

APPLICATION OF RESPONSE SURFACE METHODOLOGY (RSM) ON THE OPTIMIZATION OF HIGH AMYLOSE STARCH PRODUCTION FROM SAGO STARCH USING PULLULANASE

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ABSTRACT

Production of high amylose from sago can be considered as a high potential innovative approach of producing healthy food. High amylose starch can be produced by amylopectin debranching process. There are five parameters that could affect the debranching process during the production of the high amylose starch. The purpose of this study was to identify the optimal range of these five parameters the efficient way by applying response surface methodology (RSM). Based on the results from 2^{5-1} fractional factorial design (FFD), the most significant factors ($p < 0.05$) that affecting pullulanase debranching reaction were substrate concentration, followed by pH, duration of reaction and temperature. The *steepest ascent* analysis and central composite design (CCD), had been used to determine the optimal reaction conditions for debranching process. From our findings, substrate concentration of 4.3% (w/v), enzyme concentration of 1.5% (v/v), pH at 4.6, the duration of 83 minutes and reaction temperature at 43°C were observed able to produce the starch, which contains 52% of amylose. The actual response was closely agreeable with confirmatory test. In this study, RSM was a useful and efficient tool to guide the researchers in finding direction to the highest potential amylose production.

Keywords: High amylose starch; sago starch; response surface methodology (RSM); pullulanase, debranching enzyme

INTRODUCTION

Nowadays people are concern about healthy lifestyle especially proper diet intake. Starch is one of the important carbohydrates in diet, as an energy source. Starch is obtained from plant, normally grains such as wheat, rice and oats. Another part of plant that also consists of starch is the bulb such as potato and tapioca. However, excessive carbohydrate intake may contribute to other health circumstances such as diabetes

mellitus, obesity, hypertension and other chronic diseases. Therefore, many researchers continue to explore the development of nutritional value and improve the quality of carbohydrate sources. The study on high amylose starch is attracting researchers' attention due to its health benefits and could be considerably valuable from industrial prospective [1].

High amylose starch is an important of resistant starch (RS) resource which has similar nutrition characteristics like dietary fiber in maintaining human health [2]. RS is a portion of the starch that cannot be hydrolyzed in the upper gastrointestinal tract and acts as a substrate for bacterial fermentation in the large intestine [3]. In addition, high amylose content in wheat was found to improve the nutritional value and to have the unique functionality as end-use products. Previous studies had shown that high amylose starch in the bread making increases the amount of RS during the storage [4]. Other benefit of high amylose starch was mentioned by Morita et al. [5] as they claimed that application of high amylose starch has improved the texture of noodles. Nonetheless, high amylose starch from sago has not been widely studied compared to wheat, rice, potato and maize [2,6,7].

Literature Review

Sago starch derived from *Metroxylon spp* are widely grown in swampy geographical areas in South East Asia [8]. Sago starch is isolated from the sago palm trunk and has become an important source of local food and economic sector. Sago starch has been commercialized as sago flour and sago pearls [9]. Pure sago starch is tasteless and non-soluble in cold water. The sago starch granules are oval in shape with sizes of 20 - 60 microns. Sago starch gelatinizes at temperature 65- 70 °C. The ratio of amylose to amylopectin in sago starch is typically 27 : 73. Sago starch paste is translucent. According to Ahmad et al. [10], the composition of amylose in sago starch is around 24% - 30%, with crude protein of 0.19 - 0.25%, humidity 10.6 – 20%, crude fat (0.19 - 0.25%) fiber(0.26 - 0.32%) and ash (0.06 - 0.43%). Amylose has a linear chain structure made up of glucose molecules that are linked by α -1,4 glycosidic bond linkage. According to Zubaidah [11] estimation, one polymer chain of amylose consists of 300 units of glucose. On the other hand, amylopectin is a highly branched glucose molecules linked by α -1,4 glycosidic bonds with occasional α -1,6 glycosidic bonds which provide branching points [11]. Branching occurs at every 12 to 25 glucose units and the degree of polymerization of the outer branch chains is 24 to 30 units, depending on the species [13]. Previous researches have demonstrated the relationship between amylose and amylopectin ratio with RS content [6,14,15,7]. Many high amylose products are produced via mutation or transgenic breeding approaches [16]. Nonetheless, these techniques require high technology and skills. Another technique of producing high amylose starch is by debranching enzymatic activity which is more economic.

Pullulanase is a debranching enzyme which specifically hydrolyzes α : 1-6 -D-glucosidic linkages in pullulan and amylopectin. There are two types of pullulanase, type I and type II. The classification of pullulanase depends on its α -1, 4 glucosidic hydrolysis activities. Pullulanase types I specifically breaks in α -1,6 glucosidic linkages in pullulan to yield

maltotriose [17]. Pullulanase type II has the ability to attack both α -1, 4 glucosidic and α -1, 6 glucosidic linkages in pullulan or branched oligosaccharides such as amylopectin [18]. From the previous studies there were many parameters that have been suggested in the debranching process. The actual problems from the previous studies were their methodology approach was based on classical techniques. This is because, the optimization of parameters by classical techniques (one variable at a time on an experimental response) is not only time-consuming but the outcomes also are not as per desired. Therefore in this study, we used the response surface methodology (RSM) as an alternative optimization methodology. RSM is able to determine the relationship between the response and the independent variables by interpreting the influenced variables and the response objectives [12, 22]. RSM is more practical because it allows interactive effects among the variables and eventually overall effects of the parameters of the process are clearly elucidated [21]. In this study, the parameters that might affect the production of high amylose starch from sago were observed. Optimization of the parameters was executed by applying RSM analysis.

