



Research Article

The Optimised Statistical Model for Enzymatic Hydrolysis of Tapioca by Glucoamylase Immobilised on Mesostructured Cellular Foam Silica

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Abstract

Enzymatic hydrolysis of starches using free glucoamylase to reducing sugars have difficulties in recovering and recycling of the enzyme, hence immobilisation on inert supports were widely studied. However, effectiveness of the immobilised glucoamylase were merely observed only on soluble starches. It was considered a valuable thing to know performance of glucoamylase on Mesostructured Cellular Foam (MCF) silica in hydrolysing of tapioca. An optimised study on enzymatic hydrolysis of tapioca using glucoamylase on MCF silica (9.2T-3D) and its kinetics were described including justification of the predicted model as it was required to develop in large scale operations. Central Composite Design was used to model the process by studying effects of three factors on DE values after enzyme immobilisation. Immobilisation of glucoamylase on this support gave up to 82% efficiency with the specific activity of 1,856.78 U.g⁻¹. Its used to hydrolysis of tapioca resulted DE values of 1.740-76.303% (w/w) where the highest DE was obtained at pH of 4.1, temperature of 70 °C and agitation speed of 140 rpm. The optimisation produced a polynomial quadratic model having insignificant lack-of-fit and low standard deviation, so that it was applicable and reliable in simulating the DE with only 0.80% of data were not described. Temperature affected the process highly, but the buffer pH, agitation speed and factorial interactions were considered not important. K_M value for immobilised enzyme was better than the free glucoamylase, however, its reaction rate was slower than the free glucoamylase catalysis. Copyright © 2019 BCREC Group. All rights reserved

Keywords: Enzymatic Hydrolysis; Glucoamylase Immobilisation; Mesostructured Cellular Foam Silica; Tapioca; Central Composite Design

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1. Introduction

In the producing industries of ethanol, glucose, monosodium glutamate, nucleotides and curdlan, hydrolysis of tapioca (*Manihot esculen-*

ta) starch to give reducing sugars must be conducted at first before microbial fermentation of the substrate into these fermentative product(s) takes place. Besides acidic-based processes, enzymatic hydrolysis of tapioca is often conducted using free glucoamylase. The uses of this enzyme type often have difficulties in recovering and recycling of it back to the hydrol-

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ysis process. Therefore, immobilisation of free glucoamylase onto inert insoluble supports is studied highly. Previous experiments showed that various materials were developed to support immobilisation of free glucoamylase, such as: nanomagnetic compounds, carbon, cellulose beads, montmorillonite, polymers, gelatine, and alginate via adsorption, covalent bond, entrapment, and/or encapsulation method [1-12].

The siliceous-based compounds were one of the studied supports. For example, mesoporous silica particles were used to encapsulate free glucoamylase, which effectively hydrolysed water soluble carbohydrate and could be used for many cycles of the process [13]. Glucoamylase immobilised on mesostructured cellular foam (MCF) silica also performed well by having high activity during hydrolysis of soluble starch [14]. Later, high performance of the soluble starch hydrolysis was obtained using glucoamylase immobilised on large ordered siliceous materials such as SBA-15 and mesoporous silica either by adsorption or by covalent bonds [15,16].

As effectiveness of all silica-based supports were proved on soluble starches, it is considered a valuable thing to observe performance of glucoamylase immobilised on the MCF silica in hydrolysing of water insoluble starches. Therefore, an optimised study on the enzymatic tapioca starch hydrolysis using the MCF silica (9.2T-3D)-based glucoamylase is herewith reported including the process justification as a predicted model is required to develop the large scale operations. Kinetics of the process is also determined.

2. Materials and Methods

2.1 Materials

Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-PPG-PEG (no. 435465), 1,3,5-trimethylbenzene (>98%), orthosilicic acid tetraethyl ester (>99%) and acetic acid sodium salt (>98%) were bought from Sigma Aldrich via a domestic supplier. Fluoroammonium (>98%), hydrogen chloride (37%), sodium monohydrogen phosphate (>99%), dextrose (>99%), potassium biphosphate (>99%) and dinitrosalicylic acid (DNS) (>98%) were Merck products. Glucoamylase LYPH170122 (30,000 U.g⁻¹) was supplied by Xi'an Lyphar Biotech Co. Ltd. (China). Local tapioca starch was used. The support was synthesized following the procedures arranged by Hermida *et al.* [17].

2.2 Enzyme Immobilisation

60 mg enzyme and 500 mg support were mixed with 30 mL 100 mM pH 5.5 Sorensen PO₄ buffer. This mixture was shaken at 100 rpm and 30 °C for 5 (five) hours. After filtration of the mixture, filtrate was kept temporarily in refrigerator for protein analysis. Residue of the process was washed with 100 mM pH 5.5 Sorensen buffer (3×50 mL) where filtrate of the washing process was also kept in refrigerator before the protein checking. The residue was dried in a desiccator at room temperature overnight and then stored in refrigerator. Enzyme load (*q*) was calculated as follows:

$$q = \frac{(C_o - C_t) \times V}{W} \quad (1)$$

where *C_o* is initial free enzyme concentration (mg mL⁻¹), *C_t* is final free enzyme concentration (mg mL⁻¹), *V* is reactor working volume (mL), and *W* is weight of support (g).

