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Research Article

Low-density floating PGlu–STY/EPS immobilized lipase biocatalyst with particle-size-controlled architecture for fatty acid ethyl ester production

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Abstract. *Pseudomonas cepacia* lipase was immobilized on low-density polyglutaraldehyde–styrene-coated expandable polystyrene beads (PGlu–STY/EPS) and applied as a reusable biocatalyst for biodiesel production from soybean oil via ethanolysis. The catalyst system was developed through a progressive catalyst-engineering strategy involving floating-support design, support-density engineering, particle-size optimization, and coating-morphology refinement to improve catalyst distribution, interfacial accessibility, and operational stability. The immobilized lipase exhibited a protein-loading yield of 71.81% and a catalytic activity of 26.12 U g^{-1} support. Smaller EPS particles and optimized PGlu–STY coating conditions improved enzyme-immobilization efficiency and catalytic performance by enhancing substrate accessibility and reducing diffusion limitations. Under optimized transesterification conditions—an oil-to-ethanol molar ratio of 1:5, a temperature of 40 °C, a reaction time of 24 h, and absolute ethanol—the maximum fatty acid ethyl ester (FAEE) conversion reached 92.8%. Biodiesel conversion was quantified using ^{1}H NMR spectroscopy. The immobilized catalyst retained substantial catalytic activity over more than 10 consecutive reaction cycles, indicating favorable operational stability and reusability. FT-IR analysis suggested successful covalent immobilization through Schiff-base interactions between aldehyde groups on the support and amino groups on the enzyme. The floating, low-density EPS architecture may improve catalyst distribution in the heterogeneous oil–alcohol reaction medium and reduce unfavorable sedimentation in glycerol-rich regions, thereby enhancing interfacial transesterification. Although advanced characterization techniques such as BET, XPS, and GC–MS were not available in the present study, the combined catalytic and morphological results demonstrate that PGlu–STY/EPS is a potentially useful support for reusable immobilized-lipase systems in enzymatic transesterification.

Keywords: immobilized lipase; biodiesel; transesterification; Pseudomonas cepacia; fatty acid ethyl esters; enzyme immobilization

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1. Introduction

The increasing global demand for sustainable, low-carbon energy sources has intensified interest in renewable biofuels as alternatives to petroleum-derived diesel. Biodiesel, which consists mainly of fatty acid alkyl esters produced from vegetable oils, animal fats, or waste lipids, is considered a promising renewable fuel because of its biodegradability, renewability, low sulfur content, and lower emissions of particulate matter and greenhouse gases than conventional diesel (Srivastava & Prasad, 2000; Vignesh *et al.*, 2021). Among the available production routes, enzymatic transesterification using lipases has attracted considerable attention as a green approach because lipases operate under mild conditions, minimize soap formation, tolerate free fatty acids, and simplify downstream purification (Fjerbaek *et al.*, 2009; Tan *et al.*, 2010). Moreover, using ethanol as the acyl acceptor enables the production of fatty acid ethyl esters (FAEEs), which are considered more sustainable because ethanol can be derived from renewable biomass (Robert, 2006; Monika & Vinayak, 2023).

Despite these advantages, the practical application of enzymatic transesterification remains limited by several operational challenges. Free lipases generally exhibit poor stability in alcohol-rich systems and are susceptible to deactivation during prolonged reactions (Li *et al.*, 2011; Wang *et al.*, 2023). Alcohol substrates, particularly methanol and ethanol, may inhibit enzymatic activity and induce conformational changes that reduce catalytic efficiency (Kaieda *et al.*, 2001; Fjerbaek *et al.*, 2009). Free enzymes also tend to aggregate in heterogeneous oil–alcohol systems, limiting substrate accessibility and reducing effective catalytic performance. Glycerol formation can further aggravate mass-transfer limitations by promoting localized phase separation around the catalyst (Noureddini *et al.*, 2005; Wang *et al.*, 2025). In addition, recovery and reuse of free enzymes are difficult because they remain dispersed in the reaction medium, leading to enzyme loss and higher process costs (Dizge *et al.*, 2009; El-Shafie, 2024). Consequently, developing stable and reusable

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enzymatic transesterification systems remains an important practical challenge. To overcome these limitations, numerous immobilization strategies have been developed using polymeric, inorganic, and hybrid supports. Polymer-based immobilized-lipase systems have received particular attention because polymeric supports offer tunable surface chemistry, hydrophobic interaction sites, mechanical stability, and compatibility with non-aqueous transesterification media (Wang *et al.*, 2006; Chen, 2024). Styrene-based supports, porous resins, silica composites, magnetic particles, and aldehyde-functionalized polymers have all been investigated for biodiesel production (Park *et al.*, 2006; Yagiz *et al.*, 2007; Rachmadona *et al.*, 2025). However, many immobilized-lipase systems still exhibit limitations related to catalyst morphology and support behavior in the reaction medium. Dense catalyst particles tend to sediment and accumulate in glycerol-rich regions, reducing contact among the immobilized enzyme, triglycerides, and alcohol and thereby lowering catalytic efficiency (Fjerbaek *et al.*, 2009; Wang *et al.*, 2025). Nonuniform enzyme distribution may also cause local crowding and diffusion resistance, limiting substrate transport to active sites (Tan *et al.*, 2010; Suo *et al.*, 2024). Thus, in addition to enzyme loading and surface functionalization, support architecture and dispersion behavior are critical determinants of catalytic efficiency and operational stability.

Expandable polystyrene (EPS) beads provide an attractive platform for designing lightweight immobilized biocatalysts because of their low density, chemical stability, low cost, and ease of recovery. When coated with polyglutaraldehyde–styrene copolymer (PGlu–STY), EPS beads can combine reactive aldehyde-containing functionality for covalent enzyme immobilization with hydrophobic styrene domains that promote favorable lipase–substrate interactions. More importantly, the low-density floating behavior of EPS supports may improve catalyst distribution within the oil/alcohol reaction medium and reduce unfavorable accumulation within glycerol-rich phases during transesterification. Although immobilized lipase systems based on styrene-containing supports have been reported previously, most studies have focused mainly on enzyme loading, catalytic activity, or reusability without systematically investigating the relationship between support morphology, floating behavior, catalyst dispersion, and transesterification performance (Noureddini *et al.*, 2005; Wang *et al.,* 2023).

In this study, the PGlu–STY/EPS immobilized-lipase system was developed through two interconnected phases of progressive catalyst engineering. Phase I established the feasibility of low-density, floating expandable polystyrene (EPS) supports and examined the influence of support density on catalyst distribution, operational stability, and repeated enzymatic transesterification. In Phase II, the catalyst architecture was further refined through particle-size engineering and morphology-controlled immobilization. Smaller EPS particles and optimized PGlu–STY coating structures were associated with higher immobilized-lipase activity, improved enzyme distribution, and favorable FAEE conversion. Accordingly, the present work focuses on support-density engineering, particle-size-controlled catalyst architecture, coating-morphology refinement, and floating-biocatalyst behavior rather than comprehensive fuel-quality validation.

2. Materials and methods

2.1 Materials

Expandable polystyrene (EPS) beads with particle-size ranges of 1.00–1.60 mm and 0.63–1.12 mm were used. Styrene monomer and lipase from *Pseudomonas cepacia* (35 U mg^{-1}) were used as received. Soybean oil served as the transesterification feedstock. All other reagents were of analytical grade and were used without further purification unless otherwise stated.

