OPTIMISATION OF HYDROLYSIS CONDITIONS FOR ETHANOL PRODUCTION FROM SORGHUM STARCH

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ABSTRACT

The conversion of starch to sugar can be achieved by hydrolysis process. The two-step enzymatic hydrolysis of sweet sorghum was performed by commercially available α-amylase and glucoamylase. An optimisation study was carried out to optimise the factors of the hydrolysis process, namely, amount of substrate, liquefaction and saccharification temperature, liquefaction and saccharification time, and amount of α-amylase and glucoamylase enzymes. The screening of significant hydrolysis factors were done by using the two-level factorial design (TLFD) under the factorial design (FD). The results indicated that the liquefaction and saccharification temperature, and amount of glucoamylase enzyme were found to be the major factors for further optimisation. The major factors for hydrolysis were optimised by the central composite design (CCD) under the response surface method (RSM). The analysis of variance (ANOVA) result showed that glucoamylase enzyme ($p < 0.0021$) and saccharification temperature ($p < 0.0181$) were significant factors for hydrolysis of sorghum starch. Also, the statistical analysis showed that the optimum dextrose equivalent (69.07% (g/g)) were obtained at 90°C of liquefaction temperature, 47°C of saccharification temperature, and 0.24% (v/w) of glucoamylase enzyme.

Keywords: *α-amylase, Glucoamylase, Liquefaction, Optimisation, Saccharification, Sorghum Starch*

1.0 INTRODUCTION

The world's leading manufacturers and industries are seeking to substitute petrochemical-based feedstock with agricultural-based materials as petroleum supplies continue to decline [1]. Great attention has been given to the ethanol production using various substrates which can be classified into three main types of materials, which are sugars (from sugarcane, sugar beet, sweet sorghum, molasses, and fruits), starches (from sweet sorghum grain, cassava, corn, potato, and root crops), and cellulose materials (from agricultural residue, wood, and paper mills) [2], because of the increase in demand for ethanol which is considered as an alternative energy source [3]. Furthermore, the commercial success of amylases is associated to utilisation of starchy biomass as an industrial raw material. Agricultural substrates like corn, wheat, sorghum and other cereal grains contain around 60-75% (w/w) starch on a dry basis, hydrolysable to glucose and thus offer a good resource in fermentation processes [4].

Sweet sorghum (*Sorghum biocolor* (L.) Moench) is one of the most favourable crops for industrial applications [1]. Sorghum is a C_4 plant characterised by a high biomass- and sugar-yielding crop [5]. It contains approximately equal quantities of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) [6]. Sweet sorghum has the ability of remaining dormant during the driest periods and is often judged to be one of the most drought resistant agricultural plants [7; 8]. Thus, it can be planted primarily in semiarid and dried parts of the world, especially in areas too dry for maize [1]. Also, it has been considered as an important energy plant for the production of fuel bioethanol [9].

Sweet sorghum grain is a starch-rich grain [1]. Starch consists of two types of polysaccharides, the linear molecule, amylose and a highly branched molecule, amylopectin [10]. Amylose is a linear molecule of (1→4) linked α-D-glucopyranosyl units (α-D-(1→4)-glucan), but it is well established that some molecules are slightly branched by (1→6)-α-linkages. Meanwhile,

amylopectin is a highly branched component of starch formed through chains of α-D-glucopyranosyl residues linked together mainly by (1→4) linkages but with 5-6% of (1→6) bonds at the branch points. It is a branched polysaccharide composed of hundreds of short (1→4)-α-glucan chains, which are interlinked by (1→6)-α-linkages [11; 12]. In most common types of cereal endosperm starches, the relative weight percentages of amylose range between 18-33% and amylopectin range between 72-82% [11].

Starchy grains and effluent generated from starch processing units are the cheap feedstocks and could be used as potential raw materials for ethanol fermentation [13]. The sweet sorghum starch hydrolysis may be regarded as a first and important step in sorghum processing for bioethanol production [12]. Enzymatic hydrolysis is essential for the production of glucose syrups from starch because of the specificity of enzymes allows the sugar syrups production with well-defined physical and chemical properties and the milder enzymatic hydrolysis results in few side reactions and less "browning" [2]. Conventional process for production of bioethanol from starch basically involved a three-stage process; liquefaction of starch by α-amylase, saccharification of liquefied starch by glucoamylase and followed by fermentation of sugar to ethanol using *Saccharomyces cerevisiae* [12].

