Malaysian Journal of Chemistry, 2023, Vol. 25(3), 391-399
(Special Issue: *3rd IKMPB Online Symposium 2023 - Innovation in Chemistry Towards Sustainable Development*)

Optimization of Protease Extraction from Ridged Gourd (*Luffa acutangula)* Sarcocarp via Response Surface Methodology

Nur Fatin Nadia Mohd Dzakir[1], Erna Normaya Abdullah[1], Nur Amanina Hassanuddin[1], Syamimi Sulfiza Shamsuri[1], Anwar Iqbal[2], Mohd Bijarimi Mat Piah[3] and Mohammad Norazmi Ahmad[1]*

[1]Experimental and Theoretical Research Lab, Department of Chemistry, Kulliyyah of Science International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia
[2]School of Chemical Science, Universiti Sains Malaysia, 11800 Penang, Malaysia
[3]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, 26300 Kuantan, Pahang, Malaysia
***Corresponding author (e-mail: mnorazmi@iium.edu.my)**

Recently, the widespread use of proteases in industries, such as detergent, food, and pharmaceutical sectors, has increased the demand for proteolytic enzymes in the global market. Although the main sources of commercial proteases are animals and microorganisms, the potential of using nonconventional sources, especially plants, should not be overlooked. In this study, proteolytic enzymes were extracted from the sarcocarp of ridged gourd (*Luffa acutangula*), and the effect of the extraction process on protease activity was evaluated. The crude enzyme was optimized via response surface methodology (RSM) using a central composite rotatable design (CCRD). Four independent variables were studied, namely, the pH, the concentrations of Triton X-100 (TX-100) and 2-mercaptoethanol, and the mixing time. The optimum level of each variable based on the RSM model was determined: at pH 6.38, 4.99% (v/v) TX-100, 0.15 M 2-mercaptoethanol, and a mixing time of 4.09 min, the optimum protease activity was estimated at 1.35 U/g. A verification test revealed satisfactory agreement between the model and experimental results with 96% desirability, indicating that the quadratic model generated from RSM is significant.

Keywords: Optimization; Response Surface Methodology; Central Composite Rotatable Design; *Luffa acutangular*; protease

Received: January 2023; Accepted: April 2023

Proteases are a group of enzymes that mainly catalyze the digestion of proteins by hydrolyzing the peptide bonds to generate small groups of amino acids. These enzymes are essential components in all organisms due to their physiological function as "enzymes of digestion". In recent years, the use of proteolytic enzymes has also led to significant contributions in various industrial sectors, including food processing, pharmaceutical, leather tanning (with detergents), and chemical applications [1].

In general, four main sources of proteases can be distinguished based on their origin: microbes, animals, plants, and humans [2]. Currently, in industrial sectors, proteases are primarily derived from animal sources, such as cows and pigs, and microbial sources, including bacteria, fungi, and viruses. However, both these sources represent certain limitations, especially considering food consumers, due to certain religious requirements. Food restrictions are present within some religions; for example, pork and non-ritually slaughtered meat and their derivatives are prohibited in Judaism and Islam, while pork and beef are prohibited for Hindu and Buddhist believers [3]. Therefore, discovering alternative sources of proteases, especially plants, has become a major area of investigation among researchers to counteract the limitations.

Generally, the extraction of plant enzymes is comparatively complex due to the presence of poly-phenol oxidases and high concentrations of polyphenolic compounds [4, 5]; both these chemicals can promote enzymatic browning, resulting in enzyme inactivation and degradation [6]. Moreover, enzyme activity depends on the plant sources used, the climatic conditions for growth, and the extraction and purification methods [7]. Poor recoveries from protease extraction are further aggravated by the fact that proteases can be cellularly bound. Therefore, researchers investigating plant proteases need to design suitable media that include various variables, such as detergents, reducing agents, and activators, to optimize protease extraction.

Conventional response optimization strategies involve the change of one variable at a time; each significant factor is optimized by changing it while keeping the remaining factors constant [8]. However, this standard approach is time-consuming and costly, especially when several variables are considered. This limitation can be addressed by using an alternative method, namely, response surface methodology (RSM).

†Paper presented at the 3rd IKMPB Online Symposium 2023 - Innovation in Chemistry Towards Sustainable Development

RSM has been defined as an advanced approach useful for studying the effect of several variables on the responses by varying them simultaneously and conducting a smaller number of experiments [9]. The technique has been developed through the combination of mathematical methods and a statistical approach; it is useful for developing, enhancing, and optimizing processes and has been successfully implemented in many areas of food chemistry, including food processing and sensory analysis [10].

