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Immobilization of Laccase onto Polysulfone/Sepiolite Nanocomposite using Box-Behnken Design

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In this study, two different types of enzyme immobilization methods which were physical adsorptions (PA), and covalent bonding of cross-linked enzyme aggregates (CLEA) were used for laccase to immobilize onto raw polysulfone (PSF) and polysulfone/sepiolite (PSF/SEP) nanocomposites. After the preliminary study, a strategy for screening and optimization of laccase immobilization on PSF/SEP was then further developed by using a two-level factorial design and Box-Behnken design (BBD), respectively. Laccase (Lac) concentration, glutaraldehyde (Gla) concentration, pH values, adsorption (PA) time, PA temperature, CLEA time, and CLEA temperature were chosen in this study as important variables which they will influence the immobilization yield. It was found that Lac concentration, PA time, and Gla concentration are significant variables for the immobilization yield of Lac on PSF/SEP in the screening process. The highest immobilization yield was 75 % at 0.10 mg/mL Lac concentration, 2 h PA time, and 0.20 (v/v) % Gla concentrations, with an increase of 46.57 % over the immobilization yield before optimization.

1. Introduction

Laccase (Lac), a diverse oxidoreductase group enzyme generated by fungi was extensively exploited in the degradations of chemical pollutants due to its low specificity of substrates and substrates' monoelectronic oxidation in a wide range of complexes (Dong et al., 2023). However, the applicability of laccases is restricted by their low thermal stability, reusability, and operational activity under various industrial working environments (Hatimuria et al., 2023). In order to solve these problems, one of the most crucial, efficient, and widely used techniques is enzyme immobilization by attaching enzymes to insoluble solid supports through suitable immobilization methods such as adsorption, covalent binding, entrapment, encapsulation, and cross-linking (Zdarta et al., 2018). Inorganic and organic materials can be used as supports for enzyme immobilization. In recent years, organic polymer/nanocomposite supports had drawn much attention due to their ease of fabrication, tailor-made properties, relatively low cost, and inert property (Lyu et al., 2021).

Nowadays, the enzyme was used to immobilize into nanofilter mats to increase the anti-fouling property and mitigate the contaminants on the surface and the transport channels of the mats. Costa et al. (2019) reported that polysulfone (PSF) was chosen as a matrix due to its great thermo- and chemoresistance in a wide range of pH and carbon nanotube (CNT) as a nanofiller to form a nanocomposite for laccase immobilization. Sepiolite is also a good choice for enzyme immobilization based on its non-toxicity, excellent thermal stability, and high total surface area by the special needle-like structure which has OH functional groups at the inner surface (Mortazavi & Aghaei, 2020). Both the reported results mentioned above showed that immobilized enzymes have higher stability, reusability, and activity efficiency than free enzymes. Based on the studies before, the polysulfone/sepiolite (PSF/SEP) nanocomposites should be good support for laccase immobilization which had not yet been reported before.

Although laccase immobilization on different supports has been widely studied, a deeper understanding of using advanced statistical techniques like Box-Behnken Design (BBD) is required to optimize the immobilization process, specifically for PSF/SEP nanocomposite. It should be highlighted that improper design during enzyme immobilization might result in undesired effects since the microenvironment will have a high impact on enzyme

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sensitivity (Awad et al., 2020). In this study, BBD was used to evaluate and optimize various parameters of Lac immobilization onto PSF/SEP for the highest immobilization yield and Lac activity. Lac was immobilized on raw PSF and PSF/SEP nanocomposites by using the physical adsorption (PA) and crosslink enzyme aggregates (CLEA) methods. After the highest immobilization yield results were obtained, using a two-level factorial screening design to select the most significant factors that could affect the immobilization yield. The BBD optimization will be used to identify the optimal conditions of the immobilization yield. Lastly, the results between the immobilized laccase before and after optimization, and the error percentage between the predicted and actual value would be compared.

2. Experimental procedure

2.1 Materials

PSF pellet was purchased from Biotek Abadi SDN. BHD. Johor, Malaysia, Polyvinylpyrrolidone (PVP), 1-Methyl-2-pyrrolidinone (NMP), Sepiolite, Laccase was obtained from *Trametes versicolor* (enzyme activity ≥ 0.5 U/mg), Glutaraldehyde, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich. All other chemicals used in this study were purchased either from Sigma-Aldrich or MERCK.