METHODOLOGY

Experimental Design and Statistical Analysis.

The optimization of debranched amylopectin parameters was optimized using Design-Expert 6.0 software. From the preliminary study, there were 5 identified parameters that have significant effect on sago starch debranching process [6,7]. The parameters were substrate concentration, enzyme concentration, pH of the medium, duration of reaction and reaction temperature. Fractional factorial design (FFD) 2^{k-1} (k= number of variable) has been chosen to reduce the number of treatment combinations without efficacy argument. FFD will take into consideration of the interactions of 5 parameters. The numbers of interaction combinations for this experiment were $2^{5-1} = 16$ treatments and the samples were prepared in triplicate. Table 1 shows the range and center value of 5 parameters based on the preliminary results.

Table 1:Factor level values and variables actual values for Fractional factorial design (FFD)

Variables	Factor level		
	-1	0	+1
Substrate (X_1 , %)(w/v)	2	4	6
Enzyme (X_2 , %) (v/v)	1	1.5	2
pH (X_3 , pH)	4	5	6
Duration (X_4 , min)	30	90	150
Temperature (X_5 , °C)	40	50	60

The range of all variables was determined by using one variable at a time of experiment.

Pullulanase Debranching Process

Five parameters (substrate concentration, enzyme concentration, pH, temperature and time) for pullulanase debranching process on sago starch were determined as described previously [23] with a slight modification. The value for each variable was designated by RSM as shown in table 2. Sago starch was gelatinized in 1 ml of 0.2 M acetate buffer (with the pH value refer to table 2) at 80 °C for 20 minutes. Then, the temperature was reduced to 50°C, before adding pullulanase enzyme into the gelatinized starch. The mixture was incubated at the designated temperature and time for debranching process (table 2).

Determination of Amylose Content

Amylose content was determined based on Mc-Grance et al. [24]. A milliliter of debranched starch sample was dissolved in 1 ml of 99% DMSO for 15 minutes at 85°C in water bath. Starch was diluted in 25 ml of de-ionized water. A milliliter of dissolved starch was diluted in 50 ml of distilled water. Five milliliter of 0.0025 mol/L in 0.005 mol/L potassium iodide was added in the diluted starch. The amylose content in the solution was determined by spectrophotometer at 600nm.

Direction of Potential Optimum Variables

To determine the direction of potential improvement process, execution of *steepest ascent* methodology was used. This allows the finding of direction to optima center point and it can be used as an indicator for applying the real process of optimization. Central composite design (CCD) is the methodology to get model quadratic. The equation 1 (Eq 1) of the second order response surface is as below (1). β_0 , β_i , β_{jj} and β_{ij} are coefficient regression of the intercept, quadratic linear and χ_i is coefficient interaction and χ_j is coded as independent variables.

$$y = \beta_0 + \sum_{j=1}^k \beta_j \chi_j + \sum_{I < j=2} \sum \beta_{ij} \chi_i \chi_j + \sum_{j=1}^k \beta_{jj} \chi_j^2 + \epsilon \quad (1)$$

RESULTS AND DISCUSSION

Amylose Content

Before any treatment on sago starch, the amylose content in native sago starch was determined. In this study, the ratio of amylose : amylopectine in sago starch sample was found 25:75.

Fractional factorial design (FFD) 2^{k-1}

The FFD experimental design consists of 16 factorial points and 5 replicates of the central point (Table 2). Table 2 shows that the yield of amylose in sago starch after debranching was nearly similar to the calculated predicted response model equation value.

Table 2: FFD 2⁵⁻¹ experiment matrix with experimental values of actual high amylose (y_i) and prediction value (ŷ_i)

Test number	X ₁ Substrate	X ₂ Enzyme	X ₃ pH	X ₄ Duration	X ₅ Temperature	Amylose (%)	
						*Actual Response, (y _i)	*Predicted response, (ŷ _i)
1	-1	-1	-1	-1	-1	38.88	39.71
2	1	-1	-1	-1	-1	35.19	34.52
3	-1	1	-1	-1	-1	47.16	45.24
4	1	1	-1	-1	1	25.25	24.4
5	-1	-1	1	-1	-1	39.75	38.43
6	1	-1	-1	-1	1	26.13	26.73
7	-1	1	1	-1	1	45.42	45.81
8	1	1	1	-1	-1	22.8	23.92
9	-1	1	-1	1	-1	55.73	56.57
10	1	-1	-1	1	1	24.40	24.31
11	-1	1	-1	1	1	38.50	39.62
12	-1	-1	1	1	1	45.65	45.84
13	-1	-1	1	1	1	52.04	50.57
14	1	-1	1	1	-1	32.88	30.40
15	-1	1	1	1	-1	43.75	44.90
16	1	1	1	1	1	31.04	31.48
17 ^a	0	0	0	0	0	38.40	36.84
18 ^a	0	0	0	0	0	36.40	36.84
19 ^a	0	0	0	0	0	36.80	36.84
20 ^a	0	0	0	0	0	34.60	36.84
21 ^a	0	0	0	0	0	38.00	36.84

^a Center point. (* Data was an average of triplicate, n =3)

Half normal probability plot of effects

Half normal plot is an alternative to the normal probability plot of the factor effects. This plot depicts the absolute value of the effect estimates against their cumulative normal probabilities [25]. Figure 1 shows the half-normal plot of the effects of 5 variables on high amylose starch production. The variables and interaction factors which were far away from zero point or straight line were identified as significant. The series of significant effects of variables and interaction factors as per Eq. 2 below.

