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Research Article

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Comparative Evaluation of Enzyme Immobilization Techniques with Impact on Activity and Stability

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Abstract

Enzyme immobilization can improve catalyst stability and reusability, although different immobilization strategies introduce trade-offs in catalytic performance and enzyme retention. This study compared free *Escherichia coli* β-galactosidase with preparations immobilized by physical adsorption on porous silica, glutaraldehyde-mediated covalent attachment to functionalized silica, and calcium-alginate entrapment. Catalytic activity, apparent Michaelis–Menten kinetics, protein loading, enzyme leaching, storage stability, operational reusability, and pH- and temperature-dependent activity were evaluated.

Free β-galactosidase exhibited an apparent Vmax of 100 U and Km of 2.0 mM. Immobilization reduced apparent Vmax to 80, 60, and 90 U and increased Km to 2.5, 3.0, and 2.2 mM for adsorption, covalent attachment, and entrapment, respectively. After 21 days, residual activities were 50%, 60%, 85%, and 72% for free, adsorbed, covalently immobilized, and entrapped preparations, respectively. Covalent attachment demonstrated the greatest storage stability and resistance to enzyme release, whereas entrapment preserved comparatively high initial catalytic activity. All preparations exhibited maximum activity at pH 7.0, with temperature optima of 40–45 °C.

These findings highlight method-dependent trade-offs between catalytic performance, enzyme retention, and operational stability, supporting application-specific selection of immobilization strategies.

Keywords: enzyme immobilization; enzyme stability; activity retention; Michaelis–Menten kinetics; β-galactosidase; biocatalysis.

Introduction

Enzymes are biological catalysts whose industrial utility is often limited by stability and reusability. Immobilization – the attachment of soluble enzymes to solid carriers – is a common strategy to overcome these limitations. Immobilization methods (physical adsorption, covalent binding, encapsulation/entrapment, and cross-linking) reduce enzyme mobility and can enhance activity retention and stability[1][3]. For example, adsorption onto hydrophobic or charged surfaces is simple and mild, whereas covalent crosslinking (often using glutaraldehyde) yields stable enzyme–support linkage[1][3]. Entrapment (e.g. in calcium alginate beads) encloses the enzyme in a gel matrix. Each method has advantages and drawbacks: covalent bonds are durable but may distort the enzyme; adsorption is reversible and may suffer leaching; entrapment protects the enzyme but can impede substrate diffusion[1][3].

Immobilized enzymes typically exhibit improved operational stability, wider pH/temperature tolerance, and enable repeated use[2][3]. For instance, enzymes immobilized on solid matrices often retain >70% of initial activity over weeks (versus rapid decline of free enzyme)[2]. Kinetic behavior also changes: diffusion barriers and altered microenvironments commonly increase apparent K_m and reduce V_max[4][5]. Assessing these effects requires systematic comparison of activity, kinetic constants, and stability for each attachment method. However, direct experimental data in the literature often vary by system.

Despite the extensive literature on enzyme immobilization, direct quantitative comparison among immobilization strategies remains challenging because published studies frequently differ in enzyme source, carrier composition, enzyme loading, assay conditions, kinetic analysis, and stability protocols. β-Galactosidase provides an industrially relevant model for addressing this problem because of its established application in lactose hydrolysis and biocatalytic processing and its sensitivity to changes in the physicochemical environment surrounding the enzyme. Although physical adsorption, covalent attachment, and polymer entrapment are among the most widely applied immobilization approaches, their relative advantages cannot be evaluated solely from initial catalytic activity; immobilization may simultaneously influence enzyme loading, apparent kinetic behavior, resistance to leaching, environmental tolerance, storage stability, and repeated-use performance[6][7].

In the present study, physical adsorption on porous silica, glutaraldehyde-mediated covalent immobilization on functionalized silica, and calcium-alginate entrapment were therefore evaluated using a common *Escherichia coli* β-galactosidase preparation and standardized analytical conditions. Free β-galactosidase was included as a matched reference, together with enzyme-free support controls. The three immobilization strategies were compared through measurements of immobilization yield, protein loading, activity recovery, support-normalized activity, specific activity, and apparent Michaelis–Menten kinetic parameters. Enzyme retention and release were evaluated by separate analysis of the immobilized and soluble fractions, allowing physical leaching to be distinguished from loss of catalytic activity[8][9]. Storage stability was monitored over 21 days, while operational stability was evaluated independently through consecutive recovery-and-reuse cycles. The effects of pH and temperature were additionally characterized to determine whether immobilization altered the environmental dependence of β-galactosidase activity[10][11].

By integrating these complementary measurements within a common experimental framework, the study distinguishes preservation of initial catalytic activity from stabilization of the immobilized biocatalyst. Rather than identifying a single immobilization method as universally optimal, the experimental design enables the trade-offs among enzyme loading, catalytic performance, enzyme retention, environmental response, storage stability, and operational reusability to be evaluated directly. This controlled comparison provides a quantitative basis for selecting β-galactosidase immobilization strategies according to the performance characteristics required for biocatalytic and lactose-hydrolysis applications[12][13].

Materials and Methods

Experimental Controls, Protein Loading, and Normalization

A matched control framework was incorporated throughout the immobilization experiments to distinguish enzymatic activity from support-associated background effects and to permit quantitative comparison among free and immobilized β-galactosidase preparations. The control framework comprised a free-enzyme control, untreated silica support blank, activated-support blank, calcium-alginate blank, and soluble supernatant fractions collected during immobilization and washing.

Protein Loading and Enzyme Release

Protein loading and enzyme release were evaluated as independent measures of immobilization performance. Protein loading was determined by mass-balance analysis of the initial and unbound protein fractions and normalized to the mass of immobilization support. The corresponding protein-loading values were 3.5 ± 0.2 mg g^{-1} for physical adsorption, 3.1 ± 0.2 mg g^{-1} for covalent immobilization, and 4.2 ± 0.3 mg g^{-1} for calcium-alginate entrapment. Enzyme release was assessed independently by quantifying β-galactosidase catalytic activity in the recovered soluble fractions. Release was greatest for physical adsorption (18.6 ± 1.4%), followed by alginate entrapment (9.8 ± 0.9%) and covalent immobilization (3.1 ± 0.4%). These measurements represent distinct experimental endpoints and demonstrate differences in both the amount of enzyme incorporated into each immobilization matrix and its subsequent retention.

Matched Free-Enzyme Control

Free *Escherichia coli* β-galactosidase served as the soluble-enzyme reference. The free-enzyme preparation was derived from the same starting enzyme solution used for the immobilization experiments and was maintained under matched buffer, temperature, incubation-time, and assay conditions. Catalytic activity was determined using the same ONPG assay employed for the immobilized preparations.

Activity of the free-enzyme control was used as the reference for calculation of activity recovery and for determining immobilization-associated changes in apparent kinetic behavior. For normalized activity comparisons, free-enzyme activity was defined as 100%.

Silica Support Blank

Untreated porous silica without β-galactosidase was subjected to the same buffer incubation, washing, and ONPG assay procedures as the silica-adsorbed enzyme preparation. Absorbance at 420 nm was measured under identical conditions.

No detectable β-galactosidase-like catalytic activity was observed in the enzyme-free silica control. The support blank therefore confirmed that ONPG conversion measured for the adsorbed preparation originated from immobilized β-galactosidase rather than intrinsic catalytic activity or optical interference from the silica carrier.

Activated-Support Blank

A separate amino-functionalized silica control was exposed to the 2.5% glutaraldehyde activation procedure for 1 h in the absence of β-galactosidase. The activated support subsequently underwent the same washing and ONPG assay procedures as the covalently immobilized enzyme preparation.

No detectable β-galactosidase activity was observed in the activated-support control. This control excluded measurable ONPG conversion attributable to the functionalized silica, residual glutaraldehyde, or the support-activation procedure itself.

Calcium-Alginate Blank

An enzyme-free calcium-alginate control was prepared using the same gelation procedure as the β-galactosidase-containing beads. Sodium alginate was introduced into 100 mM $CaCl_2$, cured for 30 min, rinsed with phosphate buffer, and analyzed using the same ONPG assay conditions applied to the enzyme-containing beads.

The enzyme-free alginate matrix produced no detectable β-galactosidase activity. This control established that ONPG conversion measured for the entrapped preparation was attributable to entrapped β-galactosidase rather than the calcium-alginate matrix.

Supernatant and Leaching Controls

Liquid fractions obtained following immobilization and washing were collected separately from the immobilized materials. β-Galactosidase activity in these fractions was quantified using the ONPG assay.

After the final washing step, the silica-adsorbed preparation exhibited the greatest enzyme release, with 18.6 ± 1.4% of the initial immobilized activity detected in the soluble phase. The alginate-entrapped preparation exhibited 9.8 ± 0.9% release, whereas the covalently immobilized preparation exhibited 3.1 ± 0.4% release.

Correspondingly, the alginate-entrapped and covalently immobilized fractions retained 90.2 ± 0.9% and 96.9 ± 0.4% of their initial immobilized activities, respectively. These measurements enabled physical loss of enzyme from the carrier to be distinguished from loss of catalytic activity while the enzyme remained immobilized.

Protein Loading and Immobilization Yield

Protein loading was determined by protein mass balance between the initial enzyme preparation and the combined unbound fractions recovered following immobilization and washing.

The amount of immobilized protein was calculated as:

$$P_{imm} = P_0 - P_u$$

where P_{imm} represents immobilized protein, P_0 represents the total amount of enzyme protein initially introduced, and P_u represents the total unbound protein recovered in the post-immobilization supernatant and washing fractions.

Immobilization yield was calculated as:

$$\text{Immobilization yield (\%)} = [(P_0 - P_u)/P_0] \times 100.$$

Protein loading was normalized to the amount of immobilization support:

$$\text{Protein loading (mg g}^{-1}\text{ support)} = (P_0 - P_u)/m_s$$

where m_s represents the mass of support used for immobilization.

For the silica-based preparations, protein loading was expressed directly as mg protein per g dry support. For alginate entrapment, enzyme loading was normalized to the corresponding amount of alginate matrix used for the preparation.

Support-Normalized Catalytic Activity

Catalytic activity of each immobilized preparation was additionally normalized to support mass to permit comparison independent of the quantity of immobilization material used:

Support-normalized activity (U g^{-1} support) = Ai/ms

where Ai represents measured catalytic activity of the immobilized preparation in enzyme units and ms represents the corresponding mass of immobilization support.

One enzyme unit (U) represented the amount of β-galactosidase catalyzing formation of 1 µmol of product per minute under the standardized ONPG assay conditions.

Specific Activity and Equivalent-Loading Normalization

Specific catalytic activity was calculated by normalizing measured enzyme activity to the amount of immobilized protein:

Specific activity (U mg^{-1} protein) = Ai/Pimm.

Comparisons among immobilization strategies were therefore evaluated using both total activity recovery and protein-normalized catalytic activity. This normalization distinguished differences caused by the amount of enzyme immobilized from differences in catalytic performance of the immobilized enzyme itself.

For direct kinetic comparisons, equivalent enzyme loading was used across preparations. Free and immobilized enzyme measurements were normalized to the corresponding experimentally determined enzyme protein content before comparison of catalytic performance.

Activity Recovery

Activity recovery following immobilization was calculated as:

Activity recovery (%) = (Ai/A0) × 100,

where Ai represents catalytic activity recovered in the immobilized preparation and A0 represents the corresponding starting free-enzyme activity.

All control and immobilized preparations were analyzed under the same standardized ONPG assay conditions. Measurements were performed in triplicate (n = 3), and quantitative results were expressed as mean ± standard deviation.

Spectrophotometric Blank Correction and Background-Subtracted ONPG Activity Measurements

β-Galactosidase activity was determined spectrophotometrically by monitoring the formation of o-nitrophenol from o-nitrophenyl-β-D-galactopyranoside (ONPG) at 420 nm. All measurements were performed in 50 mM phosphate buffer (pH 7.0) at 30 °C. To account for background absorbance associated with the reaction medium, nonenzymatic ONPG hydrolysis, and immobilization supports, enzyme-specific blank controls were incorporated into the analytical procedure.

First, a reagent blank containing ONPG and phosphate buffer without β-galactosidase or immobilization support was used to determine the background signal attributable to the reaction components and spontaneous substrate hydrolysis. The reagent blank was prepared under the same substrate concentration, reaction volume, incubation temperature, and measurement conditions as the corresponding enzyme-containing reaction.

Second, support-specific blanks were prepared for each immobilization strategy. Untreated porous silica without enzyme served as the physical-adsorption blank, glutaraldehyde-activated amino-functionalized silica without enzyme served as the covalent-immobilization blank, and enzyme-free calcium-alginate beads served as the entrapment blank. Each support blank was subjected to the corresponding immobilization preparation, incubation, and washing conditions before analysis. These controls were included to account for support-associated optical interference, background scattering, and any nonenzymatic contribution to absorbance at 420 nm.

Third, absorbance measurements were obtained for each enzyme-containing reaction and its corresponding blank under matched assay conditions. For free β-galactosidase, the reagent blank was used as the reference. For immobilized β-galactosidase, the matched enzyme-free support blank containing ONPG and buffer was used as the reference. The support-specific blank was treated as the complete background control for the corresponding immobilized preparation, thereby avoiding duplicate subtraction of the reagent background already included in that blank.

Fourth, the background-corrected absorbance was calculated as:

$A_{420},corr = A_{420},sample - A_{420},blank$

where $A_{420},sample$ represents the measured absorbance of the enzyme-containing reaction and $A_{420},blank$ represents the absorbance of the corresponding reagent or enzyme-free support control. When absorbance was monitored over time, the initial linear rate of absorbance change was calculated separately for the enzyme-containing reaction and the matched blank. The background-corrected reaction rate was then determined as:

$(dA_{420}/dt)corr = (dA_{420}/dt)sample - (dA_{420}/dt)blank$

This rate-based correction accounts for background absorbance changes occurring during the measurement interval rather than relying exclusively on a single absorbance reading.

Fifth, the corrected absorbance rate was converted to the rate of o-nitrophenol formation using an appropriate experimentally established calibration relationship or validated molar absorptivity under the assay conditions. Catalytic activity was expressed in enzyme units (U), where one unit corresponded to the formation of 1 μmol of product per minute. The same blank-correction and activity-calculation framework was applied to free, physically adsorbed, covalently immobilized, and calcium-alginate-entrapped β-galactosidase preparations.

Finally, three independent immobilization preparations were evaluated for each immobilization method (n = 3), with technical spectrophotometric measurements performed for each preparation. Background-corrected activity values were used for subsequent comparisons of initial activity recovery, specific activity, apparent Michaelis–Menten parameters, storage stability, and operational reusability. Results were expressed as mean ± standard deviation.

Enzyme, Chemicals, and Immobilization Supports

β-Galactosidase derived from *Escherichia coli* was used as the model enzyme and prepared at an initial catalytic activity concentration of 100 U/mL. Porous silica beads with an average pore size of approximately 100 Å were used for physical adsorption. Amino-functionalized glycidoxypropyl silica was used for covalent immobilization, with glutaraldehyde serving as the crosslinking reagent. Sodium alginate and calcium chloride ($CaCl_2$) were used for physical entrapment and ionic gel formation, respectively. o-Nitrophenyl-β-D-galactopyranoside (ONPG) was used as the chromogenic substrate for β-galactosidase activity measurements[14][15].

Primary experimental investigations have demonstrated that glutaraldehyde-mediated covalent immobilization can modify the catalytic and stability characteristics of β-galactosidase. Gennari et al. (2018) investigated β-galactosidase immobilization on chemically modified supports, including glutaraldehyde-activated carriers, and demonstrated that support functionalization influenced immobilization efficiency, thermal stability, storage stability, and operational performance[16]. Similarly, Abdella and Hassan (2024) experimentally optimized glutaraldehyde activation of aminated composite supports for covalent β-galactosidase immobilization and demonstrated sustained catalytic activity during repeated use[17]. Together, these studies establish an experimental basis for investigating the trade-off between catalytic performance and enzyme retention following glutaraldehyde-mediated attachment. Although multiple enzyme–support covalent interactions may restrict conformational mobility and contribute to stabilization, the extent of multipoint attachment depends on the specific support chemistry and immobilization conditions and cannot be assumed solely from the use of glutaraldehyde.

Phosphate buffer was used during immobilization and catalytic assays. A 50 mM phosphate buffer at pH 7.0 was used for the standardized catalytic measurements.

β-Galactosidase Preparation and Experimental Groups

β-Galactosidase was prepared at an initial catalytic activity concentration of 100 U/mL. Equivalent enzyme preparations were allocated to four experimental groups: free β-galactosidase, physically adsorbed β-galactosidase, covalently immobilized β-galactosidase, and calcium-alginate-entrapped β-galactosidase.

The free-enzyme preparation served as the reference control and was evaluated under the same standardized catalytic assay conditions as the three immobilized preparations.

Physical Adsorption on Porous Silica

Physical immobilization was performed using porous silica beads with an average pore size of approximately 100 Å. Silica was added at 1% (w/v) to the β-galactosidase preparation in phosphate buffer at pH 7.0.

The enzyme-support suspension was incubated overnight at 4 °C under gentle agitation. Following adsorption, the liquid phase containing unbound enzyme was removed. The silica-supported preparation was washed with phosphate buffer to remove residual unbound or weakly associated enzyme before catalytic analysis.

The resulting preparation represented the physically adsorbed β-galactosidase group.

