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Capability of immobilised glucoamylase on mesostructured cellular foam silica to hydrolyse tapioca starch

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Abstract. Mesoporous size siliceous-based materials have been associated with immobilisation of free glucoamylase and soluble starches hydrolysis. As ethanol and other chemicals can be produced from insoluble feed such as tapioca, understanding of glucoamylase immobilised on mesostructured cellular foam (MCF) silica to hydrolyse the starch is required. To get the knowledge on this process for development of a large-scale operation, free glucoamylase SQzyme AGP was immobilised on MCF silica (9.2T-3D) by direct adsorption and used the product to hydrolyse tapioca. The free enzyme was able to be immobilised onto the carrier with the efficiencies of 39.20-84.84% where the operational factors of temperature, agitation speed and pH affected the immobilisation process highly. During the enzymatic hydrolysis, effects of temperature and agitation speed were high. The dextrose equivalent (DE) values of 1.21-81.70% were obtained. The optimum temperature and agitation speed were 70°C and 140 rpm. Other factors such as buffer pH, starch concentration and enzyme unit activity showed low effects on the hydrolysis process. Excellent DE were obtained at the conditions of pH, tapioca concentration and enzyme unit activity of 4.6, 3 mg mL^{-1} and 2351 U, respectively.
Keywords: Silica-based support; Enzyme immobilisation; Tapioca hydrolysis

1. Introduction

Various insoluble supports were developed for free glucoamylase via different methodologies. Nano-magnetic materials, carbon, cellulose beads, montmorillonite, polymers, gelatine, and alginate had been used to support the enzyme via adsorption, covalent bonding, entrapment or encapsulation [1-7]. The resulted immobilised glucoamylase showed better thermal and pH stability than the free type.

One of the supports having mesoporous sizes is siliceous-based materials. Mesoporous silica particles prepared from tetraethoxysilane and phenyltriethoxysilane encapsulated free glucoamylase successfully where the product could be used for many cycles in a soluble starch hydrolysis process [8]. Ordered mesoporous silicas were employed to support free glucoamylase capable of hydrolysing soluble starch highly [9, 10]. Szymańska *et al*. [11] immobilised free glucoamylase on mesostructured cellular foam silica where the immobilised glucoamylase showed higher enzymatic activity in hydrolysing a soluble starch.

Since only soluble starches were hydrolysed in order to examine capability of glucoamylase immobilised on siliceous-based supports, a study on tapioca saccharification using glucoamylase immobilised on mesostructured cellular foam (MCF) silica is considered important as knowledge on this process is needed to develop large-scale operations. Therefore, a report on this process is herewith presented to observe characteristics of the factors during the enzymatic hydrolysis.

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by Joni Agustian

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2. Experimental Method

2.1. Chemicals and Biochemical

Pluronic® P123 (435465), mesitylene (>98%), tetraethylorthosilicate (>99%) and sodium acetate (>98%) were purchased from Sigma via local suppliers. Ammonium fluoride (>98%), HCl (37%), di-sodium hydrogen phosphate dodecahydrate (>99%), D(+)-glucose monohydrate (>99%) and potassium dihydrogen phosphate (>99%) were supplied by Merck Glucoamylase SQzyme AGP (150,000 U g^{-1}) were provided by a local importer. Tapioca starch (Rose brand) was bought from a local supermarket.

2.2. Synthesis of MCF Silica (9.2T-3D)

Method developed by Hermida *et al*. [12] was used. Pluronic (4 g) was dissolved in 70 mL HCl (1.6 M) at ambient temperature in a flask. Mesitylene (3.4 mL) was added. This mixture was stirred at 750 rpm and 40°C for 2 hours. After 9.2 mL tetraethyl orthosilicate was poured, it was stirred at 1,000 rpm for 5 minutes and later kept in water bath for 20 hours at 40°C. NH_4F (5 mL, 46 mg per 5 mL) was pipetted under slow stirring. It was kept in water bath for 72 hours at 80°C and then cooled to ambient temperature. After the filtration process, the crystals were dried at 100°C for 12 hours. They were calcined at 300°C for 30 minutes and continued at 500°C for 6 hours to give the MCF silica.

