ENZYME IMMOBILIZATION
FOR BIOPROCESSING

Enzyme immobilization has been extensively explored by chemical/biochemical/biotechnology personnel for research and industrial uses. The ability to improve the stability and reusability of enzymes has driven this technique to be employed in a plethora of applications in these recent decades. *Enzyme Immobilization for Bioprocessing* offers up-to-date reviews on the current strategies and state of the art support systems involved in various bioprocesses. The highlights of this research book include:

- ✔ The latest enzyme immobilization methods and strategies – entrapment, encapsulation, adsorption and cross-linking.
- ✔ Mechanisms and interactions involved between enzyme and support.
- ✔ Kinetics and performance of immobilized enzyme in bench-top stirred reactor.
- ✔ Emerging support materials for effective immobilization, namely, smart polymer, silica, magnetic nanoparticles, graphene oxide and hollow fiber membrane.

Enzyme Immobilization for Bioprocessing also features the most recent applications of immobilized enzymes, including fingerprint visualizations.

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Contents

Preface

Enzymes are not a new subject in academic research and their applications in the industries. However, it has evolved tremendously in recent years, especially regarding the enzyme immobilization process and technology. The conventional enzyme immobilization technology and techniques are still relevant, but the new nanotechnology, modern bioinformatics, and molecular modelling have created a new landscape for enzyme immobilization work.

It is interesting to have an immobilized enzyme system successfully applied in the industries. However, factors such as cost, operational limitations and diffusion complexities imposed by substrates and the product are imminent. Therefore, the main focus of the researchers is to develop and improve on any enzyme immobilization processes to produce a stable, reusable, and robust system to adapt to the uncertain and harsh industrial environment. The immobilization technique and support system selection which are crucial prior to any applications have become our primary subject matter of interest in writing this book.

We are honoured to have all the authors who are directly involved in enzyme immobilization research to be on board in contributing to this book. We hope the readers will gain fruitful insights into enzyme immobilization and technology too.

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2022

CHAPTER 13

Hollow Fiber Membrane as a Carrier for Enzyme Immobilization

Rohaida Che Man, Shalyda Md Shaarani, Nurul Nabila Huda Baharudin, and Nur Izyan Wan Azelee

13.1 INTRODUCTION

Cyclodextrin (CD) is produced from starch by an enzymatic conversion catalyzed by cyclodextrin glucanotransferase (CGTase). CD has been used in a wide range of industries, especially in food, cosmetic, pharmaceutical, and agrochemical industries, due to its ability to improve the physicochemical properties of organic molecules, conferring greater chemical resistance to environmental factors, higher solubility, and reduced volatility (Ching *et al.* 2022). The commercialization of CGTase for industrial purposes is highly challenging due to the instability of the CGTase, sensitivity to the process condition, and high cost of isolation and purification (Guzik, Hupert-Kocurek, and Wojcieszyńska 2014; Mohamad *et al.* 2015). The instability of CGTase during the reaction process results in low yield CD. Therefore, enzyme immobilization has been applied to improve CGTase stability and achieve higher CD yields.

Immobilization is a process of fixing an enzyme to or within a support by physical or chemical methods, such as adsorption, encapsulation, entrapment and cross-linking (Pachelles *et al.* 2021). The immobilization of enzymes by entrapment and encapsulation have been proven to protect the enzyme and minimize the effects of mechanical shear, gas bubbles and hydrophobic solvents during the

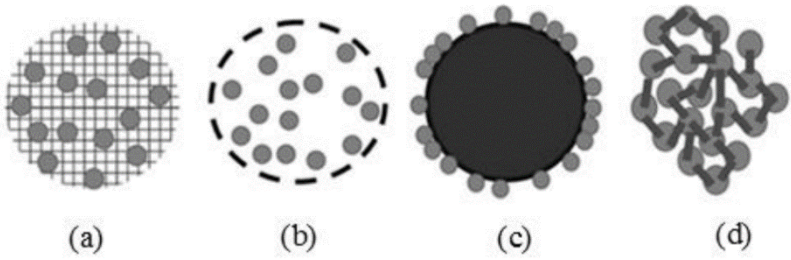
process (Brady and Jordaan 2009). However, enzymes immobilized by entrapment and encapsulation suffer from mass transfer limitations that would reduce their enzymatic activity (Rakmai, Cheirsilp, and Prasertsan 2015). Apart from that, most studies of enzyme immobilization via covalent attachment and cross-linking have shown a reduction in enzyme leaching during the immobilization process (Hara, Hanefeld, and Kanerva 2008). Nevertheless, the toxicity of the reagents is a limiting factor in applying these methods (covalent attachment and cross-linking) to an enzyme that are sensitive and can be inactivated by the cross-linker (Matijošytė *et al.* 2010). Therefore, the adsorption method remains the most attractive due to its simplicity, low cost and absence of toxic reagents (Rehm, Chen, and Rehm 2016).

Research into enzyme immobilization have exploited various supports, including alginate, chitosan, chitin, silica and hollow fiber membrane, and have reported success. Compared to other supports, hollow fiber membrane offers several distinct advantages such as a larger surface area to volume ratio, high mechanical strength, operational durability, non-toxicity, good thermal property and excellent chemical resistance (Algieri, Donato, and Giorno 2017; L. Ouyang *et al.* 2010). In addition, the hollow fiber membrane is economically attractive since this membrane is readily available and inexpensive (Man *et al.* 2015). Moreover, a membrane-type system is suitable to be employed for scale-up operation due to its simple and easy operation and maintenance (Chen *et al.* 2012). Therefore, it is suggested that the immobilization of CGTase on a hollow fiber membrane is a promising technique to improve the production of cyclodextrin (CD).

13.2 TECHNIQUE OF ENZYME IMMOBILIZATION

Enzymes can be immobilized by a variety of techniques, which may be broadly classified as physical techniques and chemical techniques. Physical techniques include physical adsorption, entrapment, and microencapsulation. Chemical techniques involve

the formation of covalent bonds between the support and the enzyme (Homaei *et al.*, 2013). Figure 13.1 shows the schematic diagram of the most common techniques of enzyme immobilization. Since the characteristics and composition of enzymes are diverse, different enzymes may require different support and technique for the best result in the enzyme immobilization process.



(*Source:* Brady & Jordaan 2009**)**

Figure 13.1 Schematic diagrams of the most common techniques in enzyme immobilization: (a) entrapment (b) encapsulation (c) adsorption (d) cross-linking

13.2.1 Entrapment

Entrapment involves trapping an enzyme in a polymer network, such as polymeric gel or sol-gel, by covalent or non-covalent bond (de Oliveira *et al.* 2018). This method protects the enzyme by preventing direct contact with the environment. Entrapment could minimize the effects of gas bubbles, mechanical shear, and hydrophobic solvents during the production process (Brady and Jordaan 2009). Popular supports used in entrapment are alginate, hydrogel, mesoporous silica, polyacrylamide, and sol-gel matrices. Even though natural polymers such as gelatin, hydrogel and alginate are frequently used for entrapment because of their simplicity, their weak mechanical properties and susceptibility to microbial

contamination presented major difficulties (Margetić and Vujčić 2016). The synthetic polymer could also better withstand the mechanical forces and chemical stress in the immobilization process. Therefore, the use of synthetic polymer in enzyme immobilization have drawn the attention of researchers.

