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Research Report

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Analysis of Enzyme Retention Using Immobilization Support for β-Glucosidase

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Abstract:

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Anish Raj Garg

An efficient carbon cycle, catalyzed by enzymes, can solve numerous industrial waste and climate issues. Enzymes, the critical biocatalysts, are both expensive and dependent on different reaction conditions, thereby limiting their large-scale industrial utility. Immobilization of these enzymes, using immobilization support materials, addresses two key technical challenges: reuse and recycling of enzymes and improved reaction chemical economy. Studies are evaluating both traditional immobilization materials and emerging materials such as biopolymers. This particular comparative study, through an experimental analysis, focuses on the retention of a widely used enzyme β-glucosidases using two traditional immobilization materials: iron oxide nanoparticles (Fe_3O_4 nanoparticles), and silica supports (SiO_2). The selection of the immobilization materials was guided by theoretical frameworks in enzyme immobilization, particularly covalent bonding theory and electrostatic interaction frameworks. Retention performance was assessed by measuring catalytic activity over successive reaction cycles and by quantifying residual protein in the supernatant using BCA assays. Initial rounds of empirical results demonstrated that silica supports exhibited the better retention of β-glucosidases, measured over multiple reaction cycles, over iron oxide nanoparticles. The expected outcome of this comparison establishes a hierarchy among commonly available immobilization materials for the widely used enzyme β- glucosidases, which can guide material selection. While this study focuses on material-driven immobilization strategies, broader approaches such as entrapment methods and affinity tags are being explored in the field to improve enzyme retention, which is beyond the scope of this research. A cost benefit-analysis needs to be undertaken after the hierarchy is established to make recommendations for the immobilization materials. This comparative framework provides a replicable methodology for further studies aiming to optimize enzyme retention for different enzymes. High retention is fundamental to ensuring operational longevity, maintaining both economic and ecological viability to achieve an efficient carbon cycle.

Keywords: Biomass, Enzyme Immobilization, Enzyme Retention, Sustainability

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Commitments on Academic Honesty and Integrity

We hereby declare that we

1. are fully committed to the principle of honesty, integrity and fair play throughout the competition.
2. actually perform the research work ourselves and thus truly understand the content of the work.
3. observe the common standard of academic integrity adopted by most journals and degree theses.
4. have declared all the assistance and contribution we have received from any personnel, agency, institution, etc. for the research work.
5. undertake to avoid getting in touch with assessment panel members in a way that may lead to direct or indirect conflict of interest.
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7. observe the safety regulations of the laboratory(ies) where the we conduct the experiment(s), if applicable.
8. observe all rules and regulations of the competition.
9. agree that the decision of YHSA(Asia) is final in all matters related to the competition.

We understand and agree that failure to honour the above commitments may lead to disqualification from the competition and/or removal of reward, if applicable; that any unethical deeds, if found, will be disclosed to the school principal of team member(s) and relevant parties if deemed necessary; and that the decision of YHSA(Asia) is final and no appeal will be accepted.

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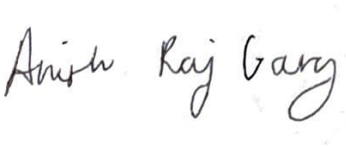

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Section 1: Introduction

Biomass and Its Energy Potential

Biomass, as the name suggests, refers to the total mass of all biological organisms in a given area or ecosystem at a specific time (Parresol, 2013). While biomass encompasses a wide variety of biological material, certain types, such as rice straw, sugarcane, and wheat straw, play a key role in the production of sustainable bioenergy and bioproducts. These lignocellulosic biomasses are processed in biorefineries using thermochemical methods to produce various outputs, such as fuel. In 2021, the global biomass energy supply reached 54 EJ, with solid biomass contributing the largest share (World Bioenergy Association, 2021). Each year, biomass stores approximately 4,500 EJ of solar energy through photosynthesis, a large sustainable potential of biomass (World Bioenergy Association, n.d.). Capturing just 5% of this efficiently could meet nearly half of the world's current total primary energy demand.

Biomass absorbs sunlight, water, and CO_2, converting them into energy-rich molecules such as sucrose ($C_{12}H_{22}O_{11}$) and glucose ($C_6H_{12}O_6$), as illustrated in Equation 1.1. This stored energy can be released through conversion into usable forms such as electricity or liquid fuel. Although biomass combustion releases CO_2, it operates within a near-closed carbon loop: plants absorb atmospheric CO_2 during growth, which is then released upon combustion, resulting in a net-zero carbon contribution when managed sustainably. This makes biofuels a promising avenue for reducing net greenhouse gas emissions (Yang et al., 2021).

$$6CO_2 + 6H_2O + \text{Light Energy} \rightarrow C_6H_{12}O_6 + 6O_2$$

Equation 1.1: *Chemical equation illustrating the reactants and products involved in the biosynthesis of glucose*

Composition and Hydrolysis Potential of Lignocellulosic Biomass

Lignin, cellulose, and hemicellulose are the three main components of lignocellulosic biomass. Lignin is the hardest layer to penetrate. Lignin is made up of phenolic compounds and is broken down efficiently using pretreatment, which comprises of water and diluted acid at around 120°C (Kaur & Goyal, 2024), allowing access to the carbohydrate polymers - cellulose and hemicellulose. Cellulose is a linear polymer, consisting of β-1,4-linked glucose units with both crystalline and amorphous regions. (Hazeena et al., 2020). On the other hand,

hemicellulose is a heteropolymer made up of several sugars, including mannose, arabinose, and xylose (Scapini et al., 2021). Using specific enzymes, enzymatic hydrolysis converts these carbohydrate polymers into fermentable sugars. (Kirti & Khora, 2023).

