STUDY THE BEST SUPPORT FOR HYDROLYSIS OF TIGER GRASS

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ABSTRACT

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Bioethanol, one of the outstanding biofuels, is not only a renewable bio-based resource, but also interesting to research for improving better properties of the enzyme needed in the hydrolysis process. The purpose of this work is to study how to reuse the enzyme used in the hydrolysis step for bioethanol production by immobilizing *Trichoderma reesei* (*T.reesei*) on various silica supports, namely, SBA-15, TUD-1, and MCM-48. The amount of the adsorbed enzymes was determined by UV-visible spectrophotometry. Among those studied supports, SBA-15 showed 100% enzymatic adsorption on the support owing to its larger pore diameter of 6.14 nm, which is large enough to accommodate *T.reesei* enzyme molecules inside the pore channel. Various parameters, viz. temperature, pH, time, and amount of the support for optimizing the immobilized enzyme were investigated. The immobilized *T.reesei* on SBA-15 support was characterized by N_2 adsorption-desorption. The amount of monomeric sugar after the hydrolysis process was measured by high performance liquid chromatography (HPLC).

บทคัดย่อ

กุลิสรา สยามนิกร: ศึกษาตัวรองรับเอนไซมที่ดีที่สุดเพื่อใช้ในปฏิกิริยาไฮโดรไลซิสหญ้าก๋ง (Study the Best Support for Hydrolysis of Tiger Grass) อ.ที่ปรึกษา : ศาสตราจารย์ ดร.สุจิตรา วงศ์เกษมจิตต์, ศาสตราจารย์ ดร. อาภาณี เหลืองนฤมิตชัย และรองศาสตราจารย์ ดร. ธัญญลักษณ์ ฉายสุวรรณ์ 62 หน้า

พลังงานทดแทนที่สำคัญอย่างหนึ่งคือ ไบโอเอทานอล นอกจากเป็นพลังงานที่มาจากพืช ยังเป็นงานวิจัยที่น่าสนใจในการศึกษาเพื่อพัฒนาเอนไซมที่ใช้ในการผลิตเอทานอลให้มีสมบัติดีขึ้นในกระบวนการไฮโดรไลซิส ในงานวิจัยนี้ ได้มีการศึกษาการนำตัวรองรับเอนไซม์ที่ใช้ในการผลิตเอทานอลในขั้นตอนการไฮโดรไลซิสกลับมาใช้ใหม่ โดยศึกษาชนิดตัวรองรับชนิด SBA-15, TUD-1, และ MCM-48 เพื่อรองรับเอมไซม์ *Trichoderma reesei* (*T. reesei*) และมีการตรวจสอบการดูดซับของเอมไซม์ด้วยเทคนิค UV-visible spectroscopy และพบว่า SBA-15 สามารถดูดซับเอมไซม์ได้ทั้งหมด เพราะเนื่องจาก SBA-15 มีขนาดรู 6.14 นาโนเมตร ที่เหมาะสมกับโมเลกุลเอมไซม์ โดยงานวิจัยนี้ มีการศึกษาปัจจัยของอุณหภูมิ เวลา ความเป็นกรดด่าง และปริมาณตัวรองรับที่เหมาะสมในการดูดซับเอมไซม์ *T. reesei* ซึ่งมีการพิสูจน์เอกลักษณ์โดยเทคนิค N_2adsorption-desorption, X-ray diffraction, และ Scanning electron microscope ปริมาณน้ำตาลหลังปฏิกิริยาไฮโดรไลซิสถูกตรวจสอบด้วย High performance liquid chromatography

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CHAPTER I
INTRODUCTION

Bioethanol, a renewable bio-based resource used as transportation fuel, is usually used as a gasoline additive to increase octane, improve combustion process, and reduce CO emission (Hansen *et al.*, 2005, Balat *et al.*, 2008). The bioethanol production process consists of pretreatment (first hydrolysis), saccharification (second hydrolysis), detoxification, fermentation, and separation. However, the important process for the bioethanol production is the hydrolysis process due to its high cost.

The hydrolysis step for converting carbohydrate polymer in lignocellulosic materials to simple sugars before fermentation can be classified into two types: chemical and enzymatic hydrolysis (Balat *et al.*, 2008). The enzymatic hydrolysis process is more attractive because it operates at a much milder condition and does not cause any corrosion (Sun and Cheng, 2002, Pan *et al.*, 2005).

Trichoderma sp. (*from T.viride, T.reesei, T.longibrachiatum*) is commonly chosen since it provides a high yield and is a good destroyer of crystalline cellulose (Zhou *et al.*, 2008). However, disadvantages of using enzyme are follows; high cost, difficult recovery, sensitive to pH and temperature, and not stable in organic solvent (Cao and Tan, 2002, Tébéka *et al.*, 2009). Immobilized enzyme is thus essential not only to overcome those drawbacks, but also for economical issue (Jain and Wikins, 1987, Jones and Vasudevan, 2009, Tébéka *et al.*, 2009).

Major three factors affecting catalytic activity, thermal stability, and additional cost of an immobilized enzyme are enzyme, matrix, and the method of attachment. The characteristics of the matrix are an important parameter in determining performance of the immobilized enzyme. Generally, supports can be either inorganic or organic component (Sheldon, 2007). Ideal support properties include high porosity, hydrophilic character, commercial availability, biocompatibility, resistance to microbial attack, and low cost. The inorganic supports provide many advantages, especially, highly stable against physical, chemical, and microbial degradation (Guisan, 2013).

Research emphasizing on the synthesis of the ordered porous materials has been rapidly grown because of the discovery of the ordered mesoporous support, e.g.,

MCM-48, SBA-15 and TUD-1. These mesoporous silica supports synthesized using template are reported as high surface area and narrow pore-size distribution mesoporous materials (Lu and Schüth, 2006), especially, SBA-15 having high surface areas, well-defined pore structure, inert framework, nontoxicity, high biocompatibility, and thermal and hydrothermal stability.

Therefore, this research is to study immobilization of the *Trichoderma ressei* using mesoporous MCM-48, SBA-15, and TUD-1 supports to determine the highest efficiency for loading enzyme on the support with the least leaching and the optimal performance of *Trichoderma reesei* immobilized on the best support for hydrolysis of Tiger grass to sugar.

CHAPTER II
LITERATURE REVIEW

2.1 Immobilized Enzyme

Historically, enzyme plays an important role in biotechnological method in energy, food and chemical industries. However, enzymes have been too expensive for economical operation in bioethanol production from biomass. An unbound (free) enzyme usually has low stability towards heat, organic solvent, and acid or base. Moreover, the enzymatic recovery is very difficult. Then, industrial enzymes must easily be handled and operated in terms of stability and reusability for profit at the industrial (Balat *et al.*, 2008).

For this purpose, enzyme immobilization is a tool to produce the industrial biocatalysts; and it has been studied over the last forty years for the successful utilization of enzymes in industrial processes (Singh *et al*., 2013). The immobilization of enzymes affects their conformation, rigidity, and aggregation state, modifying their reactivity (Palomo *et al.*, 2007, Mateo *et al.*, 2007). Thus, enzyme immobilization was studied to determine the enzyme selectivity. The immobilization of enzymes proposes the following advantages:

(i) Possibility of reusability
(ii) Increase in instability, such as resistance to high temperature, extreme pHs, high substrate concentration, polar solvents, mechanical shear
(iii) Increase in volumetric activity
(iv) Increase in selectivity, such as regio-, acyl-, chemo-, and enantioselectivity (Palomo *et al.*, 2007, Brady and Jordaan, 2009).

2.2 Enzyme

2.2.1 Degradation of Cellulose by Cellulase

Enzymatic hydrolysis of cellulose is a complete reaction by cellulase, which affects to a mixture of several functions. Three major groups are involved in the hydrolysis process:

(i) β-1-4-endoglucanase (EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber to create free chain ends;

(ii) β-1-4-exoglucanase or cellobiohydrolase (EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends;

(iii) β-glucosidase (EC 3.2.1.21.), which hydrolyzes cellobiose to produce glucose (Sun and Cheng, 2002, Cao and Tan, 2002).

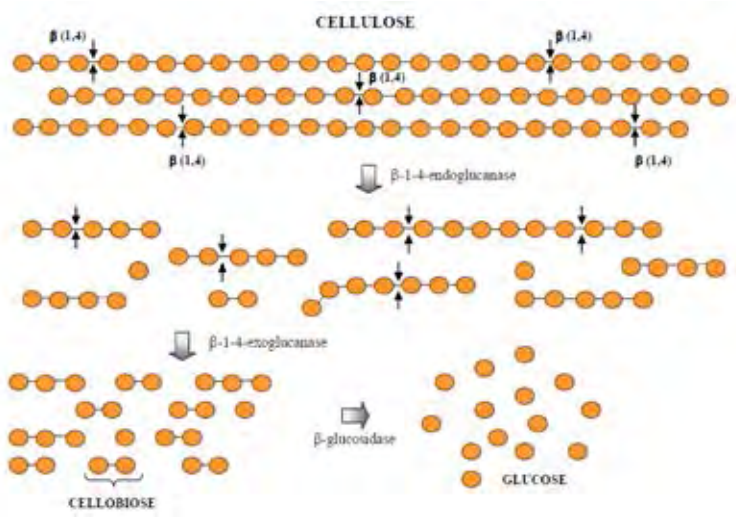

Figure 2.1 Schematic representation of the cellulase enzymes over the cellulose structure (Mussatto and Teixeira, 2010).

2.2.2 *Trichoderma reesei*

A variety of cellulolytic fungi and bacteria was reported. *Trichoderma reesei* (*T. reesei*) was intensively studied (Mussatto and Teixeira, 2010) although *T. viride, T. reesei, T. longibrachiatum* are considered to be the most productive and powerful destroyers of crystalline cellulose (Zhou *et al.*, 2008). *Trichoderma reesei*, one of the most productive cellulolytic organisms, composes of two cellobiohydrolases, CBH I and BH II (EC 3.2.1.91), at least three endoglucanases, EG I, II and III (EC 3.2.1.4), and several β-glucosidase (EC 3.2.1.21). Both EG I and CBH have about 45% sequence homology (Pentillä *et al.*, 1986). Partial proteolysis studies of CBH I and CBH II were further exposed the existence of two distinct functional domains: a binding region for insoluble cellulose and a core protein containing the active (hydrolytic) site (Tomme and Claeyssens, 1989). The binding domains were identified with terminal, glycosylated amino acid sequences (block AB (B')) which maintained in both enzymes (Knowles *et al.*, 1988).

2.3 Support or Matrix

Characteristics of the matrix are important in determining the performance of the immobilized enzyme method (Guisan, 2013). Ideal support should provide physical resistance to compression, hydrophilicity, inertness toward enzymes, ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost (Brodelius and Mosbach, 1987, Buchholz and Klein, 1987). The support (carrier) can be a synthetic polymer, a biopolymer, or an inorganic solid (Sheldon, 2007).

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) are significant for the performance of the immobilized method. In particular, pore parameters and particle size are determined the total surface area and thus critically influence the capacity for binding of enzymes (Guisan, 2013).

2.3.1 Synthetic Polymer

Eupergit C is a macroporous copolymer of N,N'-methylene-bi-(methacrylamide), glycidyl methacrylate, allylglycidyl ether, and methacrylamide with medium particle size of 170 mm and a pore diameter of 25 nm (Katchalski-Katzir and Kraemer, 2000). It is extremely hydrophilic and stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes. It bonds proteins via reaction of its oxirane moieties, at neutral or alkaline pH, with the free amino groups of the enzyme to form covalent bonds which have long-term stability within a pH range of 1 to 12 (Figure 2.2).

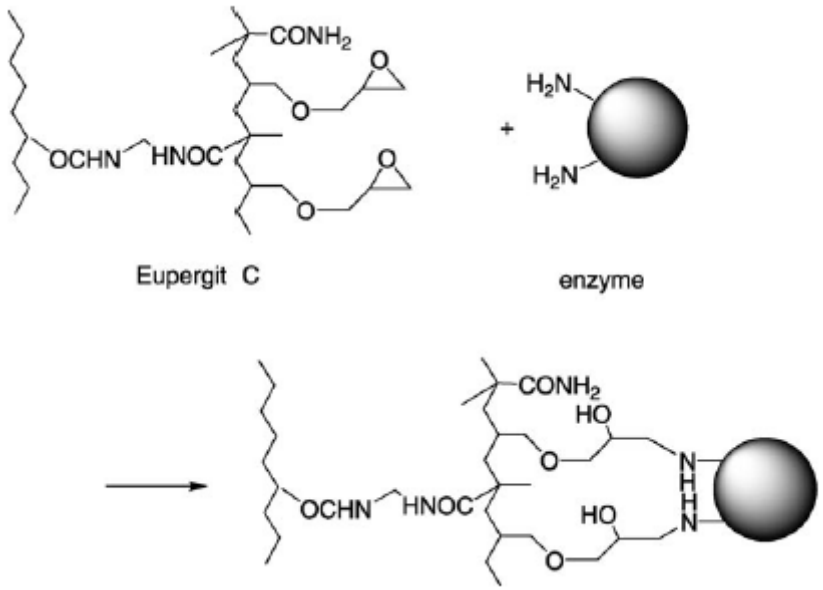

Figure 2.2 Immobilization of enzymes on Eupergit C (Sheldon, 2007).