According to Homaei *et al.* (2013), immobilization of enzyme by entrapment could avoid negative influences on the enzyme surface while granting thermal and mechanical stability, which has led to higher enzyme activity in an immobilized enzyme. For instance, CGTase immobilized in sodium alginate beads exhibited high reusability by retaining 75% of its initial activity after the seventh cycle (Arya and Srivastava 2006). The immobilized CGTase also showed lower substrate inhibition compared to the free enzyme. Santos *et al.* (2008) studied polysiloxane-polyvinyl alcohol (POS-PVA) hybrid support for the immobilization of lipase from *Candida rugosa*. With an immobilization yield of 40%, the hydrolytic activity of the immobilized lipase was 70% higher compared to the free enzyme. The results showed that the percentage of PVA in the sol-gel significantly influenced the physical properties of the particles, such as hardness and surface area.

However, most studies also found that entrapped enzymes often suffer from mass transfer limitations. A study conducted by Rakmai *et al.* (2015) on the immobilization of CGTase on alginate-gelatin mixed gel found that the entrapment methods gives a poor activity and stability towards the immobilization of CGTase. The entrapment of CGTase on an alginate-gelatin mixture may reduce the pore size of the network and lead to the diffusional limitation for the substrate and the product in the matrix of the gel. Therefore, this restriction would reduce the enzymatic activity of CGTase after repeated use.

Entrapment is a relatively simple method in enzyme immobilization, but the choice of support is critical. Unsuitable gel porosity could lead to enzyme leaching. The pore size of the support must be narrow enough to prevent enzyme leaching from the polymer network but large enough to allow the circulation of substrates and products (Margetić and Vujčić 2016). To overcome

this problem, the technique of pre-treating enzyme by a cross-linking agent has been developed. In one instance, β-Glucosidase was pre-treated with glutaraldehyde, forming an enzyme aggregation prior to entrapment in calcium alginate (Tsai and Meyer 2014). As a result, no significant loss of β-Glucosidase enzymatic activity was detected after up to 20 cycles of reactions, and more than 60% of the activity was retained, verifying that the enzyme has been stabilized by the pre-treatment process before the enzyme immobilization. The cross-linking pre-treatment with glutaraldehyde significantly minimized the leaching of the enzyme from the support.

13.2.2 Encapsulation

Encapsulation is quite similar to the entrapment technique, where the enzyme is entrapped in the internal structure of a polymer material. Compared to other immobilization techniques, the process for preparing encapsulated enzymes is straightforward and reproducible and does not require sophisticated equipment.

In a study conducted by Amud *et al.* (2007), CGTase from *Thermoanaerobacter* sp. was immobilized on three different supports, silica-glyoxyl, octadecyl-sepabeads, and sol-gel matrix, by covalent attachment, adsorption, and encapsulation, respectively. The immobilization of CGTase by encapsulation and covalent attachment showed the highest immobilization yield of 100% compared to adsorption with only 75.9%. While the immobilization yields were high, low immobilized enzyme activity was also observed, which may be due to mass transfer limitation. CGTase immobilized by covalent attachment presented the lowest enzymatic activity (5.9 U/ml), followed by encapsulation (7.4 U/ml) and adsorption (24.6 U/ml). The activity of the CGTase immobilized by encapsulation recorded in this study was lower than in another study of CGTase immobilization via covalent attachment on glyoxyl-agarose, with the enzyme activity of 42.4 U/ml (Tardioli, Zanin, and De Moraes 2006).

The loss of enzyme activity by enzyme immobilization is due to many factors such as steric hindrances, intraparticle diffusional resistance, and enzyme tridimensional conformation changes. Apart from that, the low enzymatic activity of enzyme immobilized by encapsulation may be due to the immobilization conditions and the reagents used in the sol-gel method that may have contributed to enzyme deactivation (Homaei *et al.* 2013). In addition, the highest activity of free CGTase was observed at 80 °C, whereas the highest activity of immobilized CGTase was observed at 60 °C (Amud *et al.* 2007). The shift of the optimum temperature from 80 °C to 60 °C (from free enzyme to immobilized enzyme) demonstrated that the conformal changes that occurred in the immobilized enzyme's tridimensional shape had made the enzyme more prone to thermal inactivation. More importantly, their preparation can involve the use of harsh conditions or reagents, which may be attributed to the enzyme denaturation and are detrimental to the enzyme activity (Homaei *et al.* 2013).

13.2.3 Adsorption

An enzyme can be immobilized to a solid support via surface interaction forces between the enzyme and the support material. Some of the forces involved are van der Waals, hydrogen bonding, hydrophobic interaction, electrostatic interaction, ionic bonding, and covalent bonding (Dwevedi 2016). The most popular solid supports used in adsorption include activated carbon, chitin, silica, ceramics, Eupergit C, sepharose and alumina.

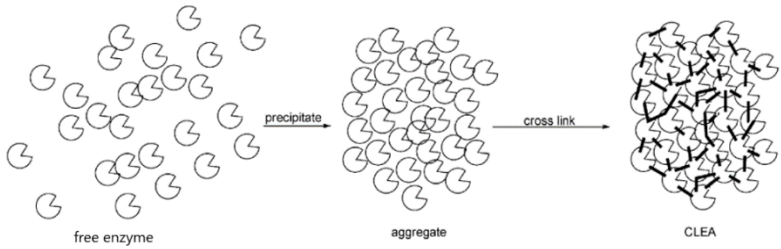
This technique also does not chemically modify the enzyme, but it has limitations as the enzyme tends to leach out, especially in aqueous solvents. This can lead to difficulties in process design and downstream processing. To minimize enzyme leaching, the choice of support and operating conditions are crucial to maximizing immobilization efficiency. For example, a study conducted by Abdel-Naby, Fouad, and Reyad (2015) showed that CGTase, obtained from *Bacillus amyloliquefaciens*, when immobilized on chitin via adsorption without requiring any chemical treatment, had

the highest enzyme activity (58.70 U/g carriers) compared to when it is immobilized on other supports such as alumina, ceramic, chitosan, polystyrene, PVC, and silica. Moreover, the optimal operating pH and temperature of the immobilized enzyme on the chitin was pH 5.0 and 63 °C, whereas the free CGTase had the optimal operating pH and temperature at 6.5 and 58 °C. Given that the immobilized CGTase can operate in a more acidic medium at a higher temperature, it has a better prospect for application as an enzyme biocatalyst for the production of the desired product.

Enzyme immobilization via adsorption generally involves weak forces such as electrostatic interaction and hydrogen bonding. To strengthen and stabilize these weak interactions, researchers often pre-treat the support with other reagents such as glutaraldehyde and ethylenediamine, which may also have the added benefit of improving the enzyme's catalytic activity (Blanco *et al.* 2013; Chen *et al.* 2012). However, the added reagent could denature the enzyme, causing drastic changes in the conformational and catalytic properties of the enzyme (Dwevedi 2016). Chieh *et al.* (2017) studied the immobilization of CGTase from *Bacillus macerans* via covalent bonding in order to build a strong interaction between CGTase and the support (bleached kenaf microfiber). Hexamethylenediamine (HMDA) and ethylenediamine (EDA) were used as spacer arms while glutaraldehyde (GA) and o-phthalaldehyde (OPA) acted as ligands. The CGTase immobilized with ethylenediamine and o-phthalaldehyde showed the highest storage stability (60°C), maintaining 60% of its activity after 15 days. The CGTase immobilized with ethylenediamine and glutaraldehyde demonstrated an enzyme activity of up to 72.72% after 12 cycles. The immobilized CGTase has improved stability at high temperatures (storage temperature at 60°C and reaction temperature at 70°C–90°C) compared to the free CGTase. The thermal stability was conferred by the structural rigidification due to the coupling agents and the multipoint covalent attachments during the immobilization process.

