

Article

Immobilization of Bioimprinted Phospholipase D and Its Catalytic Behavior for Transphosphatidylation in the Biphasic System

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Abstract

Phosphatidylserine (PS) holds considerable importance in both the food and medical sectors; however, its biosynthesis is critically dependent on phospholipase D (PLD). The practical application of PLD is constrained by pronounced side reactions in its free form and by reduced selectivity when immobilized. To address these challenges, this study employed a sequential strategy involving bioimprinting to hyperactivate PLD, followed by microencapsulation via ionotropic gelation within an alginate–chitosan matrix. This approach induced conformational rigidification, enabling PLD to maintain its hyperactivated state in aqueous environments. Under optimal conditions, the encapsulation efficiency reached 78.56%, and the enzyme activity recovery achieved 105.27%. The immobilized bioimprinted PLD demonstrated exceptional catalytic performance, achieving a 94.68% PS yield within 20 min, which significantly surpassed that of free PLD (85.82% in 150 min) and non-imprinted immobilized PLD (90.34% in 60 min). This represents 7.27-fold and 2.14-fold efficiency improvements, respectively. Furthermore, the biocatalyst exhibited outstanding storage stability, thermal stability, and reusability (77.53% yield after 8 cycles). To our knowledge, this is the first report combining bioimprinting with alginate-chitosan microencapsulation via ionotropic gelation, which yielded remarkably enhanced PLD activity. These findings highlight the strong potential of this method for efficient PS production.

Keywords: phospholipase D; bioimprint; immobilization; phosphatidylserine; microencapsulation



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1. Introduction

Phosphatidylserine (PS), a unique functional phospholipid, regulates key membrane proteins and plays a vital role in cellular signaling and physiological processes [1]. Its wide-ranging health benefits include promoting neuronal repair, enhancing cognitive functions such as memory, alertness, and focus, managing attention deficit hyperactivity disorder (ADHD) and depression, preventing Alzheimer’s disease, and reducing stress [2–4]. Beyond its neurological applications, PS functions as an effective sports supplement to alleviate post-exercise fatigue and accelerate recovery [5], and serves as a valuable component in liposomal therapies for conditions such as hypercholesterolemia, ulcerative colitis, and type 1 diabetes [6–8]. These properties underscore its considerable potential in pharmaceuticals, functional foods, and cosmetics. However, large-scale application

is constrained by its low natural abundance and high extraction costs. An increasingly preferred alternative is the enzymatic synthesis of PS via phospholipase D (PLD)-catalyzed transphosphatidylation. This approach utilizes abundant phosphatidylcholine (PC) and L-serine as substrates, offering advantages such as readily available raw materials, mild processing conditions, environmental compatibility, high yield and specificity, and minimal byproduct formation [9].

Theoretically, PS can be synthesized from low-cost PC to significantly reduce production expenses. However, experimental data reveal that PLD exhibits substrate-specific selectivity during transphosphatidylation, making PS biosynthesis the most challenging PLD-catalyzed reaction [10–12]. Bioimprinting represents a well-established technique for modulating enzyme activity and selectivity [13]. By leveraging ligand-induced structural hysteresis, this pretreatment generates hyperactivated enzyme conformations. However, such conformations exhibit significant aqueous instability, readily refolding to native states in solution. Complementary to this approach, enzyme immobilization provides spatial confinement that rigidifies hyperactivated structures [14,15]. This dual strategy synergistically reduces structural flexibility, enhances robustness against harsh conditions (extreme pH, temperature, organic solvents), and ultimately yields stabilized biocatalysts.

Typically, transphosphatidylation is performed in biphasic systems, which enhance the solubility of both the hydrophobic substrate PC and the resulting products, thereby favoring the reaction equilibrium. PLD, when interfacially imprinted, binds to and reacts efficiently with its substrates only at the oil–aqueous interface. Upon adsorption to this interface, PLD undergoes conformational rearrangements that enable interaction with interfacial molecules, resulting in interfacial activation. This activation induces the opening of the lid structure covering the active site, allowing substrate access to the catalytic center and enhancing catalytic efficiency. Thus, interfacial imprinting—occurring specifically at amphiphilic oil–water interfaces—can significantly enhance the catalytic performance of PLD. Following this principle, researchers have developed hyperactivated biocatalysts by synergizing bioimprinting with immobilization. For example, Li et al. prepared a hyperactivated PLD by stabilizing its induced conformation through intramolecular cross-linking prior to surface conjugation with nano-silica [16]. However, a notable limitation of such systems is their reaction efficiency, as achieving a phosphatidylserine (PS) yield of 97% can require up to 6 h [16]. Similarly, Qian et al. combined bioimprinting with resin adsorption and demonstrated that substrate analogs markedly enhance the performance of bioimprinted lipase [17]. This strategy of integrating bioimprinting with interfacial activation has been successfully applied to immobilized lipases, resulting in remarkable improvements in catalytic activity and stability in organic solvents [18].

To harness the advantages of interfacial activation within a robust immobilized system, the choice of an appropriate immobilization method is critical. Enzyme immobilization techniques are generally classified as chemical or physical, depending on the nature of enzyme–support interactions. Among physical approaches, microencapsulation—which confines enzymes within a polymer matrix or membrane network—is particularly attractive, as it retains enzymes while allowing the diffusion of substrates and products, thereby minimizing enzyme leaching and improving stability [19,20]. A widely used microencapsulation technique is ionotropic gelation, which relies on the cross-linking of polymers with polyvalent ions (e.g., Ca^{2+}) [21]. Alginate, a non-toxic anionic polysaccharide, undergoes gelation through the “egg-box” model involving coordination between its carboxyl groups and metal ions [22,23]. Although alginate microcapsules exhibit high porosity that facilitates diffusion [24], this characteristic may also reduce immobilization efficiency. To address this limitation, combined approaches that integrate ionic gelation with cationic polyelectrolyte complexation—such as with chitosan—are often employed [25,26]. Chi-

tosan, a polycationic polysaccharide, interacts strongly with the anionic charges of alginate, forming stable core–shell microcapsules [27–29].

Although the chitosan–alginate microspheres used in this study are inherently hydrophilic, they are expected to effectively facilitate the interfacial activation of PLD. This is attributed to the unique microenvironment formed within the porous network of the carrier, which can promote the aggregation of hydrophobic substrates, leading to the formation of localized hydrophobic microdomains that mimic the natural interfaces required for PLD activation. Consequently, even within a hydrophilic carrier, the immobilized enzyme may operate in an optimized environment. While extensive research has explored chitosan–alginate systems for immobilizing enzymes such as lipases [30], laccases [31,32], and xylanase [33], their application for PLD immobilization—particularly exploiting its interfacial activation within this matrix—remains largely unexplored. Therefore, this study aims to develop a novel immobilized PLD biocatalyst using the chitosan–alginate system, with the objective of simultaneously achieving high stability, exceptional activity, and superior synthesis efficiency.

