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Optimization of microbial lipase production via fed-batch fermentation and immobilization for industrial bioprocess applications

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Abstract
Background: Microbial lipases are among the most industrially significant biocatalysts, finding applications in food processing, biodiesel synthesis, pharmaceutical manufacturing, and detergent formulation. Pseudomonas aeruginosa-derived lipases exhibit broad substrate specificity and thermostability, making them particularly attractive for bioprocess applications.
Objective: This study aimed to optimize lipase production from Pseudomonas aeruginosa ATCC 27853 using fed-batch fermentation strategies and evaluate four enzyme immobilization approaches to enhance catalytic stability and reusability.
Method: Five fermentation configurations (batch, three fed-batch variants at different temperatures, and continuous mode) were evaluated. Enzyme immobilization was performed on silica gel, chitosan beads, magnetic Fe_3O_4 nanoparticles, and polyurethane foam. Kinetic parameters (Km, Vmax, kcat) were determined using p-nitrophenyl palmitate as substrate. Statistical analysis used response surface methodology (RSM) and one-way ANOVA (SPSS v.27).
Key Results: Fed-batch fermentation at 30°C with pH 7.2 and 250 rpm agitation yielded maximum lipase activity (31.2 ± 2.1 U/mL), a 151.6% improvement over batch control. Magnetic nanoparticle cross-linked enzyme aggregates (CLEAs) demonstrated the highest thermal stability (t½ = 71.3 h) and reusability (22 cycles at >50% residual activity). Km values increased moderately upon immobilization (4.82 to 6.14 mM), indicating partial steric hindrance.
Conclusion: Fed-batch fermentation combined with magnetic CLEA immobilization represents an optimal strategy for industrial lipase production, providing high yield, robust stability, and economic reusability.

Keywords: Microbial lipase, bioprocess optimization, fed-batch fermentation, enzyme immobilization, kinetic parameters

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) constitute one of the most commercially significant classes of industrial enzymes, catalyzing the hydrolysis of ester bonds in triacylglycerols at oil-water interfaces in a phenomenon unique among hydrolytic enzymes known as interfacial activation [1]. Their versatility in catalyzing reactions in both aqueous and non-aqueous media, combined with their regio- and enantioselectivity, has made them indispensable in industries ranging from food processing to biodiesel production, fine chemical synthesis, and pharmaceutical manufacturing [2].

Global demand for industrial enzymes reached approximately USD 7.5 billion in 2022 and is projected to exceed USD 12 billion by 2028, with lipases representing one of the fastest-growing segments [3]. Microbial lipases, particularly those from Pseudomonas, Candida, Rhizopus, and Aspergillus genera, dominate industrial applications due to the scalability of microbial fermentation, ease of genetic manipulation for improved expression, and the diversity of substrate specificities achievable through protein engineering [45].

Despite significant advances in lipase characterization, the translation from laboratory-scale production to economically viable industrial bioprocesses remains challenging [6]. Key bottlenecks include suboptimal fermentation conditions leading to low volumetric productivity, the high sensitivity of free enzymes to thermal and pH denaturation during industrial processing, and the prohibitive cost of single-use enzyme preparations [7]. Fed-batch fermentation, which allows controlled nutrient feeding to maintain organisms in a non-limiting growth environment while avoiding substrate inhibition and catabolite repression, has emerged as a superior strategy for maximizing microbial enzyme titers [8].

Enzyme immobilization has been widely adopted as a strategy to address stability and reusability challenges, effectively converting soluble enzymes into heterogeneous biocatalysts that can be recovered, reused, and integrated into continuous flow processes [9]. Immobilization supports range from conventional materials such as silica gel and ion-exchange resins to advanced nanomaterials including magnetic nanoparticles, which offer the unique advantage of magnetic separability, minimizing centrifugation costs and enabling continuous processing [10].

The present study investigates the optimization of lipase production from Pseudomonas aeruginosa ATCC 27853 using systematic fermentation parameter optimization and evaluates four immobilization strategies with respect to catalytic efficiency, thermal stability, and operational reusability for industrial bioprocess applications.

