# Hydrolysis of Starch Enzymaticaly: Immobilised Amylases on MCF Silica

Joni Agustian, Lilis Hermida

***Abstract*— As immobilisation of amylases seems to be the most promising way to obtain more stable and reusable enzymes, a review on the enzymes supports and application of Mesoporous Cellular Foam (MCF) silica as the support are presented here. Glucoamylase and alpha-amylase had been immobilized successfully in/on nanoporous, mesoporous and macroporous supports via adsorption, covalent bonding, cross-linking, entrapment and encapsulation. Factors of temperature, immobilisation time, enzyme concentration, polymer size, pH stability and reusability were studied. In general, the immobilised glucoamylase showed better thermal and pH stability and activity than the free form. Silica nanoparticles had been used to support the alpha-amylase and glucoamylase, which produced better thermal stability and enzymatic activity over its free-form. Immobilisation of the amylases in/onto gave better results than the free enzymes where quantity of the enzymes immobilised increased with the increase of the pores size.**

***Keywords*—Alpha amylase, Gluco-amylase, Nanoporous supports, Mesoporous supports, MCF silica**

## I. INTRODUCTION

Various carbohydrate sources have been associated with production of ethanol such as mineral oils, sugars and starches where starches are considered as cheap raw materials. Agricultural wastes (rice, wheat or corn straw), bagasse and lignocellulosic materials can also be used to give the compound [1]-[3]. Molasses, a by-product of sugar cane production, can be fermented to give the ethanol as well. Recently, Ji et al. [4] successfully fermented the mixture of furfural and residue of cassava. Fermentation of ethanol from starchy materials requires hydrolysis of the materials using enzymes (frequently alpha- and gluco-amylase) or acid(s) to obtain the reducing sugars, which then could be used in the fermentation processes to make the ethanol and to support the growth of microorganism(s). Therefore, availability of the reducing sugars in the beginning of the fermentation period is a must in order to support the microbial growth.

Traditionally, during the liquefaction and saccharification of starches, the alpha-amylase and gluco-amylase are supplied in the form of soluble or free enzymes. There are some reasons to replace these free enzymes. The use of soluble or free enzymes must be considered as very wasteful because the enzymes generally cannot be recovered at the end of the reaction period [5], so that they become the unreuseable enzymes [6]. Since they are soluble, their recovery from the mixture of substrate(s) and product(s) for re-used is not economically practical [7]. As the enzymes can only be used once, it is economically disadvantageous [8]. Hence, to improve their economic feasibility in many industries, the soluble enzymes are immobilized onto insoluble solid supports [9].

Immobilisation of enzymes is referred as the physical confinement or localization of enzymes in/on certain defined regions or spaces without changing their catalytic activities, thus they can be used repeatedly and continuously [10]. Other benefits offered by the immobilised enzymes are lowering the costs of enzymatic conversions [11],[12], longer enzyme half-life, less degradation and contamination, and a straightforward method for reaction control [13]. The process will certainly require fewer enzymes [12]. However, the immobilisation of enzymes also has some disadvantages such as loss or reduction in activity, limitation of enzyme diffusion and costs required for preparation of the immobilised enzymes [14]. Homaei et al. [15] show the advantages and drawbacks of the enzyme immobilization techniques, which, in general, are classified into adsorption, covalent binding, entrapment/encapsulation and cross-linking [16]-[18].

As the immobilisation of amylases seems to be the most promising way to obtain more stable and reusable enzymes [19], the paper reviews progress on the enzymes immobilisation process and presents the application Mesoporous Celullar Foam silica as support for the enzymes.

## SOURCES OF STARCHY MATERIALS

Starch is a homopolymer material consisting of glucose molecules joined with α-glycosidic bonds. The material is used by plants as storage of carbon and energy. It is a white color solid and tasteless compound, and is insoluble in cold water, alcohol and other solvents. In general, the compound contains 3 (three) main constituents i.e. amylose (≤25%), amylopectin (≤ 75%), and others (10-15%) such as lipids and proteins (Fig. 1). Glucose molecules in the amylose structure are joined by α-1,4-glycosidic bonds, whilst the amylopectin's glucoses have α-1,4-glycosidic and α-1,6-glycosidic bonds.

