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Characterization of Lipase-Immobilized Polymethacrylate-based Monolith at Different Porogen Contents, Diameter, and Number of Holes for the Immobilization Process

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ABSTRACT

Polymethacrylate-based monoliths have attracted a lot of attention due to their exceptional properties, such as reusability, solvent tolerance, and higher stability. This study investigates the physical characteristics and immobilization yield of lipase on polymethacrylate-based monoliths fabricated with varying porogen contents, hole diameters, and hole numbers. By systemically altering these structural parameters, this study aimed to elucidate their influence on monolith morphology, surface area, and pore architecture, which are critical for enzyme loading and catalytic performance. Immobilization yield was quantified via protein loading. Results revealed that the highest immobilization yield was achieved at 60% porogen concentration when varying the diameter and number of holes, with a yield of 58.67 ± 0.69%.At a diameter of 0.4 mm (18 holes) under varied porogen concentrations, immobilization yields of 57.50 ± 0.95% were obtained. In comparison, the simulation data predicted that the highest immobilization yield at a diameter of 0.3 mm (24 holes) with varied porogen concentration, yielding 55.90 ± 1.12% and immobilization yield of 58.90 ± 0.31% at 60% porogen content with different diameters and numbers of holes. These findings are crucial as they provide valuable insights into the design of tailored monolithic supports for biocatalytic applications, particularly in sustainable biodiesel production.

Keywords: Diameter; immobilization; lipase; polymethacrylate-based monoliths; porogen concentration;

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INTRODUCTION

Enzyme immobilization has been widely applied in biocatalysis due to its practicality, enhanced stability, and reusability, making it a valuable strategy for industrial processes (Parandi et al., 2022). Compared to free enzymes or other catalysts, immobilised enzymes offer improved selectivity, thermal and chemical stability, ease of recovery, and consistent activity under diverse reaction conditions (Rajnish et al., 2021; Xie & Huang, 2020). The choice of support material plays a critical role in ensuring enzyme stability and reusability, thereby determining the overall efficiency of the immobilization process.

Traditionally, supports such as silica, polymer beads, and nanomaterials have been employed, often fabricated as millimeter-sized particles with catalytic agents distributed on their surfaces or within internal pores (Trubac et al., 2001; Zhao et al., 2021). These micromaterials, polymers, and nanomaterials have been gaining a lot of attention for their potential in immobilizing enzymes for industrial applications (Rajnish et al., 2021). While effective, these particulate supports are limited by mass transfer constraints, flow dynamics, and mechanical stability, which can hinder large-scale applications pressure (Santos et al., 2020; Trubac et al., 2001). Optimal reactor performance requires supports with sufficient surface area, tailored pore structures, and mechanical robustness to sustain catalytic activity under industrial conditions (Afandizadeh & Foumeny, 2001). By understanding the underlying factors, engineers can design an optimal system with the prescribed conditions.

To overcome these limitations, monolithic supports have emerged as promising alternatives. Porous carbon monoliths with tunable porosity are more promising for various applications, such as adsorption and energy storage. A large quantity of enzymes is much easier to immobilize in a porous structure compared to a flat surface support (Gustafsson, 2012). The pore dimension of a monolith can be tailored by varying the polymerization or synthesis conditions accordingly. However, the synthesis of carbons with controlled porosity and monolithic shape is often expensive and time-consuming. Enzymes can be immobilized on a wide variety of synthetic and natural supports (carriers) through covalent or ionic bonding, adsorption, carrier-free cross-linked enzymes, and affinity binding (Sheldon et al., 2021). However, different applications need completely different technical requirements in terms of the carrier size, materials, configuration, and method of immobilization for the production process.