13.2.4 Cross-linking

Cross-linking is a support-free enzyme immobilization technique that involves the joining of enzyme molecules to each other to form a large clump, a three-dimensional complex structure, which can be achieved by chemical and physical methods (J. Ouyang *et al.* 2020). The physical aggregation of an enzyme usually involves the addition of salts, water-miscible organic solvents, or non-ionic polymers. The physical aggregates formed by this method are held together by non-covalent bonding without any perturbation to their tertiary structure. The chemical aggregation of an enzyme usually involves the formation of covalent bonds between the enzyme molecules by means of bi- or multi-functional cross-linking agents such as glutaraldehyde (Mohamad *et al.* 2015). Instead of fixing the enzyme to a carrier, the enzyme acts as its own carrier. The enzyme can be cross-linked in solution by adding precipitants such as acetone, ammonium sulfate, ethanol or 1,2- dimethoxy ethane, followed by the addition of a cross-linker. Figure 13.3 shows the schematic diagram of the establishment of cross-linking aggregation enzyme (CLEA).



(*Source:* Hanefeld, Gardossi & Magner, 2009)

Figure 13.2 Schematic diagram of aggregation and cross-linking of an enzyme to prepare a CLEA

Although there are various types of cross-linkers, such as dextran aldehyde, poly-L-lysine, and polyethyleneimine, glutaraldehyde remains popular as the cheap and versatile cross-linking reagent. However, the toxicity of the reagent is a limiting factor in applying this method to enzymes that are sensitive and can be inactivated by the cross-linker. Therefore, most studies have discussed optimizing cross-linker concentration in the enzyme immobilization process in order to minimize the amount of the reagent. A study conducted by Matijošytė *et al.* (2010) showed that higher glutaraldehyde concentrations (exceeding 10 mM) led to the inactivation of laccase from *Trametes versicolor*. On the other hand, hyperactivation of lipase B from *Candida antarctica* was observed when the glutaraldehyde concentration exceeded 150 mM (Schoevaart *et al.* 2004).

Cross-linked enzyme aggregates (CLEA) of recombinant CGTase were constructed by Zhang, Li, and Mao (2019) for high-purity β-CD production. CLEA-CGTase was prepared using 0.1% (v/v) glutaraldehyde as a cross-linker at 85 °C for 10 min with 75 U/mL of CGTase. A high proportion of β-CD (100%) was detected at 50 °C after 420 min of reaction with soluble potato starch using between 10 U/mL to 200 U/mL CLEA-CGTase. When 8000 U/mL of CLEA-CGTase was added into the reaction mixture, the proportion of β-CD remained above 90%. The CLEA technique maintained the CGTase conformation at 85 °C with the high β-CD proportion at 50 °C. Therefore, this study concluded that the CLEA technology kept the enzyme conformation for the production of the specific desired product.

CLEA of a thermostable CGTase from *Thermoanaerobacter* sp. was prepared by Rojas *et al.* (2019) for the production of the CD. The preparation of CLEA-CGTase was conducted at 20 °C for 2 hours with 75% (v/v) acetone as a precipitation reagent and 20 mM starch–aldehyde as a crosslinking reagent. The CLEA-CGTase prepared via this method was an active biocatalyst for CD production at 50°C, pH 6.0 and 6 hours of reaction time. Furthermore, the total CD yield sat at 80% of the initial value (45%

CD yield for the first batch) after five cycles of 3 hours of reaction time.

13.2.5 Summary for Technique of Enzyme Immobilization

The technique of enzyme immobilization has been studied using various methods described previously. The advantages and disadvantages of the techniques are summarized in Table 13.1.

Table 13.1 Advantages and disadvantages of enzyme immobilization techniques

Immobilization Technique	Advantage	Disadvantage	Reference
Adsorption	1. Simplicity of the immobilization process 2. Low cost 3. No addition of chemical 4. Usually does not alter original structure of enzyme	1. Weak interaction between enzyme and support	(Jamil *et al.* 2018; Jesionowski, Zdarta, and Krajewska 2014; Rehm, Chen, and Rehm 2016; Suhaimi *et al.* 2018)
Encapsulation	1. High strength bonds formed between enzyme and support 2. Reduce allosteric inhibition	1. Support required chemical activation 2. Enzyme undergoes conformational changes due to the chemical activation	(Rodrigues *et al.*, 2013; Mohamad *et al.*, 2015)

cont. **Table 13.1**

Immobilization Technique	Advantage	Disadvantage	Reference
Entrapment	1. Improve mechanical stability of the enzyme 2. No chemical interaction between enzyme and support	1. Substrate diffusional limitation for enzymatic reaction 2. Poor reusability due to leaching 3. Low loading capacity for enzyme	(Zdarta *et al.*, 2018; Mohamad *et al.*, 2015)
Cross-linking	1. Does not require support	1. Difficult to obtain large enzyme aggregate 2. Certain enzyme typically undergoes a conformational change	(Mohamad *et al.*, 2015)

Based on Table 13.1, it can be concluded that adsorption is the most attractive technique to be used in enzyme immobilization due to the low cost and simplicity of the process. Besides, this technique does not require any chemical alteration to the enzyme and support, which can prevent conformational changes of the enzyme, hence improving product formation.

13.3 MATERIAL USED FOR ENZYME IMMOBILIZATION

The characteristics of the support are important in determining the performance of the immobilized enzyme. The pertinent physical characteristics include surface area, binding density, and pore size.

The chemical characteristics include chemical composition and the binding with the enzyme molecules and the spacer that links the binding site to the support backbone. Figure 13.3 shows the classification of supports for enzyme immobilization. The advantages and disadvantages of different types of support are shown in Table 13.2.

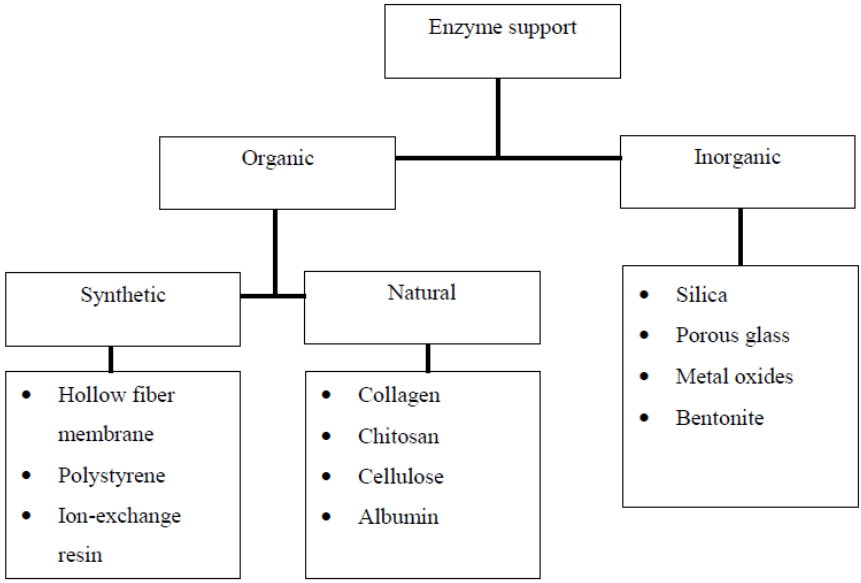


Figure 13.3 Classification of supports for enzyme immobilization

Based on Table 13.2, organic support materials have attractive traits, particularly synthetic organic polymers. This organic synthetic polymer supports typically contain reactive functional groups that can easily attach to enzymes without any addition of a chemical reagent. Other than that, synthetic polymers have good mechanical strength, so they are not easily destroyed by agitation and stirring during enzymatic reactions. The polymers are also non-toxic and would not affect the enzyme and end product during the reaction.