In this work, a high-convenience and high-efficiency approach was proposed for immobilizing PLD that has a hyperactivated structure, thereby improving the transphosphatidylolation effect to produce PS. PLD enzymatic activity was analyzed according to the structure-mediated hysteretic behavior for generating the hyperactivated 3-D structure. Thereafter, the bioimprinted PLD acquired was microencapsulated via ionotropic gelation within an alginate–chitosan matrix. A systematic comparison was subsequently conducted among three biocatalysts, namely, free PLD, immobilized PLD, and its bioimprinted variant, to evaluate their characteristics. Ultimately, the prepared bioimprinted PLD was applied to PS synthesis. Enzymatic activity tests confirmed that the bioimprinted PLD maintained the ligand-mediated structure and exhibited great transphosphatidylolation effects within aqueous systems.

2. Materials and Methods

2.1. Materials

Sodium alginate and chitosan (medium molecular weight: 190,000–310,000; viscosity: 200–800 cP, 1 wt.% within 1% acetic acid at 25 °C) were obtained from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Calcium chloride was acquired from Solarbio Tech (Beijing, China), and a BCA protein assay kit was obtained from Beyotime (Shanghai, China). Additional materials, including PLD, PC, PS, and L-serine, were prepared as described in our previous studies [34]. All reagents were analytically pure and commercially available.

2.2. PLD Immobilization

PLD hyperactivation was completed through bioimprinting as follows: 0.2 M acetate buffer (1 mL, pH 4.5) containing L-serine (300 mg) was added to PLD solution (9 mL, 1.37 mg_{protein}/mL, 0.25 U/mg_{protein}). Thereafter, this resulting mixed sample underwent 0.5 h of continuous stirring at 500 rpm in an ice bath.

Microcapsules were subsequently prepared via ionotropic pregelation using sodium alginate (SA) as the wall material, following the procedure outlined in Figure 1. Specifically, sodium alginate (SA, % *w/v*) was dissolved in 10 mL of the previously prepared bioimprinted mixed solution and stirred for 15 min to ensure complete homogeneity. For the non-imprinted immobilized PLD control, 10 mL of free PLD was used, with the subsequent immobilization steps remaining identical. The resulting biopolymer solution (10 mL) was then dripped, using a 27-gauge scalp needle attached to a 10 mL syringe, into 50 mL of an aqueous calcium chloride solution containing chitosan (CTS, % *w/v*) and CaCl₂ (M), under magnetic stirring at 500 rpm. After chitosan was dissolved in 1% (*w/v*) acetic acid

with 15 min of stirring, the pH was adjusted to 7.0 with 50%(*w/v*) NaOH prior to this step. Following gelation, those microcapsules were retained in CaCl_2 solution for a specified complexation time (CT, min). Throughout formation, the pH remained stable at about 7.0. After rigidification, the microcapsules were collected by sieving, rinsed with distilled water to remove the bioimprinting ligand, and finally lyophilized for storage. Parameters, including SA concentration (%(*w/v*)), CaCl_2 concentration (M), CTS concentration (%(*w/v*)), and CT (min), were systematically investigated via experimental design.

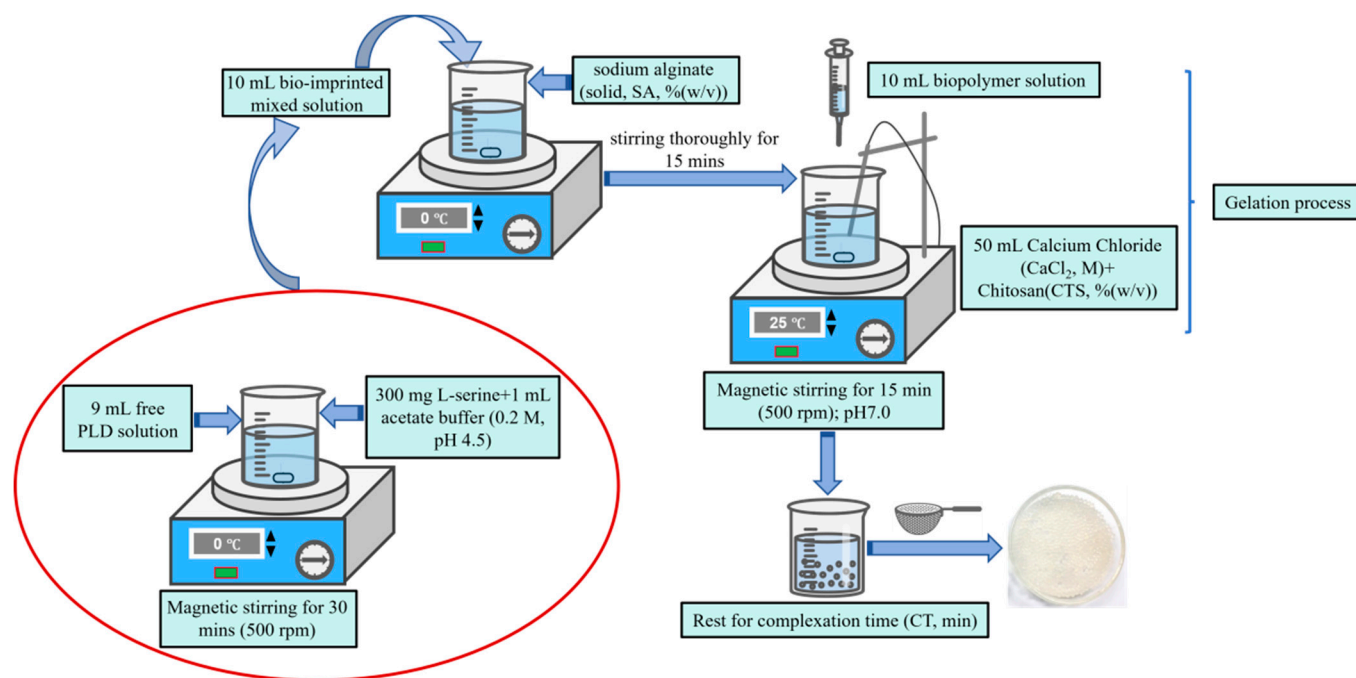


Figure 1. A sketch map showing the microencapsulation process: chitosan concentration (CTS), sodium alginate concentration (SA), calcium chloride concentration (CaCl_2), and complexation time (CT).

2.3. Optimization of Immobilized Bioimprinted PLD Microencapsulation

To maximize the immobilization efficiency of bioimprinted PLD, preliminary single-factor tests identified four key parameters: SA concentration (1–2.5% *w/v*), CTS concentration (0.3–0.9% *w/v*), CaCl_2 concentration (0.1–0.3 M), and CT (1–3 min). On the basis of these results, orthogonal optimization was conducted via an $L_9(3^4)$ array to determine the optimal preparation protocol. This experimental design systematically evaluated the four critical factors previously established to significantly influence microcapsule activity through triplicate testing. Range analysis was used to quantify each factor's impact on immobilization efficiency, ultimately defining the optimal manufacturing conditions.