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Polymethacrylate-based monoliths are attractive as a lipase carrier as they can enhance the lipase enzymes' catalytic activity due to their unique properties, which can be modified so that they can have high enzyme loading capacity and high surface-to-volume ratio, inertness against microbial and chemical degradation, biocompatibility with any enzymes, and also easy to separate at the end of the process (Haghighi et al., 2024). The monoliths are also much easier to recover and manipulate in an industrial process (Zhao et al., 2021). Their adaptability makes them particularly suitable for lipase immobilization, where enhanced catalytic activity and reusability are critical for cost-effective biodiesel production. The use of novel carriers with highly porous properties, with much easier separation in the final production process, will be very beneficial to the industry.

In this study, we prepared and modified polymethacrylate-based monoliths to serve as lipase carriers in a continuous flow system using an NGC Chromatography setup. We investigated the influence of porogen content, holes diameter, and number of holes on lipase immobilization efficiency and catalytic performance. These modifications aim to optimize monolith design for industrial applications requiring stable, reusable, and high-performance biocatalysts in continuous processes.

MATERIALS & METHODS

Chemicals and materials

Azobisisobutyronitrile (AIBN, Mw of 164.21, 98%), Cyclohexanol 99% (Mw of 100.16), ethylene glycol dimethacrylate (EDMA, 98%, Mw of 198.22), glycidyl methacrylate (GMA, 97%, Mw of 142.15), methanol AR grade ≥ 99.5%, Bradford protein assay and *Candida rugosa* lipase enzymes (L1754, BCCJ3778, type VII, hydrolytic activity 1070 U/mg, ≥700 units/mg solid) were purchased from Sigma-Aldrich, USA through Next Gene Scientific Sdn Bhd (Malaysia). Reagents such as sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate were supplied by ACCVA Solutions Sdn Bhd. All other reagents and solvents were of analytical grade and used as received, without any additional purification step.

Polymethacrylate-based Monolith Preparation

The polymethacrylate-based monolith was prepared based on a study by (Simbas & Ongkudon, 2019) methods with slight modifications. The polymethacrylate-based monolith polymerization mixture was prepared, which consisted of the initiator (AIBN), monomer (EDMA and GMA), and

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porogen (cyclohexanol). The mixture was sonicated for 15 minutes and placed in a water bath for 1 hour at 55 °C. Then the monoliths were washed with both methanol and deionized water overnight.

Physical Modification

A mechanical drilling into the surface of the polymethacrylate-based monolith was done using a High-Speed Steel (HSS) Twist Drill (Model DM-240, GUNAISI, China). The diameters of the drill used were 0.3 mm, 0.4 mm, 0.5 mm, and 0.6 mm. The number of holes drilled into the monoliths was related to the diameter of the drill. In this study, we were focused on finding the ideal average total surface area of holes for each of the drill bits based on the diameter chosen. This was determined by the number of holes that can be drilled into the monolith without any cracks occurring on the monolith's surface. The total surface area chosen specified the number of holes that were drilled into the monolith. Each diameter will have a different number of holes, but with a constant average total surface area of holes for each drill bit. The calculation was adapted from a general equation and modelling approaches for immobilization on porous materials (Kar, 2025; Maghraby et al., 2023). The calculation was expressed as follows:

$$\text{Average total surface area of holes} = 2\pi r \times t \times h$$

Where r is the radius of the holes drilled into the monolith (if the diameter of the drill is 0.3 mm, the radius is 0.15 mm), t is the thickness of the monolith (10 mm), and h is the number of holes. All analyses were performed in triplicate.

