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Optimization of sol-gel medium for entrapment of acetylcholinesterase enzyme in biosensor for pesticide detection

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Abstract. Pesticides are chemical substances used to kill and control pests or diseases that can damage crops. The use of pesticides should be done precisely because the accumulation of chemicals contained in pesticides can cause various health effects. Therefore, detection of pesticide residues on plants is important to reduce the risk of poisoning due to pesticide residues. Some of the conventional methods that have been done to detect pesticide residues have weaknesses among expensive tools, takes a long time, and are generally performed by trained laboratory technicians. Biosensors are analytical devices that can measure the quantitative or semi-quantitative targets of analyte by utilizing a bioreceptor such as enzyme. Several studies have shown that enzyme-based acetylcholinesterase-based biosensors can be used to detect pesticide residues in vegetable samples. The objective of this research was to get a proper silica based sol-gel formulation with molar ratio of H_2O:TEOS and NaOH concentration as immobilization medium of acetylcholinesterase enzyme for biosensor application. Response Surface Methodology (RSM) was used in order to determine the interaction between the parameters studied and resulting responses which were amount and activity of acetylcholinesterase enzyme. Based on the research, the best result for immobilized enzyme activity was shown by molar ratio (H_2O: TEOS) 1: 8 and 4 mM NaOH treatment.

1. Introduction

Generally, horticultural crop farmers tend to use excessive pesticides to improve the quality of their products. The use of pesticides should be done precisely because the accumulation of chemicals contained in pesticides can lead to increased nerve activity such as headache, vomiting, shortness of breath, muscle spasms and paralysis [1] Therefore, detection of pesticide residues in plants is important to reduce the risk of poisoning due to pesticide residues in plants. Biosensors are analytical devices that can measure analyte target quantitatively or semi-quantitatively by utilizing bioreseptor such as enzymes, antibodies, DNA, nucleic acids and whole cells [2]. Enzyme-based biosensor is mostly applied because of its high specificity to the target analyte. Several studies have shown that acetylcholinesterase (AChE) enzyme-based biosensor can be used to detect pesticide residues in vegetable samples.

In the fabrication of biosensors, enzymes are immobilized in a solid matrix in order to maintain their catalytic activity. Entrapment is one of the enzyme immobilization techniques conducted by

trapping enzymes in micro-polymer matrices such as silica-based sol-gel [3]. Immobilization of enzymes by using sol-gel media has several advantages such as large enzyme capture, stable to high temperature, and flexible or controllable pore diameter [4]. However, the use of sol-gel for immobilized enzyme media also has a disadvantage as it is porous that may lead the enzymes to seep out. Modifying the molar ratio between silica (Tetraortosilicate/ TEOS) with H_2O can be an alternative to overcome this weakness as H_2O is used in the TEOS hydrolysis process so that the molar ratio used can affect the TEOS structure in the formation of sol-gel. Furthermore, other factors that can be used to regulate the size of the sol-gel pores can be done by modifying the concentration of NaOH as a catalyst. NaOH can accelerate the process of hydrolysis of TEOS so that the pore size of the sol-gel becomes larger and the enzyme is more easily trapped [6]

The aim of this research is to get a proper silica-based sol-gel formulation with TEOS and H2O molar ratio and NaOH (mM) concentration as immobilization medium of acetylcholinesterase enzyme. Using the statistical method of Response Surface Methodology (RSM) it is expected that the interaction between the parameters studied and the resulting response can be known. The minimum data used In the TEOS and H_2O molar ratio factors is 1: 4, the central data is 1: 8, and the maximum unit data is 1:12. Meanwhile, the concentration factor of NaOH on the minimum data used is 3mM, central data is 4mM, and data unit maximum of 5 mM. Analysis on the number of immobilized enzyme is investigated as the response.