The aim of this study was to investigate the liquefaction and saccharification processes of sweet sorghum by commercially available α-amylase and glucoamylase. In order to achieve the optimum conditions, the present study was carried out in two stages: firstly, the two-level factorial design was applied to select the most significant conditions of starch hydrolysis such as the substrate and enzyme concentrations, and the temperature and time required for the enzymatic action, which affect glucose production. Secondly, the central composite design (CCD) was employed to obtain the optimum level of the significant factors by developing a model followed by other statistical tests such as analysis of variance (ANOVA), coefficient of determination, 2D contour and 3D surface plots for the glucose production of from sweet sorghum starch.

2.0 MATERIALS AND METHODS

2.1 Substrates

Sweet sorghum grains were obtained from Indonesian Bioenergy Foundation and blended into small size of approximately 20 μm to enhance the hydrolysis process.

2.2 Enzymes

Both α-amylase from *Bacillus subtilis* and glucoamylase from *Aspergillus niger* were obtained from enzyme industry in Jakarta, Indonesia. The activities of the two enzymes were identified to be 25,000 U/mL and 130,000 U/mL, respectively.

2.3 Hydrolysis

The shake flask was filled with 100 ml of distilled water and heated to 80°C. Then, 25 g of sweet sorghum was added to the flask (to make 25% (w/v) of substrate). After that, 0.2% (v/w) of α-amylase (from the amount of sorghum) was added and the mixture was cooked at 80 °C and mixed at 250 rpm for 1 h. After 1 h, the mixture was cooled down to 60 °C and 0.1% (v/w) of glucoamylase was added and the mixture was left for 4 h with 250 rpm agitation.

2.4 Enzymes

Samples for dextrose equivalent (DE) determinations were centrifuged at 5000 rpm for 30 min to remove the substrates. The supernatant was filtered through a 0.45 μm membrane and analysed by high performance liquid chromatography (HPLC) equipped with a refractive index detector. The column used for separation was a SUPELCOGEL C-610H column. 10 μl of sample was injected into HPLC and separation was performed at 30 °C with 0.1% H_3PO_4 as the mobile phase at a flow rate of 0.5 mL/min. Glucose was used as a standard. DE was calculated as follows:

$$DE = \frac{g\ reducing\ sugar\ expressed\ as\ glucose}{g\ dry\ solid\ weight} \times 100 \quad (1)$$

2.5 Experimental design

2.5.1 Two-level factorial design

The two-level factorial design was used to identify which factors of hydrolysis process have significant effects on the response, DE. The factors selected for the experiment were the amount of substrate (A, % (w/v)), liquefaction temperature (B, °C), liquefaction time (C, h), amount of α-amylase [D, % (v/w)], saccharification temperature (E, °C), saccharification time (F, h), and amount of glucoamylase [G, % (v/w)]. The factors were examined at two different levels (low and high) coded (1 and 2, respectively) as shown in Table 1. This design gave an output of eight experimental runs (combinations) with seven independent variables. All the experiments were performed in triplicate and the average of DE was used as the response (dependant variable). The two-level factorial design is based on the first order model which is as follows:

$$Y = b_0 + \sum b_i x_i \quad (2)$$

where Y is the response (DE value), b_0 is the model intercept and b_i is the linear coefficient, and x_i is the level of the independent variable. This model does not describe the interaction among the factors and it is used to evaluate and select the important factors that influence the response.

Table 1: Independent variables in the two-level factorial experimental design

Variables	Symbol	Coded levels	
		1	2
Amount of substrate, % (w/v)	A	25	35
Liquefaction temperature, °C	B	80	90
Liquefaction time, h	C	1	2
Amount of α-amylase, % (v/w)	D	0.1	0.2
Saccharification temperature, °C	E	50	60
Saccharification time, h	F	2	4
Amount of glucoamylase, % (v/w)	G	0.1	0.2

2.5.2 Central composite design

The central composite design was used to demonstrate the nature of the response surface in the experimental region and clarify the optimal conditions of the most significant independent variables. Three major variables namely liquefaction temperature (X_1, °C), saccharification temperature (X_2, °C), and amount of glucoamylase [X_3, % (v/w)] were included in this model. The factors were examined at five different levels (relatively low, low, basal, high, relatively high) coded (-2, -1, 0, +1, + 2) as shown in Table 2. Other variables were fixed from the result of the two-level factorial design. According to the CCD for three variables, 20 experimental runs (6 runs at centre point) were executed and the results were fitted to the following second order polynomial model:

$$Z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (3)$$

where Z is the dependent variable (dextrose equivalent); X_1, X_2 and X_3 are the independent variable (liquefaction temperature, saccharification temperature and amount of glucoamylase); β_0 is the regression coefficient at centre point; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients; and β_{12}, β_{13} and β_{23} are the second order interaction coefficients.