The efficacy of immobilized bioimprinted PLD microencapsulation was assessed by determining the immobilization efficiency and enzyme activity recovery. The immobilization efficiency (IE, %) was calculated by measuring the concentration of unencapsulated PLD protein in the calcium chloride–chitosan solution after the gelation process. The IE was determined using the following equation: $\text{IE (\%)} = (\text{PLDcontrol} - \text{PLDfree}) / \text{PLDcontrol} \times 100$, where PLDcontrol represents the total protein concentration of the 9 mL PLD solution used during bioimprinting, and PLDfree denotes the residual protein concentration measured in both the post-gelation solution (calcium chloride/chitosan mixture) and the microcapsule washing solutions. The activity recovery (AR, %) was defined as the ratio of the total catalytic activity of the immobilized bioimprinted PLD to that of the free PLD used during the immobilization process.

2.4. Characterization Methods

The surface morphologies of the samples were examined using scanning electron microscopy (SEM; SU8010, Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV. Chemical composition analysis was performed through FTIR spectroscopy (Nicolet 6700, Thermo Fisher Scientific, Waltham, MA, USA), with spectral data acquired within 400–4000 cm^{-1} . For crystallographic analysis, the dried microcapsules were examined via X-ray diffraction (D2 Phaser, Bruker Corporation, Billerica, MA, USA) connected to a LYNXEYE linear detector, with the scanning parameters set at $10^\circ/\text{min}$ over a 2θ range of $5\text{--}90^\circ$.

2.5. Free and Immobilized PLD Activity Determination

Transphosphatidylolation was conducted to measure the free and immobilized PLD catalytic abilities in synthesizing PS. PS biosynthesis was performed in a butyl acetate/water biphasic system using L-serine and PC as substrates. The reaction system was prepared by first dissolving 10 mg of PC in 5 mL of butyl acetate, followed by dissolving L-serine at various concentrations in 0.2 M acetate buffer (5 mL, pH 6.0). For enzymatic catalysis, 10 mg (dry weight) of immobilized PLD or immobilized hyperactivated PLD was added, whereas for the free PLD control, 10 μL of enzyme mixture was used. Reactions with free and immobilized PLD were conducted at 40°C for 2 h, whereas the hyperactivated PLD reaction required only 15 min of incubation at a shaking speed of 180 rpm. Process optimization studies systematically evaluated key parameters influencing PS production, including reaction time, L-serine-to-PC mass ratio, buffer pH, and temperature. In addition, the optimal pH for the enzymatic reaction was determined using 0.2 M buffer systems, including acetate buffer (pH 4.0–5.5) and phosphate buffer (pH 6.0–8.0). PS concentration was determined as previously described [34]. One unit (U) of specific activity for PLD was defined as the amount of enzyme required to produce 1 μmol of PS per minute under the specified assay conditions. Quantitative analysis involved calculating PS yield (%) as the molar percentage of PC converted to PS, with production efficiency expressed as the ratio of PS yield to reaction time.

2.6. Assessment of Thermal Stability

A comparative thermal stability assessment was performed on three PLD variants: free enzyme, immobilized form, and immobilized hyperactivated preparation. The samples were exposed to $25\text{--}65^\circ\text{C}$ in substrate-free conditions for a 1 h duration. Post-incubation, the remaining transphosphatidylolation activities were determined and expressed as percentages relative to the baseline activity measured at 25°C (set as 100% reference).

2.7. Storage Stability and Reusability

Free, immobilized, and immobilized hyperactivated PLD were stored at 4°C for various durations to detect residual transphosphatidylolation activity.

The reusability of the immobilized PLD and its bioimprinted counterpart was evaluated by monitoring PS production yields over successive reuse cycles. After each catalytic cycle, both biocatalysts were recovered and subjected to multiple washes with acetate buffer to remove residual contaminants and restore catalytic activity before reuse. Under optimized conditions, this protocol demonstrated the retained biosynthesis capacity of both biocatalyst forms through multiple reuses.

2.8. Statistical Analysis

Each experiment was implemented with three replicates. Data processing and statistical analysis were conducted via Microsoft Excel and Origin 2024 software (Origin-

Lab Corporation, Northampton, MA, USA). The quantitative data are presented as the means $\pm$ SDs.

3. Results

3.1. Variables That Affect Bioimprinted PLD Immobilization

To optimize the preparation of the bioimprinted PLD microcapsules, orthogonal optimization was performed via an $L_9(3^4)$ array. Four key parameters—the SA concentration, CTS concentration, CaCl_2 concentration, and CT—were evaluated by measuring the immobilization efficiency. Range analysis was applied to the results (the experimental design and outcomes can be found in Table 1).

Table 1. Orthogonal experiment results and range analysis.

Run	Factor				IE (%)	AR (%)
	SA (%)	CTS (%)	CaCl_2 (M)	CT (min)		
1	1.0 ₍₁₎	0.3 ₍₁₎	0.1 ₍₁₎	1 ₍₁₎	55.24	62.42
2	1.0 ₍₁₎	0.6 ₍₂₎	0.2 ₍₂₎	2 ₍₂₎	58.48	80.12
3	1.0 ₍₁₎	0.9 ₍₃₎	0.3 ₍₃₎	3 ₍₃₎	46.42	46.88
4	1.5 ₍₂₎	0.3 ₍₁₎	0.2 ₍₂₎	3 ₍₃₎	52.35	54.44
5	1.5 ₍₂₎	0.6 ₍₂₎	0.3 ₍₃₎	1 ₍₁₎	78.56	105.27
6	1.5 ₍₂₎	0.9 ₍₃₎	0.1 ₍₁₎	2 ₍₂₎	45.18	44.73
7	2.5 ₍₃₎	0.3 ₍₁₎	0.3 ₍₃₎	2 ₍₂₎	51.35	52.38
8	2.5 ₍₃₎	0.6 ₍₂₎	0.1 ₍₁₎	3 ₍₃₎	68.92	86.84
9	2.5 ₍₃₎	0.9 ₍₃₎	0.2 ₍₂₎	1 ₍₁₎	41.19	35.84
K ₁	160.15	158.93	169.34	175.00		
K ₂	176.09	205.96	152.02	155.01		
K ₃	161.46	132.80	176.33	167.69		
k ₁	53.38	52.98	56.45	58.33		
k ₂	58.70	68.65	50.67	51.67		
k ₃	53.82	44.27	58.78	55.90		
R	5.31	24.39	8.10	6.66		
Priority order		CTS > CaCl_2 > CT > SA				
Optimal level	1.5	0.6	0.3	1		
Optimal combination	SA (1.5%) CTS (0.6%) CaCl_2 (0.3 M) CT (1 min)					

SA: sodium alginate concentration (%(w/v)); CTS: chitosan concentration (%(w/v)); CaCl_2 : calcium chloride concentration (M); CT: complexation time (min); IE (%): immobilization efficiency; AR (%): activity recovery; K: sum of response values at the same factor level; k: mean response value per experiment at the same factor level.