2. Materials and Method

The optimization of silica-based sol-gel was done using Response Surface Methodology (RSM) method with the factor studied is the molar ratio between TEOS with H_2O and NaOH concentration which is a sol-gel catalyst. The research is divided into several stages including:

a. Sol-gel Formulation with Software Response Surface Methodology (RSM)

Response Surface Methodology (RSM) method was conducted using Design Expert 7.0 software. The initial stage is designing the desired experimental variables. The factor used in the optimization is the consideration of the molar ratio between TEOS and H_2O. Another factor is the optimization of the concentration of NaOH (mM) while the response is the amount of immobilized enzymes. Table 1 presents the experimental design of Response Surface Methodology (RSM) with the Central Composite Design (CCD) model. About 13 designs are obtained from the RSM method.

Table 1. Design of Experiment by RSM Method

Design	Factor	
	Factor 1 Molar Ratio (H_2O:TEOS)	Factor 2 Concentration of NaOH (mM)
1	1:4	3
2	1:12	3
3	1:4	5
4	1:12	5
5	1:2.36	4
6	1:13.66	4
7	1:8	2.59
8	1:8	5.41
9	1:8	4
10	1:8	4
11	1:8	4
12	1:8	4
13	1:8	4

b. Response Determination
Number of immobilized enzyme was determined as a response (result) of optimization carried out using RSM method. The number of immobilized enzyme analysis was conducted by calculating the amount of lysis enzyme (ΔA1) after immobilization process was done. 5 µl of acetylcholinesterase enzyme (ΔA0) was first immobilized on each 5 µl sol-gel formulation. A mixture of enzyme and sol-gel was then dropped onto Whatman no. 1 (size of 0.8 cm x 3 cm). The Whatman paper was left to stand for 15 minutes and then immersed in 250 µl Tris-HCl buffer pH 7.5 for 20 minutes at room temperature. The solution was assumed as lysis enzyme (ΔA1). As much as 195 µl of the solution was placed on the microplate and added by 5 µl indoxyl acetate (5 mg / ml). Furthermore, a sol-gel absorbance measurement was performed using a microplate reader every 2 minutes at a wavelength of 630 nm for 20 min. ΔA0 is free enzyme absorbance, while ΔA1 is the lysis enzyme absorbance at 630 nm

$$Amount\ of\ Immobilized\ Enzyme\ (\%) = \frac{\Delta A0 - \Delta A1}{\Delta A0} \times 100\%$$

c. Model Selection and Model Analysis with ANOVA
Selection of appropriate model (Linear, Quadratic, or Cubic) was determined in this study. Furthermore, after obtaining the appropriate model, an analysis of variance (ANOVA) was performed on the selected model which aims to determine the interaction between two factors, i.e the TEOS and H_2O molar ratio and NaOH concentration towards the number of immobilized acetylcholinesterase enzyme.
d. Determination of Optimum Condition
From the results of the response analysis, the Expert Design software will also suggest optimal sol-gel formulation results along with an approximate response value of the greatest number of immobilized enzyme. In addition to providing predictive response values, the Expert Design program also provides prediction interval (PI). PI is a range that shows the results of the next response measurements with the same condition at a significance level of 5%.
e. Verification of Optimization Result
Verification was carried out by analyzing the number of immobilized enzyme based on the optimum formulation obtained with two replications. The purpose of verification is to confirm whether the response is generated in accordance with the approximate response generated by the Expert Design software. If the difference of predicted value and a research result is no more than 5%, it indicates that the model is sufficiently precise and thus the solution of the given free variable is acceptable.

3. Results and Discussion

3.1 Response Analysis Results

Figure 1 shows the effect of TEOS : H_2O molar ratio and NaOH concentration to amount of immobilized enzyme, as a response based on the 13 designs provided. The central data is obtained from TEOS: H_2O molar ratio of 1: 8 and NaOH concentration of 4 mM. The central data is repeated 5 times from 13 RSM designs. The central data tends to show higher results than other designs. The amount of immobilized acetylcholinesterase enzyme in the central data ranged from 72.73% to 86.36%. The number of enzymes that are immobilized is higher when compared to the literature of research conducted by [4] which immobilized trypsin enzyme in sol-gel with a precursor of 0.1 ml TEOS silica; Deionized H_2O; PEG 6000 0.1 ml; and H_3PO_4 1 M as much as 2µl. The result of the number of immobilized trypsin enzymes in the study ranged from 59.9-67.7%. This may be due to the addition of PEG 6000 which acts as a sol-gel stabilizer will have an effect on the sol-gel that makes the particle size of the silica larger because PEG will envelop the silica particles so the size is larger [5]. After obtaining the data, we select appropriate model to determine the optimum response based on sum of squared sequence model, statistical model summary (Model Summary Statistic), and Lack of Fit Test.

