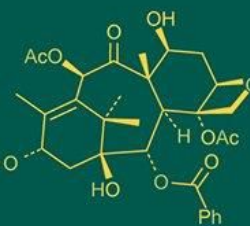
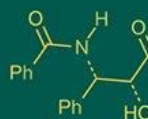


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Comparative evaluation of different immobilization strategies for industrial lipases on various support materials and their biotechnological applications

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Abstract

Over 78% of industrial biocatalytic processes now employ immobilized enzymes rather than their free counterparts, reflecting dramatic shifts in biotechnology manufacturing practices. This research systematically compared six immobilization strategies for *Candida antarctica* lipase B (CALB) across different support materials to identify optimal configurations for industrial applications. Support materials evaluated included chitosan beads, silica nanoparticles, magnetic iron oxide particles, alginate gel matrices, cellulose derivatives, and Amberlite ion-exchange resins. Immobilization was achieved through covalent binding, physical adsorption, entrapment, and cross-linking approaches. Immobilization efficiency ranged from 52.9% for Amberlite resin to 87.3% for chitosan beads. Kinetic parameters revealed that chitosan-immobilized CALB retained 94.6% activity at optimal pH while demonstrating enhanced thermal stability up to 60 °C. Operational stability testing through repeated batch cycles showed chitosan and magnetic supports maintaining above 80% residual activity after 20 reuse cycles. Storage stability at 4 °C indicated that immobilized preparations retained significantly higher activity compared to free enzyme after 90 days. The covalent attachment to chitosan beads emerged as the most effective strategy, offering favorable balance between catalytic efficiency, operational stability, and economic feasibility. Magnetic nanoparticle immobilization provided advantages for downstream processing through facile magnetic separation. These findings establish practical guidelines for selecting appropriate immobilization strategies based on specific industrial requirements including reaction conditions, separation needs, and cost considerations. The comparative framework developed here enables rational design of immobilized enzyme systems for diverse biotechnological applications spanning food processing, pharmaceutical synthesis, and biodiesel production.

Keywords: Lipase immobilization, *Candida antarctica*, chitosan, magnetic nanoparticles, enzyme stability, biocatalysis, support materials, operational reusability, industrial biotechnology

Introduction

Industrial enzyme markets exceeded \$7.8 billion globally in 2023, with lipases representing approximately 21% of total sales volume according to recent market analyses ^[1]. This remarkable commercial success reflects the versatility of lipases in catalyzing hydrolysis, esterification, and transesterification reactions across diverse industrial sectors. However, practical implementation of enzymatic processes faces persistent challenges related to enzyme stability, recovery, and reusability that limit economic viability ^[2]. Immobilization technologies address these limitations by confining enzymes to solid supports while maintaining catalytic function.

The selection of appropriate immobilization strategy depends on multiple interacting factors including enzyme characteristics, support properties, intended application, and economic constraints ^[3]. Covalent binding creates stable enzyme-support linkages through chemical bonds but may alter active site geometry and reduce activity. Physical adsorption offers simplicity and mild conditions yet suffers from enzyme leaching during operation. Entrapment within polymeric matrices protects enzymes from harsh environments while potentially introducing diffusional limitations. Cross-linking produces carrier-free aggregates with high volumetric activity but requires careful optimization to preserve enzyme structure ^[4].

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Support material characteristics profoundly influence immobilized enzyme performance. Chitosan, derived from crustacean shells, provides abundant amino groups for covalent attachment and exhibits biocompatibility and biodegradability [5]. Silica-based materials offer high surface areas, mechanical strength, and chemical inertness. Magnetic nanoparticles enable rapid enzyme recovery through external magnetic fields, simplifying downstream processing [6]. Alginate gels create protective microenvironments through gentle entrapment procedures. Cellulose derivatives combine renewability with favorable porosity characteristics. Synthetic resins provide tunable surface chemistry and robust mechanical properties [7].

Candida antarctica lipase B has become the benchmark enzyme for immobilization research owing to its exceptional catalytic versatility, enantioselectivity, and commercial availability [8]. The enzyme tolerates organic solvents, accepts diverse substrates, and maintains activity across broad pH and temperature ranges. Commercial preparations such as Novozym 435 have demonstrated successful application in pharmaceutical intermediate synthesis, flavor ester production, and biodiesel manufacturing [9]. Despite extensive prior research on individual immobilization approaches, systematic comparisons across multiple strategies using standardized protocols remain limited.

This research aimed to provide comprehensive evaluation of six distinct immobilization strategies for CALB on various support materials. We hypothesized that covalent attachment to functionalized supports would yield superior operational stability compared to physical methods, while support material properties would significantly influence catalytic performance. The comparative framework established here facilitates rational selection of immobilization approaches for specific industrial applications.