Table 1 shows the optimal levels for each factor: SA-2 ($k_2 > k_3 > k_1$), CTS-2 ($k_2 > k_1 > k_3$), CaCl_2 -3 ($k_3 > k_1 > k_2$), and CT-1 ($k_1 > k_3 > k_2$). The range values (R) revealed that the CTS concentration had the strongest influence on the encapsulation efficiency ($R_{\text{CTS}} > R_{\text{CaCl}_2} > R_{\text{CT}} > R_{\text{SA}}$), followed by the CaCl_2 concentration and complexation time, with the SA concentration having the least impact. Through comprehensive evaluation, the optimal preparation conditions for bioimprinted PLD microcapsules were established as follows: SA, CTS, and CaCl_2 concentrations of 1.5% (w/v), 0.6% (w/v), and 0.3 mol/L, respectively, with a CT of 1 min. Under these conditions, an immobilization efficiency of 78.56% and an activity recovery of 105.27% were achieved, representing the highest values observed. Compared with conventional immobilization, the enzyme in this study required greater rigidification strength to limit conformational flexibility while preserving the ligand-mediated hyperactivated form, owing to its use in a highly aqueous environment [13].

Mechanistically, CTS (wall material) and SA (core material) form a polyelectrolyte complex membrane via electrostatic interactions. Crucially, the CTS concentration most significantly enhanced capsule wall integrity and immobilization efficiency, followed by the CaCl_2 concentration. Ca^{2+} ions diffuse through the membrane to cross-link internal alginate, forming a hydrogel network; increasing the CaCl_2 concentration (0.1–0.3 M) progressively stabilizes this structure. CT moderately influences immobilization efficiency by controlling Ca^{2+} diffusion and hydrogel formation. The SA concentration has the weakest impact, primarily aiding bioimprinted PLD dispersion in the core.

The immobilized hyperactivated PLD developed in this study exhibited significantly enhanced enzymatic activity compared with its free counterpart. As summarized in Table 1, the highest encapsulation efficiency (78.56%) was accompanied by a substantial activity recovery of 105.27%. Although the maximum ratio of activity recovery to encapsulation efficiency (1.37) occurred at 58.48% encapsulation efficiency, the absolute encapsulation value was considered insufficient. Therefore, a condition providing a more balanced performance—78.56% encapsulation efficiency with 105.27% activity recovery—was selected for further investigation. Under this condition, the immobilized hyperactivated PLD achieved a maximum specific activity of 0.335 U/mg_{protein}, approximately 1.34 times higher than that of the free enzyme (0.25 U/mg_{protein}). This represents a notable improvement in specific activity compared with previously reported results. For example, Li et al. [13] reported that PLD immobilized via adsorption or covalent binding—whether bioimprinted or not—consistently exhibited specific activities lower than those of free PLD and markedly below the values obtained in this work. These findings indicate that microencapsulation effectively rigidifies the bioimprinting-induced enzyme conformation, thereby stabilizing the ligand-preferred structural state.

3.2. Characterization of Immobilized Bioimprinted PLD

3.2.1. SEM Analysis

Scanning electron microscopy (SEM) was used to examine the morphology and structure of the microcapsules. The SEM image of fully intact microcapsule particles is depicted in Figure 2A. The microcapsules exhibited a spherical morphology with surface collapse and structural heterogeneity. In the hydrogel-based microcapsule generation process, water molecules are retained within the microcapsules through molecular alignment and secondary interactions, enabling the formation of uniform spherical structures [28]. However, during the freeze-drying process, the loss of water molecules and weakening of the matrix structure destabilize this three-dimensional configuration. Consequently, the dried microcapsules deviated from their homogeneous spherical geometry, resulting in surface collapse and wrinkling, while developing extensive internal porosity [35,36]. Figure 2A illustrates the three-dimensional network structure of the microcapsules, formed through the complexation reaction between sodium alginate and calcium chloride, resulting in a calcium alginate hydrogel framework. Figure 2B,D depict the internal architecture of fractured microcapsules at varying magnifications, showing PLD adhered to the inner walls, thereby confirming the successful encapsulation of bioimprinted PLD within the chitosan-alginate microcapsules.

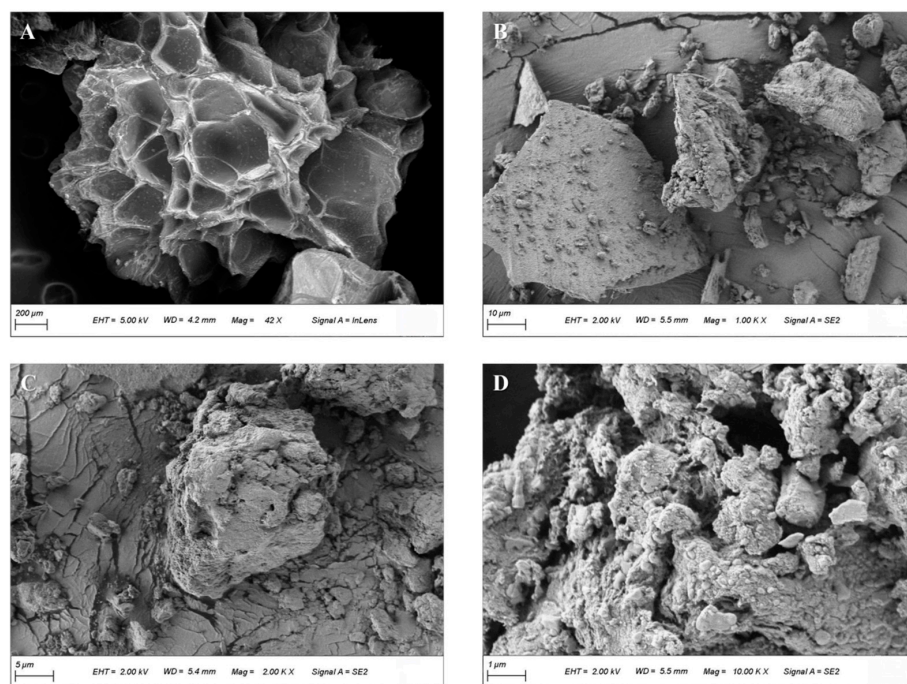


Figure 2. SEM images of the lyophilized microcapsules. (A) external structure of the microcapsules; (B–D) microstructure of the crushed microcapsules at different magnifications.