Epoxy groups can provide inactive site by using a variety of reagents (mercaptoethanol, ethanolamine, glycine, etc.) to prevent any undesired support-protein reaction. Due to the high density of oxirane groups on the surface of the beads, enzymes are immobilized at various sites of their structure (Sheldon, 2007). This "multi-point-attachment" is largely responsible for the high operational stability

of enzymes bound to Eupergit C (Bruggink *et al.*, 2003). A major drawback of Eupergit C is the diffusion limitations, which are more pronounced in kinetically controlled processes (Sheldon, 2007).

2.3.2 Natural Polymer

A variety of biopolymers, mainly water-insoluble polysaccharides, such as cellulose, starch, agarose and chitosan (Krajewska, 2004), and proteins, such as gelatin and albumin were used as supports for immobilizing enzymes (Sheldon, 2007). A superior matrix is agarose that has been extensively used. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages are hydrophilic character, ease of derivatization, absence of charged groups (which prevents nonspecific adsorption of substrate and products), and commercial availability. However, a significant limitation in the use of agarose and other porous supports is the high cost (Guisan, 2013). They are many reviews of organic support.

Trichoderma reesei Rut C30 can be immobilized using polyester cloth as support. In batch fermentations, the cellulose activity of immobilized mycelium was 1.55IU/mL, while in fed batch fermentation, the cellulose activity produced was 20% lower than that generated by free mycelium. However, the improved stability was associated with immobilized systems. It may make the immobilized system more attractive for cellulose hydrolysis (Sheldon, 1987).

Reactions of hydrolysis were carried out at specific water activities which executed by equilibrating both the enzyme preparation and the substrate solution at the desired water activity before mixing them and thereby starting the reactions. The reaction rates got at the same water activity with different supports, indicating a direct effect of the support on the enzyme. For horse liver alcohol dehydrogenase, Celite was the best support, and the reaction rate increases with increasing water activity. With the polyamide support, Accurel PA6, alcoholysis was the dominating reaction, and by using a low water activity (0.33), hydrolysis was completely suppressed while still maintaining a high alcoholysis activity. Controlled pore glass (CPG), derivatized with either hexyl or glucosyl groups, had quite

different properties as enzyme supports. For horse liver alcohol dehydrogenase, glucose-CPG was a much better support than hexyl-CPG, and in the alpha-chymotrypsin-catalyzed reactions, glucose-CPG prefered hydrolysis, and hexyl-CPG preferred alcoholysis, at water activities exceeding 0.8. The results are considered about the absorption of water on the enzymes, on the supports and the solubility of water in the reaction media; all these parameters were measured separately (Adlercreutz, 1991).

2.3.3 Inorganic Supports

A variety of inorganic solids can be applied for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silicas (Wang *et al.*, 2001), such as MCM-41 or SBA-15. One of the simplest and the cheapest methods to immobilize an enzyme was to use silica granulation. Mesoporous silicas, which are often mentioned as nanosilicas, have several advantages as supports: uniform pore diameters (2–40 nm), very high surface areas (300–1500 m^2g^{-1}) and volumes (ca. 1 mLg^{-1}), and are inert and constant at elevated temperatures (Sheldon, 2007). They are many reviews of inorganic support.

Functionalised hexagonal mesoporous SBA-15 type molecular sieves with pore sizes in the range of 51–56 Å were prepared using non-ionic block copolymers and applied for immobilization of the enzyme trypsin. Thiol, chloride, amine, and carboxylic acid functional groups were attached by siloxypropane ethers to the siliceous surface of SBA-15 via two processes, post-synthesis grafting and in situ synthesis (Yiu *et al.*, 2001).

Many studies suggest that naturally-occu0rring layered clay minerals can be applied as a class of biocompatible solid supports for immobilizing enzymes. The non-covalent immobilization induces van der Waals forces, electrostatic interactions, hydrogen bonding, and hydrophobic interactions. For avoiding desorption of enzymes, immobilization can be managed through direct covalent bonding between enzymes and clay minerals. Organic modification of clay minerals

and addition of linking molecules are made to enhance the immobilization so as to increase the loading, activity and stability of enzymes (An *et al.*, 2015).

In Simultaneous Saccharification and Fermentation (SSF), ethanol yield was 2.1 ($p=0.06$) to 2.3 ($p=0.01$) times higher than that using Separate Saccharification and Fermentation reactions of microcrystalline cellulose. Immobilizated enzyme onto solid supports can stabilize and promote enzyme activity at non-optimum reaction operation. 40-nm silica nanoparticles-supported cellulose produced glucose 1.6 times ($p=0.01$) more than free cellulase in solution in 96 h at pH 4.8 and 35 °C. There was no significant accumulation (<250 μg) of soluble cellooligomers in both free enzyme and immobilized enzyme reactions. The results present that silica-immobilized cellulase can be used to produce for increased ethanol yields in the conversion of lignocellulosic materials by SSF (Lupoi and Smith, 2011).

The immobilization of globular enzymes cytochrome C (bovine heart), papain (papaya latex) and trypsin (bovine pancreas) in the mesoporous molecular sieve MCM-41 was studied. The loading efficiency of the immobilized enzymes presents a correlation with the enzyme size. It refers that the mesopores materials are significant in the immobilization method. Silanation of the support after immobilization was affected the stopping enzyme leakage from the support without hindering interaction with large substrates. Diaz and Balkus studied the immobilization of enzymes in larger pore (> 40 a) MCM-41 structures in order to evaluate the effects of an order silica support. Additionally, the structure of the silane is an important variable for immobilizing enzyme (Diaz and Balkus, 1996).

2.4 Mode Attachment

One way of classifying various approaches to immobilize enzymes is divided in two types: irreversible and reversible methods (Gupta and Mattiasson, 1992).

2.4.1 Methods of Irreversible Enzymatic Immobilization

The idea of irreversible immobilization means that once the biocatalyst is attached to the support then it cannot be removed without destroying either the biological activity of the enzyme or the support.

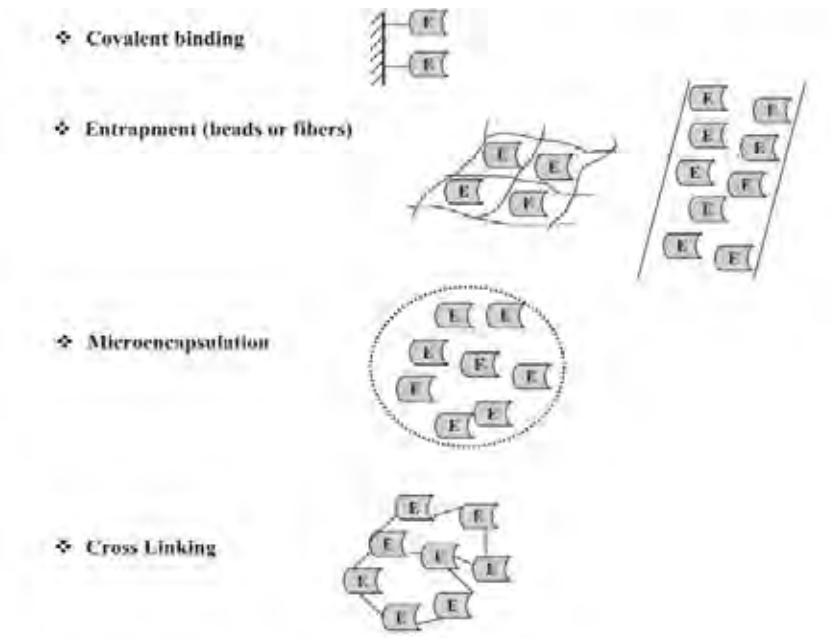

Figure 2.3 General procedures of irreversible enzyme immobilization (Guisan, 2013).

2.4.1.1 Formation of Covalent Bonds

Immobilization of proteins based on the formation of covalent bonds. This method has advantages such as the stable nature of the bonds formed between enzyme and matrix (Guisan, 2013). A general procedure is to carry out the coupling reaction in the substrate analogs (Mattiasson and Kaul, 1991). Covalent methods for immobilization are employed when there is a requirement for the absence of the enzyme in the product (Guisan, 2013). A wide variety of reactions have been developed, depending on the functional groups available on the support (Scouten, 1987). Coupling methods are classified into two main classes:

1) Activation of the matrix by addition of a reactive function to a polymer

2) Modification of the polymer backbone to produce an activated group (Table 2.2).

The processes are designed to generate electrophilic groups on the support which, reacts with the strong nucleophiles on the proteins. The most frequently used reactions occurred the following side chains of the amino acids: lysine (ε-amino group), cysteine (thiol group), and aspartic and glutamic acids (carboxylic group).

Table 2.1 Covalent coupling methods of enzymes: activation of matrix hydroxyl functions

Activation method	Group that reacts (with activated matrix)
Tresyl chloride, sulfonyl chloride	Thiol, amines
Cyanogen bromide	Amine
Bisoxiranes (epoxides)	Thiol, amine
Epichlorohydrin	Thiol, amine
Glutaraaldehyde	Amine
Glycidol-Glyoxyl	Amine
N-Hydroxy-succinimidyl	Amine

There are many financially available supports for immobilization, the best choice requires the consideration of some relevant properties of the catalyst and the intended use (Guisan, 2013). However, it is usually necessary to try more than one approach and then adapt a method to the specific condition (White and Kennedy, 1980, Taylor, 1991). The covalent reactions commonly employed enzymes bonded with to the support through either amide, ether, thio-ether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and, in many cases, it is also stabilized (Guisan, 2013).

2.4.1.2 Entrapment

The entrapment method is based on the block of an enzyme within a polymeric network that allows the substrate and products to pass through but maintains the enzyme (O'Driscoll, 1976). This method differs from the coupling methods because the enzyme is not bound to the matrix or membrane (Guisan, 2013). There are many of approaches to entrap enzymes such as gel (Bernfeld and Wan, 1963) or fiber entrapping (Dinelli *et al.*, 1976) and micro-encapsulation (Wadiack

and Carbonell, 1975). The limited is mass transfer limitations through membranes or gels.

2.4.2 Methods of Reversible Immobilization

Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be removed from the support under gentle condition. The reversible methods for enzyme immobilization are the most attractive, mostly for economic reasons because when the enzymatic activity decreases the support can be regenerated and re-loaded with fresh enzyme. In fact, the cost of support is often a primary factor in the overall cost of immobilized catalyst (Guisan, 2013). The reversible immobilization of enzymes is especially important for immobilizing labile enzymes and for applications in bioanalytical systems (Gupta and Mattiasson, 1992).

2.4.2.1 Adsorption (Noncovalent Interactions)

2.4.2.1.1 Nonspecific Adsorption

The most common immobilization method is nonspecific adsorption, which is mainly based on physical adsorption or ionic binding (Messing, 1976, Guisan, 2013). In physical adsorption, the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas the enzymes are bound by salt linkages in ionic bonding. The nature of the forces which involved in non-covalent immobilization can be reversed by changing the conditions that influence the strength of the interaction (e.g., pH, ionic strength, temperature, or polarity of the solvent). The advantage of immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. So these methods are highly attractive, but the problem is the enzyme leakage from the matrix when the interactions are relatively weak.

2.4.2.1.2 Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions which occurs an entropically driven interaction. It depends on well-known experimental variables, such as pH, salt concentration, and temperature. The

strength of interaction depends on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be controlled by the degree of substitution of the support and by the size of the hydrophobic ligand molecule.

2.4.2.2 Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme. The bond can be broken by reaction with some suitable agent, such as dithiothreitol (DTT) under mild circumtainance (Guisan, 2013). Additionally, because the reactivity of the thiol groups was modulated via pH alteration, the activity yield of the methods involving disulfide bond method was usually provided when the appropriate thiol-reactive adsorbent with high specificity was applied (Carlsson *et al.*, 1998).

2.4.2.3 Chelation or Metal Binding

Transition metal salts or hydroxides which deposited on the surface of organic carriers are bounded become bound by coordination with nucleophilic groups on the support. Generally titanium and zirconia salts have been used and the method is known as “metal link immobilization” (Kennedy and Cabral, 1985). The metal salt or hydroxide is precipitated onto the matrix (e.g., cellulose, chitin, alginic acid, and silica-based carriers) by heating or neutralization. Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal; therefore, some of the positions remain free to coordinate with enzymes. The Advantage of this method is quite simple and these immobilized specific activities obtained with enzymes have been relatively high (30–80%). However, the operational stabilities are mostly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably benefited to the existence of non-uniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be taken place on the solid supports by means of stable covalent bonds. The metal ions are then bounded by coordination to form the stable complex form (Guisan, 2013).

The methods for enzyme immobilization have studied in the term of mode of attachment including enzyme entrapment, cross-linking, and support binding. The latter includes physical bonding like weak interactions (hydrogen bond, Van der Waals interactions), ion exchange, affinity interactions and covalent bonding (Sheldon, 2007). Among all these methods, covalent immobilization mostly ensures the highest strength of the bonding between matrix and enzyme, minimizing leakage issues. Moreover, covalent attachment does not usually interfere with reagents products, and permits the high enhancement of operational stability (especially, towards heat, pH, organic solvents, and also regarding the storage). These are crucial properties in the feasibility of any industrial process. From this perspective, this review focuses on the simplest methods for functionalization of inorganic supports. Several chemical functions can be inserted on the surface of the support (*i.e.*, $–NH_2$, alcoholic –OH, –COOH, –SH) which capable of covalently reacting with enzymes under proper conditions. Typically, following functionalization, activation of supports with specific activating agents (such as organic and inorganic halides, glutaraldehyde, carbodiimides, various bifunctional agents) is necessary for achieving enzyme immobilization.

