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Vol. 13(2) DECEMEBR 2019

ISSN: 1675-7785
eISSN: 2682-8626
DOI: 10.1234/jmpc.v13i2.7526

Optimization of Immobilization Conditions for Protease Extracted from Torpedo Scad (*Megalaspis cordyla*) Viscera Using Response Surface Methodology (RSM)

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Received: 25 July 2019; Accepted: 25 October 2019; Published: 31 December 2019

ABSTRACT

Protease was extracted from the viscera of torpedo scad fish (*Megalspis cordyla*) to obtain the crude extract which was then partially purified in 70% ammonium sulphate. The collected precipitate was dialysed and subsequently immobilized in sodium alginate and calcium chloride solution. The optimum concentrations of sodium alginate and calcium chloride to produce the highest yield of immobilized protease was determined by using Response Surface Methodology (RSM). From the results, the optimum conditions obtained were 2.5% of sodium alginate and 0.25 M of calcium chloride achieving a yield of 55.52%. Thus, the utilization of 2.5% sodium alginate and 0.25 M calcium chloride as the immobilization media were able to produce yield of immobilized protease from torpedo scad viscera with the highest proteolytic activity.

Keywords: T*orpedo scad; Protease; Viscera; Immobilization; Ammonium sulphate*

INTRODUCTION

Proteases are mainly derived from plants and microbial sources where these enzymes are fundamental constituents in industrial processes [1]. Due its wide application, demand for proteolytic enzymes is continuously increasing. Fish viscera is one of the most important by-products of fish industry. Despite being discarded, fish viscera is a good source of protease exhibiting high activity over a wide range of pH and temperature [2, 3]. Previously, the visceral protease from torpedo scad has been extracted and partially purified. The optimum conditions for extraction and purification of the protease were obtained at pH 9 and 80% ammonium sulphate concentration, respectively, resulting in a proteolytic activity value of 445U [4].

The use of enzymes as catalysts for large-scale industrial processes is restricted due to its high cost and instability during storage [3]. Enzyme stability decreases due to changes in pH, temperature, friction and osmotic pressure imposed by the surrounding of their use [5]. However, immobilization improves the technological properties of this biocatalyst [6]. Generally, immobilization can be performed by

https://scilett-fsg.uitm.edu.my/
Vol. 13(2) DECEMEBR 2019

ISSN: 1675-7785
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entrapment, microencapsulation, copolymerisation, cross linking, physical adsorption, chemical attachment and covalent binding [7]. Among different immobilization techniques, entrapment in calcium alginate gel offers many advantages due to its simplicity and non-toxic characteristic [5].

Several studies have been done involving the optimization of immobilization condition. Geethanjali & Subash, [5] conducted a study on the immobilization of purified protease from rohu (*Labeo rohita*) viscera to obtain the optimum concentration of sodium alginate and calcium chloride for the formation of immobilized beads. The immobilized proteases were characterized through an assay for determination of the optimal pH, temperature and storage stability. The optimum condition for the proteases was discovered at pH 8.0, 40□C. Meanwhile, a combination of 2% sodium alginate and 0.3 M calcium chloride were the optimum concentrations producing the entrapment activity of 48.31%.

Amaral et al. [8] extracted a trypsin enzyme from the intestine of Nile tilapia (*Oreochromis niloticus*) and the protease was covalently immobilized on ferromagnetic Dacron (polyethylene terephthalate; PET). Dacron film was converted to Dacron-hydrazide powder and further magnetized. Then, the enzyme was covalently bounded to the magnetic particles. They found that the protein amount and specific activity of the immobilized enzyme on 0.6 mM alpha-benzoyl-DL-arginine-p-nitroanilide (BAPNA) at pH 8.0 and 25°C were 25.6 mg/g of particles and 18.5 ± 0.253 mU/mg protein, respectively.

Salazar-Leyva et al. [9] conducted a study on the optimum immobilization of acidic proteases obtained from Monterey sardine (*Sardinops sagax caerulea*) on partially deacetylated chitin from shrimp head waste using Response Surface Methodology (RSM) where a rotatable central composite design was applied to evaluate the effects of immobilization conditions such as enzyme loading (X1), immobilization pH (X2), and tripolyphosphate concentration (X3) on the immobilization yield. They obtained the optimum immobilization yield of 79.2% when the conditions of enzyme loading, immobilization pH and sodium tripolyphosphate concentration were 0.05 mg/mL, 3.16, and 0.75%, respectively.

Currently, there are insufficient reports on the optimization of sodium alginate and calcium chloride concentration for the formation of immobilised protease extracted from torpedo scad viscera. Hence, the objective of this study is to determine the optimum concentrations of sodium alginate and calcium chloride to produce highest yield of the immobilized protease in terms of proteolytic activity.