Recent studies have reported that certain members of the Cucurbitaceae plant family, such as melon fruit, muskmelon, and watermelon, exhibit protease activity [11, 12]. However, no research has been conducted on *Luffa acutangula*, which is highly available in subtropical and tropical Asian regions, including Malaysia. Therefore, in this study, the extraction of proteases from ridged gourd was optimized using RSM and a central composite rotatable design. The following four parameters were optimized to extract proteases from ridged gourd sarcocarp: the pH, the concentrations of Triton X-100 and 2-mercaptoethanol, and the mixing time.

EXPERIMENTAL

Chemicals and Materials

Ridged gourd *(L. acutangula)* fruits were bought from a market in Kuantan, Pahang, Malaysia. The peel was removed, and the sarcocarp of the fruits were chopped into small pieces before subjected to protease extraction. Chemicals used were Triton X-100 (TX-100) ($C_{34}H_{54}O_{11}$), trichloroacetic acid (TCA) ($C_2HCl_3O_2$), sodium hydroxide (NaOH) from R&M, Folin-Ciocalteu (Folin Reagent) from Merck, tris(hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) from Systerm, tyrosine ($C_9H_{11}NO_3$) from Fluka, sodium carbonate (Na_2CO_3), monobasic sodium phosphate (NaH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) purchased from Riedel-de Haen, and 2-mercaptoethanol ($C_2H_6SO_4$), hydrochloric acid (HCl), glycine ($C_2H_5NO_2$) and bovine casein which are purchased from Sigma Aldrich.

Extraction of Crude Extract

The enzyme was extracted based on Ahmad et al. [13] method with slight modifications. Twenty grams of the chopped fruit were blended in chilled 100 mL of appropriate buffer solution (50 mM phosphate buffer, pH 6, 50 mM Tris(hydroxymethyl)amonomethane (Tris) buffer, pH 8-, and 50-mM glycine- NaOH buffer, pH 10 respectively) containing 2- mercaptoethanol and Triton X-100 solution using a blender for 4 to 10 minutes. Then the crude extract was collected by filtering the resultant mixture. The filtrate was then centrifuged at 15000 x *g* for 20 minutes (Multifuge X1R, ThermoScientific, Langenselbold, Germany) at 4°C. The supernatant obtained was stored at 4°C until further analysis.

Optimisation of Variables using a Central Composite Rotatable Design (CCRD)

Protease extraction was carried out based on four independent variables, namely, the pH, the concentrations of Triton X-100 (TX-100) and 2-mercaptoethanol, and the mixing time. The tested variables were selected based on Ahmad et al.'s [37] parameters, with slight modifications, and were studied at three different levels (low, medium, and high), as shown in Table 1. The extraction parameters were optimized using RSM and a central composite rotatable design (CCRD) to obtain the highest protease activity of the *L. acutangula* extract. The CCRD was used to assign the treatment combinations, as shown in Table 2. The RSM method was implemented using the statistical software package Design Expert 6.01 (Stat-Ease, Minneapolis, USA).

Protease Activity Assay

The protease activity was determined based on the release of tyrosine (µmol/min), using the substrate casein and the Folin–Ciocalteu reagent [13]. A reaction mixture containing 0.9 mL of a 50 mM phosphate buffer (pH 7.5), 5 mL of a 0.65% (w/v) casein solution, and 0.1 mL of the crude enzyme was incubated at 37 °C for 10 min. The reaction was then terminated via the addition of 5 mL of 110 mM trichloroacetic acid (TCA). The unhydrolyzed casein was centrifuged at 4000 x *g* for 20 min, after which 2 mL of the supernatant was mixed with 5 mL of 500 mM sodium carbonate, Na_2CO_3, and 0.5 mL of the Folin–Ciocalteu reagent (twofold dilution). The mixture was then incubated at 37 °C for 30 min, and the absorption of the solution at 660 nm was determined using a single-beam UV-Vis spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan). One U of enzyme activity was defined as the amount of the enzyme that liberates 1.0 µmol of tyrosine/min under the assay conditions.

Table 1. Coded and actual variable levels as determined by the Central Composite Rotatable Design (CCRD).

Independent Variables	Symbol	Coded Variable level			Actual Variable level		
pH	x_1	-1	0	1	6.0	8.0	10.0
TX-100 (%)	x_2	-1	0	1	0.00	2.50	5.00
2-Mercaptoethanol (M)	x_3	-1	0	1	0.00	0.075	0.150
Mixing Time (minutes)	x_4	-1	0	1	4.0	7.0	10.0

Table 2. Treatment combinations of variables and response.

