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EFFECT OF DIFFERENT COUPLING AGENTS IN COVALENT ENZYME IMMOBILIZATION ON KENAF MICRO FIBRE

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By

NG LIN CIEH

Thesis Submitted to the school of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

January 2018

DEDICATION

This thesis is dedicated to

My family members who are always there for me and giving me their company. And of course, my beloved parents who are always showing limitless support along my studies. Thank you for your love, concern and support.

My respectable supervisor, seniors, fellow colleagues and all my dear friends.

Thank you for your concern, support and guidance

Abstract of thesis presented to the Senate of Universiti Putra Malaysian fulfilment of the requirement for the degree of Master of Science

EFFECT OF DIFFERENT COUPLING AGENTS IN COVALENT ENZYME IMMOBILIZATION ON KENAF MICRO FIBRE

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January 2018

Chairman : Associate Professor Mohd Noriznan Mokhtar, PhD
Faculty : Engineering

Enzyme immobilization by covalent binding is a technique that localizes the enzymes on a support material through the formation of covalent bonds, with retained catalytic activity. Covalent immobilization is popular for minimizing leaching of the immobilized enzymes. Therefore, using covalently immobilized enzymes enables repeated uses of the biocatalyst. It also allows easier separation between the products and the immobilized enzymes. However, many conventional support matrixes used for enzyme immobilization requires high cost. This causes the use of immobilized enzymes in industries to be less preferable. To solve this problem, researches are needed to find out alternative support materials which are more economical for industrial applications. In order to ensure the optimum performance of the immobilized enzymes in industrial operations, it is also required to study the effect of coupling agents (spacer arms and ligands) on the properties of the immobilized enzymes. Hence the objectives in this research are to study the potential of bleached kenaf bast micro fibre as the support matrix for covalent immobilization of cyclodextrin glucanotransferase (CGTase) and also to investigate the effect of different spacer arms and ligands on the properties of the immobilized CGTase. In this study, raw kenaf bast fibre was firstly bleached. After that, CGTase from *Bacillus macerans* was immobilized on the bleached kenaf bast micro fibre with the use of different coupling agents. Hexamethylenediamine, HMDA and Ethylenediamine, EDA were used as the spacer arms, while glutaraldehyde, GA and o-phthalaldehyde, OPA were used as the ligands. This is followed by determination of the immobilized CGTases properties such as storage stability and reusability. From the results, when 55.6 U/mL of free CGTase was initially added during immobilization, the recovered activity of immobilized CGTases are in the range of 0.16 to 0.24 U/(mg fibre). Besides, a shift in optimum temperature was also detected from 60^{o}C (free CGTase) to 70^{o}C (immobilized CGTases). This indicates that the thermal stability for the immobilized CGTases are higher when compared to free CGTase. For storage stability at 60^{o}C, CGTase immobilized with ethylenediamine and o-phthalaldehyde, has retained 60% of its initial activity after 15 days of storage. This highest stability was confirmed by its lowest deactivation constant, k_d (0.0361 day^{-1}). However for reusability, CGTase

immobilized using ethylenediamine and glutaraldehyde retains the highest residual activity (72.72%) after 12 cycles of batch reaction. From this study, the potential of bleached kenaf bast micro fibre has been confirmed since it can enhance the performance of all the immobilized CGTase, regardless of the coupling agents used. In addition, the present study has also proven the importance of selecting suitable coupling agents as they have different effect on the properties of the immobilized enzymes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN AGEN PERHUBUNGAN YANG BERLAINAN DALAM IMOBILISASI ENZIM KE ATAS MIKRO-SERABUT KENAF MELALUI PEMBENTUKAN IKATAN KOVALEN