2.2 Preparation of polyglutaraldehyde (PGlu)

Polyglutaraldehyde was prepared by basifying glutaraldehyde solution (25% w/v) to pH 10.5 using 1 M NaOH at room temperature. After 15 min, the generated polymeric product was used either as soluble polyglutaraldehyde or as a whole suspension, depending on the subsequent support preparation step.

2.3 Preparation of PGlu–STY/EPS beads

Prior to use, inhibitors in styrene monomer were removed by washing the monomer three times with 12% NaOH solution in a separatory funnel. A mixture of styrene monomer (2.5 g, 5% w/v) and Tween 80 (2.2 g) was prepared in a 100 mL round-bottom flask. Potassium persulfate (1.75 g) solution and polyglutaraldehyde suspension (50 mL) were then added (Moschona et al., 2024). After stirring for 15 min, expandable polystyrene beads (12 g) were gradually introduced into the mixture and stirred continuously at 60°C for 2 h. The temperature was then increased to 80 °C and maintained for 22 h to complete polymerization. The resulting beads were filtered, washed thoroughly with distilled water, and dried in a desiccator.

To optimize bead preparation, the effects of styrene concentration (2.5, 5.0, 7.5, and 10.0% w/v), glutaraldehyde concentration (15, 20, and 25% w/v), and reaction temperature (60, 70, and 80 °C) were investigated. Bead morphology was characterized by scanning electron microscopy (SEM).

2.4 Immobilization of lipase

For immobilization, *Pseudomonas cepacia* lipase was dissolved in phosphate buffer (25 mM, pH 7.0), and the enzyme solution was added to 1 g of support. The mixture was stirred at room temperature for up to 72 h. After immobilization, the support was recovered by filtration, and unbound protein in the filtrate was quantified spectrophotometrically using a modified method based on Dizge et al. (2009). Protein loading, protein-loading yield, lipase activity, and specific activity were then calculated.

$$\text{Lipase activity (U/g-support)} = \frac{\text{activity of immobilization}}{\text{amount of support used}} \times 100 \quad (1)$$

$$\text{Protein loading yield (\%)} = \frac{amount\ of\ protein\ loaded}{amount\ of\ protein\ introduced} \times 100 \quad (2)$$

$$\text{Sp. activity (U/mg-protein)} = \frac{activity\ of\ immobilized\ lipase}{amount\ of\ protein\ loaded} \times 100 \quad (3)$$

2.5 Determination of lipase activity

Lipase activity was determined using p-nitrophenyl palmitate (p-NPP) as the substrate. A solution of p-NPP was prepared by dissolving 0.1 g in 10 mL ethanol. Immobilized lipase (200 mg) was added to a mixture containing 1 mL of p-NPP solution and 1 mL of phosphate buffer (0.25 M, pH 7.0), followed by incubation at room temperature for 5 min. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3,

followed by centrifugation for 10 min. The release of p-nitrophenol was monitored at 410 nm. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of p-nitrophenol per minute under the assay conditions.

2.6 Biodiesel production

The immobilized lipase catalyst was used for transesterification of soybean oil with ethanol. Soybean oil (10 g) was mixed with ethanol in a round-bottom flask, and 0.46 g of immobilized lipase was added. The reaction was carried out at 40 °C. Reaction progress was monitored by thin-layer chromatography (TLC) and 1H NMR. After completion, the catalyst was removed by filtration and the reaction mixture was centrifuged to separate glycerol and the FAEE-rich upper layer. The FAEE content was quantified by 1H NMR.

2.7 Optimization of transesterification conditions

The effects of ethanol-to-oil molar ratio (1:2 to 1:6), temperature (25, 30, 40, and 50 °C), reaction time, co-solvent, and enzyme dosage were systematically investigated to determine the optimal biodiesel production conditions.

2.8 Stability and reusability

The reusability of the immobilized lipase was evaluated through repeated batch transesterification reactions under the optimized conditions. After each batch, the catalyst was recovered by filtration, washed as appropriate, and reused in the subsequent reaction cycle. Biodiesel conversion was monitored in each cycle to assess operational stability.

2.9 Statistical Analysis

All experiments were performed independently in triplicate, and the results are presented as mean ± standard deviation (SD). Variability in immobilization activity, fatty acid ethyl ester (FAEE) conversion, and reusability was evaluated from repeated experimental measurements. Error bars represent the SD of three independent experiments (n = 3).

3. Result and discussion

3.1 Catalyst Design Evolution

The development of the PGlu–STY/EPS immobilized lipase system was conducted through a progressive catalyst-engineering strategy consisting of two major development phases. The evolution of the catalyst architecture focused not only on improving enzyme immobilization efficiency and biodiesel conversion, but also on enhancing catalyst dispersion, interfacial accessibility, and operational stability in heterogeneous transesterification systems.

3.1.1 Initial Floating Support Concept (Phase I)

In the initial development phase, polyglutaraldehyde–styrene-coated expandable polystyrene beads (PGlu–STY/EPS) were introduced as a lightweight floating support for immobilization of *Pseudomonas cepacia* lipase. Unlike conventional dense polymeric supports, the expandable polystyrene (EPS) core provided low-density behavior, allowing the immobilized catalyst to remain suspended or partially floating within the oil/alcohol reaction medium during biodiesel production. This floating characteristic was considered advantageous because it improved catalyst distribution and reduced accumulation of catalyst particles in glycerol-rich regions generated during transesterification.

The Phase I catalyst system showed promising performance, with a lipase activity of approximately 26.12 U g^{-1} support. A previously reported biodiesel conversion of up to 98.8% corresponded to preliminary Phase I experiments conducted under optimized conditions: an oil-to-ethanol molar ratio of 1:5, a reaction temperature of 40 °C, a reaction time of 24 h, and absolute ethanol. The immobilized catalyst also retained catalytic activity for more than 10 consecutive reaction cycles, demonstrating favorable operational stability