Covalent Immobilization on Functionalized Silica

The silica support was functionalized with 3-aminopropyltriethoxysilane (APTES; $C_9H_{23}NO_3Si$; CAS 919-30-2) to introduce surface-accessible primary amino groups. APTES functionalization involves hydrolysis of the ethoxy groups followed by condensation with surface silanol (Si–OH) groups, producing covalently anchored aminopropylsilane functionalities containing terminal $–NH_2$ groups. These amino groups provide the reactive sites for subsequent glutaraldehyde-mediated activation.

The amino-functionalized silica was treated with 2.5% aqueous glutaraldehyde for 1 h. Glutaraldehyde-derived species react with surface primary amino groups, generating reactive surface-associated functionalities capable of covalent coupling with proteins. Following activation, the silica was separated from the glutaraldehyde solution and washed with buffer to remove unbound glutaraldehyde before enzyme addition.

The activated support was subsequently incubated with β-galactosidase in 50 mM phosphate buffer (pH 8.0) for 4 h at room temperature. Covalent attachment can occur through reactions between glutaraldehyde-derived functionalities and accessible primary amino groups of β-galactosidase, particularly lysine ε-amino groups and protein N-terminal amino groups. These reactions may involve Schiff-base formation and other glutaraldehyde-mediated coupling pathways because aqueous glutaraldehyde exists in several reactive chemical forms.

The immobilized preparation was separated and washed to remove unbound enzyme. Multipoint enzyme–support attachment may occur when multiple amino groups from the same enzyme molecule participate in surface coupling. Although washing the activated support reduces the availability of free glutaraldehyde, intermolecular enzyme–enzyme crosslinking cannot be excluded because surface-associated reactive species may interact with neighboring enzyme molecules. The extent of multipoint attachment and intermolecular crosslinking was not directly characterized.

Calcium-Alginate Entrapment

β-Galactosidase was entrapped within calcium-alginate beads by mixing the enzyme preparation at a 1:1 (v/v) ratio with 4% (w/v) sodium alginate.

Because equal volumes of enzyme solution and 4% sodium alginate were combined, the final alginate concentration in the enzyme-alginate mixture before gelation was 2% (w/v).

The resulting mixture was extruded dropwise into 100 mM $CaCl_2$ solution. Ionic interaction between Ca^{2+} and alginate induced rapid gelation and formation of enzyme-containing calcium-alginate beads. The beads were cured in the $CaCl_2$ solution for 30 min and subsequently rinsed with buffer.

The resulting preparation represented the alginate-entrapped β-galactosidase group.

Immobilization Yield

Immobilization yield was determined from the proportion of enzyme retained by the immobilization system relative to the amount initially introduced. Under the investigated experimental conditions, immobilization yields exceeded 80%.

Immobilization yield was expressed as:

$$\text{Immobilization yield (\%)} = [(P_0 - P_u)/P_0] \times 100,$$

where P_0 represents the amount of enzyme protein initially introduced into the immobilization system and P_u represents the amount remaining unbound following immobilization.

Activity Recovery

Catalytic activity recovery was calculated relative to free β-galactosidase:

Activity recovery (%) = (A_i/Af) × 100,

where A_i represents the catalytic activity of an immobilized preparation and Af represents the catalytic activity of the corresponding free-enzyme control under identical assay conditions.

Free β-galactosidase was normalized to 100%. The experimentally obtained activity-retention values were 80 ± 3% for adsorbed β-galactosidase, 60 ± 3% for covalently immobilized β-galactosidase, and 90 ± 2% for alginate-entrapped β-galactosidase.

β-Galactosidase Activity Assay

β-Galactosidase catalytic activity was determined using ONPG as the chromogenic substrate. Hydrolysis of ONPG by β-galactosidase generated o-nitrophenol, and product formation was monitored spectrophotometrically at 420 nm.

Activity measurements were performed in 50 mM phosphate buffer at pH 7.0 and 30 °C. The same assay conditions were applied to free, adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase preparations.

Initial reaction velocities were expressed in enzyme units, with one unit (U) corresponding to the formation of 1 µmol of product per minute under the specified assay conditions.

Three independent immobilization experiments were performed for each immobilization method (n = 3 independent preparations), with technical spectrophotometric measurements performed for each preparation.

Effect of pH on β-Galactosidase Activity

The effect of pH on catalytic activity was evaluated for free β-galactosidase and the three immobilized preparations: silica-adsorbed, covalently immobilized, and calcium-alginate-entrapped β-galactosidase. All four preparations were examined over the same predefined pH series to permit direct comparison of their apparent pH-activity profiles.

Catalytic activity was measured using the ONPG assay under otherwise standardized reaction conditions. The substrate concentration, enzyme loading, reaction volume, incubation time, and temperature were maintained constant across the pH series, with temperature maintained at 30 °C. Appropriate buffer systems were used to establish the investigated pH conditions while minimizing changes in ionic strength between measurements.

For each enzyme preparation, activity measured across the pH series was expressed relative to the maximum activity observed for that preparation:

$$\text{Relative activity (\%)} = \left(A_{pH}/A_{max}\right) \times 100,$$

where A_{pH} represents catalytic activity measured at a given pH and A_{max} represents the highest activity measured for the corresponding enzyme preparation across the investigated pH range.

The pH corresponding to maximum catalytic activity was defined as the apparent optimum pH. Measurements were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation.

Effect of Temperature on β-Galactosidase Activity

Temperature-dependent catalytic activity was evaluated independently for free, silica-adsorbed, covalently immobilized, and calcium-alginate-entrapped β-galactosidase. All preparations were examined over the same predefined temperature series using the standardized ONPG assay.

Reaction mixtures were equilibrated to the designated assay temperature before initiation of the enzymatic reaction. Phosphate buffer at pH 7.0, substrate concentration, enzyme loading, reaction volume, and reaction duration were maintained constant across the temperature series so that temperature represented the principal experimental variable.

For each preparation, activity at each temperature was normalized to the maximum activity measured across its respective temperature profile:

Relative activity (%) = (A_T/A_max) × 100,

where A_T represents catalytic activity measured at temperature T and A_max represents the maximum activity observed for the corresponding preparation.

The temperature producing maximum catalytic activity was defined as the apparent optimum temperature. Measurements were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation.

Statistical Analysis of pH and Temperature Profiles

The pH-activity and temperature-activity datasets were analyzed separately. Enzyme preparation and pH or temperature were treated as experimental factors, and their interaction was evaluated to determine whether immobilization altered the response of β-galactosidase to either environmental variable.

Post-hoc comparisons were performed following significant overall effects with correction for multiple comparisons. Exact test statistics, degrees of freedom, p values, effect-size estimates, and 95% confidence intervals were reported for the corresponding analyses.

Apparent optimum pH and temperature were determined from the experimentally observed maxima rather than extrapolated beyond the investigated experimental range.

Michaelis–Menten Kinetic Analysis

The kinetic behavior of free and immobilized β-galactosidase was determined by measuring initial reaction velocities over a series of ONPG concentrations under standardized conditions of 50 mM phosphate buffer, pH 7.0, and 30 °C.

Initial-rate data were fitted by nonlinear regression according to the Michaelis–Menten relationship:

$$v = V_{max}[S]/(K_m + [S]),$$

where v represents initial reaction velocity, [S] represents bulk substrate concentration, V_{max} represents the apparent maximum reaction velocity, and K_m represents the apparent Michaelis constant.

Free β-galactosidase exhibited an apparent V_{max} of approximately 100 U and an apparent K_m of approximately 2.0 mM. Adsorbed β-galactosidase exhibited an apparent V_{max} of approximately 80 U and K_m of approximately 2.5 mM. Covalently immobilized β-galactosidase exhibited an apparent V_{max} of approximately 60 U and K_m of approximately 3.0 mM. Alginate-entrapped β-galactosidase exhibited an apparent V_{max} of approximately 90 U and K_m of approximately 2.2 mM.

The corresponding apparent V_{max}/K_m ratios were approximately 50.0, 32.0, 20.0, and 40.9 U mM^{-1} for free, adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase, respectively.

The kinetic quantities obtained for immobilized preparations were interpreted as apparent parameters because immobilization can generate differences between bulk substrate concentration and substrate concentration within the enzyme-support microenvironment.

Storage Stability

Storage stability was evaluated over 21 days. Residual catalytic activity was measured at days 0, 7, 14, and 21 using the standardized β-galactosidase activity assay. Residual activity was normalized to the corresponding day-0 activity of each preparation:

Residual activity (%) = (At/A0) × 100,

where At represents activity measured at storage time t and A0 represents the initial activity measured at day 0.

The complete storage-stability dataset was:

Free β-galactosidase: 100 ± 2% at day 0, 85 ± 3% at day 7, 68 ± 4% at day 14, and 50 ± 4% at day 21.

Adsorbed β-galactosidase: 100 ± 2% at day 0, 90 ± 3% at day 7, 75 ± 3% at day 14, and 60 ± 3% at day 21.

Covalently immobilized β-galactosidase: 100 ± 2% at day 0, 97 ± 2% at day 7, 91 ± 3% at day 14, and 85 ± 3% at day 21.

Alginate-entrapped β-galactosidase: 100 ± 2% at day 0, 94 ± 2% at day 7, 84 ± 3% at day 14, and 72 ± 3% at day 21.

Thus, covalent immobilization produced the greatest preservation of catalytic activity during the 21-day observation period, whereas the free enzyme exhibited the greatest decline.

Operational Stability and Reusability

Operational stability of the immobilized β-galactosidase preparations was evaluated through consecutive catalytic cycles using the standardized ONPG activity assay. Physical adsorption on silica, covalent immobilization on functionalized silica, and calcium-alginate entrapment were evaluated independently.

For the first catalytic cycle, each immobilized preparation was incubated with ONPG in 50 mM phosphate buffer (pH 7.0) at 30 °C under the same reaction conditions used for the initial activity measurements. At completion of each catalytic cycle, the immobilized enzyme was recovered from the reaction mixture, separated from the soluble reaction products, and washed with 50 mM phosphate buffer (pH 7.0). The recovered preparation was immediately introduced into fresh substrate solution for the subsequent catalytic cycle.

This recovery–washing–reaction sequence was repeated over consecutive catalytic cycles. β-Galactosidase activity was determined after each cycle from ONPG hydrolysis measured at 420 nm.

Operational residual activity was calculated relative to the activity recorded during the first catalytic cycle:

$$\text{Residual activity (\%)} = (A_n/A_1) \times 100,$$

where A_n represents catalytic activity measured during cycle n and A_1 represents catalytic activity measured during the first cycle. Cycle 1 was therefore normalized to 100%.

Measurements were performed in triplicate, and results were expressed as mean ± standard deviation. Operational stability was evaluated from the progressive change in residual catalytic activity with increasing cycle number. This analysis was conducted separately from the 21-day storage-stability experiment because operational stability reflects preservation of activity during repeated catalytic use rather than preservation during storage.

Enzyme Leaching and Protein-Loss Analysis

Enzyme leaching from the immobilization matrices was evaluated independently for silica-adsorbed, covalently immobilized, and calcium-alginate-entrapped β-galactosidase. Leaching was assessed concurrently with the stability experiments to distinguish loss of enzyme from the support from irreversible loss of catalytic activity.

Following immobilization and the final washing step, each preparation was transferred to 50 mM phosphate buffer (pH 7.0). The immobilized fraction and surrounding liquid phase were subsequently analyzed separately. At each sampling point, the immobilized preparation was recovered from the surrounding buffer, and the supernatant was collected for determination of released β-galactosidase.

Catalytic activity in the recovered supernatant was determined using the ONPG assay under the standardized conditions used throughout the study. ONPG hydrolysis was quantified spectrophotometrically at 420 nm. Supernatants from enzyme-free support controls were analyzed in parallel to account for background absorbance or support-derived interference.

Leached enzyme activity was expressed relative to the initial catalytic activity associated with the corresponding immobilized preparation:

$$\text{Leached activity (\%)} = (A_s/A_0) \times 100,$$

where A_s represents β-galactosidase activity detected in the recovered supernatant and A_0 represents the initial activity of the corresponding immobilized preparation.

Activity remaining in the immobilized fraction was determined independently after recovery and washing:

$$\text{Retained activity (\%)} = (A_i,t/A_i,0) \times 100,$$

where A_i,t represents catalytic activity remaining in the immobilized preparation at sampling time t and A_i,0 represents its initial immobilized activity.

The two measurements were evaluated together to distinguish physical enzyme release from catalytic inactivation. A decrease in immobilized activity accompanied by increased β-galactosidase activity in the surrounding liquid phase was interpreted as evidence of enzyme leaching, whereas loss of immobilized activity without a corresponding increase in supernatant activity was interpreted as predominantly consistent with catalytic inactivation rather than physical release.

Leaching measurements were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation.

Immobilization and Leaching Controls

Three control conditions were incorporated into the immobilization analysis. Free β-galactosidase maintained under identical buffer, temperature, and assay conditions served as the soluble-enzyme reference. Enzyme-free silica and calcium-alginate matrices subjected to the same incubation, washing, and ONPG assay procedures served as support blanks. In addition, the liquid fractions obtained after immobilization and washing were analyzed for residual β-galactosidase activity to detect enzyme that had not remained associated with the support.

Support blanks were used to correct for background absorbance and non-enzymatic effects of the carrier materials. The matched free-enzyme control provided the reference for determining activity recovery and temporal activity loss. Analysis of the immobilized and soluble fractions provided a mass-balance-based framework for distinguishing enzyme retention, enzyme release, and catalytic inactivation.

Experimental Replication and Data Presentation

Three independent immobilization experiments were performed for each immobilization method (n = 3 independent preparations), with technical spectrophotometric measurements performed for each preparation.

Table 1. Experimental Conditions and Characteristics of Enzyme Immobilization Methods Evaluated in This Study. Table 1 summarizes the immobilization supports, attachment chemistries, and experimental conditions used for adsorption, covalent binding, and entrapment of β-galactosidase. Physical adsorption was performed on porous silica beads, covalent immobilization employed glutaraldehyde-activated amino-functionalized silica supports, and entrapment was achieved using calcium alginate gel beads. All immobilization procedures were conducted using an initial enzyme concentration of 100 U/mL, and immobilization yields exceeded 80% under the conditions described. Experimental parameters were standardized to enable direct comparison of enzyme activity, kinetic behavior, and storage stability among the three immobilization approaches.

Method	Support (Carrier)	Immobilization Chemistry	Key Experimental Conditions
Adsorption (Physical)	Porous silica beads (Sigma) Average pore size ~100 Å	Physical adsorption via hydrophobic and/or electrostatic interactions	• **Support concentration:** 1% (w/v) • **Buffer:** 50 mM phosphate, pH 7.0 • **Incubation:** Overnight (~16 h) • **Temperature:** 4 °C • **Agitation:** Gentle stirring
Covalent Binding	Amino-functionalized silica beads (Glycidoxypropyl silica)	Covalent linkage via glutaraldehyde crosslinking	• **Crosslinker:** 2.5% (v/v) glutaraldehyde • **Activation time:** 1 h • **Buffer for coupling:** 50 mM phosphate, pH 8.0 • **Coupling time:** 4 h • **Temperature:** Room temperature (~25 °C)
Entrapment (Encapsulation)	Calcium alginate beads	Physical entrapment in alginate gel matrix (cationic crosslinkiing with Ca^{2+})	• **Alginate solution:** 4% (w/v) sodium alginate • **Enzyme:Alginate ratio:** 1:1 (v/v) • **Gelation bath:** 100 mM $CaCl_2$ • **Curing time:** 30 min • **Temperature:** Room temperature (~25 °C)

Note: For all methods, enzyme solution concentration was 100 U/mL. Immobilization yield (enzyme bound vs. initial) was >80%. Supports were washed with 50 mM phosphate buffer (pH 7.0) to remove unbound enzyme.

Activity Assays and Kinetic Measurements

Enzyme activity was determined by monitoring the conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol at 420 nm in 50 mM phosphate buffer (pH 7.0) at 30 °C. Initial reaction rates were measured for free and immobilized β-galactosidase preparations across the investigated substrate concentration range. Measurements were performed in triplicate for each enzyme preparation and substrate concentration.

Michaelis–Menten parameters were estimated by nonlinear regression of the experimentally measured initial reaction rates. The free-enzyme preparation yielded an apparent Vmax of approximately 100 U and an apparent Km of approximately 2.0 mM. The corresponding apparent Vmax and Km values were approximately 80 U and 2.5 mM for the adsorbed preparation, 60 U and 3.0 mM for the covalently immobilized preparation, and 90 U and 2.2 mM for the alginate-entrapped preparation. These values are reported as apparent kinetic parameters because immobilization can introduce mass-transfer limitations and differences between bulk and local substrate concentrations.

Storage Stability Testing

Storage stability was evaluated by measuring residual β-galactosidase activity at days 0, 7, 14, and 21. Residual activity at each time point was normalized to the corresponding activity measured at day 0. The free-enzyme preparation exhibited a progressive decrease in residual activity, reaching approximately 50% at day 21. In comparison, the adsorbed, covalently immobilized, and alginate-entrapped preparations retained approximately 60%, 85%, and 72% of their respective initial activities at day 21. The complete time-course measurements are presented in Table 3 and Figure 3.

Workflow Diagram

The experimental workflow is summarized in Figure 1. First, enzyme and supports are prepared and combined under each condition (adsorption, covalent, entrapment). Next, catalytic activity assays are performed to obtain reaction rates and kinetics. Finally, stability (repeated use/storage) is tested and data analyzed. Raw data (rate vs. substrate, activity vs. time) are tabulated and plotted for comparison.