Oleh

NG LIN CIEH

Januari 2018

Pengerusi : Profesor Madya Mohd Noriznan Mokhtar, PhD
Fakulti : Kejuruteraan

Imobilisasi enzim secara kovalen merupakan satu teknik memegunkan enzim ke atas struktur sokongan dengan pembentukan ikatan kovalen, di mana aktiviti pemangkinan enzim akan dikekalkan. Teknik ini adalah terkenal kerana dapat meminimumkan pembebasan enzim terpegun daripada struktur sokongan. Ini membolehkan pengulangan penggunaan untuk enzim yang dipegunkan secara kovalen. Pengasingan produk terhasil daripada enzim terpegun juga akan menjadi lebih efektif. Walau bagaimanapun, kebanyakan struktur sokongnan lazim yang digunakan dalam pemegunan enzim memerlukan kos yang tinggi. Ini menyebabkan enzim terpegun kurang terpilih untuk penggunaan dalam industri. Justeru, kajian untuk mendapatkan struktur sokongan alternatif yang lebih ekonomikal untuk aplikasi dalam industri memang diperlukan. Untuk memastikan enzim terpegun dapat berfungsi secara optimum dalam operasi industri, kajian mengenai kesan agen penghubungan terhadap sifat enzim terpegun juga perlu dilaksanakan. Maka objektif dalam kajian ini adalah untuk mengkaji potensi mikro-serabut kenaf terluntur sebagai struktur sokongan kepada enzim siklodekstrin glukanotransferase (CGTase) yang terpegun secara kovalen dan juga menyiasat kesan pengikat dan ligan yang berlainan terhadap sifat enzim terpegun yang terhasil. Kajian ini dimulakan dengan pelunturan serabut kulit kenaf mentah untuk mendapatkan mikro-serabut terluntur. Kemudian, CGTase daripada *Bacillus macerans* dipegunkan di atas mikro-serabut tersebut dengan menggunakan agen penghubungan yang berlainan, iaitu heksametilenadiamina (HMDA) dan etilenadiamina (EDA) sebagai pengikat manakala glutaraldehid (GA) dan o-phthalaldehid (OPA) sebagai ligan. Ini diikuti dengan penentuan sifat-sifat CGTase terpegun seperti kestabilan simpanan dan kestabilan penggunaan semula. Hasil kajian menunjukkan bahawa apabila 55.6 U/ml CGTase larut ditambah semasa pemegunan, julat aktiviti pemulihan CGTase terpegun adalah di antara 0.16 dengan 0.24 U/(mg serabut). Selain itu, suhu optimum telah beralih daripada 60^{o}C (CGTase larut) kepada 70^{o}C (CGTase terpegun). Ini menunjukkan bahawa kestabilan termal CGTase terpegun adalah lebih tinggi. Dari segi kestabilan simpanan, CGTase yang dipegunkan dengan etilenadiamina dan phthalaldehid telah mengekalkan 60% daripada aktiviti asal selepas disimpan selama 15 hari pada suhu 60^{o}C. Kestabilan

simpanan yang paling baik ini telah dikenal pasti oleh nilai pemalar penyahaktifannya, k_d (0.0361 hari^{-1}) yang paling rendah. Untuk keputusan kestabilan penggunaan semula, CGTase dipegunkan dengan etilenadiamina dan glutaraldehid telah mengekalkan aktiviti enzim yang paling tinggi (72.72%) selepas 12 kitaran tindak balas. Daripada hasil kajian ini, potensi mikro-serabut kenaf terluntur sebagai struktur sokongan untuk pemegunan enzim secara kovalen telah terbukti. Ini adalah kerana prestasi setiap enzim terpegun telah ditingkatkan, walaupun agen penghubungan yang digunakan adalah berbeza. Selain itu, hasil kajian ini juga telah mengesahkan bahawa pemilihan agen penghubungan yang sesuai adalah penting. Ini adalah kerana agen penghubungan yang berlainan akan membawa kesan yang berbeza kepada sifat enzim terpegun yang terhasil.

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I certify that a Thesis Examination Committee has met on 4 January 2018 to conduct the final examination of Ng Lin Cieh on his thesis entitled "Effect of Different Coupling Agents in Covalent Enzyme Immobilization on Kenaf Micro Fibre" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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^{o}C	Degree Celsius
α-CD	α-cyclodextrin
β-CD	β-cyclodextrin
γ-CD	γ-cyclodextrin
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CGTase	Cyclodextrin glucanotransferase
CH_3COOH	Acetic acid
-CHO	Aldehyde group
-CONH-	Amide bond
-COOH	Carboxyl
E_d	Deactivation energy
EDA	Ethylenediamine
(EDA-GA)-CGTase	Immobilized CGTase using EDA as spacer arm and GA as ligand
(EDA-OPA)-CGTase	Immobilized CGTase using EDA as spacer arm and OPA as ligand
GA	Glutaraldehyde
HMDA	Hexamethylenediamine
(HMDA-GA)-CGTase	Immobilized CGTase using HMDA as spacer arm and GA as ligand
(HMDA-OPA)-CGTase	Immobilized CGTase using HMDA as spacer arm and OPA as ligand
HPLC	High performance liquid chromatography
k_d	Deactivation constant
k_d	Initial deactivation constant
L-DOPA	L-3,4-dihydroxyphenylalanine