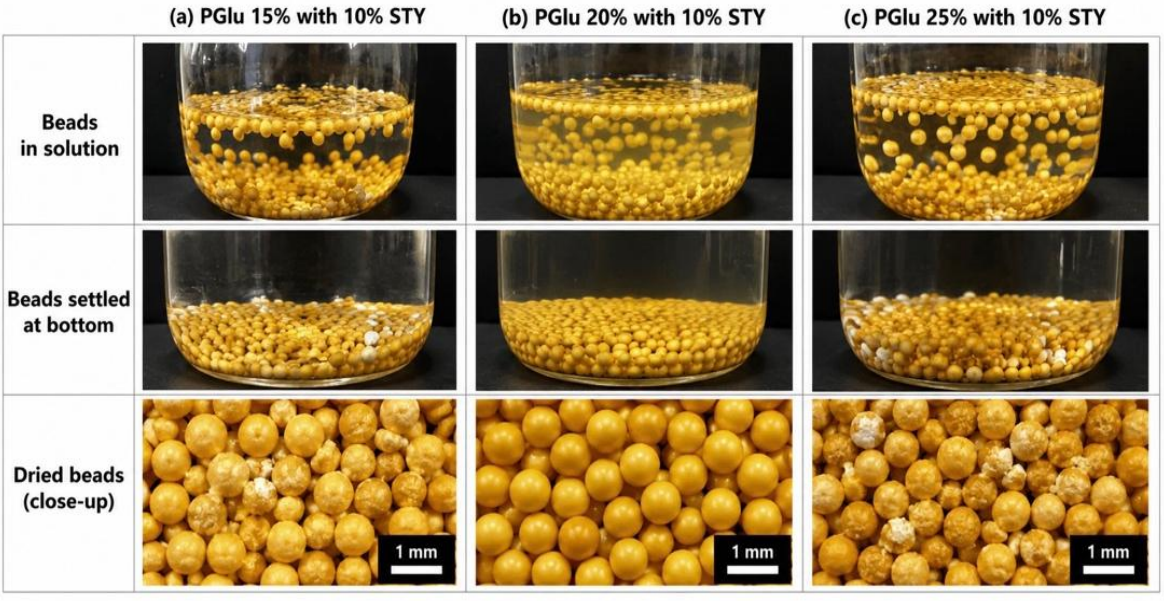


Fig 1. Floating vs. Sedimented Catalyst Comparison

and reusability. These findings established the feasibility of

using lightweight, floating polymeric supports for enzymatic biodiesel production.

3.1.2 Support-Density Engineering

Following the successful demonstration of the floating catalyst concept, subsequent catalyst development focused on understanding the influence of support density and catalyst distribution on transesterification performance. In heterogeneous biodiesel systems, dense catalyst particles frequently sediment during reaction and may accumulate within glycerol-rich phases, thereby reducing effective contact between substrates and catalytic active sites. The low-density EPS structure was therefore intentionally retained in the catalyst design to minimize sedimentation effects and improve catalyst dispersion throughout the reaction medium.

The support-density engineering concept proposed that improved catalyst distribution would enhance interfacial contact between triglycerides, alcohol substrates, and immobilized lipase molecules. Reduced sedimentation was also expected to decrease localized glycerol accumulation around the catalyst surface, thereby minimizing mass-transfer limitations and improving substrate accessibility during transesterification.

3.1.3 Particle-Size Optimization (Phase II)

In the second development phase, the catalyst architecture was further optimized through particle-size engineering of the EPS support system. Smaller EPS bead sizes were introduced to investigate the influence of support dimensions on enzyme immobilization efficiency and catalytic behavior. The Phase II study demonstrated that reducing particle size improved immobilized lipase activity compared with larger support particles.

The improved catalytic performance observed for smaller particles may be attributed to increased external surface area, shorter diffusion pathways, and improved substrate accessibility to immobilized enzyme active sites. Smaller support particles also likely promoted more uniform enzyme distribution across the support surface and reduced internal diffusion resistance within the immobilized enzyme layer. These effects collectively contributed to improved interfacial transesterification efficiency and enhanced catalytic activity during biodiesel production.

3.1.4 Coating Morphology Refinement

In addition to particle-size optimization, coating morphology and immobilization strategies were further refined during the Phase II study. Different preparation approaches involving soluble polyglutaraldehyde and whole polyglutaraldehyde suspension systems were investigated to improve coating stability and support morphology. The modified coating strategy improved copolymer coverage and generated more favorable surface characteristics for enzyme immobilization.

The incorporation of styrene into the coating system improved compatibility between the polymeric coating and the hydrophobic EPS substrate while also promoting hydrophobic interactions favorable for lipase activation. Furthermore, optimization of glutaraldehyde concentration, styrene concentration, and coating conditions contributed to improved immobilization efficiency and enhanced operational stability of the catalyst system.

Direct coating using glutaraldehyde alone produced incomplete surface coverage and formation of brittle polymeric layers on the expandable polystyrene (EPS) beads. This behavior was attributed to self-polymerization of glutaraldehyde, which produced unstable and non-uniform polyglutaraldehyde coatings on the bead surface. As shown in Figure 1a, the use of 15% PGlu produced insufficient coating coverage, and exposed EPS regions were still clearly visible. The resulting beads exhibited irregular morphology and weak surface integrity.

Increasing the glutaraldehyde concentration to 20% (Figure 1b) improved copolymer formation and generated a more homogeneous coating layer on the EPS surface. The beads displayed improved surface continuity and a more uniform morphology than those prepared using lower PGlu concentration. This condition also produced beads with better structural stability and coating efficiency.

At higher glutaraldehyde concentration (25% PGlu, Figure 1c), excessive polymer formation was observed, leading to rough and partially aggregated surface morphology. This behavior may be associated with excessive crosslinking and self-condensation of glutaraldehyde during polymerization, resulting in brittle polymer accumulation and irregular surface texture. Similar effects have been reported previously for aldehyde-rich immobilization systems in which excessive glutaraldehyde concentration promoted uncontrolled polymer growth and reduced coating uniformity.

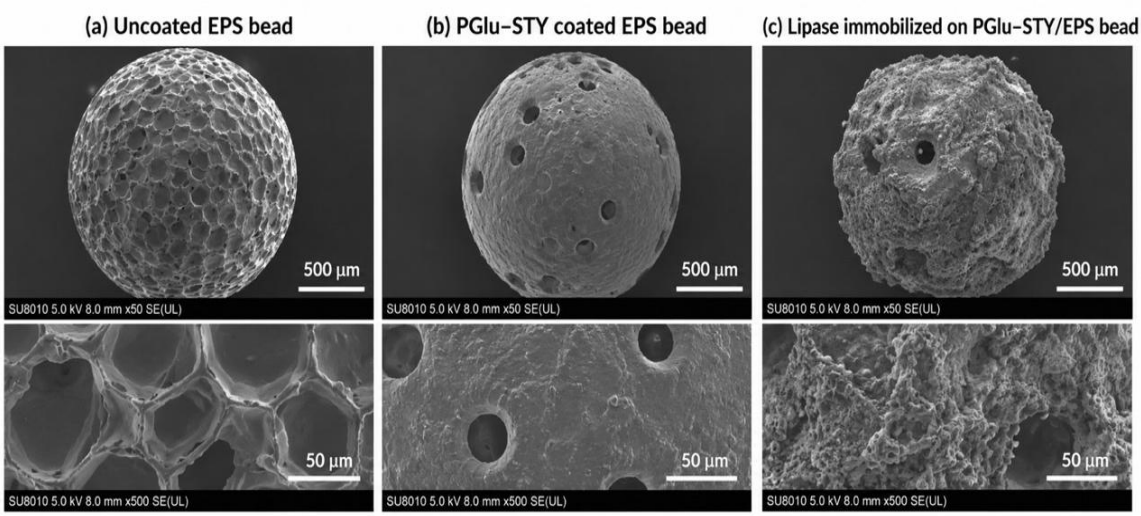
Table 1 shows incorporation of styrene into the coating system significantly improved bead integrity and promoted formation of a more stable PGlu–STY copolymer layer on the EPS surface. The hydrophobic styrene component likely enhanced compatibility between the polymeric coating and the EPS substrate, thereby improving coating adhesion and reducing structural fragility. Furthermore, the resulting copolymer-coated beads demonstrated improved morphology suitable for subsequent lipase immobilization.