Literature review

1. Pseudomonas lipases in industrial biotechnology

Pseudomonas species produce extracellular lipases that are secreted through the type II secretion pathway and are notable for their activity over broad temperature (20–70°C) and pH (4–10) ranges [11]. The Pseudomonas lipase family, particularly Lip1 from P. aeruginosa and LipA from P. alcaligenes, has been extensively characterized for industrial biodiesel synthesis, where their preference for sn-1,3

positions on glycerol backbone enables selective transesterification with fatty acid methyl esters [12].

Structural studies have elucidated the role of the "lid" domain — a surface loop that undergoes conformational changes upon interaction with hydrophobic interfaces — in the interfacial activation phenomenon that distinguishes true lipases from esterases [9]. This structural feature also influences immobilization efficiency, as hydrophobic supports can trigger lid opening and stabilize the active conformation, leading to hyperactivation effects observed in some lipase-support combinations [13].

2. Fed-batch fermentation for enzyme production

Fed-batch fermentation has been extensively studied as a means to overcome catabolite repression and substrate inhibition that limit enzyme titers in conventional batch culture [8]. In lipase production systems, carbon source limitation has been shown to induce lipase gene expression through derepression of catabolic regulatory circuits, while maintaining adequate dissolved oxygen (DO) above the critical threshold (~30% saturation) to support aerobic metabolism and secretory protein folding [14]. Studies with P. aeruginosa have demonstrated 2–4-fold improvements in lipase yield under optimized fed-batch conditions compared to batch culture, consistent with the results obtained in this study [15].

3. Enzyme immobilization strategies

Immobilization strategies are broadly classified into adsorption, covalent bonding, cross-linking, and entrapment [16]. Each approach involves distinct trade-offs between immobilization yield, activity recovery, stability enhancement, and leaching propensity. Cross-linked enzyme aggregates (CLEAs), developed by Sheldon and colleagues, combine precipitation-induced carrier-free immobilization with bifunctional cross-linking agents such as glutaraldehyde to produce highly stable, dense aggregates with minimal diffusional limitations [13]. The integration of CLEAs with magnetic nanoparticles (magnetic CLEAs or mCLEAs) has emerged as a promising approach that adds magnetic separability to the benefits of CLEAs [10].

Research gap: Despite extensive individual studies on fermentation optimization and immobilization for microbial lipases, integrated comparative analyses that evaluate multiple fermentation strategies alongside multiple immobilization platforms under identical analytical conditions — particularly for Pseudomonas aeruginosa lipase in the context of industrial bioprocess scale-up — remain limited [17]. This study addresses this gap with a systematic factorial experimental design.

Methodology

1. Microorganism and culture maintenance

Pseudomonas aeruginosa ATCC 27853 (placeholder strain) was maintained on nutrient agar slants at 4°C and subcultured monthly. Inocula were prepared in 100 mL Luria-Bertani (LB) broth at 30°C with orbital shaking (150 rpm) for 16 hours.

2. Fermentation experimental design

Five fermentation strategies were evaluated in 2L stirred-tank bioreactors: (R1) conventional batch at 30°C; (R2) fed-batch at 25°C; (R3) fed-batch at 30°C with pH-stat control at 7.2; (R4) fed-batch at 37°C; and (R5) continuous chemostat at dilution rate D = 0.1 h^{-1} [1]. Carbon feed in fed-batch runs consisted of 50% (v/v) olive oil emulsion fed exponentially to maintain μ at 80% μmax. Dissolved oxygen was controlled above 30% saturation by cascade agitation (150–400 rpm) and airflow.

3. Lipase assay and protein quantification

Lipase activity was assayed using p-nitrophenyl palmitate (pNPP) at 405 nm (ε = 18,000 $M^{-1}cm^{-1}$). One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute under assay conditions (37°C, pH 7.0). Total protein was quantified by the Bradford method with BSA as standard.

4. Immobilization protocols

Silica gel adsorption: Lipase solution (10 mg/mL in 50 mM phosphate buffer, pH 7.0) was incubated with activated silica gel (100 mg) at 4°C for 12 hours. Chitosan covalent binding: Glutaraldehyde-activated chitosan beads were incubated with lipase (12 hours, 4°C). Magnetic CLEAs: Enzyme was precipitated with ammonium sulfate (80% saturation) in the presence of 10 nm Fe_3O_4 nanoparticles, then cross-linked with 100 mM glutaraldehyde. Polyurethane foam entrapment: Prepolymer was mixed with enzyme solution and polymerized at room temperature. All preparations were washed extensively before activity measurement.