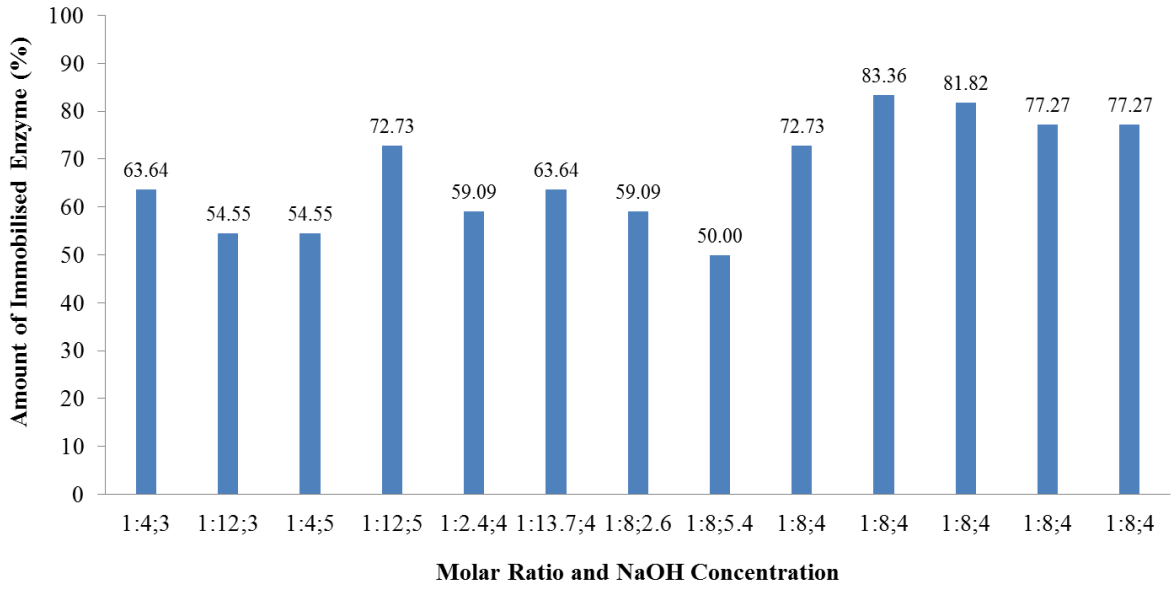

Figure 1. Amount of Immobilized Enzyme Response Result

3.2 Result of Optimization Analysis on the Number of Immobilized Enzyme

3.2.1. Analysis of Selection of Statistical Model

Analysis of statistical model selection was conducted to determine the appropriate model in describing the significance of the research results obtained. Some types of models that can be used in Design Expert program include: linear model, 2 factor interaction (2FI), Quadratic and Cubic models. The model selection analysis is based on the sum of quadratic (Sequential Model Sum of Squares), Lack of Fit Test and Summary of Statistic statistics. Based on these three methods, the Quadratic model has a significant effect on the response so that the Quadratic model is the best and suggested design.

3.2.2 Analysis of Variance (ANOVA) of RSM on the Number of Immobilized Enzyme Responses

After getting the selected model that is Quadratic model, then the analysis of variance (ANOVA) is used to analyze the accuracy level of the selected model based on the analysis value of variance to the model and the value of Lack of Fit presented in Table 2.

Table 2. Result of Analysis of Variance (ANOVA) from *quadratic* model.