Table 13.2 The advantages and disadvantages of different types of supports

Types of support		Advantages	Disadvantages
Organic	Natural polymers	● Excellent biocompatibility ● Non-toxic ● Biodegradable	● Weak mechanical stabilities ● Weak bonding between enzyme and support ● Lack of functional group for binding
	Synthetic polymers	● Good mechanical strength ● Availability of reactive functional groups ● Non-toxic	● High hydrophobicity ● Low biocompatibility
Inorganic		● Good mechanical stability ● High rigidity	● Expensive ● Low biocompatibility

(*Source*: Homaei *et al.*, 2013)

13.3.1 Organic Support

Organic supports are often related to a variety of sources, easily modified, non-toxic, and environmentally friendly, with various functional groups available to the support. Organic supports can be categorized into two types: natural and synthetic polymers. Natural polymers are mainly composed of a water-insoluble polysaccharides such as collagen, chitosan, and agarose. Cellulose has also been used as alternative natural polymer support. Supports that contain a high amount of cellulose, such as coconut fiber (Brígida *et al.*, 2008),

orange peels (Plessas *et al.* 2007) and spent grain (Castro *et al.*, 2001) can be found abundantly as agricultural and domestic waste. The immobilization of CGTase from *Bacillus licheniformis* using pineapple peel was carried out by Che Man *et al.* (2021). Pineapple is a very popular tropical fruit extensively cultivated along the tropics belt around the world, including in Malaysia. The fruit is consumed by removing the peel and crown. Therefore, a large amount of pineapple peel waste is generated from food processing plants and households. Unless this biowaste is further utilized, it will end up in a landfill. As an agricultural waste, pineapple peel is primarily composed of hemicellulose and cellulose (Aditiya *et al.* 2016), which means that it can bind enzymes for immobilization purposes. In addition, pineapple peel is an ecologically benign and inexpensive material. From the study, it showed that pH 7 was the best pH for CGTase immobilization, with a 75.97% of immobilization yield. In addition, the optimal temperature and contact time was 25°C and 24 hr with 76.80% and 75.53% of immobilization yield, respectively.

In a study conducted by Sulaiman *et al.* (2015), the CGTase was covalently immobilized on cellulose nanofiber (CNF) with 1,12-dodecanediamine as a spacer arm and glutaraldehyde as a ligand. The results showed that the process has an immobilization yield of about 62%, and the enzyme retained about 67% of its initial activity after 8 cycles. A high number of –OH functional groups were detected on the surface of the CNF, and they play a significant role in the process of enzyme immobilization. However, the immobilized CGTase showed lower enzymatic activity compared to native CGTase due to the inactivation of enzyme active sites and/or misdirection of enzyme orientation which were affected by the reaction of chemical coupling agents (spacer arm and ligand) (Cao 2006).

Enzyme immobilization yield on synthetic polymers is considerably higher than on natural polymers, mainly due to their physical rigidity. That is, the immobilization process involves immersing the support in the enzyme solution for a few hours, after which the natural support would swell and soften, affecting

immobilization yield, whereas the synthetic support works otherwise (Urbance *et al.*, 2004). The immobilization of CGTase from *Paenibacillus macerans* NRRL B-3186 on aminated polyvinylchloride (PVC) through a covalent bond with glutaraldehyde was performed by Abdel-Naby (1999). The immobilized CGTase showed a higher immobilization yield (85%) and was able to retain more than 80% of its initial catalytic activity after 14 cycles. Moreover, the immobilized CGTase demonstrated tolerance to a higher reaction temperature, resistance to chemical denaturation, and higher thermal stability compared to the free enzyme.

Another enzyme immobilization on synthetic polymer study was conducted by Junko Tomotani and Vitolo (2006), which showed that when invertase was immobilized on anion exchange resin with polystyrene derivative, the immobilization yield recorded was higher than 80%. Moreover, the anion exchange resin completely adsorbed the invertase molecules, and the enzyme retained 100% of its catalytic activity. No leaching of invertase from the anion exchange resin was detected at the end of the sucrose hydrolysis. Therefore, the immobilization of enzymes using synthetic polymer is recommended due to the high immobilization yield and high production of the desired product.

13.3.2 Inorganic Support

Inorganic supports are known for their high thermal and mechanical strength for enzyme immobilization. Furthermore, most inorganic supports provide a similar pore diameter that ensures a fixed shape of the support. Several attempts have been made by previous researchers to obtain highly active and stable immobilized enzymes using an inorganic material as a support (Carlsson *et al.* 2014; Sigurdardóttir *et al.* 2018).

Immobilization of CGTase from *Thermoanaerobacter* sp. on silica was conducted by da Natividade Schöffer *et al.* (2017). The silica was functionalized before the immobilization process by two different methods, first by disulfide bond through the cystein on the

CGTase surface (Si-SH-CGTase) and another one by amino groups using glutaraldehyde activation (Si-NH-G-CGTase). The efficiency of the Si-SH-CGTase was 11.91%, four times higher than the Si-NH-G (2.86%). The optimum pH for both Si-SH-CGTase and Si-NH-G-CGTase was 5.5, whereas the optimum temperature was 80 °C for Si-SH-CGTase, 90 °C for Si-NH-G-CGTase and 70 °C for the free CGTase, after the immobilization process. In continuous CD production, the Si-NH-G-CGTase showed higher total productivity, retaining 100% of its initial activity after 200 hr, but only 40% was observed for the Si-SH-CGTase at the same time because of the enzyme leaching during the reaction process.

A similar result was shown by Ye *et al.* (2002), whereby the microporous zeolite (inorganic support) was shown to be suitable for the immobilization of α-chymotrypsin due to the presence of hydroxyl groups. The zeolite contained hydrogen groups capable of forming strong hydrogen bonds with the enzyme. However, the industrial application of inorganic supports is dissuaded due to higher cost (Zucca and Sanjust 2014) compared with the organic support.

13.4 IMMOBILIZATION OF CYCLODEXTRIN GLUCANOTRANFERASE (CGTASE) ON HOLLOW FIBER MEMBRANE

Hollow fiber membranes have been employed mainly in biotechnology as filter aids. In the past decade, the use of hollow fiber membranes has been expanding. Initially, they were used as a means to concentrate cell streams in biomass recycle fermenters, microfiltration, pervaporation, membrane distillation, and more recently as a support for the immobilization of enzyme and the cells of microbes, animals, and plants (Algieri, Donato, and Giorno 2017). Highly porous hollow fiber membrane has been widely used for the development of immobilized enzymes because of the high surface-to-volume ratio, lower mass transfer resistance, lack of toxicity, cost-effectiveness, and high mechanical strength (Dror *et al.* 2008; Jamil

et al. 2018). One particular selling point of enzyme immobilization on hollow fiber membrane is the cylindrical configuration of the asymmetric hollow fiber. Figure 13.4 shows the basic structure of a hollow fiber membrane, which is composed of a lumen, a semipermeable membrane and a macroporous sponge.