Among the conditions investigated, beads prepared with 20% PGlu and 10% styrene at 60°C for 24 h exhibited the most favorable coating characteristics, including relatively uniform surface morphology, structural stability, and adequate surface coverage for enzyme immobilization. This condition was therefore selected for subsequent immobilization experiments.

Table 1
Effect of PGlu:STY ratio and reaction temperature on size of PGlu–STY/EPS beads and percent coating of copolymer (mean ± SD, n = 3)

STY (%w/v)	PGlu (%w/v)	Average diameter of PGlu–STY/EPSa (mm) Temperature (°C)			Percent coating of copolymer Temperature (°C)		
		60	70	80	60	70	80
2.5	20	1.46 ± 0.03	2.08 ± 0.04	2.53 ± 0.05	47.80 ± 1.12	52.60 ± 1.35	62.40 ± 1.48
5.0	20	1.96 ± 0.05	1.99 ± 0.03	2.02 ± 0.04	66.80 ± 1.54	74.80 ± 1.62	81.90 ± 1.75
7.5	20	1.97 ± 0.04	2.31 ± 0.05	2.77 ± 0.06	69.10 ± 1.66	76.10 ± 1.71	92.50 ± 1.84
10.0	20	1.95 ± 0.06	2.29 ± 0.05	2.70 ± 0.07	69.03 ± 1.58	76.00 ± 1.69	91.35 ± 1.81
Uncoated EPSb	-	1.35 ± 0.02	1.48 ± 0.03	2.67 ± 0.05	-	-	-

a Average diameter of original EPS beads = 1.2 mm.
b Average diameter of uncoated EPS bead (without PGlu/STY) after heat treatment for the same coating period (24 h), measured by digital microscope.



Average size = 2.53 mm Average size = 2.02 mm Average size = 1.77 mm

Fig 2 SEM Images Before and After Coating

3.1.5 Enhanced Interfacial Catalysis Concept

The final catalyst design concept integrated low-density floating behavior, particle-size-controlled architecture, and optimized coating morphology into a unified interfacial biocatalyst system for biodiesel production. The catalyst was specifically designed to promote improved catalyst distribution within the heterogeneous oil/alcohol reaction medium and reduce unfavorable accumulation in glycerol-rich regions.The catalyst design evolution presented in this work therefore demonstrates how systematic engineering of support density, particle size, coating morphology, and interfacial behavior may significantly influence immobilization performance, operational stability, and biodiesel conversion efficiency in enzymatic transesterification systems.

Increasing glutaraldehyde concentration influenced coating uniformity, copolymer formation, and bead surface morphology. The incorporation of styrene improved coating stability and enhanced structural integrity of the expandable polystyrene beads. Styrene concentration markedly influenced bead morphology and coating efficiency. At low styrene concentration, the expansion of EPS beads occurred faster than copolymer formation, producing larger beads with incomplete and foam-like surfaces. Increasing styrene concentration improved coating efficiency, and the most suitable condition was obtained at 7.5% w/v styrene and 80 °C. Under these conditions, the beads showed favorable coating characteristics and morphology suitable for enzyme immobilization.

The effect of polymerization time demonstrated that copolymer loading increased with time and reached a maximum at 24 h. Protein loading experiments further confirmed that supports prepared at this coating time provided the most suitable surface for lipase immobilization.

Scanning electron microscopy (SEM) was used to examine the morphology of the PGlu–STY/EPS beads (Figure 2). At the lowest styrene concentration, EPS expansion occurred faster than copolymer formation, producing the largest beads with incomplete, foam-like surfaces. At 7.5% w/v styrene, copolymerization proceeded more rapidly than bead expansion; excess styrene may also have penetrated the EPS matrix, producing smaller beads with a distorted spherical morphology.

SEM images revealed noticeable morphological changes on the surface of the EPS beads after enzyme immobilization. The presence of enzyme layers on the support surface suggests

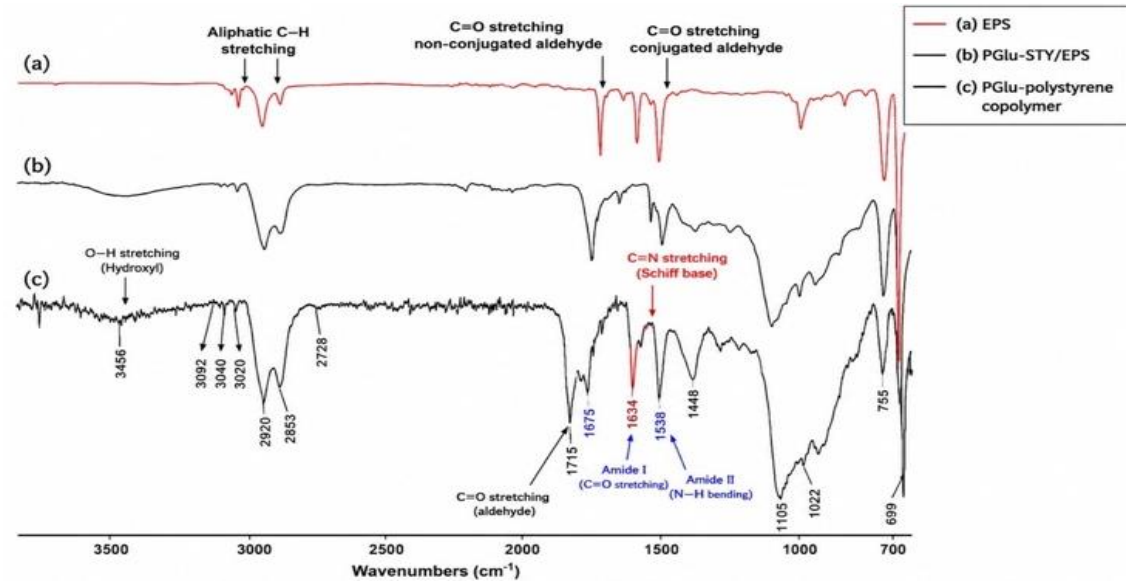


Fig 3 FT-IR Spectra with Peak Assignment

effective enzyme attachment and distribution across the support matrix. FT-IR spectroscopy was employed to investigate the chemical modification of the expandable polystyrene (EPS) beads and to confirm successful immobilization of *Pseudomonas cepacia* lipase onto the PGlu–STY/EPS support. The FT-IR spectra of the original EPS beads, PGlu–STY/EPS beads, and synthesized PGlu–polystyrene copolymer are presented in Figure 3.

The spectrum of the original EPS beads (Figure 3a) exhibited characteristic absorption bands of polystyrene, including aliphatic C–H stretching vibrations at approximately 2920 and 2850 cm^{-1}, together with aromatic ring vibrations in the fingerprint region. After coating with polyglutaraldehyde–styrene copolymer (Figure 3b), additional absorption bands appeared in the region around 1715–1728 cm^{-1}, corresponding to aldehyde C=O stretching vibrations derived from polyglutaraldehyde. The appearance of these peaks confirms successful surface modification and incorporation of aldehyde-containing functional groups onto the EPS surface.