$$(A > CE > E > DE > C > D > BC > CD > AD > B > BD) \\
(X_1 > X_3 X_5 > X_5 > X_4 X_5 > X_3 > X_4 > X_2 X_3 > X_3 X_4 > X_1 X_4 > X_2 > X_2 X_3) \quad (2)$$

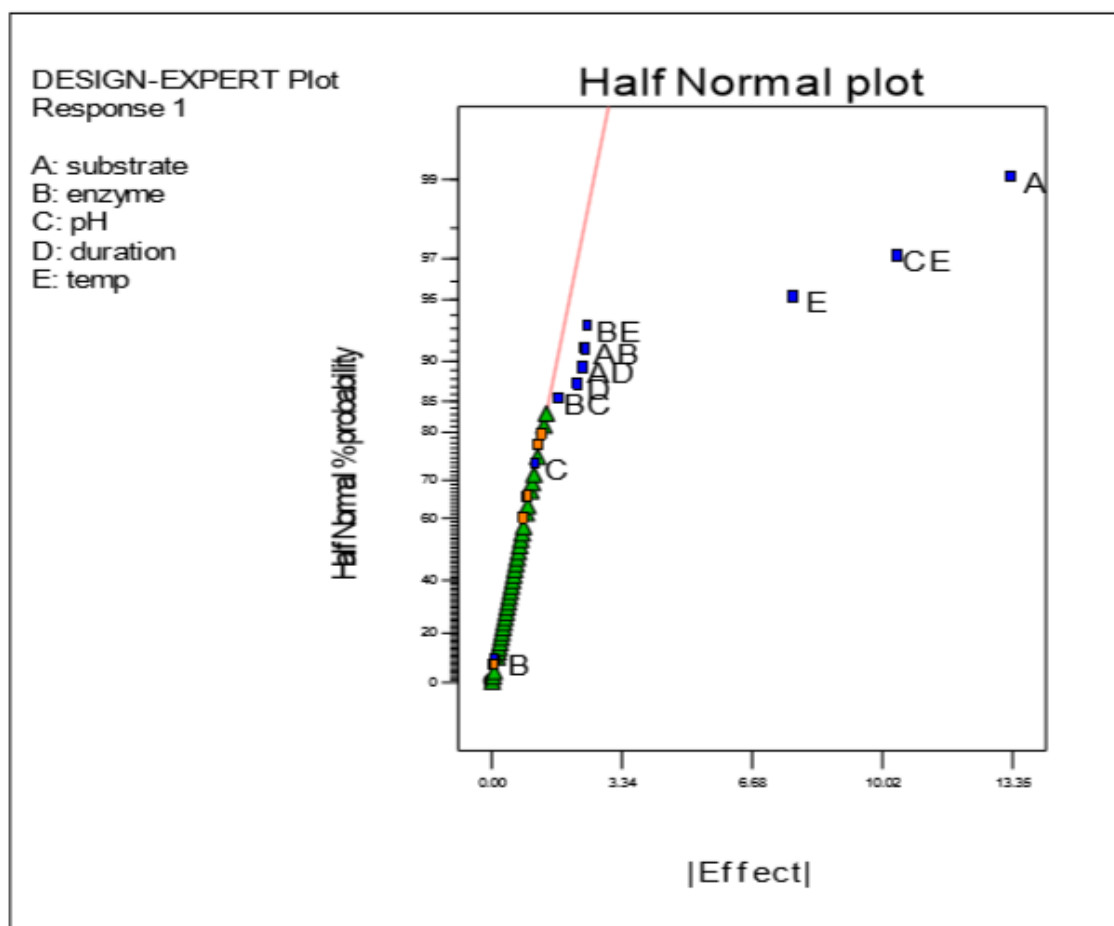


Figure 1: Half normal plot of high amylose starch

Analysis of variance (ANOVA)

Table 3 shows the ANOVA of 2^{5-1} FFD model experiment. F value (63.25) of this model was significant with R^2 , was acceptable. R^2 value is calculated from the changes value of the response, Δy divided by total of response variation on the dependent variables ($\Delta y + \sigma$). R^2 value explained the proportion of the variability in the data was able to be depicted by this model. However, the 'Lack of fit' value was not significant. This explained that the replication of the response value in this experiment was acceptable and the screening experimental design was fitted. The curvature value in this model was significant and this indicated that the center point response value of predicted model design was similar to the center point response values of the actual experiments. The significant value of curvature could lead the steepest ascent to be more effective in directing optimum point at second level model design.

Table 3: ANOVA schedule of 2^{5-1} FFD

Source	Sum of Squares	DF	Mean Square	F value	P value	
Model	3748.26	12	312.36	63.35	<0.0001	*
Curvature	266.21	1	266.21	56.51	<0.0001	*
Residuals	183.73	39	4.71			
Lack of fit	6.82	3	2.27	0.46	0.71	
Pure error	176.91	36	4.91			
Total correlation	4198.20	52				

$R^2 = 0.9318$, * Significant value ($p < 0.05$)

The regression coefficient in table 4 indicates that the effect of substrate concentration, pH, duration and temperature of the pullulanase debranching reaction were significant ($p < 0.05$). Negative value shows that all variables of regression coefficient were reduction factors. The most significant variables that affect the debranching process was substrate concentration (X_1) with the highest coefficient value, -7.04. From this value, the increment of amylose content could be performed by reducing the amount of substrate concentration. According to Wong et al. [26], pullulanase debranching yield at 5% of substrate concentration was better than 7% of substrate concentration but amylose yield decreased when the substrate concentration was reduced to 2%.

