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Optimisation of Immobilisation Condition of Protease Extracted from Silver Catfish (Pangasius sutchi) Viscera in Calcium Alginate by using Response Surface Methodology (RSM)

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ABSTRACT

Traditionally, enzymes are available both as freeze-dried or powdered form and cannot be recovered back once being used and the animal origin or background sources of the enzyme can raise doubt among the Muslims. Thus, the purpose of this study was to determine the optimum immobilisation condition of protease extracted from silver catfish (Pangasius sutchi) viscera by using response surface methodology. Optimisation of immobilisation condition is important to obtain maximum proteolytic activity thus producing high quality of immobilised enzyme that can be used and recovered for multiple times. In this study, protease from silver catfish viscera has been extracted, partially purified by acetone precipitation method and immobilised in the calcium alginate beads. Sodium alginate and calcium chloride in the range of 1–5% w/v and 0.1 – 0.5 M respectively were used for the optimisation purpose. The proteolytic activity of the protease in the alginate beads was measured as a response to the independent variables by using casein as a substrate. The highest actual and predicted proteolytic activities were 674.77 CDU/mg and 639.26 CDU/mg respectively, using sodium alginate and calcium chloride solution concentrations at 3.00% (w/v) and 0.30 M, respectively. For the experimental feasibilities, the optimum conditions that were feasible to be carried out were with sodium alginate of 2.99% (w/v) and 0.30 M calcium chloride solution. There was no significant difference ($p > 0.05$) found between the predicted (638.19

CDU/mg) and verified (699.82 CDU/mg) values. Thus, indicating that the model was significant and can be used to produce the immobilised protease under the optimum condition.

***Keywords**: silver catfish viscera, protease, immobilisation, calcium alginate, response surface methodology (RSM), proteolytic activity*

INTRODUCTION

In Malaysia, silver catfish are commonly found in Pahang River, Kelantan River and Kenyir Lake [1]. The only drawback of silver catfish is thatthe edible part is only half of its weight, another half of it is the waste [2]. Fish viscera contains several important proteolytic enzymes such as aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) [3]. Proteases are one of the most widely used enzymes and contribute about 60% of the world's total enzyme production [3]. Proteases have some characteristics that were suitable for various applications, for example in food processing, detergent, pharmaceutical and leather industries [4].

The acetone purification method has an advantage over dialysis since by precipitating the proteins in acetone, buffer or any contaminant can be removed and the protease can be concentrated into a pellet which can be re-dissolved by other solvents [5]. Besides, acetone is relatively inexpensive and available in a pure form with few contaminants that may inhibit the enzyme activity or make the enzyme poisonous [5]. Nowadays, most manufacturers of pharmaceutical, chemical and food industries have shift to use the immobilised enzymes in their production process [6]. The immobilisation method is the entrapment of enzymes in a polymeric network of natural or synthetic sources, it acts as a permeable membrane for the substrates and products pass through after reacting with the enzyme that resides inside the network [7]. According to Nawaz *et al*. [8], alginate, polyacrylamide and agar-agar are some of the sources that can be used for the immobilisation method. This method makes the separation process of biocatalyst become easier and simple, reduced processing cost and make the enzyme have greater stability in organic solvents and higher temperatures [6].

Therefore, this immobilised method was chosen to be used in this study as immobilised protease from silver catfish viscera has the potential to be utilised in diverse industrial applications and thus can contribute significantly to reduce local pollution problems. Lastly, the optimisation of the immobilisation condition by using response surface methodology (RSM) can help in obtaining optimum proteolytic activity of the protease thus producing high quality of immobilised enzyme.

MATERIALS AND METHODS

Silver catfish (Pangasius sutchi) viscera were obtained from silver catfish river cage, Paloh Hinai, Pahang. The viscera were then properly packed in a vacuum plastic bag and stored in a cooler box containing ice during transportation. The chemicals used were calcium chloride, sodium alginate, acetone, bovine serum albumin (BSA), casein, trichloroacetic acid (TCA), distilled water, Tris-HCL (pH 8.0), L-tyrosine, hydrochloric acid. All chemical reagents (New England Biolabs, USA and MP Biomedicals, Australia) which were used for analysis are of analytical grade and purchased from Next Gene Scientific Sdn Bhd, Malaysia.

Preparation of Sample

The viscera were rinsed, weighed, placed in an airtight freeze-dryer flask and stored in the freezer (-40°C) for 24 hours. Next, the frozen viscera undergo a freeze-drying process at -47°C and 0.133 bar by using a freeze dryer (ALPHA 1-4 LD plus, Christ, Germany) for seven days.