2.2 Preparation of PSF and PSF/SEP nanocomposites

Raw PSF and PSF nanocomposite with PVP and sepiolite were prepared via the phase inversion method due to its low operation cost and relatively simple process. For raw PSF samples, a casting solution was prepared by dissolving 12 wt% of PSF pallets in 88 wt% of NMP solution at 70 °C for 3 h. While the casting solution for PSF/SEP nanocomposite, 5 wt% of PVP was added in 78.45 wt% of NMP solvent and magnetically stirred until dissolve at 40 °C. The mixture was then moved into an ice-water bath, 3.55 wt% of sepiolite was added to the mixture and magnetically stirred in the ice-water bath until uniformly dispersed. After that, 13 wt% PSF pallets were added and it was stirred for another 3 h at 70 °C. After all the PSF pallets were completely dissolved, the casting solution was degassed for 30 min by using a vacuum pump. The casting was poured across the glass plate and gently glided with a glass rod. After 10 s of evaporation time, the glass plate was immersed in a distilled water bath at 25 °C. The samples formed were then washed and kept with distilled water for 24 h to remove the residual solvent. After 24 h, the samples were washed again and kept in distilled water at room temperature for further use.

2.3 Laccase immobilization procedure

For Lac to be immobilized on the raw PSF mat and PSF/SEP nanocomposites, the PA and PA together with CLEA methods were selected and used. The sample was kept in a pH 4 sodium acetate buffer solution containing 1 mg/mL of laccase solution. The vials containing the samples were then shaken by using the incubator shaker for 1 h, 25 °C at 185 rpm. The immobilizations of the PA method on PSF and PSF/SEP were finished at this moment while the CLEA method was continued by adding 0.5 % (v/v) of Gla solution into the vials and further shaking for another 1 h at 25 °C under 185 rpm. The mat was washed with pH 4.0 sodium acetate buffer multiple times to remove the unattached Lac on the samples before calculating the laccase's immobilization yield (IY) on the mats.

2.4 Screening using two-level factorial

Based on the results of the preliminary study, PSF/SEP nanocomposites were chosen as the support for laccase immobilization by using PA with CLEA methods. The 7 factors that influence the immobilization yield (IY) response are shown in Table 1. A screening process is necessary for the determination of an appropriate scope for each optimization variable. In order to determine the most significant factors among these independent variables, a two-level factorial design was used. A total of 20 run experiments were conducted, including 4 replicates at the central point.

Table 1: Independent Variables' Setting Scopes for Screening

Variables	Units	-1	+1
A: Laccase Concentration	mg/mL	0.1	1
B: pH	-	4	5.5
C: PA Temperature	°C	25	35
D: PA Time	h	1	3
E: Glutaraldehyde Concentration	(v/v) %	0.05	0.5
F: CLEA Time	h	1	6
G: CLEA Temperature	°C	4	35

2.5 Box-Behnken Design

According to the results of the two-level factorial, the significant factors were selected and Response Surface Methodology based on Box-Behnken Design was then used to determine the optimal Lac immobilized conditions on PSF/SEP. The chosen variables were Lac concentration (A), PA Time (B), and Gla concentration (C) while the IY was the response. A total of 17 run experiments were conducted which included 5 central point replications. Each independent variable's levels were set as low-level (−1) and high-level (+1) and the scopes are shown in Table 2.

Table 2: Independent Variables' Setting Ranges for Optimization

Variables	Units	-1	+1
A: Laccase Concentration	mg/mL	0.1	0.3
B: PA Time	h	0.5	2
C: Glutaraldehyde Concentration	(v/v) %	0.1	0.3

The quadratic polynomial regression model was estimated using Design-Expert 11.0 for the immobilization yield as shown in Eq(1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response value of prediction, β_0 is the constant, β_i, β_{ii}, and β_{ij} are the linear term, quadratic terms, and interaction coefficient. The variance analysis (ANOVA) was used to evaluate the results and the maximum IY response was chosen according to the solutions provided by Design-Expert 11.0.

2.6 Immobilization yield of laccase

Laccase activity was measured by monitoring the oxidation of 1 mM ABTS in pH 4 sodium acetate buffer using UV-Visible spectrophotometer at 420 nm. The cuvette contained 0.15 mL of laccase solution, 1.35 mL of 1 mM ABTS solution, and 1.5 mL of 0.1 M sodium acetate buffer. The reactions took place for 3 min and the activity was calculated using Eq(2).

$$\mathrm{U} = \frac{\Delta \mathrm{A} \times \mathrm{V} \times 10^6}{\varepsilon \times \mathrm{L} \times \mathrm{t}} \quad (2)$$

where U is the enzyme activity, ΔA is the change in absorbance at 420 nm after reaction, V is the total volume of reaction, ε is the molar extinction coefficient (M-1 cm-1), L is the optical path, and t is the time of reaction. A single unit of enzyme activity (U) is defined as the enzyme quantity that is needed to catalyze the oxidation of 1 μmol of ABTS per min. The IY is calculated using the equation as shown in Eq(3).

$$IY\ (\%) = \frac{U_i - U_f}{U_i} \times 100\ \% \quad (3)$$

where U_i is the laccase solution activity before immobilization and U_f is the laccase solution activity after immobilization.