Table 4: Regression Coefficient value and p value

Variables	Coefficient value	p value	
Intercept-y	34.89		
X_1 (substrate)	-7.04	<0.0001	*
X_2 (enzyme)	0.53	0.1013	
X_3 (pH)	-0.90	0.0064	*
X_4 (duration)	-0.85	0.0097	*
X_5 (temperature)	-3.09	<0.0001	*
$X_1 X_2$	0.53	0.0982	
$X_1 X_4$	0.71	0.0300	*
$X_2 X_3$	-0.79	0.0160	*
$X_2 X_4$	-0.63	0.0503	
$X_3 X_4$	-0.71	0.0295	*
$X_3 X_5$	3.31	<0.0001	*
$X_4 X_5$	-1.98	<0.0001	*
Center point	7.67		

* Significant value ($p < 0.05$)

The second variable with high significant value was temperature (X_5), with coefficient value -3.09. The temperature was indicated as a reduction factor. This suggested that the reaction temperature of pullulanase lower than 60°C resulted in a better amount of amylose. Our observation of the pullulanase temperature debranching process was similar to previous study [27]. In contrast, the temperatures for pullulanase debranching process were determined at 57.5°C and 37°C by Guraya et al. [7] and Praznik et al. [28] respectively. pH was the third significant variable with the coefficient value -0.9. The highest pH used in this experiment was pH 6. This result was agreeable with previous study that indicated pH for debranching was pH 5.5 [29], pH 4.7 [30] and pH 4.8-4.9 [7]. Also, the reaction duration of pullulanase on sago starch was a significant variable with coefficient value of -0.85. In this experiment, the reaction duration of pullulanase was a reduction factor. Based on the observation of regression co-efficient value, FFD model equation with coded variables was characterized as equation 3 (Eq. 3).

$$Y\% \text{ amylose} = 34.89 - 7.04 X_1 + 0.53 X_2 - 0.90 X_3 - 0.85 X_4 - 3.09 X_5 + 0.53 X_1 X_2 + 0.71 X_1 X_4 - 0.79 X_2 X_3 - 0.63 X_2 X_4 - 0.71 X_3 X_4 + 3.31 X_3 X_5 - 1.98 X_4 X_5. \quad (3)$$

The effect of Temperature and pH Interaction

Temperature and pH was the most significant interaction with the highest coefficient value of +3.31. Figure 2 shows the interaction graph of pH and temperature while other variables were disseminated at FFD center point. The straight line with triangle plot represented the highest temperature (60oC) and straight line with square plot represented the lowest temperature (40oC). Figure 2 shows that temperature lower than 60oC could enhance the production of debranched amylose. Graph dissemination at the low pH level (at the left side of the graph) was larger than high pH level (right side of graph). This indicated that temperature was significantly affected by the acidity of medium interaction. This finding was agreeable with Sheng-Jun & Jing [31] that claimed the optimal pullulanase hydrolysis conditions were 47.88 °C and pH 4.92 on pullulan. While Huanxin & Zhengyu [32] have found that the optimal hydrolysing conditions of pullulanase on maize starch were pH 5.0 and temperature at 46°C.

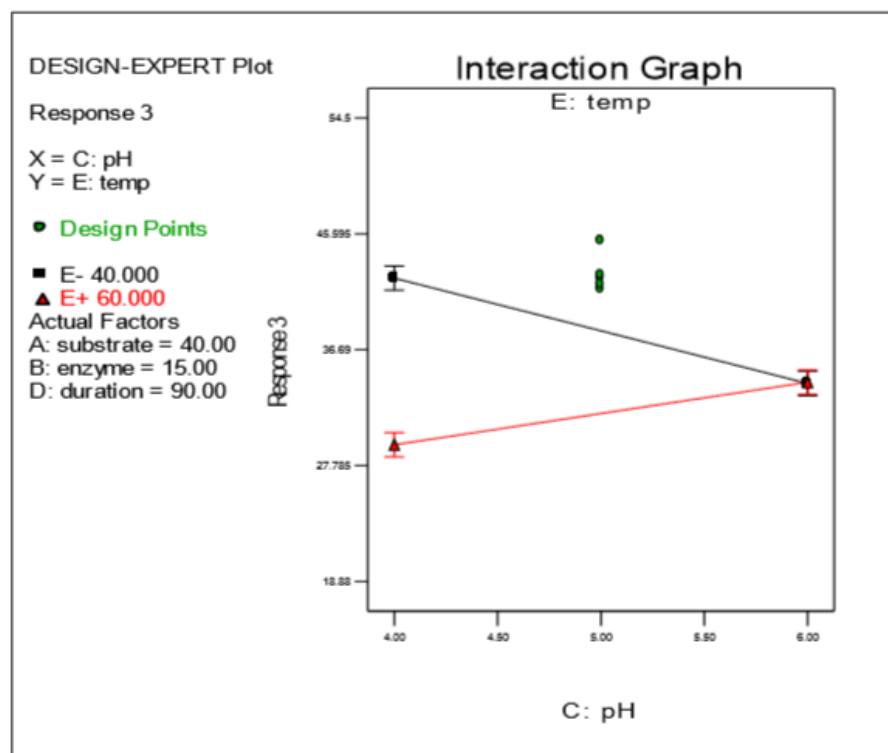


Figure 2: Interaction response between pH and temperature

The Interaction Effect of Reaction Duration and Temperature

The interaction between duration of reaction and temperature on pullulanase reaction was significant with coefficient value of -1.98. Figure 3 shows the temperature of debranching reaction was inversely proportional to time. Graph dissemination was larger at the right side (Figure 3). This outcome was in line with the previous observation that temperature and time of reaction were the reduction factors independently. This outcome is supported by previous research findings that the higher temperatures resulted in higher rates of enzyme inactivation as indicated by lower decimal reduction time (D-values) and inactivation constants (higher k values) [33]. They found that pullulanase highest stability was at 50 °C with a D- value of 76.77.