Lipase Immobilization Process

According to the process described by (Coutinho, 2020) with some modifications, Candida rugosa lipase enzymes were immobilized to the polymethacrylate-based monolith at 30 °C, 150 rpm for 72 hours (Aghabeigi et al., 2023). The immobilization methods selected for this study were adsorption to the hydrophobic surface. The monoliths were immersed in 10 mL of 0.1 M sodium phosphate buffer solution at pH 7 with a lipase concentration of 10 mg/mL. After 72 hours, the monoliths were washed with the buffer three times to remove loosely bound enzymes. The residual enzyme solution from the immobilization process was removed and stored for analysis. The filtrates or the washing solutions were collected for protein analysis, which was determined by the Bradford protein assay using the enzyme lipase as a standard reference to build the calibration curve based on the method by

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(Cheng et al., 2019; Luangon et al., 2012; Silva et al., 2023). The protein concentration in the supernatant is measured using the Bradford method. Lastly, the immobilization yield (%) for each sample was calculated based on study by (Zhang et al., 2023) and (Boudrant et al., 2020)using the following formula:

$$\text{Immobilization yield (\%)} = [\text{Immobilized enzyme} / \text{Initial amount of enzyme}] \times 100\%$$

Experimental Design

This study employed a two-stage experimental approach. First, a One-Factor-at-a-Time (OFAT) design was used to evaluate the individual effects of porogen concentration and hole diameter on lipase immobilization yield. This preliminary screening established practical ranges and identified significant factors influencing immobilization efficiency (Parandi et al., 2023). Subsequently, Response Surface Methodology (RSM) was applied to simulate and optimize the combined effects of porogen concentration, hole diameter, and number of holes. Historical Data Design of RSM was used as a statistical design to estimate and study the correlation between the porogen concentration, diameter, and amount of lipase immobilized during the immobilization process (Zulqarnain et al., 2023). The independent variables were the porogen concentration and diameter of the polymethacrylate monolith, while the immobilization efficiency was selected as the response parameter. Each experiment was performed in triplicate to reduce the chance of error (Sajjad et al., 2022). Therefore, the OFAT results served as the foundation for defining variable ranges in the RSM model.

The relationship between the independent variables and immobilization yield was described using a second-order polynomial regression equation. The regression model was evaluated using coefficient of determination (R^2) and adjusted R^2 to assess model fit. Analysis of variance (ANOVA) was also used to confirm the overall significance of the regression. All statistical analyses were performed using Design-Expert version 7.0 software (Dharmegowda et al., 2022; Panakkal et al., 2021). The model parameters and statistical outputs are reported in the Results section.

RESULTS & DISCUSSION

Polymethacrylate-based monolith synthesis

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The material interface is the key factor that determines the rate of a reaction or a process (Yeh et al., 2016). Optimizing the synthesis and fabrication of the polymethacrylate monolith will be very beneficial in increasing the productivity and maximizing the production results of the biodiesel yield in future research. The monoliths were fabricated from 0% to 60% porogen concentration to evaluate the pore morphology and mechanical stability. This preliminary screening facilitated the selection of monolith types most suitable for lipase immobilization and column drilling, ensuring reproducibility and structural integrity in subsequent experiments.

Figure 1 shown that the polymethacrylate monolith prepared has a different color and disposition according to the porogen content. After thoroughly washing with methanol and deionized water, the wall of the monolith surface and color can be differentiated. Fabricated monolith with 0% and 20% porogen concentration has a glass, opaque surface, while the monoliths with higher porogen content at 40% and 60% were white in color and more solid. However, polymethacrylate-based monoliths at higher porogen concentration were easier to break apart. This was seen during the washing steps as the wall surface of the monolith crumbled and cracked. The monoliths are washed with methanol to remove the unreacted inert porogen and monomeric reagents, and further washing with deionized water causes the samples to become firmer and solid when left to dry (Kamin et al., 2020). Methanol also has the lowest capability to dissolve the monomers, resulting in the formation of large pores and a highly porous surface for the polymethacrylate-based monolith (Hermawan et al., 2021).