M	Molar, (mol/dm^3)
$NaClO_2$	Sodium chlorite
$-NH_2$	Amino
-OH	Hydroxyl
OPA	o-Phthalaldehyde
RI detector	Infrared detector
SEM	Scanning electron microscope
-SH	Sulphydryl
TLC	Thin layer chromatography
U	Enzyme activity, (µmol substrate/minute)
w/v	Weight/Volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

1.1.1 Enzyme

Enzyme is a protein that acts as a biocatalyst to boost the rate of reactions, which includes metabolic reactions and biochemical reactions in all the cells of living organisms (Shanmugam et. al. 2009). The enzymes are of utmost importance because many biochemical reactions will require much longer time to be completed without the enzymes. This may result in the demise of living organisms since several important biochemical processes required for living fail to finish on time (Shanmugam et. al. 2009). Generally, enzymes show specificity towards the reactions they catalyse. This means that certain types of enzyme only react with certain types of substrate to produce the product. The cause of this specificity is the presence of the amino acid residues with unique features and three dimensional arrangements which build up the active site of enzymes (Furlan and Pant, 2006). Besides, an enzyme is also known to be reusable once a previous reaction it catalysed was finished. This is because the three dimensional structure of the active site will still remain the same after the enzyme has catalysed a reaction. Due to these special characteristics, the usage of enzymes has been extended into industrial applications. This includes detergent industry, food and beverage industry, textile industry and paper making industry (Kirk et. al. 2002).

Although enzymes can simplify a lot of chemical reactions, several problems also arise when soluble enzymes are applied in industries. The main problem of using soluble enzyme is that it does not allow continuous operation. This is because after the reaction, the soluble enzyme will dissolve in the products and thus, causing contamination of the products (Homaei *et. al.*, 2013). In order to separate the enzymes from the products, the industries are obliged to incur high separation cost since the separation process is technically very difficult (Nguyen and Kim, 2017). Besides that, soluble enzymes normally have low stability such as storage stability and thermal stability (Kent 2000; Nguyen and Kim, 2017). This indicates that purchase of fresh enzymes needs to be carried out more frequently by the industries. Hence, it can be seen that using soluble enzymes will always require high cost (cost of separation, purchase of fresh enzymes) for the industries.

1.1.2 Enzyme Immobilization

Enzyme immobilization is defined as the confinement and restriction of enzymes in a specific space with retained catalytic activity (Brena and Batista-Viera, 2006). The main advantage of enzyme immobilization is that it enables repetitive uses of the enzymes so

that continuous operation in industries can be achieved (Rodrigues *et. al.*, 2013; Nguyen and Kim, 2017). Moreover, the immobilization will also improve the properties of the enzymes which include the thermal stability, pH stability and storage stability (Guzik *et. al.*, 2014). Therefore, immobilization can be known as an effective remedy to the problems faced by industries as shown in section 1.1.1.

Currently, there are several methods available for enzyme immobilization. These methods can be divided into 2 main categories which are physical method and chemical method. Physical method involves the use of physical forces such as hydrogen bonding, hydrophobic interactions and van der Waals forces to immobilize enzymes on the support matrix (Dwevedi, 2016). However in chemical method, the enzymes are immobilized onto different matrices by covalent or ionic bonds (Dwevedi, 2016). Typical examples in physical method include adsorption (reversible), microencapsulation (irreversible) and also entrapment (irreversible) (Dwevedi, 2016). For chemical method, the examples are covalent attachment (irreversible), crosslinking (irreversible) and ionic binding (reversible) (Dwevedi, 2016).