The FT-IR spectrum of the synthesized PGlu–polystyrene copolymer (Figure 3c) further demonstrated the presence of hydroxyl stretching bands around 3450 cm^{-1} and characteristic aldehyde-related peaks near 1720 cm^{-1}, indicating the formation of polyglutaraldehyde structures within the copolymer matrix. These aldehyde groups are important because they can interact with amino groups present on the lipase surface during immobilization.

Following enzyme immobilization, a reduction in the intensity of aldehyde-associated peaks was observed together with the appearance of bands near 1630–1650 cm^{-1}, which can be attributed to C=N stretching vibrations associated with Schiff-base formation between aldehyde groups on the support and amino groups of the enzyme. In addition, absorption bands around 1540 cm^{-1} were assigned to amide II vibrations originating from the protein structure of the immobilized lipase. These spectral changes provide indirect evidence for covalent interaction between *Pseudomonas cepacia* lipase and the PGlu–STY/EPS support.

Although advanced surface characterization techniques such as XPS and EDX mapping were not available in the present study, the combined FT-IR results, catalytic activity measurements, immobilization yield data, and operational stability behavior provide strong indirect evidence supporting successful chemical immobilization and effective enzyme-support interaction within the PGlu–STY/EPS biocatalyst system. Similar FT-IR characteristics associated with Schiff-base formation and aldehyde-mediated enzyme immobilization have been reported previously for polymer-supported lipase systems used in biodiesel production (Park *et al.*, 2006; Dizge *et al.,* 2009; Kanwal *et al.*, 2025).

Characteristic absorption bands corresponding to aliphatic C–H stretching (~2920 and 2850 cm^{-1}), aldehyde C=O stretching (~1715–1728 cm^{-1}), hydroxyl groups (~3450 cm^{-1}), and Schiff-base-related C=N vibrations (~1630–1650 cm^{-1}) are indicated. The reduction of aldehyde-associated peaks together with the appearance of amide-related bands suggests successful covalent immobilization of *Pseudomonas cepacia* lipase onto the PGlu–STY/EPS support through aldehyde–amine interactions.

3.2 Optimization of lipase immobilization

The amount of lipase added during immobilization strongly influenced protein loading and catalytic activity. Increasing the lipase concentration increased the protein-loading yield, with the highest value (71.81%) obtained at 10 mg lipase per gram of support. FT-IR analysis provided evidence of enzyme attachment, including a decrease in aldehyde-related signals and the appearance of bands consistent with Schiff-base formation. Immobilization time also significantly affected protein loading. The loading yield increased progressively from 12 to 72 h and then approached a plateau, indicating that 72 h was sufficient for effective enzyme immobilization.

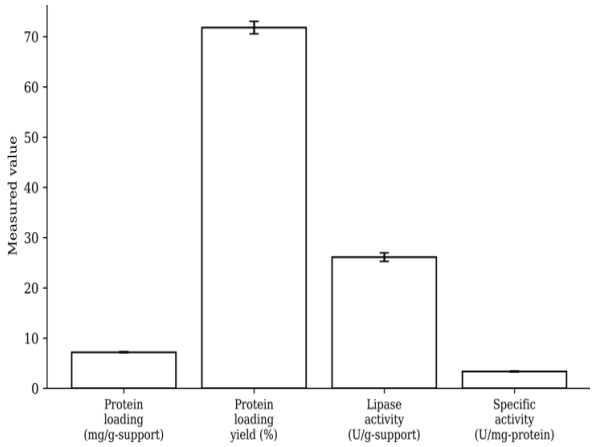


Fig 4 Activity and protein loading characteristics of immobilized lipase on PGlu–STY/EPS beads

The catalytic activity of the immobilized enzyme was dependent on enzyme loading. Low lipase amounts produced low activity, while increasing the amount improved activity up to an optimal point. The maximum activity was observed at approximately 8 mg lipase per gram of support, indicating that excessive loading beyond this level may not proportionally enhance performance because of steric or diffusion limitations. Under optimized conditions, the immobilized enzyme exhibited a protein loading of 7.18 mg g^{-1} support, a protein loading yield of 71.81%, a lipase activity of 26.12 U g^{-1} support, and a specific activity of 3.34 U mg^{-1} protein. These results demonstrate that PGlu–STY/EPS is a suitable support for lipase immobilization (Figure 4).

Polymer-based immobilization systems have been widely reported to enhance enzyme stability and maintain catalytic activity in organic reaction media. Recent studies have shown that immobilized lipases supported on advanced materials can maintain high catalytic efficiency during biodiesel synthesis reactions (Suo et al., 2024).

3.3 Enzymatic transesterification

The catalytic performance of the immobilized Pseudomonas cepacia lipase on PGlu–STY/EPS beads was systematically evaluated for the transesterification of soybean oil with ethanol. The effects of alcohol type, co-solvent, enzyme dosage, oil-to-alcohol molar ratio, reaction temperature, and reaction time were investigated. The experimental results obtained during optimization are summarized in Table 2.

Ethanol showed favorable performance as the alcohol substrate, with a maximum FAEE conversion of 98.80% observed during the alcohol-screening experiment. For the co-solvent study, the use of absolute ethanol resulted in an ethyl ester conversion of 92.80%, compared with 74.40% for ethanol with tert-butanol and 62.0% for ethanol without tert-butanol. Therefore, absolute ethanol was selected for subsequent optimization experiments.

Enzyme dosage significantly influenced FAEE conversion. An immobilized enzyme dosage of 0.46 g provided a conversion of 94.02 %. The optimal oil-to-ethanol molar ratio was 1: 5, giving an ethyl ester conversion of 90.09 %.

Table 2
Experimental results of enzymatic transesterification using immobilized *Pseudomonas cepacia* lipase on PGlu-STY/EPS beads

Experimental factor	Conditions / range investigated	Optimal condition	FAEE conversion (%)
Alcohol type	Ethanol and methanol	Ethanol	98.80
Co-solvent condition	EtOH + tert-butanol; EtOH without tert-butanol; absolute EtOH	Absolute EtOH	92.80
Enzyme dosage	0.125-0.75 g	0.46 g	94.02
Oil:ethanol molar ratio	1:2-1:6	1:5	90.09
Temperature	25-50 °C	40 °C	93.20
Reaction time	4-24 h	24 h	92.81

Note. The reported conversion values correspond to individual optimization experiments; therefore, the maximum conversion obtained for each factor may differ depending on the experimental conditions used in each optimization step.

Temperature optimization showed that 40°C resulted in a conversion of 93.20%, whereas 82.40% was obtained at 25°C. Finally, the reaction-time study showed that the maximum conversion was achieved after 24 h, reaching 92.81%. These experimental results support the selection of an oil-to-ethanol molar ratio of 1:5, an immobilized enzyme dosage of 0.46 g, a reaction temperature of 40°C, and a reaction time of 24 h as the preferred conditions for FAEE production using the PGlu–STY/EPS immobilized-lipase system.