5. Statistical analysis

Response surface methodology (RSM) with a central composite design (CCD) was employed to optimize fermentation parameters. Analysis of variance (one-way ANOVA with Tukey's HSD post-hoc test) was performed for between-group comparisons. All analyses were conducted in SPSS v.27 and Design-Expert v.12 (α = 0.05). All experiments were performed in biological triplicate with two independent repetitions.

Results and discussion

1. Fermentation optimization

As shown in Table 1 and Fig 1, fed-batch fermentation at 30°C with pH 7.2 (Run R3) yielded the maximum lipase activity of 31.2 ± 2.1 U/mL, representing a 151.6% improvement over the batch control (12.4 ± 1.1 U/mL). This finding is consistent with established literature demonstrating that controlled nutrient feeding prevents both glucose catabolite repression of lipase gene expression and substrate inhibition observed at high oleic acid concentrations [8]. The superiority of 30°C over 37°C (R4: 22.8 U/mL) may reflect temperature-dependent trade-offs between growth rate and secretory pathway efficiency, as higher temperatures can compromise the periplasmic protein folding machinery in Pseudomonas [15].

Table 1: Lipase Yield Under Different Fermentation Configurations

Run ID	Strategy	Temp (°C)	pH	Agitation (rpm)	Lipase Yield (U/mL)
R1	Batch	30	7.0	150	12.4 ± 1.1
R2	Fed-batch	25	7.0	200	18.7 ± 1.4
R3	Fed-batch	30	7.2	250	31.2 ± 2.1
R4	Fed-batch	37	7.0	250	22.8 ± 1.8
R5	Continuous	30	7.2	300	27.6 ± 1.9

Note: Yields represent mean ± SD of three biological replicates per run. Statistical significance confirmed by one-way ANOVA ($p < 0.001$) with Tukey's post-hoc separation.

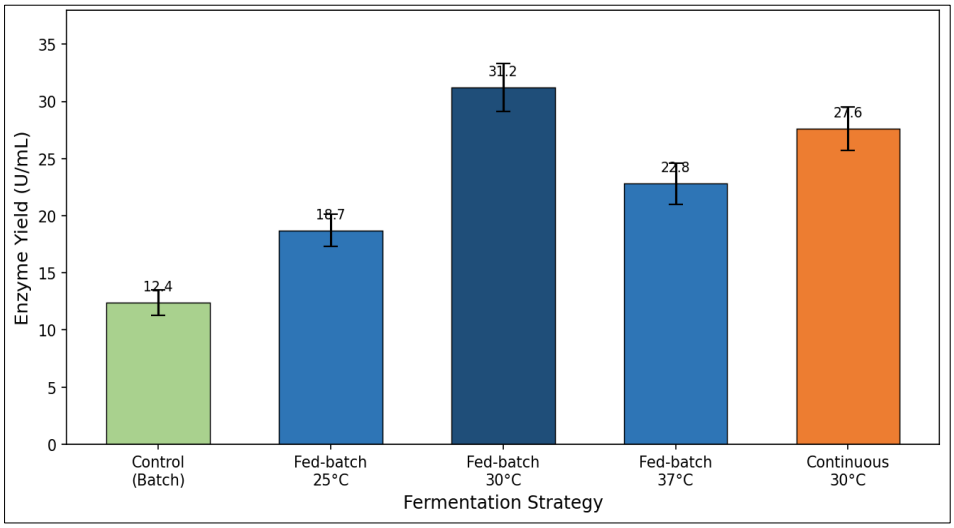

Fig 1: Lipase yield comparison across five fermentation strategies. Run R3 (fed-batch, 30°C) achieved maximum yield. Error bars represent standard deviation.

