic terpenoid-derived aldehyde, likely arise via comparable oxidative cleavage of terpenoid or carotenoid precursors that were

previously masked as glycoconjugates. Finally, 6,6-dimethyl-3-ethoxymethylene-5,6-dihydropyran-2,4-dione (+56.5%) is structurally consistent with a furanone/pyranone-type Maillard product; its increase after enzymatic treatment suggests that the galactose and shortened polysaccharide fragments liberated by β -Gal hydrolysis, in addition to feeding Maillard chemistry during subsequent thermal processing, as already noted, may also support limited non-enzymatic browning during the 10 °C incubation itself. Collectively, the concordant rise of these structurally diverse but mechanistically linked compounds reinforces the conclusion that β -Gal 1527 acts broadly on glycosidically bound aroma precursors rather than on a single compound class.

Table 4. Key aroma compounds in reconstituted-tobacco concentrate before and after β -galactosidase treatment.

No.	Compound	Blank ($\mu\text{g}/100\text{ mL}$)	β -Gal ($\mu\text{g}/100\text{ mL}$)	Increase (%)
1	Linalool	3.02 $\pm$ 0.06	3.59 $\pm$ 0.07	19.1
2	Solanone	48.98 $\pm$ 0.95	65.12 $\pm$ 1.28	33.0
3	4,7,9-Megastigmatrien-3-one	10.36 $\pm$ 0.20	12.07 $\pm$ 0.23	16.6
4	9-Hydroxy-megastigma-dien-3-one	0.38 $\pm$ 0.01	0.70 $\pm$ 0.02	84.8
5	1-Octen-3-ol	0.92 $\pm$ 0.02	1.55 $\pm$ 0.03	68.1
6	Dihydro- β -ionone	8.04 $\pm$ 0.15	12.00 $\pm$ 0.22	49.3
7	Damascenone	16.93 $\pm$ 0.32	22.13 $\pm$ 0.41	30.7
8	Dihydroactinidiolide	4.04 $\pm$ 0.08	5.34 $\pm$ 0.10	32.1
9	2-Phenylacetaldehyde	6.47 $\pm$ 0.12	7.79 $\pm$ 0.15	20.3
10	2,3-Dihydro-2,2,6-trimethylbenzaldehyde	3.76 $\pm$ 0.07	5.13 $\pm$ 0.10	36.4
11	Theaspirone	1.11 $\pm$ 0.02	1.92 $\pm$ 0.04	73.1
12	6-Methyl-5-hepten-2-one	7.66 $\pm$ 0.14	9.60 $\pm$ 0.18	25.4
13	2-Acetylpyrrole	0.89 $\pm$ 0.02	1.45 $\pm$ 0.03	61.6
14	Benzyl alcohol	4.36 $\pm$ 0.08	8.33 $\pm$ 0.16	90.9
15	6,6-Dimethyl-3-ethoxymethylene-5,6-dihydropyran-2,4-dione	1.77 $\pm$ 0.03	2.77 $\pm$ 0.05	56.5
16	2-Phenylethanol	9.12 $\pm$ 0.17	11.72 $\pm$ 0.22	28.5

Note: values are mean $\pm$ SD ($n = 3$); all increases significant at $p < 0.05$.