EXPERIMENTAL

Materials

Torpedo scad fish (*Megalaspis cordyla*) was obtained from a local market and transported in an ice box cooler to the laboratory. At the laboratory, the viscera were removed, washed with distilled water, packed in a plastic bag, sealed and then stored at -20°C. The chemicals and reagents used were of analytical grade purchased from Sigma Chemical Company (USA).

Crude enzyme preparation

Protease was extracted according to Amid et al., [10]. The viscera were thawed for 2hrs at room temperature and homogenized in 20 mM of tris-HCl buffer (pH 7.8) at a ratio of 1:1 in a blender for

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approximately 2 minutes and then centrifuged at 8,000g for 15 minutes at 4°C (KUBOTA, Model 5420, Japan).

Purification with ammonium sulphate

100 mL crude extract was gradually mixed with ammonium sulphate to a concentration of 70% (w/v) followed by 4 hrs incubation at 4°C [11]. Then it was centrifuged at 10,000g for 10 min. The precipitate was collected and dissolved in 0.02 M tris-HCI buffer, pH 7 at 4°C before desalting.

Dialysis

Desalting was performed by dialysis process in a diafiltration instrument (PR7, Shanghai) [12]. Initial salt concentration in the protease was recorded using a salinity refractometer (Model VS0052 Sealey, UK).

Immobilization of protease

30 mL of dialysed protease was immobilized in sodium alginate and calcium chloride [13]. A 1.5 to 3% sodium alginate solution was prepared by dissolving sodium alginate in 100 mL water at 80°C. The contents were stirred vigorously for 10 minutes to obtain thick uniform slurry. The dialysed protease was then mixed with sodium alginate solution. The protease-alginate mixture was then added drop wise into 0.1 to 0.4 M of calcium chloride solution from a height of 0.5 cm and was kept for curing at 4°C for 60 minutes. The cured beads were recovered by filtration and washed with distilled water and finally with 25 mM tris-HCl buffer of pH 8.0. The levels of significant factors and the interaction effects between various medium constituents which influence the yield were analysed and optimized by RSM, using a Central Composite Design (CCD) design by Design Expert® 11.0 (Stat-Ease, Inc., USA) and presented in Table 1 and 2.

Table 1: Independent factors with their codes and the actual levels used in RSM studies for optimizing immobilization conditions using sodium alginate and calcium chloride

Independent	Codes	Level correspondences		
		-1	**0**	**+1**
Sodium alginate	X_1	1.5%	2.25%	3.0%
Calcium chloride	X_2	0.1 M	0.25 M	0.4 M

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Proteolytic activity assay of immobilized protease

The proteolytic activity assay was performed according to the method of Kaneda & Uchikoba [14]. 0.1 mL protease was added into 0.9 mL of 1% (w/v) casein previously dissolved in 0.2 M sodium phosphate buffer at pH 7. The mixture was incubated at 38°C for 20 minutes. 3 mL of 5% trichloroacetic acid was then added. After 30 min, the precipitate was removed by centrifugation at 10,000g for 20 minutes using a centrifuge (KUBOTA, Model 5420, Japan). The absorbance of the supernatant was measured at 280 nm using UV-vis spectrophotometer (Model HeΛIOS-α, England). One unit of protease activity is defined as the amount of enzyme catalysing the production of tyrosine per minute at 38 C. A standard curve was prepared using tyrosine ranging from 10 to 100 µg/mL. Total proteolytic activity was determined using Equation 1 [11] as follows:

$$CDU = \left(\frac{Et - Eb}{Es}\right) X\, 50 \left(\frac{11}{10}\right) X\, (DF) \quad (1)$$

Where

Et = Absorbance of protease sample
Eb = Absorbance of blank
Es = Absorbance of tyrosine standard
DF = Dilution factor

Immobilization yield

Immobilization yield was calculated based on proteolytic activity of immobilised and dialysed protease using Equation 2 [15] as follows:

$$Yield = \frac{A}{B}\ X\ 100\% \quad (2)$$

Where

A = Total proteolytic activity of immobilized protease
B = Total proteolytic activity of dialysed protease

RESULTS AND DISCUSSION

Optimisation of immobilization parameters

Dialysed protease was immobilised in various concentrations of sodium alginate and calcium chloride prepared as suggested by RSM. The influence of sodium alginate (X1) and calcium chloride (X2) concentration on yield in terms of proteolytic activity was determined and presented in Table 2. Result shows that the highest yield obtained was 59.54% at 2.5% sodium alginate and 0.25 M calcium chloride. Meanwhile, the lowest yield obtained was 10.59% at 2.5% of sodium alginate and 0.04 M calcium

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eISSN: 2682-8626
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chloride. Response surface regression analysis was performed to obtain a second-order polynomial equation or model and the statistical analysis of the model is presented in the form of Analysis of Variance (ANOVA) (Table 3).