Starch from potato is the most widely used compound as substrate for testing capability of the immobilised hydrolytic enzymes. This starch is supplied in the form of soluble or

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Joni Agustian is with Department of Chemical Engineering, Faculty of Engineering, Universitas Lampung, Jl. Soemantri Brodjonegoro No. 1, Gedung Meneng, Rajabasa, Bandar Lampung 35145, Lampung, Indonesia, E-mail address: joni.agustian@eng.unila.ac.id

Lilis Hermida is with Department of Chemical Engineering, Faculty of Engineering, Universitas Lampung, Jl. Soemantri Brodjonegoro No. 1, Gedung Meneng, Rajabasa, Bandar Lampung 35145, Lampung, Indonesia, E-mail: lilis.hermida@eng.unila.ac.id

commercial/laboratory-prepared starch. The physicochemical properties of potato indicate that it contains higher amylose (19.50-34.52%) and amylopectin (68.6-80.5%) compared with other starches as shown in Table 1. Other starchy materials originated such as corn, cassava, wheat and rice, are also utilized. However, the starches from yam, sago, barley and arrowroot are used limitedly.

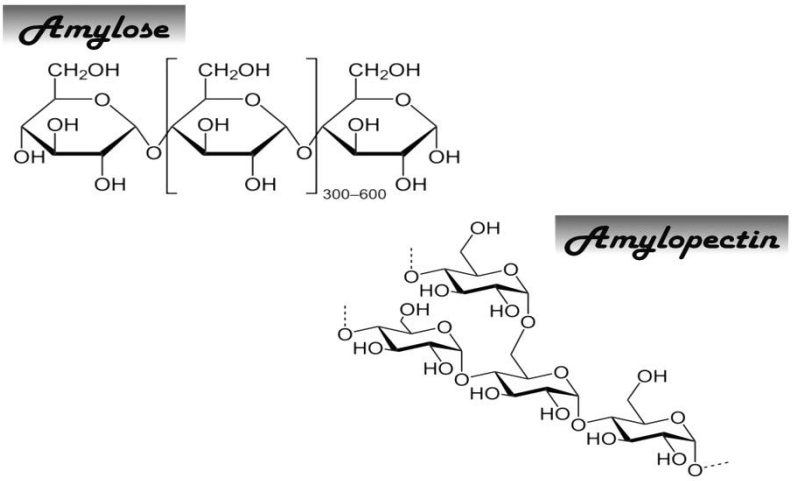


Fig. 1. Structure of amylose and amylopectin

Table 1. Characteristics of starchy materials

| Description | Potato [21]-[25] | Cassava [25]-[28] | Corn [29]-[32] | Rice [33]-[34] | Wheat [35]-[39] |
|---|---|---|---|---|---|
| Moisture (%) | 6.95-13.24 | 5.76-10.4 | 6.06-13.70 | 5.67-12.88 | 8-13 |
| Protein (%) | 0.05-0.44 | 0.10-0.88 | 0.35-8.49 | 5.28-8.16 | 0.4 |
| Ash (%) | 0.11-2.80 | 0.1-1.0 | 0.20-1.08 | 0.32-0.90 | 0.2-0.7 |
| Amylose (%) | 19.5-34.52 | 17-26.73 | 7.52-8.50 | 3.36-33.05 | 13 |
| Amylopectin (%) | 68.6-80.5 | 73.28-77.05 | no data | no data | no data |
| Phosphorus (%) | 0.01-0.075 | 0.01-0.03 | no data | no data | 0.06 |
| Fat (%) | 0.07-0.37 | 0.79-1.0 | 0.29-4.32 | 0.07-1.74 | 0.8 |
| Granules' sizes[19] | 5-100 μm | 5-45 μm | 2-30 μm | 3-8 μm | 2-35 μm |
| Type[19] | Tuber | Root | Cereal | Cereal | Cereal |
| Shape[19] | Lenticular | Spherical/Lenticular | Spherical/Polyhedral | Polyhedral | Lenticular |
| Gelatinisation Temp.[20] | 59-68°C | 58.5-70°C | 62-80°C | 58-79°C | 52-85°C |