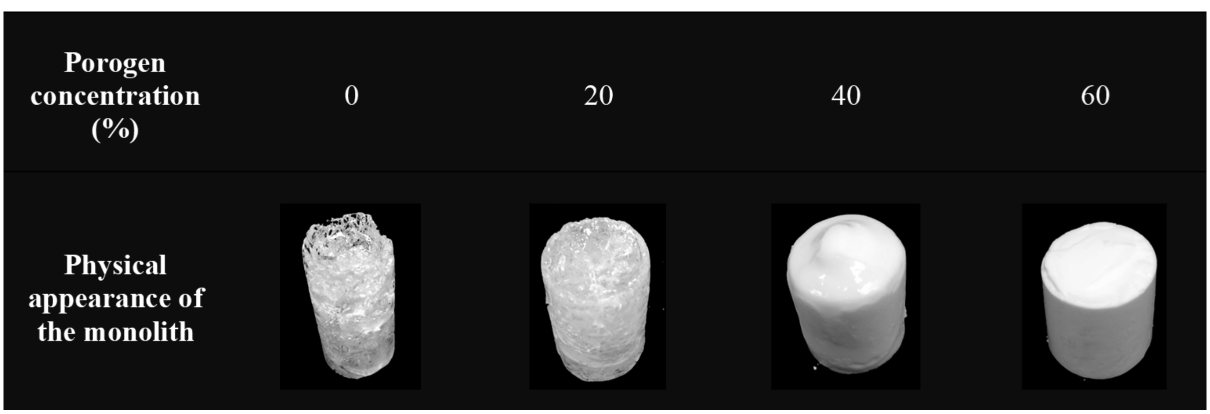

Figure 1 Polymethacrylate-based monoliths prepared at different porogen concentrations.

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Physical modification of the polymethacrylate-based monoliths through mechanical drilling

The polymethacrylate-based monoliths undergo physical modification through drilling using HSS Twist drills at several diameters. The number of holes drilled into the monolith was selected and determined by drilling each of the monoliths according to the previous Table 1. From the table, the greatest number of holes that can be drilled into the monoliths without causing a crack on the surface of the monoliths (Figure 2) was using 0.3 mm with 24 holes. Therefore, the average total surface area of holes for each drill bit chosen was 226.22 mm². Figure 2 shows some of the images of the cracked polymethacrylate-based monoliths during the drilling process. According to a study by Rahman et al., too much porogen content during polymethacrylate-based monolith preparation can cause brittle polymers that are prone to cracking and have low impact resistance (Rahman et al., 2018). As shown in Figure 2, drilling at 60% porogen concentration with a 0.3 mm bit and 28 holes resulted in visible cracks in the monolith structure. In contrast, Figure 3 illustrates monoliths prepared under the same conditions with 23 holes, where no cracks were observed. This comparison highlights the importance of porogen concentration and drilling parameters in maintaining monolith integrity for subsequent lipase immobilization.

Table 1 Different diameters and numbers of holes of the polymethacrylate-based monoliths

Diameter (mm)	**Number of holes**					
0.3	20	22	24	26	28	30
0.4	15	17	18	20	21	23
0.5	12	13	14	16	17	18
0.6	10	11	12	13	14	15
Total surface area of monolith (mm²)	188.52	207.37	226.22	245.08	263.98	282.78

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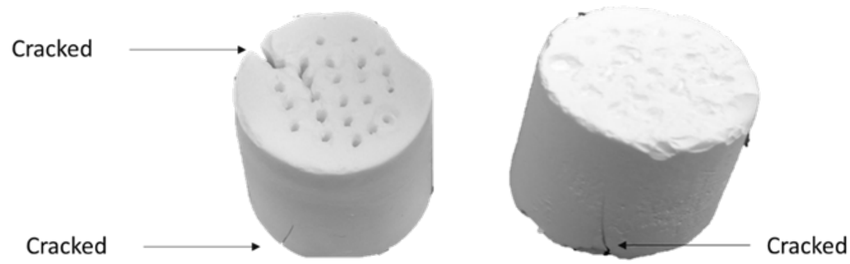

Figure 2 The cracked polymethacrylate monoliths fabricated at 60% porogen concentration during the drilling process using a 0.3 mm diameter drill bit with 28 holes.