Table 2: The actual levels of independent variables along with the observed values for the response variable, immobilization yield (%)

Experimental run	Sodium alginate concentration (%)	Calcium chloride concentration (M)	Immobilization yield (%)
1	2.50	0.25	59.94
2	2.50	0.25	57.39
3	1.50	0.4	36.25
4	1.09	0.25	10.85
5	2.50	0.46	38.05
6	2.50	0.04	10.59
7	1.50	0.10	11.40
8	2.50	0.25	58.43
9	3.50	0.10	20.85
10	2.50	0.25	52.09
11	3.91	0.25	28.60
12	3.50	0.40	22.05
13	2.50	0.25	58.03

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Table 3: ANOVA table for analysis of variance and adequacy of the quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	
Model	4475.10	5	895.02	37.20	< 0.0001	significant
X_1	51.78	1	51.78	2.15	0.1858	
X_2	526.25	1	526.25	21.87	0.0023	
X_1X_2	139.83	1	139.83	5.81	0.0467	
X_1^2	2399.37	1	2399.37	99.72	< 0.0001	
X_2^2	1842.44	1	1842.44	76.57	< 0.0001	
Residual	168.44	7	24.06			
Lack of Fit	132.58	3	44.19	4.93	0.0787	not significant
Pure Error	35.85	4	8.96			
Correlation Total	4643.54	12				

$R^2 = 0.9637$ Adjusted $R^2 = 0.9378$ Predicted $R^2 = 0.7849$

Note: X_1 = concentration of sodium alginate, X_2 = concentration of calcium chloride

The goodness of fit of the regression model was determined based on the coefficient R^2 which provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions [16]. The R^2 value is 0.9637. Meanwhile, the adjusted and predicted R^2 are 0.9378 and 0.7849, respectively. The adjusted R^2 is a corrected value for R^2 after the elimination of unnecessary model terms where the adjusted R^2 would be remarkably smaller than the R^2 if there were many non-significant terms that have been included in the model [17]. If the value of adjusted R^2 and R^2 are close to each other, it indicates a high dependence and correlation between the observed and predicted value responses [18]. Therefore, in this study, the value of adjusted R^2 is high and close to value of R^2 which indicates a high dependence and correlation between the observed and predicted value responses. The Predicted R^2 of 0.7849 is in reasonable agreement with the Adjusted R^2 which is 0.9378 where the difference is less than 0.2 (Table 3).

The significance of regression was evaluated by F and p values using Fischer's and null-hypothesis tests where the value of F predicts the quality of the entire model considering all design factors at a time whereas the p value is the probability of the factors having very little or insignificant effect on the response [18]. Larger F value signifies better fit of the RSM model to the experimental data [19]. F-value with low p value indicates the high significance of the regression model [20]. However, the p-value should be lower than 0.05 for the model to be statistically significant [21]. Therefore, based on Table 3, X2, X_1X_2, X_1^2, X_2^2 are significant model terms as the p-value is less than 0.05 and the model F-value of 37.20 with a p-value of less than 0.0001 implies that the model is significant. The linear factors such as

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concentration of sodium alginate (X_1) and concentration of calcium chloride (X_2) showed a positive coefficient. Square factors such as concentration of sodium alginate (X_1^2) and concentration of calcium chloride (X_2^2) showed negative coefficients, respectively. The quadratic or interaction factors such as concentration of sodium alginate and concentration of calcium chloride (X_1X_2) showed a negative coefficient. The equation for actual factor is Y = 105.26 * sodium alginate + 514.26 * calcium chloride - 39.42 * sodium alginate * calcium chloride – 18.57 * sodium alginate 2 – 723.3 * calcium chloride 2 – 148.62.

Lack of Fit test was also performed where the test describes the variation in the data around the fitted model [18]. Insignificant lack of fit indicates a good model and is desired as significant lack of fit indicates that there might be contributions in the regresses-response relationship that are not accounted for by the model [21]. The F-value for the lack of fit is obtained by dividing the lack of fit mean square by its pure error mean square [18]. Based on Table 3, the F-value and p-value are 4.93 and 0.0787, respectively. Thus, the p-value in this study was considered as insignificant which indicates the model was good and fitted well to the experimental data.

The interaction effects of sodium alginate and calcium chloride concentration were studied using the 3D response surface plots as shown in Figure 1. Response surface plots reveal the interaction between the response and the factors of test variables towards each other [22]. Circular or elliptical shapes of contour plot indicate whether the reciprocal interactions between the factors are significant or not between circular contour plot indicates the interactions between corresponding factors are negligible, meanwhile, elliptical contour plot indicates the interactions between corresponding factors are significant [18]. Therefore, the result of the present study showed that the contour plot was elliptical shape which indicates a significant interaction effect between concentration of sodium alginate and concentration of calcium chloride. The surface plot showed that the yield was low at the lower and higher levels of concentrations while at the middle level of concentrations, the yield was high (Figure 1).