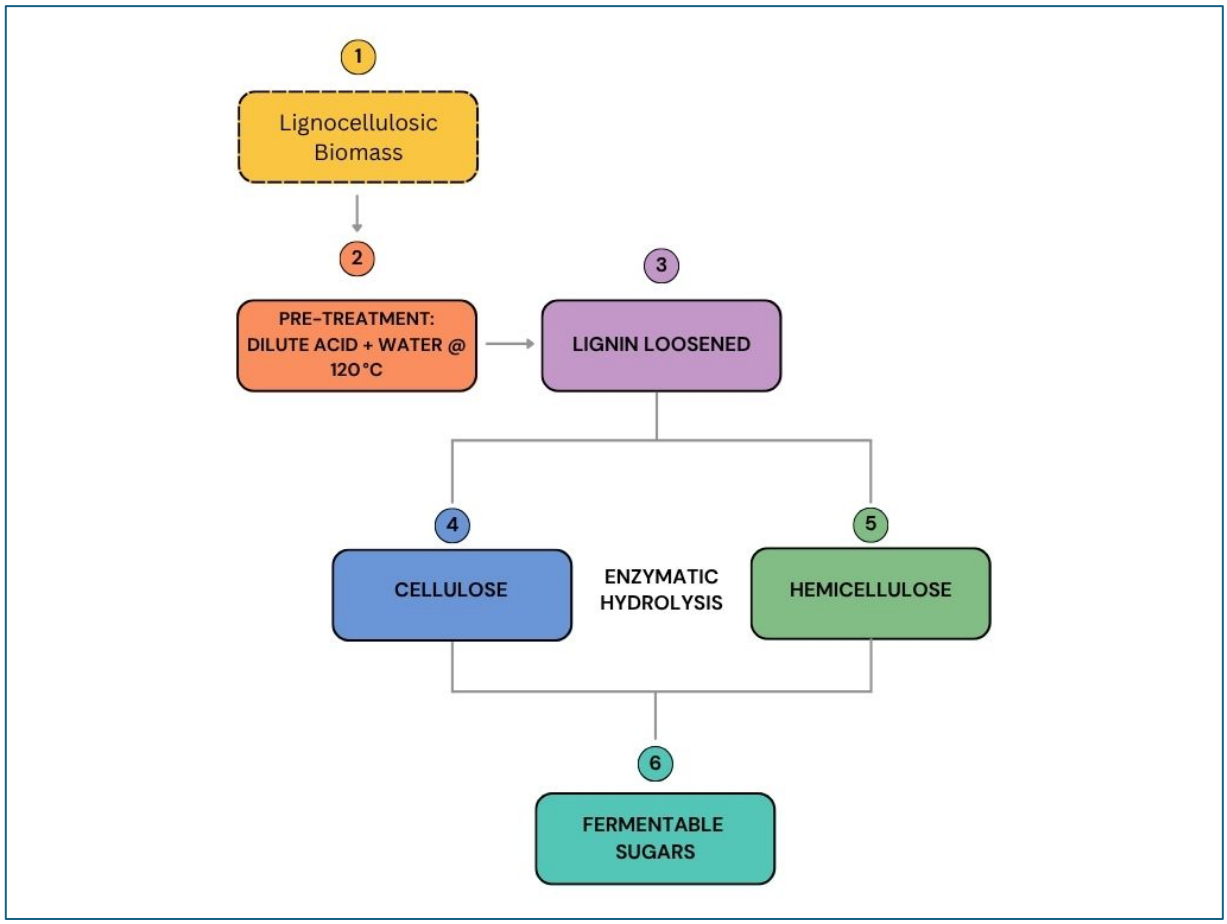

Figure 1.2: *Flowchart depicting the stepwise procedure for converting Lignocellulosic Biomass into Fermentable Sugars*

Mechanism of Enzymatic Hydrolysis and Rationale for Immobilization

Enzymatic hydrolysis of cellulose and hemicellulose is a stepwise process detailed below:

Firstly, Endoglucanase (EC 3.2.1.4) cleaves internal β-1,4-linked glucose units bonds in the amorphous cellulose, generating free chain ends. Secondly, Exoglycanase (EC 3.2.1.91) acts progressively from each of the free chain ends, releasing cellobiose units, which are essentially the two glucose molecules linked together. Finally, β-glucosidase (EC 3.2.1.21) hydrolyses the two glucose units into a glucose monomer. (Valenzuela-Ortega et al., 2023).

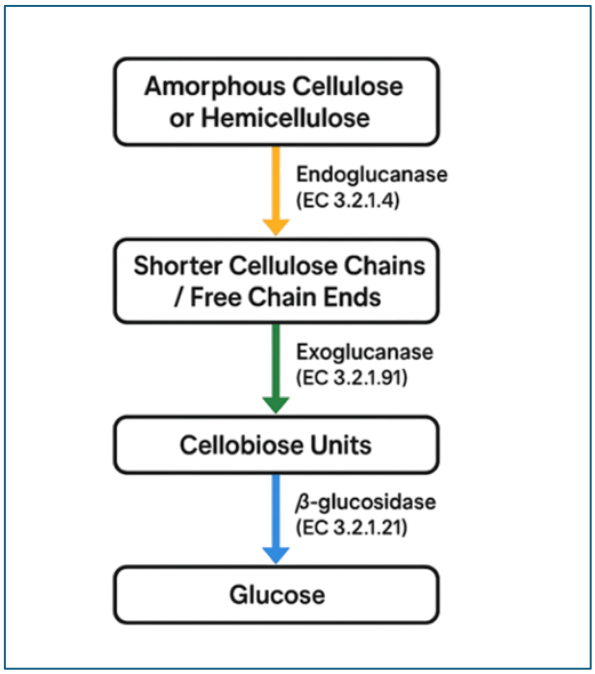

***Figure 1.3**: Flowchart depicting the step-wise enzymatic procedure for the release of glucose units*

A prominent challenge is encountered when enzymes interact with lignocellulosic biomass. Due to the *recalcitrance* of the biomass, enzymes may be absorbed into the lignin (Huang et al., 2022). This reduces catalytic efficiency and increases costs, compromising both ecological and economic sustainability(Escuder-Rodríguez et al., 2018). One promising solution is enzyme immobilization, where enzymes are confined or attached to solid supports, while retaining catalytic activity. A known advantage of enzyme immobilization is that it allows for improved operational stability and repeated use (Gao et al., 2022). However, it also introduces some physical limitations: if the enzymes are immobilized within porous particles with pore diameters around 500 nm, bulky substrates like intact cellulose fibres, which are typically several micrometres in diameter, may be too large to enter. This could prevent effective interaction between the enzyme and substrate. As a result, immobilization is often more suitable for enzymes like β-glucosidase, which act on smaller, soluble intermediates such as cellobiose, rather than directly on insoluble cellulose.