Run	x_1: pH[a]	x_2: TX-100 (%)	x_3: 2-mercaptoethanol (M)	x_4: Mixing Time (min)	Protease Activity (Unit/g)
1	6.00	5.00	0.00	10.00	0.15
2	6.00	0.00	0.00	10.00	0.12
3	10.00	5.00	0.00	10.00	0.12
4	6.00	5.00	0.150	10.00	1.35
5	10.00	5.00	0.00	4.00	0.23
6	6.00	0.00	0.00	4.00	0.12
7	8.00	2.50	0.075	7.00	0.74
8	10.00	0.00	0.00	10.00	0.12
9	6.00	5.00	0.150	4.00	1.40
10	10.00	5.00	0.150	10.00	1.24
11	8.00	2.50	0.075	7.00	0.84
12	10.00	0.00	0.00	4.00	0.16
13	8.00	2.50	0.075	7.00	0.77
14	8.00	2.50	0.075	7.00	0.84
15	6.00	0.00	0.150	10.00	1.41
16	6.00	5.00	0.00	4.00	0.20
17	10.00	0.00	0.150	10.00	1.25
18	10.00	5.00	0.150	4.00	1.33
19	10.00	0.00	0.150	4.00	1.22
20	6.00	0.00	0.150	4.00	1.31
21	8.00	2.50	0.075	4.00	0.81
22	8.00	2.50	0.075	7.00	0.85
23	8.00	2.50	0.075	10.00	0.75
24	10.00	2.50	0.075	7.00	0.75
25	6.00	2.50	0.075	7.00	0.75
26	8.00	2.50	0.00	7.00	0.11
27	8.00	5.00	0.075	7.00	0.75
28	8.00	2.50	0.150	7.00	1.23
29	8.00	0.00	0.075	7.00	0.79
30	8.00	2.50	0.075	7.00	0.75

[a] Buffers pH 6, 8 and 10 were obtained from 50 mM phosphate buffer, 50 mM Tris(hydroxymethyl)aminomethane (Tris) buffer and 50 mM glycine- NaOH buffer, respectively.

Standard curve of tyrosine was plotted against a concentration range of 0 - 100 µg/mL. Total protease activity was calculated using equation 1 below:

$$\textit{Protease activity (U/g)} = \textit{(IU) x (100/Wt) x (1/0.1) x DF} \quad (1)$$

Where IU = µmole tyrosine/min; DF = dilution factor; Wt = weight of sample in g; 0.1 = volume of enzyme used (in milliliters).

Statistical Analysis and Modelling

After the completion of RSM experiments, the optimal conditions and interaction effects of variables were analyzed using the statistical software RSM. The significance of the model was analyzed by analysis of variance (ANOVA) and the protease activity was evaluated and expressed by the equation 2:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 \quad (2)$$

Where, Y = predicted response; b_0 = intercept; b_1, b_2, b_3, b_4 = coefficient estimates of linear terms; b_{11}, b_{22}, b_{33}, b_{44} = coefficient estimates of quadratic terms; b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} = coefficient estimates of interaction terms; x_1 = pH; x_3 = 2-mercaptoethanol; x_2 = TX-100 and x_4 = mixing time

Verification Test at Optimum Level

A verification test with respect to protease activity from the same sample was conducted at optimum levels of the variables predicted by the model obtained using RSM. Triplicate experiments were conducted, and the protease activity was determined to validate the predicted optimised conditions obtained.

RESULTS AND DISCUSSION

The central composite design was used to obtain the optimal conditions of independent variables during the extraction of protease from *L. acutangula*. Thirty runs of experiments had been conducted and the results of protease activity, which is the experimental responses had been tabulated in Table 2.

The significance of the model was tested using the ANOVA (Table 3) to confirm the satisfactory adjustment of the quadratic model to the experimental data. As observed from Table 3, the regression model is significant ($p < 0.001$), while the lack-of-fit test value is insignificant, 0.9316 ($p > 0.05$), which is desirable in signifying the presence of the systematic curvature [10]. The lack-of-fit test was performed to describe the variation of the data around the model, with a good model including an insignificant lack of fit ($p > 0.05$) [14]. According to Fakhri [15], the coefficient of determination (R^2) can be used to assess the quality of the fitted model. In this study, the R^2 value of 0.9963 signifies a satisfactory goodness of fit since a value of 0.75 indicates the aptness of a model [13, 16, 17]. The adjusted R^2 ("Adj R-Squared") value obtained herein is 0.9926 (99.26%); according to Sadhukhan et al. [35], if this value is higher than 70%, the quadratic model represents a good fit and can be used to identify the optimal conditions of the process. Moreover, if the model comprises many terms and the sample size is small, the adjusted R^2 value will be smaller than R^2 [18]. The predicted R^2 explains the variability in predicting new data sets [19]. The "Pred R-Squared" of 0.9882 is in reasonable agreement with the "Adj R-Squared" of 0.9926, with a difference of less than 0.2, which indicates that the model is highly significant [20] and that the response variables can be explained accurately using the model.