2.4.2.4 Electrostatic Binding

Electrostatic binding is an important factor for attaching enzyme and metal oxide. The isoelectric point, which is approximately 9.5, provides a better micro-environment for the adsorption than low isoelectric point. Electrostatic binding is the distribution of polar and charged. Polar and charged generate the electrostatic properties by forming short-range interactions such as salt-bridges and hydrogen-bonds (Sinha and Smith-Gill, 2002). Protein interactions with electrostatics can be estimated by using electrostatic free energy which relates to protein stability, rate associations of complexes, and calculating pKa number.

2.5 Functionalization of Inorganic Supports

Typically, a generic matrix for protein immobilization should be chemically inert, otherwise, it could react with water (or buffers), and/or with substrates products arising from the enzyme-catalyzed reactions (Zucca and Sanjust, 2014). However, an ideal support should be reactive enough for achieving enzyme immobilization (Vansant *et al.*, 1995).

Restricting inspection to inorganic matrix, they usually consist of –OH groups covalently bound to the atoms. The semi-metallic or decidedly metallic feature of the elements involved in the formation of the corresponding (hydrous) oxides which affects all these supports. Moreover, those elements have empty *d* orbitals hosting electronic density from nucleophiles such as water. In S_N2-type reactions, the heteroatomic bonds like Si–O–C, Sn–O–C, Ti–O–C, Al–O–C lead to hydrolysis under the enzymatic immobilization. Therefore, these entities are classified as alkoxides. Ti–OH, Al–OH, and Sn–OH are very less acidic and more basic (in the Brønsted sense). As a consequence, some inorganic supports based on semimetal and metal oxides are more or less easily dissolved by strongly acidic or alkaline solution, which is another important limitation, (Zucca and Sanjust, 2014).

2.5.1 Silanization

General organosilanes are stable and reliable functionalization of organic supports; in principle, three main approaches could achieve support functionalization (Hoffmann *et al.*, 2006):

(i) *Grafting*: the plain support is treated under suitable situation with a chosen organosilane, forming some sort of covalently bound coating. This coating is formed by the organic functions of the starting silane;

(ii) *Co-condensation*: support particles are prepared by means of sol-gel procedures, starting from a proper mixture of tetraethyl (or tetramethyl) orthosilicate and the chosen trialkoxyorganosilane. Tetraalkylorthosilicates were replaced by other alkoxides, such as tetraethoxytitanium. However, excessive

proportions of the organosilane adversely affect the structure of the obtained particles, and disordered structures (Zucca and Sanjust, 2014).

(iii) Silsesquioxanes (general empirical formula $R_2Si_2O_3$), is oligomers derived from hydrolysis—under proper experimental conditions—of organosilanes with general formula X_3SiR, where X is an easily hydrolysable function such as Cl– or RO– (Treccani *et al.*, 2013). Bridged organosilanes produce particular silsesquioxanes that could be incorporated within the particle structure by sol-gel process, and later subjected to ammonolysis (with gaseous ammonia) at very high temperatures to break one head of the Si–C bonds bridges while inserting $–NH_2$ groups on to the organic moieties. The method is promising but requires specialty instrumentation for very high-temperature ammonolysis; certain bridged disilanes caused the collapse of the mesoporous structures when subjected to ammonolysis. On the whole, the use of silsesquioxanes (that could also be obtained as polymers of undefined degree of polymerization) is not always well distinguishable from co-condensation.

Silanization is usually performed by the means of suitable organosilanes presenting the general formula $(RO)_3Si\text{-}(CH_2)_n\text{-}X$, where R usually is $–CH_3$ or $– C_2H_5$, is 3, and X is a suitable chemical function, useful for subsequent immobilization reactions. However, the organosilanes with different structures are commercially available and could be very useful, possibly often further reaction, in immobilization procedures. The silanization reaction consists of a nucleophilic attack of a silanol group on the matrix to the silicon atom of the organosilane. When a non-silica-based matrix like (hydrous) SiO_2 or Al_2O_3 is used, a quite similar reaction takes place, and in any case a stable, covalent organosilane coating is formed. A noticeable exception is Z which, under those conditions, is incapable of forming Zr–O–Si bonds resistant against hydrolysis.

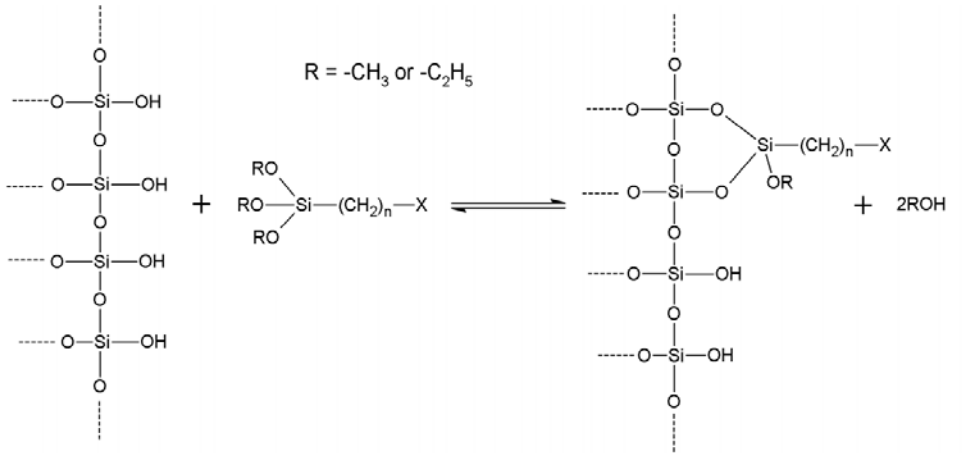

Figure 2.4 Reaction of trialkoxyorganosilanes with silanols on the surface of inorganic matrix.

In the case of silica-based supports, new siloxane bonds, bridges together the matrix and the silane, while methanol or ethanol is given off. The reaction could take place in bulk (without any added solvents, as the common silanes are nonvolatile liquids at room temperature), or in an organic solvent such as toluene or so on.

However, enzyme covalent immobilization on silanized inorganic matrixes usually implies an aqueous environment for preparation and operation of the immobilized catalyst, so the surviving alkoxide functions are unavoidably hydrolyzed, leading to additional silanols, which can in turn condense with each other when silanization of an inorganic support is carried out in an aqueous solution of the chosen organosilane. In such cases, hydrolysis of the trialkoxysilane takes place, and silanization is achieved upon condensation reactions (leading to siloxane bridges) which involving silanol groups (or more generally –OH groups, in the case of non-silica-based materials) of the matrix and those arising from the above mentioned hydrolysis.

The original structure of the plain support is only marginally affected by the grafting, although bulky silanes and/or narrow pores could lead to a substantial obstruction of the meso channels, when a mesoporous material is used as support. As grafting takes place exclusively at the surfaces of the support particles, the core

structure of the mesoporous support remains almost unaffected by the modification. Very reactive supports tend to undergo massive grafting, causing an excessive crowding of the functional groups attached to the matrix (Zucca and Sanjust, 2014).

CHAPTER III
EXPERIMENT

3.1 Materials

3.1.1 Chemicals

- Silica (SiO_2) 99.8% supplied by Nippon Aerosil, Japan.
- Triethanolamine (TEA) purchased from QRëc chemical, Thailand.
- Sodium hydroxide (NaOH) 99% purchased from Lab scan, Thailand.
- Cetyltrimethylammonium bromide (CTAB) by Fluka, Germany.
- Cellulase from *Trichoderma reesei* ATCC 26921 (Sigma Aldrich Chemical Co. Inc.,USA)
- Bradford reagent (Sigma Aldrich Chemical Co. Inc., USA)
- Sulfuric acid 95-98% purchased from Lab scan, Thailand.
- Acetronitrile 99.9% purity purchased from Lab scan, Thailand.
- Ethylene glycol (EG) 99% purity purchased from J.T. Baker, USA.
- Poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Sigma Aldrich Chemical Co. Inc., USA)
- Hydrochloric acid (HCI) purchased from Lab scan, Thailand.
- Tetraethylammonium hydroxide solution (TEAOH) (Sigma Aldrich Chemical Co. Inc., USA)

3.2 Experimental

3.2.1 Synthesis of Silatrane

The synthesis follows Wongkasemjit's synthetic method by mixing fumed silica (0.1 mole), EG (100 ml), and TEA (0.125 mole) and refluxing at 200 °C under nitrogen atmosphere for 12 hours in oil bath. The excess EG and water are removed under vacuum at 110 °C. Then, the product is washed by acetronitrile for removing TEA and EG residues. The silatrane product is vacuum-dried overnight before characterization using TGA and FT-IR.

3.2.2 Synthesis of MCM-48

Longloilert's synthetic method is followed by dissolving CTAB in 2 M NaOH solution at 50 °C. The mixture is continuously stirred while adding silatrane and the mixture is kept stirring at 50 °C for 1 hour. Subsequently, the mixture is transferred to a Teflon-lined stainless steel auto clave and treated with operating temperature of 140 °C for 16 hours. Then, the obtained solid product is collected by filtration before drying. The surfactant is removed by calcination at 550 °C for 6 hours.

3.2.3 Preparation of TUD-1

Synthesis of TUD-1 is carried out by mixing 3.22 g of water and 3.00 g of silatrane with stirring for 1 hour before adding 1.3 ml of TEAOH dropwise into the mixture with and stirring for 2 hours. After aging for 2 hours at room temperature, the synthesized solution is dried by calcination at 600 ºC for 10 hours.

3.2.4 Preparation of SBA-15

Mesoporous SBA-15 was synthesized from silatrane using poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) ($EO_{20}PO_{70}EO_{20}$) (P123) as the template and hydrochloric acid as the catalyst. A solution of 2:60:4.25:12 P123:HCl:silatrane:H_2O (mass ratio) was prepared by dissolving 4 g of P123 in 80 g of 2 M HCl (part A) and 8.8 g of silatrane in 20 g of

DI water (part B). The solution from part B was then poured into part A and stirred at room temperature for 24 hours. The product was recovered by filtration, washed with DI water, and dried overnight, followed by calcination at 550 °C for 6 hours to produce SBA-15.

3.2.5 Immobilization of Enzyme on MCM-48/TUD-1/SBA-15 Support

The enzyme is immobilized onto each support by impregnation method. *T. ressei* (0.5 ml) is mashed with the support using various contents of each support (0.1, 0.3, 0.5, 0.7, and 0.9 g). This immobilized enzyme is studied on the leaching test and hydrolysis application.

3.2.6 Leaching Testing

The immobilized enzyme is added into 15 ml of citric acid and dibasic sodium phosphate buffer and stirred for 2 hours at the room temperature. The solution mixture is filtered with Whatman filter paper No.1. The obtained filtrate (0.1 ml) is then mixed with 1 ml of Bradford reagent before analysis of the enzyme by UV-visible spectrophotometer to observe the enzyme leaching. This leaching test is performed on each support (MCM-48, TUD-1 and SBA-15) at various pHs (1-8), temperatures (room temperature, 40° and 60 °C), and times (1-5 days).

3.2.7 Microwave-assisted Pretreatment: Two-stage Microwave/Chemical Pretreatment Process

3.2.7.1 Microwave-assisted Dilute Sodium Hydroxide (NaOH) Pretreatment

Alkali pretreatment causes delignification of biomass and makes the lignocellulose swollen through saponification reactions (Pedrson and Meyer, 2010). The tiger grass powder was suspended in 1% (w/v) sodium hydroxide solution for 45 min at 160 °C using the optimal Tiger grass:NaOH solution ratio of 33g:495 ml.

3.2.7.2 Microwave-Assisted Dilute Acid Pretreatment

The objective of the dilute acid pretreatment is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes (Hendriks and Zeeman, 2009). The pretreated solid from the alkali pretreatment was treated with dilute sulfuric acid in microwave. The alkaline – pretreated Tiger grass (1g) is mixed with 15 ml of 0.5% (w/v) sulfuric acid solution. The pretreatment condition is at 200 °C for 5 minutes.

3.2.8 Hydrolysis

The two-stage pretreated hydrolysate is further enzymatically hydrolyzed and the degree of cellulose conversion is determined from the hydrolysate after the two-stage pretreatment and pH adjustment using buffer. Free enzyme, cellulase, prepared from *T.ressei* ATCC 26921 (160 µl/1g of the pretreated solid) and the immobilized enzyme (0.4033g/1g of tiger grass), are compared at various temperatures (30°, 40°, 50°, and 60 °C) and various pHs (1-8) to determine the content of the immobilized enzyme using 1g of Tiger grass to identify the optimal conditions. The sugar concentration obtained is analyzed by HPLC.