2.3 Specific Activity of Enzyme

A method described by Milosavic *et al.* [6] was adopted. A stock solution was prepared by dissolving 4 g soluble starch in 100 mL 50 mM pH 4.5 sodium acetate buffer. Then, two flasks containing 25 mL of the stock solution were prepared. The flasks were shaken in a water bath shaker at 140 rpm and 60 °C. After this temperature was reached, 50 mg immobilised enzyme and 25 mg free enzyme were added into the 1st and 2nd flasks to initiate hydrolysis of the starch. During the process, samples were withdrawn every five minutes for a 30 minutes period and centrifuged at 3000 rpm for 3 minutes before DNS analysis. One enzyme unit (U) was the amount of enzyme, which released reducing carbohydrates equivalent to one μmole glucose from soluble starch in one minute.

2.4 Experimental Design

Central Composite Design (CCD) of experiment was used to determine the statistical model of tapioca starch hydrolysis by immobilised glucoamylase on MCF silica (9.2T-3D). Three operational factors were studied in five levels (Table 1). Design Expert® v. 6.0.6 was used to design and analyse the results. Dextrose equivalent (DE) was set as response.

2.5 Enzymatic Hydrolysis Process

30 mL 0.1 M sodium acetate buffer (Table 1 provided the pH value) were placed in several

100 mL Erlenmeyer flasks. Then, 3% (w/v) of tapioca starch were dissolved in each flask. The flasks were shaken in a water bath shaker at certain agitation speed and temperature according to the operating conditions provided by CCD. After the temperature was reached, 400 mg immobilised enzyme was put into each flask to begin the hydrolysis process for 8 (eight) hours. Initial and final sample were collected to measure the DE values using DNS method.

2.6 Kinetic Constants

V_{max} and K_M was investigated by varying concentration of tapioca (1-5% w/v) in 30 mL 0.1 M sodium acetate buffer pH 4.3. The process was set at 72.4°C and 137 rpm for 3 (three) hours. 400 mg of enzyme was used. Samples were withdraw every half an hour for the DNS analysis. The initial rate data were processed with Chem SW Enzyme Kinetics! Pro.

2.7 Estimation of Protein and Glucose

The protein content was estimated using Pierce™ BCA protein assay kit. The hydrolysis of starch to glucose was followed by DNS analysis method. Shimadzu UV-VIS 1800 spectrometer was used to observe the protein and glucose content.

2.8 Characterization (FTIR, SEM)

Nitrogen adsorption-desorption isotherm data were obtained using a Quanta-chrome Autosorb 1C automated gas sorption analyzer operated at liquid nitrogen temperature to estimate average cell pore size, average window pore size, specific pore volume and specific surface area using Barrett-Joyner-Halenda (BJH) method and Brunauer-Emmett-Teller (BET) method. The enzyme and support were further observed with Zeiss EVO field emission scanning electron microscope (SEM) equipped with an Oxford INCAx act, energy dispersive X-ray (EDX) microanalysis system to obtain SEM images and chemical compositions. Samples were also observed using Frontier Perkin Elmer FTIR unit.

3. Results and Discussions

3.1 Glucoamylase on Support

Approximately 48.46 mg of the supplied free glucoamylase was adsorbed per 1 g of support (~ 82%). It was considered quite well. As the glucoamylase size (~ 80 Å) is lower than the support size (the support had specific surface area (S_{BET}) of 378 $m^2.g^{-1}$, cell diameter (d_{cell}) of 235 Å, pore volume of 2.12 $cm^3.g^{-1}$, window diameter of 158 Å), the free enzyme had high areas to attach not only on the outer surfaces, but also in the inner parts of the silica. In comparisons, nanoparticles carriers produced lower to high glucoamylase loads. For examples, the carboxyl functioned magnetic nanoparticles (diameter of ±9 nm) [2], functionalised magnetic nanoparticles (diameter <30 nm) [18] and functionalised Fe_3O_4 -chitosan hybrid (average diameter 200 nm) [19] gave the enzyme loads of 12-120 mg enzyme per gram support. However, though nanoporous materials had similar size, they tended to give relatively lower loads. Functionalised magnetic graphite nanosheets (pore sizes of ~11.71 nm, $S_{BET} = 60.07 m^2.g^{-1}$) [20], poly(amidoamine) magnetic carbon nanotubes (MCNTs-PAMAM) (CNTs diameters of 20-30 nm) [21], and $Fe_3O_4@clay$ composites (pore sizes of 13.17-22.06 nm, $S_{BET} = 36.2-48.5 m^2/g$) [22] absorbed only 9-21 mg glucoamylase per gram support. The mesoporous-type carriers had similar performances. Functionalised FeSBA-15 (pore diameter of 6-11 nm, surface area of ≥ 550 m^2/g) generally absorbed 10-22 mg glucoamylase per gram support [3]. Mesoporous carbons (Sibunit (pore diameter of 18 nm, 555 m^2/g), bulk CFC (11 nm, 162 m^2/g), activated carbon (4 nm, 1296 m^2/g) showed a little higher of enzyme absorption i.e. 5-24 mg enzyme per gram carbon [4]. Modified macroporous copolymer of glycidyl methacrylate and ethylene glycol dimethacrylate (poly(GMA-co-EGDMA)) with pore size of 53 nm and surface area of 50 m^2/g provided 35% immobilisation yield [7]. Milosavic, Prodanovic, Jovanovic, and Vujcic [6] obtained enzyme loads as high as 190 mg glucoamylase per