3.2.2. FTIR Spectra

The FTIR spectra of chitosan, sodium alginate, and free/immobilized PLD are presented in Figure 3. Infrared spectroscopy was employed to evaluate potential chemical interactions between biopolymers and biomolecules. All four samples exhibited a broad peak at about 3400 cm^{-1} , corresponding to O–H stretching vibrations arising from adsorbed water molecules and surface hydroxylation [37]. In the chitosan sample, peaks observed at 1075 cm^{-1} , 1383 cm^{-1} , and 1640 cm^{-1} were attributed to asymmetric and symmetric C–O–C bond axial deformation, C–N axial deformation, and N–H angular deformation in the amine group, respectively [38,39] (Figure 3a). In Figure 3b, the intense band at 1618 cm^{-1} and the intense band at 1414 cm^{-1} were linked with asymmetric and symmetrical C=O bond axial deformation vibrations. A peak associated with C–O bond axial deformation was detected at 1028 cm^{-1} [40,41]. As shown in Figure 3c, the peak at 1636 cm^{-1} might be related to C=O stretching of the amide I bond. The peak at 1403 cm^{-1} was related to the axial deformation of the C–N bond, whereas that at 1116 cm^{-1} may correspond to the asymmetric stretching vibration of the phosphate ester bond. For immobilized PLD (Figure 3d), the band intensity decreased at about 3400 cm^{-1} (compared with the O–H bond axial deformation), which corresponded to microcapsule component bands (free PLD, chitosan, and sodium alginate) and was related to interactions mostly among carboxylic groups. The band related to the axial deformation of the C–O bond (existing in each microcapsule component) (ranging from $1000\text{--}1150\text{ cm}^{-1}$) disappeared. This might be because secondary amides were formed after the carboxylic group was attached to the amine group in the microcapsule components. This hypothesis can be supported by a peak at 1549 cm^{-1} , which is related to secondary amides ($1500\text{--}1550\text{ cm}^{-1}$) [42]. Notably, the characteristic protein peaks of PLD at 1403 , 1116 , and 618 cm^{-1} were attenuated in the spectrum of the immobilized PLD (Figure 3d). This signal weakening likely stems from the low enzyme loading capacity inherent to the immobilization method. Therefore, successful encapsulation of bioimprinted PLD within chitosan–alginate microcapsules requires verification through complementary approaches: multiple physical characterization techniques combined with activity assays of the microcapsules.

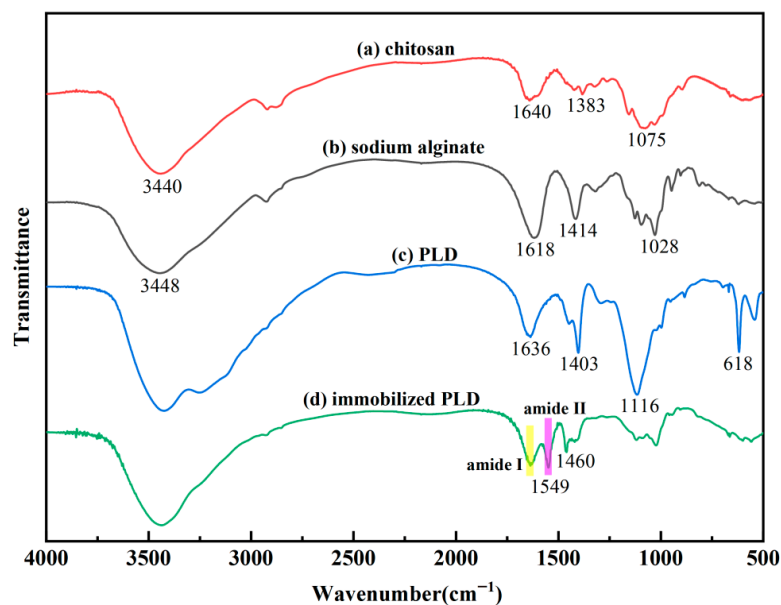


Figure 3. FTIR spectra of chitosan (a), sodium alginate (b), PLD (c), and immobilized PLD (d).

3.2.3. X-Ray Diffraction

X-ray diffraction was performed to identify amorphous and crystalline regions in the polymers (sodium alginate and chitosan) and in the microcapsules (Figure 4). For sodium alginate (Figure 4a), three peaks were observed at $2\theta = 13.2^\circ$, 22.1° , and 39.2° , consistent with the peaks at $2\theta = 13.7^\circ$, 23.0° , and 40.0° reported by Fontes et al. [40]. For chitosan (Figure 4b), a moderate peak at $2\theta = 9.1^\circ$ and an intense peak at $2\theta = 19.8^\circ$ were detected, followed by a region dominated by the amorphous form. These results are in agreement with typical diffraction patterns for semi-crystalline chitosan [38,43]. In the dried microcapsules (Figure 4c), two intense crystalline peaks appeared at $2\theta = 31.6^\circ$ and 45.4° , likely corresponding to sodium chloride. During microcapsule formation, Na^+ ions bound to the carboxylic groups of alginate are replaced by Ca^{2+} ions from the CaCl_2 gelation solution, allowing the displaced sodium ions to bind with chloride ions and form NaCl. To verify this, microcapsules were rinsed after microencapsulation, resulting in a marked decrease in crystallinity (Figure 4d), indicating the removal of a portion of sodium ions during this process.

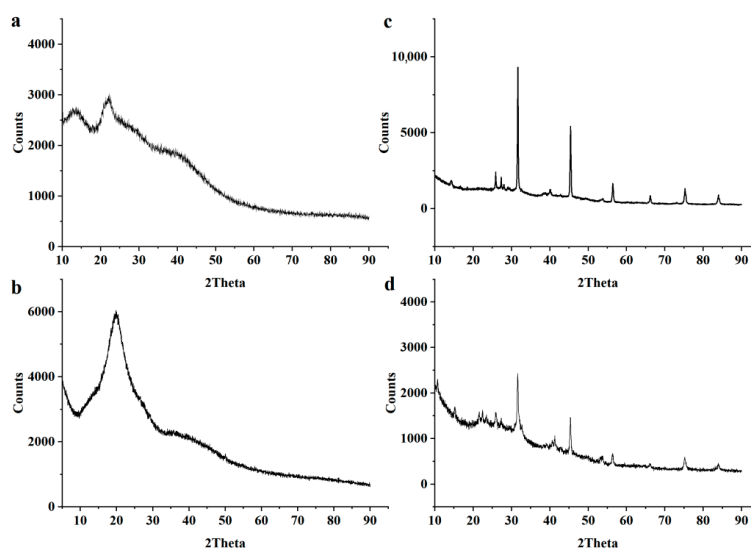


Figure 4. X-ray diffraction patterns of sodium alginate (a), chitosan (b), dried microcapsules/pre-washing state (c), and dried microcapsules/post-washing state (d).

3.3. Transphosphatidylation Reaction Optimization

3.3.1. Temperature

The reaction temperature is an important factor that affects the enzymatic activity, substance diffusion, and stability of the reaction system. Therefore, we comprehensively analyzed PS production by free PLD, immobilized PLD, and immobilized bioimprinted PLD at diverse temperatures. As shown in Figure 5a, at 30–60 °C, the production of the three PLD forms first increased but then decreased. Specifically, immobilized bioimprinted PLD had the highest PS production of 46.56% at 55 °C, immobilized PLD had the highest PS production of 37.62% at 50 °C, and free PLD had the highest PS production of 33.73% at 45 °C. When the temperature further increased to 60 °C, all three enzyme forms exhibited decreased PS production to varying degrees. Among all the tested variants, the immobilized bioimprinted PLD exhibited the highest PS yield.

