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α-amylase from white pitaya (*Hylocereus undatus* L.) peel: optimization of extraction using full factorial design

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Abstract:

Introduction. Amylase is a significant enzyme with numerous commercial applications, which is largely used to convert starches into oligosaccharides. Extraction of amylase from plant by-products or cheap sources is cost-effective. Annually, pitaya fruit juice industry produces huge amounts of peels that could be utilized as an alternative source in enzyme production industry. The work aimed to examine and optimize extraction process.

Study objects and methods. In this study, we investigated parameters of extraction to optimize the process, as well as activity of α-amylase from white pitaya fruit (*Hylocereus undatus* L.) peel. For this purpose, a two-level full factorial design was applied. Three variables, namely the pH of sodium phosphate buffer (X_1, 4.5–7.5), mixing time (X_2, 1–3 min), and a sample-to-buffer ratio (X_3, 1:3–1:5), were used to identify significant effects and interactions within the samples.

Results and discussion. The results demonstrated that the buffer pH had the most significant ($P \leq 0.05$) effect on total amylase activity. Based on full factorial design analysis, we revealed the optimal conditions for amylase enzyme extraction – pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4. Lower and higher values influenced adversely on specific activity of amylase.

Conclusion. Optimization increased the enzyme specific activity by a factor of 4.5. Thus, pitaya peel could be used in different industries as a rich natural α-amylase source.

Keywords: Extraction, peel, white pitaya, enzyme, optimization, full factorial design, specific activity

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INTRODUCTION

α-Amylase (EC 3.2.1.1, α-1,4-glucanohydrolase) is an endoacting starch-digesting enzyme that cleaves -1,4 bonds of amylose and amylopectin [1]. Thirty percent of world's enzyme market belongs to amylase, which is widely used in baking, starch liquefaction, textile, as well as detergent and paper industries [2]. Although amylases are extensively used in the food and chemical industries, their production on a large scale is just limited to specific microorganisms emphasizing the need to discover alternative sources to produce them [3]. Amylases are represented in plants, animal tissues, and microorganisms. Nowadays, bacterial α-amylases, especially from *Bacillus* genus, are the most commercially available and industrially-used enzymes [4]. However, their production does not meet the industrial needs worldwide due to their increasing demand. Furthermore, they have caused allergies in 15% of the global population. So, there is a necessity to discover novel resources to produce this valuable enzyme.

It is well documented that plants as abundant sources of α-amylases have higher productivity than bacteria and are the center of focus in developing countries due to their ubiquitous nature [5]. Thus, using plants as an alternative source of the enzyme have great advantages over microbial sources due to their cost-effective production, easy scale-up, and available natural storage organs [6].

Pitaya fruit (*Cactaceae* family) known as dragon fruit, has recently drawn much attention, not only because of their striking color and economic value as food products, but also for their health benefits [7]. Peels account for roughly 33% of the whole pitaya fruit and are the major by-products of fruit juice industry [8, 9]. They are usually discarded in roadsides, causing not only environmental problems but also are a great burden on the industry to treat wastes. So, one of eco-friendly ways to increase resource recovery is to use pitaya peels to extract novel components such as enzymes. Very few reports are accessible about enzymes in pitaya peel [10, 11]. Therefore, the peel of pitaya can be used as an economical and rich source for commercial production of beneficial and natural enzymes.

The most important stage in the recovery of enzymes from plant sources is extraction, in which an inadequate extraction procedure could change the natural structure of the enzyme and result in a reduced enzyme activity. Therefore, it is essential to optimize the extraction process to obtain enzymes with high activity. Two-level factorial design is used to minimize the number of experiments and to find out how the independent variables affect the response variables. This experimental design investigates mathematical relationships between input and output variables of a system.

To date, extraction of amylase enzyme from white pitaya (*Hylocereus undatus* L.) peel and its further optimization using FFD model have not been previously reported elsewhere. This study aimed to primarily examine and develop the optimization level of extraction variables with the minimum total protein and the highest possible total and specific activities. In this study, full factorial design was used to model the potential relationship between the variables of enzyme extraction, such as sodium phosphate buffer (pH 4.5–7.5, X_1), mixing time (1–3 min, X_2), and a sample-to-buffer ratio (1:3–1:5, X_3) which affect the responses variables of amylase derived from white pitaya peel.