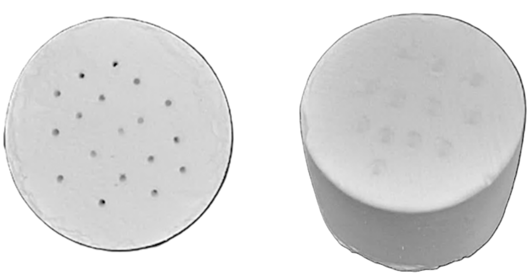

Figure 3 The polymethacrylate monoliths fabricated at 60% porogen concentration, drilled using 0.3 mm diameter drill bits without cracks, with 23 holes.

Experimental and simulation investigations of lipase immobilization process

The polymethacrylate-based monoliths were immobilized with the Candida rugosa lipase enzymes to study their immobilization yield after the monoliths underwent physical modification through drilling. The lipase immobilization process was performed on polymethacrylate at 30%, 40%, 50% and 60% porogen concentrations with diameters of 0.3 mm, 0.4 mm, 0.5 mm, and 0.6 mm. The 30%, 40%, 50% and 60% porogen content were selected for the immobilization process based on their physical surface, which is more suitable for the immobilization process. This is because at higher porogen content (>60%), monoliths produced were brittle and often collapsed into powder

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form during fabrication screening. The immobilization process can cause structural changes in the support material; therefore, choosing the right support was crucial (Cornejo, 2021).

The immobilization yield at different porogen concentrations and diameters, based on experimental results and predicted simulation values from RSM analysis for the immobilization process, is shown in Figure 4. The highest immobilization yield was obtained at 60% porogen contents under varied diameter and number of holes, with a yield of 58.67 ± 0.69%. At a diameter of 0.4 mm (18 holes) when varying the porogen concentration, immobilization yields of 57.50 ± 0.95% were achieved. In comparison, the simulation predicted the highest immobilization yield at a drill diameter of 0.3 mm with 24 holes, resulting in 55.90 ± 1.12%. At 60% porogen concentration, the model further indicated an immobilization yield of 58.90 ± 0.31% across different diameters and hole numbers.

