CROSSLINKING IMMOBILISATION OF SURFACE-ENGINEERED XYLANASE AND ITS HYDROLYSIS PERFORMANCE OF OIL PALM EMPTY FRUIT BUNCHES FOR XYLOOLIGOSACCHARIDE PRODUCTION

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ABSTRACT

Hydrolysis of hemicellulose to sugar such as xylooligosaccharide (XOS) is an alternative method to reduce the naturally abundant lignocellulose biomass waste such as oil palm empty fruit bunches (OPEFB). To achieve this, enzymatic hydrolysis has been envisioned as a highly potential method in converting hemicellulose to XOS. However, the conventional use of free enzymes is always hampered by the low stability of the enzyme, difficulty in recovery, and non-recyclability. These limitations can be solved by enzyme immobilization, such as cross-linked enzyme aggregate (CLEA), in which the immobilization occurs without a solid carrier. The interaction between the amine group at the enzyme with the crosslinker plays a significant role in determining the immobilization efficiency. Nevertheless, the low content of lysine at the surface of the enzyme could be a problem to achieve efficient cross-linking. In this study, a three-dimensional (3D) model of xylanase (rXyn) from *Aspergillus fumigatus* RT-1 was developed using Modeller v9, and surface analyzed using Swiss PDB Viewer. *In silico* mutagenesis was performed at four residues on the surface of the enzyme (mXyn) and docked with glutaraldehyde using AutoDock. Molecular Dynamic (MD) simulation was performed on all structures (rXyn, rXyn-glu, mXyn, and mXyn-glu) for 1 ns at four different temperatures, and it was found that the structures were stabilized when docked with glutaraldehyde. The recombinant xylanase (rXyn) was mutated using site-directed mutagenesis at four different residues, mainly at the back of the enzyme and away from the catalytic site. The parameters of CLEA (choice of precipitants, the concentration of precipitant, concentration of crosslinker, concentration of bovine serum albumin (BSA), and cross-linking time) were optimized. The mXyn-CLEA-BSA was found to be able to recover higher xylanase activity at 137.08 % compared to the rXyn-CLEA, rXyn-CLEA-BSA, and mXyn-CLEA, which showed lower recovery activity at 96.64%, 104.71%, and 115.48%, respectively. At 70 °C for 60 minutes, mXyn-CLEA-BSA achieved the highest stability than the other CLEAs and free enzymes. mXyn-CLEA-BSA also successfully retained more than 40% of its activity after 5 cycles, whereas in the same cycle, rXyn-CLEA lost its total activity. In comparison, rXyn-CLEA-BSA and mXyn-CLEA only retained 19.66% and 21.41% of its activity, respectively. Therefore, the performance of mXyn-CLEA-BSA was further investigated in the catalytic reaction using pre-treated OPEFB under optimized reaction conditions. Four different sizes of CLEA particles and three different sizes of OPEFB was used to study the diffusional effect. The smaller size of CLEA particle and OPEFB were found to give higher hemicellulose yield. From high performance liquid chromatography analysis, the reaction between mXyn-CLEA-BSA and OPEFB produced xylotriose and small traces of xylose, with 0.361 mg/mL and 0.044 mg/mL, respectively. These findings showed that the combination of protein surface engineering and CLEA technology could improve xylanase stability and reusability by strengthening the intermolecular linkages between xylanase and glutaraldehyde. Furthermore, the developed CLEAs offers a great advantage in synthesizing XOS from the insoluble substrate.