Adequate precision is essentially a measure of the signal-to-noise ratio (S/N), which allows one to judge or perceive if the model is "adequate" in navigating the design space and enabling the prediction of the response [10]. The adequate precision value for the model herein is 43.848, which represents an adequate signal since values greater than 4 are desirable. The coefficient of variation, CV, is the standard deviation expressed as a percentage of the mean [10]; since the CV measures the amount of variability relative to the mean, the lower its value, the more precise, dependable, and reliable the model [21]. In this study, the CV obtained is 5.35%, which can be considered a low CV value. Meanwhile, according to Bisht et al. [22], a model with a CV not greater than 10% can be considered practically reproducible. How a particular model fits each point in a design is assessed by the predicted residual error sum of squares (PRESS) value; a PRESS value of 0.072 was obtained herein. Myers and Montgomery concluded that a small PRESS value indicates that the model has good predictive ability as the smaller the PRESS value, the better the model can fit each point in the design [23, 36].

The protease activity from *L. acutangula* was expressed by the following regression equation 3:

$$Y = -0.10584 + 0.063515x_1 + 0.016422x_2 + 11.34535x_3 - 0.012677x_4 - 0.00293068x_1^2 + 0.00192436x_2^2 - 15.82626x_3^2 + 0.00196970x_4^2 + 0.000455x_1x_2 - 0.197x_1x_3 - 0.001967x_1x_4 - 0.001967x_1x_4 - 0.013467x_2x_3 - 0.00324667x_2x_4 + 0.049222x_3x_4 \quad (3)$$

Table 3. Analysis of variance for the evaluation of the quadratic model.

Source of variation	Sum of squares	Degree of freedom	Mean square	F value	*p* value
Model	6.09	14	0.43	268.38	<0.0001 significant
Residual	0.023	14	0.001615	-	-
Pure error	0.012	4	0.003114	-	-
Lack of fit	0.01	10	0.001015	0.33	0.9316 not significant
Total	6.11	29	-	-	-

*R^2 = 0.9963, coefficient of variation = 5.35%, PRESS = 0.072, Adj R-squared = 0.9926

Where, Y = predicted response; b_0 = intercept; x_1, x_2, x_3, x_4 are linear terms of pH, TX-100, 2-mercapto-ethanol and mixing time respectively, x_1^2, x_2^2, x_3^2, x_4^2 were quadratic terms of pH, TX-100, 2-mercapto-ethanol and mixing time respectively and x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4, x_3x_4 represent the interaction terms of the variables tested. A positive coefficient indicates that as the value of the independent variable increases, the mean of the dependent variable also tends to increase. A negative coefficient suggests that as the independent variable increases, the dependent variable tends to decrease.

Figure 1 shows the normal probability plot, which represents the significance of the variables toward protease activity. If the residual plot lies approximately along a straight line, then the normality assumption is satisfied [10]. Figure 1 reveals no apparent issues with normality. One can note that the experimental data are aligned with the probability graph of a straight line, indicating well-fitted data.

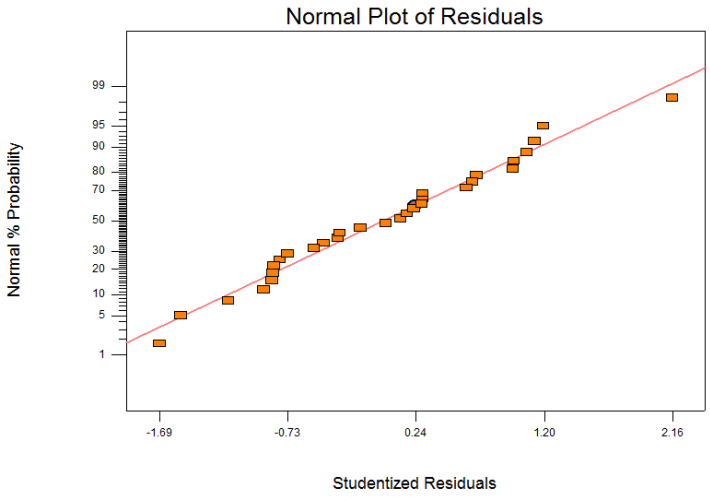

Figure 1. Normal probability.