Table 1. The factors and level of the experimental design

Factor	Symbol	Level				
		-α	Low	0	High	+α
Temperature (°C)	x_1	61.6	65	70	75	78.4
Buffer pH	x_2	4.1	4.3	4.6	4.9	5.1
Agitation speed (rpm)	x_3	123	130	140	150	157

gram poly(GMA-co-EGDMA). Functionalized mesostructured cellular foam silica (pore diameter of 22-25 nm, wall diameter of 12-14 nm, surface area of 316-620 m²/g) yielded only 3.3-26.7% immobilised glucoamylase [14].

Immobilisation of the free enzyme on the support was proved by SEM and FTIR analysis. Observations on the SEM results showed an important change on the support. The original support had smooth surfaces as shown in Figure 1-A. However, after the enzyme immobilisation was conducted, rough surfaces were observed (Figure 1-B), which indicated the presence of enzyme on it. The FT-IR spectrum of the original MCF silica (9.2T-3D) (Figure 2) showed vibrations at 1300 cm⁻¹ and 850 cm⁻¹ with peak at 1061 cm⁻¹ to indicate the Si-O-Si stretching presence. These vibrations were also found on the immobilized enzyme spectrum, but an additional vibration 1962 cm⁻¹ was discovered. The new vibration confirmed the C≡N or C≡C bounds [23]. As glucoamylase is a protein consisting of a single polypeptide chain of amino acids, this vibration indicated the presence of amino acid on the support.

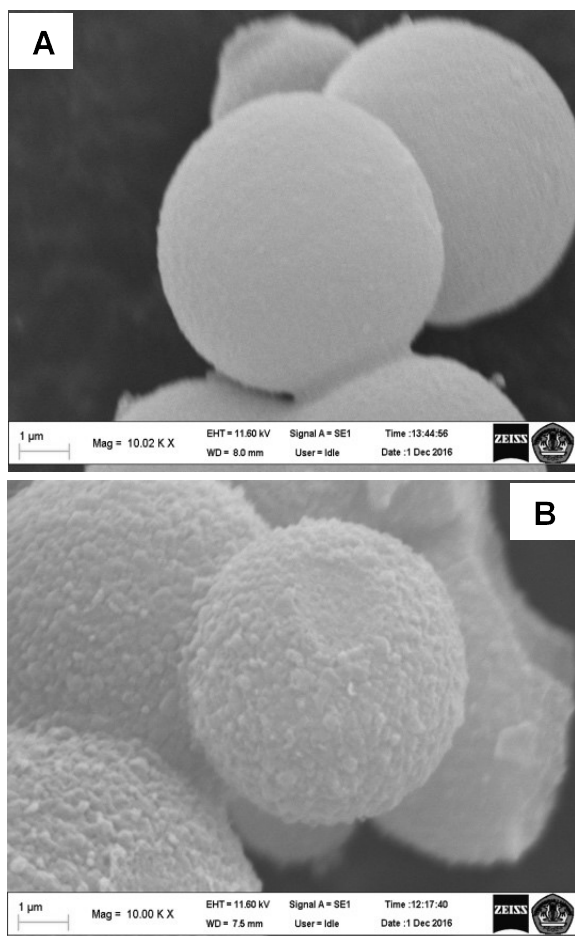


Figure 1. SEM images of supporting material (A) and glucoamylase on support (B)

Analysis of the specific activity revealed that reduction of activity occurred after the immobilisation process. The free glucoamylase activity was 30,335 U.g⁻¹, but the immobilised type had the specific activity of 1,857 U.g⁻¹. In general, immobilisation of the free glucoamylase on inorganic support tended to reduce its initial specific activity. Glucoamylase adsorbed on Sibunit retained up to 20% of the initial activity of the dissolved enzyme [4,24]. Lower glucoamylase specific activity (< 10%) was obtained when it was immobilised on polymeric compounds [6,7,25]. The specific activity as high as 55% for the immobilised glucoamylase was achieved when magnetic clays were used as supports [22]. These reductions could be caused by conformational changes on the enzyme structures during the immobilisation process as some enzyme active sites are difficult to be accessed by the substrate [26,27]. Milosevic *et al.* [7,25] described that the diffusional limitations had occurred caused by the support (polymer) that reduces the access of the substrate to the active sites of the bound enzyme. Further, Milosevic *et al.* [6] explained that the immobilization process interferes with the enzyme catalytic site and/or by diffusional problems generally produced when immobilized enzymes are acting on macromolecular substrates.

