

PAPER • OPEN ACCESS

Mesostructured cellular foam MCF-(9.2T-3D) silica as support for free α -amylase in liquefaction of tapioca starch

To cite this article: Joni Agustian and Lilis Hermida 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **509** 012059

View the [article online](#) for updates and enhancements.



IOP | ebooks™

Bringing you innovative digital publishing with leading voices to create your essential collection of books in STEM research.

Start exploring the collection - download the first chapter of every title for free.

Mesostructured cellular foam MCF-(9.2T-3D) silica as support for free α -amylase in liquefaction of tapioca starch

Joni Agustian^{1,a}, Lilis Hermida^{1,b}

¹ Department of Chemical Engineering, Universitas Lampung, Jl. S. Brodjonegoro No. 1, Gedung Meneng, Rajabasa, Bandar Lampung 35145, Lampung, Indonesia.
Email: ^a joni.agustian@eng.unila.ac.id; ^b lilis.hermida@eng.unila.ac.id

Abstract. As ethanol can be made from insoluble starches, liquefaction of tapioca using α -amylase immobilised on MCF-(9.2T-3D) silica is studied to observe characteristics of the process. The free α -amylase SQzyme BAP was immobilised on the support via adsorption. During immobilisation, temperature and agitation speed showed low effects on the process, but pH of buffer solution affected the process highly. The optimum enzyme immobilisation occurred at the temperature of 50°C, pH 6 and speed of 60-100 rpm giving the immobilisation efficiency of more than 80%. Liquefaction of the insoluble starch (tapioca) was conducted with the immobilised α -amylase. The maximum result of \pm 50% DE was obtained. The experimental factors of buffer pH, temperature and agitation speed changed the DE significantly, but concentration of immobilised enzyme and substrate affected the process lowly. The optimum pH, agitation speed, immobilised enzyme concentration and temperature were 6, 100 rpm, 84.8 U and 80°C, respectively.

Keywords: α -amylase, MCF (9.2T-3D) silica, Enzyme immobilisation, Tapioca starch, Hydrolysis

1. Introduction

Liquefaction of polysaccharides in ethanol production from starches using free α -amylase is frequently difficult to recover and to recycle the free enzyme back to the process, so that immobilisation of free α -amylase onto inert supports is developed highly. The enzyme can be immobilised via adsorption, covalent bonding, entrapment and cross-linking onto magnetic nanoparticles, polyvinyl alcohol, polyglycidylmethacrylate, Fuller mineral, polyaniline, carboxymethyl cellulose-gelatine [1-8].

One of the useful supports for free α -amylase is silica-based materials. For example is silica-coated nanoparticles had been applied in hydrolysis of soluble starches where the immobilised α -amylase produced better enzymatic activity than the free enzyme [9, 10]. The encapsulated α -amylase by mesoporous silica showed higher thermal stability than its free form in soluble starch hydrolysis [11]. Free α -amylase immobilised onto mesoporous cellular foam (MCF) silicas (MCF-135 and MCF-335) had converted soluble starch to maltose where the covalently bond immobilised α -amylase had 80% of the free enzyme activity [12]. However, the immobilised α -amylase on these supports were merely used to hydrolyze the soluble starchy materials.

As ethanol can be prepared not only from soluble starches but also from insoluble starches such as cassava or tapioca, a study on liquefaction of the tapioca using the α -amylase immobilised on a siliceous support is required in order to observe characteristics of this process. Therefore, this paper describes immobilisation of the free α -amylase SQzyme BAP on MCF-(9.2T-3D), a new type of the MCF silicas



having similar characteristics with the MCF-335, and the enzymatic hydrolysis of tapioca starch using this immobilised enzyme.

2. Experimental Section

2.1. Materials

The main chemicals were bought from Sigma Aldrich (Pluronic® P123 (435465); trimethylbenzene, >98%; tetraethoxysilane, >99%; CH₃COONa) and Merck (NH₄F, >98%; hydrochloric acid, 37%; Na₂HPO₄·12H₂O, >99%; dextrose monohydrate, >99%; KH₂PO₄, >99%). α-amylase (SQzyme BAP; 4,000 U/g) and tapioca flour were provided by local importers.