2.3. Immobilisation Process

The process was conducted via adsorption of free glucoamylase onto MCF silica (9.2T-3D) surfaces. The free enzyme (30-150 mg) was dissolved in some Sorensen phosphate buffer 100 mM (pH of 5-7). Then, 500 mg MCF silica was added in. The mixture was placed in water bath shaker (Medline BS-31) and shaken (80-140 rpm). Various shaker temperatures were employed (30-50°C). After 5 hours, the mixture was filtered (Whatman#42 filter paper). The filtrate was kept in refrigerator before protein analysis was conducted, whilst residue was washed with Sorensen buffer (3x50 mL) based on pH of the immobilisation process. The residue was dried at temperature of 35-40°C overnight and after that kept in desiccator. The filtrate from the washing process was also kept in refrigerator before analysing the protein content. The dried immobilised enzyme was stored in a refrigerator. Amount of free enzyme on support was calculated as follow:

$$\text{Immobilised Enzyme (\%)} = \frac{(C_0 - C_t)}{C_0} \text{ x V x } 100 \quad (1)$$

$$q = \frac{(C_0 - C_t) \text{ x V}}{W} \quad (2)$$

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), q is amount of enzyme onto MCF silica (mg g^{-1}) and W is weight of support (g). Buffer pH, temperature, agitation speed and enzyme concentration were varied using one-factor-at-a-time (OFAT) design.

2.4. Batch studies on hydrolysis of tapioca starch using immobilized α-amylase

OFAT was used to study activity of the immobilised glucoamylase in tapioca starch hydrolysis. Sodium acetate buffer (30 mL, 100 mM, pH 4.0-6.0) was poured into some 100 mL Erlenmeyer. Tapioca powder was added at concentration of 3-16 mg mL^{-1}. The immobilised glucoamylase (2351-7053 U) was mixed with the starch solution. The solution was shaken in water bath shaker at constant speed (140 rpm) for 24 hours at a certain temperature (35-60°C). Samples were withdrawn at certain interval times and then observed their reducing sugar. To compare the immobilised enzyme, the tapioca hydrolysis using free glucoamylase were also conducted under the same conditions.

2.5. Estimation of protein and glucose

Pierce™ BCA protein assay kit was used to estimate protein content by using the standard method provided by the kit manufacturer. Hydrolysis of starch to glucose was determined by the DNSA

(dinitrosalicyclic acid) method. The enzyme contents and glucose samples were observed using spectrometer Shimadzu UV-VIS 1800.

2.6. Characterization (FTIR, SEM, EDX)

Nitrogen adsorption-desorption isotherm data were obtained using a Quanta-chrome Autosorb 1C automated gas sorption analyser operated at liquid nitrogen temperature to estimate average cell pore size, average window pore size, specific pore volume and specific surface area (S_{BET}). Average cell pore size was evaluated using Barrett-Joyner-Halenda (BJH) method from the adsorption branch of the isotherm data. Average window pore size was evaluated using BJH method from the desorption branch. S_{BET} was calculated using Brunauer-Emmett-Teller (BET) method. Samples (support and enzyme) were analysed using 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 by Frontier Perkin Elmer FTIR.

3. Results and Discussion

Textural properties of MCF silica (9.2T-3D) matrix are shown in **Table 1**. The average window pore and cell sizes of it were 158 Å and 235 Å, respectively, confirming the mesoporous structure of the matrix. The window pores and cell sizes should allow favourable conditions for access and immobilization of the glucoamylase enzyme due to the enzyme size (usually 80 Å) is lower than the support size. Adsorption of the enzyme onto the support surfaces was confirmed by SEM (**Fig. 1**). The figure indicated that the support was in the form of spherical particles with smooth surfaces. Immobilization of the free enzyme on the support resulted in rough surfaces on the spherical material (**Fig. 1-B**). Such morphological changes could be due to the organic material of the enzyme that was adsorbed on these surfaces. The adsorption of the enzyme was also determined by FT-IR.

Table 1. Nitrogen adsorption-desorption result.

S_{BET} ($m^2\ g^{-1}$)	V_{pore} ($cm^3\ g^{-1}$)	d_{cell} (Å)	d_{window} (Å)
378	2.12	235	158

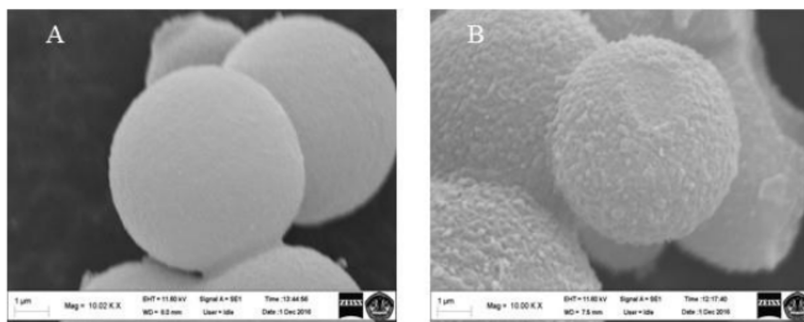

Figure. 1. SEM images: **A**. MCF (9.2T-3D) silica, **B**. Glucoamylase on support.