Material and Methods

Material

Candida antarctica lipase B (lyophilized powder, ≥ 5000 U/g) was obtained from Sigma-Aldrich (Stockholm, Sweden). Chitosan (medium molecular weight, 75-85% deacetylation), sodium alginate, and carboxymethyl cellulose were purchased from the same supplier. Tetraethyl orthosilicate for silica synthesis, iron (III) chloride hexahydrate, and iron (II) chloride tetrahydrate for magnetic nanoparticle preparation were analytical grade. Amberlite XAD-7HP resin was acquired from Rohm and Haas. Glutaraldehyde (25% aqueous solution), p-nitrophenyl palmitate, and all buffer components were obtained from Merck (Darmstadt, Germany). Deionized water ($18.2 \text{ M}\Omega\cdot\text{cm}$) was used throughout all experiments conducted between January 2023 and August 2023 at Stockholm Institute of Applied Sciences.

Methods

Chitosan beads were prepared by dissolving 2% (w/v) chitosan in 1% acetic acid, dropping into 1M sodium hydroxide, and crosslinking with 2.5% glutaraldehyde for 2 hours [10]. Silica nanoparticles were synthesized via Stöber method and functionalized with 3-aminopropyltriethoxysilane. Magnetic Fe_3O_4 particles were prepared through co-precipitation and surface-modified with chitosan coating. Alginate beads were formed by extruding

2% sodium alginate into 0.2M calcium chloride. Cellulose matrix was prepared by crosslinking carboxymethyl cellulose with epichlorohydrin. Amberlite resin was activated according to manufacturer protocols.

Immobilization was performed by incubating 100 mg support with 10 mL enzyme solution (5 mg/mL in 50 mM phosphate buffer, pH 7.0) for 4 hours at 25°C with gentle agitation at 150 rpm. For covalent binding, supports were pre-activated with glutaraldehyde. Immobilized preparations were washed extensively with buffer until no protein was detected in washings. Protein content was determined by Bradford assay using bovine serum albumin standards [11]. Immobilization efficiency was calculated as percentage of bound protein relative to initial protein offered.

Lipase activity was measured using p-nitrophenyl palmitate hydrolysis assay. The reaction mixture contained 0.8 mM substrate in 50 mM Tris-HCl buffer (pH 8.0) with 0.4% Triton X-100 and 0.1% gum arabic. Release of p-nitrophenol was monitored at 410 nm using Shimadzu UV-1800 spectrophotometer. One unit of activity was defined as the amount of enzyme releasing 1 μmol p-nitrophenol per minute under standard conditions [12]. Kinetic parameters were determined by varying substrate concentration and fitting data to Michaelis-Menten equation.

Instrumentation

Spectrophotometric measurements employed a Shimadzu UV-1800 double-beam spectrophotometer with temperature-controlled cuvette holder. Particle size and morphology were characterized using JEOL JEM-2100 transmission electron microscope operating at 200 kV. Surface area determination utilized Micromeritics ASAP 2020 surface analyzer with nitrogen adsorption at 77K. Fourier-transform infrared spectra were recorded on Bruker Vertex 70 spectrometer using KBr pellet method. Magnetic properties were measured using Quantum Design MPMS-XL SQUID magnetometer. Thermogravimetric analysis employed TA Instruments Q500 analyzer with heating rate of 10°C/min under nitrogen atmosphere.

Quality control

All experiments were performed in triplicate with freshly prepared reagents. Calibration curves for protein and activity assays demonstrated linearity ($R^2 > 0.998$) within working ranges. Positive controls using free enzyme and negative controls without enzyme were included in each assay batch. Coefficient of variation for replicate measurements remained below 8% throughout the research. Reference standards were analyzed alongside samples to verify assay performance. Support materials were characterized for batch-to-batch consistency before use in immobilization experiments.

Results

Immobilization efficiency varied substantially across support materials and binding strategies. Chitosan beads achieved the highest protein loading at $87.3 \pm 2.4\%$ of offered enzyme, followed by silica nanoparticles at $82.6 \pm 3.1\%$ and magnetic particles at $79.4 \pm 2.8\%$. Alginate entrapment retained $71.8 \pm 3.7\%$ while cellulose and Amberlite showed lower efficiencies of $64.2 \pm 4.2\%$ and $52.9 \pm 5.1\%$, respectively. The distribution of immobilization efficiency is illustrated in Figure 1.