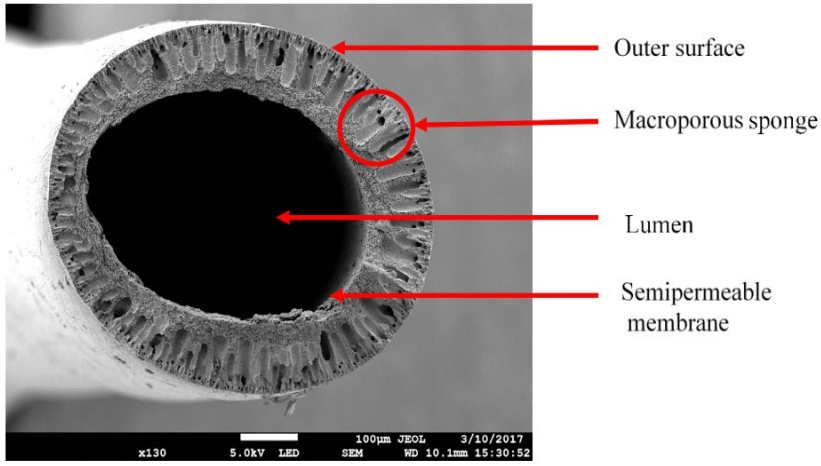


Figure 13.4 Cross-sectional area of a hollow fiber membrane at 5000x magnification

The hollow center of the fiber, known as the lumen, is where the substrate flows continuously. The outer layer of the hollow fiber is a porous sponge with a larger hydraulic permeability that serves as a mechanical support for the ultrathin semipermeable membrane (Tan *et al.* 2006). The microporous sponge also provides a large surface-to-volume ratio for enzymes that require surface attachment (Kang and Cao 2014). These properties have been demonstrated by Suhaimi *et al.* (2018) and Ye *et al.* (2005), in which a single enzyme such as CGTase and lipase could be retained within the macroporous matrix of a hollow fiber membrane.

Several studies have shown that high enzymatic activity can be observed when using a hollow fiber membrane as a support. For example, the immobilization of esterase enzyme from pig liver on hollow fiber membrane has shown higher retention of enzymatic

activity for a longer period of time (Sousa *et al.* 2001). The enzymatic activity of immobilized pig liver esterase increased by 62% compared to the free enzyme. Moreover, the short diffusional distance between the substrate and the enzyme is one of the factors that facilitate heat and mass transfer, which could enhance the production process.

Hollow fiber membranes also have high chemical, biological, and mechanical stability, which makes them more attractive as a support by enhancing enzyme stability and increasing production yield. A study conducted by Chen *et al.* (2012) observed that the immobilized lipase on a hollow fiber membrane could retain a higher initial activity of 97% compared to the free lipase (70%) after 10 repeated uses. The findings from the immobilization of tyrosine on polyvinylidene fluoride (PVDF) hollow fiber membrane showed that the enzyme could retain 90% of its initial activity after 6 cycles (Algieri, Donato, and Giorno 2017). These observations add to the consensus on the possibility of reusing immobilized enzymes in a bioreactor without significant loss of performance. Apart from that, there was very little difference between the specific activity obtained from the immobilized tyrosine (0.71 U/mg) and the free enzyme (0.75 U/mg). It could be concluded that the adsorption of an enzyme on a hollow fiber membrane does not alter the catalytic properties of the enzyme.

There are numerous types of membranes that have been utilized in various enzyme immobilization processes, as summarized in Table 13.3. Synthetic polymers such as polypropylene, polyacrylonitrile, and nylon have been used as supports in enzyme immobilization. The hydrophobicity of the membrane surface could also affect the adsorption of enzymes on the support. Shamel *et al.* (2007) has studied the immobilization of lipase via adsorption onto two different supports, namely polysulfone membrane, which is a hydrophobic polymer, and regenerated cellulose, which is a hydrophilic polymer. The results showed that the polysulfone membrane had adsorbed more lipase (21.59 LU/cm^2) compared to the regenerated cellulose (0.5 LU/cm^2).

Table 13.3 Enzyme immobilized on different types of membranes

Enzyme	Types of Membrane	Product	References
Laccase	Polyethylene hollow fiber membrane	Oxidation of bisphenol A	Mokhtar *et al.* (2019)
β-galactosidase	Disc-shaped polyethersulfone ultrafiltration membrane	Glucose	Sen *et al.* (2016)
Carbonic anhydrase	Polymethyl-pentene hollow fiber membrane	Paranitrophenol acetate	Arazawa *et al.* (2012)
CGTase	PVDF hollow fiber membrane	Cyclodextrin	Suhaimi *et al.* (2018)
Halohydrin dehalogenase	Glass fiber membrane	Halohydrins	Gul *et al.* (2020)

CGTase has been successfully immobilized on various types of support such as chitosan, silica, alumina, sepharose, glyoxyl-agarose, and Eupergit C. However, there have not been as many studies on the immobilization of CGTase on a hollow fiber membrane. Therefore, in this study, a polyvinylidene fluoride (PVDF) hollow fiber membrane, hydrophobic polymer support, has been chosen due to the higher contact angle it presents that may assist in the enzyme immobilization process (Aksoy and Hasar 2021). PVDF hollow fiber membrane contains negatively charged fluoride ions and positively charged hydrogen ions (Man *et al.* 2015) that could interact with both the positively and negatively charged sites of CGTase. Moreover, the hydrophobic surface of the support tends to bind more protein due to the better protein-adsorption affinity. The membrane packaging also allows for easy separation and maintenance of the bioreactor and low mass transfer resistance (Chen *et al.* 2012; Man *et al.* 2016).

The CGTase immobilization yield is strongly affected by temperature, increasing significantly from 20 °C to 25 °C and decreasing from 30 °C to 40 °C, reaching a maximum of 70.66% at 25 °C. The reduction in adsorption of CGTase on hollow fiber membrane at increased temperature was in good agreement with the

Langmuir adsorption isotherm, whereby the adsorption was a spontaneous and exothermic process, and the decrease in temperature would benefit the electrostatic interaction between the support and the enzyme (Kim *et al.* 2001; C. Wang *et al.* 2016).

Figure 13.5 (A) shows the surface of a hollow fiber membrane before the immobilization of CGTase, whereas Figure 13.5 (B) shows the surface of the hollow fiber membrane after the immobilization of CGTase. As seen in Figure 13.5 (B), CGTase were visible on the surface of the hollow fiber membrane, indicating that CGTase has been successfully immobilized on it. The immobilization was occurred by adsorption, which is known to be simple and inexpensive. The high amount of enzyme adsorbed onto the support was due to the electrostatic interaction between the enzyme particles and the PVDF membrane surface. The PVDF membrane used in this study contain positively charged hydrogen atoms and negatively charged fluoride atoms (Fontananova *et al.*, 2015). Meanwhile, the enzyme contains positively charged amino groups and negatively charged carboxyl groups (Lei *et al.*, 2008). The electrostatic interactions between the positively charged PVDF and the negatively charged enzyme as well as between the negatively charged fluoride atoms of PVDF and the negatively charged carboxyl groups may contribute to the strong attachment of the CGTase molecules onto the hollow fiber membrane. Interestingly, the adsorption of CGTase on the PVDF membrane occurred through electrostatic interaction without requiring any chemical treatment. Thus, the hollow fiber membrane could be safely used as a support without using any chemical alteration that could potentially damage or alter the characteristics of the enzyme and the end product.