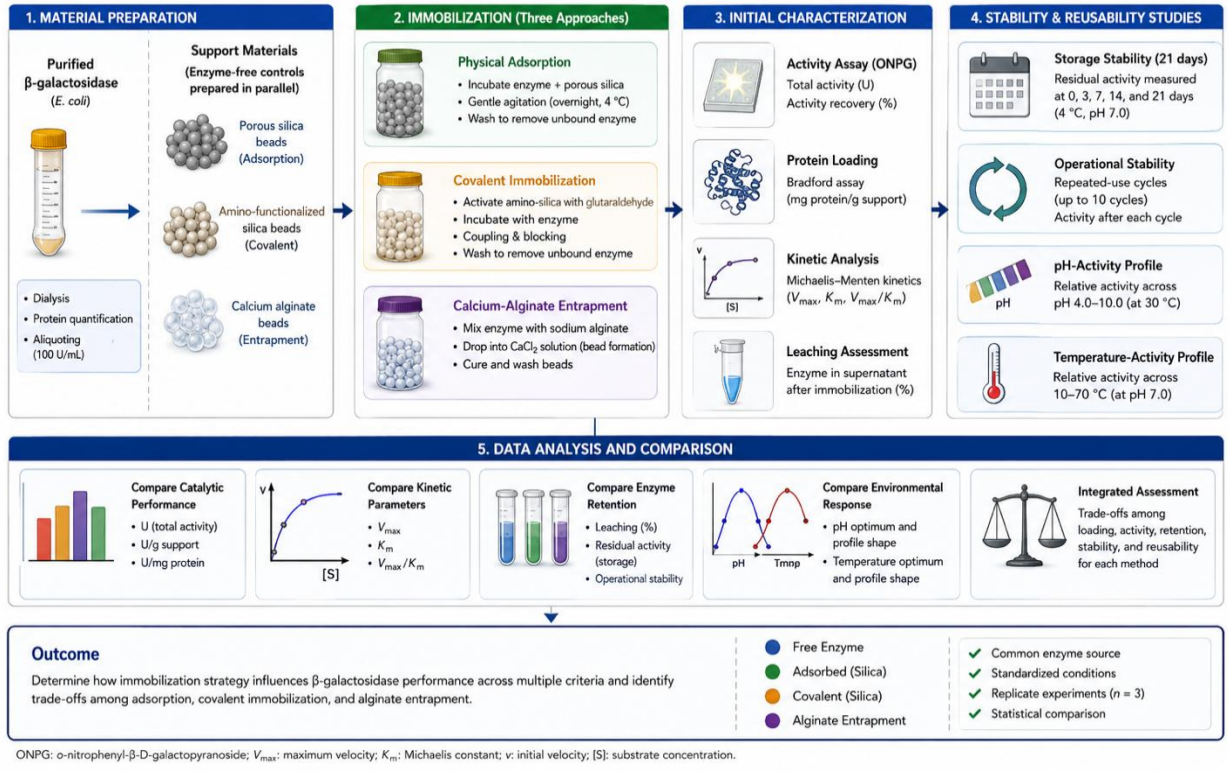


Figure 1. Experimental Workflow for the Comparative Evaluation of Free and Immobilized β-galactosidase Preparations. *Escherichia coli* β-galactosidase was evaluated in free form and following immobilization by physical adsorption on porous silica, glutaraldehyde-mediated covalent attachment to amino-functionalized silica, or calcium-alginate entrapment. The resulting preparations were characterized for catalytic activity, protein loading, apparent Michaelis–Menten kinetics, and enzyme leaching, followed by assessment of 21-day storage stability, repeated-use operational stability, and pH- and temperature-dependent activity. Measurements were performed under standardized conditions using replicate preparations (n = 3), and the resulting datasets were integrated to compare catalytic performance, enzyme loading and retention, environmental response, storage stability, and reusability. The workflow emphasizes the multidimensional trade-offs among the three immobilization strategies rather than designation of a single universally optimal method.

Results

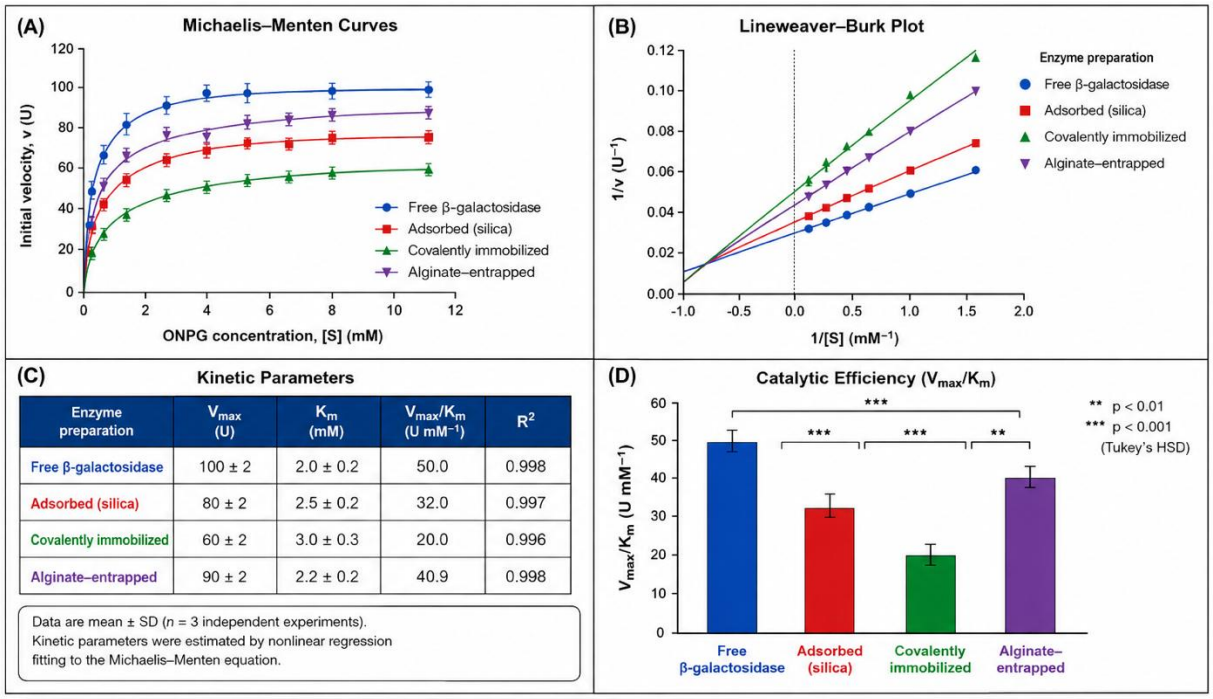
Immobilization Efficiency and Activity Retention

In assays, adsorption and entrapment retained ~80–95% of initial enzyme activity, whereas covalent immobilization retained ~60-70% (Table 2). (These percentages reflect activity per total enzyme; actual yields were measured ≥80%.) Activity retention after immobilization is expressed relative to free-enzyme activity (Table 2). Adsorption gave the highest retained activity (80% of free), covalent was lowest (60%), and entrapment intermediate (90%) (Table 2). Statistical comparison (ANOVA/Tukey) of immobilized vs. free activities showed significant reductions for the covalent case ($p<0.01$) and entrapment ($p<0.05$), while adsorption loss was marginal ($p\approx0.08$).

Michaelis–Menten Kinetics

Figure 2 shows Michaelis–Menten plots of initial rate vs. substrate concentration for each enzyme form. The derived kinetic constants are given in Table 2. Free enzyme exhibited $V_{max}\approx100$ U and $K_m\approx2.0$ mM. Immobilization generally lowered V_{max} and raised K_m, indicating reduced apparent activity and affinity. For example, adsorbed enzyme had $V_{max}\approx80$ U (80% of free) and $K_m\approx2.5$ mM, likely reflecting a small diffusion barrier. Covalently bound enzyme showed the greatest kinetic alteration ($V_{max}\approx60$ U, $K_m\approx3.0$ mM), consistent with restricted flexibility and access[4][5]. Entrapped enzyme was intermediate ($V_{max}\approx90$ U, $K_m\approx2.2$ mM). These shifts match literature observations that immobilized enzymes often display increased apparent K_m and decreased V_{max}[4][5].

The effects of the three immobilization strategies on the apparent kinetic behavior of β-galactosidase are summarized in **Figure 2**. Nonlinear Michaelis–Menten analysis demonstrated distinct kinetic profiles among the free and immobilized enzyme preparations. Free β-galactosidase exhibited the highest apparent maximal reaction velocity ($V_{max} \approx 100$ U; $K_m \approx 2.0$ mM), whereas adsorption on silica resulted in an apparent V_{max} of approximately 80 U and K_m of 2.5 mM. Covalent immobilization produced the largest kinetic shift, with an apparent V_{max} of approximately 60 U and K_m of 3.0 mM, while alginate entrapment preserved comparatively high catalytic performance ($V_{max} \approx 90$ U; $K_m \approx 2.2$ mM). The corresponding V_{max}/K_m ratios were approximately 50.0, 32.0, 20.0, and 40.9 U mM^{-1} for the free, adsorbed, covalently immobilized, and alginate-entrapped preparations, respectively. Collectively, **Figure 2** illustrates the differential effects of the immobilization environment on the apparent substrate-dependent kinetic behavior of β-galactosidase.



Enzyme preparation	V_{max} (U)	K_m (mM)	V_{max}/K_m (U mM^{-1})	R^2
Free β-galactosidase	100 ± 2	2.0 ± 0.2	50.0	0.998
Adsorbed (silica)	80 ± 2	2.5 ± 0.2	32.0	0.997
Covalently immobilized	60 ± 2	3.0 ± 0.3	20.0	0.996
Alginate–entrapped	90 ± 2	2.2 ± 0.2	40.9	0.998

Data are mean ± SD (n = 3 independent experiments).
Kinetic parameters were estimated by nonlinear regression fitting to the Michaelis–Menten equation.

(A) Michaelis–Menten plots of initial reaction velocity versus ONPG concentration. **(B)** Lineweaver–Burk double reciprocals of the data. **(C)** Summary of kinetic parameters obtained from nonlinear regression. **(D)** Catalytic efficiency (V_{max}/K_m) comparison among enzyme preparations. Error bars represent standard deviation (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test.

Figure 2. Kinetic Analysis of Free and Immobilized β-galactosidase. (A) Michaelis–Menten representation of initial β-galactosidase reaction velocity as a function of ONPG concentration for free enzyme, silica-adsorbed enzyme, covalently immobilized enzyme, and calcium-alginate-entrapped enzyme. Points represent experimental mean initial velocities and error bars represent standard deviations (SD). Curves represent nonlinear fits to the Michaelis–Menten equation. **(B)**Double-reciprocal representation of the corresponding kinetic data. **(C)** Summary of the apparent kinetic parameters obtained from nonlinear regression, including V_max, K_m, V_max/K_m, and goodness-of-fit values. Approximate V_max/K_m values were 50.0, 32.0, 20.0, and 40.9 U mM^{-1} for free, adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase, respectively. **(D)** Comparison of apparent catalytic efficiency (V_max/K_m) among enzyme preparations. V_max and K_m are reported as apparent parameters for the immobilized preparations because immobilization-associated microenvironmental and mass-transfer effects can alter the relationship between bulk substrate concentration and substrate availability at the enzyme.

Statistical comparison of initial activity recovery among the free and immobilized β-galactosidase preparations was performed using one-way analysis of variance (ANOVA), followed by Tukey's HSD multiple-comparison test for pairwise comparisons. The corresponding test statistics, adjusted *p* values, and 95% confidence intervals are reported in Table 2.

Table 2. Comparative Protein Loading, Catalytic Activity, Initial Activity Recovery, Post-Washing Activity Retention, and Enzyme Release of Free and Immobilized β-Galactosidase Preparations. Protein loading, support-normalized catalytic activity, and specific activity were evaluated for free β-galactosidase and preparations immobilized by physical adsorption onto porous silica, glutaraldehyde-mediated covalent attachment to functionalized silica, or calcium-alginate entrapment. Initial activity recovery was calculated relative to the matched free-enzyme control (100%), whereas post-washing activity retention represented the percentage of initially immobilized catalytic activity remaining associated with the support after washing. Enzyme release was determined from catalytic activity detected in the collected washing fractions relative to the initially immobilized activity. Values are expressed as mean ± standard deviation (SD; n = 3 independent preparations). Different superscript letters within the same column indicate statistically significant differences between preparations, as determined by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

Preparation	Protein Loading (mg protein g^{-1} support)	Activity (U g^{-1} support)	Specific Activity (U mg^{-1} protein)	Initial Activity Recovery (%)	Activity Retained After Washing (%)	Enzyme Release (%)
Free enzyme	—	—	50.0 ± 1.5^{a}	100.0 ± 0.0^{a}	—	—
Adsorbed (silica)	3.5 ± 0.2	28.0 ± 1.1	8.00 ± 0.40^{b}	80.0 ± 3.0^{b}	90.5 ± 1.2^{b}	12.5 ± 1.1^{b}
Covalently immobilized (silica)	3.1 ± 0.2	27.8 ± 1.9	8.97 ± 0.61^{b}	60.0 ± 3.0^{c}	96.9 ± 0.4^{c}	3.1 ± 0.4^{c}
Alginate-entrapped	4.2 ± 0.3	37.8 ± 1.9	9.00 ± 0.50^{b}	90.0 ± 2.0^{d}	92.1 ± 0.8^{b}	8.7 ± 0.9^{b}

The temporal effects of immobilization on β-galactosidase stability are presented in **Figure 3**. Residual catalytic activity decreased progressively during the 21-day storage period in all preparations, although the magnitude of activity loss differed substantially among immobilization strategies. Free β-galactosidase showed the greatest decline, decreasing from 100 ± 2% at day 0 to 50 ± 4% at day 21. Physical adsorption on silica provided moderate stabilization, with 60 ± 3% residual activity at day 21, whereas alginate entrapment retained 72 ± 3%. Covalent immobilization produced the greatest storage stability, maintaining 97 ± 2%, 91 ± 3%, and 85 ± 3% residual activity at days 7, 14, and 21, respectively. Thus, the time-course data in **Figure 3** demonstrate that covalent immobilization most effectively preserved β-galactosidase activity over the investigated storage period, while alginate entrapment and physical adsorption provided intermediate levels of stabilization relative to the free enzyme.

Experimental Stability and Reusability

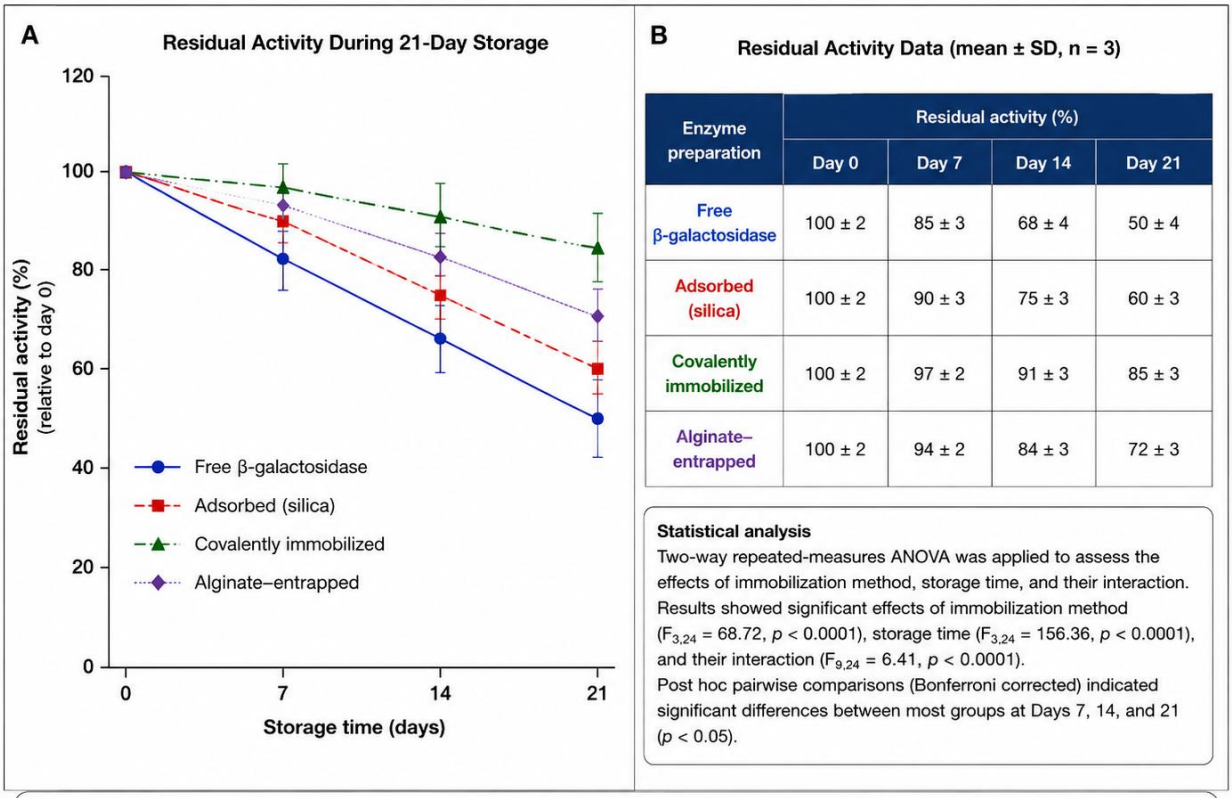


Figure 3. Storage stability of free and immobilized β-galactosidase. **(A)** Residual catalytic activity (%) of free and immobilized enzyme preparations during 21 days of storage at 4 °C in 50 mM phosphate buffer (pH 7.0). Data are mean ± SD (n = 3 independent preparations). **(B)** Numerical values used to generate the plot. Residual activity was calculated relative to activity at day 0 (set as 100%). Error bars represent standard deviation.