FT-IR spectrum of the immobilized glucoamylase was compared with that of original support (**Fig. 2**). The spectrum of the support showed its vibrations at 1300 cm^{-1} and 850 cm^{-1} with peak at 1061 cm^{1} to indicate Si–O-Si stretching vibrations. The vibrations were also observed on the immobilized glucoamylase, but additional vibrations at 2020 cm^{-1}, 1962 cm^{-1} and 1900 cm^{-1} were found, which were not noticed on the support. Based-on the reference, the vibration at 1962 cm^{-1} confirmed the C≡N or C≡C bounds [13]. As glucoamylase is a protein consisting amino acids, this vibration indicated the presence of amino acid on the support. EDX analysis on the support verified that it contained Si and O

elements at 1.75 keV and 0.35 keV, respectively (**Fig. 3**). However, the immobilized enzyme showed elements of C and K besides Si and O. Based on both spectra, a successful glucoamylase immobilization was obtained.

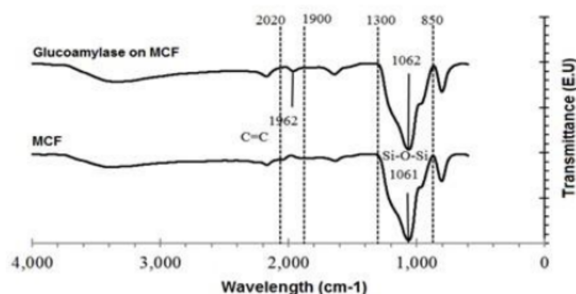

Figure. 2. FTIR spectra of support and glucoamylase.

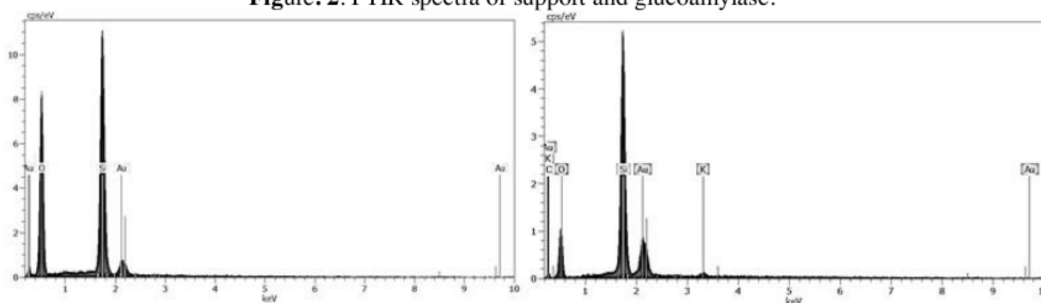

Figure. 3. EDX spectra (***left***: MCF (9.2T-3D); ***right***: glucoamylase on support).

3.1. Immobilisation of glucoamylase

All immobilisation factors show high effects on the process as shown in **Table 2** where the efficiency varied highly. The increase of pH tended to decrease the result. The optimum efficiency was achieved at pH 5.5 to result 84.84% (101.81 mg g^{-1}). The optimum pH is in agreement with the previous observations that used the acetate buffer pH 5.5 to obtain high immobilised glucoamylase [2, 14] When the free glucoamylase concentrations were arranged at 0.5-3 mg mL^{-1}, the efficiencies were found at 26.26-84.84% where the optimum value was given by the initial concentration of 2.0 mg mL^{-1}. The initial concentrations of 2.0-2.5 were frequently used to immobilise this enzyme on carbon or a magnetic support [2, 15]. The efficiency decreased when the temperature was increased. The best result was found at 30°C where 84.84% (~102 mg g^{-1}) of free enzyme was adsorbed. High temperatures tended to give low results. At temperatures of 40°C, the result only differed slightly with the highest value (< 6%), so that the process could be run at this temperature if it is difficult to operate the process at 30°C. Generally, the highest efficiencies of free glucoamylase immobilisation on inorganic supports were obtained at 30°C [2, 9]. The optimum result from various agitation speeds was obtained at 100 rpm. High agitation speeds reduce the efficiency. Although high contact between free enzyme and the MCF silica could occur at high speeds, as the process was based-on the direct adsorption that frequently has weak bonds, high speeds could disrupt the attached free enzymes and move them away from the support. Similarly, at low speeds, low contacts occurred, hence the free enzyme was difficult to land on the support, which resulted low efficiencies. Previous works used speed of 200 rpm. George *et al*. [9] and George and Sugunan [10] employed this speed to immobilise free glucoamylase on amino functionalised mesoporous silica compounds such SBA-15 and MPS where the free glucoamylase bonded covalently onto the supports.