Compared with other carriers, the glucoamylase immobilised on the MCF silica (9.2T-3D) had better specific activity. The functionalised magnetic nanoparticles having larger diameter (< 30 nm) obtained the specific activity as high as of 1,408.6 U.g⁻¹ [18]. The mesoporous carbons, which had relatively higher surface area as described above, only gave the specific activity of 84-540 U.g⁻¹ [4,24]. Although the poly(GMAco-EGDMA) had higher pore sizes, the glucoamylase immobilised on this support showed lower specific activity (700-1,100 U.g⁻¹) [6,25], which could be caused by this support had lower surface area than the MCF silica.

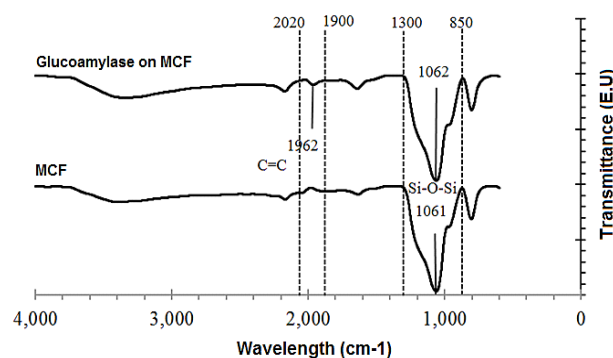


Figure 2. The FTIR spectra

3.2 Statistical DOE

DE values produced by the CCD from combination of 3 (three) operational factors in 5 (five) levels were found in the range of 1.740-76.303% (w/w) as shown in Table 2. The highest result was obtained at pH of 4.1, temperature of 70°C and agitation speed of 140 rpm, whilst the lowest response was observed at temperature of 61.6°C, pH of 4.6 and agitation speed of 140 rpm. To obtain the optimised model and its justifications, the experimental results were processed with Design Expert® v. 6.0.6 software.

$$\begin{aligned} \ln(DE) = & -194.16232 + 5.10662(\text{temperature}) + 0.29678(\text{pH}) \\ & + 0.21039(\text{agitation speed}) - 0.03565(\text{temperature})^2 \\ & - 0.66333(\text{pH})^2 - 0.00063(\text{agitation speed})^2 \\ & + 0.049431(\text{temperature} \times \text{pH}) \\ & - 0.001298(\text{temperature} \times \text{agitation speed}) \\ & + 0.012313(\text{pH} \times \text{agitation speed}) \end{aligned} \quad (2)$$

A polynomial quadratic model, which related all operational factors and their interactions, was developed by the statistical analysis as described in Equation (2). As described, all individual factors have positive effects on DE,

Table 2. Central composite design experiments

Run	x ₁ (°C)	x ₂	x ₃ (rpm)	DE (%) (w/w)	
				Actual	Predicted
1	61.6	4.6	140	1.740	1.42
2	65	4.9	150	8.243	9.55
3	70	4.6	140	68.406	68.72
4	75	4.9	150	44.830	48.79
5	70	4.6	157	64.161	58.05
6	75	4.3	130	71.169	68.84
7	70	5.1	140	51.852	42.41
8	75	4.3	150	59.985	56.74
9	70	4.6	140	68.406	68.72
10	70	4.1	140	76.303	79.43
11	75	4.9	130	49.602	51.06
12	70	4.6	140	68.406	68.72
13	78.4	4.6	140	20.598	21.44
14	65	4.3	150	13.725	14.94
15	70	4.6	140	68.406	68.72
16	65	4.3	130	13.580	13.98
17	70	4.6	140	68.406	68.72
18	70	4.6	123	60.612	57.05
19	65	4.9	130	6.508	7.71
20	70	4.6	140	68.406	68.72

but most quadratic forms show the opposite effects. Temperature and pH of buffer were the significant factor as their (*P>F*) values were less than 0.05 (Table 3).

The obtained model is reliable and can be applied to simulate the process. It can describe relationship between response and factorial interactions during the enzymatic hydrolysis process with the (*P>F*) value is < 0.0001 (Table 3). The lack-of-fit test indicates that the model has insignificant lack-of-fit and a low standard deviation value (i.e. 0.13). Analysis of variance (ANOVA) produces coefficient of determination (*R*²) of 99.20% with the adjusted and predicted *R*² are 98.48% and 93.93%, respectively. The *R*² is acceptable as the closer the *R*² value to unity, the better the models fits the data [28,29]. Previous observations on polysaccharides hydrolysis using CCD gave the *R*² of 86.36-96.94% [30-33]. Based-on the resulted *R*², more

Table 3. Statistical analysis of the model

	Source	Sum of Squares	DF	Mean Square	p-value Prob > F
Sequential Model Sum of Squares	Mean	250.33	1	250.33	
	Linear	9.36	3	3.12	0.0217
	2 FI	0.089	3	0.030	0.9916
	Quadratic	11.47	3	3.82	< 0.0001
	Cubic	0.10	4	0.026	0.1619
	Residual	0.065	6	0.011	
Lack of Fit Test	Total	271.41	20	13.57	
	Linear	11.72	11	1.07	
	2 FI	11.64	8	1.45	
	Quadratic	0.17	5	0.034	
	Cubic	0.065	1	0.065	
Analysis of Variance for Quadratic Model	Pure Error	0.000	5	0.000	
	Model	20.91	9	2.32	< 0.0001
	x ₁ -T	8.88	1	8.88	< 0.0001
	x ₂ -pH	0.48	1	0.48	<0.0003
	x ₃ -rpm	0.0004	1	0.0004	0.8859
	x ₁ x ₂	0.044	1	0.044	0.1369
	x ₁ x ₃	0.034	1	0.034	0.1874
	x ₂ x ₃	0.011	1	0.011	0.4392
	x ₁ ²	11.45	1	11.45	< 0.0001
	x ₂ ²	0.051	1	0.051	0.111
	x ₃ ²	0.057	1	0.057	0.0961
Residual	0.17	10			
Lack of Fit	0.17	5			
Pure Error	0.000	5			
Cor Total	21.08	19			