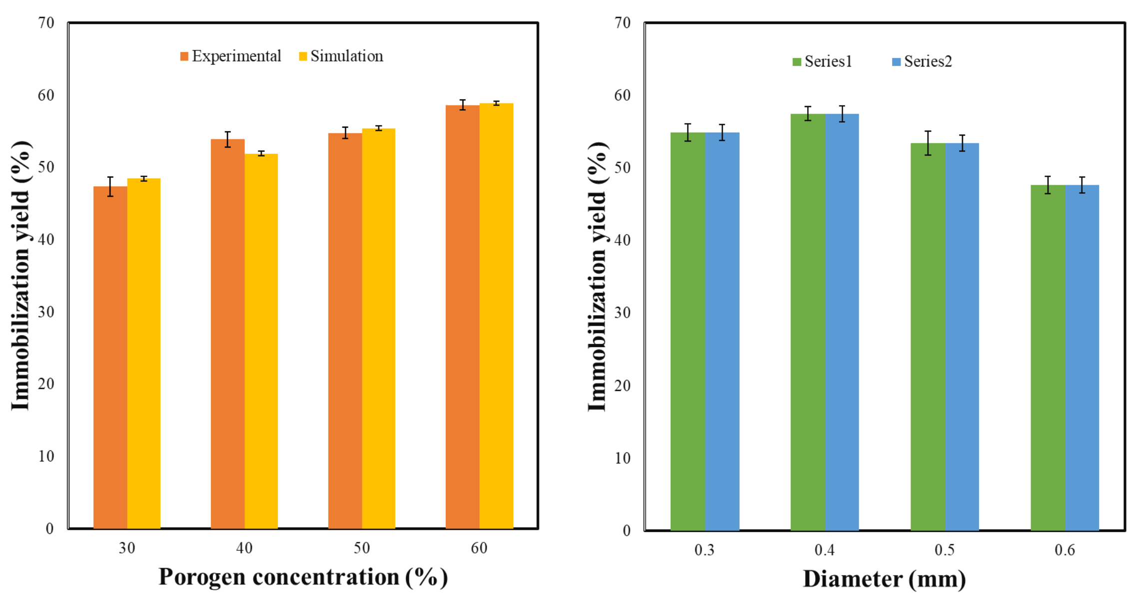

Figure 4 The immobilization yield at different (A) porogen concentration and (B) Diameter based on the experimental results and predicted simulation value from RSM analysis for the immobilization process.

In this study, Design Expert and Response Surface Methodology (RSM) data analysis were adopted to analyze the interaction between parameters and investigate the immobilization efficiency of lipase enzymes attached to the polymethacrylate-based monolith (Table 2 and Figure 5). From the

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analysis of variance (ANOVA) analysis in Table 2, the Model F-value of 4.76 implies that the model is significant, in which there is only a 1.48% chance that the a "Model F-Value" this large could occur due to noise. Linear model was suggested as the model because the p-value was statistically significant with p-value of <0.001. RSM is used to analyze and evaluate the input variables based on the confidence level and the coefficient of determination (Dharmegowda et al., 2022). In this model, significance of each parameter was also determined using the p-value. From the table, only parameter A, the porogen concentration is significant. Different porogen concentrations can changed the overall polymerization kinetics resulted in distinct pore structures (Chan et al., 2017). Table 2 shows that the predicted R^2 of 0.1880 is in reasonable agreement with the adjusted R^2 of 0.3723. The interaction of input and output parameters for a process is the most critical parameters which affect the response of that particular process (Zulqarnain et al., 2023). Hence, experimental responses and the prediction value are essential for an accurate optimum conditions' prediction of the immobilization process.

Table 2 Analysis of Variance (ANOVA)

Source	**Sum of Squares**	**Df**	**Mean square**	**F value**	**p-value (Prob> F)**
Model	340.42	3	113.47	4.76	0.0148
A	301.50	1	301.50	12.64	0.0026
B	0.46	1	0.46	0.019	0.8917
C	0.79	1	0.79	0.033	0.8583
Residual	381.73	16	23.86		
Cor Total	722.15	19			

Standard Deviation	4.88	R-Squared	0.4714
Mean	53.69	Adj R-Squared	0.3723
C. V. %	9.10	Pred R-Squared	0.1880
PRESS	586.40	Adeq precision	6.587

Regression Equation (Model: Linear)

Immobilization yield = 53.91 + 5.21 (A, porogen concentration) + 0.86 (B, holes diameter) + 1.12 (C, number of holes)