3.2.9 Characterization

The phase of the support is characterized on a Rigaku DMAX 2200HV X-ray diffractometer (XRD) with a scanning speed of 1 °C/min and CuKα source (λ= 0.154 Å) in a range of 2θ = 20°–80°. The specific surface area is measured by the Brunauer-Emmett-Teller (BET) method and the pore size distribution is calculated by the Barrett-Joyner-Halenda (BJH) on a Quantasorb Jr. (Autosorb-1). Prior to each analysis, the product is degassed at 250 °C for 12 hours. Thermal properties are analyzed by Thermogravimetry (TGA) on Pyris Diamons Perkin Elmer using a heating rate of 10 °C/min under nitrogen atmosphere. The structure and the formation of Siltrane are determined by a Nicolet Nexus 670 Fourier transform infrared spectrometer (FT-IR) at room temperature. UV-visible spectrophotometer (Shimadzu UV-1800) is employed. The morphology of the supports is investigated on Field emission scanning electron microscope (FE-SEM,

Hitachai S-4800) with an accelerating voltage of 2kV. High performance liquid chromatography (HPLC, Shimadzu Corp.) equipped with a refractive index detector (RID-10A, Shimadzu Corp., Kyoto, Japan) and Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA) using 50 µl injection volume, 0.005 M sulfuric acid (HPLC grade) as mobile phase with a 0.6 ml/min flow rate, 65 °C column temperature, and 20 min run time.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Characterization of Silatrane Precursor

FTIR spectrum in Figure 4.1 shows the formation of silatrane complexes, consistent with-the results cited elsewhere (Charoenpinijkarn *et al.*, 2001), consisting of C-H stretching (2800–2976 cm^{-1}, C-H bending (1380–1460 cm^{-1}, C-N (1270 cm^{-1}), Si-O-CH_2 (1015-1085 cm^{-1}), C-O (1013-1070 cm^{-1}), Si-O-CH (970,883 cm^{-1}) and Si-N stretching (560-590 cm^{-1}).

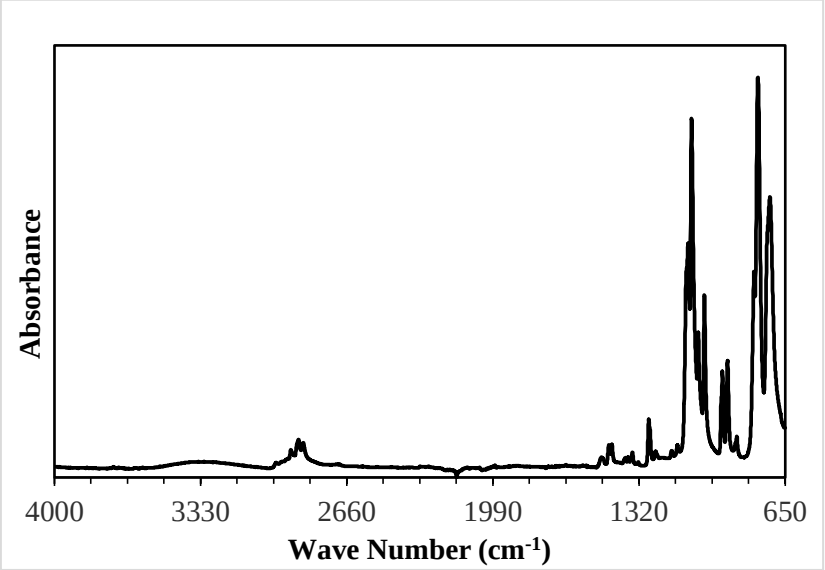

Figure 4.1 FTIR spectrum of Silatrane.

Thermatogram of silatrane by TGA shown in Figure 4.2 shows mass loss at 350 °C and gave the SiO_2 residue or ash yield of 22.7 %.

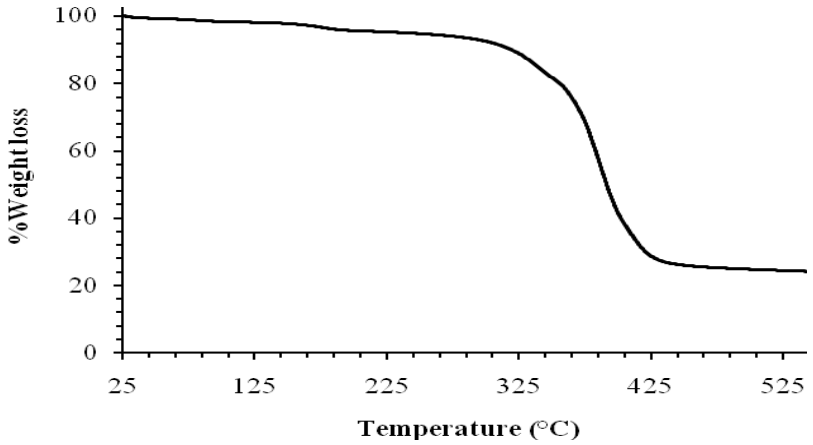

Figure 4.2 TGA thermogram of Silatrane precursor**.**

4.2 Characterization of MCM-48

4.2.1 X-Ray Diffraction (XRD)

XRD pattern of the calcined MCM-48 (Figure 4.3) exhibits 4 diffraction peaks at {211}, {220}, {420}, and {332}, corresponding to interwoven three-dimensional channel structure (Longloilert *et al.*, 2011).

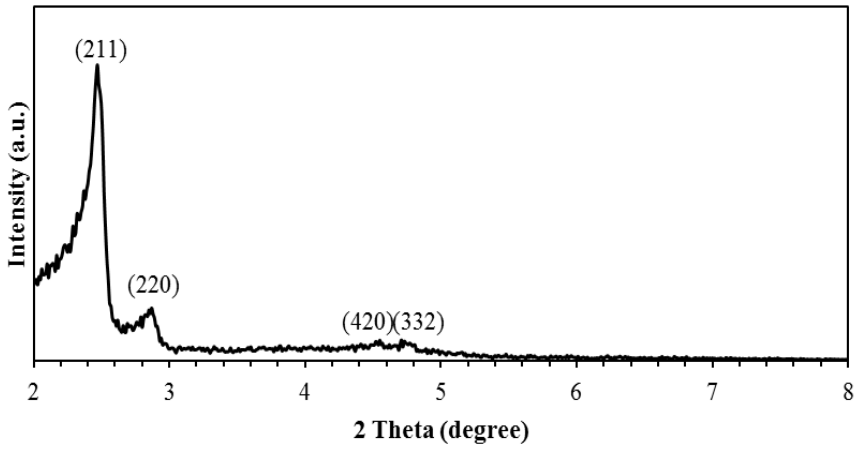

Figure 4.3 Small-angle XRD pattern of MCM-48.

4.2.2 N_2 Adsorption/Desorption Isotherms

N_2 Adsorption/desorption isotherms in Figure 4.4 shows a type IV isotherm with hysteresis loop and surface area around 1870 m^2/g. These results

indicate that MCM-48 has mesoporous structure. Moreover, the synthesized MCM-48 also shows a narrow pore size distribution around 2.82 nm, as seen in Figure 4.5.

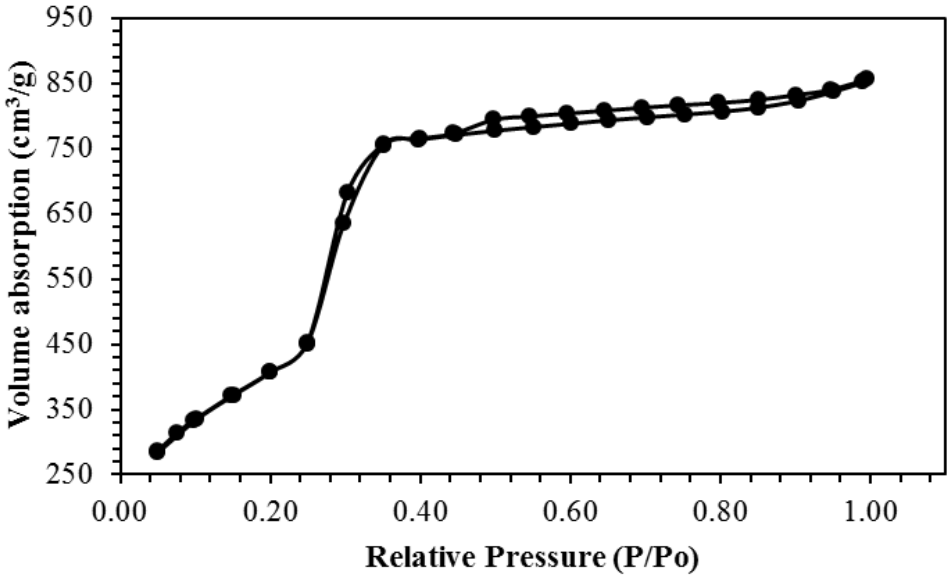

Figure 4.4 Nitrogen adsorption-desorption isotherm of MCM-48.

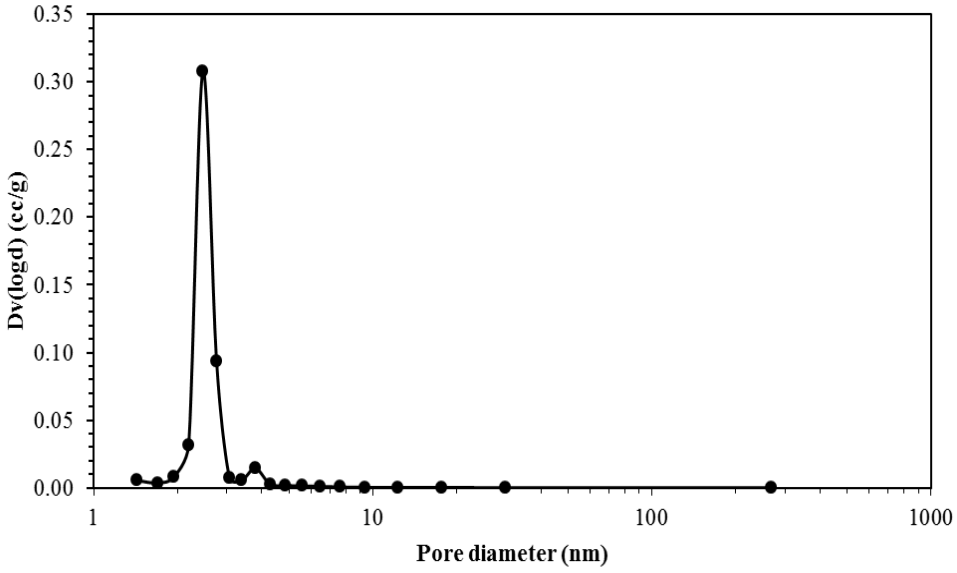

Figure 4.5 Pore size distribution of MCM-48.

4.2.3 Scanning Electron Microscopy (SEM) of MCM-48

The sample synthesized in this work was examined by FE-SEM to investigate their morphology. The images in Figure 4.6 are the MCM-48. It exhibits the truncated octahedral shape of aggregated MCM-48 particle, consistent with the results observed by Longloilert *et al*., 2011.

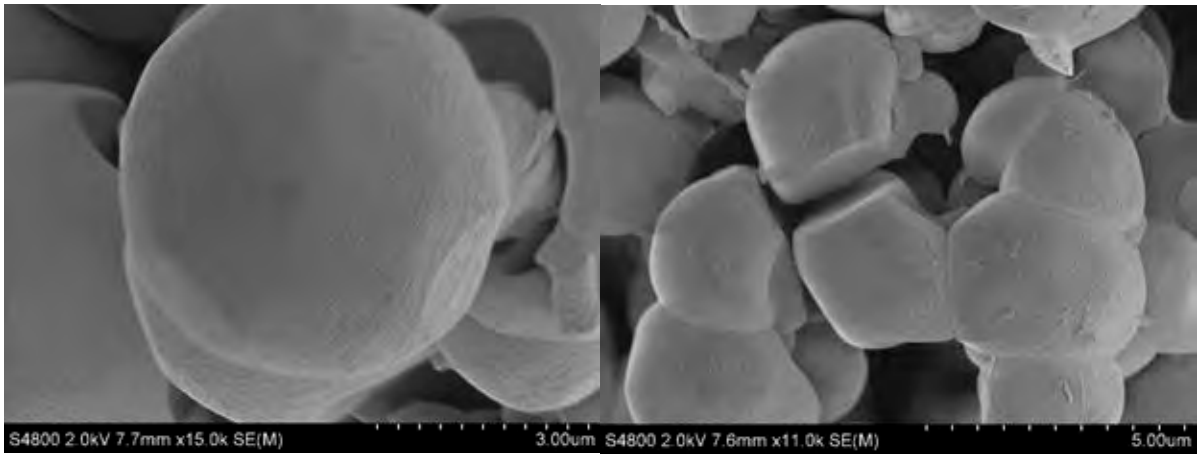

Figure 4.6 SEM images of MCM-48.

4.3 Characterization of SBA-15 Support

The morphological and structural characterization of the SBA-15 was performed by using SEM, N_2 Adsorption/Desorption Isotherms and SAXS.

4.3.1 Scanning Electron Microscopy (SEM) of SBA-15

Scanning electron microscopy (SEM) was employed to investigate the morphology of SBA-15, as shown in Figure 4.7. The micrographs showed that the SBA-15 possessed a sinuous morphology at a scale of a few hundred nanometers coexisting with poorly ordered mesoporus fragments (Samran *et al.*, 2011). The twisted bundles of silica tubes are seen to coexist with less crystalline and aperiodic material in the right-handed side.