3. Results and discussion

3.1 Preliminary study of laccase immobilization on PSF and PSF/SEP using PA and CLEA method

Two different immobilization methods were investigated which are the PA method and the CLEA method. For the PA method, laccase was directly immobilized on PSF and PSF/SEP mats. From the results, laccases were able to adsorb on the surface of PSF and PSF/SEP mats with a 32.70 % and 43.42 % immobilization yield respectively. PSF/SEP nanocomposites have higher immobilization yield due to the introduction of hydrophilic hydroxyl (OH) groups on the surface of PSF (Wasim et al., 2017). The addition of sepiolite and polyvinylpyrrolidone had increased the porosity and surface roughness of the mats. This increment in total surface area of PSF/SEP nanocomposite for laccase adsorption had led to an increment of laccase immobilization yield (Khodami et al., 2020). The OH group increases the hydrophilicity of PSF/SEP and more hydrogen bonding is formed between laccase and the supports, PSF/SEP mats have a greater immobilization yield compared to raw PSF mats. The CLEA immobilization method was further applied to the PSF/SEP nanocomposites for the second method. It was found that the value of the immobilization yield was 51.17 %, about 1.18 times greater than that of the PA method. The presence of Gla had formed a multipoint linkage

between the enzyme and increasing the laccases attached to the PSF/SEP nanocomposites (Lee et al., 2016). In order to optimize the immobilization yield of laccase on PSF/SEP nanocomposites, a screening and optimization study had done by using the two-level factorial and the Box-Behnken design.

3.2 Two-level factorial screening

Table 3 showed that there are 5 variables having p-values less than 0.05 including Lac concentration (A), pH (B), PA time (D), Gla concentration (E), and CL temperature (G) which denoted that they are significant variables. Based on the ANOVA table, the determination R^2 coefficient was 0.9932, nearly close to 1, indicating that the prediction and actual values were nearly fit. The F value (93.15) and the p-value (<0.0001, which is less than 0.05) of this model, both implied that this is a significant model. Three model terms (A, D, and E) that showed the most significant effect on the IY response (p-value ≤ 0.0001) were selected from the ANOVA table for the optimization process. The mathematical equation for these model terms was presented in Eq(4).

$$IY\ (\%) = 46.023 + 67.967Lac - 1.237pH - 3.977PA\ Time - 8.640Gla + 1.431CL\ Temp - 9.146Lac * pH - 24.606Lac * PA\ Time - 16.327Lac * Gla - 2.002Lac * CL\ Temp - 1.466pH * PA\ Time + 6.709Lac * pH * PA\ Time \quad (4)$$

According to Eq.(4), a solution with the highest IY response (73.15 %) whose desirability value is 1 was chosen for the new scopes of the optimization significant variables. These new scopes of the significant independent variables were selected as shown in Table 2. The other variables such as pH, PA temperature, CLEA time, and CLEA temperature were maintained at 4, 25 °C, 1.0 h, and 34.8 °C, respectively.

Table 3: The screening ANOVA of laccase immobilization preparation conditions for laccase immobilization on PSF/SEP nanocomposites using two-level factorial

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5029.93	11	457.27	93.15	< 0.0001	Significant
A	64.92	1	64.92	13.23	0.0083	
B	29.78	1	29.78	6.07	0.0433	
D	771.87	1	771.87	157.24	< 0.0001	
E	251.46	1	251.46	51.23	0.0002	
G	418.51	1	418.51	85.26	< 0.0001	
AB	33.26	1	33.26	6.78	0.0353	
AD	170.89	1	170.89	34.81	0.0006	
AE	43.73	1	43.73	8.91	0.0204	
AG	3118.94	1	3118.94	635.38	< 0.0001	
BD	44.52	1	44.52	9.07	0.0196	
ABD	82.04	1	82.04	16.71	0.0046	
Curvature	1075.97	1	1075.97	219.19	< 0.0001	Significant
Residual	34.36	7	4.91			
Lack of Fit	3.43	4	0.86	0.08	0.9820	Not Significant
Pure Error	30.93	3	10.31			
Cor Total	6140.26	19				
R-squared	0.9932					