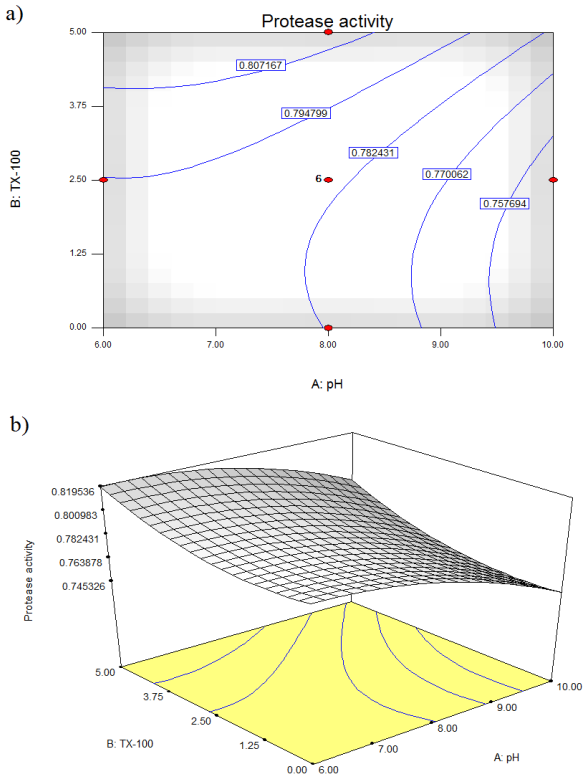

Figure 2. Effects of pH and TX-100.

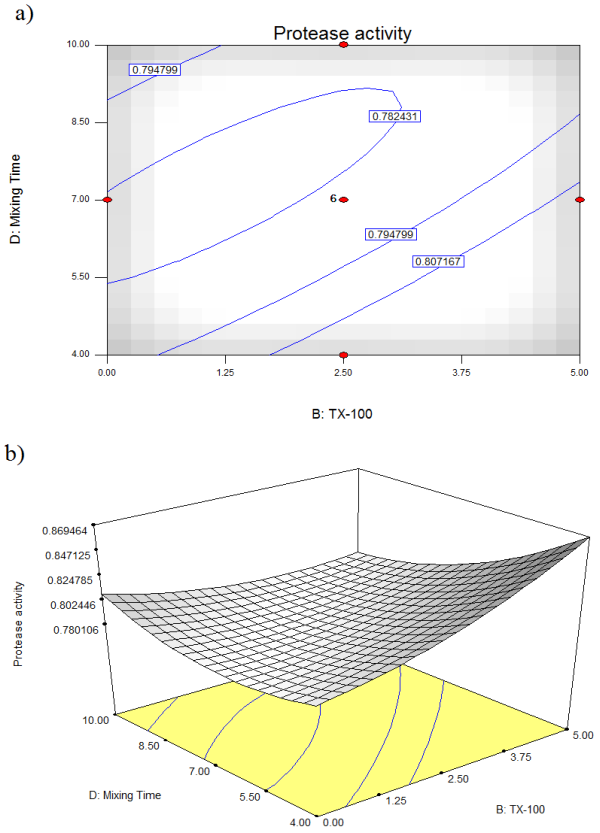

Figure 3. Effect of TX100 and Mixing time.

The results were also analyzed using 3D response surface and contour plots (Figures 2 and 3) to determine the optimum level of the variables by considering their effects on protease activity. The optimum protease activity obtained from the RSM model is 1.36 U/g, and the optimum values obtained for each parameter are x_1 (pH) = 6.38, x_2 (TX-100 concentration) = 4.99%, x_3 (2-mercaptoethanol concentration) = 0.15 M, and x_4 (mixing time) = 4.01 min. A verification test (n = 3) of the protease extraction was performed at the optimum conditions, yielding an experimental value of 1.31 ± 0.17 U/g for protease activity, which is close to the predicted value of 1.36 U/g. The proximity of the experimental and predicted values (differing by only 0.05 U/g) represents a satisfactory agreement between the values and supports the model's validity. These results can also serve to confirm the applicability and accuracy of RSM in optimizing protease extraction from *L. acutangula* sarcocarp.

According to Ahmad et al. [13], the t-value also influences the strength of the variable effect on the response; the larger the t-value, the stronger the effect of the variable on the response. The variables are divided into three groups: main (pH, TX-100, 2-mercaptoethanol, and mixing time), quadratic (pH^2, $TX\text{-}100^2$, $2\text{-mercaptoethanol}^2$, and mixing time^2), and interaction effects (pH.TX-100, pH.2-mercaptoethanol, pH.mixing time, TX-100.2-mercaptoethanol, TX-100. mixing time, and 2-mercaptoethanol.mixing time). The significance of these variables regarding the protease activity can be arranged in ascending order as x_2 (TX-100) < x_4 (mixing time) < x_1 (pH) < x_3 (2-mercaptoethanol) for the main factors; x_1^2 (pH^2) < x_2^2 ($TX\text{-}100^2$) < x_4^2 (mixing time^2) < x_3^2 ($2\text{-mercaptoethanol}^2$) for the quadratic factors; and x_1x_2 (pH.TX-100) < x_2x_3 (TX-100.2-mercaptoethanol) < x_3x_4 (2-mercaptoethanol.mixing time) < x_1x_4 (pH. mixing time) < x_2x_4 (TX-100.mixing time) < x_1x_3 (pH.2-mercaptoethanol) for the interaction terms.