## II. IMMOBILISED AMYLASES

### GLUCOAMYLASE

Glucoamylase had been immobilized successfully using various methods and supports e.g. adsorption on nano-magnetic material, carbon, cellulose bead and montmorillonite [40]-[44], covalent bonding on polymers, gelatin and montmorillonite [44], [45]-[50], entrapment in alginate [51], and encapsulation in calcium alginate-clay beads [52]. Factors of temperature, immobilisation time, enzyme concentration, polymer size, pH stability and reusability were studied. The immobilised glucoamylase showed better thermal and pH stability than the free form [40], [42], [48]. Milosavic et al [46] summarized that Dextrose Equivalent (DE) produced by the immobilised glucoamylase was higher than the free enzyme. However, study on the kinetic effects indicated that the $K_M$ value of the immobilised enzyme was larger than the free glucoamylase [48], [52].

### ALPHA-AMYLASE

Some ways have been developed to immobilize α-amylase. The enzyme can be immobilised via adsorption, covalent bonding, entrapment and cross-linking in pH 3-12 buffer solutions onto soluble or inorganic supports such as magnetic nanoparticles, polyvinyl alcohol, polyglycidyl methacrylate, Fuller mineral, poly aniline, carboxymethyl cellulose-gelatine [53]-[60]. Akkaya et al. [57] concluded that the immobilised alpha amylase has higher stability and kinetic constant than its free-form. Ashly et al. [58] observe the efficient immobilisation process was affected by the immobilisation media, contact time and free enzyme quantity. The immobilised alpha amylase could retain 75% original activity after 5 (five) cycles of application [59]. The alpha amylase immobilised onto $Fe_2O_3$ nanoparticle retained its activity as high as 94% of the initial value [60]. Increase of thermal stability of the alpha amylase immobilised onto polyvinyl alcohol fibers was observed, but its activity decreased as high as 25% after stored for 30 days [54]. These

previous results indicated that the immobilised alpha amylase has better stability and activity.

## III. SILICA SUPPORTS

Immobilisation of the alpha amylase covalently on silica-coated modified magnetite nanoparticles also produced better thermal stability and enzymatic activity over its free-form [61]. Soleimani et al. [62] used silica nanoparticle to observe the enzyme performance in starch hydrolysis process under various temperature and pH. They found the immobilised alpha amylase was better as well than the free-form. Encapsulation of this enzyme on mesoporous silica produced higher thermal stability enzyme than the free enzyme where quantity of the alpha amylase immobilised onto the support increased with the increase of silica pores size [63]. However, Pandhya et al. [64] using 3 (three) stages immobilisation process of the alpha amylase onto mesoporous silica MCF-335 summarized that the immobilised lipase only retained 80% of its initial activity.

The immobilisation of glucoamylase on mesostructured cellular foam (MCF) silica via the adsorption and covalent bond method resulted higher enzyme activity than the free enzyme where the pores structure of the MCF silica is the critical factor during the immobilisation process [65]. Encapsulation of the glucoamylase on mesoporous silica gave the $K_M$ value higher than the free glucoamylase and could be used for many cycles [66].

Recently, Hermida et al. [67] had developed the MCF silica 9.2T-3D that has similar characteristics with the MCF-335. As a limited resource is associated with the immobilisation of the amylases onto the MCF silica material and in order to examine this newly synthesized MCF silica, this MCF silica 9.2T-3D was used to immobilize the amylases.