3.3 Box-Behnken Design

The F value, p-value, and lack of fit (LOF) from the BBD ANOVA (as shown in Table 4) of this model were 14.21, 0.0010, and 0.9819 respectively, which indicated that this model was significant. The results showed that A, A^2, C^2, and AB had a significant effect towards the IY response. The model was also analyzed as a fit model if the LOF showed an insignificant result. The relationship between the independent variables and the IY response was established by utilizing the mathematical model formulated based on this data as demonstrated in Eq(5).

$$IY\ (\%) = 48.016 - 286.064Lac + 5.365PA\ Time + 244.79Gla - 176.867Lac * PA\ Time - 234.25Lac * Gla + 56.7PA\ Time * Gla + 974.65Lac^2 + 8.163PA\ Time^2 - 714.6Gla^2 \quad (5)$$

Table 4: The BBD ANOVA of laccase immobilization on PSF/SEP nanocomposites

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	3670.94	9	407.88	14.10	0.0011	Significant
A	2155.29	1	2155.29	74.53	<0.0001	
B	13.60	1	13.60	0.47	0.5149	
C	23.19	1	23.19	0.80	0.4003	
A^2	399.98	1	399.98	13.83	0.0075	
B^2	88.77	1	88.77	3.07	0.1232	
C^2	215.01	1	215.01	7.44	0.0295	
AB	703.84	1	703.84	24.34	0.0017	
AC	21.95	1	21.95	0.76	0.4125	
BC	72.34	1	72.34	2.50	0.1578	
Residual	202.43	7	28.92			
Lack of Fit	8.53	3	2.84	0.06	0.9789	Not Significant
Pure Error	193.90	4	48.47			
Cor Total	3873.37	16				
R-squared	0.9477					

Based on the results, the 3D contour plots of the independent variables' mutual effects were produced as shown in Figure 1. From Figure 1 (a) and (b), Lac concentration showed a significant effect on IY response as proved by ANOVA in Table 4 which the p-value was lower than 0.05. The negative value of Lac in Eq(5) denoted that at the highest Lac concentration (0.3 mg/mL), the IY of Lac on PSF/SEP will show negative results. As the Lac concentration increased, the IY response slightly decreased as shown in Figure 1 (a) and (b). This can be due to the higher competition for the excessive laccases to adsorp on the limited adsorption space on the carrier surface which results in a lower immobilization yield (Fortes et al., 2017).

The effect of PA time and the Gla concentration were not significant towards the immobilization yield of laccases on PSF/SEP which only showed a slight change at the optimum 0.1 mg/mL Lac concentration. For PA time, the longer the PA time, the more laccases enable to be adsorped on PSF/SEP, the greater the immobilization yield as shown in Figure 1 (a) and (c). For Gla concentration, the immobilization yield increased gradually as the Gla concentration increased until the highest point was reached, then started to show a downward trend. The initial low immobilization yield might be due to the lack of Gla to crosslink and form CLEA. Conversely, the decrease in immobilization yield after highest results might be due to the excessive amount of Gla in the solution which would cause laccases' active sites damaged (Wang et al., 2021).

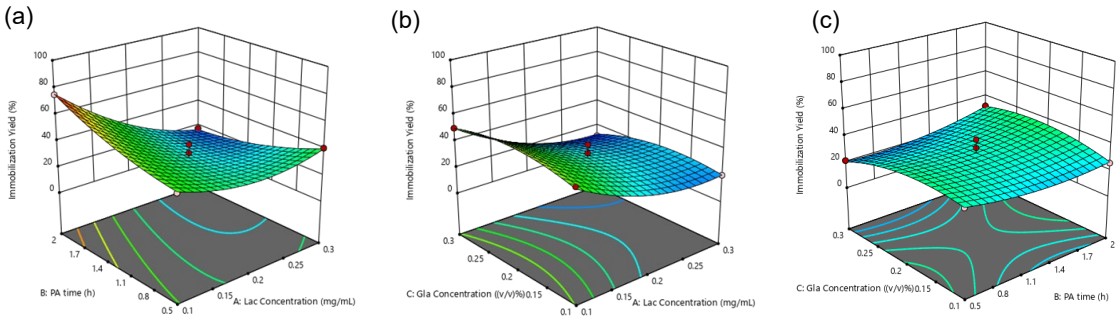

Figure 1: 3D surface plots for the reaction between (a) Lac concentration and PA time, (b) Lac concentration and Gla concentration, and (c) PA time and Gla concentration on the IY

3.4 Confirmation of the best preparing conditions for laccase immobilization

A solution of 0.1 mg/mL Lac concentration, 2 h of PA time, and 0.2 (v/v) % Gla concentration with the highest immobilization yield (75.53 %) which the desirability value is 1 was chosen from the BBD. The results from the preliminary, screening, and optimization studies were shown in Table 5. The error value between the predicted and the actual value for the two-level factorial and the BBD were 4.83 % and 0.71 % respectively. This showed that the actual results are close to the predicted value from the equation and the models are validated.