2.2. Synthesis and characterization of MCF silica

The procedure as developed by Hermida *et al.* [13] was followed.

2.3. Immobilisation process

Immobilisation was conducted via adsorption of free α-amylase (1-3 mg mL⁻¹) dissolved in Sorensen buffer 100 mM (pH of 5-7) on/in 500 mg support. The mixture was shaken Medline BS-31 shaker at the required speed and temperature (60-140 rpm and 30-55°C) for 5 hours. The mixture was then filtered where the filtrate was analyzed for its protein content. The formed residue was rinsed with buffer (3x50 mL) according to the process pH. The immobilised enzyme was dried at 35°C overnight and then put in desiccator. Filtrates collected from the rinsing process were also determined their protein contents. The immobilised α-amylase was calculated using equation 1 and 2 [14-16] :

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

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

C₀ and C_t are initial and final free enzyme concentration (mg mL⁻¹), V is reactor volume (mL), q is amount of enzyme onto the support (mg g⁻¹) and W is weight of support (g).

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

CH₃COONa (30 mL, 100 mM, pH 5-7) was pipetted into some 100 mL flasks. Tapioca starch (3-20 mg mL⁻¹) was then added in. Immobilised α-amylase (42.4-254.4 U) was put into the solution to start the hydrolysis process. The mixture was shaken in water bath at various speeds (100-160 rpm) and temperatures (50-80°C) for 24 hours. Samples were collected at certain interval times and observed their dextrose equivalent (DE).

2.5. Estimation of protein and glucose

To calculate the protein content, Pierce™ BCA protein kit was used, whilst hydrolysis of starch to glucose was determined by DNS (dinitrosalicylic acid) method. Both observations were estimated using the Shimadzu UV-VIS 1800 spectrometer.

2.6. Characterization of immobilized α-amylase using FTIR, SEM and EDX

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 (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 analyzed 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 evaluated by Frontier Perkin Elmer FTIR.

3. Results and Discussions

3.1. Characterization of support

Textural properties of MCF silica (9.2T-3D) based on nitrogen adsorption-desorption analysis are shown in **Table 1** and **Fig. 1**. Average pore size and specific surface area (S_{BET}) were evaluated using BET method. Total pore volume (V_{pore} , $\text{cm}^3 \text{g}^{-1}$) was calculated as amount of nitrogen adsorbed at $P/P_0 = 0.98992$. The average window pores and cell sizes were 158 Å and 235 Å, respectively, confirming the mesoporous structure of the silica. The window pores and cell sizes should allow favorable conditions for access and immobilization of α -amylase enzyme due to the enzyme size (~ 40 Å) is lower than the support size.

Table 1. Nitrogen adsorption-desorption result.

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

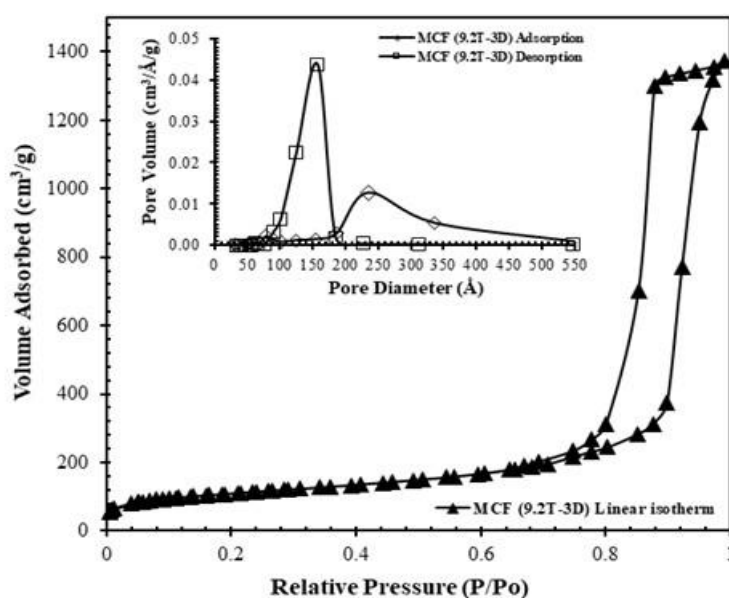


Figure 1. N_2 adsorption/desorption isotherms and pore size distribution plots (inset).