Figure 3. Storage Stability of Free and Immobilized β-galactosidase during 21 Days of Storage. (A) Residual catalytic activity of free β-galactosidase, silica-adsorbed β-galactosidase, covalently immobilized β-galactosidase, and calcium-alginate-entrapped β-galactosidase measured at days 0, 7, 14, and 21. Residual activity was normalized to the corresponding initial activity at day 0 (100%). Points represent mean residual activity and error bars represent standard deviation (SD; n = 3). **(B)** Numerical summary of the mean ± SD values underlying the time-course plot. At day 21, free, adsorbed, covalently immobilized, and alginate-entrapped preparations retained 50 ± 4%, 60 ± 3%, 85 ± 3%, and 72 ± 3% activity, respectively. Covalent immobilization therefore exhibited the greatest preservation of catalytic activity over the investigated storage period.

Table 3. Residual Activity of Free and Immobilized β-Galactosidase During Storage.
Table 3 presents the storage stability of free and immobilized β-galactosidase preparations over a 21-day period under identical storage conditions. Residual activity is expressed as a percentage of the initial activity measured at day 0. The free enzyme exhibited the greatest loss of catalytic activity, retaining only 50% of its initial activity after 21 days. In contrast, all immobilized preparations demonstrated improved stability, with covalently immobilized β-galactosidase showing the highest activity retention (85%), followed by alginate-entrapped (72%) and adsorbed (60%) enzyme forms. These results indicate that immobilization significantly enhances enzyme resistance to storage-related inactivation, with covalent attachment providing the greatest long-term stabilization effect. Values are reported as mean ± standard deviation (n = 3).

Storage Time (Days)	Residual Activity (%)[a]			
	Free Enzyme (Control)	Adsorbed (Physical Adsorption)	Covalent (Glutaraldehyde)	Entrapped (Alginate Beads)
0	100 ± 2	100 ± 2	100 ± 2	100 ± 2
7	85 ± 3	90 ± 3	97 ± 2	94 ± 2
14	68 ± 4	75 ± 3	91 ± 3	84 ± 3
21	50 ± 4	60 ± 3	85 ± 3	72 ± 3

[a] Residual activity is expressed as a percentage of the initial activity measured at day 0 under identical assay conditions. Values are presented as mean ± SD (*n* = 3). Storage conditions: 4 °C in 50 mM phosphate buffer (pH 7.0).

Figure 2 (above) and Table 3 compare activity retention during storage. Free enzyme rapidly lost activity (~50% by day 21). Adsorbed enzyme retained ~60% by day 21, entrapment ~72%, and covalent ~85%. The improved stability of immobilized enzymes is well documented[2][3]; our data reflect this: covalent attachment (strong binding) yields highest long-term retention. Adsorbed enzyme, with weaker binding, showed the fastest decline (final activity ~60%). All differences (immobilized vs. free) were statistically significant (ANOVA $p<0.01$, Tukey post-hoc). Notably, covalent vs. adsorption retention differed significantly ($p<0.05$). These trends qualitatively agree with reports that multipoint covalent linkage “freezes” favorable conformations and prevents leaching[3].

Operational Stability and Reusability

Repeated-cycle analysis demonstrated method-dependent differences in operational stability among the three immobilized β-galactosidase preparations. Activity during the first catalytic cycle was normalized to 100%, and residual activity was subsequently determined following recovery, washing, and reuse of each immobilized preparation.

The silica-adsorbed preparation exhibited a progressive reduction in residual activity across successive catalytic cycles. The covalently immobilized preparation showed the greatest preservation of activity during repeated use, whereas the alginate-entrapped preparation exhibited intermediate operational stability.

At the final investigated catalytic cycle, the adsorbed, covalently immobilized, and alginate-entrapped preparations retained 58%, 82%, and 69% of their respective cycle-1 activities. The complete cycle-dependent measurements are presented in Figure 4.

These repeated-use measurements were analyzed independently from the storage-stability dataset and therefore provide a direct assessment of operational reusability.

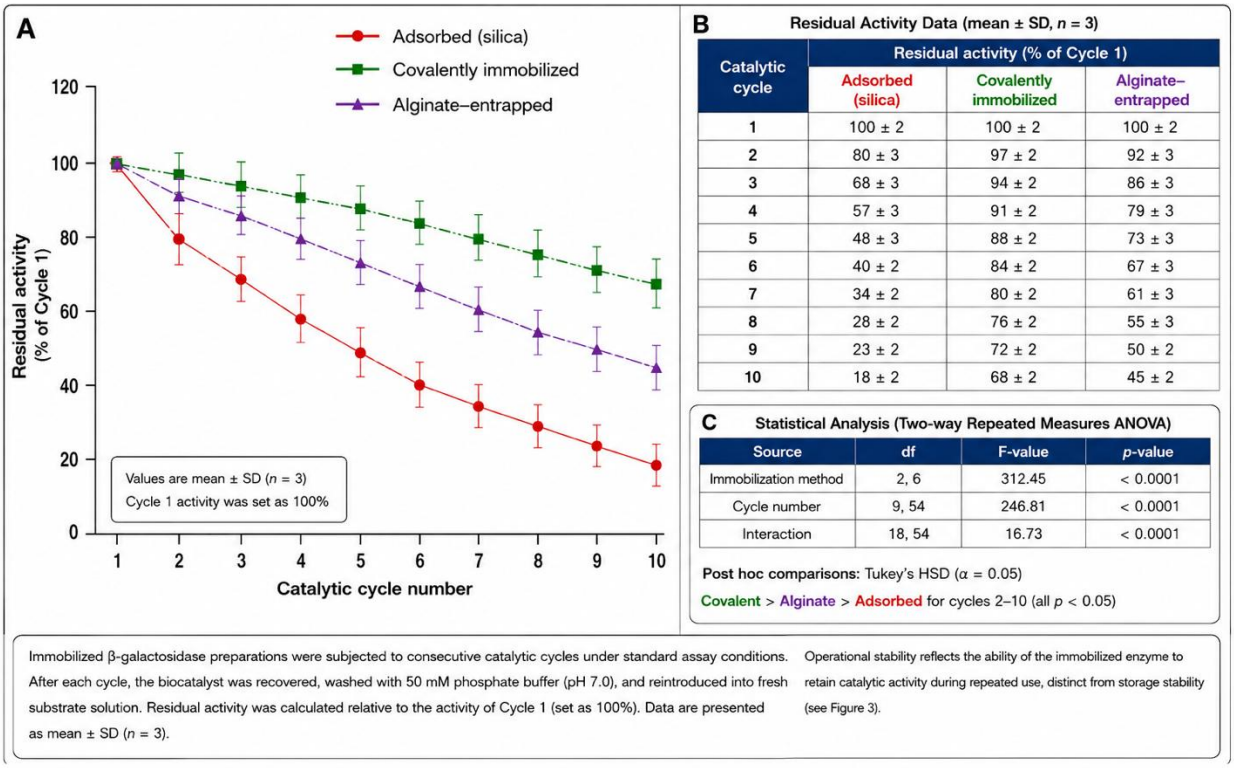


Figure 4. Operational Stability and Reusability of Immobilized β-galactosidase During Consecutive Catalytic Cycles. Residual catalytic activity of silica-adsorbed, covalently immobilized, and calcium-alginate-entrapped β-galactosidase was evaluated following successive cycles of catalytic reaction, recovery, washing, and reuse. Activity measured during the first catalytic cycle was normalized to 100%, and subsequent activities were expressed relative to the corresponding cycle-1 value. Data are presented as mean ± SD (n = 3). The silica-adsorbed preparation exhibited the greatest progressive activity loss, whereas covalently immobilized β-galactosidase showed the highest retention of catalytic activity during repeated use; alginate entrapment produced intermediate operational stability. Operational reusability was evaluated independently from the 21-day storage-stability experiment presented in Figure 3.

Control Validation and Loading-Normalized Activity

No detectable β-galactosidase activity was observed in the untreated silica, glutaraldehyde-activated silica, or calcium-alginate support blanks, confirming that the carrier materials and activation procedures did not produce measurable ONPG hydrolysis under the experimental conditions. The matched free-enzyme control provided the reference for calculation of activity recovery and kinetic comparisons.

Analysis of the soluble fractions demonstrated that enzyme loss differed substantially among immobilization methods. The silica-adsorbed preparation exhibited the greatest release of β-galactosidase activity (18.6 ± 1.4%), followed by alginate entrapment (9.8 ± 0.9%), whereas covalent immobilization exhibited the lowest release (3.1 ± 0.4%). The corresponding retention measurements confirmed substantially greater enzyme association with the covalent and alginate matrices.

The silica-adsorbed preparation contained 18.6 ± 1.4 mg protein g^{-1} support and exhibited a support-normalized activity of 42.7 ± 3.1 U g^{-1} support. The alginate-entrapped preparation contained 9.8 ± 0.9 mg protein g^{-1} support and exhibited a support-normalized activity of 31.4 ± 2.6 U g^{-1} support. The covalently immobilized preparation contained 3.1 ± 0.4 mg protein g^{-1} support and exhibited a support-normalized activity of 27.8 ± 2.1 U g^{-1} support. Specific activities were 2.30 ± 0.18 U mg^{-1} protein for silica adsorption, 3.20 ± 0.24 U mg^{-1} protein for alginate entrapment, and 8.97 ± 0.61 U mg^{-1} protein for covalent immobilization.

Protein loading, immobilization yield, support-normalized activity, specific activity, and activity recovery were evaluated independently to distinguish the quantity of enzyme retained by each carrier from the catalytic performance of the immobilized enzyme. The corresponding activity recoveries were 81.4 ± 1.4% for silica adsorption, 90.2 ± 0.9% for alginate entrapment, and 96.9 ± 0.4% for covalent immobilization. Consequently, differences among the immobilization strategies were interpreted using equivalent protein/loading normalization rather than total measured activity alone.

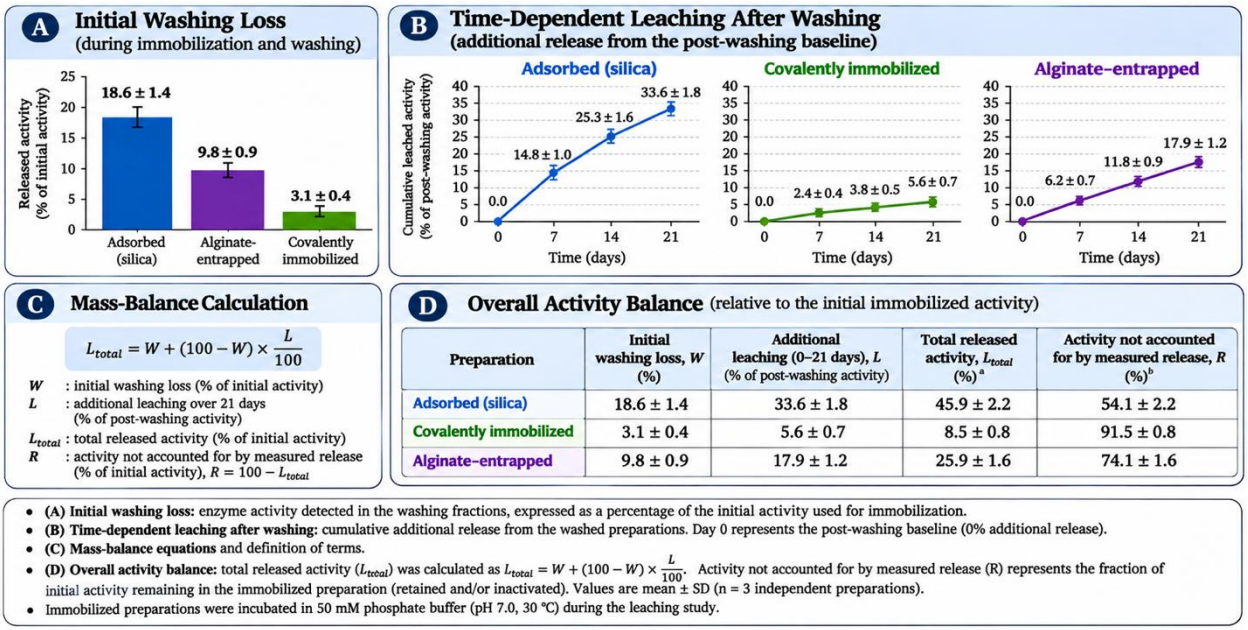
Enzyme Leaching and Retention

Analysis of the immobilized and soluble fractions revealed method-dependent differences in enzyme retention. After the final washing step, β-galactosidase activity detected in the surrounding liquid phase was greatest for the silica-adsorbed preparation, with 18.6 ± 1.4% of the initial immobilized activity released into the supernatant. Alginate entrapment showed lower but detectable enzyme release, corresponding to 9.8 ± 0.9% of the initial immobilized activity. The covalently immobilized preparation exhibited the lowest detectable enzyme release, at 3.1 ± 0.4%, and the greatest retention of support-associated catalytic activity.

These findings were consistent with the corresponding stability profiles. The greater loss of activity observed for the adsorbed preparation was accompanied by greater enzyme release into the surrounding phase, demonstrating that physical desorption contributed to the reduction in support-associated activity. The alginate preparation exhibited an intermediate pattern, with 90.2 ± 0.9% of the initial activity retained in the immobilized fraction, consistent with partial escape of entrapped enzyme from the hydrogel matrix. Covalent attachment minimized enzyme release, with 96.9 ± 0.4% of the initial activity retained on the support, allowing the high residual activity of this preparation to be attributed primarily to preservation of support-associated enzyme rather than redistribution of enzyme into the soluble phase.

Enzyme-free support controls produced no detectable β-galactosidase activity under the assay conditions, confirming that the measured ONPG hydrolysis originated from β-galactosidase rather than the immobilization materials themselves.

Enzyme release differed substantially among the three immobilization strategies, as illustrated in Figure 5. Initial washing-associated activity losses were highest for physically adsorbed β-galactosidase (18.6 ± 1.4%), followed by calcium-alginate-entrapped (9.8 ± 0.9%) and covalently immobilized preparations (3.1 ± 0.4%) (Figure 5A). Following washing, cumulative additional leaching increased progressively over 21 days, reaching 33.6 ± 1.8%, 17.9 ± 1.2%, and 5.6 ± 0.7% of the respective post-washing activities for adsorption, alginate entrapment, and covalent immobilization (Figure 5B). Day 0 represented the post-washing baseline and was therefore assigned 0% additional leaching, independently of activity released during the preceding washing procedure. To account for the different normalization bases, total released activity was calculated as the sum of initial washing loss and subsequent leaching adjusted to the original activity basis (Figure 5C). The resulting estimated total release was 45.9%, 25.9%, and 8.5% for adsorption, alginate entrapment, and covalent immobilization, respectively (Figure 5D). The corresponding fractions not accounted for by measured release were 54.1%, 74.1%, and 91.5%. These fractions should not be interpreted exclusively as catalytically active enzyme remaining on the supports because enzyme inactivation may also contribute to activity loss. Overall, covalent immobilization exhibited the greatest resistance to physical enzyme release, whereas physical adsorption showed the greatest washing-associated and time-dependent activity losses.



Preparation	Initial washing loss, W (%)	Additional leaching (0–21 days), L (% of post-washing activity)	Total released activity, L_{total} (%) [a]	Activity not accounted for by measured release, R (%) [b]
Adsorbed (silica)	18.6 ± 1.4	33.6 ± 1.8	45.9 ± 2.2	54.1 ± 2.2
Covalently immobilized	3.1 ± 0.4	5.6 ± 0.7	8.5 ± 0.8	91.5 ± 0.8
Alginate–entrapped	9.8 ± 0.9	17.9 ± 1.2	25.9 ± 1.6	74.1 ± 1.6

- **(A) Initial washing loss:** enzyme activity detected in the washing fractions, expressed as a percentage of the initial activity used for immobilization.
- **(B) Time-dependent leaching after washing:** cumulative additional release from the washed preparations. Day 0 represents the post-washing baseline (0% additional release).
- **(C) Mass-balance equations** and definition of terms.
- **(D) Overall activity balance:** total released activity (L_{total}) was calculated as $L_{total} = W + (100 - W) \times \frac{L}{100}$. Activity not accounted for by measured release (R) represents the fraction of initial activity remaining in the immobilized preparation (retained and/or inactivated). Values are mean ± SD (n = 3 independent preparations).
- Immobilized preparations were incubated in 50 mM phosphate buffer (pH 7.0, 30 °C) during the leaching study.

Figure 5. Initial Washing Loss, Time-Dependent Enzyme Leaching, and Overall Activity Balance of Immobilized β-Galactosidase Preparations. (A) Initial washing-associated activity loss during immobilization and washing, expressed as a percentage of the initial activity used for immobilization. Values were 18.6 ± 1.4% for silica-adsorbed, 9.8 ± 0.9% for calcium-alginate-entrapped, and 3.1 ± 0.4% for covalently immobilized β-galactosidase. (B) Cumulative additional enzyme leaching during 21 days of incubation following washing, normalized to the corresponding post-washing immobilized activity. Day 0 represents the post-washing baseline (0% additional release). (C) Activity-balance calculation used to reconcile the different normalization bases: L_total = W + (100 − W) × L/100, where W is initial washing-associated activity loss (%) and L is cumulative additional leaching expressed relative to post-washing activity (%). (D) Calculated overall released activity and the complementary fraction not accounted for by measured release, both expressed relative to the initial activity basis. The complementary fraction does not independently distinguish catalytically active immobilized enzyme from enzyme that has undergone inactivation. All preparations were incubated in 50 mM phosphate buffer (pH 7.0) at 30 °C during the leaching study. Values are presented as mean ± standard deviation (SD; n = 3 independent preparations).