Apart from that, the physicochemical properties of both the enzyme and the membrane surface also contributed to the immobilization of CGTase on the hollow fiber membrane. According to Wang *et al.* (2012), there were some properties of the support surface that would affect the adsorption of the enzyme on the support, such as topography, surface composition, hydrophobicity and surface potential. In the present study, PVDF

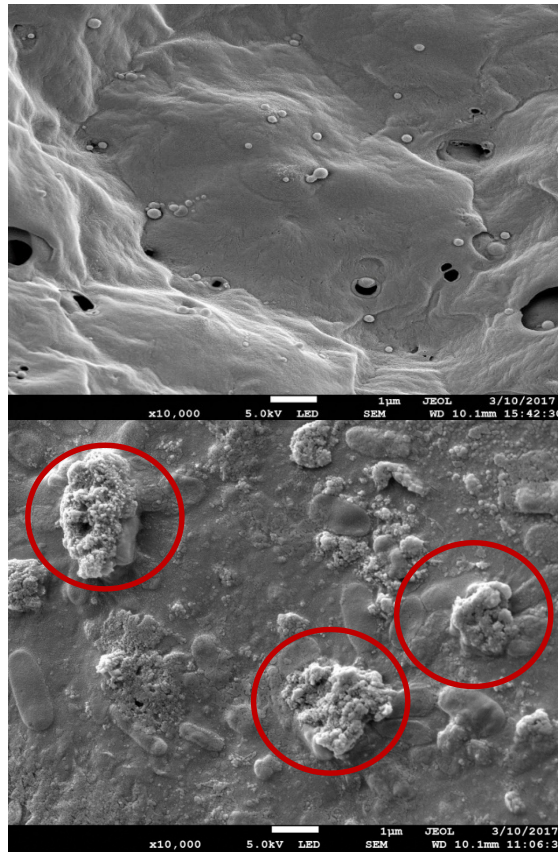


Figure 13.5 FESEM images of a hollow fiber membrane under 10,000x magnification. (A) before enzyme immobilization (B) after enzyme immobilization. The red circles show the immobilized CGTase on the surface of the hollow fiber membrane

membrane acts as a hydrophobic polymer due to the higher contact angle that has assisted in the immobilization of CGTase on the hollow fiber membrane (Algieri, Donato, and Giorno 2017; Chen *et al.* 2012). The hydrophobic PVDF hollow fiber membrane showed 28% higher immobilization yield (68.40%) compared to the

polyethylenimine (PEI) hollow fiber membrane that acted as a hydrophilic membrane (40.26% immobilization yield). The adsorption of CGTase on PEI membrane only involved electrostatic interaction of the negatively charged CGTase surface and the positively charged PEI surface (Man *et al.* 2015). Therefore, the higher immobilization yield on the PVDF hollow fiber membrane might be due to the strong interactions between the CGTase and the support (two electrostatic interactions and one hydrophobic interaction), compared to the PEI which only involve one electrostatic interaction.

Response surface methodology (RSM) was applied to determine the optimum immobilization condition in the immobilization of CGTase on a hollow fiber membrane. Under the optimized immobilization condition, the maximum immobilization yield was 88.25% when the temperature, pH and contact time was 24 °C, pH 6.7 and 24 hr, respectively. The results revealed that the immobilization of CGTase on the hollow fiber membrane was successfully optimized and improved about 4.6-fold compared to before the optimization process (25°C, pH 4 and 24 hr).

13.5 PRODUCTION OF CYCLODEXTRIN (CD) BY IMMOBILIZED CGTASE ON HOLLOW FIBER MEMBRANE

The production of CD by the immobilized CGTase was conducted using the optimized conditions for every one hour in seven successive batches. The highest yield of CD achieved by the immobilized CGTase was 12.21 mg/ml, whereas the highest yield of CD achieved by the free CGTase was 10.32 mg/ml, both at 6 hours of reaction time. Meanwhile, the cumulative yield of CD by the immobilized and free CGTase was 76.33 mg/ml and 67.63 mg/ml in 7 hours, respectively. This showed that using the immobilized CGTase instead of the free enzyme has improved CD yield by 12.86%. The increased CD yield achieved by the immobilized CGTase could be due to the adsorption of CGTase on

the hydrophobic support modifying the conformation of the enzyme such that it is more open (active site open), thus increasing substrate access to the enzyme active sites (Chen *et al.* 2012; Secundo 2013). According to Secundo (2013), immobilization could improve the activity of the enzyme if the interaction between the enzyme and the support surface had enhanced the enzyme unfolding mechanism.

The reusability of the immobilized CGTase is one of the important factors when considering this enzyme system for industrial application. The immobilized CGTase was recycled ten times to evaluate the production of CD in the consecutive batch reactions under the optimized conditions. The immobilized CGTase could retain 37.7% of its initial activity after 10 catalytic cycles. The loss of relative activity during the repeated use cycle of the immobilized enzyme was due to the gradual leaching of the bounded CGTase during the reaction process (Blanco *et al.* 2013; Xie and Ma 2010). The result presented in this study showed that the hollow fiber membrane performed well compared to other supports in CGTase immobilization systems. For instance, the immobilized CGTase on magnetic support was only able to keep 20% of its initial activity after being reused for four cycles (Blanco *et al.* 2013). In addition, the immobilization of CGTase from *Thermoanarebacter* sp. via covalent attachment on Eupergit C depicted that the immobilized CGTase was only able to retain 50% of its initial activity after five cycles of repeated use (Martín *et al.*, 2003).

The cumulative yield of CD achieved by the immobilized CGTase after 10 cycles of reuse was 26.43 mg/ml. Meanwhile, the yield of CD achieved by the free CGTase was only 2.21 mg/ml, as the free CGTase was difficult to reuse due to its solubility. These results demonstrated that the immobilization of CGTase on the hollow fiber membrane substantially improved the yield of CD by allowing for the reuse of the CGTase. The reusability of the immobilized enzyme was facilitated by the easy separation of the enzyme from the bulk solution, thus enabling multiple uses of the immobilized CGTase and leading to a higher cumulative yield of CD compared to the yield achieved by the free CGTase. Therefore, the reusability of the immobilized CGTase would optimize cost

efficiency and provide high potential use of the immobilized enzyme in industrial processes.

13.6 CONCLUSION

CGTase was successfully immobilized on polyvinylidene fluoride (PVDF) hollow fiber membrane via adsorption. The hollow fiber membrane appeared to be a suitable support for the immobilized CGTase for the production of CD with 12.21 mg/ml at 6 hr of reaction time. The biodegradable and environmentally friendly hollow fiber membrane showed good operational stability in this condition. The optimization of the conditions for the immobilization of CGTase on the hollow fiber membrane had substantially improved the production of CD by allowing for the reusability of the enzyme. The immobilized CGTase has excellent reusability and ease of recovery (easy separation from the product), which is valuable for the continuous production of CD in industrial applications. The simple immobilization technique used in this study could contribute to the lowering of the operating cost of industrial-scale α-CD production.

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REFERENCES

Abdel-Naby, M. A. 1999. “Immobilization of Paenibacillus Macerans NRRL B-3186 Cyclodextrin Glucosyltransferase and Properties of the Immobilized Enzyme.” *Process*

Biochemistry, 34(4): 399–405.

Abdel-Naby, M. A, A. Fouad, and R. M. Reyad. 2015. "Catalytic and Thermodynamic Properties of Immobilized Bacillus Amyloliquefaciens Cyclodextrin Glucosyltransferase on Different Carriers." *Journal of Molecular Catalysis B: Enzymatic,* 116: 140–47.