Table 2. Immobilization efficiency and enzyme loading.

Operational Factors		Immobilisation Efficiency (%)	Enzyme Loading (mg g^{-1})
Temperature[a]	30°C	84.84	101.81
	35°C	78.80	94.56
	40°C	77.67	93.21
	45°C	66.00	79.2
	50°C	56.73	68.08
Agitation Speed[b]	80 rpm	58.91	70.69
	100 rpm	84.84	101.81
	120 rpm	58.08	69.70
	140 rpm	59.21	71.05
Buffer pH[c]	5.0	62.91	75.49
	5.5	84.84	101.81
	6.0	57.22	68.67
	6.5	47.47	56.96
	7.0	39.21	47.05
Enzyme Concentration[d]	0.5 mg mL^{-1}	26.60	7.98
	1.0 mg mL^{-1}	67.43	40.46
	2.0 mg mL^{-1}	84.84	101.81
	3.0 mg mL^{-1}	54.73	97.86

[a]: agitation speed: 100 rpm, buffer pH: 5.5, enzyme concentration: 2 mg mL^{-1}; [b]: temperature: 30°C, buffer pH: 5.5, enzyme concentration: 2 mg mL^{-1} ; [c]: temperature: 30°C, agitation speed: 100 rpm, enzyme concentration: 2 mg mL^{-1} ; [d]: temperature: 30°C, agitation speed: 100 rpm, buffer pH: 5.5

3.2. The enzymatic hydrolysis of tapioca starch

Direct conversions of tapioca into glucose molecules using glucoamylase immobilised on the MCF silica (9.2T-3D) working under various Sorensen phosphate buffer pHs are shown in **Fig.4-*a***. Dextrose Equivalent results (DE) were low. The highest DE was 20.11% obtained at pH 4.6. The DE increase of < 10% was developed when the reaction time was changed from 5 hours to 24 hours. Better results were found at pH ≤ 5.0; hence increasing buffer pH would decrease tapioca conversion (the optimum pH for glucoamylase enzyme is 4.5-5.0. The maximum DE given by the immobilised enzyme was less than half of the conversion produced by free enzyme that resulted DE as high as 57.36%. During the glucoamylase immobilisation via adsorption, the random immobilisation caused all enzyme catalytic sites may not be available for the substrate [16] that cause in low activity of the enzyme [17] as their active sites may be blocked from substrate accessibility, multiple point-binding may occur, or the enzyme may be denatured [18] or the glucoamylase gets inactivated partially [10]. The optimum pH found in the experiments differed slightly with the previous observations. Hydrolysis of 30% (w/w) cassava maltodextrin using glucoamylase immobilised on chicken bone was conducted in 50 mM sodium acetate buffer pH 4.5 [19]. Kovalenko *et al.* [15] and Kovalenko and Perminova [4] used 50 mM acetate buffer pH of 4.5-5.0 in hydrolysis of dextrin using glucoamylase immobilised on inorganic supports.

Tapioca concentration had low effects on DE for both glucoamylase forms (**Fig. 4-*left***). The immobilised enzyme still produced lower DE than its opponent. DE values resulted by it were 9.29-19.39%, whilst free enzyme gave DE of 23.79-56.80%. These results indicated that activity of the immobilised glucoamylase in direct hydrolysis of tapioca flour (meaning no gelatinisation was conducted previously) decreased after immobilisation. But, both enzymes showed the same phenomenon: reductions of conversion were observed when tapioca concentrations were increased. Maximum DE given by the immobilised enzyme was developed by the lowest tapioca concentration (3 mg mL^{-1}), but the free enzyme developed high DE at the starch concentration of $\leq$ 5 mg mL^{-1}. The DE were almost constant when high tapioca concentrations were used (8-16 mg mL^{-1}), although small changes were observed. Previous experiments had indicated effects of substrate concentrations on activity of immobilised glucoamylase. High reaction rate of hydrolysis of soluble starch increased rapidly at low starch concentrations [1, 5, 20].