3.2. Characterization of immobilized enzyme using SEM, EDS and FTIR

Adsorption of the enzyme onto the support was firstly confirmed by SEM. **Fig. 2** shows images of MCF silica (9.2T-3D) and immobilized α -amylase. **Fig. 2-A** indicates that the support was in the form of spherical particles with smooth surfaces. Immobilization of α -amylase on the support resulted in rough surfaces on the particles (**Fig. 2-B**). Such morphological changes could be due to organic materials of the α -amylase that was adsorbed by the support.

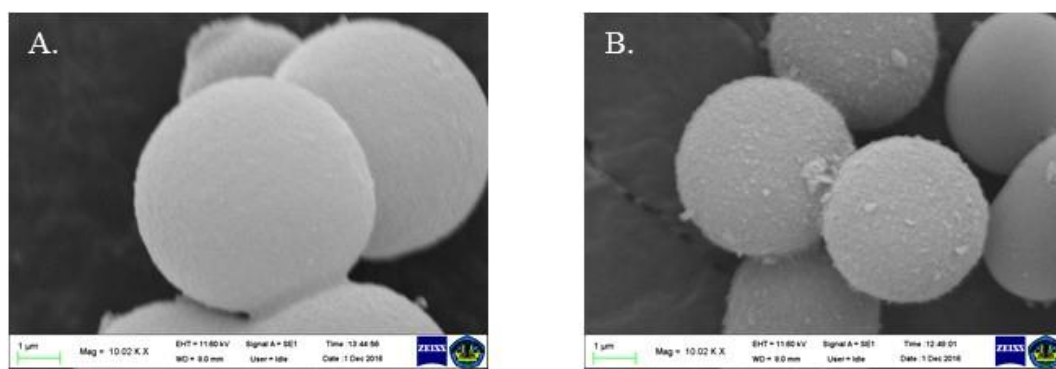


Figure 2. SEM images: **A.** MCF (9.2T-3D) silica, **B.** α -amylase on MCF (9.2T-3D) silica.

The adsorption of enzyme on the support was then observed with FT-IR (**Fig. 3**). FT-IR spectrum of the support showed its vibrations at around $1,062$ - $1,260$ cm^{-1} and 804 cm^{-1} to indicate Si–O–Si stretching vibrations. These vibrations were observed as well on the immobilised α -amylase spectrum. An additional vibration at 2260 cm^{-1} was found on the immobilized enzyme, which was not noticed in the support spectrum. The vibration at 2260 cm^{-1} confirmed C \equiv N or C \equiv C bounds (Baiz et al., 2013). As α -amylase is a protein consisting of a single polypeptide chain of amino acids, this vibration indicated the presence of amino acid structures on the support. EDX analysis was conducted on the support and immobilized α -amylase where both materials were initially coated with high pure gold (**Fig. 4**). The results verified that the support contained Si and O elements at 7.5 keV and 3.5 keV, respectively. The spectrum of the immobilized α -amylase showed elements of C and K besides Si and O. Both spectra indicated successfulness of α -amylase immobilization on MCF silica (9.2T-3D).

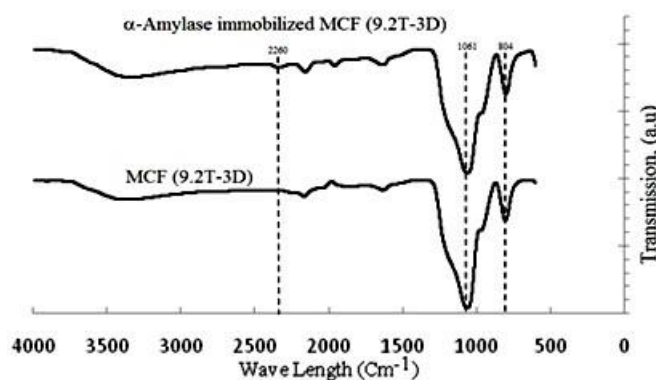


Figure 3. FTIR spectra of support and immobilized enzyme.