When a sufficient temperature is applied, a structural rearrangement of the conformation of the active center is induced [44]. Following immobilization, the PLD molecular structure becomes effectively rigid, resulting in enhanced thermal resistance. Compared with non-bioimprinted immobilized PLD, the immobilized hyperactivated PLD demonstrated a higher optimum temperature. This finding indicates that, compared with conventional immobilization methods, the immobilized hyperactivated PLD exhibits greater rigidification capacity, more effectively restricts enzyme flexibility, and stabilizes the ligand-induced hyperactivated conformation. These attributes allow the enzyme to withstand elevated temperatures, shift its optimal temperature range to higher values, and sustain high PS yields over a broader temperature range.

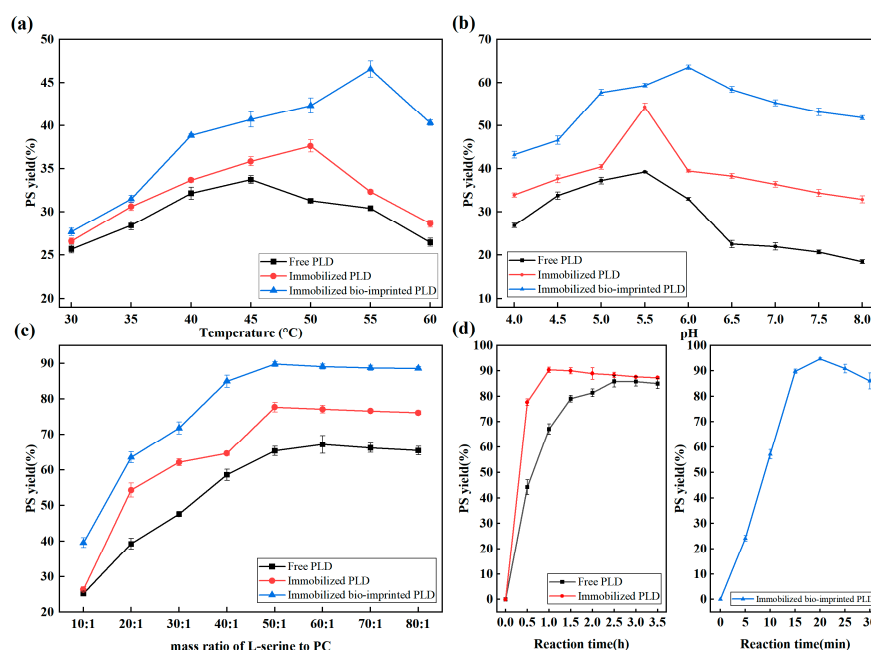


Figure 5. Optimization of PS synthesis catalyzed by three biocatalysts under various conditions: (a) impact of the reaction temperature; (b) impact of the pH; (c) impact of the L-serine-to-PC mass ratio; (d) impact of the reaction time. Biocatalysts: free PLD, non-imprinted immobilized PLD, and immobilized bioimprinted PLD.

3.3.2. pH

Transphosphatidylation was performed to determine the pH dependence of PLD with/without immobilization at a pH of 4.0–8.0. As shown in Figure 5b, the optimal pH values for the biocatalysts of free and immobilized PLD were identical, with PS production peaking at pH 5.5, reaching 39.25% and 54.31%, respectively. Increased PS production

was detected with the use of immobilized PLD. When the pH further increased to 6.5, the PS yield of free PLD decreased sharply, followed by a gradual decrease. In contrast, the immobilized bioimprinted PLD had the highest PS production of 63.52% at pH 6.0, an increase of 1.62-fold relative to that of free PLD. Additionally, the immobilized bioimprinted PLD had different PS production rates as the pH changed, with a production rate of 51.8% at pH 8.0, which was nearly comparable to the best PS production achieved by the non-bioimprinted immobilized PLD counterparts. The enhanced performance can be attributed to the greater rigidification strength achieved in the immobilized hyperactivated PLD preparation [13]. This robust structural stabilization effectively limits conformational flexibility while conferring reduced pH sensitivity and markedly improved pH tolerance compared with conventional preparations.

3.3.3. Substrate Mass Ratio

The substrate mass ratio governs the shift of the reaction equilibrium toward the target product in reversible transphosphatidylation [45]. Consequently, maintaining a sufficient substrate concentration was identified as a critical prerequisite for enhancing the reaction efficiency. Figure 5c presents the PS production of free PLD, immobilized PLD, and immobilized bioimprinted PLD under diverse L-serine-to-PC mass ratios. The three systems showed similar trends; however, among the diverse systems, the PS production of immobilized PLD catalysis always increased relative to that of free PLD. Moreover, PS production rapidly increased with increasing L-serine production and reached a plateau when the L-serine-to-PC mass ratio was 50:1. At the critical ratio, maximum PS yields of 77.65% and 89.74% were attained from the non-bioimprinted and bioimprinted immobilized PLD variants, respectively. Unlike the immobilized variants, free PLD gradually increased the PS yield (50:1–60:1 substrate ratio) until reaching maximum conversion (67.14%), after which all three enzyme forms entered stationary phases with sustained yields. These results confirm that optimal L-serine–PC ratios enhance phosphatidylserine synthesis, confirming established substrate-dependent mechanisms [34].

In the transphosphatidylation reaction, PLD, whether in free or immobilized form, operates via a two-step ping-pong mechanism. Initially, a phosphatidyl–enzyme intermediate is generated, accompanied by the release of choline. This intermediate subsequently undergoes nucleophilic attack, wherein serine competes with water molecules to react with the intermediate, resulting in the formation of either the desired product, PS, or the by-product, phosphatidic acid (PA). Consequently, the concentration of serine critically determines both the efficiency and directionality of the transphosphatidylation process. A high serine–PC ratio enhances the reaction performance by kinetically favoring serine over water as the nucleophile, thereby directing the process toward PS synthesis while effectively suppressing the hydrolytic side reaction. This effect is particularly important for immobilized PLD, where elevated serine concentrations alleviate mass transfer limitations, ensuring sufficient access of serine—rather than water—to the enzyme active site and thus promoting efficient PS production.

3.3.4. Reaction Time

It is necessary to analyze the reaction time during biocatalytic processes to identify the best time needed to achieve the maximum production to increase economic viability.