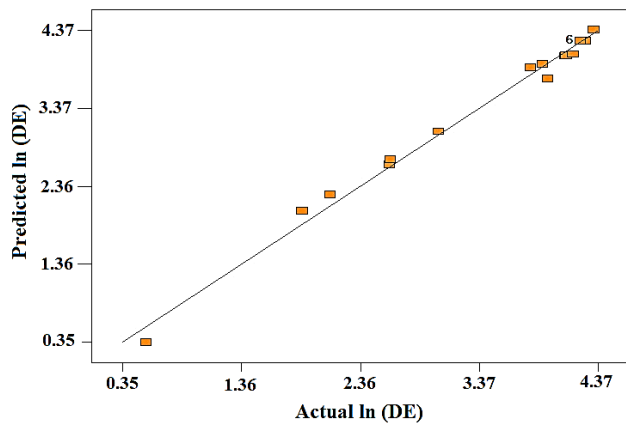


Figure 3. Plot of actual versus predicted for DE

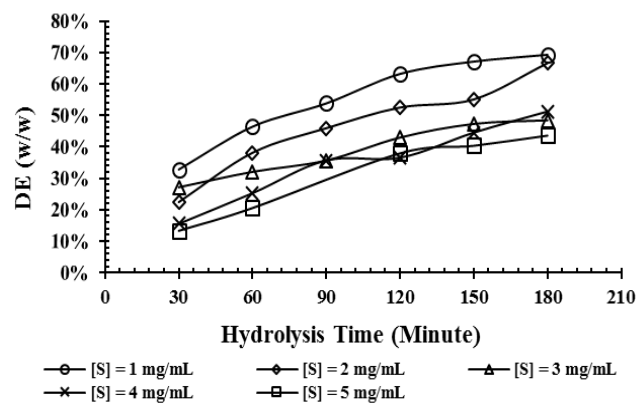


Figure 5. Progress of DE during initial rate measurements

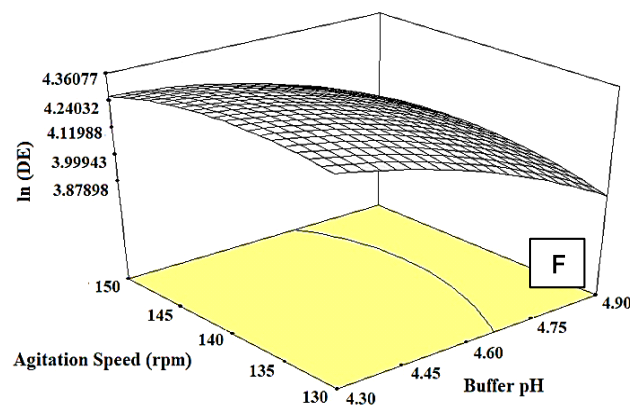
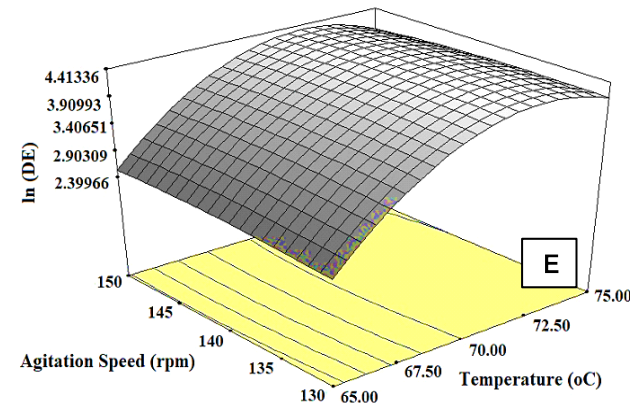
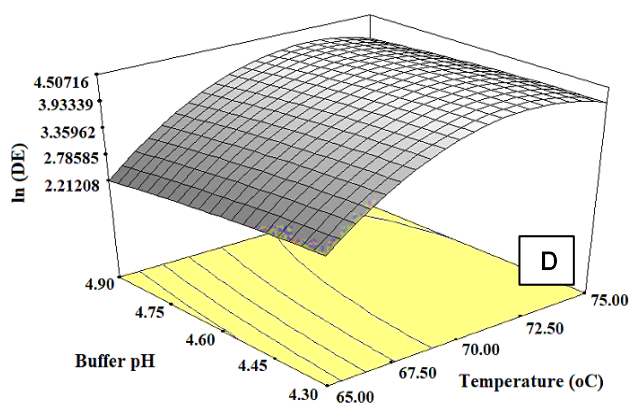
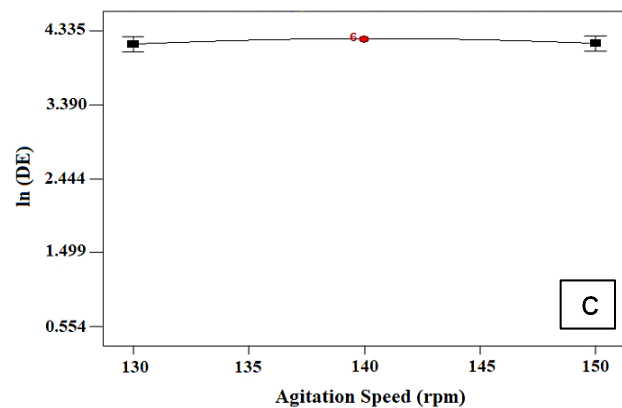
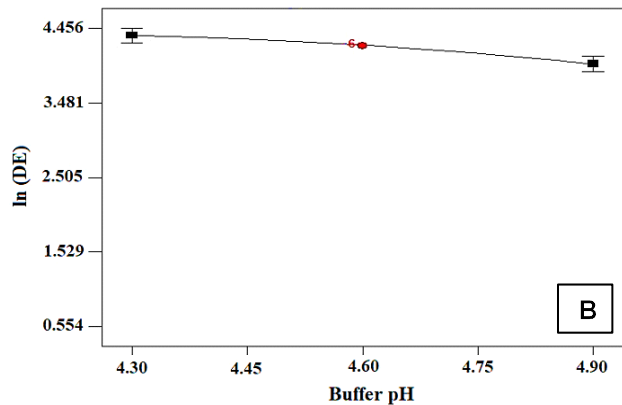
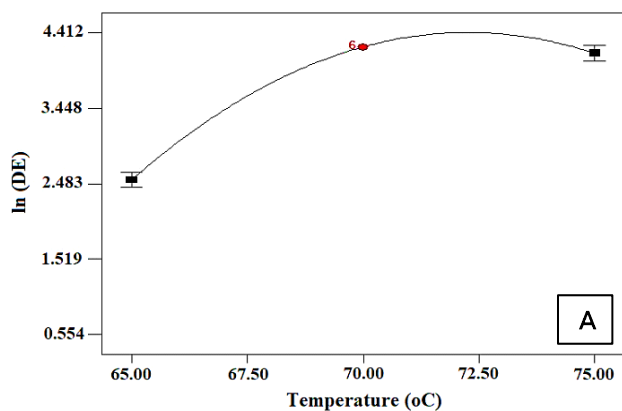


Figure 4. Effects of the factors and their interactions

than 95% of actual data can be explained by the model [34]. Only 0.80% of total variations were not described by the model. Hence, the predicted responses match the experimental results reasonably well as shown in Figure 3.

3.3 Effect of Operational Factors

Factor of temperature affected the process highly as shown in Figure 4-A. Great increases of the response were observed up to temperature of 70 °C to reach a peak at 72.5 °C, but the effects of the factor were relatively low starting from temperature of 70 °C to 72.5 °C. Decreases of the DE values occurred at temperatures of more than 72.5 °C, hence it is not required to operate the process above it. Compared with previous studies, the optimum temperature found in the experiments showed different behaviours. Free glucoamylase immobilised on activated carbon, bulk catalytic filamentous carbon, Sibunit and polymeric supports worked optimally at slightly lower operational temperatures i.e. 65-70 °C [4,6,7,24,25]. The same sit-

uation was observed on the immobilised glucoamylase on magnetic chitosan nanocarriers where the optimal temperature was 65 °C [19]. Zhao *et al.* [21,22] concluded that the optimum temperatures for the glucoamylase on magnetic carbon and clays were between 55 °C and 65 °C. However, immobilisation of the free glucoamylase on ordered mesoporous silica supports operated optimally at temperatures of 30-40 °C during hydrolysis of 3% (w/v) soluble starch at pH of 5.5 [16]. This big difference related to the changing of the physical and chemical properties of the enzyme during the immobilisation process where the creation of conformational limitations on the movement of the enzyme as a consequence of the formation of covalent bonds between the enzyme and the support [6,7].

Although pH of buffer solution tended to decrease the response (DE) as shown in Figure 4-B, its effects were considered quite high as the differences of DE value found at the low pH condition (i.e. 71.169%) and high pH condition (i.e. 49.602%) were more than 20%. In the ex-