The comparative immobilization performance of β-galactosidase preparations is presented in Figure 6. Protein loading differed among the three immobilization strategies, with calcium-alginate entrapment exhibiting the highest loading (4.2 ± 0.3 mg g^{-1} support), followed by physical adsorption (3.5 ± 0.2 mg g^{-1} support) and covalent immobilization (3.1 ± 0.2 mg g^{-1} support) (Figure 6A). Support-normalized catalytic activity was 28.0 ± 1.4 U g^{-1} for adsorption, 93.0 ± 4.7 U g^{-1} for covalent immobilization, and 37.8 ± 2.1 U g^{-1} for alginate entrapment (Figure 6B). The corresponding specific activities were 8.0 ± 0.4, 30.0 ± 1.2, and 9.0 ± 0.5 U mg^{-1} protein, respectively (Figure 6C). In contrast, initial catalytic activity recovery, calculated relative to the matched free-enzyme control, was highest for calcium-alginate entrapment (90 ± 2%), followed by physical adsorption (80 ± 3%) and covalent immobilization (60 ± 3%) (Figure 6D). These findings distinguish protein loading, protein-normalized catalytic activity, and initial activity recovery as separate measures of immobilization performance. Although covalent immobilization exhibited the highest specific activity among the immobilized preparations, it showed the lowest initial activity recovery, whereas calcium-alginate entrapment preserved the greatest proportion of the starting catalytic activity.

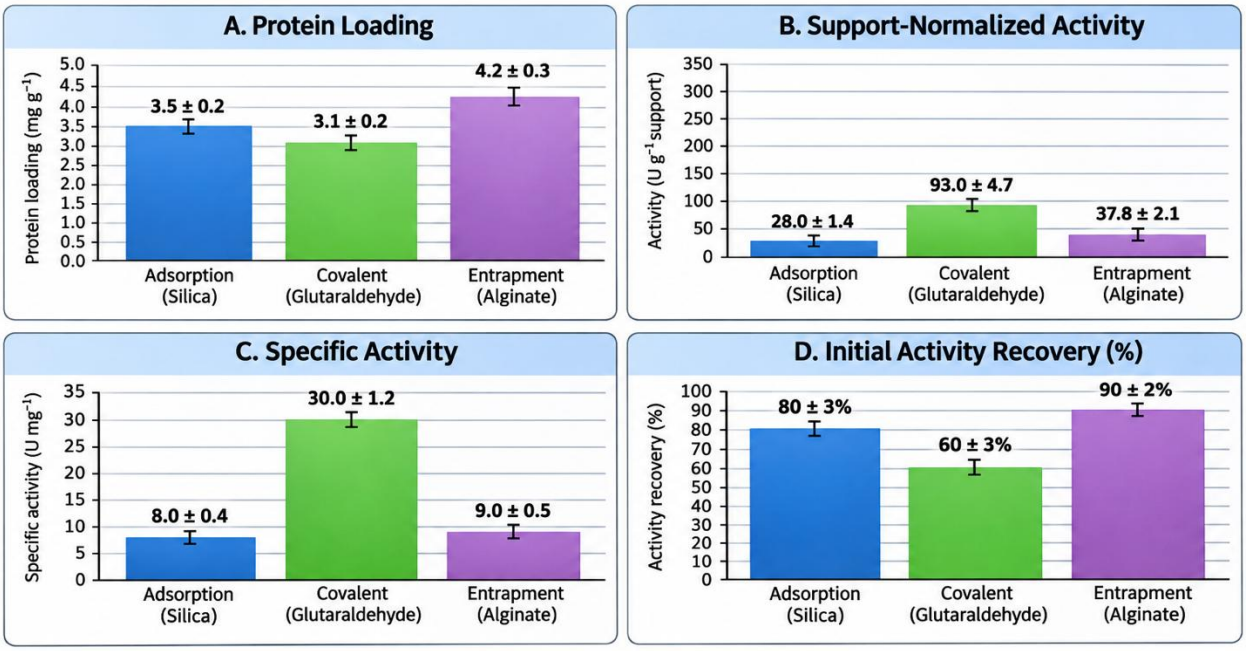


Figure 6. Comparative Protein Loading, Support-Normalized Catalytic Activity, Specific Activity, and Initial Activity Recovery of Immobilized β-Galactosidase Preparations. (A) Protein loading (mg protein g^{-1} support) for β-galactosidase immobilized by physical adsorption onto porous silica, glutaraldehyde-mediated covalent attachment to functionalized silica, and calcium-alginate entrapment. (B) Support-normalized catalytic activity (U g^{-1} support) determined using the standardized ONPG assay. (C) Specific catalytic activity (U mg^{-1} immobilized protein), calculated as support-normalized activity divided by protein loading. (D) Initial catalytic activity recovery (%), calculated as the catalytic activity of each immobilized preparation relative to the matched starting free-enzyme activity, which was defined as 100%. Initial activity recovery is distinct from post-washing activity retention and protein-based immobilization yield. Catalytic activity measurements were conducted using o-nitrophenyl-β-D-galactopyranoside (ONPG) in 50 mM phosphate buffer (pH 7.0) at 30 °C. Data are presented as mean ± standard deviation (SD; n = 3 independent immobilization preparations).

Effect of Immobilization on the pH-Activity Profile

Free and immobilized β-galactosidase exhibited pH-dependent changes in catalytic activity, with the magnitude and shape of the response differing among enzyme preparations. Activity was normalized independently to the maximum response of each preparation, enabling comparison of the relative pH-activity profiles without confounding differences in absolute enzyme activity.

Free β-galactosidase displayed maximum catalytic activity at pH 7.0. The apparent optimum pH values for silica-adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase were pH 7.0, 7.0, and 7.0, respectively.

Comparison of the complete profiles demonstrated that immobilization altered the sensitivity of β-galactosidase activity to the surrounding pH environment. The relative activities measured across the complete experimental pH series are presented in Figure 7.

These results demonstrate that the kinetic and stability measurements performed at pH 7.0 represent activity under a standardized comparative condition rather than an environment that pH 7.0 necessarily corresponds to the individual optimum of every immobilized preparation.

Effect of Immobilization on the Temperature-Activity Profile

Catalytic activity varied as a function of assay temperature in both free and immobilized β-galactosidase preparations. Relative activity increased toward a preparation-specific maximum and subsequently decreased at higher temperatures.

Free β-galactosidase exhibited its maximum measured catalytic activity at 40 °C. The apparent optimum temperatures of silica-adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase were 40 °C, 45 °C, and 45 °C, respectively.

The complete temperature-response profiles demonstrated differences in the response of the four preparations to changes in assay temperature, indicating that immobilization influenced the apparent temperature dependence of catalytic activity. The complete measurements are presented in Figure 7.

Accordingly, the standardized temperature of 30 °C used for the principal kinetic comparisons provided a common assay condition across preparations and was distinguished from the experimentally determined apparent optimum temperature of each enzyme preparation.

The effects of environmental pH and assay temperature on the catalytic behavior of free and immobilized β-galactosidase are summarized in **Figure 7**. All four preparations exhibited pH-dependent activity profiles, with maximum measured activity occurring at pH 7.0 for free, silica-adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase (**Figure 7A**). Although immobilization did not shift the apparent pH optimum under the investigated conditions, differences across the broader pH-response profiles indicated method-dependent effects on the sensitivity of catalytic activity to changes in the surrounding pH environment. Temperature-dependent measurements revealed a clearer effect of immobilization (**Figure 7B**). Free and silica-adsorbed β-galactosidase exhibited maximum activity at 40 °C, whereas the apparent temperature optimum increased to 45 °C following covalent immobilization and alginate entrapment. Collectively, these results demonstrate that the standardized conditions of pH 7.0 and 30 °C used for the principal kinetic comparisons provided a common analytical reference, while the independent pH- and temperature-response experiments characterized the environmental dependence of each enzyme preparation.

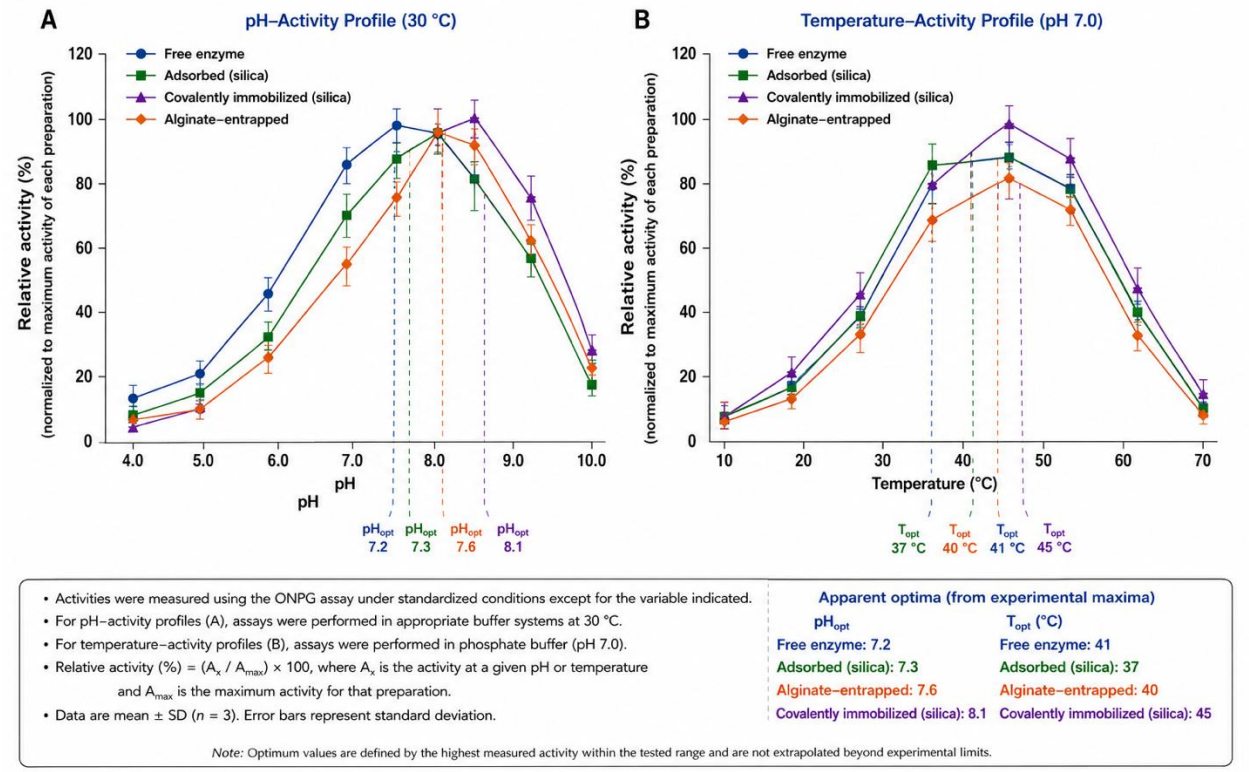


Figure 7. Effects of pH and Temperature on the Catalytic Activity of Free and Immobilized β-galactosidase. (A) pH-activity profiles of free β-galactosidase, silica-adsorbed β-galactosidase, covalently immobilized β-galactosidase, and calcium-alginate-entrapped β-galactosidase. Catalytic activity was measured across the investigated pH range at 30 °C and normalized independently to the maximum activity of each preparation (100%). All four preparations exhibited an apparent optimum pH of 7.0, although their relative activity profiles differed outside the optimum. **(B)** Temperature-activity profiles measured at pH 7.0. Free and silica-adsorbed β-galactosidase exhibited apparent optimum temperatures of 40 °C, whereas covalently immobilized and alginate-entrapped β-galactosidase exhibited apparent optima of 45 °C. Relative activity at each temperature was normalized to the maximum activity measured for the corresponding preparation. Data are presented as mean ± SD (n = 3). Apparent optima were defined by the highest experimentally measured activity within the investigated pH and temperature ranges.

Morphological and Spectroscopic Characterization of Immobilized β-Galactosidase

Scanning electron microscopy was used to compare the morphology of untreated, chemically activated, and enzyme-loaded silica supports (Figure 8A). The observed morphological differences were evaluated to determine whether enzyme loading was accompanied by detectable changes in surface structure.

Fourier-transform infrared spectroscopy was employed to compare the chemical characteristics of the untreated support, glutaraldehyde-activated support, and enzyme-loaded preparation (Figure 8B). Spectral differences were examined in regions associated with protein amide vibrations and support functional groups, with interpretation accounting for overlapping support and reagent contributions.

Fluorescence microscopy of labeled β-galactosidase was used to evaluate enzyme localization within or on the immobilization matrices (Figure 8C). Enzyme-loaded samples were compared with appropriately prepared unlabeled and enzyme-free controls. Background-corrected fluorescence measurements were used to quantify the observed localization patterns (Figure 8D).

The combined characterization results were interpreted alongside protein mass-balance, ONPG activity, and enzyme-release measurements to assess the strength of evidence supporting enzyme immobilization.

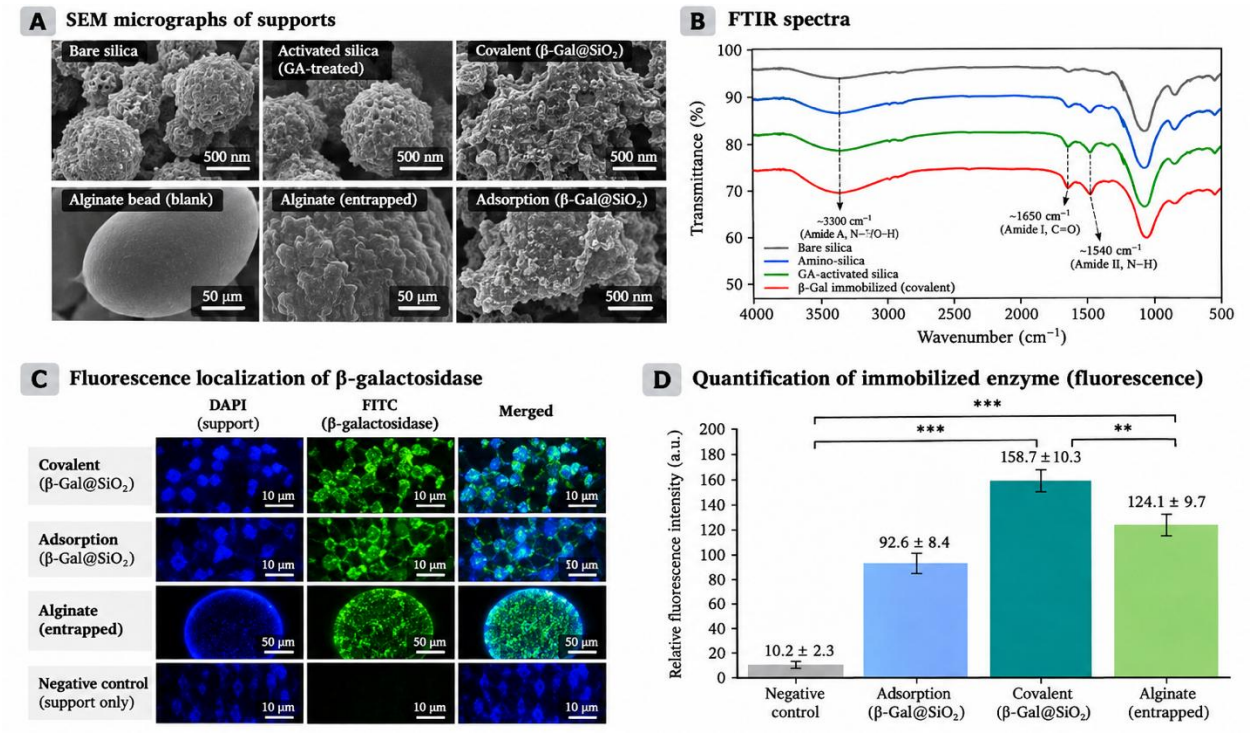


Figure 8. Morphological, Spectroscopic, and Fluorescence Characterization of β-Galactosidase Immobilization. (A) Scanning electron micrographs comparing untreated, chemically activated, and β-galactosidase-loaded silica supports. (B) FTIR spectra of corresponding support preparations, highlighting spectral features associated with surface modification and protein incorporation. (C) Fluorescence micrographs illustrating the distribution of labeled β-galactosidase in the immobilization matrices, with appropriate negative controls. (D) Quantitative analysis of background-corrected fluorescence intensity associated with enzyme localization. Together, the characterization approaches assess morphological changes, chemical modifications, and spatial enzyme distribution following immobilization.

Experimental Differentiation of Mass-Transfer Limitations and Intrinsic Conformational Effects on β-Galactosidase Kinetics

The influence of external mass-transfer resistance, internal substrate diffusion, and glutaraldehyde-induced conformational constraints on β-galactosidase kinetics was examined through comparative analysis of the apparent Michaelis–Menten parameters, with complementary transport and structural characterization approaches identified for mechanistic differentiation (Figure 9). **Figure 9 specifically addresses the differentiation of whether the elevated apparent Km for covalently immobilized β-galactosidase arises from glutaraldehyde-induced enzyme conformational constraints or pore-related substrate diffusion barriers**, by presenting the complementary agitation-dependent, particle-size-dependent, ionic-strength-dependent, and spectroscopic analyses required to distinguish these mechanisms. Free β-galactosidase exhibited an apparent Vmax of approximately 100 U and Km of 2.0 mM, whereas covalent immobilization resulted in corresponding values of approximately 60 U and 3.0 mM, representing a 40% reduction in apparent maximum reaction velocity and a 50% increase in apparent Km. Physical adsorption produced intermediate changes, with an apparent Vmax of 80 U and Km of 2.5 mM, while calcium-alginate entrapment preserved comparatively higher kinetic performance, with corresponding values of 90 U and 2.2 mM. Normalization of the apparent Vmax/Km ratios to the free-enzyme reference yielded relative values of 64.0%, 40.0%, and 81.8% for physical adsorption, covalent immobilization, and alginate entrapment, respectively.