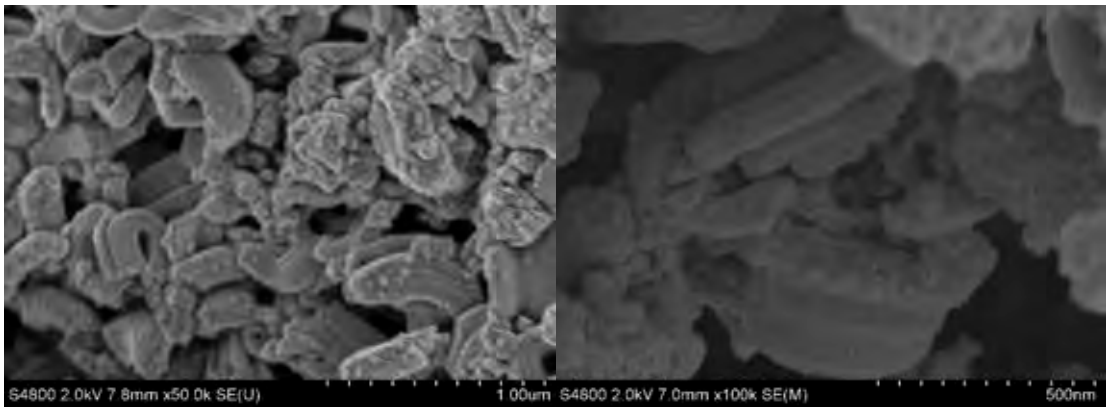

Figure 4.7 SEM images of SBA-15.

4.3.2 Small Angle X-Ray Diffraction (SAXS**)**

The SAXS patterns of SBA-15 in Figures 4.8 show three well-resolved peaks, which can be indexed {100}, {110}, and {200}, as also obtained by Samran and coworkers (Samran *et al.*, 2011).

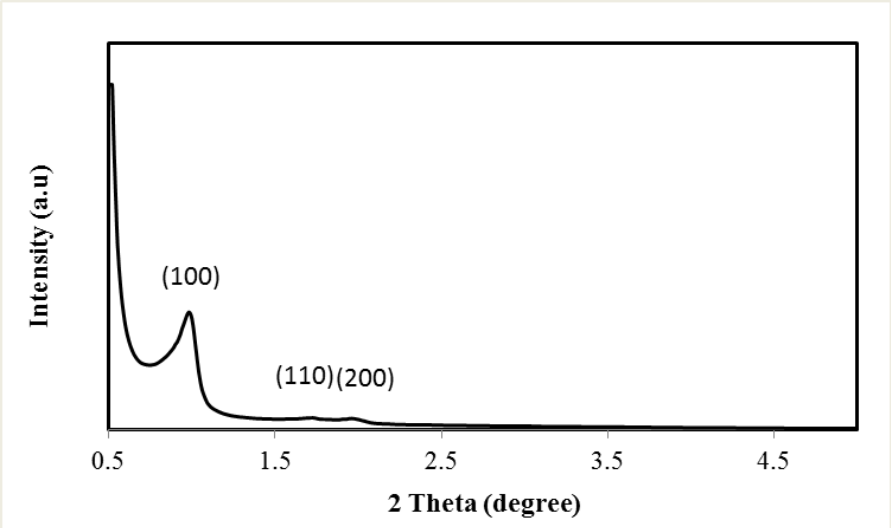

Figure 4.8 SAXS Pattern of SBA-15 support.

4.3.3 N_2 Adsorption/Desorption Isotherms

N_2 Adsorption/desorption isotherms in Figure 4.9 shows the IV isotherm with hysteresis loop and surface area around 827.7 m^2/g. Moreover, the

synthesized SBA-15 also shows pore size distribution around 6.137 nm (61.37 Å), indicating that SBA-15 has mesoporous structure, as seen in Figure 4.10 (Samran *et al.*, 2011).

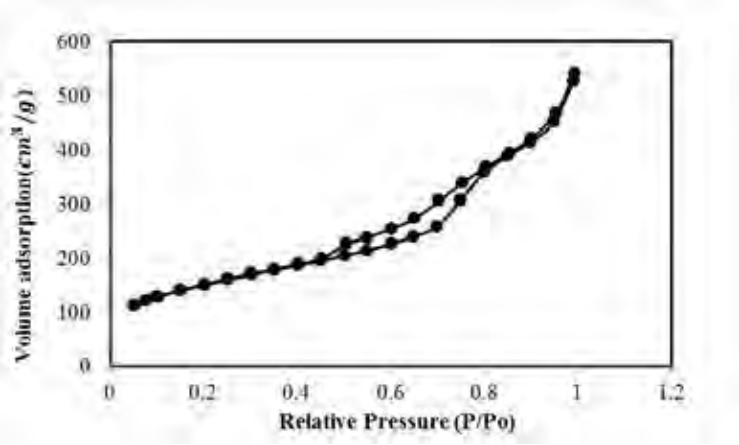

Figure 4.9 Nitrogen adsorption-desorption isotherm of SBA15 support.

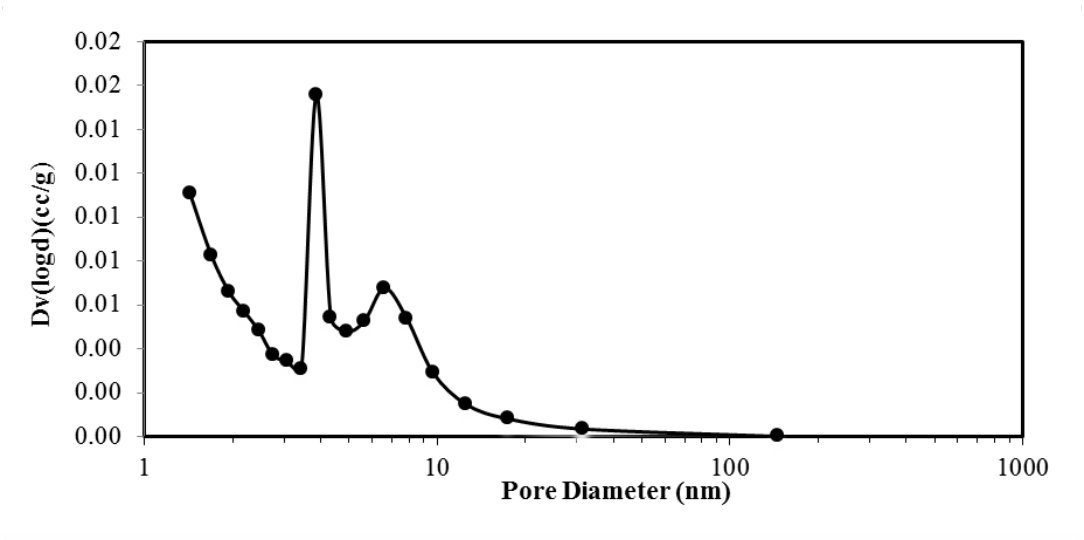

Figure 4.10 Pore size distribution of SBA-15 support.

4.4 Characterization of TUD-1 Support

4.4.1 N_2 Adsorption/Desorption Isotherms

N_2 Adsorption/desorption isotherms in Figure 4.11 also shows a type IV isotherm with hysteresis loop and surface area around 935.5 m^2/g. Moreover, the synthesized TUD-1 also shows pore size distribution around 4.476 nm (44.76 Å), indicating that TUD-1 has mesoporous structure as seen in Figure 4.12.

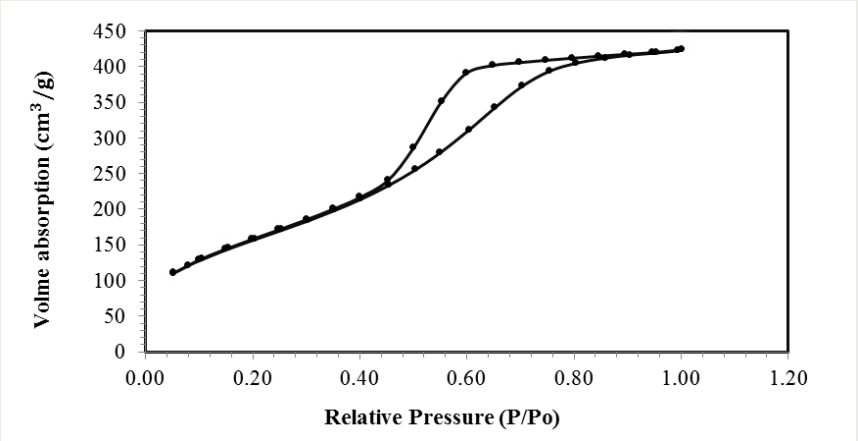

Figure 4.11 Nitrogen adsorption-desorption isotherm of TUD-1 support.

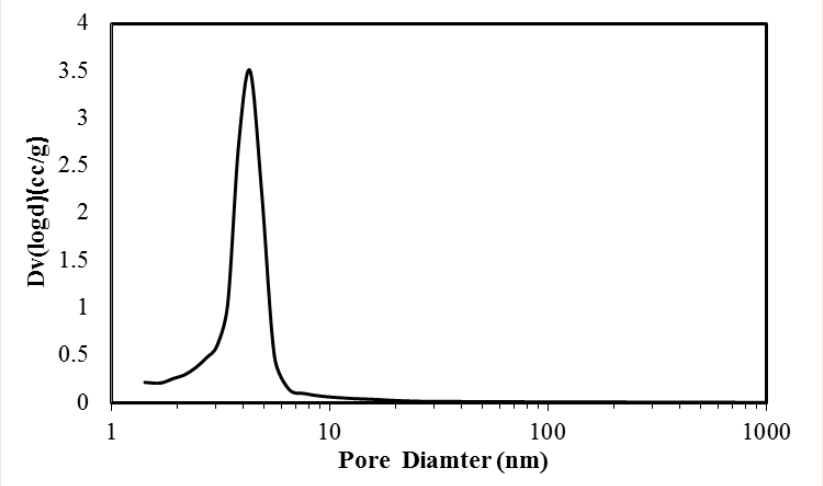

Figure 4.12 Pore size distribution of TUD-1 support.

4.4.2 Scanning Electron Microscopy (SEM) of TUD-1

SEM Images in Figures 4.13 of TUD-1 synthesized by sol gel technique show irregularly-shaped particle without well-defined morphology. Such morphology is similar to the results described.

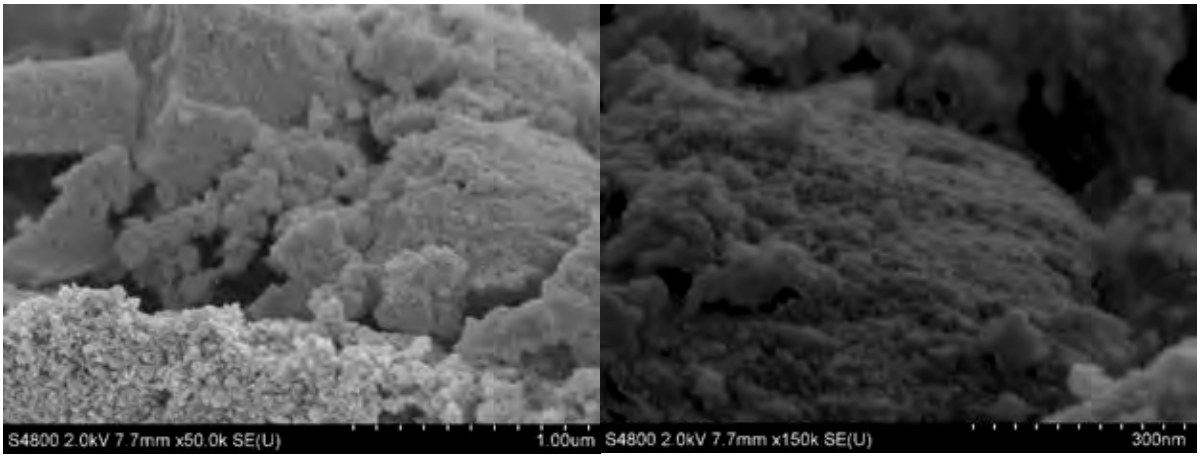

Figure 4.13 SEM images of TUD-1.

4.5 Calibration of Standard Protein Concentration

Protein assay is used to characterize protein concentration by mixing sample with bradford reagent and using a spectrophotometer to measure the absorbance. Five standard points (0.1, 0.2, 0.3, 0.4, 0.5 ml of enzyme) were measured. The absorbance of each concentration shows 0.56, 0.72, 0.81, 0.92 and 1.00. The calibration curve of the standard protein is shown in Figure 4.14. The R-square is 0.99, thus, this graph is used to calculate the concentration of adsorb protein.

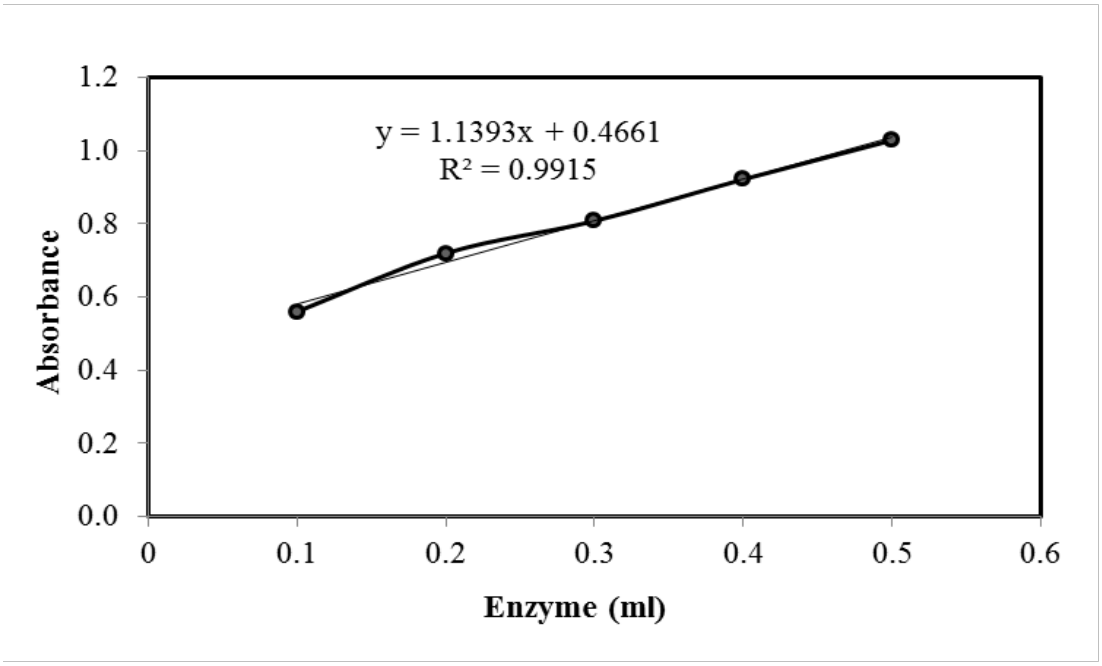

Figure 4.14 Calibration curve of the protein standard.