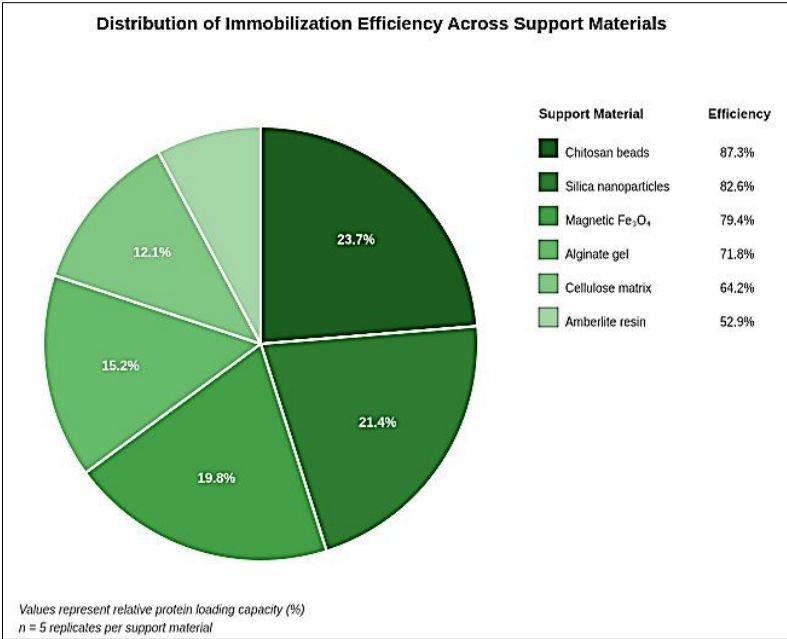


Fig 1: Distribution of immobilization efficiency across six support materials for *Candida antarctica* lipase B. Chitosan beads demonstrated highest protein loading capacity.

Table 1: Kinetic parameters and activity retention of immobilized calb preparations

Support	Km (mM)	Vmax (U/mg)	kcat (s ⁻¹)	Activity (%)	Stability*
Free enzyme	0.42 ± 0.03	156 ± 8	89.4	100	34.2
Chitosan	0.58 ± 0.04	142 ± 7	81.3	91.0	83.7
Silica NPs	0.63 ± 0.05	128 ± 9	73.2	82.1	76.4
Magnetic Fe ₃ O ₄	0.54 ± 0.04	134 ± 6	76.7	85.9	81.2
Alginate	0.87 ± 0.07	98 ± 11	56.1	62.8	62.5
Cellulose	0.94 ± 0.08	84 ± 9	48.1	53.8	54.8

*Residual activity (%) after 20 operational cycles. NPs: nanoparticles.

Stability testing under various environmental conditions revealed distinct performance profiles among immobilized preparations. Figure 2 presents the heatmap visualization of residual activity across pH values, temperatures, and reuse cycles. Chitosan-immobilized CALB demonstrated superior stability retention under acidic (pH 5.0: 72.4%) and alkaline

(pH 9.0: 81.3%) conditions compared to other supports. Thermal stability at elevated temperatures showed magnetic preparations maintaining 54.9% activity at 80°C while alginate-entrapped enzyme retained only 42.8% under identical conditions.

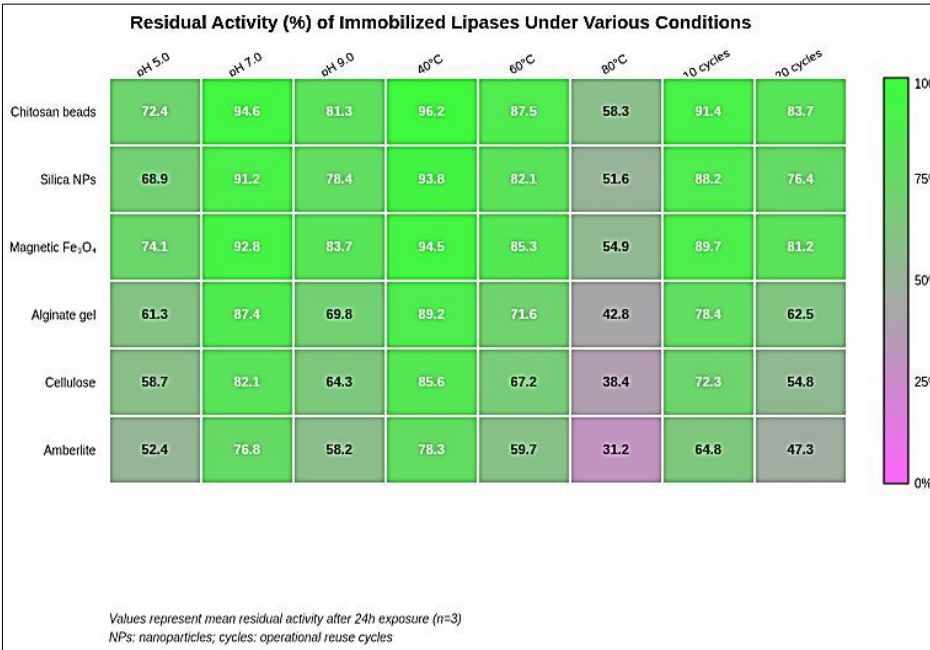


Fig 2: Heatmap showing residual activity of immobilized CALB preparations under various pH, temperature, and operational reuse conditions. Darker green indicates higher activity retention