Table 2: Independent variables in the central composite experimental design

Variables	Symbol	Coded levels				
		-2	-1	0	+1	+2
Liquefaction temperature, °C	X_1	75	80	85	90	95
Saccharification temperature, °C	X_2	40	45	50	55	60
Amount of glucoamylase, % (v/w)	X_3	0.10	0.15	0.20	0.25	0.30

The developed regression model was calculated by analysing the values of regression coefficients, analysis of variance (ANOVA), *p*- and *F*-values. The quality of fit of the model equation was confirmed by the coefficient of determination, R^2. The statistical software package Design-Expert®6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to identify the experimental design as well as to establish a regression model to predict the optimum combinations considering the effects of linear, quadratic and interaction on dextrose equivalent value.

3.0 RESULTS AND DISCUSSION

3.1 Screening of significant hydrolysis parameters for glucose production using two-level factorial design

Seven hydrolysis parameters were screened by the two-level factorial design, which showed eight experimental runs for glucose production (Table 3). The main effect of each parameter on dextrose equivalent was estimated as the difference between the average of the measurements made at the low (1) and high level (2) of the factors. The main effects of each hydrolysis parameter are shown in Figure 1.

Table 3: The two-level factorial design for the screening of hydrolysis parameters

Run	*Factors*							*Response*
	A [% (w/v)]	*B (°C)*	*C (h)*	*D [% (v/w)]*	*E (°C)*	*F (h)*	*G [% (v/w)]*	*Y [% (w/w)]*
1	25	80	1	0.2	60	4	0.1	27.70
2	35	80	1	0.1	50	4	0.2	51.97
3	25	90	1	0.1	60	2	0.2	54.28
4	35	90	1	0.2	50	2	0.1	51.26
5	25	80	2	0.2	50	2	0.2	61.62
6	35	80	2	0.1	60	2	0.1	20.67
7	25	90	2	0.1	50	4	0.1	52.04
8	35	90	2	0.2	60	4	0.2	41.73

The screening results were analysed using the analysis of variance (ANOVA) as appropriate to the experimental design used as shown in Table 4. The computed *F*-value (46.87) indicates that the model was highly significant at high confidence level. The probability *p*-value was also relatively low (*p*-value > *F* = 0.0210) which indicates the significance of the model. The Fisher variance ratio, the *F*-value is a statistically valid measure of how well the factors describe the variation in the mean of data. The greater the *F*-value indicates that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. Also, the *F*-value is inversely proportional to *p*-value > *F*. Higher *F*-value will result to lower *p*-value > *F*.

Table 4: Analysis of variance (ANOVA) for screening

Source	Sum of Squares	*F*-value	*p*-value > *F*
Model	1389.47	46.87	0.0210
A	112.59	18.99	0.0488
B	174.31	29.40	0.0324
E	657.19	110.83	0.0089
F	25.88	4.36	0.1719
G	419.51	70.75	0.0138

For the independent variables, the *p*-value for saccharification temperature is the lowest (0.0089), followed by amount of glucoamylase (0.0138), liquefaction temperature (0.0324), amount of substrate (0.0488), and lastly saccharification time (0.1719). The *p*-values were used to check the significance of each coefficient. The lower the *p*-value indicates the more significant correlation of coefficients (*p*-value < 0.05 indicate the model terms are significant; *p*-value < 0.01 indicate the model terms are highly significant).

When the factor is highly significant, the small change in the factor (either increase or decrease) will give big impact on the response. Positive effect means increasing the factor will result to an increase in the response while negative effect means reducing the factor will result to an increase in the response. Linear and quadratic effects of parameters were significant, meaning that they can act as limiting factor and little variation in their value would change either the growth rate or the product formation rate or both to a considerable extent [14].

From the plot in Figure 1, three factors, which are liquefaction temperature, the amount of α-amylase and glucoamylase gave positive effect to the response. On the other hand, the amount of substrate, liquefaction time, saccharification temperature and time, gave negative effect to the response. The results showed that the highest value represents the most significant factor, by considering the absolute value only (neglect the positive and negative sign). The saccharification temperature gave highest impact on dextrose equivalent, followed by amount of glucoamylase, liquefaction temperature, amount of substrate, saccharification time, liquefaction time, and lastly amount of α-amylase enzyme. Positive linear coefficient means positive effect and vice versa. Therefore, the most important factors that affect the hydrolysis process of sweet sorghum are the liquefaction and saccharification temperature, and glucoamylase enzyme.