Extraction of Crude Protease

The freeze-dried viscera were grounded and homogenized at 1:1 ratio with 25 mM Tris–HCl (4°C, pH 8.0, 30 seconds) [9]. Then centrifuged at 10, 000 rpm (15 minutes, 4°C) by using a microcentrifuge instrument (Centrifuge 5418, Eppendorf, Germany) [10]. The supernatant (crude protease) was collected.

Purification using Acetone

Crude protease (0°C) and acetone (precooled to -20°C for at least 1 hour) were used at ratio 1:1. The cold acetone was slowly added into the supernatant, agitated gently at least for 10 to 20 minutes to allow precipitation and followed by centrifugation at 10, 000 rpm (10 minutes, 4°C) using a microcentrifuge instrument (Centrifuge 5418, Eppendorf, Germany). The precipitate was air-dried and carefully blotted with filter paper to remove the excess liquid. The pellets were dissolved with 0.1 M Tri-HCl buffer (pH 8.0) and centrifuged again at 10, 000 rpm (20 minutes) then the supernatant was collected [11].

Optimisation of Immobilisation Condition with Calcium Alginate

Response surface methodology (RSM) with central composite design (CCD) was used to generate the factorial design (two factors, five levels, and single block) by using Design-Expert software version 11.0 (StatEase, USA) The CCD is composed of 13 treatments. The two independent variables; sodium alginate solution (X_1,% w/v) and calcium chloride solution (X_2, M) were employed at five levels (-a, -1, 0, +1, and +a) for the determination of the optimum immobilisation condition for the protease. The test variables' values listed in Table 1 were applied to Design-Expert Software Version 11 to obtain the experimental design. Proteolytic activity was measured as a response to the independent variables. Immobilisation was performed according to Geethanjali and Subash [7].

Table 1: Independent Variables, Coded Values, Actual Levels and Ranges of Parameters used in the RSM Design in Optimising the Immobilisation Condition of Protease Extracted from Silver Catfish (Pangasius sutchi)

Factor	Levels				
	(-a)	-1	0	1	(a)
X1	1	2	3	4	5
X2	0.1	0.2	0.3	0.4	0.5

X_1: sodium alginate concentration (%w/v), X2: calcium chloride concentration (M)

Verification

The optimum condition predicted by the Design Expert version 11.0 software was used to repeat the immobilisation step. The verified value must be testified to be no significant difference at 5% level when compared to the predicted value of proteolytic activity by using *t*-Test (SPSS) Statistic 21.0 version.

Analysis of the Proteolytic Activity

Proteolytic activity of immobilised protease obtained under the optimum condition was determined according to Drapeau [12] with slight modification in which for immobilised protease, 0.5 g of beads was mixed with 10 ml enzyme diluent to make the enzyme solution. The total activity was determined by using the casein digestion unit analytical method (CDU) and was calculated according to the following formula [9] [12].

$$Casein\ Digestion\ Unit\ (CDU/mg) = \frac{Et - Eb}{Es}\ x\ 50\ x \frac{11}{10} x\ DF$$

Et = Absorbance of sample
Eb = Absorbance of blank
Es = Absorbance of standard (Tyrosine)
DF = Dilution factor of enzyme solution in mg

Analysis of Variance (ANOVA) was used to analyse the data at a 5% level and all measurements were carried out in triplicate. Differences between means were identified by using Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Optimisation of Immobilisation Condition of Protease using Response Surface Methodology (RSM)

In this study, immobilisation was conducted by entrapment of protease within an enclosed gel of calcium alginate beads. Table 2 shows the highest

actual and predicted responses were at run 12 with 674.77 CDU/mg and 639.26 CDU/mg, respectively, under predetermined factors, in which the sodium alginate concentration was 3.00% (w/v) and 0.30 M calcium chloride solution. The lowest actual and predicted responses were at run 2 with 77.35 CDU/mg and 71.53 CDU/mg, respectively, whereby factors include using sodium alginate of 4.00% (w/v) and 0.20 M of calcium chloride solution.