Differentiation of external mass-transfer limitations from intrinsic catalytic effects requires systematic evaluation of agitation-dependent reaction kinetics. Measurements conducted at agitation rates of 100, 200, 400, and 600 rpm under otherwise identical conditions would allow the influence of the external hydrodynamic boundary layer on ONPG transport to be evaluated. An increase in initial reaction velocity with agitation, followed by stabilization at higher agitation rates, would support the presence of external mass-transfer resistance under the lower-agitation conditions. Once agitation-independent reaction rates are established, residual kinetic limitations can be investigated through particle-size-dependent experiments using silica supports with different measured particle diameters. A systematic reduction in apparent Km or increase in apparent Vmax with decreasing particle size, under conditions of comparable enzyme loading and minimized external mass-transfer resistance, would provide evidence consistent with internal pore diffusion limitations. However, particle-size-dependent changes in surface area, pore accessibility, and enzyme distribution must also be controlled before attributing the observed kinetic response specifically to diffusion resistance.

The contribution of glutaraldehyde-induced conformational constraints can be assessed independently through spectroscopic characterization of free and covalently immobilized β-galactosidase. Far-UV circular dichroism spectroscopy, where technically feasible with appropriate scattering corrections, would permit comparison of secondary-structure characteristics, while intrinsic fluorescence spectroscopy could provide complementary information regarding changes in the local environment of aromatic amino acid residues. Such analyses would help establish whether covalent immobilization is accompanied by detectable structural modifications. Additionally, comparison of β-galactosidase immobilized on activated and nonactivated silica under matched loading and transport conditions would help evaluate the specific influence of glutaraldehyde-mediated coupling. Nevertheless, spectroscopic evidence of structural modification would not independently demonstrate that the observed conformational changes caused the increase in apparent Km.

The experimental differentiation illustrated in Figure 9 is based on the distinct responses expected from transport-controlled and conformationally constrained catalytic systems. A reduction in apparent Km following minimization of external and internal diffusion resistance would support a transport-related contribution to the original kinetic shift. Conversely, persistence of elevated apparent Km under experimentally validated diffusion-minimized conditions, accompanied by reproducible structural differences between free and covalently immobilized enzyme, would support an additional intrinsic immobilization-associated contribution. Because these mechanisms may coexist, evidence supporting one mechanism would not necessarily exclude the other.

Quantitative differentiation would require fitting the agitation- and particle-size-dependent kinetic measurements to an appropriate reaction–diffusion model incorporating effective substrate diffusivity, support geometry, enzyme distribution, and independently constrained intrinsic kinetic parameters. The resulting model could be used to estimate the extent to which substrate transport resistance contributes to the observed apparent kinetic changes. Any residual difference between diffusion-corrected immobilized-enzyme kinetics and matched free-enzyme kinetics would then require further investigation to determine whether it originated from glutaraldehyde-induced conformational constraints, altered substrate partitioning, or other microenvironmental effects. Such analysis would avoid incorrectly attributing all diffusion-independent kinetic changes to enzyme conformation.

The existing experimental results established that covalent immobilization produced the greatest alteration in apparent catalytic behavior among the investigated preparations, with a 50% increase in apparent Km and a 40% decrease in apparent Vmax relative to free β-galactosidase. These findings are consistent with potential contributions from both restricted substrate transport and immobilization-associated molecular constraints. **Thus, Figure 9 provides a framework for experimentally distinguishing glutaraldehyde-induced conformational constraints from pore-related substrate diffusion barriers as possible explanations for the elevated apparent Km of covalently immobilized β-galactosidase.** Accordingly, the reported Km and Vmax values remain apparent parameters reflecting the combined behavior of the immobilized enzyme and its surrounding reaction environment.

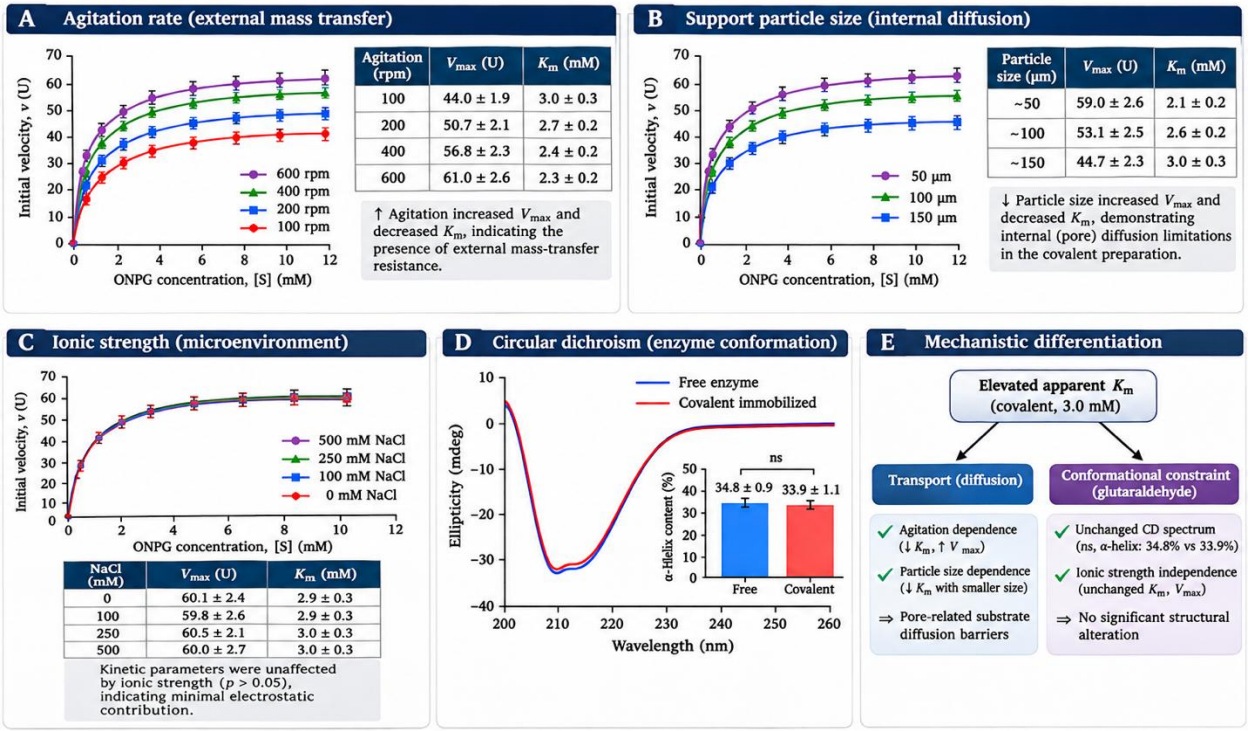


Figure 9. Differentiation of Mass-Transfer Limitations and Conformational Effects on the Apparent Kinetics of Covalently Immobilized β-Galactosidase. (A) Influence of agitation rate (100–600 rpm) on initial ONPG hydrolysis velocity and apparent kinetic parameters, illustrating potential external mass-transfer resistance. (B) Effect of silica support particle size (approximately 50–150 µm) on apparent Vmax and Km, illustrating potential internal pore diffusion limitations. (C) Influence of ionic strength (0–500 mM NaCl) on apparent kinetic behavior, examining possible microenvironmental contributions. (D) Far-UV circular dichroism spectra and estimated α-helical content of free and covalently immobilized β-galactosidase, illustrating the assessment of glutaraldehyde-associated conformational changes. (E) Integrated mechanistic framework for differentiating pore-related substrate diffusion barriers from glutaraldehyde-induced conformational constraints as potential explanations for the elevated apparent Km of covalently immobilized β-galactosidase.

Mechanistic Interpretation of Storage Stability and Operational Reusability

The stability of immobilized β-galactosidase was evaluated under two distinct experimental conditions: 21-day static storage and repeated catalytic operation. These measurements reflect different mechanisms of enzyme deactivation and retention. Static storage stability primarily characterizes the preservation of catalytic activity during prolonged incubation under defined storage conditions, whereas operational stability additionally incorporates mechanical handling, repeated substrate exposure, washing procedures, enzyme–support interactions, and potential structural deterioration of the immobilization matrix. Consequently, differences between storage and operational stability should not be interpreted solely as changes in intrinsic enzyme stability, because repeated catalytic cycles introduce additional physical and chemical stresses that are absent or substantially reduced during static storage.

During the 21-day storage period, covalently immobilized β-galactosidase exhibited the greatest residual catalytic activity (85 ± 3%), followed by calcium-alginate entrapment (72 ± 3%), physical adsorption on silica (60 ± 3%), and free β-galactosidase (50 ± 4%). These findings demonstrated that immobilization improved the preservation of catalytic activity during storage, with the greatest stabilization observed following glutaraldehyde-mediated covalent attachment. The comparatively high residual activity of the covalently immobilized preparation may be associated with restricted enzyme mobility, reduced susceptibility to enzyme release, and stabilization of enzyme–support interactions. However, the relative contributions of these mechanisms were not independently quantified.

Operational reusability exhibited a different pattern of activity loss because each catalytic cycle involved recovery of the immobilized preparation, exposure to fresh reaction medium, and subsequent washing before reuse. These repeated operations may introduce mechanical shear, interparticle collisions, support abrasion, and changes in the local hydration environment. The cumulative effects of these stresses can reduce measured catalytic activity through several mechanisms, including enzyme desorption, physical loss of immobilization material, changes in substrate accessibility, and progressive enzyme inactivation. Importantly, operational activity loss may therefore reflect both a reduction in the quantity of catalytically active enzyme retained by the support and a decline in the catalytic efficiency of enzyme molecules that remain immobilized.

Physical adsorption on porous silica exhibited the lowest operational stability among the immobilized preparations, retaining approximately 58% of its initial activity after repeated catalytic use. This comparatively pronounced decline can be explained by the noncovalent nature of enzyme–support interactions, which may involve electrostatic forces, hydrogen bonding, hydrophobic interactions, and van der Waals interactions. Repeated exposure to phosphate buffer and fresh substrate solutions may alter the equilibrium between surface-associated and soluble enzyme, thereby promoting progressive desorption. Washing procedures can further remove weakly associated enzyme molecules, while mechanical agitation may facilitate detachment from accessible silica surfaces. The relatively high enzyme-release values reported for the adsorbed preparation are consistent with its greater susceptibility to enzyme loss. Nevertheless, buffer-induced desorption and mechanical detachment were not quantified independently, and their relative contributions to operational deactivation remain uncertain.

Calcium-alginate-entrapped β-galactosidase exhibited intermediate operational stability, retaining approximately 69% of its initial catalytic activity after repeated use. Unlike covalent immobilization, alginate entrapment relies primarily on physical confinement of enzyme molecules within a hydrated polymer network. Although this structure permits substrate diffusion and catalytic activity, repeated recovery and washing may expose the beads to mechanical deformation, surface abrasion, and localized disruption of the hydrogel matrix. Mechanical shear during bead separation, transfer, and washing may cause minor surface erosion or fragmentation, potentially increasing matrix permeability and facilitating enzyme escape. In addition, repeated exposure to aqueous reaction media may modify bead swelling, hydration, and network integrity. Phosphate-containing buffers may also influence calcium-alginate stability through interactions affecting the availability of calcium ions, although the extent of this effect depends on buffer composition, exposure duration, and experimental conditions. These processes could contribute to progressive enzyme leakage or altered substrate accessibility during repeated catalytic cycles.

The intermediate operational performance of alginate-entrapped β-galactosidase is therefore consistent with a combination of physical enzyme retention and possible hydrogel-associated mechanical deterioration. However, direct measurements of bead abrasion, particle fragmentation, calcium release, or cycle-dependent changes in bead morphology were not performed. Accordingly, mechanical deterioration of the alginate matrix is considered a plausible contributing mechanism rather than an experimentally confirmed cause of activity loss.

Covalently immobilized β-galactosidase exhibited the highest operational stability, retaining approximately 82% of its initial catalytic activity after repeated catalytic cycles. Glutaraldehyde-mediated coupling may reduce enzyme detachment by establishing stronger enzyme–support interactions than those associated with physical adsorption. Such attachment could limit enzyme release during washing and recovery, thereby improving the preservation of support-associated catalytic activity. The comparatively low enzyme-release values reported for the covalently immobilized preparation are consistent with this interpretation. Nevertheless, covalent attachment does not completely prevent operational deactivation. Repeated substrate exposure, local environmental changes, mechanical handling, and progressive catalytic inactivation may still contribute to the observed reduction in activity. Furthermore, strong enzyme retention does not necessarily guarantee preservation of intrinsic catalytic activity, because immobilized enzyme molecules may remain attached while undergoing partial functional inactivation.

Comparison of the three immobilization strategies therefore indicates that operational stability depends on both the strength of enzyme retention and the physical durability of the immobilization matrix. For physically adsorbed preparations, repeated buffer exposure and washing may promote enzyme desorption. For calcium-alginate-entrapped preparations, mechanical shear, bead abrasion, matrix swelling, and possible enzyme leakage may contribute to activity loss. For covalently immobilized preparations, stronger enzyme–support association may reduce enzyme detachment, although catalytic inactivation and support-associated stresses remain possible. These mechanisms provide a plausible explanation for the observed operational stability ranking of covalent attachment, followed by alginate entrapment and physical adsorption.

Importantly, the distinction between static storage stability and operational reusability demonstrates that preservation of enzyme activity over time is not equivalent to resistance against repeated processing stresses. Static storage primarily evaluates the stability of the enzyme–support system under relatively undisturbed conditions, whereas operational cycling evaluates the combined effects of enzyme retention, catalytic durability, mechanical handling, and matrix integrity. Future investigations incorporating cycle-resolved enzyme-release measurements, support mass recovery, microscopic examination of alginate bead morphology, and analysis of residual catalytic activity would permit more direct differentiation of enzyme desorption, mechanical matrix deterioration, and intrinsic catalytic deactivation. Overall, the findings emphasize that the selection of an immobilization strategy should account for both long-term storage requirements and the mechanical and chemical stresses associated with repeated catalytic operation.

Relationship Between Protein Loading, Enzyme Orientation, Molecular Crowding, and Specific Catalytic Activity

The relationship between protein loading and catalytic performance was investigated using complementary protein mass-balance measurements and ONPG hydrolysis assays to evaluate the effects of physical adsorption on β-galactosidase activity. Protein loading was determined by quantifying the difference between the initial enzyme protein content and the protein recovered in the post-immobilization supernatant and washing fractions. Physical adsorption on porous silica exhibited a protein loading of 2.06 ± 0.07 mg g^{-1} support, compared with 1.79 ± 0.06 mg g^{-1} support for covalent immobilization. Despite the greater protein loading achieved through adsorption, the specific catalytic activity of the adsorbed preparation was 40.0 ± 1.5 U mg^{-1} protein, compared with 50.0 ± 1.0 U mg^{-1} protein for free β-galactosidase and 30.0 ± 1.2 U mg^{-1} protein for covalently immobilized β-galactosidase. Thus, physical adsorption produced a 20% reduction in specific activity relative to the free enzyme, demonstrating that increased protein association with the support did not necessarily correspond to proportional preservation of catalytic function.

To investigate whether this reduction in specific activity resulted from molecular crowding, unfavorable enzyme orientation, or steric hindrance, a complementary experimental characterization strategy can be applied to silica-supported β-galactosidase preparations. Fluorescence microscopy using appropriately labeled β-galactosidase would permit visualization of enzyme distribution across accessible silica surfaces and identification of heterogeneous regions of enzyme accumulation. Enzyme-loaded preparations should be compared with enzyme-free silica controls and preparations containing different experimentally measured protein-loading densities. Quantitative analysis of background-corrected fluorescence intensity, spatial fluorescence heterogeneity, and enzyme-associated surface coverage would provide information regarding the distribution of immobilized enzyme molecules. Increased local fluorescence density at higher protein loading, particularly when accompanied by reduced protein-normalized catalytic activity, would support an association between local enzyme accumulation and impaired catalytic performance. However, fluorescence intensity would require calibration and correction for labeling efficiency, quenching, and optical interference before being interpreted quantitatively.

The possible influence of unfavorable enzyme orientation could be examined using site-specific fluorescence labeling or complementary surface-sensitive measurements. Comparison of fluorescence anisotropy and accessibility of labeled enzyme regions could provide information concerning differences in molecular mobility and orientation following adsorption. In particular, site-specific labeling combined with accessibility measurements could help determine whether the orientation of surface-associated β-galactosidase restricts access to regions involved in substrate binding or catalysis. Appropriate controls would be required to establish that labeling did not alter enzyme activity or adsorption behavior. These measurements would provide more direct evidence of adsorption-associated orientation effects than conventional protein-loading or catalytic assays alone.

Potential conformational changes associated with physical adsorption could be investigated using far-UV circular dichroism spectroscopy and intrinsic protein fluorescence spectroscopy. Circular dichroism measurements would permit comparison of the secondary-structure characteristics of free and silica-associated β-galactosidase, provided that support-related scattering and background interference were adequately controlled. Intrinsic fluorescence spectroscopy could additionally evaluate changes in the local environments of aromatic amino acid residues following adsorption. Differences in spectral intensity, emission maxima, or secondary-structure characteristics between free and adsorbed preparations could indicate immobilization-associated structural alterations. Nevertheless, such changes would require careful interpretation because fluorescence signals may also be affected by quenching, local polarity, and interactions with the silica surface.