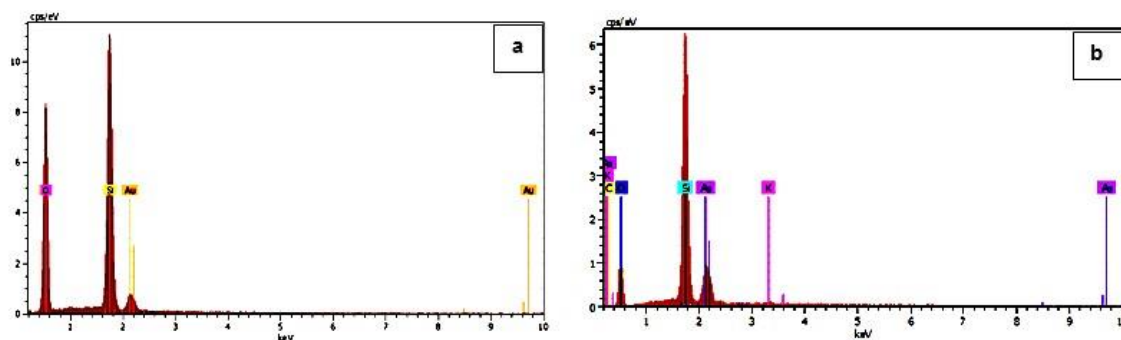


Figure 4. EDX spectra: a) Support, b) immobilized enzyme.

3.3. Immobilisation of enzyme

Immobilisation efficiencies and enzyme loadings are given in Table 2. 4 (four) factors were studied during the immobilisation, which included temperature, agitation speed, buffer pH and initial enzyme concentration. High effect on the immobilisation process was developed by pH of buffer solution. The optimum efficiency of the process was achieved at pH 6.0 where 94.45% (113.34 mg g⁻¹) of free α -amylase was adsorbed by the support. Other operational pH tended to decrease the efficiency significantly. In these experiments, the initial free enzyme concentration as high as 2.5 mg mL⁻¹ could be used as more than 90% free enzyme were immobilised, which indicated that the immobilisation of free α -amylase SQzyme-BAP on the support.

Effect of temperature was considered low. The efficiencies were in the range of 75.49-84.88% where the highest and lowest value were obtained at temperature of 30°C and 50°C, respectively. The optimum temperature could be set at the temperature of 45-55°C as they gave more than 80% immobilised enzyme with < 5% difference. It was a little higher than the previous observations. Temperature of 40°C was used in the immobilisation of α -amylase on carboxymethyl cellulose-gelatine-silica nano-hybrid support [4]. Many immobilisation processes of α -amylase on organic-based or magnetite materials via covalent bonds occurred at 25-35°C [2, 5, 9, 17-21]. Strong supports of MCF silica occurred at high adsorption temperatures that could be caused by the silanols (-OH) are easier to attached by the enzyme.

Table 2. Immobilization efficiency and enzyme loading

Operational Factors	Immobilisation Efficiency (%)	Enzyme Loading (mg g ⁻¹)
Temperature ^a	30°C	75.49
	35°C	79.59
	40°C	77.41
	45°C	81.05
	50°C	84.88
	55°C	83.60
Agitation Speed ^b	60 rpm	83.38
	80 rpm	83.79
	100 rpm	84.88
	120 rpm	80.97
	140 rpm	82.29
Buffer pH ^c	5.0	74.59
	5.5	84.88
	6.0	94.45
	6.5	80.10
Enzyme Concentration ^d	7.0	83.38
	1.0 mg mL ⁻¹	96.98
	1.5 mg mL ⁻¹	97.64
	2.0 mg mL ⁻¹	94.72
	2.5 mg mL ⁻¹	93.59
	3.0 mg mL ⁻¹	88.54