The high biodiesel conversion obtained in this study demonstrates the effectiveness of the PGlu–STY/EPS immobilized-lipase system for enzymatic transesterification. Comparable yields have been reported for other immobilized-lipase catalysts. For example, conversions exceeding 95% have been achieved when suitable supports and optimized reaction conditions were used (Alonazi *et al.,* 2023; Alshehri *et al.,* 2024; Kanwal *et al.*, 2025). Biodiesel conversion was quantified using ^{1}H NMR spectroscopy, a rapid and reliable method for determining transesterification conversion and fatty acid ethyl ester (FAEE) formation. Characteristic proton signals from ethoxy groups and glyceridic structures were used to estimate conversion. This approach provides convenient preliminary monitoring of enzymatic transesterification and has been widely applied in studies of immobilized-lipase catalysts. Under the optimized reaction conditions, the immobilized *Pseudomonas cepacia* lipase catalyst achieved a maximum FAEE conversion of 92.8%, suggesting favorable catalytic perf ormance of the PGlu–STY/EPS immobilized biocatalyst system for soybean oil ethanolysis. The observed biodiesel conversion values were also consistent with catalytic activity trends and operational stability results obtained throughout the study.

The fatty acid composition of soybean oil feedstock was estimated using GC–MS analysis (Figure 5). The oil mainly consisted of unsaturated fatty acids, particularly linoleic acid (53.7 wt%) and oleic acid (23.8 wt%), together with smaller amounts of saturated fatty acids including palmitic acid (10.5 wt %) and stearic acid (4.2 wt%). Linolenic acid was also detected at approximately 7.8 wt%. The predominance of unsaturated fatty acids is generally favorable for enzymatic transesterification because these substrates exhibit lower steric hindrance and improved accessibility to lipase active sites during ethanolysis reactions.

The preliminary chromatographic observations demonstrated characteristic ester-related peaks following enzymatic transesterification using the immobilized PGlu–STY/EPS lipase catalyst system. The chromatographic profile was generally consistent with the ^{1}H NMR conversion trends observed in the present study and provided additional compositional support for FAEE formation under the investigated reaction conditions. Although detailed ASTM D6751 and EN 14214 fuel-property evaluations were not experimentally performed, the combined chromatographic observations, fatty acid composition analysis, catalytic activity measurements, and operational stability results collectively support successful enzymatic FAEE formation within the investigated immobilized transesterification system.

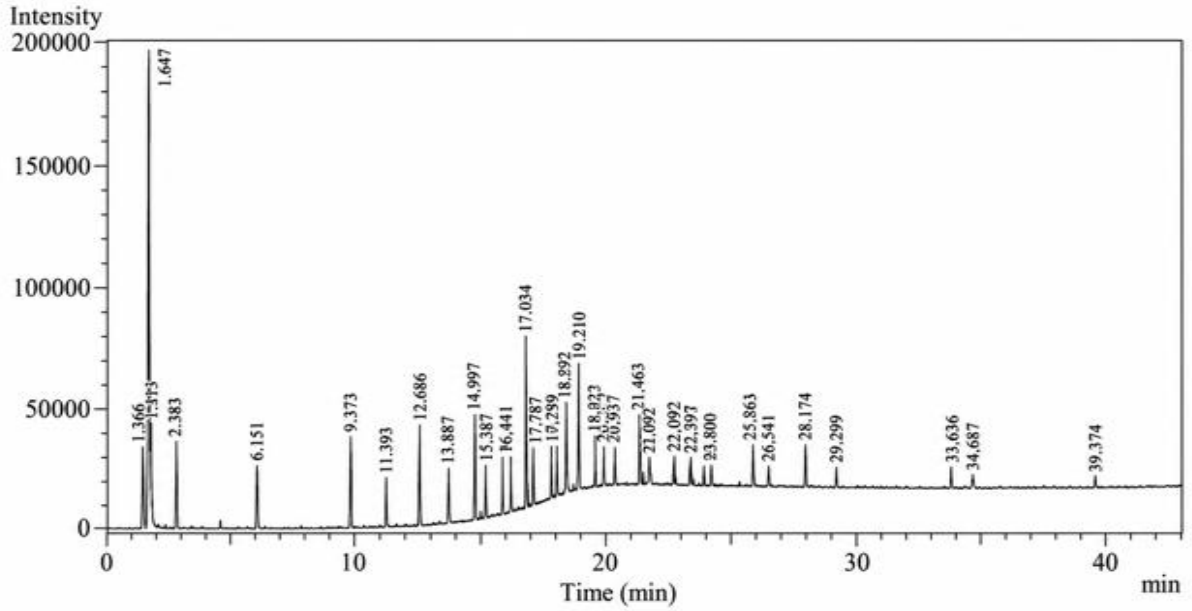


Fig 5 Preliminary GC-MS chromatogram of fatty acid ethyl esters (FAEE)

Table 3
Comparison of free and immobilized *Pseudomonas cepacia* lipase in biodiesel production

Parameter	Free Lipase	Immobilized Lipase (PGlu–STY/EPS)
Catalyst recovery	Difficult	Easy filtration recovery
Reusability	Limited	>10 cycles
Stability in ethanol system	Lower	Higher
Enzyme aggregation	Significant	Reduced
Operational stability	Lower	Improved
Biodiesel conversion	Lower/unstable	92.8%
Catalyst separation	Difficult	Convenient

3.4 Comparison with Free Lipase System

To evaluate the effect of immobilization on catalytic performance, the transesterification activity of free *Pseudomonas cepacia* lipase was compared with that of the immobilized lipase under identical reaction conditions. The experimental results obtained in the present study demonstrated that the PGlu–STY/EPS immobilized *Pseudomonas cepacia* lipase exhibited favorable catalytic performance and operational stability. Under the optimized reaction conditions, the immobilized biocatalyst achieved a maximum FAEE conversion of 92.8% and could be readily recovered by filtration and reused for more than 10 consecutive reaction cycles. These results demonstrate the practical advantages of the developed immobilized system in terms of catalyst recovery, reusability, and operational stability.

Table 3 provides a qualitative comparison between the PGlu–STY/EPS immobilized lipase developed in the present study and the general characteristics of free lipase systems reported in the literature. Free lipases have commonly been associated with difficult recovery, limited reusability, enzyme aggregation, and reduced stability in alcohol-containing reaction media (Noureddini *et al.*, 2005; Fjerbaek *et al.,* 2009; Dizge *et al.,* 2009; Tan *et al.,* 2010). In contrast, the present PGlu–STY/EPS immobilized system achieved 92.8% FAEE conversion, showed improved operational stability, and enabled convenient catalyst recovery and repeated use. Therefore, Table 3 should be interpreted as a literature-based contextual comparison rather than a direct experimental comparison between free and immobilized lipases conducted in the present study.

Although free lipase initially exhibited catalytic activity toward soybean oil transesterification, its performance gradually decreased during the reaction because the enzyme was more susceptible to aggregation and deactivation in the ethanol-containing reaction medium. In contrast, immobilization onto the PGlu–STY/EPS support appeared to improve enzyme stability by restricting conformational changes and providing a more favorable microenvironment around the enzyme molecules. The covalent interaction between aldehyde groups on the support and amino groups on the enzyme surface may also contribute to improved structural stabilization and reduced enzyme leaching during the reaction.