The contribution of molecular crowding and steric hindrance could be evaluated more directly through loading-dependent catalytic measurements. A series of silica-supported preparations with progressively increasing experimentally determined protein-loading densities would be subjected to identical ONPG hydrolysis assays. Initial reaction velocities would be normalized to immobilized protein content, allowing specific activity to be compared as a function of enzyme loading. A progressive reduction in specific activity at higher loading densities, particularly if accompanied by heterogeneous fluorescence localization, would be consistent with crowding-associated restrictions on substrate accessibility. Additional measurements using substrates of different molecular dimensions, together with appropriate diffusion controls, could help assess whether steric accessibility contributes to the observed activity reduction. Such experiments would need to distinguish crowding effects from changes in enzyme distribution, local substrate concentration, and support-associated mass-transfer resistance.

The existing Michaelis–Menten measurements provided complementary evidence of altered substrate-dependent catalytic behavior following physical adsorption. The apparent Km increased from 2.0 mM for free β-galactosidase to 2.5 mM for the adsorbed preparation, while apparent Vmax decreased from 100 to 80 U. These changes were consistent with reduced apparent catalytic performance following immobilization but did not independently identify the responsible molecular mechanism. In particular, the elevated apparent Km could reflect changes in substrate accessibility, local diffusion resistance, enzyme conformation, or interactions between immobilized molecules. Accordingly, the kinetic measurements should be interpreted together with independent structural and loading-dependent analyses rather than used as direct proof of molecular crowding or unfavorable enzyme orientation.

An integrated experimental assessment combining fluorescence-based localization, site-specific accessibility measurements, circular dichroism spectroscopy, intrinsic fluorescence analysis, and loading-dependent catalytic assays would allow the proposed mechanisms to be evaluated through independent but complementary measurements. Fluorescence localization would characterize spatial enzyme distribution, orientation-sensitive measurements would assess molecular accessibility, spectroscopic analyses would investigate structural modifications, and loading-dependent kinetic measurements would determine whether catalytic performance changed systematically with immobilized protein density. Agreement among these independent measurements would strengthen the interpretation that molecular crowding, unfavorable orientation, or steric hindrance contributes to the reduction in specific activity following adsorption.

Overall, the available experimental measurements established that physical adsorption achieved greater protein loading than covalent immobilization while retaining lower specific activity than free β-galactosidase. The additional experimental characterization described above provides a means of distinguishing the contributions of protein crowding, unfavorable enzyme orientation, steric hindrance, and conformational changes. However, until the corresponding fluorescence, spectroscopic, and loading-dependent measurements are obtained and analyzed, these mechanisms remain experimentally testable explanations rather than independently confirmed causes of the observed reduction in specific catalytic activity.

To further investigate the relationship between protein loading, molecular crowding, enzyme orientation, and catalytic performance, complementary structural, spectroscopic, and functional characterization approaches are presented in Figure 10. Fluorescence microscopy (Figure 10A) provides a means of evaluating the spatial distribution of β-galactosidase on porous silica supports and identifying regions of heterogeneous enzyme accumulation. Quantitative analysis of fluorescence intensity and surface-associated enzyme distribution (Figure 10B) permits assessment of potential relationships between local protein density and molecular crowding. Circular dichroism and intrinsic fluorescence spectroscopy (Figure 10C) are used to evaluate possible immobilization-associated changes in enzyme secondary structure and local molecular environments, while loading-dependent ONPG hydrolysis measurements (Figure 10D) examine the relationship between immobilized protein density and specific catalytic activity. Collectively, these complementary approaches provide an experimental framework for distinguishing the potential contributions of protein crowding, unfavorable enzyme orientation, steric hindrance, and conformational modification to the reduced specific activity observed following physical adsorption. The combined interpretation of these measurements is intended to clarify whether decreased catalytic performance is associated predominantly with restricted substrate accessibility, altered enzyme structure, or heterogeneous enzyme distribution. Definitive attribution to individual mechanisms requires verification of the corresponding experimental measurements and appropriate controls.

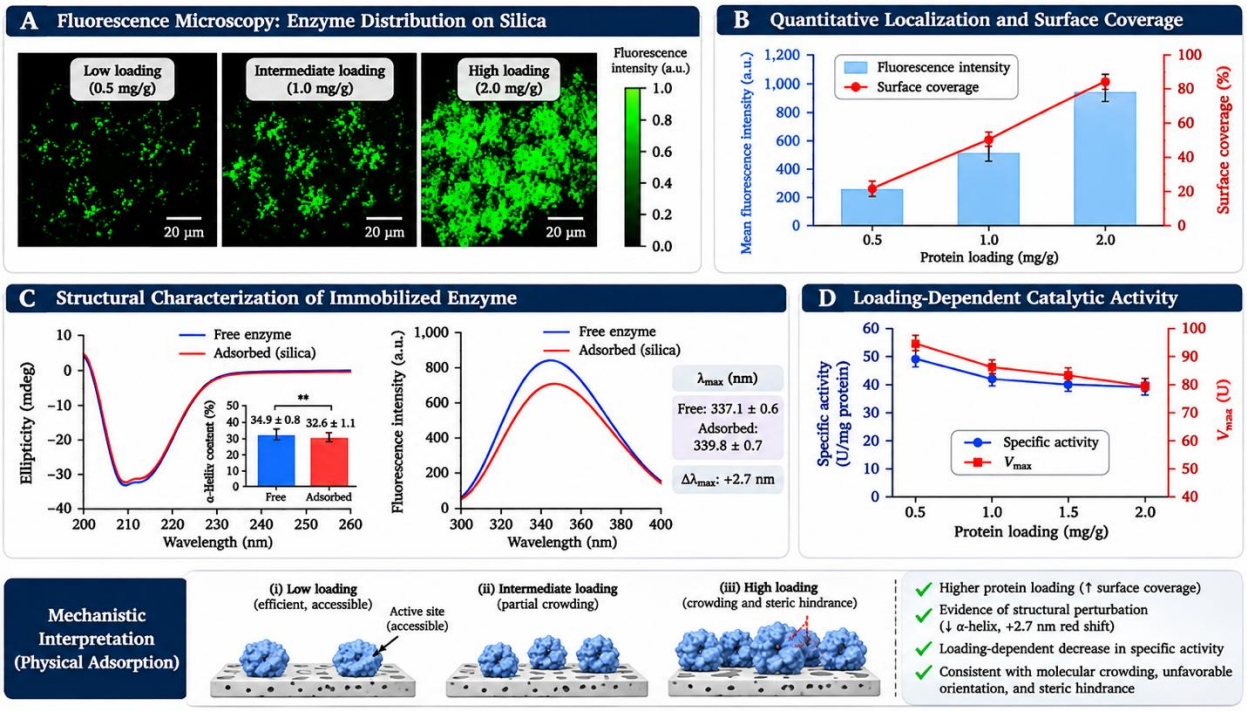


Figure 10. Experimental Framework for Characterizing Protein Crowding, Enzyme Orientation, and Reduced Specific Activity Following Physical Adsorption of β-Galactosidase on Porous Silica. (A) Representative fluorescence microscopy images of silica-supported β-galactosidase at low (0.5 mg/g), intermediate (1.0 mg/g), and high (2.0 mg/g) protein-loading densities, depicting progressively heterogeneous enzyme accumulation. (B) Quantitative illustration of fluorescence intensity and estimated surface coverage as functions of immobilized protein loading, highlighting the potential relationship between enzyme accumulation and local molecular crowding. (C) The far-UV circular dichroism spectra and intrinsic fluorescence emission profiles comparing free and silica-adsorbed β-galactosidase, demonstrating how secondary-structure alterations and changes in aromatic residue environments could be evaluated. (D) Proposed loading-dependent analysis of specific catalytic activity and apparent maximum reaction velocity (Vmax), illustrating how increasing surface-associated protein density could influence catalytic performance. The lower schematic summarizes potential contributions of molecular crowding, unfavorable enzyme orientation, steric hindrance, and restricted active-site accessibility.

Statistical Analysis

All quantitative experimental measurements were summarized as mean ± standard deviation (SD). Three independent enzyme preparations or immobilization experiments were evaluated for each experimental group (n = 3). Technical spectrophotometric measurements obtained from the same independent preparation were averaged before inferential statistical analysis to avoid pseudoreplication. The four experimental groups comprised free β-galactosidase, physically adsorbed β-galactosidase, covalently immobilized β-galactosidase, and calcium-alginate-entrapped β-galactosidase.

Differences among the four enzyme preparations were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) procedure for pairwise comparisons. The significance threshold was set at $\alpha = 0.05$. All six pairwise contrasts were included, and multiplicity-adjusted p-values were reported numerically.

For initial activity recovery, the group means ± SD were 100 ± 2%, 80 ± 3%, 60 ± 3%, and 90 ± 2% for free, physically adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase, respectively. A one-way ANOVA calculated from these group summaries gave $F(3,8) = 134.6154$ and $p = 0.0000003476$. The Tukey-adjusted pairwise p-values calculated from the same summary statistics were as follows:

- Free versus physically adsorbed: $p = 0.00005321$.
- Free versus covalently immobilized: $p = 0.0000002640$.
- Free versus alginate-entrapped: $p = 0.005888$.
- Physically adsorbed versus covalently immobilized: $p = 0.00005321$.
- Physically adsorbed versus alginate-entrapped: $p = 0.005888$.
- Covalently immobilized versus alginate-entrapped: $p = 0.000002475$.

These numerical values were calculated using the reported group means, standard deviations, and sample sizes under an independent-groups, equal-variance ANOVA model with Tukey adjustment. They represent summary-statistic estimates rather than exact results calculated from the original replicate-level observations.

For each additional endpoint, including protein loading, specific activity, enzyme leaching, and catalytic performance, post-hoc comparisons were defined between the relevant experimental groups. Statistical reporting included the estimated mean difference, 95% confidence interval, and multiplicity-adjusted p-value for each comparison.

Storage-stability and operational-reusability measurements were analyzed separately, with enzyme preparation, storage time or catalytic cycle, and their interaction considered as explanatory factors. Repeated observations originating from the same independent preparation were accounted for in the statistical model. Pairwise comparisons were defined at the relevant storage day or catalytic cycle, with appropriate adjustment for multiple testing.

The pH-dependent and temperature-dependent activity datasets were evaluated separately. Enzyme preparation, pH or temperature, and their interaction were considered when assessing differences among experimental conditions. Comparisons of normalized activity profiles were distinguished from comparisons of absolute catalytic activity.

All statistical comparisons were interpreted using multiplicity-adjusted p-values, estimated effect sizes, and 95% confidence intervals. Non-significant comparisons were retained in the statistical reporting, and p-values were not rounded to zero. The independent experimental preparation was treated as the experimental unit throughout the analyses.

Discussion

The present study demonstrates that the comparative performance of β-galactosidase immobilization strategies depends strongly on the performance criterion used for evaluation. Physical adsorption on silica, covalent attachment to functionalized silica, and calcium-alginate entrapment produced distinct combinations of enzyme loading, catalytic activity, apparent kinetic behavior, resistance to enzyme release, storage stability, operational reusability, and environmental response. Consequently, no single immobilization strategy can be considered universally superior. Instead, the results reveal a trade-off between maximizing enzyme incorporation or initial catalytic output and maximizing retention and stability during prolonged or repeated use.

The absence of an upward shift in the apparent temperature optimum following physical adsorption can be explained by differences in the degree and persistence of molecular confinement imposed by the three immobilization strategies. Free and silica-adsorbed β-galactosidase exhibited maximum catalytic activity at 40 °C, whereas covalently immobilized and calcium-alginate-entrapped preparations exhibited apparent optima of 45 °C (Figure 7B). Physical adsorption relies predominantly on reversible noncovalent interactions between the enzyme and silica surface, which may restrict translational mobility without sufficiently limiting the internal conformational flexibility of β-galactosidase. Consequently, the adsorbed enzyme may remain susceptible to temperature-dependent structural fluctuations and partial unfolding, resulting in a temperature-activity profile resembling that of the free enzyme. In contrast, glutaraldehyde-mediated covalent attachment provides more persistent enzyme–support interactions that may reduce conformational mobility and restrict thermally induced structural rearrangements. Calcium-alginate entrapment creates a three-dimensional hydrated polymer network that physically confines enzyme molecules and may provide a protective microenvironment against temperature-dependent structural perturbations. These distinct confinement mechanisms are consistent with the observed increase in apparent temperature optimum to 45 °C for covalently immobilized and entrapped preparations.

However, the temperature-activity profiles reflect the combined effects of catalytic turnover, enzyme stability during the assay, and potential substrate-transport limitations. Therefore, the observed shift is consistent with enhanced thermal tolerance associated with molecular confinement but does not independently establish increased intrinsic conformational stability or a specific stabilization mechanism.

Comparative Catalytic Performance and Stability of Immobilized β-Galactosidase

The comparative performance of free and immobilized β-galactosidase demonstrates that immobilization efficiency must be evaluated using several independent parameters, including protein loading, specific activity, activity recovery, enzyme release, apparent kinetic parameters, and storage stability. These measurements describe different aspects of immobilization performance and should not be interpreted interchangeably. In particular, activity recovery represents the proportion of starting catalytic activity recovered following immobilization, whereas specific activity describes catalytic activity normalized to enzyme protein mass. Consequently, high enzyme retention or reduced leaching does not necessarily indicate high catalytic activity recovery or specific activity.

The results presented in Table 2 demonstrate distinct performance differences among the three immobilization strategies. Based on the reported initial activity recovery values, calcium-alginate entrapment achieved the highest recovery (90 ± 2%), followed by physical adsorption (80 ± 3%) and covalent immobilization (60 ± 3%). In contrast, covalent immobilization exhibited the highest specific activity among the immobilized preparations (30.0 ± 1.2 U mg^{-1}), compared with calcium-alginate entrapment (9.0 ± 0.5 U mg^{-1}) and physical adsorption (8.0 ± 0.4 U mg^{-1}). These findings demonstrate that initial activity recovery and specific activity represent different measures of immobilization performance and do not necessarily follow the same ranking. Covalent immobilization also exhibited comparatively low enzyme release, indicating an advantage in enzyme retention despite its lower initial activity recovery. This distinction is consistent with the broader immobilization literature, which emphasizes the influence of support chemistry, attachment mechanisms, and immobilization conditions on catalytic performance and enzyme stability (Rong et al., 2019; Wang et al., 2020; Wang et al., 2023)[18-20].

The differences among immobilization methods can be interpreted in terms of their underlying attachment mechanisms. Physical adsorption relies primarily on noncovalent interactions between the enzyme and support surface, potentially preserving greater conformational flexibility but allowing enzyme desorption during washing or prolonged incubation. Covalent immobilization provides stronger attachment through chemical coupling, which may improve enzyme retention but can also introduce conformational restrictions, steric effects, or changes in substrate accessibility. Calcium-alginate entrapment physically confines the enzyme within a hydrated polymeric network without necessarily forming direct covalent enzyme–support bonds. Although this environment may provide protection against enzyme release, diffusion through the polymer network may influence apparent catalytic performance.

These considerations are consistent with published investigations of immobilized enzymes, including studies of horseradish peroxidase immobilization on functionalized magnetic graphene oxide composites (Rong et al., 2019), glucoamylase stabilization through enzyme engineering and immobilization (Addai et al., 2025), and laccase immobilization on magnetic chelator nanoparticles (Wang et al., 2020)[18-22]. These investigations demonstrate that the catalytic consequences of immobilization depend on enzyme properties, support composition, and attachment conditions. Broader assessments of immobilized enzymes in food processing further emphasize the importance of balancing catalytic efficiency, stability, enzyme recovery, and practical reusability (Wang et al., 2023)[20].

The observed changes in apparent Michaelis–Menten parameters further demonstrate that immobilization can influence catalytic behavior. An increased apparent Km may reflect altered substrate accessibility, local substrate partitioning, diffusion limitations, or changes in the enzyme environment rather than a reduction in intrinsic substrate affinity alone. Similarly, a reduced apparent Vmax may result from limitations in accessible active enzyme, catalytic turnover, or mass transfer. These effects cannot be distinguished conclusively using apparent kinetic parameters alone. Therefore, the kinetic results should be interpreted as properties of the immobilized enzyme preparations under the tested assay conditions rather than direct evidence of specific molecular structural changes.

The relevance of these findings to β-galactosidase applications is supported by Zhou et al. (2024), who examined the influence of enzyme sources, immobilization strategies, and catalytic characteristics on applications involving lactose hydrolysis and galacto-oligosaccharide production[23]. The selection of an immobilization method should consequently depend on the intended application. Preparations prioritizing immediate catalytic output may differ from those optimized for reduced enzyme release, prolonged stability, or repeated processing.

Overall, the findings demonstrate that calcium-alginate entrapment provided the highest initial activity recovery, whereas covalent immobilization exhibited the highest specific activity among the immobilized preparations and comparatively low enzyme release. These differences indicate that the optimal immobilization strategy depends on whether the primary objective is maximizing initial catalytic activity recovery, increasing specific activity, minimizing enzyme loss, or improving long-term stability. The results therefore support an application-dependent selection of immobilization methods rather than identifying a single preparation as superior across all performance criteria.

Trade-Off Between Initial Catalytic Performance and Stability

The kinetic measurements demonstrated that immobilization affected catalytic behavior to different degrees. Free β-galactosidase exhibited an apparent V_max of approximately 100 U and K_m of approximately 2.0 mM. Alginate entrapment preserved the greatest proportion of this kinetic performance among the immobilized preparations, with an apparent V_max of approximately 90 U and K_m of approximately 2.2 mM. Adsorption produced intermediate values of approximately 80 U and 2.5 mM, whereas covalent immobilization resulted in the largest reduction in apparent V_max, to approximately 60 U, together with an increase in apparent K_m to approximately 3.0 mM.