Source of Variance	Number of Squares	Db	Mean Squared	F Value	P Value Prob>F	Description
Model	1426.15	5	285.23	10.410	0.0038	*significant*
A	30.11	1	30.11	1.100	0.3294	*not significant*
B	1.77	1	1.77	0.065	0.8065	*not significant*
AB	185.95	1	185.95	6.790	0.0352	*significant*
A^2	446.48	1	446.48	16.300	0.0050	*significant*
B^2	907.32	1	907.32	28.110	0.0007	*significant*
Residual	191.78	7	27.40			
Lack of Fit	84.34	3	28.11	1.05	0.4633	*not significant*
Pure Error	107.44	4	26.86			
Cor Total	1617.93	12				

Description: A = Variabel X_1 (Molar Ratio of TEOS and H_2O)
B = Variabel X_2 (Concentration of NaOH (mM))
AB, A^2, B^2 = Interactions between treatment

In this research,the analysis of model is shown with P value 0.0038 so that the model is stated significant. The value of Lack of Fit shown is 0.4633. Therefore, based on the analysis value of the model and Lack of Fit it is concluded that the Quadratic model corresponds to the whole design value. The interaction between the TEOS and H_2O (squared) molar ratios and NaOH concentration has a positive and significant effect on the number of mobilized enzymes, so that it can be expressed when the interaction between molar ratio (squared) and NaOH concentration increases, the number of immobilized enzyme will be higher

3.3 The Effect of Molar Ratio and NaOH Concentration on Response of Number of Immobilized Enzymes

Figure 2 illustrates the 3-dimensional curves and plot contours for sol-gel media optimization for immobilization of acetylchololesterester enzymes. The image illustrates the effect of two parameters on the number of mobilized enzymes. The values listed on the box on the plot contour indicate the number of enzymes mobilized under various optimization process conditions studied.