Different concentrations of sodium alginate will affect the degree of cross-linking of the gelling agent thus will also affecting the pore size of the calcium alginate beads. The pore size of the beads is the crucial factors that allow diffusion of substrate and product in and out from the beads or the permeable membrane in order to react with the enzyme resides within the polymeric network of the alginate [7]. Based on Table 2, it can be observed that when the sodium alginate was used at the highest concentration of 4.00 (run 2) and 4.41% (w/v) (run 6) the proteolytic activity of the immobilised protease decreased drastically to 77.35 and 109.15 CDU/mg respectively. While, when the lowest concentration of 1.59% (w/v) sodium alginate (run 5) was used, the proteolytic activity was 422.10 CDU/mg but it is still considered to have low proteolytic activity.

Several researchers reported that sodium alginate concentration in the range of 2.00 to 3.00% was suitable to be used for immobilisation of keratinase, lipase and proteases [13] [14]. Keerti *et al*. [14] added that when sodium alginate was used below 3.00% (w/v), the cross-linked between the gels become less tighten and thus causes larger pore sizes to develop. Meanwhile, above 3.00% (w/v), the enzyme alginate matrix becomes more viscous and causes a lack of uniform pore size and the beads have less porosity. Based on Keerti *et al*. [14] and Geethanjali and Subash [7], it can be concluded that the proteolytic activity in this study was decreased due to enzyme leakage from the beads through the large pore during washing or storage when 1.59% (w/v) of sodium alginate was used. On the other hand, with 4.00 and 4.41% (w/v) sodium alginate, the smaller size of the pore hindered the diffusion of the substrate to react with the enzyme within the beads thus minimum reaction occurred and lower proteolytic activity was observed.

Ahmed *et al*. [15] also found that immobilisation of enzyme invertase from *Bacillus macerans* was the best when using 0.30 M calcium chloride.

Geethanjali and Subash [7] also reported that 0.30 M calcium chloride had the highest % immobilisation (48.3%) at the end of the immobilisation assay when compared to 0.10 M (29%), 0.20 M (36%), 0.40 M (31%) and 0.5 M (24%) calcium chloride. Adinarayana *et al*. [16] stated that with 0.375 M calcium chloride, the gelling process of the beads was too rapid and case hardening effect was noticed. Besides, the number and the size of pores also decreases as the concentration of calcium chloride increased [16].

Below 0.30 M calcium chloride, the formed beads had an irregular shape and take a longer time to harden in the calcium chloride solution and thus may affect the amount of entrapped enzyme within the beads. This can be observed by comparing the proteolytic activity at run 7 (333.70 CDU/mg) and 12 (674.77 CDU/mg), which used 0.16 M and 0.30 M of calcium chloride, respectively. This observation aligned with Geethanjali and Subash [7] who found an irregular shape of beads formation when calcium chloride solution was used below 0.30 M.

Response Surface Regression for the Optimisation of Immobilisation Condition of Protease

Based on Table 2, the second-order polynomial equation for optimisation of immobilisation condition of protease was derived and illustrated in the following uncoded equation:

$$Y = -900 + 782X_1 + 2833X_2 - 195.1X_1^2 - 7764X_2^2 + 888X_1X_2 \quad \text{(Equation 1)}$$

Where X_1 = sodium alginate (%w/v), X_2 = calcium chloride (M), Y = activity

In this study, adjusted R2 (93.78%) and R^2 (96.37%) value were obtained and it indicates a high dependency and interaction between the observed and predicted values of response and this result was aligned with Henseler *et al*. [17] and Moore *et al*. [18] studies. The adjusted R^2 is the corrected value of R^2 after some unimportant model terms were removed. If there are many insignificant terms incorporated in the model, the value of adjusted R^2 would be smaller than R^2. Next, the R^2 value of 96.37% means that the variability in the observed response values could be interpreted by the model and only 3.63% of the variability in the observed response values cannot be interpreted by the model obtained and could be due to others element which were not incorporated in the model. The model is

fit and reliable to be used as the R2 value (96.37%) being nearer to 1, that indicated that the model has a better interaction between the experimental and predicted values.

Analysis of Variance (ANOVA) for the Optimisation of Immobilisation Condition of Protease

Analysis of variance (ANOVA) was used to test the suitability and fitness of the experimental variables on the linear, quadratic and interaction terms. The results are shown in Table 3. Regression, linear and quadratic factors were highly significant as indicated by higher F values of 37.22, 33.33 and 53.75 respectively, with a low *p*-value of 0.000 when compared to interaction factors that are least significant as it only has F value of 11.92 and *p*-value of 0.011. In general, a larger F value indicates a better fit of the RSM model to the experimental data as its forecasts the quality of the whole model with regards towards all the design factors all at the same time [19]. The *p*-value is the probability of the factors having a non-significant effect on the response [20]. Any significance in the lack-of-fit test indicates the model needs to be rejected, while an insignificant result indicates a good model [19]. In this study, the selected model showed an insignificant outcome ($p>0.05$) as the *p*-value is 0.161 indicating that the model is acceptable and suits well with the experimental data and there is a significant consequence of the parameters on the output response.