Conditions: ^a: agitation speed = 100 rpm, Sorensen PO₄ buffer = 100 mM pH 5.5, enzyme concentration = 2 mg mL⁻¹; ^b: temperature = 50°C, Sorensen PO₄ buffer = 100 mM pH 5.5, enzyme concentration = 2 mg mL⁻¹; ^c: temperature = 50°C, agitation speed = 100 rpm, enzyme concentration = 2 mg mL⁻¹; ^d: temperature = 50°C, agitation speed = 100 rpm, buffer solution = 100 mM pH 6

A low effect of agitation speed on immobilisation was considered. Although more than 80% of free α -amylase was immobilised when agitation speed was varied (60-140 rpm), the results differed slightly with each other. The agitation could be run at the speed of 60-100 rpm, though the speeds of > 80 rpm also gave good efficiencies, but it is not required to operate the process using high speeds. Relatively low agitation speeds were used to adsorb free α -amylase on MCF silica with high immobilisation efficiencies. Wang *et al.* [22] used agitation speed of 150 rpm to obtain 75% immobilised α -amylase on polystyrene pellets with pentaethylenhexamine and pentaethyleneglycol spacers. Beyler-Çiğil *et al.* [21] obtained 570.9 mg immobilised enzyme from 2,000 mg initial free enzyme at speed of 200 rpm on after 12 hours immobilisation process.

3.4. The enzymatic hydrolysis of tapioca starch

Effects of buffer pH and initial enzyme concentration during the enzymatic hydrolysis process are shown in **Fig. 5-a**. The effect caused by pH of sodium acetate was considered high as Dextrose Equivalent (DE) differed highly (i.e. 22.75-51.16%) where the highest value was found at pH 6.0. However, there is only a small DE difference between the sodium acetate buffer pH 6.0 and 6.5 (i.e. < 3%), so that the buffer pH in the range of 6.0-6.5 could be operated. The optimum pH followed previous observations where the activities were optimized at pH of 6-6.5 [2, 9, 12]. Compared with free α -amylase, the immobilized enzyme showed higher activities (DE) at buffer pH of ≥ 6.5 , however, it had a little lower activity at its optimum pH than free enzyme where 93% of the hydrolytic activity was maintained after immobilisation, which closed to original α -amylase activity (free enzyme). In contrast, the immobilised α -amylase concentration had relatively a low effect on the enzyme activity. An increase trend was observed when quantity of the enzyme was varied in the range of 42.4-254.4 U to give 41.99-51.16% DE. The highest and lowest DE were obtained at 84.8 U and 42.4 U, respectively. The enzyme concentrations of > 84.8 U produced slightly lower DE values, though the number of immobilised enzyme had been three or four times higher after 24 hours observation indicating that maximum reaction velocity was achieved at a lower immobilised enzyme concentration. Compared with free α -amylase activities, the immobilised enzyme performed lower enzymatic activities. At the optimum concentration, the immobilised type produced as high as 93% enzyme activity, which was lower than the free type.

Fig. 5-b describes effects of temperature and agitation speed on tapioca hydrolysis. At various hydrolysis temperatures, their effect on the process was low at high operational temperatures ($\geq 60^\circ\text{C}$). DE values were 26.99-52.06% where the highest DE was obtained at 80°C . DE value was doubled when temperature was changed from 50°C to 60°C , which indicated high effect occurred at low operational temperature(s). Though the highest result was obtained at 80°C , the temperature of 60°C gave DE value of 51.55%, which closed to the highest DE. Hence, the operating temperature could be operated at 60°C as high temperatures only produced a small increase. The free α -amylase achieved its optimum DE at 60°C (57.52%). Although the immobilised α -amylase had relatively lower activity than the free type ($\pm 90\%$ original activity), it had better thermal stability as it still retained high activity at high hydrolysis temperatures. This could be caused by free enzyme is connected to a surface of solid in a multipoint way [18]. The optimum temperatures for enzymatic starch hydrolysis using α -amylase immobilised on various supports were found in the range of 60 - 80°C [2, 19, 21]. Agitation speed had high effect on the immobilised α -amylase performance. The DE values were 35.10-51.55% where the highest and lowest DE were given by speed of 140 rpm and 100 rpm, respectively. The free α -amylase reached the optimum speed the same as the immobilised type. Compared with the free enzyme, the immobilised α -amylase showed $\pm 90\%$ the original activity.