Enzyme Immobilization Advantages

Immobilized enzymes offer several advantages:

- They can be easily separated from the reaction mixture,
- They can be reused over multiple cycles, reducing enzyme consumption, and
- They exhibit enhanced resistance to heat, pH changes, and denaturation (Mulinari et al., 2020)

Enzyme Immobilisation Supports

There are various types of supports with each having a type of binding mode. The binding mode refers to how the enzyme is attached to the support, which affects the enzyme's stability, and reusability. Each of the binding modes has been described in detail in the literature review (Mulinari et al., 2020). The choice of inorganic and magnetic supports was made as one the objective of this research was to test the reusability of enzymes; further illustrated in Figure 2.3, demonstrating covalent surface chemistry creating highest reusability.

Support Type	Example	Binding Mode	Key Benefit
Inorganic Materials	Silica, glass, zeolites	Covalent	Thermal stability, high surface area
Magnetic Supports	Iron Nanoparticles	Covalent	Easy separation, reusable
Natural Polymers	Alginate, Chitosan	Entrapment, ionic	Biodegradable, mild conditions
Synthetic Polymers	PVA, polystyrene	Covalent	Stable, customizable chemistry

***Table 1.4**: Illustration of different enzyme binding models and their specific benefits*

Aim of the Study

The present study evaluates the retention and reusability of β-glucosidase using two traditional immobilization materials: chitosan-coated iron oxide (Fe_3O_4) nanoparticles and silica (SiO_2) supports. By comparing their performance, the research aims to identify cost-

effective and efficient strategies for enhancing enzymatic biomass conversion. This study contributes to advancing green technologies by promoting low-waste, energy-efficient catalytic systems. Enhanced reusability and stability of immobilized enzymes can significantly reduce operational costs and support climate mitigation and sustainable industrial growth.

Structure of this Research Paper

The rest of this paper is organized as follows: Section 2 reviews existing research on enzyme immobilization. Section 3 outlines the experimental methodology and presents the results. Section 4 presents the cost-benefit analysis. Section 5 addresses unresolved questions and directions for future work.

Section 2: Literature Review

Enzymatic and Microbial Degradation

Enzymatic degradation is an approach in controlled bioconversion systems. It involves applying isolated or purified enzymes to pretreated biomass to selectively cleave glycosidic bonds in cellulose and hemicellulose. The end products, mainly glucose and xylose, can then be fermented into biofuels or other bioproducts (Wu et al., 2022).

Microbial degradation employs whole organisms such as fungi or bacteria, which colonize biomass surfaces and secrete a suite of hydrolytic enzyme. These microbes also metabolize the sugars produced, making the approach well-suited to natural or composting systems, though less controllable than enzymatic hydrolysis (Okal et al., 2023).

Scenario	Method
High purity product generation	Enzymatic
Low-cost, large-scale breakdown	Microbial
Controlled laboratory studies	Enzymatic
Composting or natural degradation	Microbial

***Table 2.1**: Different scenarios and the corresponding methods used.*

Structural Recalcitrance of Lignocellulosic Biomass

As shown in Table 2.1, that enzymatic hydrolysis is more beneficial for industrial applications; however, there are several challenges to address. Main among these is the inherent structural recalcitrance of lignocellulosic biomass - a consequence of its chemically heterogeneous architecture. This resistance to enzymatic hydrolysis arises from the spatial organization and bonding of its constituent polymers (Zoghlami & Paës, 2019).

- Lignin acts as a chemical and physical barrier, forming a rigid hydrophobic matrix that impedes enzyme penetration. It forms a tough, water-repellent layer that makes it difficult for enzymes to reach the cellulose and hemicellulose. Lignin's structure might deactivate them and reduce their effectiveness (Mariana et al., 2021).

- Hemicellulose, which is, undoubtably, more amorphous and easier to hydrolyze than cellulose, also, has significant structural challenges. Its polymer chains are often substituted with various side groups, which can prevent enzyme access and reduce effectiveness. Moreover, its close position with lignin makes hemicellulose degradation more difficult. As a result, a *cocktail of enzymes* are required to efficiently hydrolyze hemicellulose (Qaseem et al., 2021).
- Cellulose chains are grouped together into tightly packed, crystalline structures. These are held together by strong hydrogen bonds, which make it hard for enzymes to reach and break the glucose down. (Ramanathan et al., 2022).

Challenges of Enzymatic hydrolysis

Despite the efficiency of enzymes, several bottlenecks hinder the viability of enzymatic hydrolysis at scale:

- Enzyme Instability: Enzymes often lose activity under industrial conditions or are degraded over time, limiting their reusability,
- Non-Productive Binding: Enzymes, particularly cellulases and β-glucosidases, can irreversibly adsorb onto lignin or other residues, reducing the active enzyme pool, and
- Product Inhibition – Accumulated sugars like cellobiose and glucose inhibit enzyme activity, slowing the reaction rate and reducing sugar yield (Ndochinwa et al., 2024).

Different types of Immobilisation methods

Different type of immobilized material is typically accompanied by an immobilization method, which is the surface chemistry on the material, typically acting as an indicator of how effective the immobilization might be (Datta et al., 2013).

Immobilization Method	Description with Advantages and Disadvantages
Entrapment	Enzymes are physically confined within a polymeric matrix. Offers mild conditions but may limit substrate diffusion.
Covalent Binding	Enzymes are chemically bonded to functionalized surfaces. Strong and reusable but may reduce activity due to active site modification.
Ionic Binding	Uses electrostatic interactions between charged enzyme groups and supports. Gentle and reversible but sensitive to pH and ionic strength.
Adsorption	Involves weak interactions. Simple and does not modify the enzyme, but prone to enzyme leaching.