Figure 5d shows the time course of the alterations in PS production during transphosphatidylation with the 3 biocatalysts. During the initial stages, PS production increased quickly in the three catalyst systems. The PS production of immobilized PLD sharply increased, peaking at 90.34% at 1 h, whereas that of free PLD peaked at 67.14%. Following this, the PS production of free PLD decreased and reached a maximum (85.82%) at 2.5 h, and the yield of

immobilized PLD slightly decreased. In stark contrast, the yield of immobilized bioimprinted PLD increased sharply within a short period, peaking at 94.68% as early as 20 min, followed by a declining trend. On the basis of these results, the selectivity of the bioimprinted PLD was markedly improved. The optimized immobilized bioimprinted system achieved robust performance, demonstrating an exceptionally high transphosphatidylation rate.

3.4. Stability and Reusability

3.4.1. Thermal Stability

Thermal stability represents an important performance metric enabling the scale transition of carrier-fixed biocatalysts. We assessed the thermal stability of immobilized PLD via a 1 h incubation across a 25–65 °C gradient, benchmarking against free enzyme counterparts. As illustrated in Figure 6, immobilized PLD and its bioimprinted counterpart exhibited parallel thermal stability trends until 50 °C was reached. Divergence initiated within the 50–65 °C range, where the bioimprinted variant demonstrated significantly greater residual activity than conventional immobilized PLD under thermal denaturation conditions. Thermal treatment at 65 °C for 1 h yielded residual activities of 71.65% for standard immobilized PLD and 78.75% for the bioimprinted derivative. Unlike immobilized enzymes, free PLD underwent continuous thermal deactivation, with only 59.86% residual activity remaining at 50 °C for 1 h. Subsequent 65 °C exposure caused near-complete inactivation (9.79% residual activity). The experimental results clearly indicate that the immobilized PLD exhibits markedly enhanced thermal stability compared with its free counterpart. This pronounced thermal stability is primarily attributed to the synergistic effects of substrate-induced stabilization and carrier-enabled rigidification. The high serine/PC ratio in the reaction system ensures effective occupation and binding of serine molecules to the enzyme active site [18], thereby stabilizing the open (active) conformation, reducing conformational flexibility, and improving resistance to thermal denaturation. In parallel, the interaction between the enzyme and the carrier provides additional structural rigidity, physically restricting unfolding at elevated temperatures [23,46]. Consequently, the combined effects of “conformational selection and stabilization” through substrate binding and “spatial confinement” arising from immobilization play a pivotal role in enhancing thermal stability.

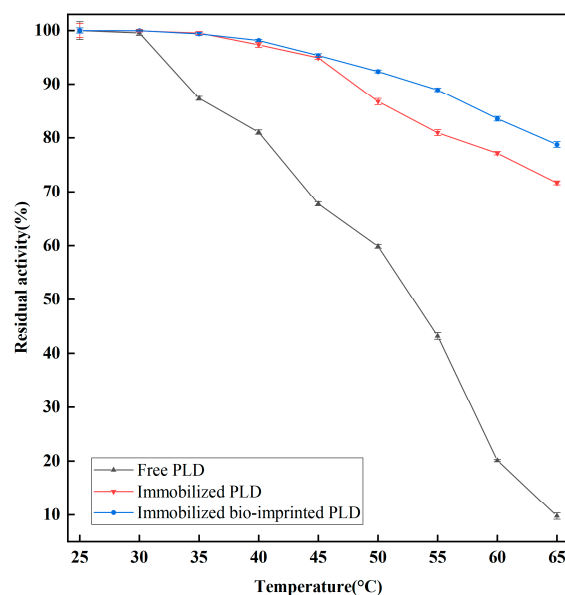


Figure 6. Effect of temperature on the enzyme stability of three biocatalysts: free PLD, non-imprinted immobilized PLD, and immobilized bioimprinted PLD.

3.4.2. Storage Stability

We evaluated the storage stability by observing the residual activities of free PLD, immobilized PLD, and its bioimprinted variant during storage at 4 °C, with the initial activity normalized to 100%. As shown in Figure 7, compared with the immobilized PLD, the free PLD significantly accelerated the decay of the activity. After 40 days, free PLD retained merely 52.55% of the initial activity, whereas immobilized and bioimprinted PLD maintained 82.57% and 89.6%, respectively. Extending storage to 80 days resulted in 41.99% (immobilized) and 47.64% (bioimprinted) residual activity, which contrasted sharply with the 15.77% retention of free PLD. These results demonstrate the markedly superior long-term stability of the immobilized systems, confirming their industrial applicability.

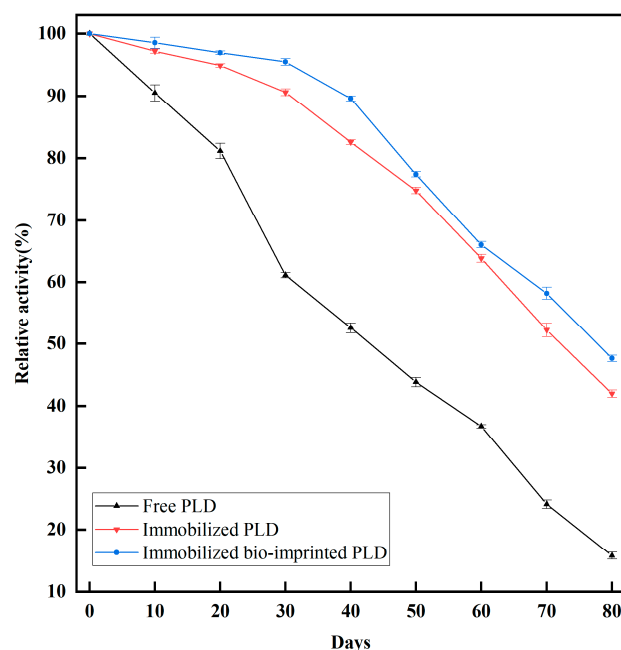


Figure 7. Storage stabilities of three biocatalysts: free PLD, non-imprinted immobilized PLD, and immobilized bioimprinted PLD.

3.4.3. Reusability

Industrial biocatalysis leverages immobilized enzymes for recyclability and facile separation—key attributes that substantially reduce operational costs. This study specifically assessed the operational stability of recycled immobilized PLD and its bioimprinted counterpart, addressing the critical need for sustainable enzyme reuse in cost-sensitive production systems. To evaluate reusability, we performed sequential synthesis batches of PS with identical enzyme preparations. The PS yield was monitored following every reaction cycle. As shown in Figure 8, after four reuse cycles, the PS yield of the immobilized bioimprinted PLD remained as high as 88.92%, whereas that of the non-bioimprinted immobilized PLD was 73.53%. While the immobilization matrix shields the enzyme during catalysis, repeated cycling progressively modifies the microenvironment of PLD, causing partial enzyme denaturation and activity loss. After eight consecutive reaction cycles, the immobilized bioimprinted PLD still achieved a notable PS yield of 77.53%, while the non-bioimprinted immobilized PLD retained only 22.72% of its initial yield.