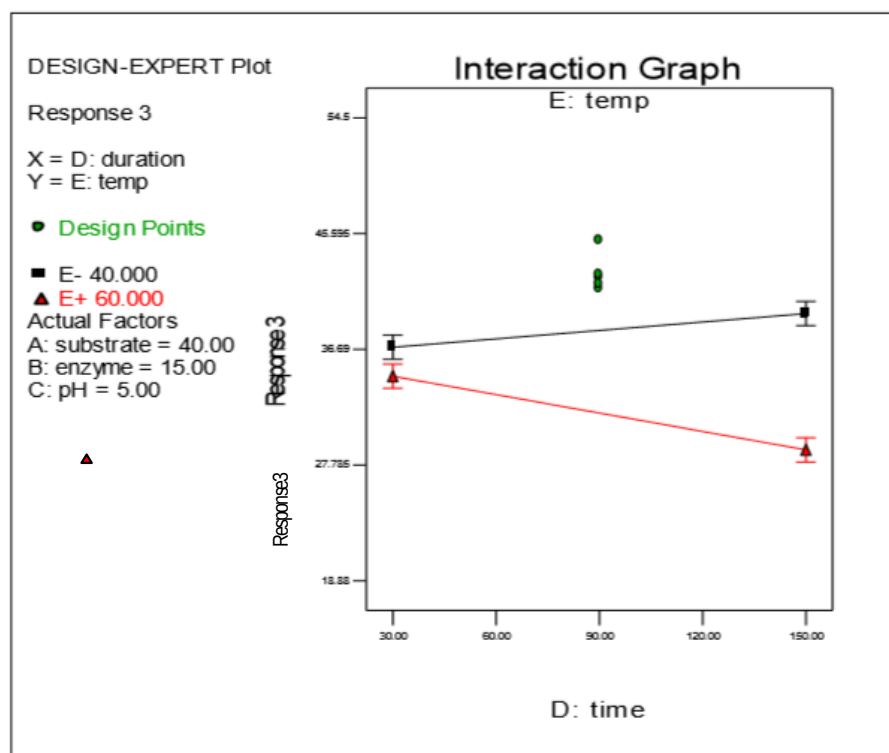


Figure 3: Interaction response between time and temperature

The Interaction Effects of Enzyme Concentration and pH

The interaction effect between enzyme concentration and pH was significant with coefficient value – 0.79. The straight line with triangle plot represented the highest pH value (pH6) and straight line with square plot represented the lowest pH value (pH4). From the figure 4, dissemination of graph was wider at the right side. pH was indicated as significant variable but was not significant to enzyme concentration (Table 4). Therefore this interaction was affected by pH value only. Comparing this findings with other study, which found that enzyme concentration proportionately increased from 8 to 14 U/g starch with the degree of pullulanase hydrolysis. However, there was no significant increase in the degree of hydrolysis for the high amylose rice starch samples treated with 16 U/g starch [35].

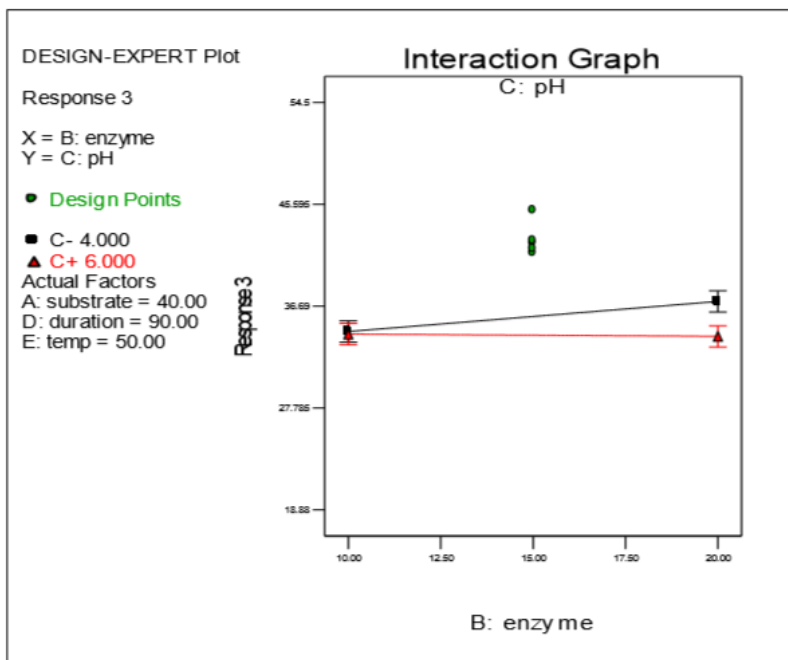


Figure 4: Interaction response between enzyme concentration and pH

The Interaction Effect of Reaction Duration and pH

The interaction between duration of reaction and pH was indicated as significant with coefficient value -0.71. There was no significant difference between 30 minutes time duration and 150 minutes reaction duration at pH 4 (Figure 5). But at pH 6, the difference of response was observed significantly between 30 minutes to 150 minutes. Therefore the graph dissemination was slightly wider at the right side of the graph. This result is almost similar to the previous research outcomes that debranching time reached at maximum about 3 to 6 hours with high consumption of pullulanase and decreased thereafter in the condition of constant pH value [34].