4.6 Parameters Influencing on Leaching of Immobilized *Trichoderma ressei* from the Support

4.6.1 Effects of Various Supports and Contents on % Adsorption of Immobilized *Trichoderma ressei* on the Supports

Since the support is one important parameter for immobilizing enzyme, the type of the support was studied in this work. Only suitable characteristics of the support provide an excellent adsorption for the immobilized enzyme, such as physical resistance to compression, resistance to acid and base, biocompatibility, and good heat stability (Guisan, 2013). Generally, a porous support is a good candidate for being used as a support due to its high specific pore volume and surface area, especially, mesoporous materials commonly studied because of their suitable pore size and high surface area for immobilizing *Trichoderma ressei* (Lee and Brown, 1997).

In this work, there are three types of mesoporous silica used to observe and study the suitable content for impregnating *Trichoderma ressei* enzyme on each support, namely, MCM-48, TUD-1, and SBA-15. The immobilized enzyme on the support was added into a mixture of citric acid and dibasic sodium phosphate buffer

(pH = 5) and stirred for 2 h at room temperature. The results are shown in Figure 4.15.

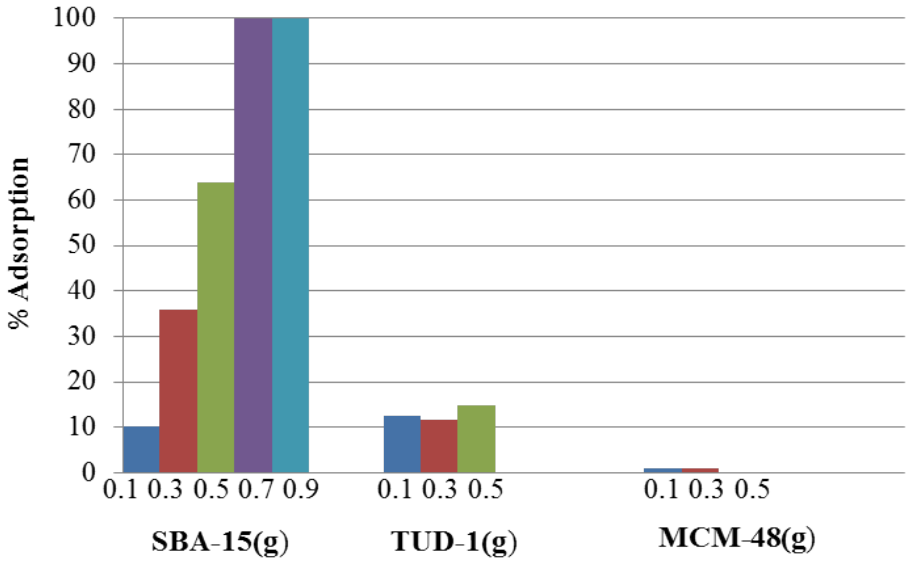

Figure 4.15 Influence of various supports and contents on % adsorption of immobilized *Trichoderma ressei* on the support.

The results show that SBA-15 gives the highest % adsorption for immobilization and the higher content provides the higher %adsorption up to 0.7g using 0.5 ml of *Trichoderma ressei*. The MCM-48 support hardly shows the adsorption whereas the TUD-1 support shows better adsorption. This result can be explained from the pore diameter of the supports, as summarized in Table 4.1.

Table 4.1 Pore diameter (nm) of the studied supports

Support	Pore diameter (nm)
SBA-15	6.14
MCM-48	2.82
TUD-1	4.48
Trichoderma ressei	4.50

As can be clearly seen, the pore diameter of the support is the key role of the immobilization process. The head with an average diameter of 4.5±0.5nm is the size of *Trichoderma ressei* (Lee and Brown, 1997) while only SBA-15 having the biggest and bigger pore size than the enzyme can allow the enzyme molecules to penetrate into the pore (Karimi *et al*., 2013). Both TUD-1 and MCM-48 having smaller pore sizes than the enzyme only allow the enzyme to stay on the surface, resulting in less enzyme activity when comparing to SBA-15 which is the best, suitable host for *Trichoderma ressei* with the suitable content of 0.7 g of SBA-15 in 0.5 ml of *Trichoderma ressei.* The higher amount of SBA-15 provides the higher volume for the enzyme to incorporate into the pores. The higher content than 0.7g does not seem to have better incorporation of the enzyme since both 0.7 and 0.9 g of SBA-15 result in the same 100 % adsorption. Thus, 0.7 g of SBA-15 in 0.5 ml of *Trichoderma ressei* is the optimal condition to give 100% adsorption of the enzyme on the support and used for further study.

4.6.2 Effect of pH on % Adsorption of Immobilized *Trichoderma ressei* on the SBA-15 Support

The next factor studied to observe the adsorption ability is pH of the buffer solution. The SBA-15 support of 0.7 g in 0.5 ml of *Trichoderma ressei* is stirred with the buffer mixture of citric acid and dibasic sodium phosphate for 2 hours at room temperature. In this work, eight different pHs (1, 2, 3, 4, 5, 6 ,7 and 8) of the buffer are studied for the leaching test of immobilized enzyme from the support and the results are illustrated in Figure 4.16.

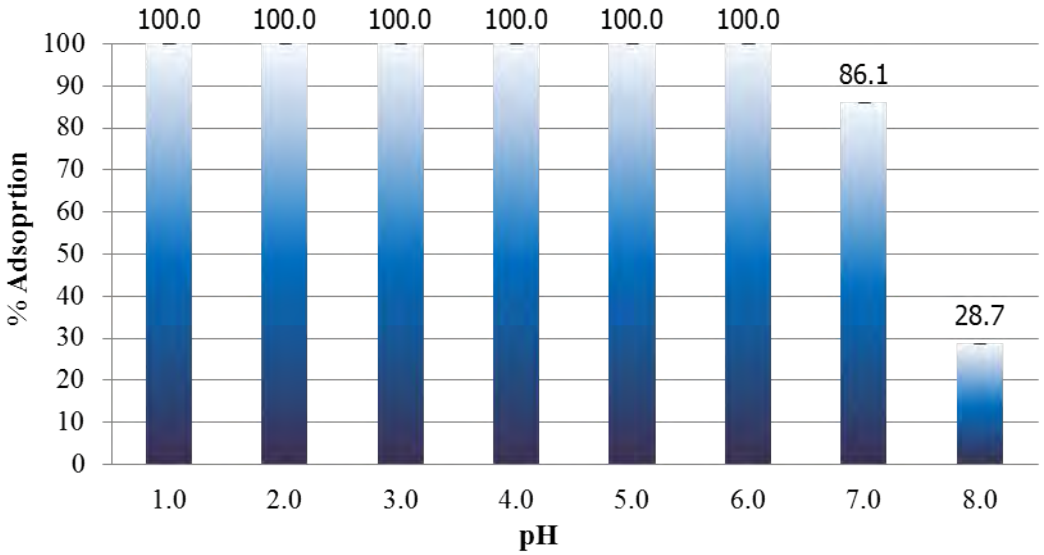

Figure 4.16 Influence of pH on % adsorption**.**

The results show that pHs = 1 to 6 provide 100% adsorption of the enzyme while the % adsorption decreases at the pH higher than 6. This phenomenon can be explained by the isoelectric points of both enzyme and support, see Table 4.2.

Table 4.2 Isoelectric points of the support and the enzyme

Sample Name	Isoelectric Point
SBA-15 support	3.7
Trichoderma ressei	5.5-6.5

Trichoderma ressei, having two functional groups (carboxylic acid and amine), has the isoelectric point (IP) at 5.5-6.5, where the net charge is zero (Vinzant *et al*., 2001, Reshmi *et al*., 2007). Enzyme molecules are, thus, always amphoteric, containing carboxylate and ammonium ions. When pH is lower than IP, proton predominantly protonates at more basic carboxylate groups. The ammonium ions are thus active toward the support by forming H-bonding at oxygen of hydroxyl group. At higher pH than IP, carboxylate groups are negative, hydroxyl ion in the solution thus strictly deprotonates the ammonium group to form water. The reactions of enzyme at lower and higher than IP are shown in equations 4.1 and 4.2, respectively.

$^{+}NH_3\text{-}R\text{-}COO^{-} \longrightarrow {}^{+}NH_2\text{-}R\text{-}COOH$ Equation 4.1

$^{+}NH_3\text{-}R\text{-}COO^{-} \longrightarrow NH_2\text{-}R\text{-}COO^{-}$ Equation 4.2

The ranges of IP of SBA-15 are 3.7 (Karimi *et al*., 2013). When pH is higher than IP, the surface of SBA-15 becomes negative. At pH lower than IP, the surface of SBA-15 becomes positive. The reactions of SBA-15 at lower and higher than IP are shown in equations 4.3, 4.4, respectively.

$S\text{-}OH + H^{+}A^{-} \longleftrightarrow S\text{-}OH_2{}^{+}A^{-}$ Equation 4.3

$S\text{-}OH + B^{+}OH^{-} \longleftrightarrow S\text{-}O^{-}\ B^{+} + H_2O$ Equation 4.4

The support becomes positive charge at pH = 1-3 and less positive at pH = 4-6 because the IP of the support is 3.7. In the case of the enzyme, having IP = 5.5-6.5, at pH=1-5 the enzyme dominantly stays positive, making the ammonium ion part form H-bonding with the support, resulting in 100% adsorption. At higher pHs of 7-8, both the enzyme and the support are dominantly negative, causing less binding between the enzyme and the support and resulting in the decrease of the %adsorption

4.6.3 Effect of Time on % Adsorption of Immobilized *Trichoderma ressei* on the Support

In this study, the immobilized enzyme is used in the hydrolysis application which takes 1 to 5 days to observe, using 0.7 g of SBA-15, 0.5 ml of *Trichoderma ressei*, pH = 5 of citric acid and dibasic sodium phosphate buffer for at room temperature. The results are shown in the Figure 4.17.

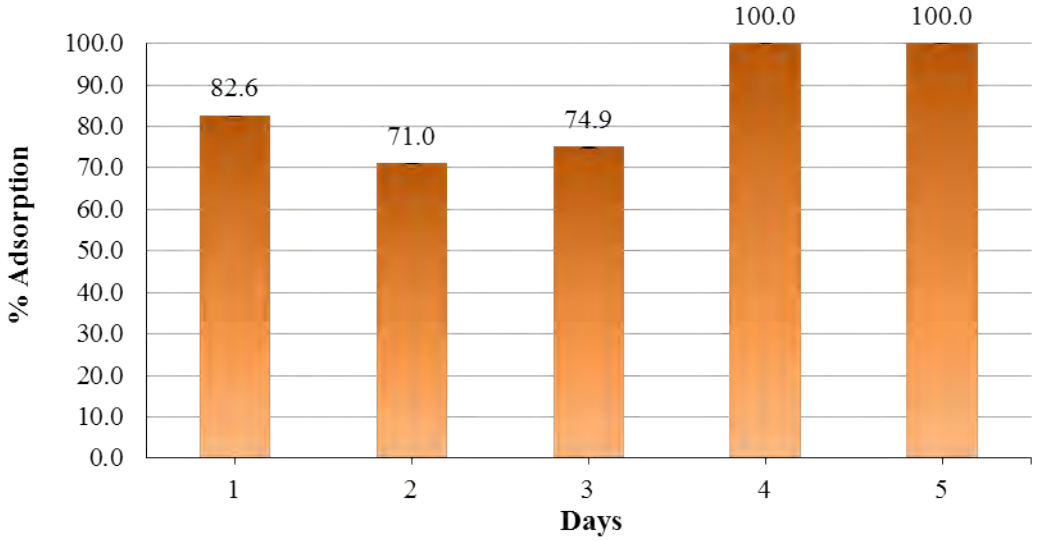

Figure 4.17 Influence of time on % adsorption.

As can be seen, after 1, 2, and 3 days, % adsorptions obtained were 82.6, 71.0, and 74.9, respectively. Remarkably, 100 %adsorption was observed after 4 and 5 days. During the first 1-3 days, some of the enzyme seems to be in the reversible state of forming H-bonding until the fourth and the fifth days show strong H-bondings, resulting in 100% adsorption and staying no leaching. As a result, the 100% enzyme can immobilize on to the support for 4 days in the hydrolysis.