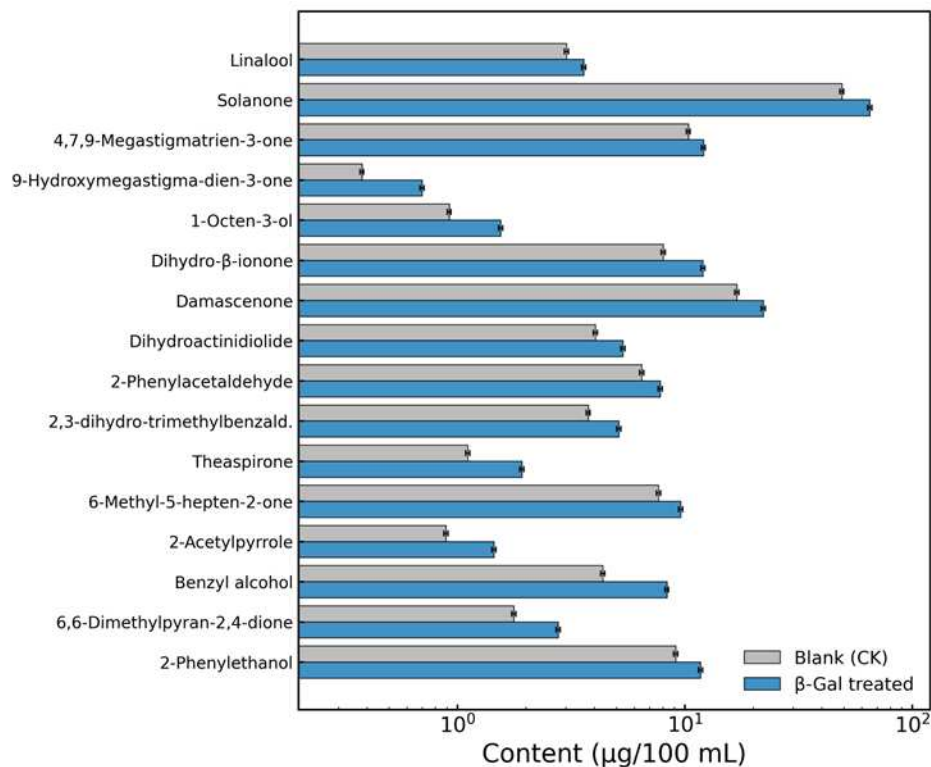


Figure 10. Key aroma compounds in reconstituted-tobacco concentrate before (blank) and after β -galactosidase treatment, measured by HS-SPME-GC-MS (log scale). Values are mean $\pm$ SD (n = 3).

3.5.3 Time-course of volatile classes

Daily profiling of the treated concentrate showed that esters were the most abundant class and peaked within the first two days before declining, while phenols, ketones, and benzene derivatives peaked on day 1–2 and then decreased; aldehydes, heterocyclic compounds, and hydrocarbons remained comparatively low throughout (Fig. 11). The full-time-course values are provided in Supplementary Table S1.

This transient, early-peaking pattern parallels observations in other *K. variicola*-associated reconstituted-tobacco systems. Macrotranscriptomic profiling of *K. variicola* H8 during fermentation of reconstituted-tobacco concentrate showed that several aroma-active metabolites, including linalool, 2-acetylpyrrole, and benzyl alcohol, peaked around 24 h before declining as fermentation continued^[49]. A metagenomic analysis of *K. variicola*-driven reconstituted tobacco concentrate processing likewise reported that norisoprenoid and other aroma-enhancing compounds increased and then declined as readily available nutrients were progressively depleted^[50]. Because the reconstituted-tobacco concentrate used here is itself a fermentation-derived matrix that retains a background microbiota, a comparable dynamic rapid release of esters, phenols, ketones, and benzene derivatives from glycosidic and other bound precursors during the first 1–2 days, followed by their consumption or further biotransformation once readily

accessible precursors are exhausted offers a plausible explanation for the early peak and subsequent decline in Fig. 11. However, the present design did not distinguish the relative contributions of continued β -Gal catalysis, residual microbial metabolism, and simple volatilization over the extended incubation. The comparatively low and stable levels of aldehydes, heterocyclic compounds, and hydrocarbons throughout the 5-day window suggest that these classes are either minor products of β -galactosidase-mediated deglycosylation in this matrix or are rapidly transformed further (e.g., aldehydes into the corresponding alcohols or acids); their dynamics merit targeted profiling in future work.