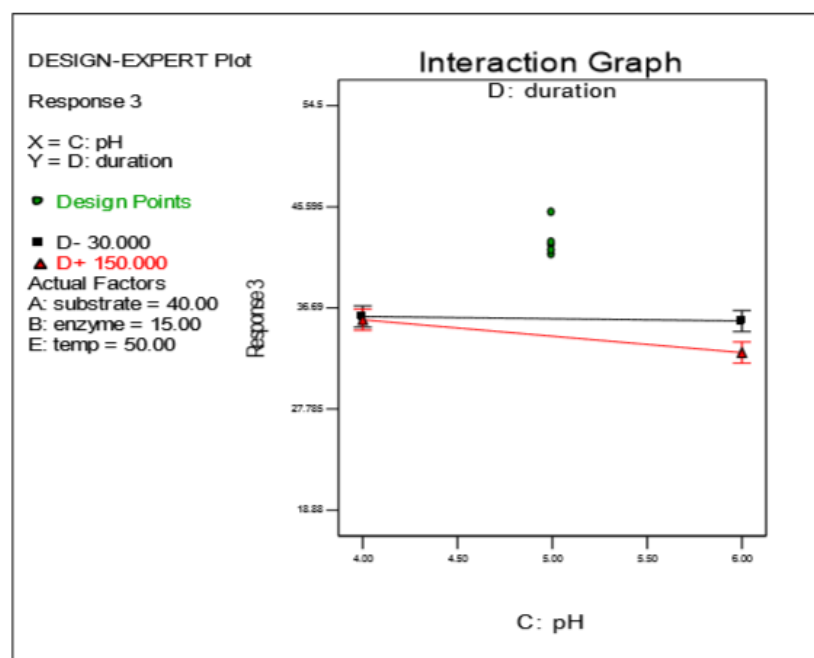


Figure 5: Interaction response between duration of reaction and pH

The Interaction Effect of Substrate Concentration and Duration of Reaction

The final observation that indicated positive significant interaction was substrate concentration and the duration of reaction with coefficient value of + 0.71. The straight line with square plot represented minimum 30 minutes reaction duration and straight line with triangle plot represented maximum 150 minutes of reaction duration. Figure 6 shows that there was a significant difference of response at different level of substrate concentration. But at the same amount of substrate, there was no significant difference between minimum and maximum duration of reaction. This indicates that the reduction of substrate concentration increased the yield of debranched amylose. This might be due to that gelatinization was greater at low concentration and thus enzyme simply can react with the starch. Previous study on pullulanase activity effect of different concentrations of pullulan, soluble starch and dextran with 4.0 mg/mL, 11.1 mg/mL and 40.0 mg/mL respectively, showed that pullulan is the best substrate for pullulanase with the highest V_{max}/K_m ratio [33]

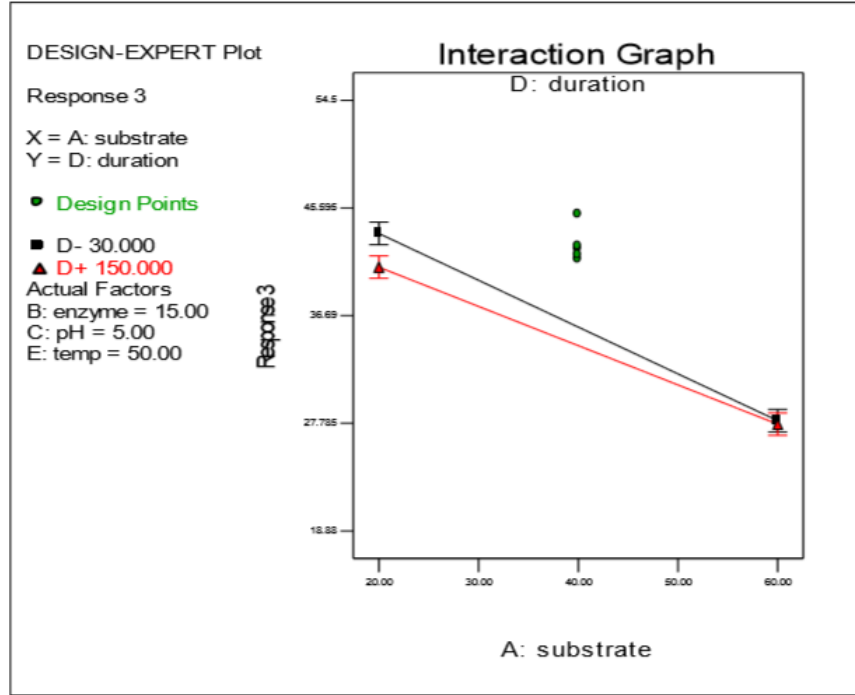


Figure 6: Interaction between substrate concentration and duration of reaction

Steepest Ascent

Steepest ascent is a method to find a direction of potential process improvement [25]. All significant variables would be the indicator to find the direction of *steepest ascent*. Based on the Eq.3, the substrate concentration was marked as a standard in the path of *steepest ascent*. *Steepest ascent* path calculation for each variable was shown in table 5. The step size for each original unit was too small to be measured in the experiment. Therefore it was suggested to convert the value of each factor into 10 unit of step size.