Factor of tapioca starch concentration had low effect on the immobilised α -amylase performance (**Fig. 5-c**) as the difference between the highest and lowest DE was only $\pm 6\%$. Variation on the factor gave DE in the range of 46.0-52.1%. The highest result was obtained at 3% (w/v) starch concentration; however, it was slightly higher than DE produced by the concentration of 5% (w/v) and 8% (w/v). Hence, the enzymatic hydrolysis process could be run at the range concentration of 3-8% in order to get 50% DE. Singh and Ahmad [4], Sohrabi *et al.* [9] and Swarnalatha *et al.* [20] found that activity of

immobilised α -amylase was almost unchanged at high starch concentrations, though the concentrations were increased. Activities of α -amylase immobilised on silica materials were almost linear at starch concentration of $> 10 \text{ mg mL}^{-1}$ [12]. High activity of the α -amylase immobilised on various supports occurred at low starch concentrations [12, 23, 24]. Compared with free α -amylase, the immobilised enzyme only had as high as 90% the original activity.

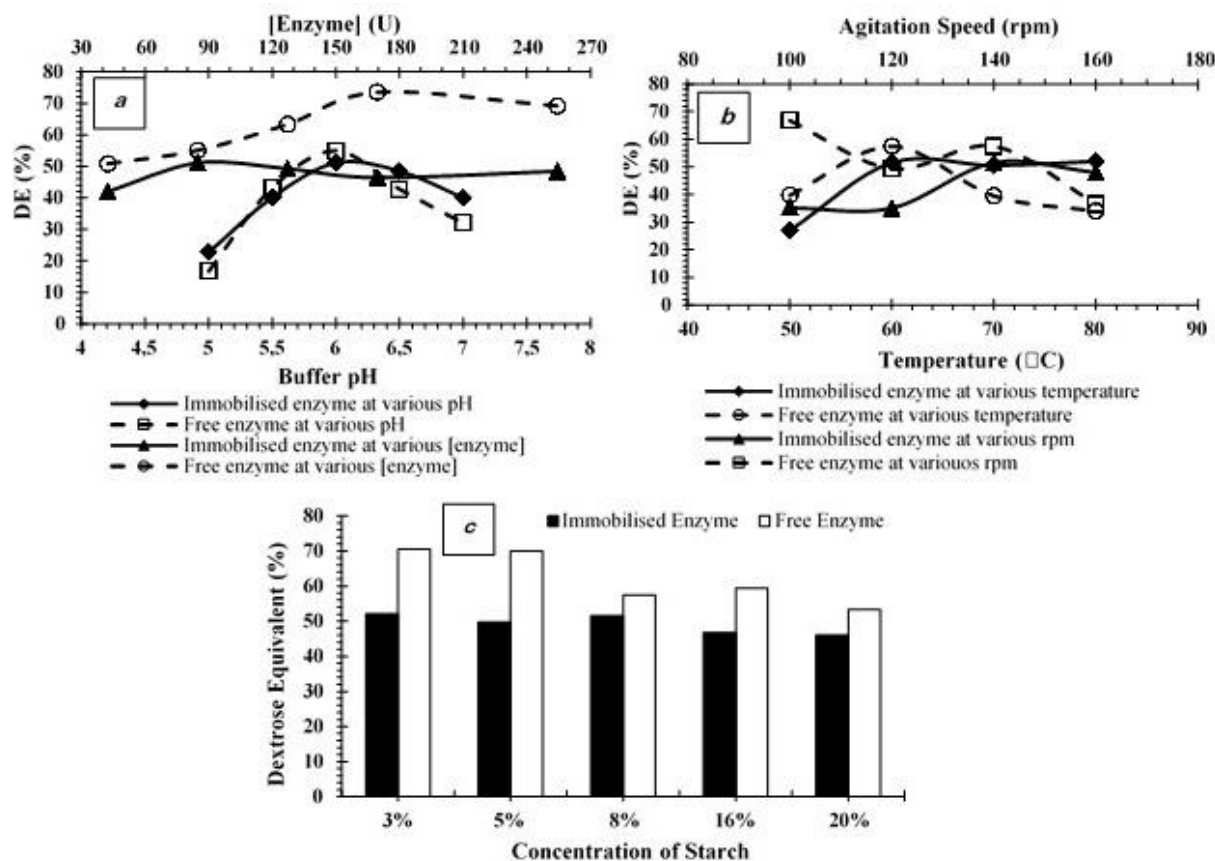


Figure 5. Effects of hydrolysis parameters: *a*) pH and enzyme concentration (pH: 60°C, 140 rpm, 3 mg mL⁻¹ starch, 84.8 U enzyme; enzyme: 60°C, 140 rpm, buffer pH 6, 3 mg mL⁻¹ starch; 24 hours); *b*) temperature and agitation speed (temperature: 8 mg mL⁻¹, 140 rpm, buffer pH 6, 84.8 U enzyme; agitation speed: 60°C, 8 mg mL⁻¹, buffer pH 6, 84.8 U enzyme; 24 hours); *c*) starch concentration (60°C, 140 rpm, buffer pH 6, 84.8 U enzyme, 24 hours observation).

4. Conclusion

Free α -amylase SQzyme BAP was immobilised successfully on MCF silica (9.2T-3D) with high efficiencies. The product functioned well in hydrolysis of tapioca starch. During the immobilisation process, effects of temperature and agitation speed were low, but pH of media affected this process highly. The optimum temperature, agitation speed and medium pH were found at 50°C, 60-100 rpm and pH, respectively, to give $> 80\%$ efficiency. In tapioca hydrolysis, variation of buffer pH, immobilised enzyme concentration and temperature had high effects on the process, however, the starch concentration affect it lowly. Optimum DE were obtained at pH 6, enzyme of concentration of 84.8 U, temperature of 60-80°C and agitation speed of 100 rpm. The process could operate at starch concentrations of 3-8% (w/v) with $\pm 50\%$ DE.

Acknowledgment

These works were supported by Fundamental Research Grant Scheme provided by Ministry of Research, Technology and Higher Education of Indonesia (No. 76/UN26/8/LPPM/2016). We thank for the financial support given to support these works. We deeply appreciated the SEM-EDX analyses provided by the Integrated Laboratory and Centre of Technology Innovation (UPT LTSIT) Universitas Lampung.

References