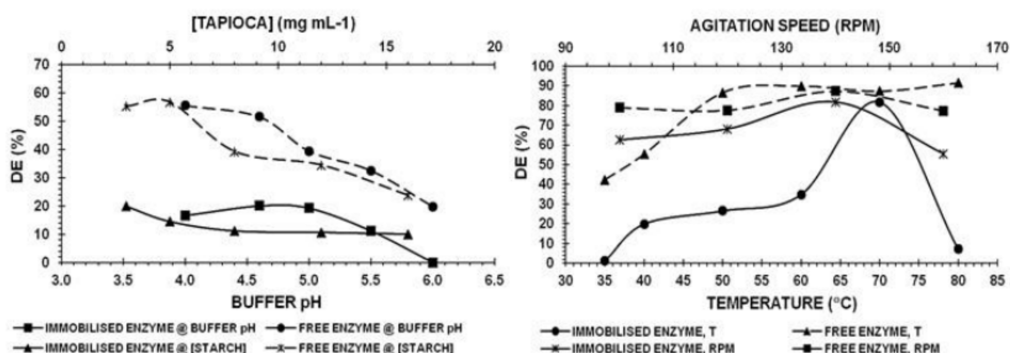

Figure 4. Effects of hydrolysis parameters after 24 hours: **pH** (T: 40°C, agitation: 140 rpm, immobilised enzyme: 2351 U, free enzyme: 7053 U), **starch concentration** (T: 40°C, agitation: 140 rpm, buffer: 100 mM pH 4.6, immobilised enzyme: 2351 U, free enzyme: 7053 U), **temperature** (agitation: 140 rpm, buffer: 100 mM pH 4.6, tapioca: 3 mg mL^{-1}, immobilised enzyme: 2351 U, free enzyme: 7053 U), **agitation speed** (T: 70°C, buffer: 100 mM pH 4.6, tapioca: 3 mg mL^{-1}, immobilised enzyme: 2351 U, free enzyme: 7053 U).

The agitation speed showed quite high effect on the hydrolysis process using immobilised glucoamylase (**Fig. 4-*right***). The highest DE was 81.70% obtained at the speed of 140 rpm. Activity of the immobilised glucoamylase was still lower than its free enzyme. Temperature affected the process highly. DE changed sharply when temperature was increased where the highest DE was found at temperature of 70°C (81.70%). Higher temperatures than the optimum value caused denaturation of the immobilised enzyme as DE dropped to below 10% at 80°C. The optimum DE produced by the immobilised enzyme closed to the free enzyme results, which had conversions of 86.61-91.51% at temperatures of 50-80°C. The optimum temperature for the immobilised glucoamylase found in the experiments differed slightly with the previous observations. George *et al*. [9] found the optimum temperatures of 30-40°C after evaluating performance of free glucoamylase immobilised onto silica materials via covalent method on soluble starch. Zhao et al[3] concluded the activity of immobilised glucoamylase on metal–ligand functionalized magnetic FeSBA-15 during hydrolysis of soluble starch was higher at temperatures of 55-65°C. Tardioli *et al.* [21] observed thermal inactivation of the glucoamylase immobilised onto highly activated glyoxyl-agarose supports covalently occurred at the temperatures of > 45°C.

Variation of initial enzyme concentrations resulted DE values of 9.59-19.58% (**Fig. 5**). Better DE were produced by the concentrations of 2351-4702 U. They increased rapidly using the free enzyme, but the immobilised glucoamylase showed low growth.

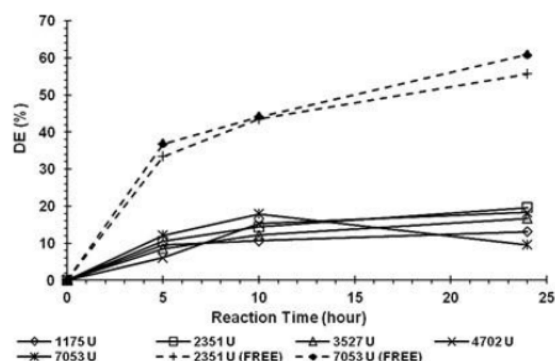

Figure. 5. Values of DE at some enzyme concentrations (T: 40°C, agitation: 140 rpm, buffer: 100 mM pH 4.6, tapioca: 3 mg mL^{-1}).

4. Conclusions

The efficiencies of immobilisation of free glucoamylase on MCF silica (9.2T-3D) were found in the range of 39.20-84.84%. Factors of temperature, agitation speed and buffer pH showed high effects on this process. The optimum immobilisation temperature, agitation speed and buffer phosphate Sorensen pH were 30°C, 100 rpm and 5.5, respectively. The immobilised enzyme hydrolysed the tapioca starch, but its activity was lower than the free enzyme. From five factors studied during the enzymatic hydrolysis using immobilised glucoamylase, temperature and agitation speed gave high effects. The optimum buffer pH, tapioca flour and enzyme concentration, temperature and agitation speed were 4.6, 3 mg mL^{-1}, 2351 U, 70°C and 140 rpm.

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