Table 2: Actual Levels of Independent Variables Used in Optimising the Immobilisation Condition of Protease Extracted from Silver Catfish (Pangasius sutchi) using Calcium Chloride and Sodium Alginate Solution and the Observed and Predicted (FITS1) Proteolytic Activity Values as Response

Run No.	Factors		Response (Proteolytic Activity (CDU/mg)	
	X1	X2	Y	FITS1
1	2.00	0.20	523.76	494.13
2	4.00	0.20	77.35	71.53
3	2.00	0.40	445.00	483.98
4	4.00	0.40	353.59	416.38
5	1.59	0.30	422.10	422.35
6	4.41	0.30	109.15	75.73
7	3.00	0.16	333.70	365.64
8	3.00	0.44	667.40	602.31
9	3.00	0.30	632.04	639.26
10	3.00	0.30	579.00	639.26
11	3.00	0.30	667.40	639.26
12	3.00	0.30	674.77	639.26
13	3.00	0.30	643.09	639.26

Where: X_1= sodium alginate concentration (% w/v), X_2= calcium chloride concentration (M)

Table 3:ANOVA for Optimisation of Immobilisation Condition of Protease Extracted from Silver Catfish (Pangasius sutchi) using Sodiu Alginate and Calcium Chloride Solution

Source	DF	Adj SS	Adj MS	F	P	Status
Regression	5	491763	98353	37.22	0.000	Significant
Linear	2	176160	88080	33.33	0.000	Significant
Quadratic	2	284097	142048	53.75	0.000	Significant
Interaction	1	31506	31506	11.92	0.011	Significant
Residual Error	7	18499	2643			
Lack of Fit	3	12749	4250	2.96	0.161	Not significant
Pure Error	4	5751	1438			
Total	12	510263				

Where: DF = degree of freedom, Adj SS = adjusted sum of square, Adj MS = adjusted mean square, F = fischer, P = probability

Response Optimiser and Overlaid Contour Plots at the Optimum Condition and Its Feasibility

Figure 1 shows the results at the optimum condition for the target goal after the response optimiser was acquired. Based on Figure 1, the optimal desirability for the target goal is acceptable as it is close to 1, which is 0.968 and is in agreement with Amdoun *et al*. [21] who had stated that if several responses fall within the unacceptable intervals, the desirability will be close to 0, while if within the acceptable interval the desirability will be closed to 1. Next, based on Figure 2 and Table 4, the optimum conditions for the target goal (△) with sodium alginate of 2.99% (w/v) and 0.30 M calcium chloride were feasible to be carried out. As based on the overlaid contour plots in Figure 2, the optimum conditions for the target goals (△) were situated in the white part or within the feasible zone while the optimum condition for the maximum (⯎) and minimum goals (☆) were located in the grey area or non-feasible region. The feasible zone is an area that the acceptable values for each response are between their specific contours and the possible combination of variable settings can be acquired [22]. Therefore, the optimum condition for the target goal (△) was selected as it is feasible to be carried out.

Contour and Surface Plots of Proteolytic Activity at a Feasible Optimum Condition

The 2D contour and 3D surface plots, as presented in Figure 3 and 4, respectively, showed the effects of sodium alginate (% w/v) and calcium chloride (M) on the proteolytic activity of the immobilised protease. The appearance of the contour plot whether it is circular or elliptical will specify the correlative relationship between the test variables as insignificant or significant, respectively [23]. In this study, an elliptical shape for the contour plot was obtained and indicates a significant relationship between the sodium alginate (% w/v) and calcium chloride (M) on the proteolytic activity of the immobilised protease. Thus, the model is acceptable and fits well with the experimental data. Furthermore, the surface plot showed that the proteolytic activity of the immobilised protease increased at the middle level of sodium alginate (% w/v) and calcium chloride (M). By analysing the contour and surface plots (Figure 3 and 4), the predicted proteolytic

activity is observed to be 638.19 CDU/mg and lies in the following ranges of the examined variables such as sodium alginate 2.99% (w/v) and calcium chloride 0.30 M.