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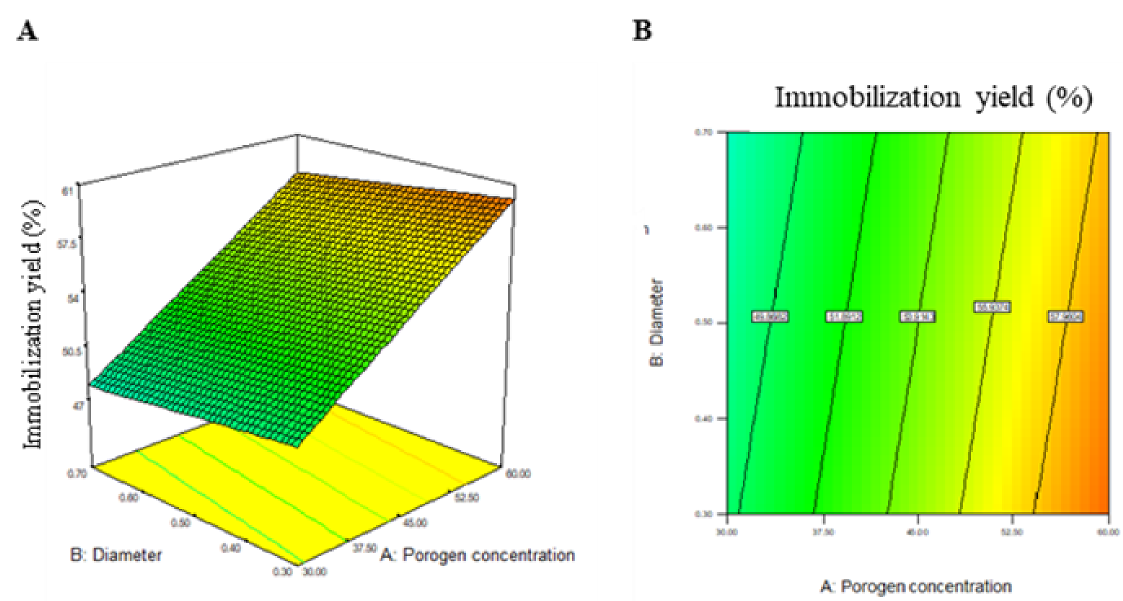
Figure 5 (A and B) shows that the highest immobilization yield was achieved at 0.30 mm diameter and 60% porogen concentration, with 57.96%. Meanwhile, for the interaction of the number of holes and diameter, predicted that the highest immobilization yield of 60.45% at a higher number of holes with a smaller diameter of 0.3 mm (Figure 5, C and D). Lastly, the interaction of the number of holes and porogen concentration in Figure 5 (E and F) shows the highest immobilization yield at 24 holes and 60% porogen concentration, with 58.14%. These findings highlight that 60% porogen concentration with diameters of 0.3 mm and a number of holes of 24 constantly yields the highest immobilization yield for the lipase immobilization process.

The higher the porogen concentration, the larger the pore size and porosity of the polymethacrylate-based monolith (Hermawan et al., 2021). Thus, this allows unrestricted interaction of a lipase enzyme with the surface of the monolithic material, which leads to a higher immobilization yield (Volokitina et al., 2017). The pores must have a large enough size to allow the binding of the lipase enzymes inside and outside the monolithic material. The polymethacrylate-based monolith with higher porogen content forms a more porous matrix with increased surface area and interconnected pore networks, which facilitates the enzyme binding and diffusion (Bié et al., 2022; Serial et al., 2026). A greater number of micropores increases the total available surface area. Therefore, enzymes can spread more evenly, which reduces crowding and steric hindrance during the immobilization process.