***Table 2.2**: Description of different types of immobilization methods*

Key Performance Metrics for Immobilized Enzymes

Immobilized enzymes can be evaluated using four key performance metrics:

1. Activity retention after immobilization: This measures the percentage of the enzyme's original catalytic activity that is preserved immediately after being immobilized onto a support material. It reflects how well the immobilization method preserves the enzyme's functional integrity.
2. Reusability (reuse cycles): This indicates the number of catalytic cycles the immobilized enzyme can undergo before experiencing a significant decline in activity. It is critical for assessing cost-effectiveness in industrial applications.
3. Degradation rate: This refers to the rate at which the immobilized enzyme loses its structural integrity or catalytic function over time, whether due to denaturation, leaching, or mechanical stress.
4. Binding capacity: This represents the amount of enzyme (by weight) that can be immobilized per gram of support material. It directly impacts the overall catalytic efficiency and scalability of the immobilized system (Rodrigues et al., 2021).

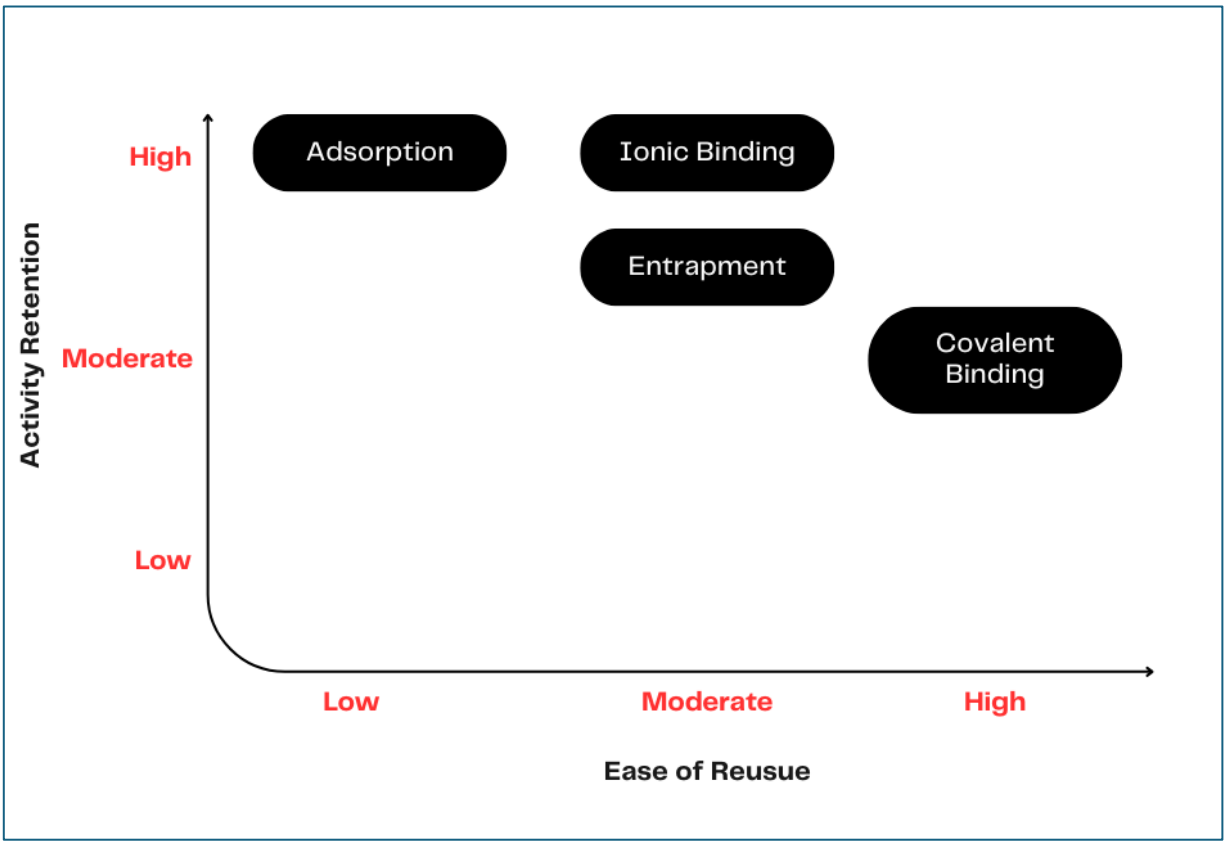

***Figure 2.3**: Matrix comparing ease of reuse and activity retention for different immobilization methods.*

Research Gap

While enzyme immobilization has shown great potential for improving reusability and operational stability, relatively few studies have systematically compared the performance of β-glucosidase across different support materials under standardized conditions. Most existing research focuses on optimizing a single support type or immobilization method in isolation, making it difficult to draw meaningful comparisons between systems. This limits our understanding of how support chemistry, structure, and surface interactions influence enzyme activity and stability. In order to find an effective solution, this research compares β-glucosidase immobilized on chitosan-coated iron oxide nanoparticles and mesoporous silica supports, using identical reaction conditions. nanoparticles and mesoporous silica supports, using identical reaction condition.

Section 3: Methodology and Result

Quantifying the Beta-glucosidase

Before initiating the immobilization process, quantifying the concentration of β-glucosidase (Bgl 0.38A) was essential. The β-glucosidase 0.38A used was a mutant β-glucosidase derived from *Penicillium funiculosum* and had been developed in the lab (Kereke et al., 2023).

Protein quantification was carried out using the BCA assay. A standard curve was generated using known concentrations of BSA ranging from 62.5 to 1000 µg/mL. The reaction mixture contained 20 µL of each BSA standard or enzyme sample, combined with 200 µL of the BCA working reagent. This mixture was pipetted onto a 96-well microplate reader. The mixture was incubated at room temperature for 30 minutes to allow for colour development, and absorbance was measured at 562 nm using a microplate reader.