Considering the orders of the variables mentioned above, the 2-mercaptoethanol concentration is the major factor in *L. acutangula* protease extraction, which can be explained by the larger t-value of x_3 (57.683) compared to x_1 (-2.157). This chemical helps reduce the oxidation that can occur within a protein, specifically on cysteine residues, which can otherwise lead to denaturation. According to Ho [24], disulfide bonds present in protein residues, including cysteine, are native structural elements of a majority of proteins and help stabilize their molecular structures. Based on the experimental results, the protease activity increases as the concentration of 2-mercaptoethanol is increased and approaches 0.15 M. This result agrees with the report of Ahmad et al. [37], where the addition of a reducing agent enhanced the protease activity. Since proteases consist of proteins, the presence of disulfide bonds must be preserved in order to maintain protease

activity. Therefore, 2-mercaptoethanol was used as a reducing agent to reduce the disulfides via interchange reactions, hence maintaining the protease activity and enhancing the total protease availability.

The pH of the extraction medium also results in a significant model term. A suitable pH (under optimum conditions) can allow one to control the activity of proteases and prevent them from being easily denatured. In addition, a suitable pH will increase the protease activity in the crude extract. According to Ahmad et al. [38], the natural pH for plant cells usually ranges between 6.0 and 7.0; the optimum pH of 6.38 in this study falls within this range. The protease activity increases as the pH increases until the optimum point before declining with further increases in the pH. Furthermore, the enzymatic process is strongly affected by even small changes in the hydrogen ion concentration [25]. Notably, the pH exerts a more significant effect (t = -2.157) than the mixing time (t = -1.563) on the protease activity. Therefore, reaching the optimum pH is important to stabilize the hydrogen ion concentration and allow for maximum enzyme activity.

Next, the concentration of TX-100 was included as one of the variables; TX-100 acts as a detergent during protease extraction due to its ability to isolate proteases from the membrane constituents via solubilization during the extraction process [13, 26, 27]. Some anionic detergents damage enzymes by altering their structure [28]; however, TX-100, which is less harmful as a nonionic detergent, helps maintain and preserve the structure of enzymes and their biological activities [29, 30, 31]. In this study, the optimum protease activity was achieved at a TX-100 concentration of 4.99%. This is aligned with the findings of Ahmad et al. [19], who used TX-100 as one of the parameters.

The mixing time, one of the variables considered in the protease extraction, is more significant than the TX-100 concentration; the absolute value of the t-value of the former (-1.563) is larger than that of the latter (1.486). Previous studies have also mentioned that the mixing time constitutes one of the significant factors that helps increase protease activity [32, 33]. The optimum mixing time in this study is 4.01 min; the protease activity decreases with further increases in mixing time, where longer times can cause protease denaturation due to intensive agitation. The shear forces of the Waring blender used can significantly damage the tertiary structure of proteins in solution if the mixing time is unsuitable. Furthermore, an inappropriate mixing time may damage the natural structure of the enzyme and lead to a decline in enzymatic activity [34]. As revealed by Ahmad et al. [19, 37], the optimum mixing time for protease activity is 5.35 min for cashew protease and 2 min for *Ambarella* protease; with longer mixing times, the vigorous shearing of the sample would lead to heat dissipation from the blades and denature the protease [33].

The optimum protease activity extracted from ridged gourd plant obtained in this research is 1.36 U/g which is in comparable with other sources. Table 4 shows the protease activity exhibited by ridged gourd plants in comparison to other protease extraction.

CONCLUSION

The protease activity of ridged gourd (*L. acutangula*) sarcocarp was optimised by central composite rotatable design in RSM. The crucial parameter affects the protease activity of crude extract were pH, Triton X-100, 2-mercaptoethanol and mixing time. From the results, it was found that the optimum conditions for this extract to produce high protease activity were pH 6.38, 4.99% Triton X-100(v/v), 0.15 M 2-mercapto-ethanol and 4.01 minutes of mixing time. The protease obtained in the verification test (n = 3) was 1.31 ± 0.17 unit/g, while the calculated value obtained by the RSM model was 1.36 U/g. Thus, this represents a satisfactory agreement of model's validity that is in between 96%. Therefore, it can be concluded that *L. acutangula* can be included as a source of protease extraction.

ACKNOWLEDGEMENT

This paper is a part of a project that was funded by the Malaysia Ministry of Higher Education (FRGS/1/2021/STG04/UIAM/02/2) and Royal Society of Chemistry (R21-0378989050).