- [1] Baskar G, Banu N A, Leuca G H, Gayathri V and Jeyashree N 2015 Magnetic immobilization and characterization of α -amylase as nanobiocatalyst for hydrolysis of sweet potato starch *Biochem. Eng. J.* **102** 18-23
- [2] Oktay B, Demir S and Kayaman-Apohan N 2015 Immobilization of α -amylase onto poly (glycidyl methacrylate) grafted electrospun fibers by ATRP *Mater. Sci. Eng. C* **50** 386-93
- [3] Nakagawa K and Goto Y 2015 Preparation of α -amylase-immobilized freeze-dried poly (vinyl alcohol) foam and its application to microfluidic enzymatic reactor *Chem. Eng. Process. Process Intensification* **91** 35-42
- [4] Singh V and Ahmad S 2014 Carboxymethyl cellulose-gelatin-silica nanohybrid: An efficient carrier matrix for alpha amylase *Int. J. Biol. Macromol.* **67** 439-45
- [5] Akkaya B, Yenidunya A F and Akkaya R 2012 Production and immobilization of a novel thermoalkalophilic extracellular amylase from bacilli isolate *Int. J. Biol. Macromol.* **50** 4 991-5
- [6] Ashly P, Joseph M and Mohanan P 2011 Activity of diastase α -amylase immobilized on polyanilines (PANIs) *Food Chem.* **127** 4 1808-13
- [7] Rath P, Tripathi P and Kayastha A M 2012 Immobilization of α -amylase from germinated mung beans (*Vigna radiata*) on Fuller's earth by adsorption *J. Plant Biochem. Biotechnol.* **21** 2 229-34
- [8] Khan M J, Husain Q and Azam A 2012 Immobilization of porcine pancreatic α -amylase on magnetic Fe₂O₃ nanoparticles: Applications to the hydrolysis of starch *Biotechnol. Bioprocess Eng.* **17** 2 377-84
- [9] Sohrabi N, Rasouli N and Torkzadeh M 2014 Enhanced stability and catalytic activity of immobilized α -amylase on modified Fe₃O₄ nanoparticles *Chem. Eng. J.* **240** 426-33
- [10] Soleimani M, Khani A and Najafzadeh K 2012 α -Amylase immobilization on the silica nanoparticles for cleaning performance towards starch soils in laundry detergents *J. Mol. Catal., B Enzym.* **74** 1-2 1-5
- [11] Hisamatsu K, Shiomi T, Matsuura S-i, Nara T Y, Tsunoda T, Mizukami F and Sakaguchi K 2012 α -Amylase immobilization capacities of mesoporous silicas with different morphologies and surface properties *J. Porous Mater.* **19** 1 95-102
- [12] Pandya P H, Jasra R V, Newalkar B L and Bhatt P N 2005 Studies on the activity and stability of immobilized α -amylase in ordered mesoporous silicas *Microporous Mesoporous Mater.* **77** 1 67-77
- [13] Hermida L, Abdullah A Z and Mohamed A R 2013 *Materials and processes for energy: communicating current research and technological developments*, ed A Méndez-Vilas (Badajoz Spain: Formatex Research Center) pp 312-9
- [14] Kamal H, Sabry G M, Lotfy S, Abdallah N M, Rosiak J and Hegazy E s A 2007 Immobilization of glucoamylase on polypropylene fibers modified by radiation induced graft copolymerization *J. Macromol. Sci. A Pure Appl. Chem.* **45** 1 65-75