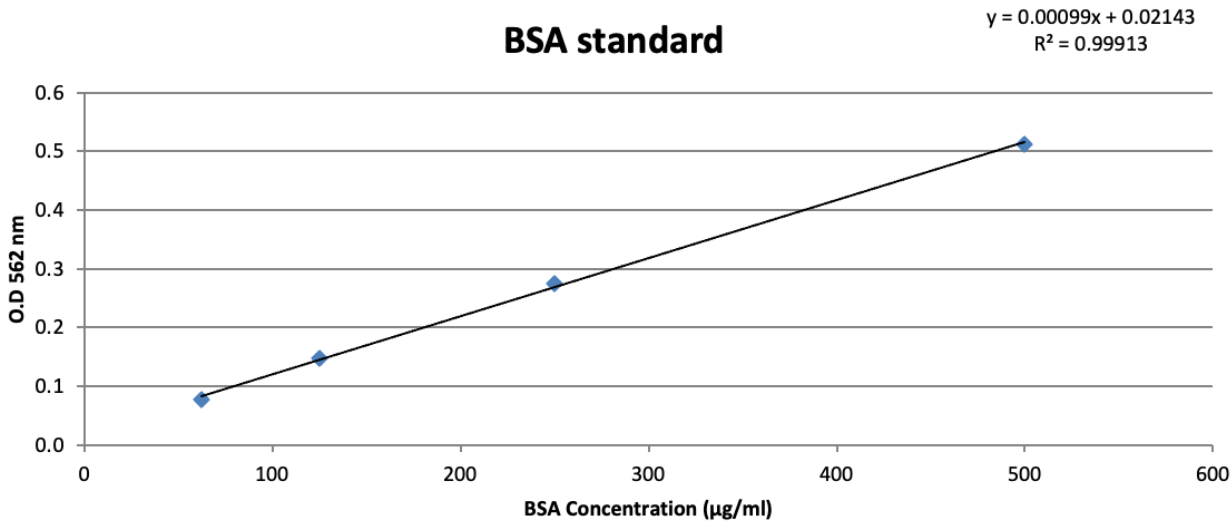

***Table 3.1**: Standard curve for BCA assay showing BSA concentration vs. absorbance*

The standard curve exhibited excellent linearity and the R^2 is close to 1. The regression equation is:

$$y = 0.0099x + 0.02143\ (R^2 = 0.9913)$$

where Y is the absorbance at 562 nm and X is the protein concentration in µg/mL.

Using this equation, the protein concentration of Bgl 0.38A was calculated from three independent sets. The final average concentration was determined to be 5.84 mg/mL.

Immobilisation of Beta-glucosidase on chitosan-coated iron nanoparticles

Synthesis of Fe_3O_4 Nanoparticles

Fe_3O_4 nanoparticles were synthesized by co-precipitation. 5.4 g of $FeCl_3$ and 1.99 g of $FeCl_2 \cdot 2H_2O$ were dissolved in 50 mL of autoclaved Milli-Q water under magnetic stirring. The solution was heated to 80 °C with continuous stirring, followed by the dropwise addition of 28% NH_4OH until the pH reached 10. The reaction was maintained at 80 °C for an additional 2 hours to allow complete precipitation of Fe_3O_4 nanoparticles. The resulting black precipitate was washed repeatedly with Milli-Q water until a neutral pH was achieved, then dried overnight at 55°C to obtain solid Fe_3O_4 nanoparticles powder. The yield of the nanoparticles was approximately 500 mg (Babbal et al., 2024).

Preparation of Chitosan-Coated Fe_3O_4 Nanoparticles

Chitosan solution was prepared by dissolving 0.25 g of chitosan in 50 mL of 1% (v/v) acetic acid (prepared from 0.5 mL glacial acetic acid and 49.5 mL Milli-Q water) under magnetic stirring for 5 minutes. The dried Fe_3O_4 nanoparticles were dispersed into this chitosan solution with magnetic stirring for 30 minutes. Subsequently, 50 mL of 1 M NaOH was added dropwise under stirring to precipitate the chitosan onto the nanoparticles. The mixture was centrifuged at 13,000rpm for 5 minutes, and the resulting pellet was washed repeatedly with Milli-Q water until the pH was neutral.

Figure 3.2: Chitosan-coated Fe_3O_4 Nanoparticles after drying

Glutaraldehyde Activation of Chitosan-Coated Nanoparticles

For surface activation, 15 mg of semi-dried chitosan-coated Fe_3O_4 nanoparticles were added to 1 mL of 5% (v/v) glutaraldehyde (GDA) solution in each of 20 vials. The suspensions were incubated on a rotospin at room temperature for 2 hours. After incubation, the particles were washed three times with 50 mM sodium citrate buffer to remove excess GDA.

However, in future preparations, a lower GDA concentration (1% v/v) and more extensive washing (10 times) may be preferable, as free glutaraldehyde in solution can inactivate enzymes. Using milder conditions helps ensure that GDA remains surface-bound, minimizing potential loss of enzymatic activity during downstream applications.

Immobilization of β-Glucosidase (BGL)

β-Glucosidase was immobilised by incubating 100 µL of the enzyme solution with 20 mg of glutaraldehyde-activated nanoparticles in separate vials. The mixtures were left on a rotospin overnight at room temperature. After incubation, the immobilised enzyme was magnetically separated and washed three times with buffer to remove any unbound enzyme. The supernatant was collected and tested using the BCA assay to detect any remaining free enzyme. No significant colour change was observed, which could suggest either that most of the enzyme had successfully immobilised onto the nanoparticles, or that the immobilisation process had failed due to issues such as enzyme denaturation or errors during the assay.