Table 4. Protease activity extracted from ridged gourd in comparison to other sources.

Sources of Protease Extraction	Protease Activity
Ridged Gourd plant (*L. acutangula*) sarcocarp	1.36 U/g
B. cereus spp. isolated from fermented cabbage [38]	0.395 - 2.539 U/g
Horse mango (*Mangifera foetida* Lour)[13]	25.4 U/g

REFERENCES

1. Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., Priji, P., Josh, M. S. & Benjamin, S. (2013) Versatility of microbial proteases. *Advance in Enzyme Research*, **1(3)**, 39–51.

2. Motyan, J. A., Toth, F. & Tozser, J. (2013) Research applications of proteolytic enzymes in molecular biology. *Biomolecules*, **3***,* 923–942.

3. Bonne, K. & Verbeke, W. (2008) Muslim consumer trust in halal meat status and control in Belgium. *Meat Science*, **79***,* 113–123.

4. Westermeier, R. & Gorg, A. (1998) Protein purification: principles, high resolution methods and applications. *New York: John Wiley and Sons*.

5. Brueske, C. H. & Dropkin, V. H. (1980) Plants enzymology and histo enzymology. *New Delhi: Kalyani Publishers.*

6. Lattanzio, V., Linsalata, V., Palmeiri, S. & Sumere, C. F. V. (1989) The beneficial effect of citric and ascorbic acid on the phenolic browning reaction in stored artichoke (*Cynara scolymus* L.) heads. *Food Chemistry,* **33(2)**, 93–106.

7. Kadiri, D. D. & Anand, P. S. (2016) Optimization of protease extraction from *Billbergia pyramidalis* (Sims) *Lindl.* by response surface methodology. *International Journal of Pharmaceutical Sciences Review and Research,* **36(1)**, 267–276.

8. Tari, C., Genckal, H. & Tokatli, F. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus sp. L21. Process Biochemistry*, **41(3)**, 659–665.

9. Adinarayana, K. & Ellaiah, P. (2002) Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus sp. Journal of Pharmacy and Pharmaceutical Sciences*, **5(3)**, 272–278.

10. Myers, R. H. & Montgomery, D. C. (2002) Response surface methodology: Process and product optimization using designed experiments (2nd ed.). *Canada: John Wiley and Sons*.

11. Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T. & Iwasaki, T. (1994) *Cucumisin*, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *Journal of Biological Chemistry,* **269(52)**, 32725–32731.

12. Chitturi, S., Talatam, V. G. & Vuppu, S. (2013) Studies on protein content, protease activity, antioxidants potential, melanin composition, glucosinolate and pectin constitution with brief statistical analysis in some medicinally significant fruit peels. *Der Pharmacia Lettre*, **5(1)**, 13–23.

13. Ahmad, M. N., Liew, S. L., Yarmo, M. A. & Said, M. (2012) Optimization of protease extraction from horse mango (*Mangifera foetida Lour*) kernels by a Response Surface Methodology. *Bioscience, Biotechnology and Biochemistry*, **76(8)**, 1438–1444.

14. Patel, D., Kothari, D. & Goyal, A. (2011) Enhancement of dextransucrase activity of *Pediococcus pentasaceus SPAm1* by Response Surface Methodology. *Indian Journal of Biotechnology*, **10**, 346–351.

15. Fakhri, A. (2014) Application of response surface methodology to optimize the process variables for fluoride ion removal using maghemite nanoparticles. *Journal of Saudi Chemical Society*, **18(4)**, 340–347.

16. Roberta, C. S. T., Guzzon, S. O., Cladera-Olivera, F. & Brandelli, A. (2006) Optimization of protease production by *Microbacterium sp*. in feather meal using response surface methodology. *Process Biochemistry*, **41***,* 67–73.

17. Haddar, A., Fakhakh-Zouri, N., Hmidet, N., Frikha, F. & Nasri, M. (2010) Application of statistical experimental design for optimization of keratinase production by *Bacillus pumillus* A2 grown on chicken feather and some biochemical properties. *Process Biochemistry,* **45(5)**, 617–626.

18. Potumarthi, R., Sudhakar, C., Pavani, A. & Jetty, A. (2008) Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus lincheniformis NCIM-2042* using statistical methods. *Bioresources Technology*, **99**, 1776–1786.

19. Norazmi, M., Shahrain, M., Normaya, E., Nor, M., Iqbal, A. & Halim, K. (2020) The Applicabi lity of Using a Protease Extracted from Cashew Fruits (*Anacardium occidentale*), as Possible Me at Tenderizer: An Experimental Design Approac h. *Journal of Texture Studies*.