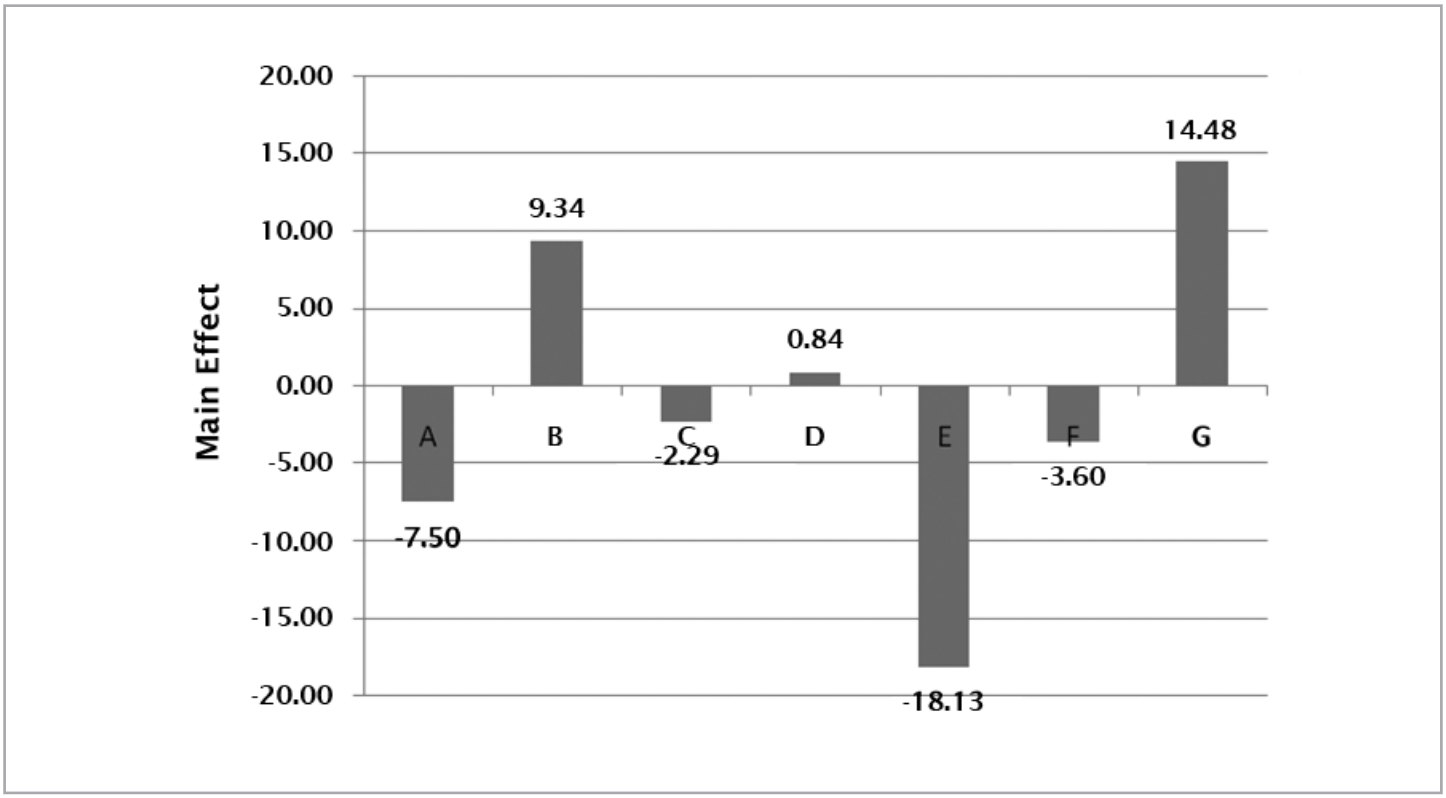

Figure 1: Main effects of the hydrolysis parameters on dextrose equivalent

In this study, the substrate concentration has negative effect on hydrolysis process, which means lower amount of substrate gives higher amount of dextrose equivalent. According to Mojović et al. [12], the substrate concentration had a pronounced effect on the starch hydrolysis and the ethanol fermentation. Regarding the yields, lower amount of substrate is more appropriate as substrate inhibition could be avoided. Furthermore, Aggarwal *et al*. [15] mentioned that 25% (w/v) of sorghum slurry was more appropriate for liquefaction process. This is because, at 35% (w/v), the mixing and homogenization were less efficient as the viscosity of the slurry is very high. Thus, the maximum concentration of sorghum slurry that suitable for liquefaction was observed to be 25% (w/v). This is because at lower substrate concentration, mixing and homogenisation of the reaction mixture was possible [16].

The liquefaction time has slightly negative effect on the amount of DE. As the liquefaction time increases, the amount of DE decreases. This is because the longer exposure of the enzyme to high temperatures, which are needed for gelatinisation of the starch granules and for achieving a good susceptibility to enzyme action, could lead to slight enzyme deactivation [12]. According to Shewale and Pandit [16], liquefaction of sorghum slurry 25% (w/v) was completed in only 1 hour.