- [15] Zhao G, Wang J, Li Y, Huang H and Chen X 2012 Reversible immobilization of glucoamylase onto metal–ligand functionalized magnetic FeSBA-15 *Biochem. Eng. J.* **68** 159-66
- [16] Arica M Y, Yavuz H and Denizli A 2001 Immobilization of glucoamylase on the plain and on the spacer arm-attached poly (HEMA-EGDMA) microspheres *J. Appl. Polym. Sci.* **81** 11 2702-10
- [17] Homaei A A, Sariri R, Vianello F and Stevanato R 2013 Enzyme immobilization: an update *J. Chem. Biol.* **6** 4 185-205
- [18] George R, Gopinath S and Sugunan S 2013 Improved Stabilities of Immobilized Glucoamylase on Functionalized Mesoporous Silica Synthesised using Decane as Swelling Agent *Bull. Chem. React. Eng. Catal.* **8** 1 70-6
- [19] Wu Z, Qi W, Wang M, Wang Y, Su R and He Z 2013 Chelate immobilization of amylase on metal ceramic powder: Preparation, characterization and application *Biochem. Eng. J.* **77** 190-7
- [20] Swarnalatha V, Esther R A and Dhamodharan R 2013 Immobilization of α -amylase on gum acacia stabilized magnetite nanoparticles, an easily recoverable and reusable support *J. Mol. Catal., B Enzym.* **96** 6-13
- [21] Beyler-Çiğil A, Çakmakçı E, Danış Ö, Demir S and Vezir M 2013 Alpha-amylase immobilization on modified Polyimide material *Chemical Engineering Transactions* **32** 1687-92
- [22] Wang F, Gu Z, Cui Z and Liu L 2011 Comparison of covalent immobilization of amylase on polystyrene pellets with pentaethylenehexamine and pentaethylene glycol spacers *Bioresour. Technol.* **102** 20 9374-9
- [23] Kumari A and Kayastha A M 2011 Immobilization of soybean (Glycine max) α -amylase onto Chitosan and Amberlite MB-150 beads: optimization and characterization *J. Mol. Catal., B Enzym.* **69** 1-2 8-14
- [24] Torabizadeh H, Tavakoli M and Safari M 2014 Immobilization of thermostable α -amylase from *Bacillus licheniformis* by cross-linked enzyme aggregates method using calcium and sodium ions as additives *J. Mol. Catal., B Enzym.* **108** 13-20