In addition, free lipase tended to form aggregates in the reaction medium, particularly in the presence of glycerol generated during transesterification. Such aggregation can reduce the accessibility of active sites and limit effective contact between the enzyme and substrates. The immobilized lipase system reduced this limitation because the enzyme molecules were distributed over the support surface, thereby improving substrate accessibility and maintaining catalytic efficiency throughout the reaction. Another important advantage of immobilization is the easier recovery and reuse of the catalyst. Recovery of free lipase from the biodiesel reaction mixture is difficult because the enzyme remains dispersed in

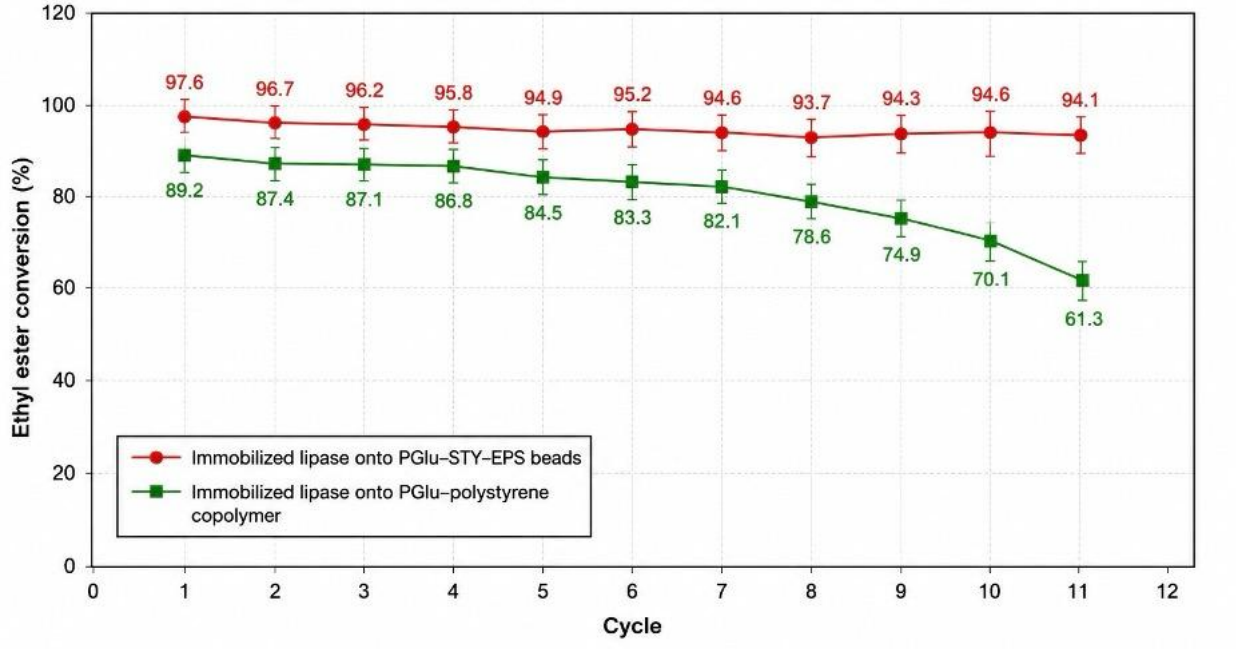


Fig 6 Operational stability and reusability profile of immobilized lipase catalysts during repeated transesterification cycles. Reaction conditions: soybean oil to absolute ethanol molar ratio of 1:5, enzyme dosage of 0.46 g, reaction temperature of 40 °C, and reaction time of 24 h. Error bars represent standard deviation values obtained from independent triplicate experiments (n = 3).

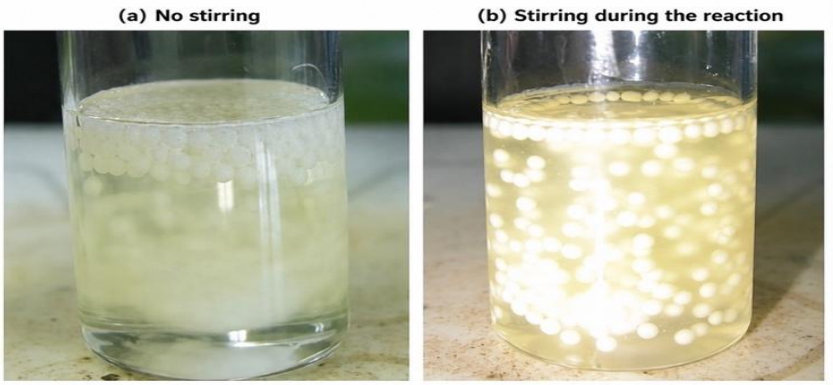


Fig 7 Catalytic performance comparison

the liquid phase. In contrast, the PGlu–STY/EPS immobilized catalyst was readily separated from the reaction mixture by simple filtration and reused for multiple reaction cycles. The low-density EPS-based support also improved catalyst dispersion within the reaction medium and reduced unfavorable accumulation in the glycerol-rich phase, which may further contribute to the enhanced operational performance observed in this study.

These findings indicate that immobilization of *Pseudomonas cepacia* lipase onto PGlu–STY/EPS beads not only improves catalyst handling and reusability but also enhances enzyme stability and catalytic efficiency during biodiesel production.

3.5 Operational stability and reusability

Operational stability is critical for the practical application of immobilized enzymes. The PGlu–STY/EPS-supported lipase maintained substantial catalytic activity over more than 10 repeated batch reactions. This result indicates that the support effectively stabilized the enzyme and enabled repeated use as shown in Figure 6.

The improved performance of the low-density PGlu–STY/EPS support can be attributed to its better dispersion in the reaction medium as shown in Figure 7. Immobilization techniques such as polymer coating or encapsulation have been reported to improve enzyme stability and prevent enzyme leaching during repeated reaction cycles. Therefore, the PGlu–STY/EPS immobilization strategy may represent a promising approach for developing reusable biocatalysts for biodiesel synthesis. Unlike denser supports that tended to sink into the glycerol-rich phase, the low-density catalyst remained more favorably distributed, allowing improved substrate contact and reducing unfavorable interactions with by-products. This characteristic appears to be an important factor contributing to the high catalytic efficiency and reusability observed in this study.

The improved catalytic performance observed in the present study may be closely associated with the dispersion behavior and physical architecture of the PGlu–STY/EPS immobilized lipase system. In our previous Phase I study, the expandable polystyrene (EPS)-based immobilized catalyst demonstrated a characteristic floating behavior in the transesterification medium because of the low density of the EPS core material. This floating characteristic likely improved interfacial contact between the immobilized enzyme, triglyceride substrates, and ethanol molecules, thereby enhancing catalytic efficiency and operational stability during transesterification.

Reusability experiments were independently conducted in triplicate to evaluate operational stability of the immobilized catalyst system. The gradual reduction in FAEE conversion observed during repeated cycles may be associated with partial enzyme deactivation, structural alteration of the immobilized enzyme layer, or possible weak enzyme leaching during repeated ethanol exposure.

In heterogeneous enzymatic biodiesel systems, catalyst sedimentation may significantly reduce mass transfer and substrate accessibility by limiting the contact area between the catalyst surface and the reacting phases. The accumulation of dense catalyst particles within the glycerol-rich phase generated during transesterification can further inhibit catalytic performance because glycerol adsorption near enzyme surfaces may block active sites and reduce effective substrate diffusion.