For α-amylase enzyme, it provides very low positive effect on the DE. In one study, Shewale and Pandit [16] found that liquefaction of 25% (w/v) sorghum slurry was completed in 1 h with B. *licheniformis* α-amylase (BLA) concentration of 0.08% (v/w) of flour in the absence of $CaCl_2$ supplementation. However, Aggarwal *et al*. [15] mentioned that the optimum α-amylase enzyme concentration was 0.15% (v/w) for 1 h of liquefaction time, which is higher than the concentration used in this study.
The saccharification time has slightly lower negative effect on DE production compared to the effect of saccharification temperature. As the saccharification time increases, the amount of DE produced decreases. Ejiofor *et al*. [17] saccharified cassava starch at 55 °C only for 2 h.

3.2 Optimisation of hydrolysis parameters for glucose production using central composite design

The significant hydrolysis parameters such as liquefaction and saccharification temperature, and glucoamylase enzyme as independent variables were optimised for the maximum glucose production from sorghum starch. Experiments were carried out as designed by using central composite design (CCD) (Table 5), and the average glucose production obtained was used as the response. The optimal values of glucose produced within the experimental constrains were predicted by fitting a second order polynomial model to the experimental results for the dextrose equivalent by the Design-Expert software (v 8.0). The regression model developed relating the variables are as follows:

$$Z = 1492.04 + 17.68X_1 + 27.94X_2 + 897.52X_3 - 0.09X_1^2 - 0.22X_2^2 - 1782.18X_3^2 - 0.06X_1X_2 + 2.54X_1X_3 - 5.68X_2X_3 \quad (4)$$

where the dextrose equivalent (Z) is a function of liquefaction temperature (X_1), saccharification temperature (X_2) and amount of glucoamylase (X_3).

Table 5: The central composite design for the optimization of hydrolysis parameters

Run	Factors			Response	
	X_1 (°C)	X_2 (°C)	X_3 (% (v/w))	Z (% (w/w))	
				Observed	Predicted
1	80	45	0.15	38.62	44.73
2	90	45	0.15	54.98	52.39
3	80	55	0.15	44.54	42.69
4	90	55	0.15	47.24	44.11
5	80	45	0.25	52.26	57.96
6	90	45	0.25	63.74	68.15
7	80	55	0.25	45.08	50.24
8	90	55	0.25	57.74	54.19
9	75	50	0.20	55.92	49.64
10	95	50	0.20	57.54	61.25
11	85	40	0.20	55.20	49.67
12	85	60	0.20	30.70	33.67
13	85	50	0.10	32.48	34.49
14	85	50	0.30	62.38	57.80
15	85	50	0.20	62.24	63.97
16	85	50	0.20	62.12	63.97
17	85	50	0.20	62.36	63.97
18	85	50	0.20	66.14	63.97
19	85	50	0.20	70.40	63.97
20	85	50	0.20	63.12	63.97

At the model level, the correlation measures for the estimation of the regression equation are the determination coefficient R^2. The correlation between the observed and predicted values is better when the value of R^2 is closer to 1. In this experiment, the value of R^2 was 0.8636. This value indicates a high degree of correlation between the observed and predicted values. The value of R^2 indicates that 86.36% of the variables: liquefaction temperature, saccharification temperature and amount of glucoamylase play an important role to the response. The value of R^2 is also a measure of fit of the model and it can be mentioned that only about 13.64% of the total variations were not explained by the dextrose equivalent [18].

Table 6: Analysis of variance (ANOVA) for optimisation

Source	Sum of Squares	*F*-value	*p*-value > *F*
Model	2038.28	7.04	0.0026
X_1	134.79	4.19	0.0679
X_2	256.00	7.95	0.0181
X_3	543.36	16.88	0.0021
X_1^2	114.12	3.55	0.0891
X_2^2	781.58	24.29	0.0006
X_3^2	499.11	15.51	0.0028
X_1X_2	19.47	0.60	0.4547
X_1X_3	3.23	0.10	0.7581
X_2X_3	16.13	0.50	0.4951

The optimization results were analysed using the analysis of variance (ANOVA) as appropriate to the experimental design used as shown in Table 6. The computed *F*-value (7.04) indicates that the model was significant at high confidence level. The probability p-value was also relatively low (p-value > F = 0.0026) indicates the significance of the model. It was observed that the linear and square terms of both saccharification temperature (X_2) and glucoamylase (X_3) were significant ($p < 0.05$). Meanwhile, the linear and square terms of liquefaction temperature (X_1), and also the interactive terms between liquefaction temperature and saccharification temperature (X_1X_2), liquefaction temperature and glucoamylase (X_1X_3) and saccharification temperature and glucoamylase (X_1X_3) shown in the ANOVA analysis were not significant ($p > 0.05$).