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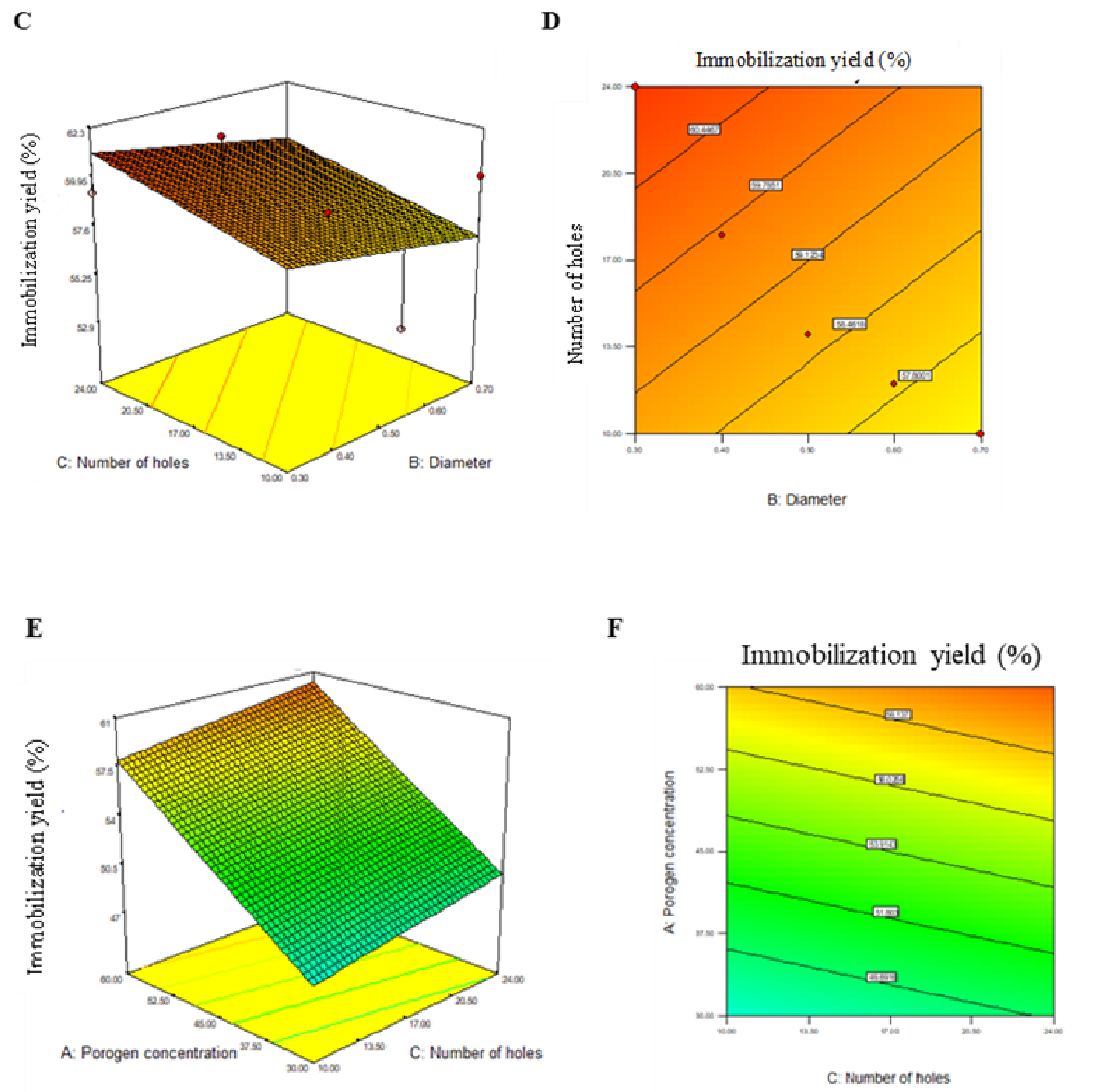

Figure 5 The 3-D surface and contour plot of the immobilization yield, (A-B) interaction between diameter and porogen concentration, (C-D) interaction between diameter and number of holes, and (E-F) interaction between porogen concentration and number of holes after the lipase immobilization process.

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CONCLUSION

This study underscores the pivotal role of structural parameters, such as porogen concentration and monolith diameter (corresponding to the number of holes), in optimizing the lipase immobilization process on porous polymethacrylate-based monoliths. The experimental and simulation results consistently highlight that 60% porogen concentration is the most favorable condition, with diameters of 0.4 mm and 0.3 mm yielding the highest immobilization yield for the experimental and simulation data. The immobilization yield increases with smaller pore diameters, a higher number of holes, and greater porogen contents because of the improved surface area and accessibility for lipase enzyme attachment, enhancing the loading efficiency. These findings not only validate the tunability of monolith architecture for enhanced enzyme loading but also pave the way for designing robust biocatalytic platforms tailored for industrial applications such as biodiesel production. By continuing to refine the polymethacrylate-based monolith design and expanding its application scope, this research makes a meaningful contribution to the advancement of eco-friendly and efficient biocatalytic technologies.

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