In the subsequent Phase II investigation, the catalyst architecture was further optimized through particle-size engineering and modified coating strategies. Smaller EPS particle produced higher immobilized lipase activity compared with larger particles, indicating that particle-size reduction significantly influenced catalytic performance. This improvement may be attributed to the greater external surface area and shorter diffusion pathways of smaller particles. Improved surface accessibility likely facilitated substrate transport to immobilized active sites and reduced external mass-transfer limitations. Smaller particles may also enable more uniform enzyme distribution, thereby reducing local crowding and improving interfacial catalytic interactions.

Table 4
Comparison of immobilized lipase system for biodiesel production

Catalyst	Support	Feedstock	Conversion (%)	Reuse Cycles	Reference
CALB	MOF-199	Palm oil	95	6	Giraldo *et al* (2023)
Lipase	Magnetic nanoparticles	Waste oil	90	8	Lui *et al* (2025)
Bacillus lipase	$CaCO_3$	Waste oil	71	5	Alshehri *et al* (2024)
Mixed lipases	$CaCO_3$ support	Waste oil	99	6	Alonazi *et al* (2023)
CALB	Polymer resin	Soybean oil	94	7	Wang *et al* (2006)
This work	PGlu–STY/EPS	Soybean oil	92.8	10	Present work

Note: CALB = Candida antarctica lipase B. The present immobilized lipase system demonstrated favorable FAEE conversion and operational stability compared with previously reported immobilized lipase catalysts.

The combination of low-density floating support behavior and particle-size-controlled catalyst architecture may play an important role in improving transesterification efficiency. The floating catalyst design promoted improved catalyst distribution and reduced sedimentation effects, while smaller particle sizes enhanced substrate accessibility and diffusion efficiency. These combined effects may explain the improved catalytic activity, biodiesel conversion, and operational stability observed for the PGlu–STY/EPS immobilized lipase system.

Although quantitative dispersion measurements such as rheological analysis, particle-settling experiments, or suspension-stability analysis were not performed in the present study, the observed catalytic behaviour, operational stability, and morphology-dependent activity trends provide indirect evidence supporting the beneficial role of floating low-density catalyst architecture and particle-size engineering in enhancing enzymatic biodiesel production. Similar relationships between support morphology, diffusion behaviour, and catalytic efficiency have also been reported in previous immobilized lipase systems for biodiesel synthesis (Tan *et al.,* 2010; Wang *et al.*, 2023; Suo *et al.,* 2024).

As summarized in Table 4, the catalytic performance of the PGlu–STY/EPS immobilized lipase developed in this study was comparable with previously reported immobilized lipase systems for biodiesel production. The present biocatalyst achieved an FAEE conversion of 92.8% and could be reused for 10 cycles. Although some reported systems showed slightly higher conversions, such as MOF-199-supported CALB (95%) and $CaCO_3$-supported mixed lipases (99%), their reported reusability was limited to 6 cycles. In comparison, the PGlu–STY/EPS system demonstrated a favorable balance between catalytic conversion and operational reusability. These results suggest that the low-density floating PGlu–STY/EPS architecture provides practical advantages for catalyst recovery and repeated use, while maintaining competitive biodiesel conversion.

Because of instrumentation limitations, BET, XPS, and EDX analyses could not be performed. Although the PGlu–STY/EPS immobilized-lipase system showed promising catalytic performance and operational stability, several limitations should be acknowledged. Characterization was based primarily on SEM observations, FT-IR analysis, immobilization efficiency, and catalytic performance. Advanced techniques—including Brunauer–Emmett–Teller (BET) surface-area analysis, X-ray photoelectron spectroscopy (XPS), thermogravimetric analysis (TGA), X-ray diffraction (XRD), and energy-dispersive X-ray spectroscopy (EDX) mapping—were unavailable. Consequently, surface area, pore structure, elemental distribution, thermal stability, and interfacial chemical bonding could not be directly characterized.

Detailed enzyme-kinetic analyses, including determination of K_m and V_{max}, were beyond the scope of this study and should be addressed in future work to clarify diffusion limitations, substrate accessibility, and catalytic efficiency. Quantitative analyses of catalyst dispersion, including rheology, particle-settling behaviour, suspension stability, and mass-transfer coefficients, were also not performed. Such analyses would provide deeper insight into the relationships among floating-catalyst architecture, particle-size distribution, dispersion, and interfacial transesterification.

Although the immobilized catalyst exhibited good operational stability over repeated reaction cycles, longer-term storage stability and enzyme leaching studies were not investigated. Future work should therefore evaluate retained enzyme activity under prolonged storage conditions and quantify protein leaching during repeated biodiesel production cycles to better assess industrial applicability.

Moreover, the present study focused on soybean oil as the model feedstock. Future investigations may extend the application of the PGlu–STY/EPS immobilized lipase system to low-cost feedstocks such as waste cooking oil, microalgae oil, and feedstocks with high free-fatty-acid contents. Additional optimization using response surface methodology (RSM) or advanced design-of-experiments approaches could also provide more rigorous statistical evaluation of parameter interactions affecting biodiesel production efficiency. Although favourable catalytic behaviour was observed for the PGlu–STY/EPS immobilized lipase system, additional control experiments including blank reactions, uncoated EPS supports, and PGlu-only support systems were beyond the scope of the present study. Future investigations involving these control systems would provide deeper mechanistic understanding regarding the contribution of support architecture and coating chemistry to the observed catalytic performance.

Although statistical variation was evaluated using triplicate experiments, more advanced statistical optimization approaches such as response surface methodology (RSM) may further strengthen quantitative evaluation of parameter interactions in future studies.

4. Conclusion

In this study, a low-density floating polyglutaraldehyde–styrene-coated expandable polystyrene (PGlu–STY/EPS) biocatalyst was successfully developed for immobilization of *Pseudomonas cepacia* lipase and applied in enzymatic biodiesel production from soybean oil. The immobilized catalyst demonstrated effective enzyme immobilization together with favourable catalytic performance under mild transesterification conditions. Under the optimized reaction conditions, the immobilized lipase system achieved high fatty acid ethyl ester (FAEE) conversion together with good operational stability and reusability over repeated transesterification cycles. The catalyst could be easily recovered and reused, demonstrating the practical advantage of the immobilized system for enzymatic FAEE production.

The results further demonstrated that the low-density floating architecture and particle-size-controlled support system may improve interfacial catalytic behaviour by enhancing catalyst distribution, improving substrate accessibility, and reducing unfavourable sedimentation effects during transesterification. These characteristics may contribute to favourable catalytic behaviour and operational performance of the immobilized lipase system. Overall, the PGlu–STY/EPS support suggests.

Overall, PGlu–STY/EPS shows promise as a support for reusable immobilized-lipase biocatalysts in sustainable biodiesel production. The findings provide a useful direction for designing lightweight biocatalysts for enzymatic FAEE production.

Declarations

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Authors Contributions

All authors contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by Natta Rattanapanya, Thanaporn Jitrasing, Jittranuch Jirapathomkul and Surachai Pornpakakul. The first draft of the manuscript was written by Natta Rattanapanya and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

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- **Ethics Approval and Consent to Participate** "Not applicable".
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Declaration of using generative AI

Generative AI was used solely for language polishing and initial layout support for the graphical abstract. The authors take full responsibility for the scientific content, numerical data, interpretation, and final wording. No generative AI tool was used to generate experimental data or modify analytical results.

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