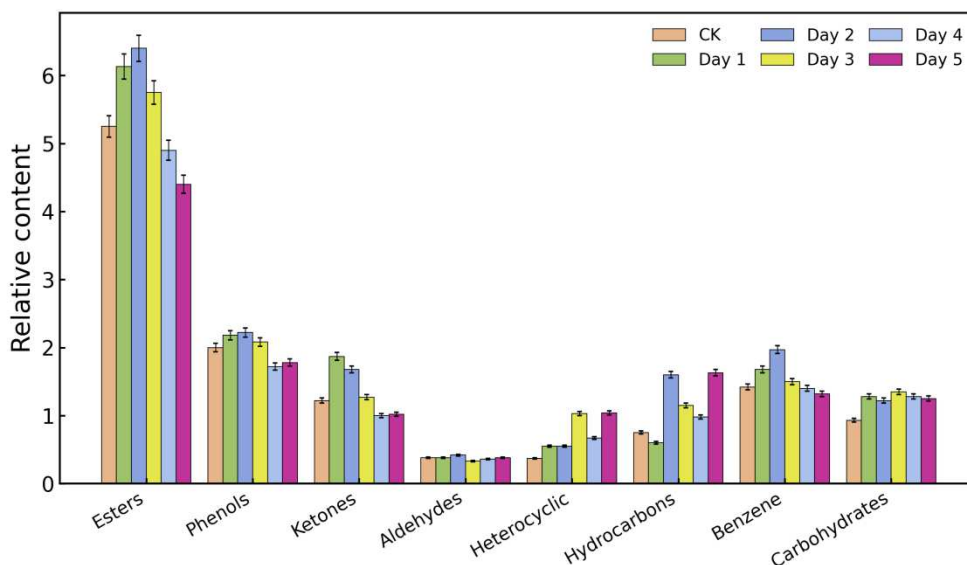


Figure 11. Time-course of volatile-compound classes in reconstituted-tobacco concentrate over five days of β -galactosidase treatment (relative content). Values are mean $\pm$ SD ($n = 3$).

3.6 Estimated economic and environmental impact

Because protein 1527 is fully active at 10 °C, it can drive the same β -galactosidic hydrolysis without the heating that a conventional mesophilic or thermostable β -galactosidase (for example, the GH42 enzyme Gan42B, optimum $\approx$ 50 °C) requires, an intrinsic advantage of cold-active biocatalysts. For an illustrative 10 m³ aqueous batch (specific heat 4.18 kJ kg⁻¹ K⁻¹) warmed from 25 °C to 50 °C at 85 % heating efficiency, the sensible-heat demand is $\approx$ 342 kWh per batch ($\approx$ 34 kWh m⁻³); operating at 10 °C avoids all of this heating and the subsequent re-cooling of the product. At an industrial electricity price of US\$0.10 kWh⁻¹ and the Chinese national-grid emission factor of 0.5703 kg CO₂e kWh⁻¹^[51], this equals $\approx$ US\$34 and $\approx$ 195 kg CO₂e saved per batch ($\approx$ US\$3.4 and $\approx$ 19.5 kg CO₂e m⁻³); a 3 000 m³ yr⁻¹ line would save $\approx$ 100 MWh, $\approx$ US\$10 000, and $\approx$ 58 t CO₂e per year (Table 5). The 10 °C activity optimum, Fe²⁺ activation (+44.6%) and 16.6–91.0% aroma-compound increases at 10 °C are experimentally demonstrated, whereas the energy, cost and CO₂ values are engineering projections: they scale roughly linearly with batch volume and with

the ambient-to-operating temperature gap ($\approx$ US\$13–109 and $\approx$ 41–355 kg CO₂e per 10 m³ batch across a 37–65 °C conventional optimum and US\$0.08–0.20 kWh⁻¹) and fall further on lower-carbon grids.

Table 5. Illustrative per-batch energy, cost, and carbon-footprint comparison of a conventional ($\approx$ 50 °C) and the cold-active (10 °C) β -galactosidase for a 10 m³ aqueous reaction (25 °C ambient).