4.6.4 Effect of Temperature on % Adsorption of Immobilized *Trichoderma ressei* on the SBA-15 Support

Another parameter studied is the temperature, using the 0.5 ml of *Trichoderma ressei* immobilized on 0.7g of SBA-15 in the pH = 5 buffer mixture of citric acid and dibasic sodium phosphate for 4 days. In this research, three different temperatures (room temperature, 40°, and 60 °C) are evaluated to study the leaching test for 2 hours in an incubator, and the results are shown in the Figure 4.18.

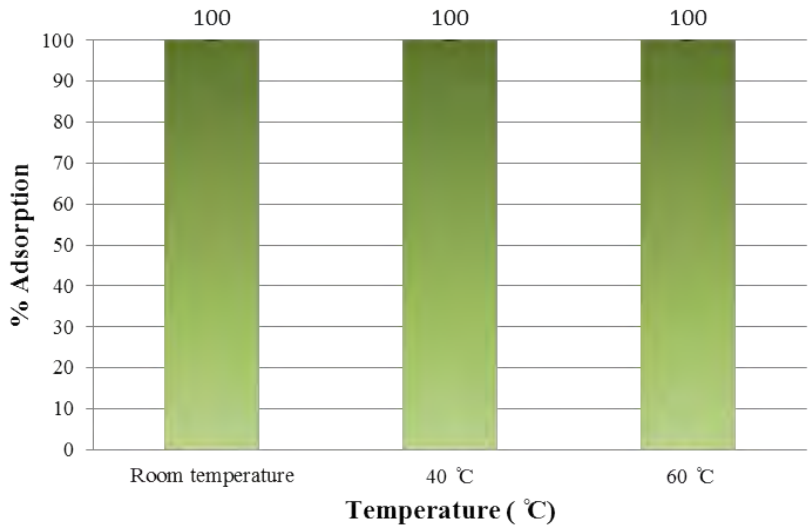

Figure 4.18 Influence of temperature on % adsorption.

All studied temperatures (room temperature, 40 and 60℃) provide 100% adsorption of immobilized enzyme without leaching. These impressive results could be from the fact that SBA-15 is the support having well-defined pore structure with high thermal stability up to 1453K (1180 °C), thus, the SBA-15 structure is not changed at these temperatures, resulting in no enzyme destruction.

4.7 Use of Immobilized Enzyme on SBA-15 in Hydrolysis of Tiger Grass

4.7.1 Evaluation of the Optimal pH in the Hydrolysis of Tiger Grass Using Immobilized Enzyme Comparing with Free Enzyme

Hydrolysis process is the step that changes cellulose to glucose using enzyme as biocatalyst. In this work, comparison of the immobilized enzyme and the free enzyme is studied to observe the efficiency of the immobilized enzyme. Again, the immobilized enzyme is prepared from 0.7g of SBA-15 with 0.5 ml of *Trichoderma ressei* enzyme by impregnation. Tiger grass (1g) after treated in the acid pretreatment is added into 2 different flasks containing 0.4033g of immobilized enzyme and 160 µL of free enzyme before placing them in an incabator for 4 days at 60 °C, and the results are illustrated in Figure 4.19.

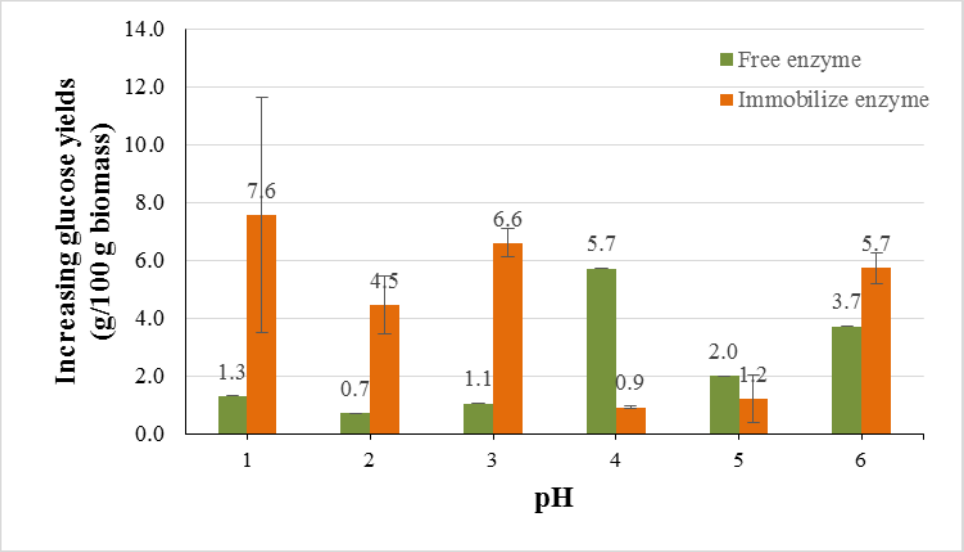

Figure 4.19 Influence of pH using immobilized (blue) and free (red) enzyme on glucose yield.

From the results, the immobilized enzyme seems to give higher efficiency than the free enzyme at all pH values, except pH = 5 which is consistent with our previous study finding that the optimal pH of *Trichoderma ressei* is the 4.8 (Komolwaich *et al*., 2016). At lower and higher pHs, the free enzyme can denature while the immobilized enzyme is stable in this studied pH range. If the concentration of hydronium or hydroxide ions is too high, it will disrupt the hydrogen bonds of the enzyme structure, thus, the enzyme can no longer be active toward the cellulose, resulting in low hydrolysis activity (Bey *et al*., 2016). The immobilized enzyme system can suffer higher concentrations of hydronium or hydroxide ions, as compared to the free enzyme, due to the presence of only the carboxylate groups which can be protonated.

4.7.2 Evaluation of the Optimal Temperature in the Hydrolysis of Tiger Grass using Immobilized Enzyme Comparing with Free Enzyme

In this work, various temperatures (30°, 40°, 50°, and 60 °C) were studied using the immobilized enzyme, comparing with the free enzyme, in the hydrolysis step for converting cellulose in Tiger grass to glucose, using the following

conditions: the immobilized enzyme prepared from 0.7g of SBA-15 with 0.5 ml of *Trichoderma ressei* enzyme by impregnation, 160 µL of the free enzyme, 4 days incabation at various tempertures. The results illustrating in Figure 4.20 show the glucose yield versus the reaction temperature.

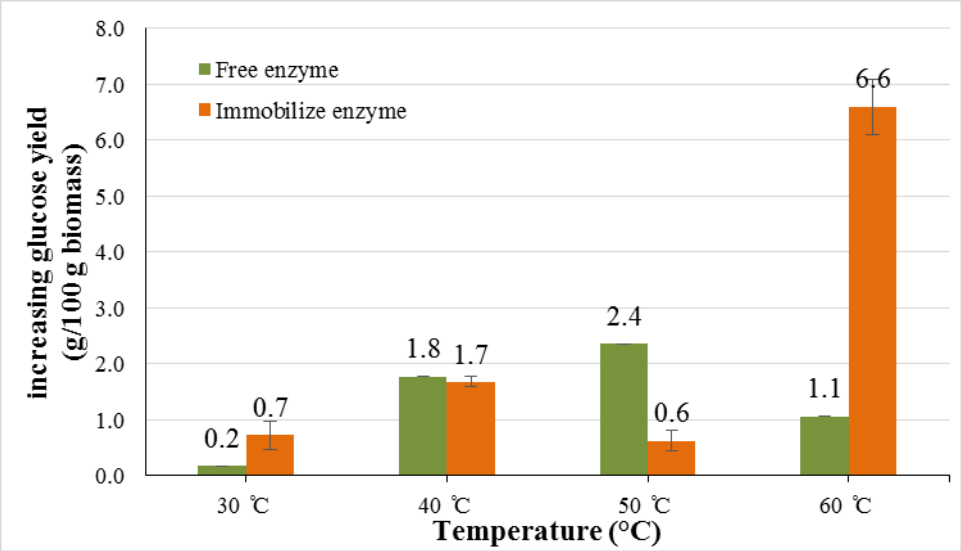

Figure 4.20 Influence of temperature using immobilized (blue) and free (red) enzyme on increasing glucose yield.

At the temperature of 30 °C, the immobilized enzyme has similar increasing glucose yield to the free enzyme. At the temperature of 40°, 50 °C, the free enzyme can produce the higher glucose yield than the immobilized enzyme. However, at 60 °C, the immobilized enzyme produces much higher amount of the glucose than the free enzyme. From the study of Lupoi and coworkers, they obtained the optimal hydrolysis temperature using the free cellulase at 50 °C (Lupoi and Smith, 2011) whereas Komolwaich and coworkers obtained the optimal hydrolysis temperature using the free cellulase at 45 °C (Komolwaich *et al*., 2016). Therefore, *Trichoderma ressei* immobilized on SBA-15 gave the highest glucose at the higher temperature of 60 °C. The immobilized enzyme was also reported to shift the optimal temperature as well as enable overall activity retention over a broader range of working temperature due to the higher thermal stability by modification of the

enzyme structure that resistance to the denaturation under extreme condition (Wong *et al*., 2017).

4.7.3 Effect of the Immobilized Enzyme Content on the Glucose Yield

In this work, various amounts of the immobilized enzyme (0.4033, 0.8066, 1.2099, 1.6132 and 2.0165 g), using 1 g of Tiger grass, were studied in the hydrolysis step for converting cellulose to glucose by incabating for 4 days at 60 ºC. The results illustrating in Figure 4.21 and showing the glucose yield indicate that, as expected, the higher amount of the immobilized enzyme provides the higher glucose yield.

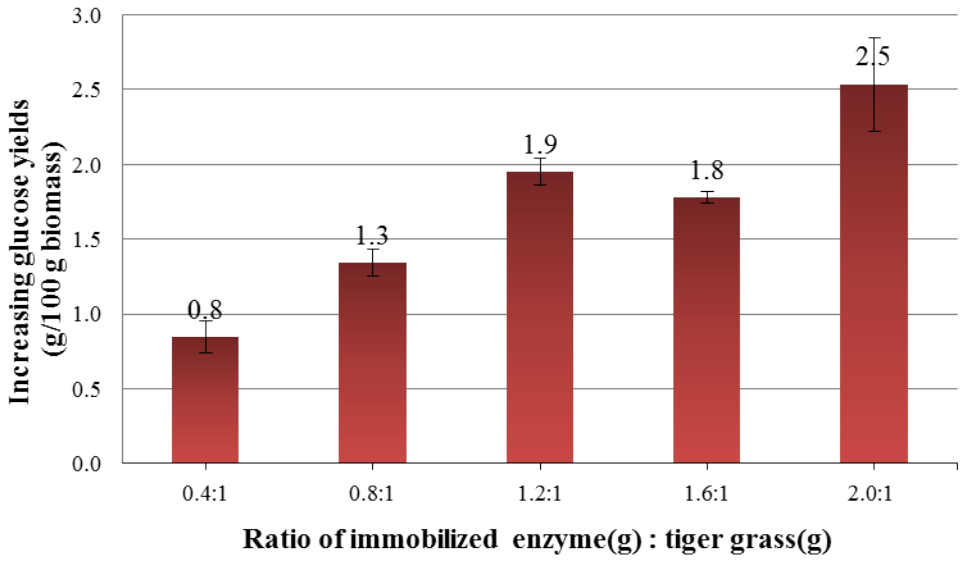

Figure 4.21 Influence of the immobilized enzyme content on the glucose yield in the hydrolysis process.

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Trichoderma ressei immobilized on different supports, MCM-48 ,TUD-1 and SBA-15, was prepared by impregnation method. The leaching test was observed to determine % adsorption, and SBA-15 with the highest pore size resulted in the highest % adsorption, causing *Trichoderma ressei* to be able to incorporate in the pore. The content of 0.7g of SBA-15 in 0.5 ml of *T. ressei* provided 100% adsorption, giving 100% immobilized enzyme.

The immobilized *T.ressei* on SBA-15 in the hydrolysis step at pH conditions of 1-3 and 4 provided the higher enzyme activity than that using the free enzyme. Immobilization prevents the heat inactivation of the cellulase at high temperatures. The optimal conditions for using the immobilized enzyme in hydrolysis are follows; pH=3, temperature=60 °C, and 2.1065 g of the immobilized enzyme.

5.2 Recommendations

1. Study of the immobilized enzyme in recycling for reducing the cost of the hydrolysis process.

2. The enzymatic activity method of the immobilized enzyme should also be studied to ensure the efficiency of the enzyme activity.

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APPENDIX

Appendix A Calibration Curve Protein Standard

Protein assay is used to characterize protein concentration by mixing sample with bradford reagent and using a spectrophotometer to measure the absorbance. Five standard points (0.1, 0.2, 0.3, 0.4, 0.5 ml of enzyme) were measured. The absorbance of each concentration shows 0.56, 0.72, 0.81, 0.92 and 1.00. The calibration curve of the standard protein is shown in Figure A.1. This graph is used to calculate the concentration of adsorb protein

Enzyme (ml)	Absorbance	SD
0.1	0.6	0.0
0.2	0.7	0.0
0.3	0.8	0.0
0.4	0.9	0.1
0.5	1.0	0.0

Table A1 The value of Absorbance and Enzyme concentration

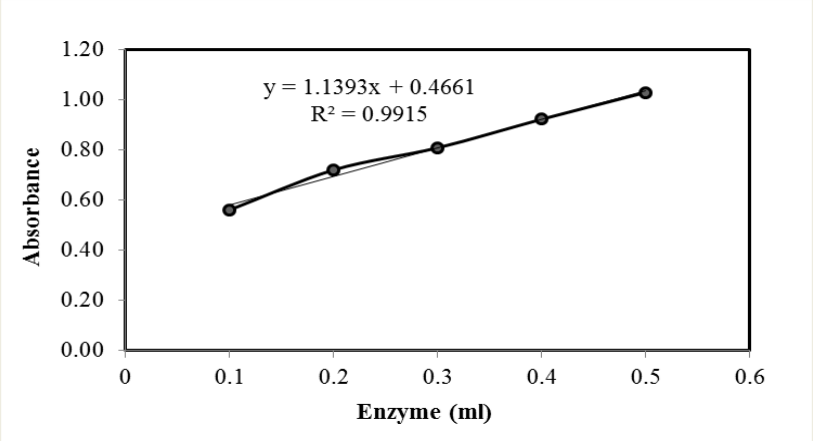

Figure A.1 Calibration curve of the protein standard.