STUDY OBJECTS AND METHODS

Plant material. The study objects were white pitaya fruits (*Hylocereus undatus* L.) with the weight range of 400–500 g were obtained from the local market in Selangor, Malaysia. Fruits of the same size, with no visual defects, and under commercial maturity stage were selected for experiments. The fruits (2 kg) were washed with distilled water, drained off, and kept at 4°C prior to extraction.

Chemicals and reagents. 3,5-dinitrosalicylic acid and bovine serum albumin were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Bradford reagent was purchased from Amresco (AMRESCO LLC, Solon, OH, USA). Dibasic sodium phosphate ($Na_2HPO_4{\cdot}2H_2O$), monobasic sodium phosphate ($NaH_2PO_4{\cdot}H_2O$), and maltose were obtained from Merck (Darmstadt, Germany). Sodium potassium tartrate ($NaKC_4H_4O_6{\cdot}4H_2O$) and soluble starch were purchased from Fisher Scientific. All chemicals and reagents were of analytical grade.

Enzyme extraction. The fruits peels were separated, cut into tiny pieces by a stainless steel knife and blended using a Waring blender (32BL 80, Dynamic Corporation of America, New Hartford, Connecticut, USA). Afterwards, 20 g of the mixed sample was weighed and homogenized with a 0.01 M sodium phosphate buffer (pH 4.5–7.5) with a sample-to-buffer ratio of 1:3 to 1:5 for mixing time of 1 to 3 min at 4°C. The homogenized sample was filtered using a cheese cloth and centrifuged at 10 000 rpm for 15 min at 4°C using a refrigerated centrifuge (SIGMA 3-18K, Sartorius, Göttingen, Germany). The obtained supernatant containing the crude enzyme was used to assay amylase activity and protein concentration [12]. The extraction procedures were carried out in triplicate.

Determination of amylase activity. Amylase activity was measured based on the method of Bernfeld with slight modifications [13]. The mixture of enzyme sample containing crude extract (0.5 mL) and 1% (w/v) soluble starch (0.5 mL) was prepared in a 0.1 M phosphate buffer (pH 6). After incubation of the mixture (55°C, 10 min), the reaction was stopped by 1 mL n.d. 3,5-dinitrosalicylic acid. Then, it was heated, cooled, and the volume was adjusted to 12 mL before reading absorbance at 540 nm. A unit of enzyme activity is defined as a quantity of enzyme that releases 1 μmol of maltose per minute.

Determination of protein concentration. A colorimetric protein assay was carried out based on the method of Bradford to measure protein concentration using bovine serum albumin as the standard protein [14].

Determination of specific activity of amylase. Specific activity of amylase was calculated by dividing the enzyme unit per mL (U/mL) to the protein concentration (mg/mL) according to the following formula [15]:

$$\text{Specific activity (U/mg)} = \text{Total activity (U)/total protein (mg)} \quad (1)$$

Experimental design and statistical analysis. In the present work, the effect of three extraction variables, namely the pH of buffer (pH 4.5–7.5), mixing time (1–3 min), and a sample-to-buffer ratio (1:3–1:5) on total protein, total activity, and specific activity of extracted amylase from white pitaya peel was studied using a two-level three-factor (2^3) full factorial design. Twenty-eight experiments were conducted based on the two-level full factorial design with three independent variables and each variable with three levels (Table 1). Analysis of the data was conducted by multiple regressions with the least-square method. The polynomial equation describes the behavior of the present system:

$$Y_r = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i \neq j=1}^{n} b_{ij} X_i X_j \quad (2)$$

where Y_r represents the measured response variable, while X_i, and X_j are the levels of independent variables; b_0 is coefficient constant for offset term (predicted response at the center); b_i, and b_{ij} are coefficient constants for linear and interactions effects.

R^2 with at least 0.80 demonstrated a good fitness of model [16]. If the absolute F-value became larger and the P-value became smaller, the related variables were found to be more significant ($P \leq 0.05$). The design of experimental data analysis and optimization procedure was carried out with Minitab v.16 statistical package (Minitab Inc., State College, PA, USA).