The 2D contour plots and 3D response surface are the graphical representation of the regression model used to determine the optimum values of the parameters within the considered ranges [19]. The 2D and 3D plots for the interaction between two variables among three the variables are shown in Figure 2 to Figure 4. The purpose of response surface is to determine the optimum values of the variables, which mean the response is at maximum value [19]. The contour plot represents an infinitive number of combinations of the two test variables while the other variable maintained at zero level (centre). The maximum predicted value is obtained from the surface confined in the smallest ellipse in the contour plot. Elliptical contours are obtained when there is a perfect interaction between the two independent variables [20]. Meanwhile, the 3D surface plot shows whether the ellipse in the contour plot is at maximum or minimum.

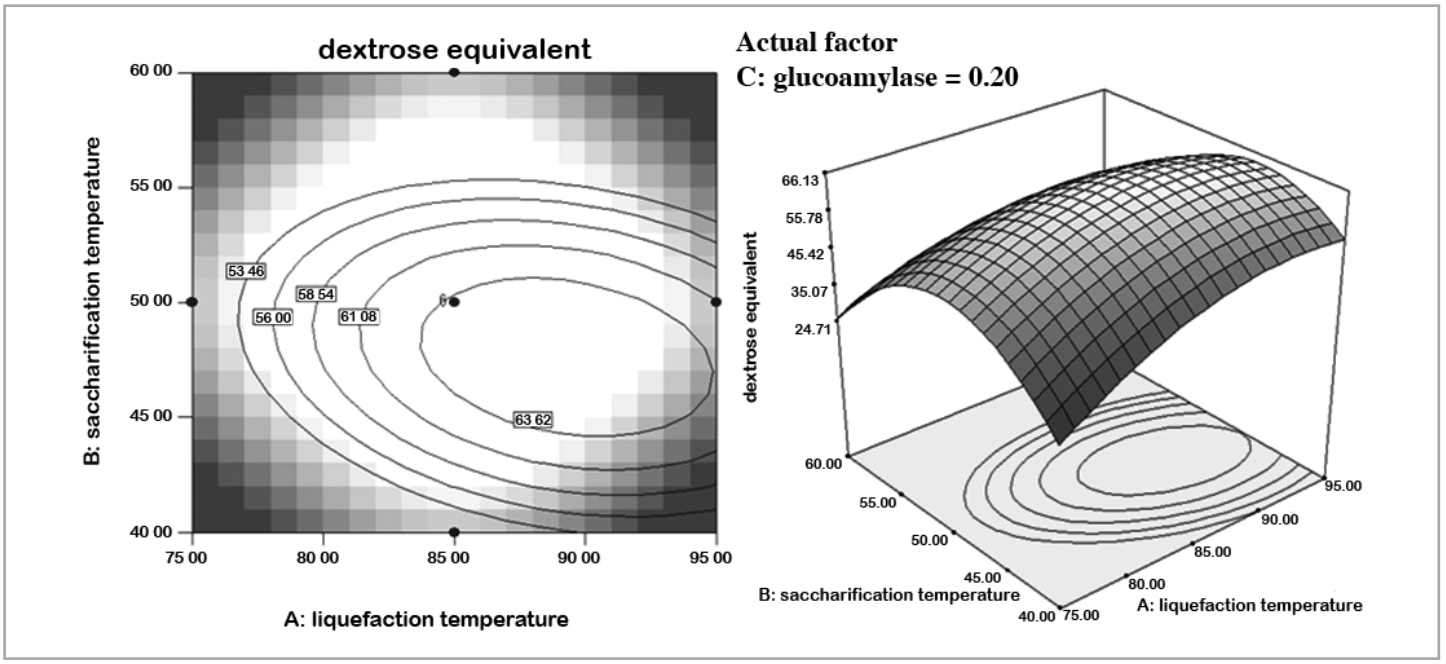

Figure 2: 2D contour plot and 3D response surface show the effect of liquefaction temperature (°C) and saccharification temperature (°C) on the dextrose equivalent [% (g/g)] [glucoamylase was 0.20 % (v/w)]

Figure 2 illustrates the elliptical response surface of dextrose equivalent from the interaction of liquefaction temperature and saccharification temperature. The predicted dextrose equivalent decreased at lower and higher values of ranges for both liquefaction and saccharification temperature. The maximum dextrose equivalent of about 66.13% (g/g) was predicted at liquefaction and saccharification temperature around 89 °C and 47 °C while glucoamylase concentration was 0.20% (v/w).