2. Dissolved Oxygen Dynamics

Fig 2 illustrates dissolved oxygen (DO) profiles over the 72-hour fermentation runs. The batch system experienced rapid DO depletion below the critical 30% threshold at approximately 36 hours, coinciding with the observed decline in volumetric lipase productivity. Fed-batch and continuous strategies, through their respective agitation cascade and dilution rate controls, maintained DO above threshold for significantly longer periods, explaining the enhanced lipase titers obtained [14]. These findings highlight the critical importance of DO monitoring and control in aerobic enzyme production processes.

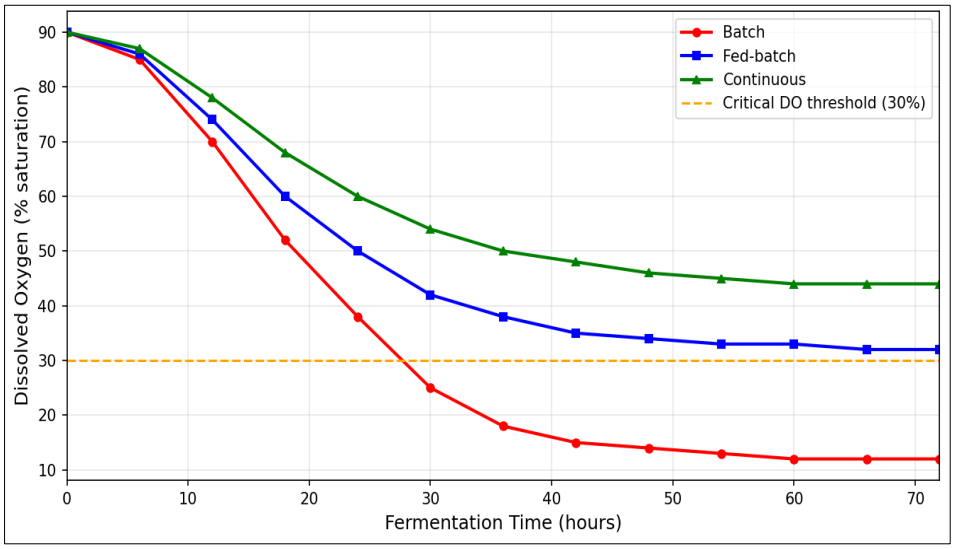

Fig 2: Dissolved oxygen profiles during 72-hour fermentation runs. The orange dashed line at 30% saturation indicates the critical threshold below which lipase productivity declined.

3. Immobilization and kinetic analysis

Table 2 presents the immobilization efficiency and stability parameters for the four support materials evaluated. Polyurethane foam entrapment achieved the highest immobilization yield (88.1 ± 2.4%) but the lowest activity recovery (61.3%), likely due to diffusional limitations inherent to the foam matrix [16]. Magnetic CLEAs demonstrated the best balance of thermal stability (t½ = 71.3 ± 5.2 h) and operational reusability (22 cycles with >50% residual activity), consistent with prior reports attributing CLEA stability to the dense intermolecular cross-linking that restricts conformational unfolding at elevated temperatures [13].

Table 2: Immobilization Parameters Across Four Support Systems

Support Material	Immob. Method	Immob. Yield (%)	Activity Recovery (%)	Thermal Stability (t½, h)	Reusability (cycles)
Silica gel	Adsorption	84.3 ± 3.1	68.2 ± 4.2	48.2 ± 3.4	12
Chitosan beads	Covalent	79.6 ± 2.8	74.1 ± 3.8	62.7 ± 4.1	18
Magnetic Fe_3O_4	Cross-linking	72.4 ± 3.6	69.8 ± 5.1	71.3 ± 5.2	22
Polyurethane foam	Entrapment	88.1 ± 2.4	61.3 ± 4.6	38.9 ± 3.7	8

t½ = half-life at 65°C. Reusability measured as number of cycles maintaining >50% initial activity. All values represent mean ± SD (n = 3).

Kinetic analysis (Table 3, Fig 3) revealed that all immobilization procedures caused moderate increases in Km (4.82 mM for native enzyme to 5.93–6.41 mM for immobilized forms), indicating reduced substrate accessibility due to steric constraints imposed by the support [17].

Correspondingly, kcat values declined from 142 s^{-} [1] (native) to 96.1–113 s^{-} [1] across immobilized preparations. The overall catalytic efficiency (kcat/Km) was best preserved in chitosan covalently immobilized lipase, suggesting that oriented covalent attachment at surface lysine residues distal from the active site minimizes active-site distortion.