Optimization and validation procedures. A numerical optimization was used to determine accurate optimal levels of independent parameters that led to obtaining the desired response targets and concurrent multiple optimizations using the response optimizer function in the Minitab software. For validation, a comparison between experimental data and predicted values from the model helps to determine if the regression equation precisely anticipates the values or not [17].

Table 1 The matrix of full factorial design

Treatment runs	Block	pH of buffer (X_1)	Mixing time (X_2)	A sample-to-buffer ratio (X_3)
1	1	4.5	3	5
2	1	7.5	3	3
3[c]	1	6.0	2	4
4[c]	1	6.0	2	4
5[c]	1	6.0	2	4
6	1	7.5	1	5
7	1	4.5	1	3
8	2	4.5	1	5
9	2	4.5	3	3
10	2	7.5	1	3
11	2	7.5	3	5
12[c]	2	6.0	2	4
13[c]	2	6.0	2	4
14[c]	2	6.0	2	4
15[c]	3	6.0	2	4
16	3	7.5	3	3
17[c]	3	6.0	2	4
18	3	6.0	1	3
19[c]	3	6.0	2	4
20	3	7.5	1	5
21	3	4.5	3	5
22[c]	4	6.0	2	4
23	4	6.0	1	5
24[c]	4	6.0	2	4
25	4	4.5	3	3
26	4	7.5	3	5
27[c]	4	6.0	2	4
28	4	7.5	1	3

[c] center point; X_1 – pH of buffer; X_2 – mixing time, min; X_3 – a sample-to-buffer ratio

RESULTS AND DISCUSSION

Fitting the full factorial design. In the preset study, multiple regression analysis was done using full factorial design to fit mathematical models to the experimental data and to attain an optimal region for the response variable. The estimated regression coefficient of the full factorial model, along with the corresponding R^2 and lack-of-fit tests, is presented in Table 2. Significance of the factors studied was confirmed by both P-value and F-ratio as statistical parameters. Results indicated that the R^2 values for total activity, total protein, and specific

Table 2 Regression coefficient R^2, adjusted R^2, probability value, lack of fit, and significance level for extract variables

Source	Total activity (Y_1, U)	Total protein (Y_2, mg)	Specific activity (Y_3, U/mg)
b_0	43.932	16.831	2.5851
b_1	13.408	1.360	0.6706
b_2	3.166	0.902	0.0803
b_3	11.222	3.867	0.0219
b_1b_2	3.084	0.832	–
b_1b_3	2.293	1.916	–0.3178
b_2b_3	–	–	– 0.1879
Regression coefficient R^2	0.992	0.939	0.974
Adjusted regression R^2	0.989	0.909	0.967
P-value	0.000[a]	0.000[a]	0.000[a]
Lack of fit (P-value)	0.073[b]	0.233[b]	0.788[b]

Table 3 Main and interaction effects of pH (X_1), mixing time (X_2), and a sample-to-buffer ratio (X_3) on response variables for extracted amylase

Variables	Main effect			Interaction effect		
	X_1	X_2	X_3	X_1X	X_1X_3	X_2X_3
	Total activity					
P-value	0.000[a]	0.003[a]	0.000[a]	0.004[a]	0.023[a]	–
F-ratio	210.53	11.74	147.49	11.14	6.16	–
	Total protein					
P-value	0.000[a]	0.008[a]	0.000[a]	0.013[a]	0.000[a]	–
F-ratio	20.19	8.89	163.24	7.56	40.05	–
	Specific activity					
P-value	0.000[a]	0.326[b]	0.786[b]	–	0.001[a]	0.028[a]
F-ratio	70.51	1.01	0.08	–	15.83	5.53

b_i – the estimated regression coefficient for the main linear effects. b_{ij} – the estimated regression coefficient for the interaction effects. 1 – pH of buffer; 2 – mixing time; 3 – a sample-to-buffer ratio

X_1, X_2 and X_3 – the main effect of pH of buffer, mixing time, and a sample-to-buffer ratio, respectively; X_1X_2 – the interaction effect of pH of buffer and mixing time; X_1X_3 – the interaction effect of pH of buffer and a sample-to-buffer ratio; and X_2X_3 – the interaction effect of mixing time and a sample-to-buffer

[a] – significant ($P \leq 0.05$)

[b] – not significant ($P > 0.05$)

activity were higher than 0.80, which showed the regression model fits the experimental data.