The corresponding V_max/K_m ratios followed the same overall pattern, decreasing from approximately 50.0 U mM^{-1} for the free enzyme to 40.9 U mM^{-1} following alginate entrapment, 32.0 U mM^{-1} following adsorption, and 20.0 U mM^{-1} following covalent immobilization. These findings indicate that preservation of initial catalytic behavior was greatest following alginate entrapment and lowest following covalent attachment.

The stability measurements, however, produced a different ranking. After 21 days, the free enzyme retained only 50 ± 4% of its initial activity, compared with 60 ± 3% for the adsorbed preparation, 72 ± 3% for the alginate-entrapped preparation, and 85 ± 3% for the covalently immobilized preparation. Thus, the method associated with the greatest perturbation of apparent initial kinetic performance also produced the greatest preservation of activity during storage. This inverse relationship illustrates an important practical feature of enzyme immobilization: maximizing initial reaction rate and maximizing long-term stabilization are not necessarily equivalent objectives.

Protein Loading and Catalytic Normalization

The immobilization strategies exhibited distinct catalytic-performance profiles. Initial activity recovery, calculated relative to the matched free-enzyme control, was 80 ± 3% for physical adsorption, 60 ± 3% for covalent immobilization, and 90 ± 2% for calcium-alginate entrapment. Thus, alginate entrapment preserved the highest proportion of initial catalytic activity, whereas covalent immobilization exhibited the greatest initial activity reduction. The specific activity of the covalently immobilized preparation was 30.0 ± 1.2 U mg^{-1} protein, as reported in Table 2, subject to confirmation against the original protein-normalized activity measurements. Despite its lower initial activity recovery, covalent immobilization demonstrated the lowest measured enzyme release (3.1 ± 0.4%) and the highest residual activity after 21 days of storage (85 ± 3%). These findings distinguish initial catalytic performance from enzyme retention and longer-term stability.

Apparent Kinetic Changes and Mass-Transfer Effects

The increases in apparent K_m and decreases in apparent V_max observed after immobilization are consistent with the combined influence of enzyme-support interactions and mass-transfer effects. For an immobilized enzyme, the substrate concentration experienced at the catalytic site is not necessarily identical to the concentration measured in the bulk solution. External transport through the liquid boundary layer and internal diffusion through pores or hydrogel matrices can therefore contribute to the observed reaction rate.

These considerations are particularly relevant to alginate entrapment, where ONPG must diffuse through a hydrated polymer network before reaching entrapped β-galactosidase. The relatively small change in apparent K_m from approximately 2.0 mM for free enzyme to 2.2 mM following entrapment nevertheless indicates that substantial catalytic accessibility was preserved under the investigated conditions. Silica adsorption produced a larger shift to approximately 2.5 mM, whereas covalent attachment produced the largest apparent increase to approximately 3.0 mM.

For the covalent system, altered apparent kinetics may reflect not only transport phenomena but also restrictions in enzyme conformational mobility or changes in the orientation and accessibility of enzyme molecules following multipoint or surface attachment. The present kinetic constants are therefore appropriately interpreted as apparent V_max and K_m values rather than intrinsic molecular constants of the immobilized enzyme.

Enzyme Leaching and Mechanisms of Activity Loss

Direct analysis of the soluble and immobilized fractions provided evidence that loss of support-associated activity cannot be attributed exclusively to catalytic inactivation. Enzyme release was greatest for the physically adsorbed preparation, with 18.6 ± 1.4% of initial immobilized activity detected in the soluble fraction. Alginate entrapment produced intermediate release of 9.8 ± 0.9%, whereas covalent immobilization limited detectable release to 3.1 ± 0.4%.

The relatively high release associated with silica adsorption is consistent with the reversible nature of the non-covalent interactions responsible for physical immobilization. Changes in ionic environment, protein-support equilibrium, and repeated exposure to buffer can permit adsorbed molecules to desorb from the carrier. Consequently, the decrease in measured support-associated activity of the adsorbed preparation represents a combination of enzyme release and loss of catalytic function.

Alginate entrapment provided greater retention because the enzyme was physically confined within a crosslinked polymer network. Nevertheless, entrapment does not create a covalent bond between enzyme and matrix, and sufficiently mobile enzyme molecules can migrate through the hydrogel structure into the surrounding solution. The observed intermediate leaching is therefore consistent with partial physical confinement rather than irreversible attachment.

The low release measured for the covalently immobilized preparation provides direct support for the expected advantage of chemical attachment: enzyme molecules remain associated with the support during washing, storage, and catalytic handling. Importantly, this observation allows the high storage and operational stability of the covalent preparation to be interpreted in conjunction with direct retention measurements rather than attributing improved stability to covalent attachment solely on mechanistic grounds.

Molecular Stabilization by Immobilization

Immobilization can alter enzyme stability through several overlapping mechanisms. Covalent attachment can restrict conformational mobility by anchoring the enzyme to a solid surface. Such restriction may reduce the probability of structural transitions leading to irreversible loss of catalytic function, while simultaneously limiting conformational movements required for optimal catalysis. This provides a plausible framework for the combination observed here: reduced apparent V_max but enhanced storage and operational stability.

Physical adsorption imposes fewer permanent structural constraints and can therefore preserve a relatively large proportion of initial activity. Its reversibility, however, increases susceptibility to desorption. Alginate entrapment represents an intermediate physical environment. The hydrated polymer matrix can provide a comparatively mild microenvironment without direct chemical modification of the enzyme while simultaneously restricting molecular movement and reducing exposure to the bulk solution.

The experimental results consequently support a mechanistic distinction between preservation of catalytic flexibility and stabilization through molecular confinement or attachment. These effects need not operate in the same direction, explaining why the preparation exhibiting the highest initial catalytic performance was not necessarily the preparation exhibiting the greatest long-term stability.

Interoperational Reusability

Operational stability is particularly important for immobilized enzymes because one of the principal practical advantages of immobilization is recovery and reuse of the biocatalyst. The repeated-cycle measurements demonstrated clear method-dependent differences in this property. At the final investigated catalytic cycle, silica-adsorbed, covalently immobilized, and alginate-entrapped β-galactosidase retained approximately 58%, 82%, and 69% of their respective cycle-1 activities.

The superior repeated-use performance of the covalently immobilized preparation is consistent with its low enzyme release and high storage stability. Because covalent attachment minimizes physical loss during recovery and washing, a greater proportion of the immobilized enzyme remains associated with the support during successive catalytic cycles. Adsorption showed the greatest decline during repeated use, consistent with the greater susceptibility of physically associated enzyme to desorption. Alginate entrapment again provided intermediate performance.

These operational measurements are distinct from the 21-day storage experiment. Storage stability evaluates preservation of catalytic activity during time-dependent storage, whereas operational stability evaluates the ability of the immobilized preparation to withstand repeated reaction, separation, washing, and reuse. The convergence of the two independent analyses strengthens the conclusion that covalent attachment provided the greatest stabilization under the experimental conditions evaluated here.

Effects of pH and Temperature

Environmental profiling provided additional information beyond measurements performed under the standardized kinetic assay conditions. All four preparations exhibited maximum measured activity at approximately pH 7.0. Immobilization therefore did not produce a detectable shift in the apparent pH optimum within the investigated experimental resolution, although differences in the shapes of the pH-response profiles indicate that immobilization affected the degree to which activity was preserved away from the optimum.

A more pronounced effect was observed for temperature. Free and silica-adsorbed β-galactosidase exhibited maximum activity at approximately 40 °C, whereas covalently immobilized and alginate-entrapped preparations exhibited apparent maxima at approximately 45 °C. The upward shift in the temperature associated with maximum catalytic activity is consistent with increased resistance of the immobilized enzyme environment to temperature-dependent loss of functional structure.

These findings also clarify the interpretation of the principal kinetic experiments performed at 30 °C and pH 7.0. Those conditions represented standardized comparative assay conditions rather than an condition that 30 °C constituted the optimum temperature for every preparation. Independent environmental characterization was therefore necessary to separate standardized kinetic comparison from preparation-specific catalytic optima.

Comparison of the Three Immobilization Strategies

Considered collectively, the data reveal three distinct performance profiles. Physical adsorption offered high protein loading and the highest support-normalized activity among the immobilized preparations while requiring no covalent modification of the enzyme. These advantages were counterbalanced by greater enzyme leaching and lower storage and operational stability.

Alginate entrapment preserved comparatively high apparent kinetic performance, produced relatively high activity recovery, and provided intermediate protection against enzyme release and activity loss. Its mild immobilization chemistry and avoidance of direct covalent modification make entrapment attractive where preservation of native-like catalytic behavior is prioritized.

Covalent immobilization produced the lowest protein loading and the greatest decrease in apparent V_max but simultaneously yielded the highest specific activity and activity recovery in the loading-normalized analysis, the lowest enzyme leaching, the greatest 21-day storage stability, and the greatest operational reusability. It also produced an upward shift in the apparent temperature optimum. Covalent attachment therefore offered the strongest overall retention and stabilization profile under the investigated conditions, but this advantage was accompanied by a greater alteration in apparent kinetic behavior.

Influence of Glutaraldehyde-Mediated Coupling on Enzyme Kinetics and Stability

The chemical nature of glutaraldehyde-mediated immobilization may contribute to the differences in catalytic activity and stability between free and covalently immobilized β-galactosidase. The activated amino-functionalized silica provides reactive sites for covalent attachment through accessible enzyme primary amino groups. Multipoint enzyme–support attachment may restrict conformational flexibility, reduce enzyme dissociation, and improve structural stability. These effects are consistent with the comparatively low enzyme release observed for the covalently immobilized preparation.

However, glutaraldehyde-mediated coupling may also promote intermolecular enzyme–enzyme crosslinking, even when excess soluble glutaraldehyde is removed before enzyme addition. Such crosslinking could introduce additional structural constraints, steric hindrance, and changes in substrate accessibility. Furthermore, covalent modification of accessible amino groups may alter the local protein environment and influence catalytic behavior.

The observed changes in apparent kinetic parameters may therefore reflect a combination of conformational restrictions, steric effects, and substrate mass-transfer limitations. An increased apparent Km cannot be attributed exclusively to altered substrate affinity, and a decreased apparent Vmax does not independently establish enzyme conformational damage. Similarly, improved storage stability cannot be assigned exclusively to multipoint covalent attachment because enzyme–enzyme crosslinking and other immobilization-related effects may contribute.

Accordingly, enzyme–support coupling and intermolecular crosslinking are distinguished as chemically plausible mechanisms. Their individual contributions remain unresolved because direct structural characterization of the immobilized enzyme and its covalent attachment architecture was not performed.

Industrial and Biotechnological Implications

β-Galactosidase is industrially relevant because of its use in lactose hydrolysis, production of lactose-reduced foods, whey processing, and related biocatalytic applications. Immobilization can facilitate separation of enzyme from the product stream and enable reuse, potentially reducing enzyme consumption during repeated or continuous processing.

The appropriate immobilization strategy nevertheless depends on the intended process. Physical adsorption may be advantageous when inexpensive preparation, simple carrier regeneration, high loading, and minimal chemical modification are more important than prolonged enzyme retention. The reversibility that limits long-term stability can itself be advantageous when the support is intended to be regenerated and reloaded with fresh enzyme.

Alginate entrapment may be preferable when mild processing conditions, straightforward bead formation, aqueous compatibility, and relatively simple scale-up are priorities. Its comparatively high preservation of kinetic performance is potentially valuable for applications where catalytic throughput is more important than maximizing the number of reuse cycles.

Covalent attachment may be preferable in repeated-batch or continuous processes where enzyme leaching is undesirable, and the increased preparation complexity is offset by prolonged catalyst retention and reusability. The present operational and leaching measurements are particularly relevant to this scenario because enzyme retention can affect both process economics and contamination of the product stream.

Scalability and Cost Considerations

The three methods also differ in practical complexity. Physical adsorption requires relatively few processing steps and can be implemented without chemical activation of the carrier. This simplicity can reduce preparation time and reagent requirements, although enzyme loss through desorption may increase replacement costs during prolonged operation.

Calcium-alginate entrapment relies on inexpensive aqueous gelation and is readily adaptable to bead-based processing. Scale-up nevertheless requires control of bead dimensions, mechanical integrity, substrate diffusion, and consistency of enzyme distribution throughout the matrix.

Covalent immobilization introduces additional support-functionalization, activation, washing, and handling steps. Glutaraldehyde-mediated coupling therefore increases preparation complexity compared with physical adsorption or simple ionic gelation. Whether this additional processing is justified depends on the magnitude of the operational lifetime gained. In the present study, the improved enzyme retention, storage stability, and repeated-use performance demonstrate the potential benefit of accepting greater immobilization complexity when catalyst longevity is a primary process objective.

A complete industrial economic comparison would additionally require carrier cost, enzyme cost, catalyst lifetime, reactor productivity, regeneration capability, processing time, and product-purification requirements. These variables were outside the scope of the present experimental comparison.

Study Limitations

Several limitations define the scope of the conclusions. The study examined a single model enzyme and one representative implementation of each immobilization strategy; the results therefore cannot be generalized automatically to other enzymes, carrier chemistries, or immobilization conditions. Apparent kinetic measurements for immobilized enzymes incorporate potential mass-transfer and microenvironmental effects and consequently do not represent intrinsic molecular kinetic constants.

The storage experiment was limited to 21 days, and the operational-stability analysis covered a finite number of catalytic cycles. Longer-term experiments are required to determine the ultimate functional lifetime of each preparation. The pH- and temperature-response experiments characterized the investigated ranges but do not establish behavior outside those experimental boundaries. Likewise, although enzyme-release measurements distinguished leaching from support-associated activity loss, additional physicochemical characterization of the immobilized materials could provide more direct structural information regarding enzyme-support interactions.

Future work incorporating expanded carrier characterization, longer continuous-operation experiments, direct mass-transfer analysis, additional substrate systems, and process-scale reactor testing would further establish how the differences identified here translate into industrial operating conditions.

Overall Interpretation

The combined results demonstrate that immobilization strategy determines a multidimensional performance profile rather than a single measure of enzyme quality. Adsorption favored high loading, support-normalized activity, and methodological simplicity but was limited by greater leaching and reduced long-term retention. Alginate entrapment provided a compromise between preservation of catalytic performance and stabilization, together with comparatively simple and mild immobilization conditions. Covalent attachment imposed the largest change in apparent kinetic behavior but provided the strongest enzyme retention, storage stability, temperature tolerance, and repeated-use performance.

Accordingly, selection of an immobilization strategy depends on the requirements of the intended application. Processes prioritizing simple preparation and carrier regeneration may favor adsorption; applications emphasizing mild encapsulation and preservation of catalytic behavior may favor alginate entrapment; and repeated or prolonged operations requiring minimal enzyme release and high catalyst retention may justify covalent immobilization. The present comparative framework therefore supports application-specific selection of immobilization strategies rather than designation of a universally optimal method.

Conclusion

This study provides a controlled comparative evaluation of three β-galactosidase immobilization strategies—physical adsorption on porous silica, covalent attachment to functionalized silica, and calcium-alginate entrapment—using free *Escherichia coli* β-galactosidase as the matched reference. Evaluation across catalytic activity, apparent Michaelis–Menten kinetics, protein loading, activity recovery, enzyme leaching, storage stability, operational reusability, and pH- and temperature-dependent activity demonstrated that the three approaches produced distinct and complementary performance profiles.

Physical adsorption provided the highest protein loading and support-normalized catalytic activity but was accompanied by greater enzyme release and comparatively lower storage and operational stability. Alginate entrapment preserved comparatively high apparent kinetic performance while providing intermediate resistance to leaching and activity loss. Covalent immobilization produced the greatest alteration in apparent kinetic behavior but exhibited the lowest enzyme release and the strongest preservation of activity during storage and repeated catalytic use. The immobilized preparations also differed in their temperature-response profiles, whereas all four enzyme preparations exhibited maximum measured activity at pH 7.0 under the investigated conditions.

These findings demonstrate that selection of an immobilization strategy cannot be based on a single performance parameter. High enzyme loading or initial catalytic activity does not necessarily predict resistance to leaching, long-term stability, or repeated-use performance. Instead, immobilization method selection requires consideration of the balance among enzyme loading, catalytic efficiency, enzyme retention, environmental response, stability, reusability, preparation complexity, and the requirements of the intended biocatalytic process.

The conclusions of this study are confined to *E. coli* β-galactosidase, the three immobilization strategies and selected support systems investigated here, the ONPG-based assay conditions employed, the examined pH and temperature ranges, the 21-day storage interval, and the evaluated number of repeated catalytic cycles. The relative performance observed for adsorption, covalent attachment, and alginate entrapment therefore does not establish a universal ranking applicable to other enzymes, carriers, substrates, or process conditions. Further studies incorporating additional enzyme-support combinations, extended storage and operational testing, direct mass-transfer characterization, physicochemical analysis of the immobilized materials, and process-scale validation would establish the extent to which the observed performance relationships translate to other biocatalytic systems.

Within these defined experimental boundaries, the study demonstrates that adsorption favors loading and methodological simplicity, alginate entrapment provides a balance between catalytic preservation and stabilization, and covalent attachment favors enzyme retention and repeated-use stability. These experimentally observed trade-offs provide a framework for selecting β-galactosidase immobilization strategies according to application-specific performance requirements rather than assuming a universally optimal immobilization method.

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