Table 5: *Steepest ascent* path of variables

	Step size	Conversion to natural unit (10 unit)
Substrate concentration, X_1	$\Delta X_1 = \frac{\beta_{X_1}}{\beta_{X_1}} = \frac{-7.04}{7.04} = -1$	$-1 \times 10 = 10\text{mg/ml}$
pH, X_3	$\Delta X_3 = \frac{\beta_{X_3}}{\beta_{X_1}} = \frac{-0.90}{7.04} = -0.13$	$-0.13 \times 10 = -1.3$
Duration, X_4	$\Delta X_4 = \frac{\beta_{X_4}}{\beta_{X_1}} = \frac{-0.85}{7.04} = -0.12$	$-0.12 \times 10 = -1.2 \text{ min}$
Temperature, X_5	$\Delta X_5 = \frac{\beta_{X_5}}{\beta_{X_1}} = \frac{-3.09}{7.04} = -0.44$	$-0.44 \times 10 = -4.4 \text{ }^\circ\text{C}$

Table 6 shows the path and the response of *steepest ascent*. Test 1 shows that amylose content increased from 25% to 42.2%. In the test 2, the measurements of all parameters were reduced and amylose content decreased to 35.8%. Same response was observed in test 3, the amylose content produced was only 5%. From this result, parameter in test 1 has been chosen as the center point in the next Central Composite Design, CCD.

Table 6: Step of *steepest ascent*

Test	Step	X ₁	X ₃	X ₄	X ₅	Substrate concentr ation (mg/ml)	pH	Durati on (min)	Temp (°C)	Response (% amylose)
	Source (center point)	0	0	0	0	40	5	90	50	
	Δ	-1	-0.13	-0.12	-0.44	-10	-1.3	-1.2	-4.4	
1.	Source	0	0	0	0	40	5	90	50	42.2
2.	Source - Δ	-1	-0.13	-0.12	-0.44	30	3.7	88.8	45.6	35.8
3.	Source -2 Δ	-2	-0.26	-0.24	-0.88	20	2.4	87.6	41.2	5

Optimization Stage: Central Composite Design, CCD

Central composite design (CCD) is an easy technique to derive the function relationship of variable value to the response surface and to fit a second order model [29]. CCD is a recommended methodology to reduce the large numbers of factor and level in the experiments. CCD consists of 2^k factorial, where k is the number of factor [25]. Based on the result of test 1 in *steepest ascent* path, center point for CCD was identified. The value of non-significant variables X₂ (enzyme concentration) was standardized at center value in CCD experiment. Table 7 shows the CCD coded value and the factor level of each variable. Center point was coded as 0, -1 was coded as set of low factor and +1 was coded as high level. Factor levels at axial point were coded as -2 and +2. There were 30 experiments including 16 factorials, 8 axial and 6 center points that were completed for CCD in table 8).

Table 7: Factor Level and CCD coded value

Variables /Factor	Factor Level				
	-2	-1	0	+1	+2
Substrate concentration, X ₁	2	3	4	5	6
pH, X ₃	3	4	5	6	7
Duration, X ₄	30	60	90	120	150
Temperature, X ₅	30	40	50	60	70

Table .: CCD experimental with actual responses and predicted responses of amylose percentage calculated from model equation

Experiment	Substrate concentration [mg/ml]	pH	Duration [min]	Temp [°C]	*Actual response (% amylose)	*prediction response (% amylose)
1	-1	-1	-1	-1	41.56	39.22
2	1	-1	-1	-1	40.77	40.59
3	-1	1	-1	-1	31.24	31.63
4	1	1	-1	-1	36.49	34.92
5	-1	-1	1	-1	35.26	34.29
6	1	-1	1	-1	37.88	36.84
7	-1	1	1	-1	34.59	32.56
8	1	1	1	-1	35.27	37.02
9	-1	-1	-1	1	35.43	34.02
10	1	-1	-1	1	34.88	33.91
11	-1	1	-1	1	23.59	21.62
12	1	1	-1	1	22.1	23.41
13	-1	-1	1	1	30.8	29.38
14	1	-1	1	1	30.48	30.44
15	-1	1	1	1	22.3	22.83
16	1	1	1	1	26.49	25.81
17	-2	0	0	0	0	3.28
18	2	0	0	0	8.26	7.63
19	0	-2	0	0	38.43	41.29
20	0	2	0	0	29.27	29.07
21	0	0	-2	0	45.9	47.94
22	0	0	2	0	44.77	45.4
23	0	0	0	-2	46.4	48.03
24	0	0	0	2	30.61	31.61
25	0	0	0	0	52.32	50.4
26	0	0	0	0	49.65	50.4
27	0	0	0	0	49.07	50.4
28	0	0	0	0	50.21	50.4
29	0	0	0	0	51.71	50.4
30	0	0	0	0	49.43	50.4

* Data were average from triplicate

Model selection

Sequential model summary of analysis in table 9 has recommended quadratic model (F value <0.0001 and $p <0.0001$) to explain the data relationship of this design. Lack of fit test of quadratic model was not significant with p value 0.1819 ($p >0.0001$). Model summary statistics in table 9 showed that adjusted R^2 value 0.9115 was near to 1.

Table 9: Model summary statistics

Sources	Std Deviation	R-Squared, R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	11.01	0.1844	0.1397	0.0810	9963.52	
2FI	11.36	0.2024	0.0833	0.0792	9982.63	
Kuadratik	3.53	0.9376	0.9115	0.8885	1208.35	Suggested
Kubik	3.6	0.9342	0.9079	0.8641	1473.29	Aliased

PRESS: predicted error sum of squares

Analysis of Variance (ANOVA). Analysis of variance in table 10 shows that CCD model was significant with R^2 value of 0.9276. This R^2 represented 92.76% of response variance could be explained by this model. Lack of fit test was not significant and p value was 0.1817. This observation explained that the quadratic model was accurate for CCD.