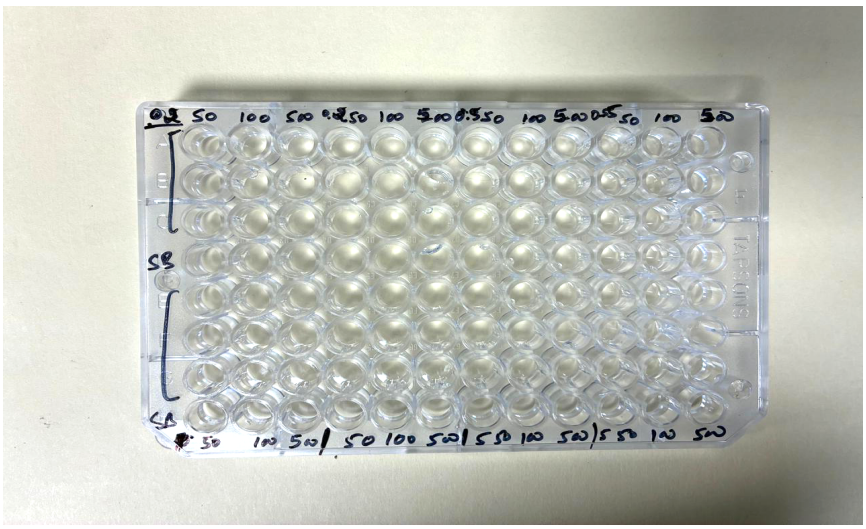

***Figure 3.3**: BCA assay to detect the amount of enzyme leftover in the supernatant after immobilisation*

Catalytic Reusability of Immobilized of β-Glucosidase (BGL)

The catalytic reusability of immobilized BGL was assessed over five cycles. In each cycle, 10 mg of immobilized BGL supports were incubated with 1 mL of p-nitrophenyl-β-D-glucopyranoside (pNPG) substrate solution (10 mM in sodium citrate buffer) at 37 °C for 30 minutes. The reaction was terminated by magnetic separation of the supports, and the supernatant was transferred to a clean tube. A stop solution consisting of 1 mL of 1 M Na_2CO_3 was added to develop the yellow color of p-nitrophenol (pNP). Absorbance was measured at 405 nm using a spectrophotometer.

After each reaction, the supports were washed three times with sodium citrate buffer, using a volume equivalent to 5 times the bed volume of the immobilized supports per wash. The supports were then reused in the subsequent cycle. A 24-hour interval was maintained between each cycle, during which the supports were stored at 4 °C to preserve enzymatic activity.

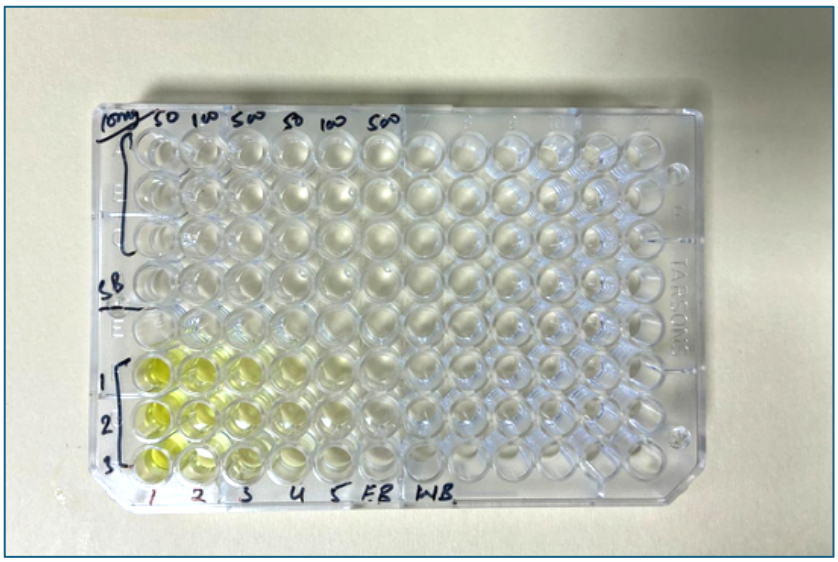

***Figure 3.4**: BCA assay to detect the amount of enzyme immobilized after reusability cycles*

Immobilised β-Glucosidase activity on magnetic nanoparticles

Cycle	Mean Absorbance				Standard Deviation	pNP (mg/ml)	Yield (mg pNP/ mg BGL)	% Activity Retained
	Trial 1	Trial 2	Trial 1	Average				
1	1.342	1.389	1.400	1.377	0.03	10.84	2.17	-
2	1.140	1.156	1.181	1.159	0.02	9.22	1.84	84.7926
3	1.002	0.989	1.072	1.021	0.04	7.59	1.52	67.4013
4	0.837	0.850	0.824	0.837	0.01	5.96	1.19	45.6908
5	0.583	0.594	0.578	0.585	0.01	4.34	0.87	18.8

***Table 3.5**: Table depicting the result of the catalytic activity of immobilised BGL over iron nanoparticles*

Cycle vs Percentage Change

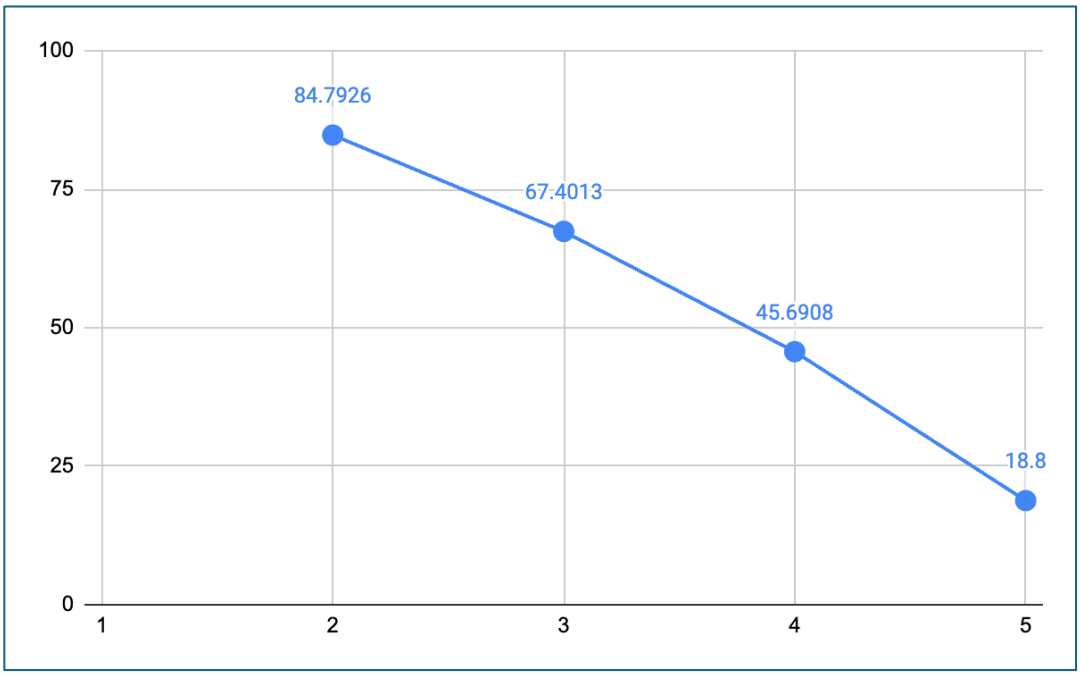

***Figure 3.6:** Graph depicting the percentage decrease of the catalytic activity of immobilised BGL over iron nanoparticles*