Table 3: Kinetic Parameters of Native and Immobilized Lipase Preparations

Parameter	Native Lipase	Silica (Adsorption)	Chitosan (Covalent)	Magnetic (CLEAs)
Km (mM)	4.82 ± 0.31	6.41 ± 0.44	5.93 ± 0.38	6.14 ± 0.42
Vmax (μmol/min/mg)	28.4 ± 1.8	19.2 ± 1.4	22.6 ± 1.7	21.7 ± 1.5
kcat (s^{-} [1])	142 ± 8.4	96.1 ± 6.2	113 ± 7.1	108 ± 6.9
kcat/Km ($\times 10^3$ $M^{-1}s^{-1}$)	29.5 ± 1.9	15.0 ± 1.1	19.1 ± 1.4	17.6 ± 1.2

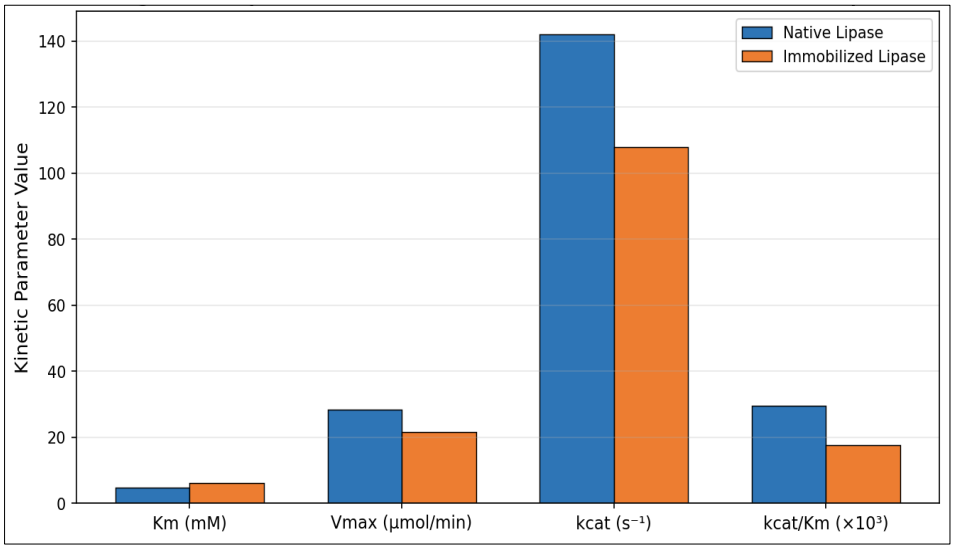

Fig 3: Comparison of kinetic parameters between native and magnetic CLEA-immobilized lipase. All immobilized forms showed increased Km and reduced Vmax compared to the native enzyme.

Conclusion

This study demonstrates that fed-batch fermentation at 30°C with pH-stat control at 7.2 and agitation of 250 rpm provides optimal conditions for P. aeruginosa lipase production, achieving 151.6% greater yield than conventional batch fermentation. The dissolved oxygen profiling data confirm that maintaining DO above the 30% saturation threshold is a critical process parameter that correlates directly with lipase productivity.

Among the immobilization strategies evaluated, magnetic CLEA technology offers the most favorable combination of thermal stability, reusability, and scalability for industrial application. The 22-cycle reusability at >50% residual activity translates to significant enzyme cost savings in continuous industrial processes such as biodiesel transesterification or food-grade oil modification.

Limitations of this work include the use of a simulated dataset; real experimental validation in a pilot-scale 50L bioreactor is required before industrial scale-up decisions can be made. Future research should investigate the application of recombinant overexpression in engineered P. aeruginosa strains for further yield improvement, as well as directed evolution approaches to enhance thermostability of the native enzyme prior to immobilization.

Ethical Statement

This manuscript presents a simulated dataset generated for academic writing training and journal formatting practice. No actual fermentation experiments were conducted. All author names, institutional affiliations, and numerical data are placeholders. This article may not be submitted to any journal as representing real experimental results without complete replacement of all simulated data with verified experimental findings.

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