Table 3 shows the main and interaction effects of pH (X_1), mixing time (X_2) and a sample-to-buffer ratio (X_3) on each response variable for the extracted amylase. The main effect of pH showed the highest significant ($P \leq 0.05$) effects on the total activity of amylase, and the interaction effect of time with ratio had the lowest significant ($P \leq 0.05$) effects on the specific activity of amylase.

Effect of extraction variables on total protein. As shown in Table 2, the total protein value was significantly ($P \leq 0.05$) affected by pH of buffer, mixing time, and a sample-to-buffer ratio, as well as by the interaction effect of pH with a sample-to-buffer ratio and mixing time. As shown in Figs. 1a and 1b, the main and interaction effects of target extraction variables positively affected total protein of amylase. In this study, a sample-to-buffer ratio followed by its interaction effect with pH had the most significant ($P \leq 0.05$) effect on the total protein (Fig. 1c).

The results also indicated that pH 6 of sodium phosphate buffer was the optimum pH for extraction of amylase from white pitaya (*Hylocereus undatus* L.) peel. Indeed, any changes in the pH of buffer can effect on the protein structure by lowering protein solubility and increasing protein hydrophobic interactions, which finally leads to denaturation and precipitation of the enzyme [18, 19].

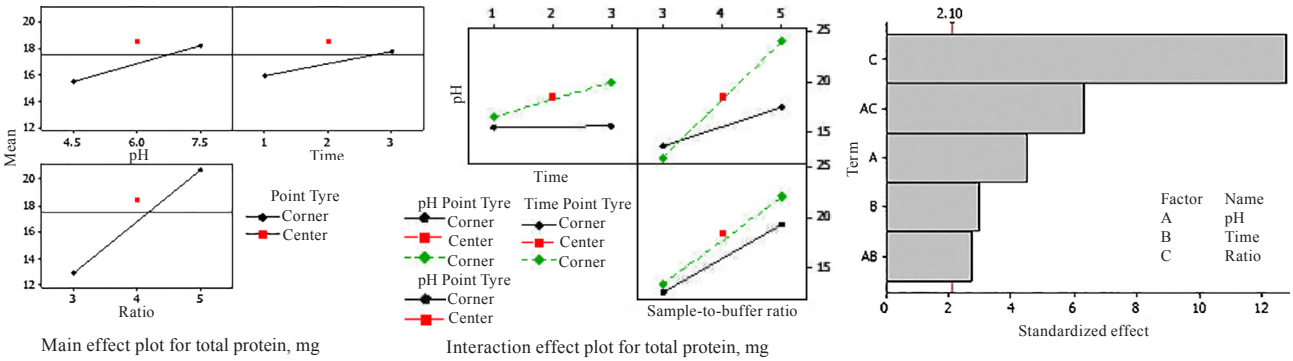
It should be noted that the extraction time also significantly ($P \leq 0.05$) affected the total protein; however, its effect was significantly ($P \leq 0.05$) lower than a sample-to-buffer ratio and pH effects. The most desirable time to mix sodium phosphate buffer with peels was 2 min. Tang *et al.* reported that protein yield increased as the extraction time increased, and decreased with increasing time above certain value [20]. Thus, a prolonged extraction process can lead to protein denaturation, and affect the enzyme function [21].

Figure 1b clearly indicates that interaction between pH and a sample-to-buffer ratio was stronger than the interaction of pH and mixing time. The effect of a sample-to-buffer ratio was more significant ($P \leq 0.05$) at a higher pH, but total protein was lower at a lower pH. The individual optimum region with minimum total protein value (Y_2 = 18.45 mg) was predicted to be achieved at pH 6, mixing time of 2 min, and a 1:4 a sample-to-buffer ratio.

Effect of extraction variables on total amylase activity. As shown in Table 2, the main effect of pH, mixing time, a sample-to-buffer ratio, and the interaction effect of pH with a sample-to-buffer ratio and pH with mixing time had significant ($P \leq 0.05$) effects on the total amylase activity. As given in Figs. 2a and 2b, the main and interaction effect variables positively affected the total activity of amylase enzyme. Main and interaction effects had higher mean total activity at their center point. As seen in Fig. 2c, the main effect of the pH of buffer had the most significant ($P \leq 0.05$) effect on the total activity followed by a sample-to-buffer ratio, time, interaction of pH with mixing time, and interaction of pH with a sample-to-buffer ratio.