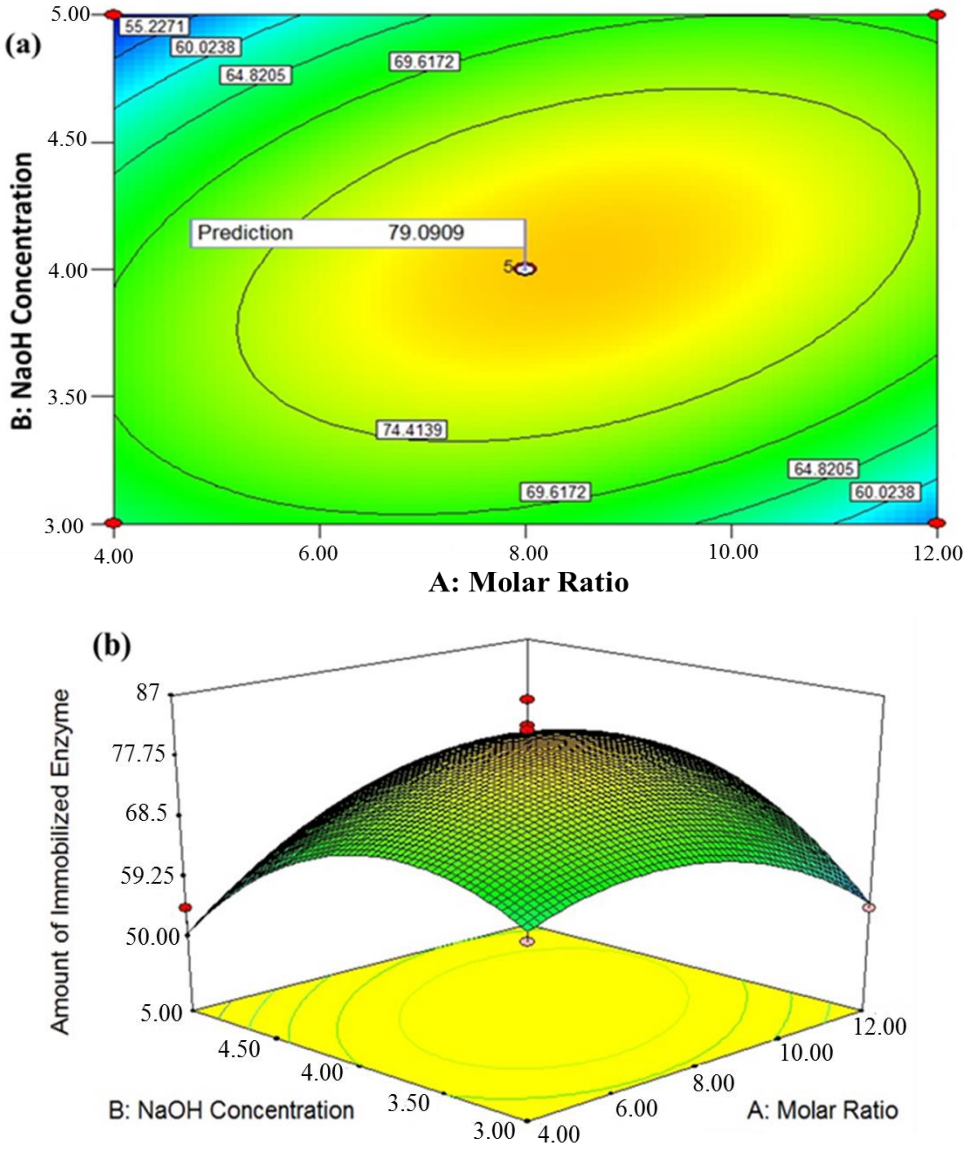

Figure 2. (a) Contour plot (b) surface response (3D) curve of molar ratio and NaOH concentration against the amount of immobilized enzyme response

Figure 2 shows that the curve shape of the saddle represents the possibility of the variable at maximum and minimum points. Such contour systems are called saddle or minimax system. The x and y axes in Figure 2 (a) show the optimized variables. The x-axis shows the variables of the TEOS and H_2O molar ratios, whereas the y-axis shows the NaOH concentration variable. The circular lines indicate the response. Optimal response is indicated by the flag in the center of the contour indicating the optimal point information located at the point (node) shown in the flag. On the plot contour, the number of optimized mobilized enzymes is shown at the point (node) of 79.0909%.

3.4 The Determination of Amount of Immobilized Enzymes Optimum Response

Software Design Expert 7.1.5 is used to identify the best combination of sol-gel formulation parameters used to optimize the number of immobilized enzymes and the relative activity of immobilized enzymes. Desirability is a method used to explain how well the optimum solution is offered to match the objectives of the response . The desirability value 1 indicates the perfect case, but the desirability value 0 indicates the response that should be discarded. In this study, the optimal solution offered by the model is the molar ratio of 1: 8.58 (TEOS: H_2O) and the NaOH concentration of 4.09 (mM) for a response prediction of 79.155%, with a desirability value of 0.77. The optimum point of each variable is the stationary point which is supposed to be the optimum response.

3.5 The verification on optimization result

The verification of optimization results needs to be done to prove the predicted results and response values of the optimum formula solution suggested by the Design Expert program. Verification is done by comparing the value of response analysis on the research with the response value of the results of Design Expert software calculations. Verification and prediction results are shown in Table 3.

Based on the data, the comparison of predicted results of Design Expert program with actual calculation on all responses shows that the value of verification is in accordance with the prediction value, because the response is still around the range of PI.

Table 3. Comparison of Verification Result and Predictions.

	Molar Ratio TEOS:H_2O	NaOH Concentration (mM)	Amount of Immobilized Enzyme (%)
Prediction	1:8.58	4.09	79.15
Verification	1:8.58	4.09	84.09 ± 3.21
95% PI low	1:8.58	4.09	65.61
95% PI high	1:8.58	4.09	92.70

4. Conclusions

Based on this research, it is found that the result of sol-gel optimization for TEOS molar ratio factor: H_2O is 1: 8.58 while for NaOH concentration factor is 4.09 mM. From the sol-gel optimization, the verification result of immobilized enzyme is 84.09 ± 3.21%.

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