### SILICA MCF 9.2T-3D

The discovery of mesostructured cellular foam (MCF) silicas allows a much wider choice of supports to be studied for the catalysts' incorporation. These materials have pore sizes in the range of 150-500 Å [68], which are larger than that of SBA-15 and MCM-41. Their structure consists of spherical cells and windows [68] where the cells (pore sizes: 200-500 Å) are framed by a disordered array of silica struts (**Fig. 1**) and the windows (pore sizes: 100-150 Å) interconnect the cells to form a continuous three-dimensional (3D) pore system [69]. They reduce diffusional restriction of reactants or substrates, and enables reactions involving bulky molecules to occur. The MCF silicas also have ultra-large pore sizes within the continuous 3D pore system. The functionalization of the MCF is possible as well since they exhibit very similar chemical properties to the MCM-41 and SBA-15. They are hydrothermal robust materials. Their characteristics make applications of these silicas emerging.

The MCF silica is formed by adding a hydrophobic swelling agent such as 1,3,5 trimethylbenzenexylene (TMB) in sufficiently large amount during synthesis of the SBA-15 to induce a phase transformation from highly ordered hexagonal (*P*6*mm*) symmetry to the MCF structure [70]. The formation of SBA-type materials is a result of interaction of P123 i.e. amphiphilic block copolymer (EO*x*PO*y*EO*x*) and inorganic siliceous species through hydrogen bonding [71]. The synthesis is performed under acidic conditions in which the hydrophilic head groups (EO units) and positively charged silica species are assembled together by electrostatic interactions mediated by negatively charged chloride ions. By changing size of the EO group using TMB as hydrophobic swelling agent, the SBA-type materials differ in the pore diameter [72].

Addition of TMB plays the important role in determining the final structures of the mesoporous silicas. It was found that the SBA-type materials were still formed at ratio of TMB/P123 less than 0.2. At the ratio of TMB/P123 in the range of 0.2-0.3, mixed phase silicas in the form of SBA-15 and MCF were formed. The phase silica of MCF type materials was synthesized at the ratio of TMB/P123 > 0.3. The proposed evolution of mesoporous silica structures with the increase of the TMB/P123 ratio and the TEM micrographs is described by Lettow et al. [73]. It was reported that at the (TMB/P123) ratio of 0.2-0.3, walls of cylindrical pores began to buckle that formed spherical nodes down the length of the pores. Besides mono-disperse spheres characteristic of MCF material, SAXS (small-angle X-ray scattering) pattern of samples synthesized at the TMB/P123 ratio of 0.2-0.3 matched hexagonal (*P*6*mm*) characteristic of SBA-15 material. At the TMB/P123 ratio higher than 0.3, the SAXS pattern only related to mono-disperse spheres characteristic of MCF materials

**Fig. 3** shows mechanistic pathway for formation of MCF material as described suggested in the literature [68]. The process is as follow: firstly, formation of an oil-in water micro-emulsion consisting of P123/TMB droplets by mixing aqueous HCl, P123 and TMB. Then, TEOS is hydrolyzed to form hydrophilic cationic silica species. Thirdly, condensation of the cationic silica species generates a "soft" silica coating through hydrogen bonding between the cationic silica species with the P123-coated TMB droplets to form a composite phase. Next, aging the mixture at elevated temperatures is carried out for the formation of large window pores with narrow size distribution in the composite materials. The aging treatment would also lead to agglomeration and packing of the composite materials. Finally, spherical particles of MCF material are produced by filtering the mixture, and then precipitated composite droplets obtained is dried and calcined.

Hydrothermal stability of MCF silica has been thoroughly studied as explained by Li et al. [74]. A higher aging temperature in MCF silica preparation resulted in a larger micro-porosity in the MCF silica, whilst a higher calcination temperature contributed to a stability of MCFs in higher-temperature steam. MCF silica prepared at the aging temperature higher than 100 °C and calcination temperature at 550 °C displays high hydrothermal stability at 600 °C under 100% steam, however, at 800 °C under 100% steam, the mesostructure of MCF completely collapsed. When the calcination temperature is increased to 900 °C, the mesostructure was stable under the pure steam at 800 °C. It can be concluded that the high aging temperature and high calcination temperature in the MCF preparation were in favor of the hydrothermal stability of MCF.