Our results revealed that the maximum total activity occurred at extraction pH of 6. Pires *et al.* reported that extraction pH determined the net charge of proteins [22]. The considerable difference in extraction efficiency with nominal changes in aqueous phase pH showed the sensitivity of system to any pH changes. These changes might disrupt the efficiency of enzyme in hydrolyzing the protein, considered as the main cause of the results obtained for pH range. Chen *et al.* showed that fluctuations in the pH of many enzyme reactions normally could result in some reactants changes such as denaturation of protein structure or disturbance of enzyme ionic character related to its active site [21].

We found that the best ratio for amylase extraction was 1:4. A decreased volume of buffer reduced the total activity of amylase, which was due to insufficiency of buffer to infiltrate the solid mass. On the other hand, the increase in the buffer volume beyond the optimum level resulted in an immediate reduction in the total amylase activity due to an excessive dilution of the solution. In



(a) (b) (c)

Figure 1 Main effects (a), interaction effects (b), and Pareto chart of extraction variables (c) on total protein of amylase from white pitaya peel (c)

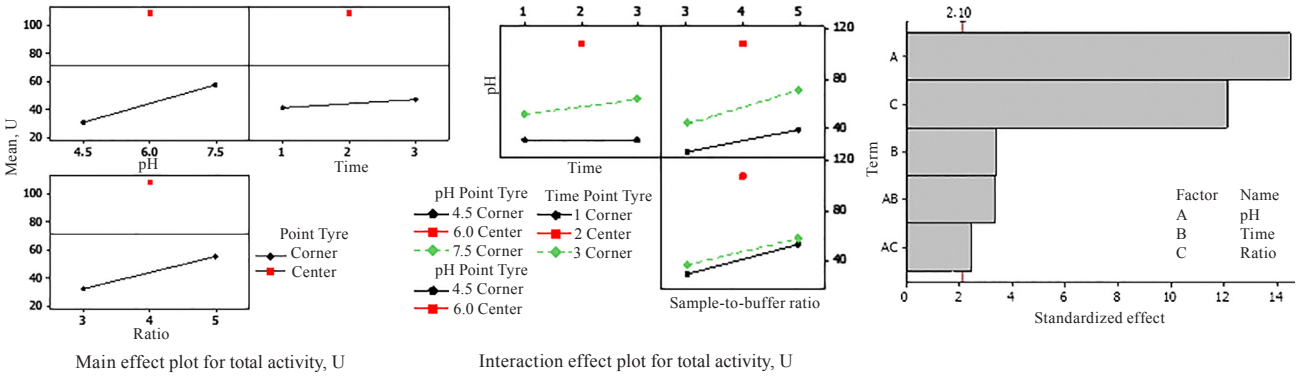


Figure 2 Main effects (a), interaction effects (b), and Pareto chart of extraction variables (c) on total activity of amylase from white pitaya peel (c)

the present study, using more buffers to the center point released more enzymes from the sample, and a lower volume of buffer reduced the enzyme activity. Similarly, other studies found that the amount of extracted enzyme increased with the rise in the buffer volume and vice versa [23–26].

Time is one of the important physical parameters in the enzyme extraction that significantly ($P \leq 0.05$) affected the total activity of amylase. However, its effect was significantly ($P \leq 0.05$) lower than pH and a sample-to-buffer ratio effects. Mixing time of more than 2 min had negative effects on the total activity of amylase. In fact, buffer contact with the enzyme rises when the extraction time increases, although the higher shear forces during mixing with blender could denature and deactivate the enzyme. Our results consist with those of Amid *et al.* who reported that the optimum time required for extraction of amylase from mango peel was 2 min [27].

All the interactions had statistical significance ($P \leq 0.05$) in influencing on the total activity of amylase except for the interaction of mixing time with ratio. The interaction plot illustrates that interaction of pH and mixing time was stronger than that of pH and a sample-to-buffer ratio (Fig. 2b). It was observed that the interaction effect of pH with a sample-to-buffer ratio had the lowest significant ($P \leq 0.05$) effect on the activity of enzyme. The highest total activity (Y_1 = 108.2 U) of amylase was obtained at buffer pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4.