The results demonstrate a progressive decline in enzymatic activity across cycles, as expected due to partial deactivation. The activity decreased from approximately 2.17 mg pNP/mg BGL in Cycle 1 to 0.87 mg pNP/mg BGL by Cycle 5. Moreover, the results were triplicated to ensure greater accuracy. This indicates that while BGL remains reusable, catalytic performance diminishes over time.

Immobilization of β-Glucosidase Using Silica Supports

Activation of Mesoporous Silica Supports with Glutaraldehyde

Amino-functionalized mesoporous silica supports (100 mg; Sigma-Aldrich, CAS No. 126850-14-4) were first washed three times with 50 mM sodium citrate buffer (pH 5.0) to remove surface impurities. Then, the supports were incubated with 1 mL of 5% (v/v) glutaraldehyde solution. In order to allow for GDA activation, the supports were placed on a rotospin for 2 hours. Following activation, the supports were washed, using a centrifuge, three times with sodium citrate buffer to remove unreacted glutaraldehyde (Babbal et al., 2024).

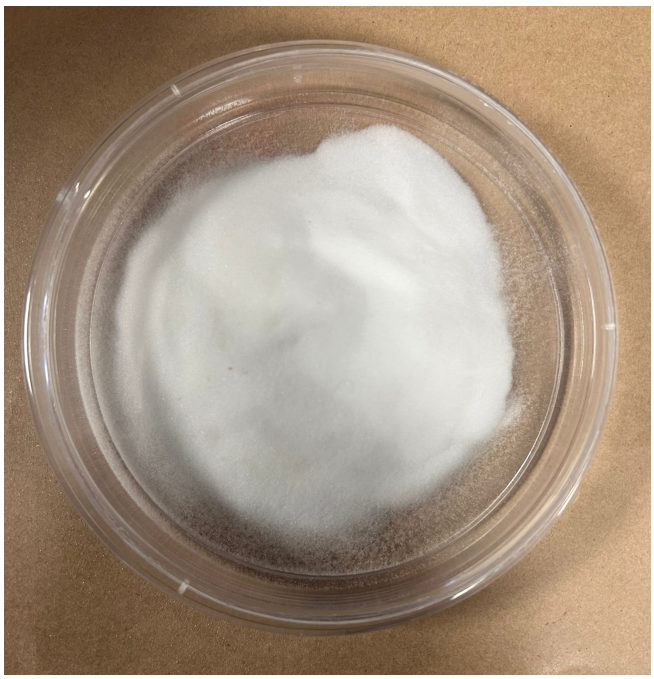

Figure 3.7: *Image of the silica supports used for immobilization*

Immobilization of β-Glucosidase (BGL)

To immobilize the enzyme, a stepwise procedure was used. Firstly, 100 µL of the enzyme solution was dissolved in the GDA-activated mesoporous silica supports (100 mg). In order to immobilize the enzyme, this solution was placed on a rotospin overnight. After the rotospin, the immobilized enzyme supports were separated by centrifugation and washed three times with sodium citrate buffer to remove any unbound enzyme.

Catalytic Reusability of Immobilized of β-Glucosidase (BGL)

The reusability of immobilized β-glucosidase on silica supports was evaluated over five cycles. In each cycle, 10 mg of immobilized enzyme supports were incubated with 1 mL of p-nitrophenyl-β-D-glucopyranoside (pNPG) substrate solution (5 mM in sodium citrate buffer) at 37 °C for 30 minutes using a shaker. After incubation, the supports were separated by centrifugation and the supernatant was transferred to a clean tube. To stop the reaction and develop the yellow p-nitrophenol (pNP) color, 1 mL of 1 M Na_2CO_3 was added. Absorbance was measured at 405 nm using a spectrophotometer to quantify pNP production. The supports were washed three times with sodium citrate buffer before being reused for subsequent assay cycles. For each cycle, 10 vials containing individual 10 mg portions of immobilized enzyme supports were used to ensure reproducibility and statistical accuracy of the assay.

Immobilized β-Glucosidase activity on silica supports

Cycle	Mean Absorbance				Standard Deviation	pNP (mg/ml)	Yield (mg pNP/ mg BGL)	% Activity Retained
	Trial 1	Trial 2	Trial 3	Average				
1	0.92	1.05	1.123	1.031	0.08	8.12	2.40	-
2	0.917	1.01	1.067	0.998	0.06	7.85	2.32	96.777
3	0.85	1.054	1.023	0.975	0.08	7.62	2.25	93.753
4	1.006	0.94	0.824	0.923	0.07	7.18	2.12	87.978
5	0.715	0.863	0.934	0.837	0.09	6.53	1.93	80.412