Among all the immobilization methods available up to date, covalent binding is one of the most popular methods in enzyme immobilization (Mohamad et. al., 2015). As the name suggests, covalent bonds are formed between the functional groups of the support and the functional groups of the enzymes during the immobilization. Usually, the functional groups on enzyme involved in covalent binding are unimportant for the catalytic activity of enzymes. This includes the amino group from lysine side chain, thiol group from cysteine side chain and carboxylic group, imidazole group and phenolic group from aspartic and glutamic acids side chain (Mohamad et. al., 2015). The main reason for covalent binding to be famous in enzyme immobilization is due to the advantages that it can provide. The prime advantage provided by covalent binding is the minimized leaching of immobilized enzymes from the support matrix. As a result, mixing of the enzymes with the products after the reaction will not occur. This improves the operational stability of the immobilized enzymes as well as prevents the requirement to separate the enzyme from the products in industrial application (Brena and Batista-Viera, 2006). In addition, covalent binding can also improve the thermal stability of the immobilized enzymes since during the immobilization, the 3D confirmation of the enzymes will be rigidified by the covalent bonds formed (Deng and Li, 2010).

At the present, the covalently immobilized enzymes have also been applied in continuous flow bioreactor in industries. This includes continuous stirred tank reactor, continuous packed bed reactor, continuous fluidized bed reactor and continuous membrane reactor. Among these reactors, packed bed column reactors are the most frequently used for immobilized enzymes (Illanes and Altamirano, 2008). In a packed bed reactor, the immobilized enzymes are packed in the column of the reactor system where the substrate will flow across the bed of immobilized enzymes for reactions (Zhang et. al., 2016). Recirculation of unreacted substrates is also performed to ensure higher percentage of yield. Besides packed bed reactors, the immobilized enzymes have also been applied in other bioreactor system such as in a continuous stirred tank reactor. In a continuous stirred tank reactor, the substrate and the immobilized enzymes are mixed well together in the tank where the reaction takes place (Novick and Rozzell, 2005). In order to retain the immobilized enzymes in the reactor, a filter is normally included at the exit. There is

another alternative way which is by connecting a long enough tube at the exit so that the gravity is able to retain the immobilized enzymes in the reactor (Novick and Rozzell, 2005). Due to the use of these bioreactors, several advantages have been brought to the industries which include increased production yield and reduction in wastage of enzymes.

1.1.3 Kenaf Fibre As Support Matrix

In enzyme immobilization, selecting suitable support matrix is also very important. This is because the properties of the immobilized enzymes are often affected by the support matrix selected (Brena and Batista-Viera, 2006). The characteristics considered when choosing a support matrix includes hydrophilicity, porosity, non-toxicity, biodegradability and available at low cost (Mohamad et. al., 2015; Brena and Batista-Viera, 2006).

Kenaf (*Hibiscus* cannabinus) is a type of plant which is widely cultivated in Malaysia (Chen and Liu, 2010). The fibres in kenaf can be divided into the bast fibre (40% of the plant) and also the core fibre (60%) of the plant (Raju *et. al.*, 2008). The kenaf bast fibre has a very good potential to be used as the support matrix for enzyme immobilization, especially after it is bleached. This is because in addition to those characteristics mentioned above, kenaf bast fibre also possesses several other attractive characteristics such as good mechanical properties (high tensile strength) (Salit 2014). Besides that, kenaf bast fibre is also well known for its good thermal properties (Alexopoulou *et. al.*, 2013). These two characteristics are important because a strong support matrix can increase the stability of the immobilized enzymes produced. Furthermore, kenaf bast fibre consists of hydroxyl (-OH) functional groups on the surface of its cellulose. After being activated, these hydroxyl (-OH) functional groups will act as the ideal sites for covalent binding with enzymes (Sulaiman *et. al.*, 2014). When kenaf bast fibre is bleached, more hydroxyl (-OH) functional groups on the surface of cellulose can be exposed since unwanted lignin and hemicellulose are removed. Thus, the intensive presence of these abundant hydroxyl (-OH) functional groups on the surface of the fibre will further increase the chances of covalent binding with the enzymes. Due to these special characteristics, the concept of applying bleached kenaf bast fibre as support matrix for enzyme immobilization seems very attractive and promising.