Verification of the Optimum Condition of Protease Immobilisation

In Table 5, the suitability of the model equation for predicting the optimum response value was evaluated for the optimum condition of protease immobilisation under conditions where the sodium alginate is 2.99% (w/v) and calcium chloride is 0.30 M. Optimisation using actual experimental values was tested using the *t*-Test (SPSS) and there was no significant difference ($p > 0.05$) between predicted and verified values. Thus, indicating that the model was significant and can be used to produce the immobilised protease under optimum condition.

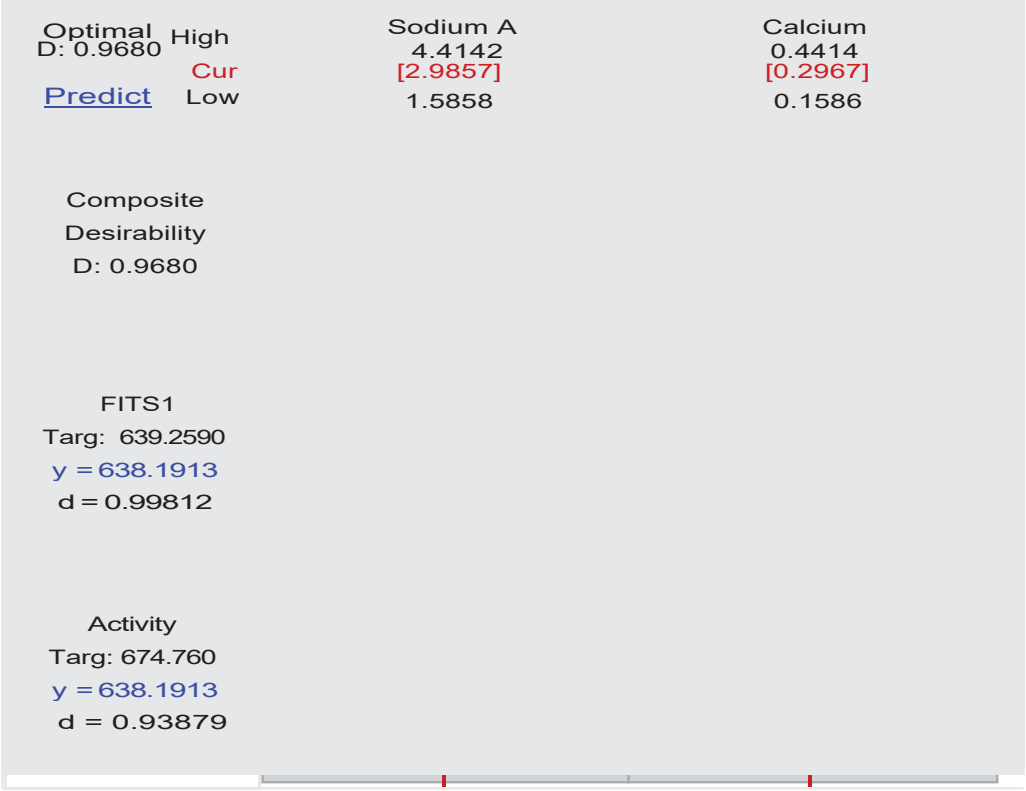

Figure 1: The Response Optimiser at the Optimum Condition for the Target Goal

Table 4: Comparison Values of Target and Predicted Responses for DiGerent Optimum

Goal		Lower	Target	Upper	Optimum condition		Predicted Response	/NF
					X1	X2	(FITS)	
Target	Activity (CDU/ mg)	77.35	674.76	674.77	2.9857	0.2967	638.1913	F
	FITS 1	71.531	639.259	639.260				
Maximum	Activity (CDU/ mg)	77.35	674.77	674.77	2.7857	0.3414	670.0220	NF
	FITS 1	71.531	639.260	639.260				
Minimum	Activity (CDU/ mg)	77.35	77.35	674.77	4.4142	0.1586	375.3930	NF
	FITS 1	71.531	71.531	639.260				

Where: X_1= sodium alginate (%w/v), X_2= calcium chloride (M), FITS= predicted response (%), F= feasible, NF= not feasible