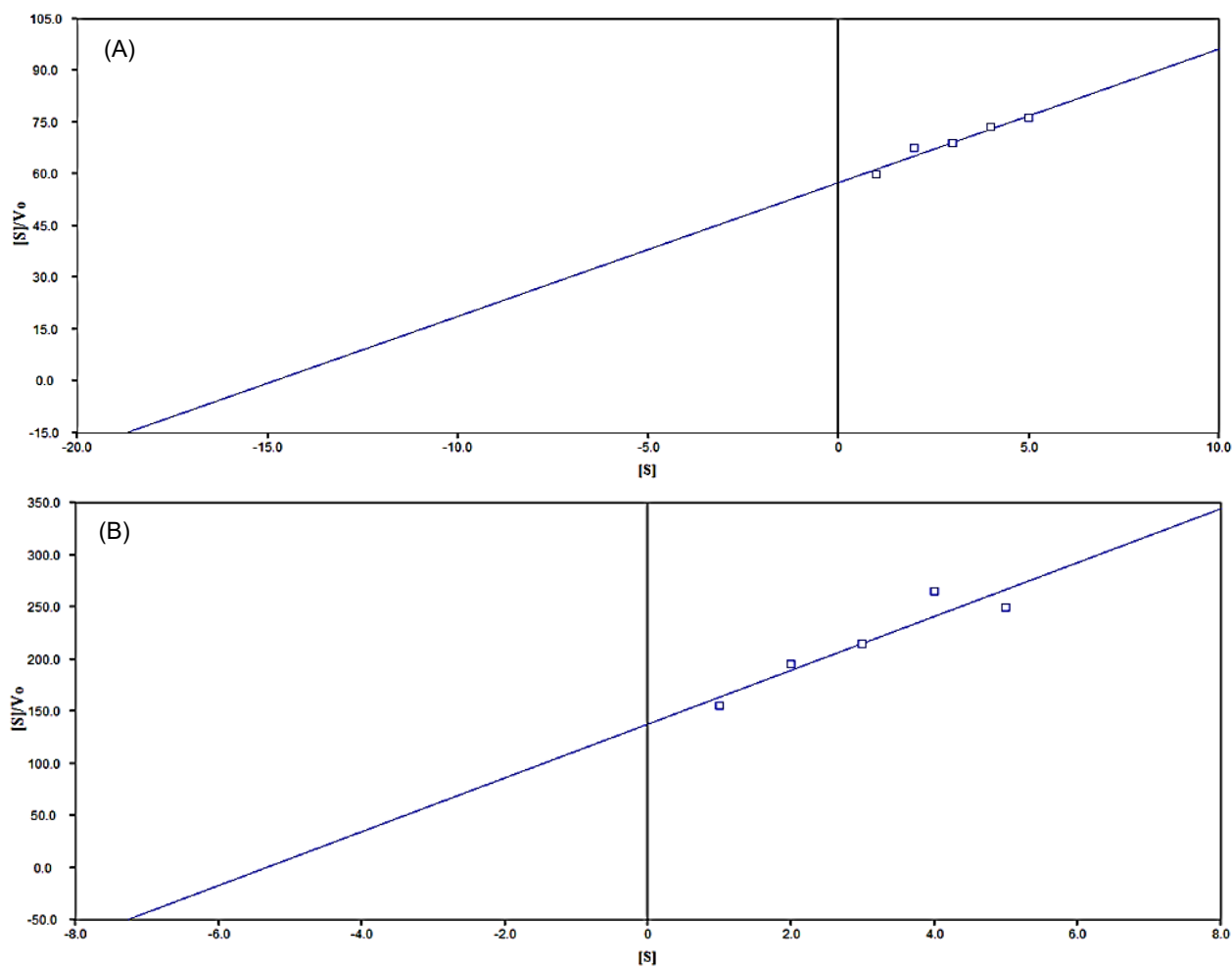


Figure 6. Plot of the kinetics (A: Free enzyme; B: Immobilised enzyme)

periments, the maximum DE was found at pH of 4.30, however, the pH of 4.6 gave the DE value as high as 68.406%, which differed slightly with the maximum value. Hence, operations of the hydrolysis process in the range of 4.3-4.6 were tolerable. The pHs found in this process were following some earlier observations. The maximum glucoamylase activity immobilised in the supports as described before were discovered at pH of 4.5 [6,7,18,25]. The cassava maltodextrin hydrolysis using the glucoamylase immobilised on chicken bones worked highly in sodium acetate buffer pH of 4.5 [35]. Kovalenko and Perminova [4] and Kovalenko *et al.* [24] concluded that the free glucoamylase from several manufacturers has the optimum pH of 4.5-4.6, however, from their immobilised glucoamylase experiments, the obtained pH range was 4.5-5.0 in dextrin hydrolysis. They suspected that the purity of the utilized enzyme related to the pH change. However, a higher pH range for immobilised glucoamylase was also observed. The free glucoamylase immobilised in ordered mesoporous silicas and carboxyl-functionalised magnetic nanoparticles had optimum pHs of > 5.5 [2,16].

Factor of the agitation speed and the factorial interactions are considered low and not significant matters. The DE values were almost constant during variation of the agitation speed (Figure 4-C). A high DE value (71.169%) was given by the low agitation speed (i.e. 130 rpm), whilst the high speed produced the DE value as high as 59.985%. The speed of 140 rpm developed the DE value of up to 68.406%, which did not differ highly with the speed of 130 rpm. Hence the speeds of 130-140 rpm were tolerable during the process operations. Three interactions in the process, i.e. temperature-buffer pH (x_1x_2), temperature-agitation speed (x_1x_3) and buffer pH-agitation speed (x_2x_3) (Figure 4-D to F), gave the ($P>F$) values of > 10%.

