Tri-Enzyme Immobilized on Magnetized Multiwall Carbon Nanotubes for Single Pot Lignocellulosic Biomass Hydrolysis

by

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LIST OF ABBREVIATIONS

abs	absorbance
ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
APTS	(3-Aminopropyl)triethoxysilane
BMSW	Biogenic Municipal Solid Waste
Br	Bromine
C	Carbon
CBM	Carbohydrate-Binding Molecules
CLEAs	Cross-Linked Enzyme Aggregates
CMC	Carboxymethyl Cellulose
C≡N	Nitrile group
C=O	Carbonyl group
CO-NH	Peptide linkage
COOH	Carboxylic acid
DI	Deionize Water
dil	Dilution
DNS	3,5-Dinitrosalicylic acid
DWCNTs	Double-wall Carbon Nanotubes
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDX	Energy Dispersive X-ray
Fe	Iron
FPase	Filter-Paper cellulase
Fe_3O_4	Iron oxide
FTIR	Fourier Transform Infrared
H	Hydrogen
HCL	Hydrochloric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen Peroxide
H_3PO_4	Phosphoric acid
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulphuric acid
IOMNP	Iron Oxide Magnetic Nanoparticles
IR	Infrared Radiation

KBr	Potassium Bromide
$KMnO_4$	Potassium permanganate
MCM	Mesoporous Material
m-MWCNTs	Magnetized Multiwall Carbon Nanotubes
MNPs	Magnetic Nanoparticles
MWCNTs	Multiwall Carbon Nanotubes
N	Nitrogen
Na	Sodium
NHS	N-hydroxysuccinimide
NPs	Nanoparticles
O	Oxygen
OFAT	One-Factor-At-A-Time
OH	Hydroxide
O-MWCNTs	Oxidized Multiwall Carbon Nanotubes
pH	Potential of hydrogen
PMMA	Poly(methyl methacrylate)
p-MWCNTs	Pristine Multiwall Carbon Nanotubes
P_2O_5	Phosphorus Pentoxide
PPA	Polyphosphoric acid
PS	Paddy Straw
PVA	Polyvinyl Alcohol
SEM	Scanning Electron Microscopy
SF	Submerged Fermentation
SFF	Solid-State Fermentation
Si	Silicon
sp.	Species
SWCNTs	Single-Wall Carbon Nanotubes
UHR-SEM	Ultra-High Resolution Scanning Electron Microscope
UV-Vis	Ultraviolet–Visible
w/v	Weight over volume

LIST OF SYMBOLS

β	Beta
°C	Degree celcius
cm	Centimetre
g	Gram
h	Hour
IU	International unit
kHz	One thousand hertz
m	Metre
mg	Milligram
min	Minutes
mL	Milliliter
mm	millimeter
nm	Nanometer
rpm	Revolutions per minutes
USD	United States Dollar
µm	Micromolar
µmol	Micromole
%	Percentage

Tri-Enzim Tidak Bergerak pada Nanotiub Karbon Berbilang Dinding Bermagnetuntuk Hidrolisis Biojisim Lignoselulosa Periuk Tunggal