20. Ahmad, M. N., Mohd Azli, N. H., Ismail, H., M ohamed Iqbal, M. A., Mat Piah, B., Normaya, E. (2020) Inhibitory Effects of *Manihot esculenta* E xtracts on Food-Borne Pathogens and Their Anti oxidant Properties: Supercritical Fluid Extractio n, Statistical Analysis and Molecular Docking S tudy. *Journal of Food Process Engineering*.

21. Joardar, N., Mukherjee, S., Babu, S. P. S. (2018) Thioredoxin reductase from the bovine filarial parasite Setaria cervi: studies on its localization and optimization of the extraction. *International Journal Biological Macromolecule*, **107**, 2375–2384.

22. Bisht, D., Yadav, S. K. & Darmwal, N. S. (2013) Computation of interactive effects and optimization of process parameters for alkaline lipase production by mutant strain of *Pseudomonas aeruginosa* using response surface methodology. *Brazilian Journal of Microbiology*, **44(1)**, 245–252.

23. Hunter, W. G. & Hunter, J. S. (1987) Statistic for experimenters: An introduction to design, data analysis and model building. *New York: John Wiley*.

24. Ho, J. G. S. (2003) The likelihood of aggregation during protein Renaturation can be assessed using the second virial coefficient. *Protein Science*, **12**, 708–716.

25. Suberu, Y., Akande, I., Samuel, T., Lawal, A. & Olaniran, A. (2019) Optimization of protease production in indigenous bacillus species isolated from soil samples in Lagos, Nigeria using response surface methodology. *Biocatalysis and Agricultural Biotechnology*, **18**, 101011.

26. Uchikoba, T., Hosoyamada, S., Onjyo, M., Arima, K., Yonezawa, H. & Kaneda, M. (2001) A serine endopeptidase from the fruits of *Melothria japonica* (Thunb.) maxim. *Phytochemistry*, **57**, 1–5.

27. Anderson, M. J. (2005) RSM simplified: Optimizing process using Response Surface Methods for design of experiments. *New York: Productivity Press*.

28. Fatima, S., Ajmal, R., Badr, G., & Khan, R. H. (2014) Harmful effect of detergents on lipase. *Cell Biochemistry and Biophysics*, **70**, 759–763.

29. Trinh, T. H. T., Kim, J., Lee, C. -H. & Ryou, C. (2019) Non-ionic detergents Nonidet P-40 and Triton X-100 increase enzymatic activity of Plasmin. *Biochemical and Biophysical Research Communications*, **512**, 314–318.

30. Ezgimen, M. D., Mueller, N. H., Teramoto, T., Padmanabhan, R. (2009) Effects of detergents on the West Nile virus protease activity. *Bioorganic Medicinal Chemistry*, **17**, 3278–3282.

31. Furth, A. J. (1980) Removing unbound detergent from hydrophobic proteins. *Analytical Biochemistry,* **109**, 207–215.

32. Amid, M., Tan, C. P., Mirhosseini, H., Aziz, N. A. & Ling, T. C. (2011) Optimization of serine protease extraction from mango peels (*Mangifera Indica Cv. Chokanan*). *Food Chemistry*, **124**, 666–671.

33. Amid, M., Abd Manap, Y. & Zohdi, N. (2014) Optimization of Processing Parameters for Extraction of Amylase Enzyme from Dragon (Hylocereus polyrhizus) Peel Using Response Surface Methodology. *The scientific world journal*, 2014, 1–12.

34. Amid, M., Mustafa, S., Sarker, M. Z. I. & Yazid, A. M. M. (2011) Optimization of the conditions for extraction of serime protease from kesinai plant (*Streblus asper*) leaves using response surface methodology. *Molecules*, **16**, 9245–9240.

35. Sadhukhan, B., Mondal, N. K. & Chattoraj, S. (2016) Optimisation using central composite design (CCD) and the desirability function for sorption of methylene blue from aqueous solution onto Lemna major. *Karbala International Journal of Modern Science*, **2**, 145–155.

36. Myers, R. H. & Montgomery, D. C. (2016) Response surface methodology (2nd ed.). *New York, NY: John Wiley and Sons Inc*.

37. Ahmad, M. N., Noh, N. A., Abdullah, E. N., Yarmo, M. A., Piah, M. B. & Bulat, K. H. (2019) Optimization of a protease extraction using a statistical approach for the production of an alternative meat tenderizer from Spondias cytherea roots. *Journal of Food Processing and Preservation*, **43**, 1–14.

38. Kotlar, C., Ponce, A. & Roura, S. (2015) Characterization of a novel protease from *bacillus cereus*and evaluation of an eco-friendly hydrolysis of a brewery byproduct. *Journal of the Institute of Brewing*, **121(4)**, 558–565.