1.1.4 Selection of Coupling Agents

In covalent immobilization, another important consideration is the selection of suitable coupling agents. Numerous previous studies have shown that the use of different coupling agents can affect the properties of the immobilized enzymes particularly in the enzyme activity, stability and reusability (Rueda et. al., 2016; de Albuquerque et. al., 2016). One of the reasons that cause deviation in immobilized enzyme properties is due to the difference in microenvironment of support matrix created when different coupling agents are used. For example, when comparing glycidol and glutaraldehyde, the support matrix activated with epoxy group by using glycidol will acquire higher hydrophobicity. However for activation with aldehyde group by using glutaraldehyde, the activated support obtained will be more hydrophilic (Torres-Salas *et. al.*, 2011). Thus, this will

help in obtaining relatively higher activity recovery of the immobilized enzymes since the enzymes tend to denature at hydrophobic support surface due to dehydration (Sulaiman *et. al.*, 2014). Another reason which accounts for difference in properties of the immobilized enzymes is due to the unequal bond stability obtained when different coupling agents are used. For instance, when cyanogen bromide is used as the coupling agent, the stability of bond formed between the coupling agent and the enzyme is considered low (Zucca and Sanjust, 2014). However for activation with glutaraldehyde, the bond stability can be very high (Zucca and Sanjust, 2014). Due to the difference in bond stability, the degree of stabilization of enzyme structure will also be different. Hence, this will affect their properties such as thermal stability, since the immobilized enzyme properties are determined by the stability of their structure (Rodrigues *et. al.*, 2013).

In many cases, the length of coupling agents used is also a key factor which affects the immobilized enzyme properties. This is because it will affect the intensity of multipoint covalent attachment that occurs during the immobilization (dos Santos et. al., 2015; Barbosa et. al., 2013). When a smaller size coupling agent (such as a shorter spacer arm) is used, the immobilized enzyme can acquire higher rigidity through multipoint covalent attachment (dos Santos et. al., 2015). Conversely, the effect of rigidification through multipoint covalent attachment will be lower if a larger size coupling agent (such as a longer spacer arm) is used (dos Santos et. al., 2015). However in terms of reducing steric hindrance for the reaction between enzyme and support, using a larger size coupling agent will be more effective (Barbosa et. al., 2013). This is because it can create greater distance between the support and the enzyme when compared to using a smaller coupling agent. Thus, greater distance will prevent the support matrix from covering the active site of enzyme, which is crucial for enzymatic reaction (Barbosa et. al., 2013). As a result, it can be seen that coupling agents used for support activation will greatly influence the properties of the immobilized enzyme produced. Due to this, selection of suitable coupling agents is really important and should be carefully considered before performing enzyme immobilization.

1.2 Problem Statement

Enzyme immobilization is a good technique which enables maximal use of the enzyme. This is because it allows continuous operation and repeated use of the enzymes especially in industrial sector where the use of bioreactor is involved. However, a major challenge faced by the industries is the high cost involved in using the immobilized enzyme for industrial processes and operations. This is resulted from the reason that most of the conventional support matrixes such as synthetic polymers, silica based carriers, active membranes and acrylic resin are very costly (Mohamad *et. al.* 2015). Therefore, the cost required for using immobilized enzymes in industries is increased and because of this, use of immobilized enzymes is rarely preferred (Hubner *et. al.*, 2015).

To circumvent this problem, a useful solution is to search for an alternative and economical support matrix. The use of bleached kenaf bast micro fibre as the support matrix is highly recommended. This is because cellulose inside kenaf bast fibre has abundant amount of hydroxyl (-OH) groups on its surface (Sulaiman *et. al.*, 2014). When

kenaf bast fibre is bleached, a high amount of these surface hydroxyl (-OH) groups will be exposed due to removal of unwanted lignin and hemicellulose. The presence of these hydroxyl (-OH) groups will enable covalent binding of the coupling agents and the enzymes on the surface of the fibre (Sulaiman *et. al.*, 2014). Thus, this makes bleached kenaf bast micro fibre a readily available support matrix for covalent enzyme immobilization. Furthermore, kenaf bast fibre is also well known for its good mechanical properties and thermal stability (Alexopoulou *et. al.*, 2013; Salit, 2014). This is an added value because these properties can help increase the lifespan of the immobilized enzymes produced since industrial operations usually involve high operating temperature and pressures. Besides that, using bleached kenaf bast micro fibre will require a much lower cost when compared to those conventional support matrixes. This will solve the problem of high cost involved for using immobilized enzymes and thus, promote the use of immobilized enzymes in industrial application. Other advantages which help bleached kenaf bast micro fibre to be selected as support matrix include its biodegradability, easy availability and non-toxic (Sen and Reddy, 2011; Bharath *et. al.*, 2015).