Table A.2 Effects of various supports and content on % adsorption of immobilized *Trichoderma ressei* on the supports

Support(g): *T.ressei*(0.5ml)	Absorbance						Abs total	Enzyme (ml)	% Non-adsorption	% Adsorption	SD
	1	**2**	**3**	**4**	**5**	**6**					
TUD-1 0.1g	1.0	1.1	1.1	0.0	0.0	0.0	1.1	4.4	87.6	12.4	0.6
TUD-1 0.3g	1.0	1.1	1.1	0.0	0.0	0.0	1.1	4.4	88.3	11.7	0.6
TUD-1 0.5g	1.0	1.1	1.1	1.0	1.0	1.1	1.0	4.3	85.1	14.9	0.0
MCM-48 0.1g	1.1	1.1	1.2	0.0	0.0	0.0	1.1	5.0	99.1	0.9	0.0
MCM-48 0.3g	1.1	1.2	1.2	0.0	0.0	0.0	1.1	5.0	99.1	0.9	0.0
MCM-48 0.5g	1.2	1.2	1.2	0.0	0.0	0.0	1.2	5.3	100.0	0.0	0.0
SBA-15 0.1g	1.0	1.1	1.1	1.0	1.1	1.1	1.1	4.4	87.6	12.4	0.0
SBA-15 0.3g	0.8	0.9	0.9	0.9	0.9	0.9	0.9	3.2	64.0	36.0	0.0
SBA-15 0.5g	0.6	0.7	0.7	0.6	0.7	0.7	0.7	1.8	36.1	63.9	0.0
SBA-15 0.7g	0.1	0.1	0.1	0.0	0.0	0.0	0.1	-2.0	0.0	100.0	0.1
SBA-15 0.9g	0.2	0.2	0.2	0.0	0.0	0.0	0.2	-1.5	0.0	100.0	0.1

Table A.3 Effect of pH on % adsorption of immobilized *Trichoderma ressei* on the SBA-15 support

pH of Buffer	Absorbance						Abs total	Enzyme (ml)	% Non-adsorption	% Adsorption	SD
	1	2	3	4	5	6					
ENZ 0.5 ml	0.9	1.0	1.0	1.0	1.0	0.0	1.0	0.5	-	-	-
1.00	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.5	0.0	100.0	0.0
2.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.5	0.0	100.0	0.0
3.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.5	0.0	100.0	0.0
4.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.5	0.0	100.0	0.0
5.00	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-0.4	0.0	100.0	0.0
6.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.4	0.0	100.0	0.0
7.00	0.6	0.7	0.7	0.5	0.6	0.6	0.6	0.1	13.9	86.1	0.0
8.00	0.7	0.8	0.8	0.8	0.9	0.9	0.9	0.4	71.3	28.7	0.0

Table A.4 Effect of temperature on % adsorption of immobilized *Trichoderma ressei* on the SBA-15 support

Temperature	Absorbance						Abs total	Enzyme (ml)	% Non-adsorption	% Adsorption	SD
	1	**2**	**3**	**4**	**5**	**6**					
ENZ 0.5 ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	-	-	0.0
40℃	0.0	0.0	0.0	0.1	0.1	0.1	0.1	-0.4	0.0	100.0	0.0
60℃	0.0	0.0	0.1	0.1	0.1	0.0	0.0	-0.4	0.0	100.0	0.0
Room temperature	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-0.3	0.0	100.0	0.0

Table A.5 Effect of time on % adsorption of immobilized *Trichoderma ressei* on the SBA-15 support

pH of Buffer	Absorbance						Abs total	Enzyme (ml)	% Non-adsorption	% Adsorption	SD
	1	2	3	4	5	6					
Day 1	0.5	0.5	0.6	0.6	0.6	0.6	0.6	0.1	17.4	82.6	0.0
Day 2	0.2	0.2	0.2	0.6	0.6	0.6	0.6	0.1	29.0	71.0	0.0
Day 3	0.6	0.6	0.6	0.1	0.1	0.1	0.6	0.1	25.1	74.9	0.0
Day 4	0.3	0.3	0.3	0.3	0.4	0.4	0.4	-0.1	0.0	100.0	0.0
Day 5	0.2	0.2	0.2	0.4	0.4	0.4	0.4	-0.1	0.0	100.0	0.0
ENZ 0.5 ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	98.1	1.9	0.0

Table B.1 Effect of pH in hydrolysis by using immobilized *Trichoderma ressei* on the SBA-15 support compare with *Trichoderma ressei* solution

Sample Name	pH	Glucose	Xylose	Arabinose	monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	SD
Before free enzyme	1	14.1	2.6	0.0	8.5	0.2	0.0
After free enzyme	1	14.8	3.3	0.0	8.7		
Before Immo enzyme No1	1	9.8	0.9	0.0	7.9	0.5	0.7
After Immo enzyme No1	1	14.2	1.2	0.0	8.4		
Before Immo enzyme No2	1	14.5	1.4	0.0	6.0	0.9	0.0
After Immo enzyme No2	1	20.2	6.1	0.0	6.9		
Before free enzyme	2	7.9	0.9	0.0	14.4	1.8	0.0
After free enzyme	2	8.7	0.9	0.0	16.1		
Before Immo enzyme	2	13.6	0.7	0.0	13.6	1.6	1.7
After Immo enzyme No1	2	16.7	1.4	0.0	15.2		
Before Immo enzyme No2	2	12.9	0.6	0.0	15.4	1.7	0.0
After Immo enzyme No2	2	16.8	1.9	0.0	17.1		
Before free enzyme	3	14.7	1.6	0.0	11.0	2.4	0.0
After free enzyme	3	15.3	2.1	0.0	13.3		

Sample Name	pH	Glucose	Xylose	Arabinose	monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	SD
Before Immo enzyme No1	3	15.2	1.5	0.0	16.3	0.8	0.6
After Immo enzyme No1	3	19.7	3.2	0.0	17.0		
Before Immo enzyme No2	3	14.2	1.5	0.0	6.9	0.5	0.0
After Immo enzyme No2	3	20.0	2.8	0.0	7.4		
Before free enzyme	4	13.0	1.1	0.0	16.3	1.1	0.0
After free enzyme	4	18.0	1.8	0.0	17.4		
Before Immo enzyme No1	4	8.7	0.8	0.0	16.6	6.3	6.6
After Immo enzyme No1	4	9.1	1.3	0.0	22.9		
Before Immo enzyme No2	4	14.1	1.5	0.0	15.8	7.0	0.0
After Immo enzyme No2	4	14.7	1.9	0.0	22.7		
Before free enzyme	5	11.5	1.1	0.0	8.5	0.2	0.0
After free enzyme	5	13.0	1.6	0.0	8.7		
Before Immo enzyme No1	5	7.3	0.8	0.0	7.9	0.5	0.7
After Immo enzyme No1	5	7.8	0.9	0.0	8.4		
Before Immo enzyme No2	5	14.5	1.9	0.0	6.0	0.9	0.0
After Immo enzyme No2	5	16.3	2.0	0.0	6.9		
Before free enzyme	6	12.0	1.2	0.0	14.4	1.8	0.0
After free enzyme	6	15.1	1.9	0.0	16.1		
Before Immo enzyme No1	6	13.4	0.6	0.0	13.6	1.6	1.7
After Immo enzyme No1	6	17.7	1.7	0.0	15.2		
Before Immo enzyme No2	6	12.9	1.3	0.0	15.4	1.7	0.0
After Immo enzyme No2	6	17.4	2.9	0.0	17.1		

Table B.2 Effect of temperature in hydrolysis by using immobilized *Trichoderma ressei* on the SBA-15 support compare with *Trichoderma ressei* solution

Sample Name	Temperature	Glucose	Xylose	Arabinose	Monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	SD
Before free enzyme	30℃	7.6	0.9	0.0	8.5	0.2	0.0
After free enzyme	30℃	7.8	0.9	0.0	8.7		
Before Immo enzyme No1	30℃	7.0	0.9	0.0	7.9	0.5	0.3
After Immo enzyme No1	30℃	7.4	0.4	0.6	8.4		
Before Immo enzyme No2	30℃	5.2	0.8	0.0	6.0	0.9	0.6
After Immo enzyme No2	30℃	6.0	0.3	0.5	6.9		
Before free enzyme	40℃	13.1	1.2	0.0	14.4	1.8	0.0
After free enzyme	40℃	14.8	1.4	0.0	16.1		
Before Immo enzyme No1	40℃	12.7	0.9	0.0	13.6	1.6	0.1

Sample Name	Temperature	Glucose	Xylose	Arabinose	Monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	SD
After Immo enzyme No1	40℃	13.2	1.9	0.0	15.2		
Before Immo enzyme No2	40℃	11.4	3.8	0.0	15.4	1.7	0.0
After Immo enzyme No2	40℃	11.9	3.5	0.0	17.1		
Before free enzyme	50℃	9.9	1.0	0.0	11.0	2.4	0.0
After free enzyme	50℃	11.6	1.0	0.8	13.3		
Before Immo enzyme No1	50℃	13.5	2.7	0.0	16.3	0.8	0.2
After Immo enzyme No1	50℃	14.5	2.5	0.0	17.0		
Before Immo enzyme No2	50℃	5.9	1.0	0.0	6.9	0.5	0.0
After Immo enzyme No2	50℃	6.6	0.9	0.0	7.4		
Before free enzyme	60℃	14.7	1.6	0.0	16.3	1.1	0.0
After free enzyme	60℃	15.3	2.1	0.0	17.4		
Before Immo enzyme No1	60℃	15.2	1.5	0.0	16.6	6.3	0.5

Sample Name	Temperature	Glucose	Xylose	Arabinose	Monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	SD
After Immo enzyme No1	60℃	19.7	3.2	0.0	22.9		
Before Immo enzyme No2	60℃	14.2	1.5	0.0	15.8	7.0	0.0
After Immo enzyme No2	60℃	20.0	2.8	0.0	22.7		

Table B.3 Effect of ratio (SBA-15:1gTiger grass) in hydrolysis by using immobilized *Trichoderma ressei* on the SBA-15 support compare with *Trichoderma ressei* solution

Ratio (SBA-15 (g) : Tiger Grass (g)	Glucose	Xylose	Arabinose	Total monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	S.D.
Before 0.4g:1g	12.9	1.8	0.0	14.7	0.9	0.1
0.4g:1g	13.5	2.1	0.0	15.5		
Before 0.4g:1g	14.1	2.2	0.0	16.3	0.9	
0.4g:1g	14.8	2.4	0.0	17.2		
Before 0.4g:1g	11.8	1.7	0.0	13.5	0.7	
0.4g:1g	12.3	1.9	0.0	14.2		
Before 0.8g:1g	7.0	0.9	0.0	7.9	1.3	0.1
0.8g:1g	8.0	0.4	0.8	9.2		
Before 0.8g:1g	13.7	1.7	0.0	15.4	3.0	
0.8g:1g	16.1	2.3	0.0	18.4		
Before 0.8g:1g	5.6	0.3	0.6	6.5	1.4	
0.8g:1g	6.6	0.4	0.8	7.9		
Before 1.2g:1g	11.7	1.3	0.0	13.0	2.0	0.1
1.2g:1g	13.2	0.8	1.0	15.0		

Ratio (SBA-15 (g) : Tiger Grass (g)	Glucose	Xylose	Arabinose	Total monomeric sugar yields (g/100 g biomass)	Increasing glucose yields (g/100 g biomass)	S.D.
Before 1.2g:1g	7.3	5.9	0.0	13.2	0.9	
1.2g:1g	7.9	6.2	0.0	14.1		
Before 1.2g:1g	13.8	1.6	0.0	15.5	1.9	
1.2g:1g	15.3	2.1	0.0	17.4		
Before 1.6g:1g	9.8	1.1	0.0	10.9	1.8	0.0
1.6g:1g	11.1	0.6	1.0	12.7		
Before 1.6g:1g	9.0	1.1	0.0	10.1	1.8	
1.6g:1g	10.2	0.6	1.1	11.9		
Before 1.6g:1g	14.6	1.7	0.0	16.3	4.2	
1.6g:1g	18.0	2.6	0.0	20.5		
Before 2.0g:1g	7.1	6.2	0.0	13.3	2.2	0.3
2.0g:1g	8.4	7.1	0.0	15.5		
Before 2.0g:1g	14.5	2.2	0.0	16.7	2.8	
2.0g:1g	16.7	2.8	0.0	19.5		
Before 2.0g:1g	14.0	2.1	0.0	16.1	2.7	

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