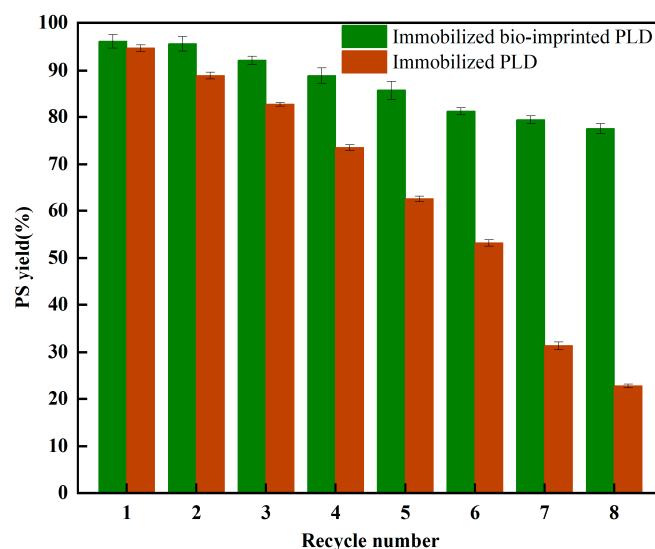


Figure 8. Operational stability of immobilized bioimprinted PLD.

4. Discussion

The present study proposed a method for producing a potent biocatalyst by further engineering PLD through integrated bioimprinting and immobilization. This dual modification induced a hyperactivated enzyme conformation, which was structurally rigidified via chitosan-alginate microencapsulation. The immobilized hyperactivated PLD produced in this study exhibited higher activity than both free PLD and immobilized PLD without bioimprinting. Moreover, control experiments revealed that sequential immobilization followed by L-serine addition failed to activate PLD via bioimprinting. This outcome highlights the necessity of structural flexibility for inducing hysteretic conformational memory. Pre-immobilization imposes excessive rigidity, preventing the ligand-induced structural reorganization essential for imprinting efficacy. Successful bioimprinting requires ligand binding during enzyme structural adaptation, thereby “freezing” PLD in a hyperactivated state complexed with L-serine. This conformation persists after ligand removal due to intramolecular rigidification, resulting in sustained enhancement of transphosphatidylase activity and selectivity. Ligand binding customizes the enzyme’s catalytic properties by inducing specific conformational changes [13]. Notably, the immobilized hyperactivated PLD maintained superior PS production in a biphasic system, confirming the retention of the ligand-induced conformation.

The transphosphatidylase selectivity and activity could be markedly enhanced after the use of the immobilized hyperactivated PLD. Moreover, the production efficiency of immobilized PLD (PS yield per unit time) depends critically on the enzyme source, the chosen immobilization method, and the specific reaction conditions. This work revealed a 90.34% PS yield for immobilized PLD at 60 min and a 94.68% PS yield for immobilized bioimprinted PLD at 20 min, reflecting 2.14-fold and 7.27-fold greater efficiency than free PLD, respectively. In addition, the experimental results demonstrated a significant increase in both the thermal stability and storage stability of PLD following immobilization. The immobilized PLD maintained its structural conformation at elevated temperatures and during extended storage, with its stability attributed to the combined effects of substrate-induced stabilization and carrier-mediated rigidification [46]. Encapsulation of the PLD molecules provided substantial structural reinforcement, effectively preventing conformational alterations. As a result, greater energy input was required to disrupt this rigid structure before the immobilized PLD could undergo dissolution and subsequent denaturation. This improved stability directly contributed to enhanced catalytic efficiency in PS synthesis, as the

enzymatic reactions proceeded at accelerated rates under higher temperatures, ultimately yielding superior product outputs [47].

pH has an essential effect on the transphosphatidylolation reaction, significantly influencing both the enzymatic activity and aggregation state. As demonstrated in Figure 5b, the optimal catalytic performance for free PLD, immobilized PLD, and bioimprinted PLD occurred under mildly acidic conditions (pH 5.5, 5.5, and 6.0, respectively). This pH-dependent behavior arises from PLD's dual catalytic capacity: in addition to catalyzing transphosphatidylolation, the enzyme also mediates hydrolytic side reactions. Under alkaline conditions (pH > 7), hydrolysis is promoted due to the increased availability of hydroxyl groups, whereas an acidic microenvironment preferentially suppresses hydrolysis and enhances transphosphatidylolation efficiency [16]. As a result, PLD exhibits superior activity and reaction specificity within this acidic pH range.

Biocatalyst selectivity accounts for a key performance indicator for use. In fact, PLD is capable of catalyzing transphosphatidylolation and hydrolyzing the substrate PC into PA [48]. PA, as a byproduct, was blended into the desired product within the organic solvent, which could hardly be removed because they have similar physicochemical characteristics. For our bioimprint-prepared PLD, its selectivity markedly increased in this work. A fast reaction rate was attained, while the transphosphatidylolation reaction was accomplished in 20 min. PS production was 94.68%, indicating that PC hydrolysis was minimized in this system. The above phenomenon can be explained by the positive synergy between the composite carrier and bioimprint. Typically, PLD immobilized with L-serine maintains the ligand-mediated conformation; therefore, PLD can beneficially interact with L-serine. This positively affected PLD selectivity, thereby facilitating the transphosphatidylolation reaction while suppressing PC hydrolysis.

As summarized in Table 2, PLD has been immobilized onto various carriers using diverse methodologies reported in the literature. Collectively, these strategies enhance enzyme stability, enable reuse, and improve PS production. Although the PLD in this study did not achieve the highest immobilization rate among the cited works, its performance remains competitive. Our approach uniquely combined bioimprinting with immobilization, inducing the formation of a hyperactivated PLD structure that was subsequently immobilized via microencapsulation. This method, which is relatively simple compared with cross-linking and covalent binding, effectively preserved enzyme activity. As a result, the immobilized hyperactivated PLD exhibited high catalytic potency, achieving a PS productivity of 5.68 g/L/h and 94.68% PS production within only 20 min of bioconversion. These results highlight the strong competitiveness of this system in key performance metrics. Overall, this study presents a high-performance immobilized catalyst with potential for large-scale industrial PS production.

Table 2. Performance characteristics of PLD immobilized with different supports.

Support/Method	Immobilization Efficiency (%)	PS Yield (%)	Time	Ref.
Chitosan–sodium alginate/microencapsulation	78.56	94.68	20 min	this study
Amino hollow mesoporous silica cube/cross-linking	87.15	90.40	10 h	[49]
Fe ₃ O ₄ @SiO ₂ –GO/adsorption	84.4	95.1	90 min	[50]
non-porous SiO ₂ /cross-linking	/	97	6 h	[16]
Ordered mesoporous silica cube/adsorption	76.27	91.2	2 h	[51]
Epoxy resin hierarchical porous polymer/adsorption	/	95.5	40 min	[52]
Janus-poly-polystyrene/adsorption	/	93	2 h	[53]
Cellulose nanofibrils/cellulose-binding domain	56.3	95.4	2 h	[54]

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