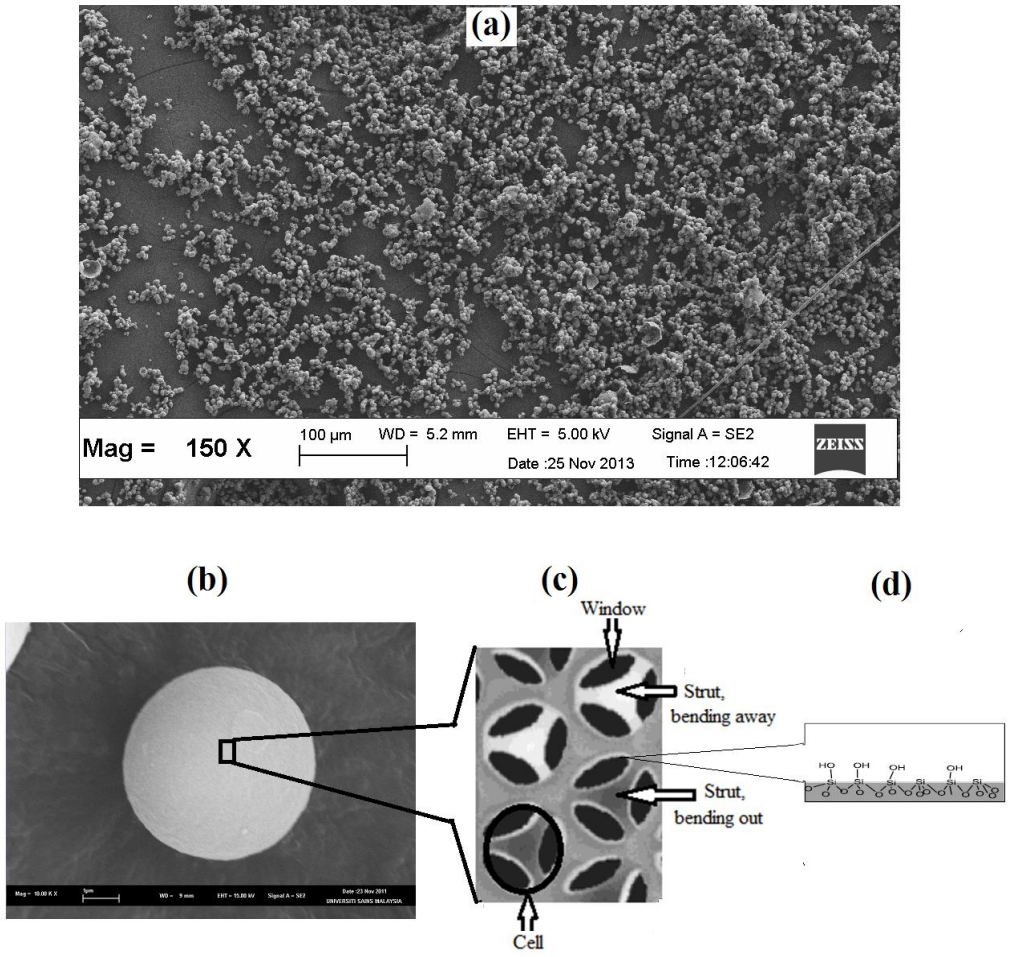


**Fig. 1** SEM image of (a) spherical MCF silica particles; (b) a single spherical MCF particle at higher magnification; (c) Schematic cross section of the structure exhibited by MCF silica; (d) Silanol groups (≡*Si-OH*) on wall of MCF silica