Aditiya, H. B. *et al.* 2016. "Second Generation Bioethanol Potential from Selected Malaysia's Biodiversity Biomasses: A Review." *Waste Management,* 47: 46–61.

Aksoy, Y., and H. Hasar. 2021. "Fabrication of Gas-Permeable Polyvinylidene Fluoride (PVDF) Hollow-Fiber Membrane by Dry-Jet Wet Spinning and Its Application in Membrane Biofilm Reactors." *Journal of Water Process Engineering,* 40: 101879.

Algieri, C., L. Donato, and L. Giorno. 2017. "Tyrosinase Immobilized on a Hydrophobic Membrane." *Biotechnology and Applied Biochemistry,* 64(1): 92–99.

Amud, A. E. *et al.* 2007. "Methods and Supports for Immobilization and Stabilization of Cyclomaltodextrin Glucanotransferase from Thermoanaerobacter." In *Biotechnology for Fuels and Chemicals.* New York: Humana Press: 309–21.

Arazawa, D. T. *et al.* 2012. "Immobilized Carbonic Anhydrase on Hollow Fiber Membranes Accelerates CO2 Removal from Blood." *Journal of Membrane Science,* 403–404: 25–31.

Arya, S. K., and S. K. Srivastava. 2006. "Kinetics of Immobilized Cyclodextrin Gluconotransferase Produced by Bacillus Macerans ATCC 8244." *Enzyme and Microbial Technology,* 39(3): 507–10.

Blanco, K. C. *et al.* 2013. "Reuse of Cyclodextrin Glycosyltransferase through Immobilization on Magnetic Carriers." *Enzyme Engineering*: 1–5.

Brady, D., and J. Jordaan. 2009. "Advances in Enzyme Immobilization." *Biotechnology Letters,* 31(11): 1639–50.

Brígida, A. I. S. *et al.* 2008. "Immobilization of Candida Antarctica Lipase B by Adsorption to Green Coconut Fiber." *Applied Biochemistry and Biotechnology,* 146(1–3): 173–87.

Cao, L. 2006. "Covalent Enzyme Immobilization." *Carrier-bound Immobilized Enzymes*: 169–316.

Carlsson, N. *et al.* 2014. "Enzymes Immobilized in Mesoporous Silica: A Physical – Chemical Perspective." *Advances in Colloid and Interface Science,* 205: 339–60.

de Castro, H. F., R. de Lima, and I. C. Roberto. 2001. "Rice Straw as a Support for Immobilization of Microbial Lipase." *Biotechnology Progress,* 17(6): 1061–64.

Che Man, R., *et al.* 2021. "Effect of Process Parameters on Immobilization of Cyclodextrin Glucanotranferase (CGTase) from *Bacillus licheniformis* on Pineapple Waste." *Materials Today: Proceedings*.

Chen, G.-J. *et al.* 2012. "Effect of Membranes with Various Hydrophobic/Hydrophilic Properties on Lipase Immobilized Activity and Stability." *Journal of bioscience and Bioengineering* 113(2): 166–72.

Ching, C. *et al.* 2022. "Identifying the Physicochemical Properties of β-Cyclodextrin Polymers That Determine the Adsorption of Perfluoroalkyl Acids." *Water Research* 209: 117938.

Cieh, N. L., *et al.* 2017. "Bleached Kenaf Microfiber as a Support Matrix for Cyclodextrin Glucanotransferase Immobilization via Covalent Binding by Different Coupling Agents." *Process Biochemistry,* 56: 81–89.

Dror, Y. *et al.* 2008. "Encapsulation of Enzymes in Biodegradable Tubular Structures." *Macromolecules,* 41(12): 4187–92.

Dwevedi, A. 2016. "Basics of Enzyme Immobilization." In *Enzyme Immobilization*, Cham: Springer, 21–44.

Gul, I. *et al.* 2020. "Enzyme Immobilization on Glass Fiber Membrane for Detection of Halogenated Compounds." *Analytical Biochemistry,* 609: 113971.

Guzik, U., K. Hupert-Kocurek, and D. Wojcieszyńska. 2014. "Immobilization as a Strategy for Improving Enzyme Properties-Application to Oxidoreductases." *Molecules,* 19(7): 8995–9018.

Hanefeld, U., L. Gardossi, and E. Magner. 2009. "Understanding Enzyme Immobilization." *Chemical Society Reviews,* 38(2):

453–68.

Hara, P., U. Hanefeld, and L. T. Kanerva. 2008. "Sol–Gels and Cross-Linked Aggregates of Lipase PS from Burkholderia Cepacia and Their Application in Dry Organic Solvents." *Journal of Molecular Catalysis B: Enzymatic,* 50(2–4): 80–86.

Homaei, A. A. *et al.* 2013. "Enzyme Immobilization: An Update." *Journal of Chemical Biology,* 6(4): 185–205.

Jamil, N. *et al.* 2018. "Effect of Enzyme Concentration and Temperature on the Immobilization of Cyclodextrin Glucanotransferase (CGTase) on Hollow Fiber Membrane." *Materials Today: Proceedings,* 5 (10, Part 2): 22036–42.

Jesionowski, T., J. Zdarta, and B. Krajewska. 2014. "Enzyme Immobilization by Adsorption: A Review." *Adsorption,* 20(5–6): 801–21.

Junko T. E., and M. Vitolo. 2006. "Method for Immobilizing Invertase by Adsorption on Dowex® Anionic Exchange Resin." *Revista Brasileira de Ciencias Farmaceuticas/Brazilian Journal of Pharmaceutical Sciences,* 42(2): 245–49.

Kang, G., and Y. Cao. 2014. "Application and Modification of Poly (Vinylidene Fluoride) (PVDF) Membranes–a Review." *Journal of Membrane Science,* 463: 145–65.

Kim, D. W., *et al.* 2001. "Effect of Metal Ions on the Degradation and Adsorption of Two Cellobiohydrolases on Microcrystalline Cellulose." *Bulletin of the Korean Chemical Society,* 22(7): 716–20.

Lei, C. *et al.* 2008. "Enzyme Specific Activity in Functionalized Nanoporous Supports." 125102.

Man, R. C. *et al.* 2015. "Effects of the Immobilization of Recombinant *Escherichia coli* on Cyclodextrin Glucanotransferase (CGTase) Excretion and Cell Viability." *Biochemical Engineering Journal,* 98: 91–98.

Man, R. C. *et al.* 2016. "Effects of Culture Conditions of Immobilized Recombinant *Escherichia coli* on Cyclodextrin Glucanotransferase (CGTase) Excretion and Cell Stability." *Process Biochemistry,* 51(4): 474–83.

Margetić, A., and Z. Vujčić. 2016. "Immobilization of Cell Wall Invertase in a Polyacrylamide Hydrogel for Invert Sugar Production." *Journal of the Serbian Chemical Society,* 81(12): 1359–69.

Martín, M. T. *et al.* 2003. "Immobilization on Eupergit C of Cyclodextrin Glucosyltransferase (CGTase) and Properties of the Immobilized Biocatalyst." *Journal of Molecular Catalysis B: Enzymatic,* 21(4–6): 299–308.

Matijošytė, I. *et al.* 2010. "Preparation and Use of Cross-Linked Enzyme Aggregates (CLEAs) of Laccases." *Journal of Molecular Catalysis B: Enzymatic,* 62(2): 142–48.

Mohamad, N. R. *et al.* 2015. "An Overview of Technologies for Immobilization of Enzymes and Surface Analysis Techniques for Immobilized Enzymes." *Biotechnology and Biotechnological Equipment,* 29(2): 205–20.