An elliptical response surface in Figure 3 shows the variation of dextrose equivalent as a function of liquefaction temperature and glucoamylase by making saccharification temperature a constant. About 67.14% (g/g) of maximum dextrose equivalent was obtained from the response surface at liquefaction temperature, glucoamylase concentration, and saccharification temperature was about 89 °C, 0.24% (v/w) and 50 °C, respectively.

Figure 4 is the response surface plot for the dextrose equivalent with the interaction of saccharification temperature and glucoamylase concentration. The maximum dextrose equivalent was predicted at given ranges of both saccharification temperature and glucoamylase concentration. Thus, the maximum dextrose equivalent of about 66.94% (g/g) was obtained when saccharification temperature around 47 °C, glucoamylase concentration was 0.24% (v/w), and liquefaction temperature was about 85 °C.

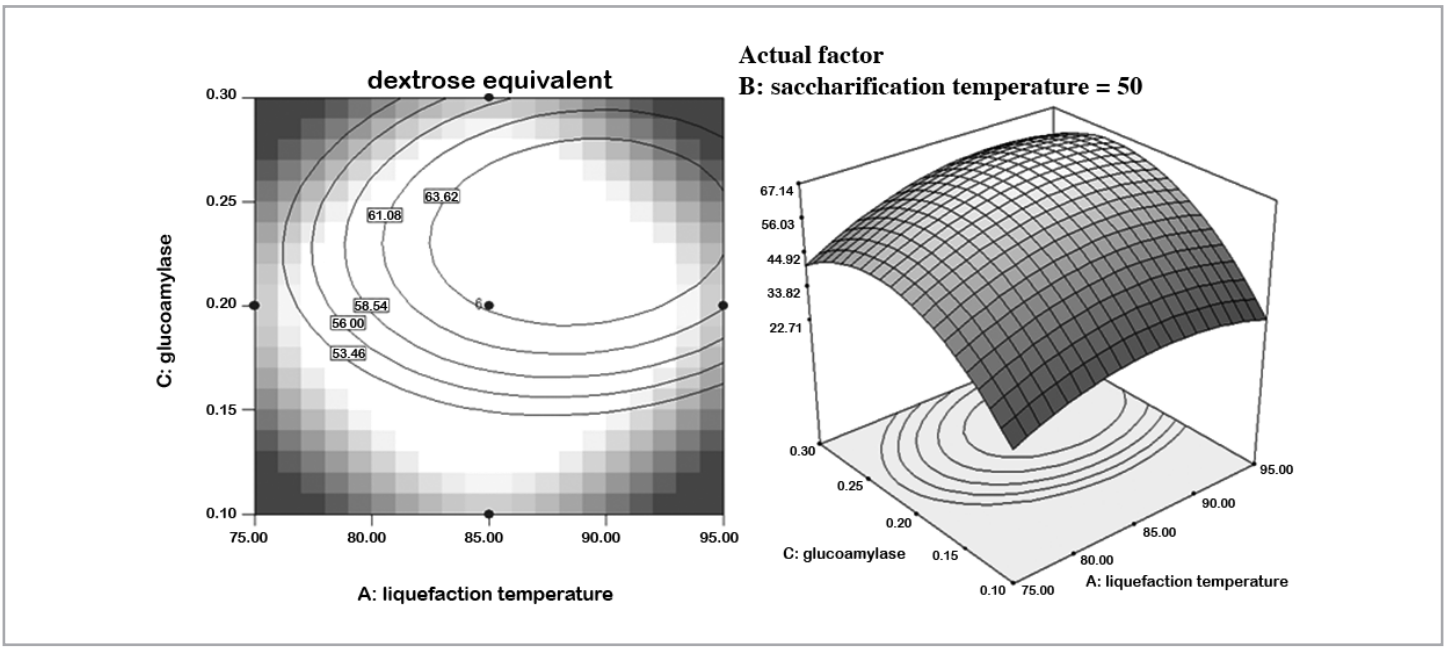

Figure 3: 2D contour plot and 3D response surface show the effect of liquefaction temperature (°C) and glucoamylase [% (v/w)] on the dextrose equivalent [% (g/g)] (saccharification temperature was 50 °C)