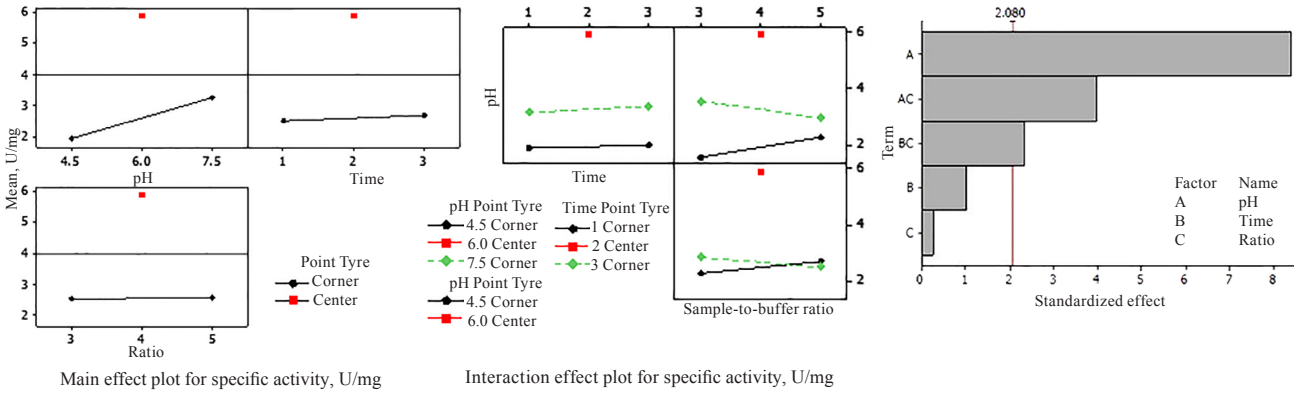
Effect of extraction variables on specific activity of amylase. The specific activity value of the amylase was significantly ($P \leq 0.05$) affected by pH of buffer and interaction effect of a sample-to-buffer ratio with pH and mixing time (Table 2). Figure 3a demonstrates that mixing time and a sample-to-buffer ratio were insignificant ($P > 0.05$), and only pH of buffer exhibited a significant ($P \leq 0.05$) effect on the specific activity.

In this study, the main term of pH indicated the most significant ($P \leq 0.05$) effect followed by interaction of pH with a sample-to-buffer ratio and interaction of mixing time with a sample-to-buffer ratio on the specific activity of amylase (Fig. 3c). The specific activity of amylase significantly ($P \leq 0.05$) decreased at pH buffer of 4.5 and 7.5 due to the changes in enzyme structure at acidic and alkaline pH. The highest specific activity was obtained at pH of 6 that confirmed the enzyme was active at slightly acidic pH, so, extraction efficiency depended on the pH of buffer. Similar observation was made by Amid *et al.*, who investigated the effect of pH on the specific activity of serine protease from Kesinai plant leaves [28]. It was indicated that the activity of enzyme decreased beyond the center point at higher and lower pH of buffer.

In addition, one of the most significant ($P \leq 0.05$) interaction effects was the interaction between pH of buffer and a sample-to-buffer ratio. It was observed that the specific activity increased with increasing the ratio from 1:3 to 1:4, whereas at a higher ratio (1:5) no considerable changes in the specific activity were observed when the pH of the buffer was changed (Fig. 3b). This could be attributed to the excessive dilution of the enzyme extraction solution.

In other words, at the lower ratio (1:5), variation in the pH of buffer had a significant effect on the specific activity possibly due to an adequate volume of buffer required to infiltrate the solid mass. Aikat and Bhattacharyya reported the same observation of the effect of buffer volume on the activity of pectinase enzyme [29]. The interaction effect of mixing time with a sample-to-buffer ratio showed the least significant ($P \leq 0.05$) effect on the specific activity. Overall, the highest amount of specific activity (Y_3 = 5.89 U/mg) was predicted when amylase was extracted under the following conditions: buffer pH of 6, mixing time of 2 min, and a sample-to-buffer ratio of 1:4.