Table 10: ANOVA of CCD

Sources	Sum of squares	Degree of freedom	Mean square	F value	P value	
Model	10 056.94	14	7118.35	5767	<0.0001	*
Residual	784.69	63	12.46			
Lack of fit	169.23	10	16.92	1.46	0.1817	
Cor total	10 841.63	77				

Table 11 shows the regression coefficient value and p value for each study variables. The variables that observed to be significant were substrate concentration (X_1), pH (X_3) and temperature (X_5). Substrate concentration was indicated as positive significant factor whereas pH and temperature were negative significant factors for debranching process. There were two significant interactions of among two variables. Interaction of pH and duration of reaction (X_3 X_4) was directed to positive interaction. pH and temperature interaction was directed to negative interaction. Second order polynomial model from regression analysis can be quote as equation 4 (Eq. 4). From the Eq. (4), temperature was the most significant factor followed by pH and substrate concentration. This experiment suggested that temperature has an important role on pullulanase debranching activity.

$$Y = 50.4 + 1.09 X_1 - 3.06 X_3 - 0.63 X_4 - 4.10 X_5 - 11.23 X_1^2 - 3.80 X_3^2 - 0.93 X_4^2 - 2.64 X_5^2 + 0.48 X_1 X_3 + 0.30 X_1 X_4 - 0.37 X_1 X_5 + 1.47 X_3 X_4 - 1.20 X_3 X_5 + 0.072 X_4 X_5 \quad (4)$$

Table 11: Regression coefficient value of CCD

Variables	Coefficient value	P value	
Intercept-y	50.40		
X_1 (substrate, mg/ml)	1.09	0.011	*
X_3 (pH)	-3.06	<0.0001	*
X_4 (Duration, minit)	-0.63	0.1320	
X_5 (temperature, °C)	-4.10	<0.0001	*
X_1^2	-11.23	<0.0001	*
X_3^2	-3.80	<0.0001	*
X_4^2	-0.93	0.0603	
X_5^2	-2.64	<0.0001	*
$X_1 X_3$	0.48	0.3509	
$X_1 X_4$	0.30	0.5640	
$X_1 X_5$	-0.37	0.4683	
$X_3 X_4$	1.47	0.0055	*
$X_3 X_5$	-1.20	0.0212	*
$X_4 X_5$	0.072	0.8879	

* significant ($p < 0.05$)

The generated 3 dimensional response surface from CCD explained the quadratic relationship of debranched amylose with substrate concentration and pH (Figure 7). Optimization suggested that substrate concentration of 4.3% (mg/ml), pH 4.6 at temperature of 43°C for 83 minutes produce 52% of high amylose sago starch. Further experiment has been conducted to confirm the suggestion from this model. Result showed that the response value produced 52.6% of amylose in sago starch (n=4). This study proved that the model was appropriate and accurate.

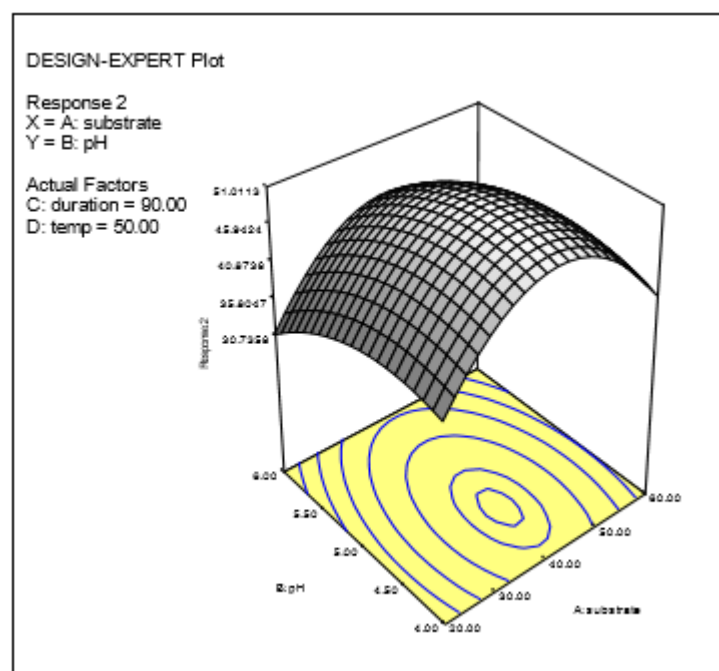


Figure 7: Response surface of high amylose starch yield

CONCLUSIONS

Utilization of RSM is effective for the identification of the factors and the optimal conditions significantly affecting pullulanase enzymatic activity. Response surface methodology (RSM) has been recognized as a relevant optimization method for many years [19, 20, 21]. Screening methodology using fractional factorial design (FFD) has identified the number of variables from five to four significant variables that influence the pullulanase debranching activity on sago starch. Steepest ascent pathway recommended adopting center point of four significant variables in central composite design (CCD) experiment. Optimization of factor level for pullulanase debranching process on sago starch was achievable by CCD implementation. This mathematical analysis methodology discovered optimal condition for pullulanase debranching activity to produce high amylose sago starch by reacting 4.3% (w/v) of substrate concentration with 1.5% of pullulanase (v/v) at pH of 4.6 for 83 minutes at 43°C. Confirmation experiment has been conducted and the response proved that amylose content increased from 25% of amylose in native sago starch to 52.6% of amylose after debranching with pullulanase.

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