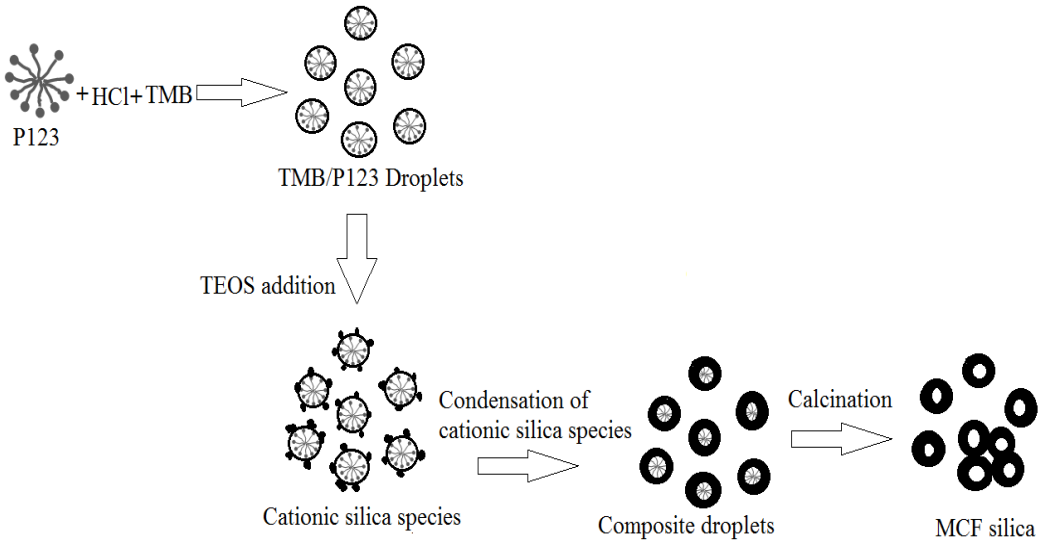


**Fig. 2.** Formation of MCF silica particles

## AMYLASES IMMOBILISATION ON SILICA MCF 9.2T-3D

Preliminary experiments of the amylases immobilisation in/on silica MCF 9.2T-3D are given in Fig. 3 and Fig. 4 where efficiencies of immobilisation (%ET) were observed at various temperatures (T).

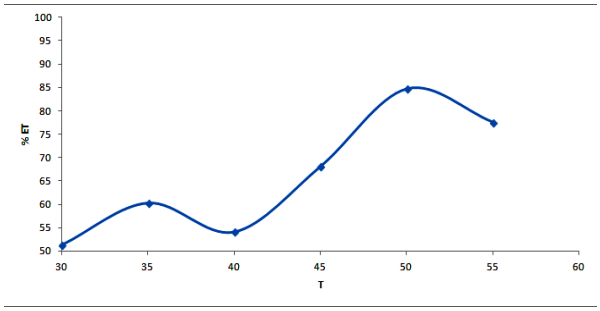


**Fig. 3** Immobilisation efficiency (% ET) of alpha-amylase at various temperatures (T)

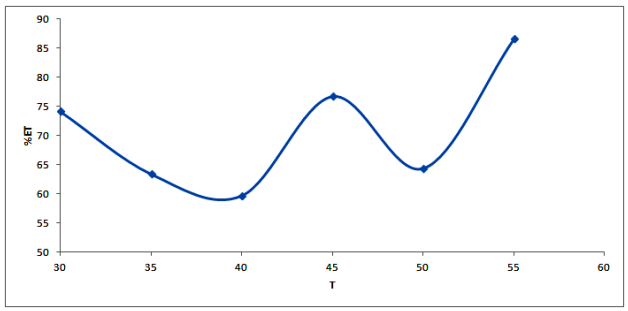


**Fig. 4** Immobilisation efficiency (% ET) of glucoamylase at various temperatures (T)

The alpha-amylase immobilisation efficiency tended to increase when the temperature was raised. The %ET reached a peak at the temperature of 50°C (85%ET). Low %ET was found at the lowest immobilisation temperature (51%ET). A fluctuation of %ET was found when the glucoamylase was immobilised in/on this support. The %ET deceased when the immobilisation temperatures were set in the range of 30-40°C. High %ET were obtained at the temperature of 40-55°C where the highest value was observed at 55°C (86%ET). Both results indicate that the silica MCF 9.2T-3D could accommodate the immobilization of amylases.

## IV. CONCLUSION

Immobilisation of amylase enzymes has used nanoporous, mesoporous and macroporous as supports via various immobilisation methods. The preliminary observations on the silica MCF 9.2T-3D showed that the support could immobilize the enzymes highly.

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