ABSTRAK

Periuk tunggal ialah idea inovatif dengan menggabungkan pelbagai enzim dalam satu proses untuk hidrolisis biojisim lignoselulosa. Enzim asli mempunyai beberapa kelemahan dalam persekitaran industri, termasuk aktiviti enzim yang rendah, kestabilan yang lemah, sifat tidak ekonomik, dan pemisahan yang mencabar daripada produk akhir. Kajian baru-baru ini menangani imobilisasi bersama xilanase, laccase dan selulase pada nanotiub karbon berbilang dinding bermagnet yang telah disintesis dan difungsikan menggunakan kaedah mesra alam yang menggabungkan sintesis berasaskan air difungsikan dengan pengoksidaan asid ringan karbon berbilang dinding murni tiub nano. Enzim yang tersekat gerak telah dicirikan dari segi kestabilan dan kebolehgunaan semula sebelum penubuhan sistem periuk tunggal untuk hidrolisis jerami padi kepada gula penurun (glukosa dan xilosa). Melalui rawatan asid ringan, 8 M asid, 8 jam, dan 80 ℃ masa dan suhu refluks telah diperiksa sebagai keadaan kerja yang ideal. Penubuhan sistem berasaskan air dalam sintesis oksida besi pada f-MWCNTs telah disahkan oleh spektroskopi sinar-X (EDX) penyebaran tenaga, dengan 10.49% besi dikesan pada permukaan MWCNTs. Imobilisasi enzim pada m-MWCNTs berjaya dicapai melalui kaedah penjerapan dengan kecekapan pengikatan > 95% untuk semua enzim, yang selanjutnya disahkan dengan bantuan puncak spektroskopi inframerah (FTIR) transformasi, mengimbas imej mikroskop elektron (SEM), dan analisis EDX. Kepekatan enzim optimum untuk imobilisasi direkodkan pada 5 mg/mL untuk kedua-dua selulase dan xilanase dan 7 mg/mL untuk laccase. Berdasarkan kajian kestabilan, suhu optimum selulase tidak bergerak, laccase, dan xilanase masing-masing sepadan dengan 50, 60, dan 70 ℃. pH optimum 5 direkodkan dengan selulase tidak bergerak dan laccase, manakala pH 6 direkodkan dengan xilanase tidak bergerak. Kajian kebolehgunaan semula pada substrat model menunjukkan semua enzim tidak bergerak mengekalkan lebih daripada 50% aktiviti relatif selepas lima kitaran analisis, manakala sehingga 30% aktiviti relatif masih dikekalkan dengan selulase tidak bergerak dan xilanase yang tertakluk kepada hidrolisis jerami padi. Pengesanan puncak FTIR pada 1657 cm^{-1} menggunakan jerami padi yang dirawat dengan laccase tidak bergerak pada m-MWCNTs menunjukkan delignifikasi berjaya. Bagi sistem periuk tunggal, operasi telah dijalankan pada suhu 50 ℃, pH 5, dan 100 rpm selama 16 jam menggunakan jerami padi yang tidak dirawat dan diberi asid. Biojisim yang tidak dirawat menunjukkan kandungan glukosa dan xilosa yang lebih tinggi semasa setiap kitaran analisis. Daripada analisis kromatografi cecair (HPLC) berprestasi tinggi, 1.03 g/L dan 1.04 g/L glukosa dan xilosa dirembes daripada biojisim yang tidak dirawat melalui sistem periuk tunggal selepas kitaran ketiga analisis, menunjukkan enzim tidak bergerak pada m-MWCNTs disintesis. menggunakan sistem berasaskan air dan menonjolkan potensinya untuk hidrolisis biojisim lignoselulosa.

Tri-Enzyme Immobilized on Magnetized Multiwall Carbon Nanotubes for Single Pot Lignocellulosic Biomass Hydrolysis

ABSTRACT

Single pot is an innovative idea by combining multi-enzyme in single process for hydrolysis of lignocellulosic biomass. Native enzymes have several drawbacks in the industrial setting, including low enzyme activity, poor stability, uneconomical nature, and challenging separation from the final product. This recent study addresses the co-immobilization of xylanase, laccase, and cellulase on magnetized multiwall carbon nanotubes that have been synthesized and functionalized using an environmentally friendly method that combines water-based synthesis of functionalized multiwall carbon nanotubes with mild acid oxidation of pristine multiwall carbon nanotubes. The immobilized enzymes were characterized in terms of stability and reusability prior to the establishment of a single-pot system for the hydrolysis of paddy straw into reducing sugars (glucose and xylose). Through mild acid treatments, 8 M of acid, 8 h, and 80 °C of reflux time and temperature were examined as ideal working conditions. The establishment of a water-based system in the synthesis of iron oxides on p-MWCNTs was confirmed by energy-dispersive X-ray spectroscopy (EDX), with 10.49% iron detected on the surface of MWCNTs. Immobilization of enzymes on m-MWCNTs was successfully achieved via the adsorption method with > 95% binding efficiency for all enzymes, which was further confirmed with the aid of Fourier-transform infrared spectroscopy (FTIR) peaks, scanning electron microscopy (SEM) images, and EDX analysis. The optimum enzyme concentration for immobilization was recorded at 5 mg/mL for both cellulase and xylanase and 7 mg/mL for laccase. Based on the stability study, the optimum temperature of immobilized cellulase, laccase, and xylanase corresponds to 50, 60, and 70 °C, respectively. The optimum pH of 5 was recorded with immobilized cellulase and laccase, while pH 6 was recorded with immobilized xylanase. A reusability study on model substrate showed all immobilized enzymes retained more than 50% of relative activity after five cycles of analysis, while up to 30% of relative activity was still retained with immobilized cellulase and xylanase that were subjected to the hydrolysis of paddy straw. The detection of a FTIR peak at 1657 cm^{-1} using paddy straw treated with immobilized laccase on m-MWCNTs indicates successful delignification. As for the single-pot system, the operation was conducted at 50 °C, pH 5, and 100 rpm for 16 h using untreated and acid-pretreated paddy straw. Untreated biomass exhibited higher glucose and xylose content during each cycle of analysis. From high-performance liquid chromatography (HPLC) analysis, 1.03 g/L and 1.04 g/L of glucose and xylose were secreted from untreated biomass through a single-pot system after the third cycle of analysis, highlight the potential of immobilized enzymes on m-MWCNTs synthesized using a water-based for lignocellulosic biomass hydrolysis.