From the above descriptions, it is known that the temperature factor affected the immobilised glucoamylase activity highly as it increased the DE rapidly from the low temperature condition within a low temperature range,

whilst the pH of buffer solution developed less high effects on the tapioca hydrolysis process. Both factors indeed were significant for the polynomial process model as described in the previous section. However, the agitation speed has relatively low effects on the process, hence it was concluded as a non-significant factor. These results followed most of the previous studies as mentioned above where 2 (two) important factors were frequently observed during the glucoamylase activity evaluation i.e. temperature and pH of buffer in which the temperature factor usually worked at a broad range of operational conditions than the pH factor. Hence, the temperature plays a high role during the enzymatic hydrolysis process of tapioca starch using immobilised glucoamylase on MCF silica as concluded in Table 3.

3.4 Model Verification

To observe similarity between the experimental and predicted DE, justification of the model was conducted by testing some proposed solutions provided by the software. The results are shown in Table 4. Differences between both DE were less than 5%, which are acceptable as they are in the range of 95% confidence level. This comparison confirmed validity and adequacy of the model.

3.5 Reaction Kinetics

The enzymatic kinetics data were determined by observing the reaction progress at tapioca concentrations of 1-5 mg mL⁻¹ in 30 minutes time interval for 3 (three) hours as drawn in Figure 5. All concentrations almost have the same reaction rates. In the first 30 minutes, 13-32% (w/w) DE were formed where higher tapioca concentrations tended to give low DE values. After 120 minutes, DE values were increased to 36.55-63.18% (w/w) indicating the active sites of the immobilised glucoamylase were not difficult to be accessed by the substrate. At least more than 45% (w/w) DE were obtained after 3 hours.

Table 4. The actual DE versus predicted DE

Solution	Temperature (°C)	Buffer pH	Agitation Speed (rpm)	%DE		
				Prediction	Actual	Difference
1	66.7	4.1	127	31.78	34.90	3.12
2	72.4	4.3	137	91.84	90.12	-1.72
3	73.5	4.3	129	84.86	88.53	3.67
4	73.2	4.6	143	78.65	75.78	-2.87

K_M and V_{max} value calculation were based on Michaelis-Menten equation (Equation (3)). The formula was analysed using Hanes-Woolf plot (Equation (4)) as it gives low deviations from true K_M and V_{max} [36]. The errors resulted by this plot are distributed more evenly than the Lineweaver-Burk and Eadie-Hofstee plot [37].

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (3)$$

$$\frac{[S]}{v} = [S] \left(\frac{1}{V_{max}} \right) + \frac{K_M}{V_{max}} \quad (4)$$

where v is rate of reaction, K_M is the Michaelis-Menten constant, V_{max} is the maximum reaction rate and $[S]$ is concentration of substrate. Figure 6 describes the Hanes-Woolf plot for both glucoamylase types.

Value of K_M for the immobilised glucoamylase is lower than the free enzyme. The immobilised type had the K_M value of 5.33 mg.mL⁻¹, whilst the K_M for the free glucoamylase was 14.86 mg.mL⁻¹. The increase of the K_M value after the immobilisation indicated that the enzyme had better affinity on the substrate. This could be caused by the change of the enzyme structure that increased the accessibility of the enzyme active sites [38]. V_{max} for the process catalyzed by the immobilised and free glucoamylase were 0.04 and 0.26 mg⁻¹.mL⁻¹.minute⁻¹, respectively. Although 82% of free glucoamylase was adsorbed by the support, the specific activity of the immobilised enzyme was lower than the free type meaning reduction of the free glucoamylase activity indeed occurred after the immobilisation process as described previously. The glucoamylase immobilised on MCF silica (9,2T-3D) had similar V_{max} value with the glucoamylase encapsulated by calcium-alginate-clay in tapioca starch hydrolysis, but the K_M result indicated that this siliceous support was more resistance. Rahim *et al.* [12] obtained the V_{max} and K_M of 0.0762 mg.mL⁻¹.minute⁻¹ and 2.1708 mg.mL⁻¹, respectively, whilst their free glucoamylase gave the V_{max} and K_M of 0.2247 mg.mL⁻¹.minute⁻¹ and 1.8326 mg.mL⁻¹, which were better than the free glucoamylase used in the experiments.

4. Conclusion

Immobilisation of glucoamylase on MCF silica (9,2T-3D) gave up to 82% efficiency with the immobilised enzyme specific activity of 1,856.78 U.g⁻¹. Its uses to hydrolysis of tapioca starch resulted DE values of 1.740-76.303% (w/w) where the highest DE was obtained at pH of 4.1, temperature of 70 °C and agitation

speed of 140 rpm. The statistical optimisation of the process produced a polynomial quadratic model, which has insignificant lack-of-fit and low standard deviation, so that it is applicable and reliable in simulating the results in which only 0.80% of the data were not described. Temperature affected the process highly, but the buffer pH, agitation speed and factorial interactions were considered not important. K_M value for immobilised enzyme was better than the free glucoamylase, however, its reaction rate was slower than the free glucoamylase catalysis.

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