Parameter	Conventional β -Gal ($\approx$ 50 °C)	Cold-active β -Gal 1527 (10 °C)
Operating temperature	$\approx$ 50 °C	10 °C (demonstrated optimum)
ΔT from 25 °C ambient	+25 K (heating)	0 K (no heating)
Heating energy, 10 m ³ batch (85 % eff.)	$\approx$ 342 kWh	$\approx$ 0
Energy cost (US\$0.10 kWh ⁻¹)	$\approx$ US\$34	$\approx$ 0
CO ₂ e (0.5703 kg kWh ⁻¹)	$\approx$ 195 kg	$\approx$ 0
Post-reaction cooling	required	not required
Reaction time	12 h	12 h (demonstrated)
Aroma-precursor thermal degradation	higher	lower (16.6–91.0 % aroma gain, demonstrated)

Note: values are engineering estimates (assumptions: specific heat 4.18 kJ kg⁻¹ K⁻¹, 85 % heating efficiency, electricity US\$0.10 kWh⁻¹, grid factor 0.5703 kg CO₂e kWh⁻¹); the cold-active values assume operation at or below ambient with negligible heating, and savings scale approximately linearly with reaction volume and temperature gap.

4. Conclusion

A cold-active GH42 β -galactosidase (protein 1527) from *K. variicola* GB3 was expressed in *E. coli*, purified, and characterized. The enzyme is a 685-residue, 77.5 kDa protein that is optimally active at 10 °C and pH 6.5, and stable at 0–20 °C and pH 6.0–7.0. It is strongly activated by Fe²⁺ and inhibited by Ca²⁺. Nonlinear regression kinetics yielded $K_m = 3.05$ mM, $V_{\max} = 222.2$ $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, $k_{\text{cat}} \approx 287$ s⁻¹, and $k_{\text{cat}}/K_m \approx 9.4 \times 10^4$ M⁻¹·s⁻¹, indicating efficient catalysis at low temperature.

When applied to tobacco strands and reconstituted tobacco concentrate at 10 °C, the enzyme was associated with improved sensory attributes and increases of 16.6–91.0% in 16 aroma compounds. These findings identify a cold-active β -galactosidase from *K. variicola* and indicate its potential for low-temperature, aroma-preserving processing of reconstituted tobacco. Because the application experiments used a water control, causal attribution to catalysis awaits confirmation with the heat-inactivated-enzyme control and direct measurement of released aglycones or free galactose; scale-up and mechanistic studies are warranted. Beyond tobacco, the same catalytic chemistry supports a wider role for cold-active GH42 β -galactosidases as green, energy-saving biocatalysts for the low-temperature release of glycosidically bound aroma compounds in the flavor and fragrance sector, and complements their established value in lactose-free dairy processing and prebiotic GOS synthesis.

Protein 1527 remains a promising starting point for further improvement through protein engineering. Future studies could focus on enhancing catalytic efficiency and operational stability and on fine-tuning the temperature optimum for specific industrial applications, guided by the conserved GH42 catalytic residues E157 and E314 and the predicted active-site architecture.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The sequence information supporting the findings of this study is available in GenBank under accession number PZ320841. (<https://www.ncbi.nlm.nih.gov/nuccore/PZ320841>)

Author Contributions

LX: conceptualization, methodology, investigation, formal analysis, writing – original draft. JH: recombinant vector construction, protein expression and purification, and enzymatic characterization. KI: sequence analysis, structural modeling, and writing – review and editing. SS: tobacco-treatment experiments and sensory evaluation. YiW: GC-MS analysis and volatile-compound quantification. YoW: data curation, statistical analysis, and validation. DM: conceptualization, supervision, project administration, validation, writing – review and editing. SH: bioinformatics analysis, sequence alignment, and structural interpretation. BD: resources, industrial application design, and sensory evaluation. CC: methodology, data visualization, validation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Ethics Approval and Informed Consent

All methods involving human participants were carried out in accordance with the relevant institutional, national, and international guidelines and regulations. The sensory evaluation of cigarettes was conducted by a trained expert panel in accordance with the Chinese National Standard GB 5606.4-2005, Cigarettes—Part 4: Technical Requirements for Sense Evaluation. The experimental protocols involving human participants were reviewed and approved by the Ethics Committee of Zhengzhou University of Light Industry. Written informed consent was obtained from all participants prior to their participation in the study. All participants were fully informed of the nature and procedures of the sensory evaluation and voluntarily agreed to participate.

Conflict of Interest

Authors Lei Xue, Shixi Song, Ying Wang, Yonghong Wang, and Baoan Deng were employed by China Tobacco Shaanxi Industrial Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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