***Table 3.8**: Table depicting the result of the catalytic activity of immobilised BGL over silica supports*

Cycle vs Percentage Change

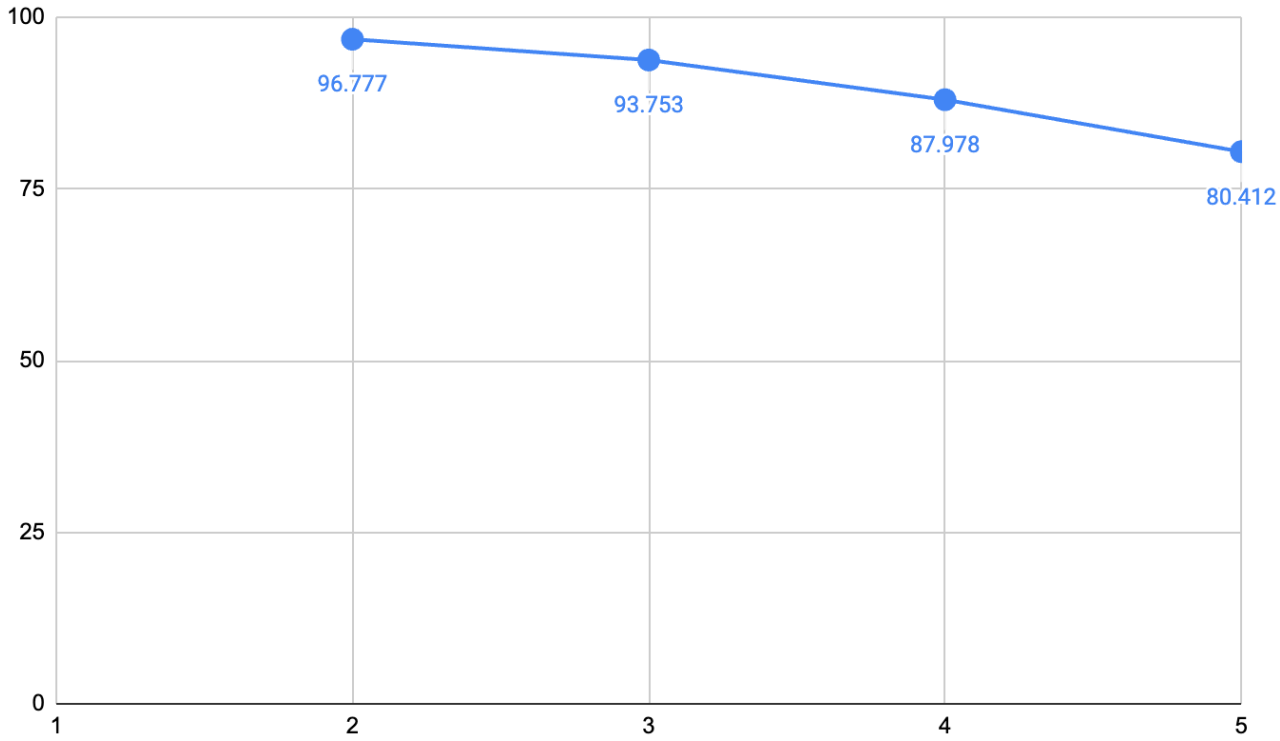

***Figure 3.9**: Graph depicting the percentage decrease of the catalytic activity of immobilised BGL over silica supports*

Like with iron nanoparticles, there was a decrease in enzymatic activity across cycles due to partial deactivation. However, this decrease was far less than that observed with iron. The specific activity decreased only from 2.40 to 2.32 over five cycles. Moreover, the results were obtained in triplicate to ensure greater accuracy. This indicates that BGL was much more effective in retaining activity on silica

Section 4: Cost Benefit Analysis

From a cost perspective, silica supports are relatively inexpensive, chemically stable, and widely available, making them attractive for large-scale enzyme immobilization. For the research, the silica support was bought commercially, and their activation with GDA is a straightforward, low-cost technique. One of silica's prominent physical properties is its thermal and mechanical stability under extreme reaction conditions. As seen in the experiment above, they can withstand repeated reaction and washing cycles. Moreover, in 5 cycles they preserved enzyme activity well, allowing for optimistic scope for more cycles and lowering the costs attached with enzyme immobilization.

In contrast, chitosan-coated iron oxide nanoparticles are significantly more expensive to synthesize, especially when considering industrial-scale production. For the experiment, the synthesis of Iron Oxide using those chemicals resulted in high costs, however, these costs can be reduced if greater amounts of Iron Oxide is being made. Moreover, the additional step of chitosan coating, made it more expensive, leading to scalability challenges. Moreover, as seen in the results, the enzyme retention was relatively low over just 5 reaction cycles, making their benefit compared to the cost not beneficial.

Section 5: Discussion

Comparative Study

After conducting comprehensive experimentation, the results demonstrated a clear distinction in enzyme retention of the two immobilization materials tested. Silica supports retained over 80% of enzyme activity after five cycles, from 2.40 to 1.93 mg pNP/mg BGL. In contrast, the chitosan-coated Fe_3O_4 nanoparticles retained only 18.8% activity after the same number of cycles. This could indicate that chitosan-coated iron oxide may be inefficient with retention. One factor that contributed to the such a difference between iron-oxide nanoparticles and silica supports is that the silica can can physically entrap enzymes within their pores. This provides structural protection from reaction condition. On the other hand, enzymes immobilized on the surface of iron oxide nanoparticles remain fully exposed, making them more susceptible to unfolding, leaching, or deactivation over time.

Future Scope and Evaluation

This comparative study reinforces the importance of selecting appropriate immobilization supports for optimizing enzymatic biomass conversion. The findings suggest that mesoporous silica provides an effective balance for β-glucosidase applications. Future experiments could compare the efficacy of β-glucosidase immobilized through other scalable approaches, such as covalent immobilization via epoxy-functionalized supports or ionic immobilization through amine-functionalized surfaces. The latter is particularly promising, as it may enable enzyme stripping and reuse of the support material. Additional studies should also aim to:

- Assess long-term performance over more than 10 reuse cycles
- Evaluate performance at elevated temperatures
- Explore alternatives to covalent immobilization strategies

Although epoxy-functionalized supports were mentioned in the initial abstract, they were not included in this study due to procurement delays. Additionally, since these supports are typically silica-based, they would have overlapped with the existing mesoporous silica system, limiting the comparative value in this context.

Finding the right immobilization method for β-glucosidase will enhance yields and reduce the cost of enzymes used. This will make the enzymatic production of sugars from common feedstocks more economically feasible. Ultimately, it will support circular bioeconomy goals by advancing sustainable biomass conversion systems.

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