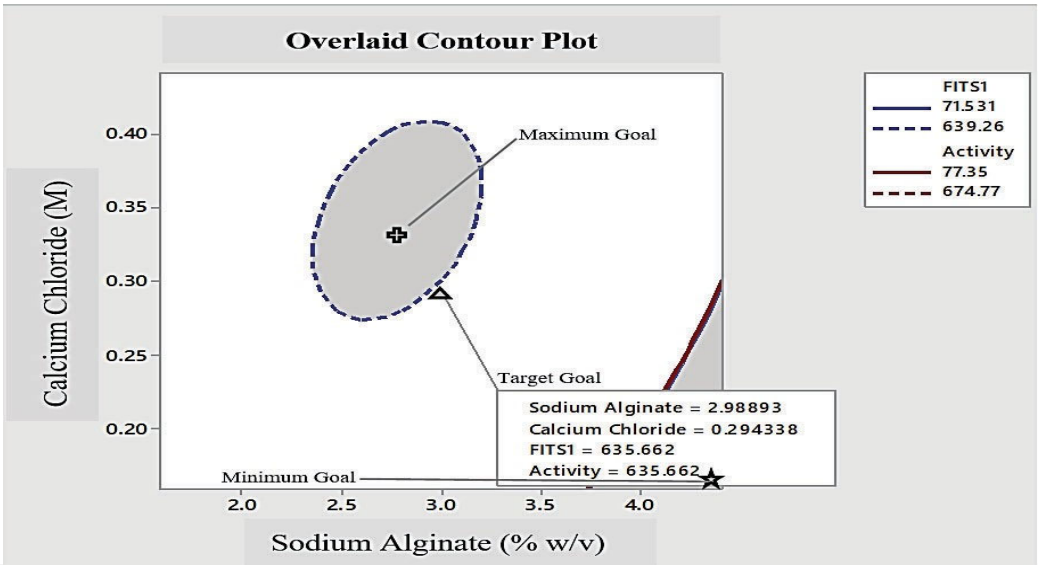

Figure 2 Overlaid Contour Plot at the Optimum Condition for the Target Goal(△), Maximum Goal(✚)and Minimum Goal (☆)

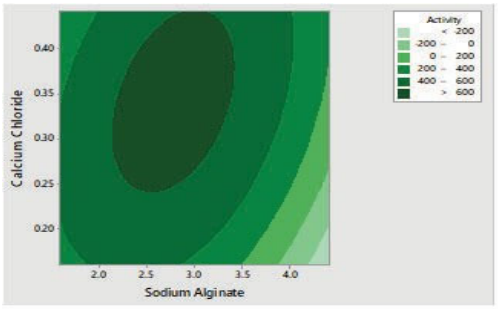

Figure 3 Contour Plot of Proteolytic Activity at a Feasible Optimum Condition. With a Sodium Alginate of 2.99% (w/v) and Calcium Chloride of 0.30 M.

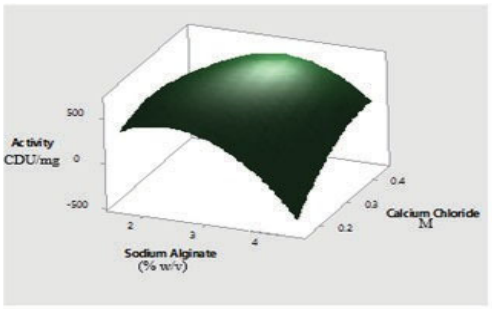

Figure 4 Surface Plot of Proteolytic Activity at a Feasible Optimum Condition. With a Sodium Alginate of 2.99% (w/v) and Calcium Chloride of 0.30 M

Table 5: Comparison of the Verified and Predicted Values of Proteolytic Activity (CDUlmg) at Feasible Optimum Conditions

Optimum condition		Proteolytic activity (CDU/mg)	
X1	X2	V	P
2.99	0.30	699.82a	638.19a

Where: X1= sodium alginate (%w/v), X2= calcium chloride (M), V= verification value, *P= predicted value*

CONCLUSION

The feasible optimum conditions obtained in this study was with sodium alginate of 2.99% (w/v) and 0.30 M calcium chloride solution. Verification for the optimum condition showed no significant diRerence ($p > 0.05$) between predicted (638.19 CDU/mg) and verified (699.82 CDU/mg) values. Thus, the polynomial equation model derived can be used to produce the immobilised protease. The immobilised protease can be utilised in diverse industrial applications and thus can contribute significantly to reduce local pollution problems. Besides, the optimisation process is important in order to obtain optimum proteolytic activity and producing high-quality and economical immobilised protease. Lastly, by using fresh water fish such as silver catfish (*Pengasius sutchi*) viscera, the Muslim society's doubtfulness will be relieved as the process of slaughtering and its source will not be an issue.

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