CHAPTER 1 : INTRODUCTION

1.1 Research Background

Lignocellulosic biomass is a complex substituent composed of two major components: polysaccharides (cellulose and hemicellulose) and aromatic polymers (lignin) (Zoghlami and Paës, 2019). The biodegradation of this structure required a series of enzymes, which include cellulase, xylanase, and laccase. Cellulase are multicomponent enzymes that are responsible for cellulose bioconversion into glucose subunits by hydrolyzing β-1,4-glycosidic bond in the cellulose structure (Behera *et al.*, 2017). Meanwhile, laccase and xylanase are responsible for lignin decomposition and hemicellulose degradation, respectively (Kumar and Chandra, 2020).

These enzymes (cellulase, laccase, and xylanase) are well known as industrial enzymes as they show great potential for industrial utilization. This statement can be proven by the broad range of industrial implementations such as cellulase, laccase, and xylanase commonly applied in biofuel production, the pulp and paper industry, and food, which is mainly for the extraction of fruit extracts as well as animal nutrition (Rajeeva and Soni, 2015; Akram *et al.*, 2018; Bhardwaj *et al.*, 2019). At present, according to "Future Market Insight," cellulase dominates the global enzyme market compared to xylanase and laccase with USD 1621 million and is expected to soar to nearly USD 3153.1 million by 2032 (Ilić *et al.*, 2023). According to Behera and Ray, (2016), the growing demand for industrial enzymes is due to intensive studies in biofuel second-generation production.

Microorganisms such as fungi and bacteria are well-known sources for these industrial enzymes (cellulase, laccase, and xylanase) production. Although both classes (fungi and bacteria) of microorganisms are capable of secreting particular enzymes, fungi are always preferred over the bacterial strain due to their capability to secrete large concentrations of the enzymes (Imran *et al.*, 2016). Fungal *Aspergillus* strains are known as the best industrial enzyme producers (Cunha *et al.*, 2018; Iqbal *et al.*, 2018; Siva *et al.*, 2022). In real-life applications, industrial processes consume a huge amount of enzymes, thus contributing to high production costs. Thus, an idea to reuse or recycle an enzyme has come to light (Siva *et al.*, 2022).

Enzyme immobilization is an essential and powerful technique in biotechnology where confinement of enzyme molecules happens on or with a carrier, resulting in better stability of the enzyme and retaining most of its activity (Basso and Serban, 2019). Through immobilization, enzymes can be reused in multiple cycles, which is anticipated to lower the production cost and overcome technical bottlenecks. According to Xin *et al.*, (2018), the selection of carrier plays a vital role in immobilized enzyme efficiency, which includes the physical properties of the carrier, surface chemistry, and inner morphology. To date, nanotechnology has evolved rapidly in the science and technology sectors due to its beneficial applications (Singh, 2017). In specific, magnetic carriers have gained an overwhelming response as the perfect choice to provide a support system in immobilization due to their lower toxicity, small size, and large surface area while allowing convenient separation and recovery of enzymes from the solution mixture (Jose *et al.*, 2020).

Co-precipitation is a common technique for producing magnetic nanoparticles where iron oxide (Fe_3O_4) is commonly used (Mireles *et al.,* 2016). This method is highly preferable due to its simplicity, but reaction parameters such as pH, nanoparticle concentration, metal ions, and temperature are key to a successful co-precipitation process (Mosayebi *et al.,* 2017). According to Daoush, (2017), co-precipitation methods usually involve multiple solvents and even high temperatures for some procedures. Unfortunately, uncontrollable solvent usage could create a potential risk to the environment and human health (Wang *et al.,* 2020). Thus, the urge to establish a green synthesis method is always desired.

The discovery of more effective and stable support materials with targeted industrial utilization including nanotubes, activated carbon, nanoparticles and nanofibers (Costa *et al.,* 2019). Among them, carbon nanotubes (CNTs) are commonly used in immobilization due to good biocompatibility, high enzyme loading and cheaper (Soni *et al.,* 2020). Unfortunately, these materials demand a functionalization process to enhance the adsorption capacity while also promoting a sufficient functional group on the carrier surface (Jiang *et al.,* 2020). An acid, such as nitric (HNO_3), hydrochloric (HCl), or a combination of sulphuric (H_2SO_4) and nitric acid (HNO_3), is typically used in functionalization. The main problems with using this kind of acid are the cytotoxicity, disruption of the molecular network of the CNTs, and contribution to toxic waste in the environment (Hoa, 2018; Garnica-Gutiérrez *et al.*, 2018). As a result, there is a growing interest in developing a mild acid oxidation condition or finding an alternative by using weak acids.