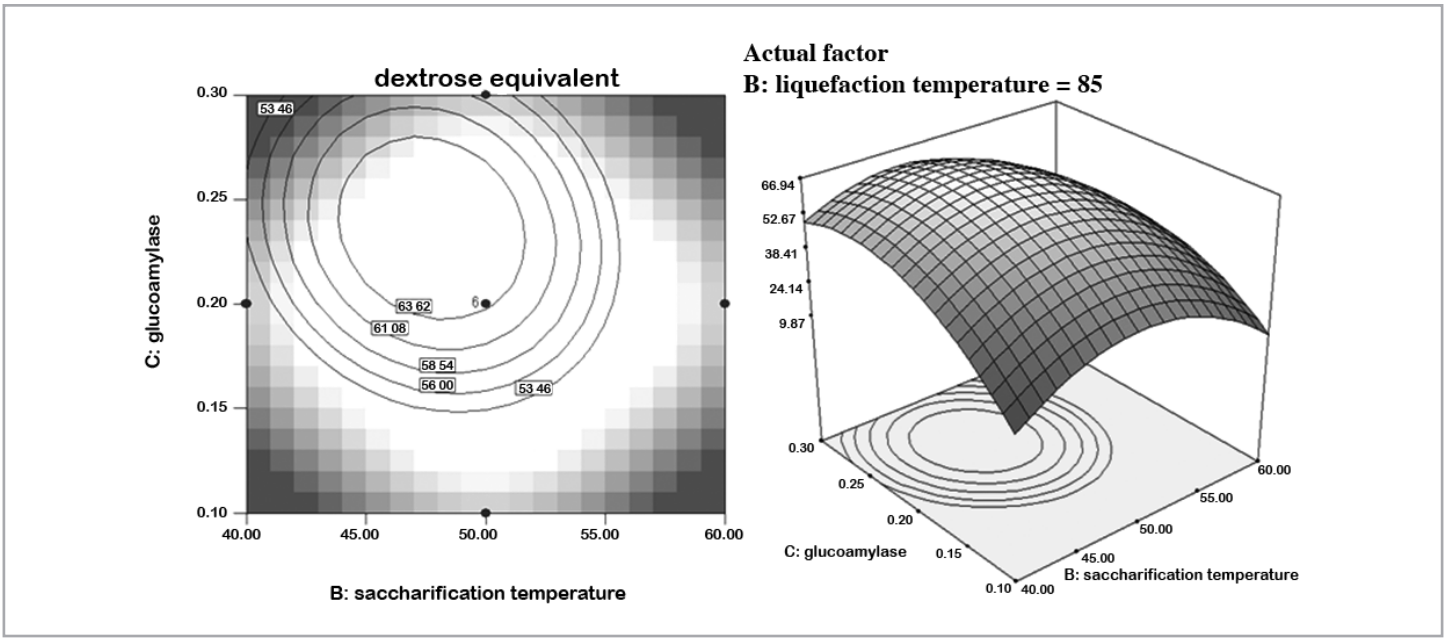

Figure 4: 2D contour plot and 3D response surface show the effect of saccharification temperature (°C) and glucoamylase [% (v/w)] on the dextrose equivalent [% (g/g)] (liquefaction temperature was 85 °C)

The optimum hydrolysis conditions for maximum dextrose equivalent of 69.07% (g/g) were predicted at 90 °C of liquefaction temperature, 47 °C of saccharification temperature, and 0.24% (v/w) of glucoamylase enzyme. Even though the liquefaction temperature is not significant, the interaction of all factors are significant since the probability *p*-value of the whole model was very low (*p*-value > *F* = 0.0026).

According to Shewale and Pandit [16], there are two effects occur simultaneously as the liquefaction temperature was increased from 75 to 95°C. The first one is when the gelatinisation rate of starch increases, and the second is when the dextrinisation rate of the starch molecules decreases due to enzyme deactivation at high temperatures. Therefore, there exists an optimum temperature for the liquefaction process. Moreover, for amylases to attack starch, the suspension should be brought to high temperatures (90-110 °C) for the breakdown of starch kernels [21]. The increase in hydrolysis of starch could also be connected to the effect of high temperature or heat on the weaker areas on the starch granule, allowing the enzyme to break the starch granules more extensively [10]. Aggarwal *et al*. [15] found that the maximum saccharification occurred at 45 °C as the rate of saccharification reduced substantially at higher temperature. Meanwhile, Shewale and Pandit [16] mentioned that 55 °C is the optimum temperature for saccharification to occur. Also, the glucoamylase enzyme activity increased progressively with an increase in temperature from 20 °C and reaching maximum at 60 °C [19].

4.0 CONCLUSION

In this study, three hydrolysis parameters, liquefaction and saccharification temperature, and amount of glucoamylase enzyme were selected by the two-level factorial design as the significant factors for dextrose equivalent. These factors were further optimized by using the central composite design. The dextrose equivalent of 61.62% (g/g) found during the screening process by two-level factorial design was increased to a predicted value of 69.07% (g/g) during optimisation using central composite design. The variables of saccharification temperature and glucoamylase concentration showed significant effects on the dextrose equivalent. ■

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