Optimization procedure. The Multiple response optimizations were conducted to evaluate the optimum set level of independent variables to obtain the required results. The findings showed that extraction with buffer of pH 6 for 2 min mixing time in a sample-to-buffer ratio of 1:4 resulted in an optimal condition for total



(a) (b) (c)

Figure 3 Main effect (a), interaction effects (b), and Pareto chart of extraction variables (c) on specific activity of amylase from white pitaya peel (c)

protein, total activity and specific activity of amylase from white pitaya peel. Under optimal conditions, the corresponding expected response variables for total protein, total activity, and specific activity for the extracted amylase were predicted to be 18.45 mg, 108.2 U, and 5.89 U/mg, respectively. The results showed the accuracy of the statistical model for amylase extraction (Fig. 4).

Verification of the fitted model. The acceptability of full factorial design was verified by comparing the experimental and expected values. If these two results are closer, the regression equation can explain the acceptability better. The comparison between experimental values of the responses and the expected values measured employing the models can be done by performing a two-sample *t*-test. There was not a significant ($P > 0.05$) difference between the experimental and predicted values. There was a close correspondence between those values.

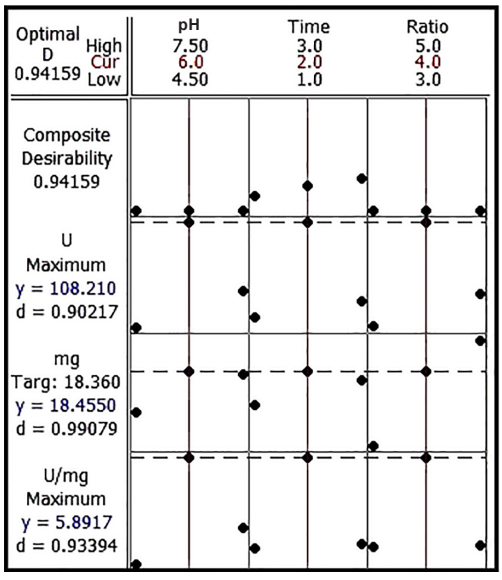


Figure 4 Response optimizer plots for interaction effects of extraction variables on total protein, total activity, and specific activity of amylase

The validation of the models was performed using buffer with pH 6 for 2 min of mixing time in a sample-to-buffer ratio of 1:4. Under optimal extraction conditions, the predicted values for total protein, total activity and specific activity were 18.45 mg, 108.2 U and 5.89 U/mg, respectively. A supplementary experiment under the selected optimal conditions demonstrated the experimental values of total protein, total activity, and specific activity to be 18.82 mg, 105.8 U, and 5.62 U/mg, respectively, which were in close agreement with the expected values indicated the suitability of the corresponding model.

CONCLUSION

The present study revealed that the peel of white pitaya (*Hylocereus undatus* L.) could be a good source of amylase. The results indicated that the activity of the extracted amylase was significantly ($P \leq 0.05$) influenced by main extraction variables. pH had the most significant ($P \leq 0.05$) effect on amylase activity. The analysis using full factorial design demonstrated that the maximum amylase extraction could be achieved at pH of 6. Amylase had a lower enzymatic activity at pH 4.5 and 7, which might be due to denaturation of amylase at such low or high pH.

Among all main extraction variables, time had the least significant ($P \leq 0.05$) effect on the total activity and total protein of amylase. The results exhibited that the highest activity was achieved at the sample-to-buffer of 1:4, but lower or higher ratios would negatively affect the enzymatic activity of amylase. This might be caused by the insufficient buffer volume to infiltrate the sample.

Therefore, the extraction at pH 6 for 2 min using a sample-to-buffer ratio of 1:4 was the optimal extraction condition for amylase. This optimization increased the specific activity of the enzyme by a factor of 4.5 (to a value of 5.89 U/mg). On the basis of such findings, the main effect of pH of buffer, mixing time, and a sample-

to-buffer ratio were the principal factors affecting the extraction of amylase from white pitaya peel. Amylase extracted from white pitaya peel can be used as a potential economical enzyme in different industries and for biotechnological applications.

CONTRIBUTION

Z. Shad, the main author, performed the experiments. H. Mirhosseini and A.S. Meor Hussin designed the work, processed and analyzed the data, and wrote the manuscript. M. Motshakeri managed lab procedures and supervised the project. M.R. Sanjabi performed enzyme experiments, edited the manuscript, and contributed experimental design and data analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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