Besides that, single-pot systems for saccharification of biomass also have been researched recently as an attempt to establish an economical system, which is always preferred. A single pot can be defined as a pretreatment and saccharification process that combines the use of several enzymes in a single process with the goal of improving system efficiency and simplicity (Althuri and Mohan, 2019). However, establishing a complete system is not an easy task as each enzyme has a different ideal condition. Till date, there are multiple published works related to mild acid oxidation on CNTs and individual immobilized system (cellulase, laccase and xylanase) on magnetic carrier (Nadar and Rathod, 2019; Chen *et al.*, 2020; Amaro-Reyes *et al.*, 2019). Nevertheless, to the best of our knowledge, there is limited studies that focused into great detail on greener functionalization processes and single-pot systems for hydrolyzing biomass using magnetic CNTs. Therefore, with an objective to establish an eco-friendly single pot system, this study attempted to use functionalized CNTs synthesized using a mild acid as a carrier. Subsequently, the magnetic properties of CNTs were incorporated using iron oxide through a water-based method. This was followed by the /implementation of a single-pot research system comprising tri-enzymes (cellulase, laccase, and xylanase) immobilized on magnetized multiwall carbon nanotubes (m-MWCNTs).

1.2 Problem Statement

Enzymes are highly sought-after in the industrial market because of their variety of uses. Native enzymes, on the other hand, difficult to reuse or recycle, exhibit poor stability, and high production costs (Habimana *et al.*, 2021). The immobilization of enzymes can enhance the properties of the enzymes, increase the effectiveness of the catalytic process, and reduce the cost of production (Basso and Serban, 2019). In order to achieve more effective commercial application, screening for the optimal working

condition of an enzyme has a significant impact on the enzyme's performance. Thus, in order to create an efficient system of immobilized enzyme that functions to the fullest extent possible, the effects of pH and temperature on immobilized enzyme must be investigated (Popescu *et al.,* 2021) using a one-factor at-a-time (OFAT) analysis. Adsorption seems to be the most widely utilized immobilization technique, as it produces stable enzymes and maintains the original characteristics of the enzyme without impairing its function. Nevertheless, it is crucial to emphasize that even with the lucrative advantages, the immobilized enzyme performance is still limited in the absence of appropriate screening for the enzyme optimal working conditions on pH and temperature.

Recently, the rapid evolution of technology has led to the discovery of more effective and stable support materials with targeted industrial utilization, including nanotubes, activated carbon, nanoparticles, and nanofibers (Costa *et al.,* 2019). Due to their high enzyme loading, good biocompatibility, and affordability, carbon nanotubes (CNTs) are frequently used in immobilization (Soni *et al.,* 2020). However, CNTs have limited solubility in solvents, which is considered their main drawback and can be solved through functionalization to increase dispersibility (Abdulhameed *et al.,* 2021).

Previously, the dispersion efficiency of CNTs could be increased by applying a harsh acid treatment, such as using acid with a high concentration or raising the working temperature and duration. However, due to the harsh acid treatment, CNTs lengths were decreased, and acid waste could contribute to water and soil pollution as a result of the high acidity (Xiu *et al.,* 2019). Therefore, it is important to address the need to resolve these problems. Despite this, only a small number of studies focused on CNTs treatment, as the majority focused on biocatalytic advancement (Abdulhameed *et al.,* 2021).

Separation of the carrier from the reaction medium is another significant issue with earlier immobilization studies (Mehnati-Najafabadi *et al.,* 2018). The recovery of immobilized enzyme is typically accomplished using filtration and centrifugation techniques. This process is regarded as inconvenient (Liu *et al.,* 2018) when it comes to hydrolysis of lignocellulosic biomass as it is insoluble in liquid medium, thus separation during the recovery process is difficult. As a result, the idea of using magnetic nanoparticles as a carrier has drawn a lot of attention because magnetic bars make it simple to separate and recycle them from reaction medium. The immobilized enzymes recovered from the reaction medium reported could be used for several cycles (reusability), which would suggest the practicality and economic feasibility of the delignification and saccharification processes of biomass (Kharazmi *et al.,* 2020).

Previous studies have successfully individually immobilized the enzymes cellulase, laccase, and xylanase on magnetic nanoparticles (Patel *et al.,* 2020; Abdul Manaf *et al.,* 2021). Because of their wide range of industrial applications, particularly in the pulp and paper and biofuels industries, these enzymes are highly preferred in enzymatic studies (Sharma et al., 2020). It is crucial to note that the synthesis of CNTs with iron oxide is typically associated with high chemical and energy consumption, which made the process unprofitable (Karimi, 2016). Therefore, an effort has been made in the current research to develop an eco-friendly synthesis method with the goal of creating a green environment system. Additionally, to the best of our knowledge, not much work has been done on the immobilization of enzymes on environmentally friendly, magnetized multiwall carbon nanotubes (m-MWCNTs). Furthermore, there is a paucity of well-established studies on single pot bio-conjugates, which employ lignocellulosic biomass as a substrate and integrate xylanase, laccase, and cellulase to function as a