Table 5: The evaluation of the experimental findings prior to and following optimization

	Preliminary	Two-level factorial	BBD
Predicted Value (%)	-	73.15	75.53
Experimental Value (%)	51.17	69.78	75
Error (%)	-	4.83	0.71
Increment (%)	46.57	6.96	

4. Conclusion

The PSF/SEP nanocomposites were successfully used as support for Lac immobilization and showed higher immobilization yield (IY) compared to the raw PSF. The CLEA method using Gla with PSF/SEP showed the best IY (51.17 %) and this method was chosen for the further optimization process. The IY of the preliminary study was then further enhanced by 46.57 % after using the BBD method (75 %). This study showed that the IY can be enhanced by modifying the immobilized condition of enzymes. However, there are no clear optimum points obtained from the 3D plots of BBD which the optimum point might fall outside the study range. Another study with a greater range and the enzyme activity and stability tests are recommended for future study in order to prove and convince that Lac immobilized on PSF/SEP has a great potential to be used in industrial.

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References

Awad, G. E., Ghanem, A. F., Abdel Wahab, W. A., Wahba, M. I., 2020, Functionalized κ-carrageenan/hyperbranched poly(amidoamine)for protease immobilization: Thermodynamics and stability studies, International Journal of Biological Macromolecules, 148, 1140–1155.

Costa, J. B., Lima, M. J., Sampaio, M. J., Neves, M. C., Faria, J. L., Morales-Torres, S., Tavares, A. P., Silva, C. G., 2019, Enhanced biocatalytic sustainability of laccase by immobilization on functionalized carbon nanotubes/polysulfone membranes, Chemical Engineering Journal, 355, 974–985.

Dong, C. D., Tiwari, A., Anisha, G. S., Chen, C. W., Singh, A., Haldar, D., Patel, A. K., Singhania, R. R., 2023, Laccase: A potential biocatalyst for pollutant degradation, Environmental Pollution, 319, 120999.

Fortes, C. C., Daniel-da-Silva, A. L., Xavier, A. M., Tavares, A. P., 2017, Optimization of enzyme immobilization on functionalized magnetic nanoparticles for laccase biocatalytic reactions, Chemical Engineering and Processing: Process Intensification, 117, 1–8.

Hatimuria, M., Das, J., Gavvala, K., Bag, S., Pabbathi, A., 2023, Recent advances in the use of laccase enzyme in deep eutectic solvents, Sustainable Chemistry and Pharmacy, 33, 101148.

Khodami, S., Mehdipour-Ataei, S., Babanzadeh, S., 2020, Preparation, characterization, and performance evaluation of sepiolite-based nanocomposite membrane for desalination, Journal of Industrial and Engineering Chemistry, 82, 164–172.

Lee, J. Y., Kwak, H. W., Yun, H., Kim, Y. W., Lee, K. H., 2016, Methyl cellulose nanofibrous mat for lipase immobilization via cross-linked enzyme aggregates, Macromolecular Research, 24(3), 218–225.

Lyu, X., Gonzalez, R., Horton, A., Li, T., 2021, Immobilization of Enzymes by Polymeric Materials. Catalysts, 11(10), 1211.

Mortazavi, S., Aghaei, H., 2020, Make proper surfaces for immobilization of enzymes: Immobilization of lipase and α-amylase on modified Na-sepiolite, International Journal of Biological Macromolecules, 164, 1–12.

Wang, Y., Yang, Y., Wang, J., Wang, Z., Zhao, S., 2023, Supramolecular enzymatic membranes comprising of β-cyclodextrin and laccase for efficient removal and desalination of small molecules, Desalination, 546, 116198.

Wang, Z., Su, T., Zhao, J., 2021, Immobilization of Fusarium solani Cutinase onto Magnetic Genipin-Crosslinked Chitosan Beads, Catalysts, 11(10), 1158.

Wasim, M., Sabir, A., Shafiq, M., Islam, A., Azam, M., Jamil, T.,2017, Mixed matrix membranes: Two step process modified with electrospun (carboxy methylcellulose sodium salt/sepiolite) fibers for nanofiltration, Journal of Industrial and Engineering Chemistry, 50, 172–182.

Zdarta, J., Meyer, A., Jesionowski, T., Pinelo, M., 2018, A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility, Catalysts, 8(2), 92.