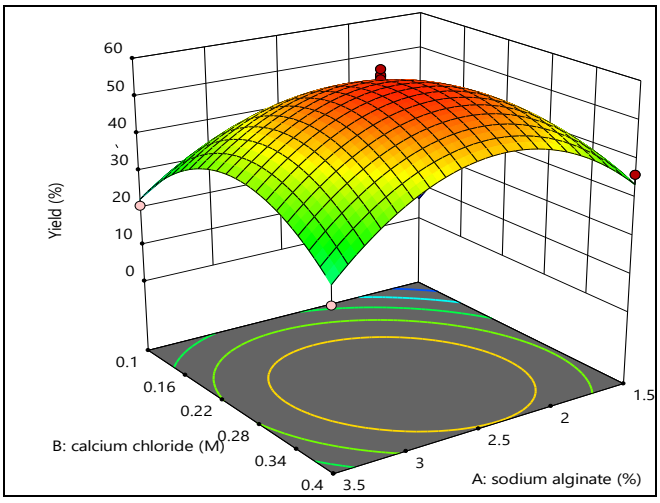

Figure 1: Response surface plot as a function of the interactions of two variables (concentration of sodium alginate and concentration of calcium chloride) on immobilization yield (%).

https://scilett-fsg.uitm.edu.my/
Vol. 13(2) DECEMEBR 2019

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Verification of concentration of sodium alginate and concentration of calcium chloride at the feasible optimum condition was performed and the result is shown as in Table 4 where the desirability obtained was 1.0. The verification value of the yield of immobilised enzyme at the feasible optimum condition was 55.52% which was very close to the predicted value of 57.18%. Since the difference between the verification and predicted values was less than 5%, therefore the feasible optimum condition of the concentration of sodium alginate and concentration of calcium chloride predicted by Design Expert® Software Version 11.0 (Stat-Ease, Inc., USA) was acceptable.

Table 4: Comparison between the verified and predicted values of yield of immobilised protease at feasible optimum condition

Optimum condition		**Verification value (%)**	**Predicted value (%)**
X_1	X_2		
2.5	0.25	55.52	57.18

Note: X_1= concentration of sodium alginate (%), X_2= concentration of calcium chloride (M)

Based on this study, it was found that the optimum condition to achieve the highest yield of immobilized protease was utilization of 2.5% sodium alginate and 0.25 M calcium chloride resulting in the yield of 59.94%. According to Farag & Hassan [23], sodium alginate ranging from 2 to 3% was suitable for immobilization of keratinase, lipase, and protease. Meanwhile, the mechanical strength of alginate beads was highly dependent on the calcium chloride concentration of the gelation solution and the used of concentrated calcium chloride solution has more effect on the efficiency of immobilized systems as it improved some of the bead characteristics, such as thickness and percentage of cell leakage [24]. The gel formation was a result of the crosslinking which occur with divalent cations of sodium alginate and calcium chloride where they became lodged among the polymer chains, forming the net structure [25]. Thus, the concentration of calcium chloride is important for the stability and pore size of the beads [26].

Geethanjali & Subash [5] conducted a study on the immobilization of purified protease from rohu (*Labeo rohita*) viscera obtained by employing various concentrations of sodium alginate ranging from 1 to 5% and calcium chloride from 0.1 to 0.5 M to optimize the best concentration for beads formation. A combination of 2% sodium alginate and 0.3 M calcium chloride was discovered as the optimum concentrations with the entrapment activity of 48.31%. It was concluded that the entrapped enzyme activity increased as the concentration of sodium alginate increased. This was attributed to decreased in pore size at concentration above 3%. Meanwhile, as the concentration of calcium chloride increased, the activity of entrapped enzyme increased only up to 0.3 M. They suggested that at low calcium chloride concentration the entrapped enzyme leaked out, while at higher concentration the beads formed irregular shape which reduced the activity.

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CONCLUSIONS

The optimum condition for immobilization yield obtained using Response Surface Methodology (RSM) was 2.5% of sodium alginate and 0.25 M of calcium chloride concentration achieving a yield of 55.52%. Observation from this study suggested that immobilization of purified visceral protease extracted from Torpedo scad (*Megalaspis cordyla*) with 2.5% sodium alginate and 0.25 M calcium chloride concentration formed the most stable beads preserving the proteolytic activity. Significant interaction between sodium alginate and calcium chloride concentration was observed.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to the Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Shah Alam for use of the laboratory facilities.

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