When an enzyme is covalently immobilized on a new support, its properties may either be improved or deteriorated (Cao *et. al.*, 2005). In some cases, the stability of the immobilized enzymes will reduce after covalent immobilization since the three dimensional conformation of the enzyme has been altered (Sambamurthy and Kar, 2006). This also becomes an obstacle for the industrial application of the immobilized enzymes. Therefore to solve this problem, there is a need to investigate the effect of coupling agents on the properties of the immobilized enzymes which are covalently bound to the bleached kenaf bast micro fibre. As shown in section 1.1.4, use of different coupling agents will significantly affect the properties of the immobilized enzymes due to difference in microenvironment of the support matrix, difference in stability and also the distance of bond between the support and the enzyme, which can affect the steric hindrance. When the effect of coupling agents is investigated, the properties of the covalently immobilized enzymes on bleached kenaf bast micro fibre will be optimized. This will then prevent deterioration of the properties of the enzymes after covalent immobilization and ensure their optimum performance when they are applied in industrial operations.
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Hence in this study, bleached kenaf bast micro fibre is adopted as the support matrix for covalent immobilization of CGTase due to its excellent characteristics, as mentioned previously. The effect of coupling agents is also investigated in this study since there is still insufficient information regarding how different coupling agents can affect the properties of the enzymes immobilized on bleached kenaf bast micro fibre. The strategy of selecting the coupling agents is to fulfil the purpose of covalently immobilize CGTase through the amine group ($-NH_2$) from lysine residue on the enzyme surface. The reason for binding through ($-NH_2$) group from lysine is due to its easy availability on the surface of enzyme molecules (Zucca and Sanjust, 2014). Besides, the ($-NH_2$) group has high reactivity for immobilization even though it does not affect the enzyme activity (Zucca and Sanjust, 2014). Hence, this will allow optimum activity retention of the immobilized enzyme.

To enable covalent binding with ($-NH_2$) group from lysine on enzyme surface, glutaraldehyde and o-phthalaldehyde (both ligands contain 2 aldehyde functional group)

have been selected as the ligands in this study. This is because the aldehyde groups on these ligands are very reactive towards the (-NH_2) group from lysine and thus, these ligands can react readily with the enzymes with high stability of the covalent bonds formed (Zucca and Sanjust, 2014; Sulaiman *et. al.*, 2014). As for the spacer arms used in this study, hexamethylenediamine and ethylenediamine were selected. This is because hexamethylenediamine and ethylenediamine can form covalent bonds with aldehyde group (Portaccio *et. al.*, 2007) from the ligand and also the surface hydroxyl (-OH) (from carboxyl group) which is present on the fibre after bleaching (Gabrovska *et. al.*, 2008; Abdel-Halim, 2012). Therefore, it means that the spacer arms can act as a bridge to connect the fibre to the ligand and enzyme firmly after the formation of covalent bonds. Thus by using the chosen spacer arms and ligands, the enzymes will be immobilized to the fibre through the (-NH_2) group of lysine.

1.3 Objective Of Study

i) To study the potential of bleached kenaf microfibre as support matrix for CGTase immobilization via covalent binding
ii) To investigate the effect of different spacer arms and ligands on the properties of the immobilized CGTase

1.4 Scope Of Study

This research focuses on 2 parts which are the investigation on the potential of bleached kenaf microfibre as support matrix for CGTase immobilization via covalent binding and also the effect of different spacer arms and ligands on the performance of the immobilized CGTase. To perform these investigations, the experiments consisted of,

1. Preparation of bleached microfibre from raw kenaf fibre by acidified sodium chlorite bleaching.
2. Surface modification of the support matrix by covalent coupling with different spacer arms and ligands, which was followed by CGTase immobilization.
3. Study and comparison on the properties of the immobilized CGTases with different spacer arms and ligands. This includes storage stability, thermal stability and reusability.

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