Mokhtar, A. *et al.* 2019. "Novel Biodegradation System for Bisphenol a Using Laccase-Immobilized Hollow Fiber Membranes." *International Journal of Biological Macromolecules,* 130: 737–44.

da Natividade S. J. *et al.* 2017. "Directed Immobilization of CGTase: The Effect of the Enzyme Orientation on the Enzyme Activity and Its Use in Packed-Bed Reactor for Continuous Production of Cyclodextrins." *Process Biochemistry,* 58: 120–27.

de Oliveira, R. L. *et al.* 2018. "Thermodynamic and Kinetic Studies on Pectinase Extracted from Aspergillus Aculeatus: Free and Immobilized Enzyme Entrapped in Alginate Beads." *International Journal of Biological Macromolecules,* 115: 1088–93.

Online, View Article *et al.* 2015. "RSC Advances."

Ouyang, J. *et al.* 2020. "Enzymatic Hydrolysate of Geniposide Directly Acts as Cross-Linking Agent for Enzyme Immobilization." *Process Biochemistry,* 99: 187–95.

Ouyang, L. *et al.* 2010. "Catalytic Hollow Fiber Membranes Prepared Using Layer-by-Layer Adsorption of Polyelectrolytes and Metal Nanoparticles." *Catalysis Today,*

156(3–4): 100–106.

Pachelles, S. *et al.* 2021. "Combine Strategy of Treated Activated Charcoal and Cell Surface Protein Curli Induction for Enhanced Performance in *Escherichia coli* Immobilization." *Process Biochemistry,* 110: 26–36.

Plessas, S. *et al.* 2007. "Use of Saccharomyces Cerevisiae Cells Immobilized on Orange Peel as Biocatalyst for Alcoholic Fermentation." *Bioresource Technology,* 98(4): 860–65.

Rakmai, J., B. Cheirsilp, and P. Prasertsan. 2015. "Enhanced Thermal Stability of Cyclodextrin Glycosyltransferase in Alginate–Gelatin Mixed Gel Beads and the Application for β-Cyclodextrin Production." *Biocatalysis and Agricultural Biotechnology,* 4(4): 717–26.

Rehm, F. B. H., S. Chen, and B. H. A. Rehm. 2016. "Enzyme Engineering for in Situ Immobilization." *Molecules,* 21(10): 1370.

Rodrigues, R. C. *et al.* 2013. "Modifying Enzyme Activity and Selectivity by Immobilization." *Chemical Society Reviews,* 42(15): 6290–6307.

Rojas, M. J. *et al.* 2019. "Preparation of Crosslinked Enzyme Aggregates of a Thermostable Cyclodextrin Glucosyltransferase from *Thermoanaerobacter* Sp. Critical Effect of the Crosslinking Agent." *Catalysts,* 9(2): 120.

Santos, J. C. *et al.* 2008. "Morphological and Mechanical Properties of Hybrid Matrices of Polysiloxane–Polyvinyl Alcohol Prepared by Sol–Gel Technique and Their Potential for Immobilizing Enzyme." *Journal of Non-Crystalline Solids,* 354(42–44): 4823–26.

Schoevaart, R. *et al.* 2004. "Preparation, Optimization, and Structures of Cross-linked Enzyme Aggregates (CLEAs)." *Biotechnology and Bioengineering,* 87(6): 754–62.

Secundo, F. 2013. "Conformational Changes of Enzymes upon Immobilization." *Chemical Society Reviews,* 42(15): 6250–61.

Sen, P. *et al.* 2016. "Studies On Hydrolysis of Skimmed Milk Using Immobilized β-Galactosidase in a Membrane Reactor."

Materials Today: Proceedings 3(10, Part A): 3403–17.

Shamel, M. M. *et al.* 2007. "Hydrolysis of Palm and Olive Oils by Immobilized Lipase Using Hollow Fiber Reactor." *Biochemical Engineering Journal,* 34(3): 228–35.

Sigurdardóttir, S. B. *et al.* 2018. "Enzyme Immobilization on Inorganic Surfaces for Membrane Reactor Applications: Mass Transfer Challenges, Enzyme Leakage and Reuse of Materials." *Advanced Synthesis and Catalysis,* 360(14): 2578–2607.

Sousa, H. A. *et al.* 2001. "Immobilization of Pig Liver Esterase in Hollow Fiber Membranes." *Enzyme and Microbial Technology,* 29(10): 625–34.

Suhaimi, S. *et al.* 2018. "Stability and Reusability of Cyclodextrin Glucanotransferase Immobilized on Hollow Fiber Membrane." *{IOP} Conference Series: Earth and Environmental Science,* 185: 12002.

Sulaiman, S. *et al.* 2015. "Study on the Preparation of Cellulose Nanofiber (CNF) from Kenaf Bast Fiber for Enzyme Immobilization Application." *Sains Malaysiana,* 44(11): 1541–50.

Tan, X. *et al.* 2006. "Polyvinylidene Fluoride (PVDF) Hollow Fiber Membranes for Ammonia Removal from Water." *Journal of Membrane Science,* 271(1–2): 59–68.

Tardioli, P. W., G. M. Zanin, and F. F. D. Moraes. 2006. "Characterization of Thermoanaerobacter Cyclomaltodextrin Glucanotransferase Immobilized on Glyoxyl-Agarose." *Enzyme and Microbial Technology,* 39(6): 1270–78.

Tsai, C., and A. S. Meyer. 2014. "Enzymatic Cellulose Hydrolysis: Enzyme Reusability and Visualization of β-Glucosidase Immobilized in Calcium Alginate." *Molecules,* 19(12): 19390–406.

Urbance, S. E. *et al.* 2004. "Evaluation of Succinic Acid Continuous and Repeat-Batch Biofilm Fermentation by Actinobacillus Succinogenes Using Plastic Composite Support Bioreactors." *Applied Microbiology and Biotechnology,* 65(6): 664–70.

Wang, C. *et al.* 2016. "Effect of Sulfonated Lignin on Enzymatic

Activity of the Ligninolytic Enzymes Cα-Dehydrogenase LigD and β-Etherase LigF." *Enzyme and Microbial Technology,* 93: 59–69.

Wang, K. *et al.* 2012. "A Review of Protein Adsorption on Bioceramics." *Interface Focus,* 2(3): 259–77.

Xie, W., and N. Ma. 2010. "Enzymatic Transesterification of Soybean Oil by Using Immobilized Lipase on Magnetic Nanoparticles." *Biomass and Bioenergy,* 34(6): 890–96.

Ye, P. *et al.* 2005. "Chitosan-Tethered Poly (Acrylonitrile-Co-Maleic Acid) Hollow Fiber Membrane for Lipase Immobilization." *Biomaterials,* 26(32): 6394–6403.

Ye, Y. *et al.* "Immobilization of α -Chymotrypsin on Zeolite for Peptide Synthesis in Organic Solvent." 21–25.

Zdarta, J. *et al.* 2018. "A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility." *Catalysts,* 8(3): 92.

Zhang, J., M. Li, and H. Mao. 2019. "Cross-linked Enzyme Aggregates of Recombinant Cyclodextrin Glycosyltransferase for High-purity B-cyclodextrin Production." *Journal of Chemical Technology & Biotechnology,* 94(5): 1528–33.

Zucca, P., and E. Sanjust. 2014. "Inorganic Materials as Supports for Covalent Enzyme Immobilization: Methods and Mechanisms." 14139–94.

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