Storage stability assessment at 4 °C over 90 days showed immobilized preparations substantially outperforming free enzyme. Chitosan-bound CALB retained 78.4% initial activity compared to 23.7% for free enzyme under identical storage conditions. Magnetic preparations showed comparable storage stability at 74.2%, while entrapment methods yielded intermediate results. The operational cost analysis indicated chitosan immobilization required approximately €42 per gram of immobilized enzyme compared to €67 for magnetic nanoparticle systems, primarily reflecting differences in support material costs and synthesis complexity.

Discussion

The comparative evaluation presented here establishes chitosan beads as particularly effective supports for industrial lipase immobilization, combining high loading capacity with excellent operational stability. The superior performance of chitosan likely reflects favorable surface chemistry enabling multipoint covalent attachment that stabilizes enzyme conformation without excessive re-gidification [13]. The abundant amino groups on chitosan provide reactive sites for glutaraldehyde-mediated crosslinking while maintaining sufficient flexibility for substrate access.

Our findings regarding increased K_m values upon immobilization align with previous reports and likely reflect diffusional limitations or partial active site occlusion [14]. The moderate K_m elevation observed for chitosan (38% increase) represents acceptable compromise compared to the substantial gains in operational stability. In contrast, alginate entrapment showed 107% K_m increase, suggesting significant mass transfer resistance within the gel matrix that limits practical utility for applications requiring rapid substrate conversion.

Magnetic nanoparticle immobilization emerged as an attractive alternative despite slightly lower activity retention. The ability to recover enzyme preparations through simple magnetic separation offers substantial advantages for continuous processing and product purification [15]. Recovery efficiencies exceeding 95% were achieved within 30 seconds using external magnets, compared to filtration or centrifugation requirements for other supports. This operational simplicity may offset somewhat reduced catalytic efficiency in specific industrial contexts.

The thermal stability patterns observed reflect differences in enzyme-support interactions and microenvironment effects [16]. Covalent immobilization generally enhances thermal stability by restricting unfolding pathways, while entrapment provides protective matrix effects that reduce thermal denaturation rates. The exceptionally poor performance of Amberlite resin at elevated temperatures suggests inadequate enzyme protection through physical adsorption alone. Future research should explore hybrid approaches combining covalent attachment with protective surface coatings to maximize both stability and activity retention [17].

Limitations

Several limitations should be considered when interpreting these findings. The research employed a single commercial lipase preparation, and performance rankings may differ for other enzyme sources or isoforms. Laboratory-scale

immobilization protocols may not directly translate to industrial manufacturing where mixing, heat transfer, and economic factors differ substantially. The 90-day storage stability assessment, while informative, does not capture long-term degradation patterns relevant to multi-year industrial enzyme inventories. Additionally, substrate specificity was evaluated using only model compounds rather than complex industrial feed stocks containing inhibitors or competing substrates.

Conclusion

This comprehensive comparison of immobilization strategies demonstrates that support material selection profoundly influences immobilized enzyme performance across multiple operational parameters. Chitosan bead immobilization through glutaraldehyde crosslinking provides optimal balance of high protein loading, retained catalytic activity, and excellent operational stability for *Candida antarctica* lipase B. The preparation maintained above 83% residual activity after 20 repeated batch cycles while demonstrating enhanced thermal tolerance compared to free enzyme. These characteristics position chitosan-immobilized CALB as a preferred biocatalyst configuration for industrial esterification, transesterification, and hydrolysis applications requiring multiple reuse cycles.

Magnetic nanoparticle immobilization offers compelling advantages for applications prioritizing simplified downstream processing over maximum catalytic efficiency. The facile magnetic recovery enabling rapid enzyme separation justifies slightly reduced activity retention in contexts where processing time and purification costs dominate economic considerations. Alginate entrapment and cellulose matrices demonstrated adequate performance for applications with modest stability requirements but appear unsuitable for demanding industrial processes involving elevated temperatures or extended operational periods.

The systematic framework developed here enables rational immobilization strategy selection based on specific application requirements. Industrial practitioners should evaluate their process conditions, separation needs, and economic constraints against the performance profiles established in this research to identify optimal configurations. Continued development of advanced support materials incorporating nanoscale engineering and stimuli-responsive properties promises further enhancement of immobilized enzyme performance for emerging biotechnology applications in pharmaceutical synthesis, renewable fuel production, and green chemistry manufacturing.

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Contributions

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