ABSTRAK

Hidrolisis hemisellulosa kepada gula seperti xilooligosakarida (XOS) adalah antara kaedah alternatif untuk mengurangkan limpahan buangan biojisim lignosellulosa seperti tandan buah kosong kelapa sawit (OPEFB). Untuk mencapainya, hidrolisis enzimatik adalah dilihat sebagai kaedah berpotensi tinggi dalam menukarkan hemisellulosa kepada XOS. Walaubagaimanapun, penggunaan konvensional enzim bebas kebiasaannya terbantut disebabkan oleh kestabilan enzim yang rendah, kesukaran dalam perolehan dan sifatnya yang tidak boleh diguna semula. Permasalahan ini boleh diselesaikan dengan menggunakan immobilisasi enzim seperti agregat enzim terpaut silang (CLEA) di mana immobilisasi berlaku tanpa penggunaan pembawa pepejal. Walaupun begitu, kandungan lisin yang kurang pada permukaan enzim boleh menyebabkan masalah untuk mencapai pautan silang yang cekap. Interaksi antara kumpulan amina pada enzim dengan pemaut silang memainkan peranan yang penting dalam menentukan kecekapan immobilisasi. Dalam kajian ini, model 3 dimensi xilanase (rXyn) dari *Aspergillus fumigatus* RT-1 telah dihasilkan menggunakan Modeller v9 dan permukaan enzim telah dianalisis menggunakan Swiss PDB Viewer. Mutasi *in silico* telah dijalankan pada empat baki pada permukaan enzim (mXyn) dan telah didokkan bersama glutaraldehida menggunakan AutoDock. Simulasi Dinamik Molekular (MD) telah dilakukan pada semua struktur (rXyn, rXyn-glu, mXyn, mXyn-glu) selama 1 ns pada empat suhu berbeza dan didapati stuktur telah distabilkan apabila didokkan bersama glutaraldehida. Xilanase rekombinan (rXyn) telah dimutasi menggunakan kaedah mutasi teraruh pada empat baki berbeza khususnya pada bahagian belakang enzim dan jauh dari tapak bermangkin. Paramater untuk CLEA (pemilihan agen pemendakan, kepekatan agen pemendakan, kepekatan agen pemaut silang, kepekatan albumin serum bovin (BSA) dan masa pemaut silang) telah dioptimasi. mXyn-CLEA-BSA telah didapati mampu untuk memulihkan aktiviti xilanase yang tinggi pada 137.08% berbanding rXyn-CLEA, rXyn-CLEA-BSA dan mXyn-CLEA yang menunjukkan kepulihan aktiviti yang rendah pada 96.64%, 104.71% dan 115.48%. Pada suhu 70°C selama 60 minit, mXyn-CLEA-BSA telah mencapai kestabilan yang paling tinggi berbanding CLEA yang lain dan enzim bebas. mXyn-CLEA-BSA juga berjaya mengekalkan lebih 40% aktiviti enzim setelah 5 kitaran, sedangkan pada kitaran yang sama, rXyn-CLEA telah hilang semua aktiviti. Sebagai perbandingan, rXyn-CLEA-BSA dan mXyn-CLEA masing-masing hanya mengekalkan 19.66% dan 21.41% aktiviti. Oleh itu, prestasi mXyn-CLEA-BSA telah dikaji dengan lebih lanjut di dalam tindakbalas bermangkin menggunakan OPEFB terawat dibawah keadaan tindakbalas yang optimum. Empat saiz zarah CLEA yang berbeza dan tiga saiz OPEFB yang berbeza digunakan untuk mengkaji kesan resapan. Saiz zarah CLEA dan OPEFB yang lebih kecil didapati mampu menghasilkan hemisellulosa yang tinggi. Berdasarkan analisis kromatografi cecair prestasi tinggi, tindakbalas antara mXyn-CLEA-BSA dan OPEFB menghasilkan xilotriosa dan jejak kecil xilosa dengan nilai masing-masing sebanyak 0.361 mg/mL and 0.044 mg/mL. Dapatan ini menunjukkan kombinasi kejuruteraan permukaan protin dan teknologi CLEA dapat memperbaiki kestabilan dan kebolehan guna semula xilanas dengan memperkuatkan jalinan antara molekul di antara xilanas dan glutaraldehida. Tambahan lagi, CLEA yang dihasilkan menawarkan kelebihan besar dalam menghasilkan XOS dari substrat tak larut.

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LIST OF ABBREVIATIONS

3D	-	Three Dimensional
A	-	Absorbance
APTES	-	(3-Aminopropyl)triethoxysilane
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine Serum Albumin
CLEs	-	Cross-Linked Enzyme
CLEAs	-	Cross-Linked Enzyme Aggregate
CLECs	-	Cross-Linked Enzyme Crystal
dH_2O	-	Distilled water
DP	-	Degree of Polymerization
DNA	-	Deoxyribonucleic Acid
DNS	-	3,5-Dinitrosalicylic Acid
dNTPs	-	Deoxynucleoside Triphosphate
E. coli	-	*Escherichia coli*
EC	-	Enzyme Control
EDTA	-	Ethylenediaminetetraacetic Acid
Ea	-	Enzyme activation energy
e.g.	-	“for example”
etc	-	et cetera/ “and so forth”
FM	-	Feather Meal
FTIR	-	Fourier-Transform Infrared Spectroscopy
g	-	gram
g/L	-	gram per liter
GO	-	Graphene Oxide
GRAS	-	Generally Recognized As Safe
h	-	hour
HCl	-	Hydrochloric acid
HPLC	-	High Performance Liquid Chromatography
HPAEC	-	High Performance Anion-Exchange Chromatography
HMF	-	Hydroxymethyl Furfural

HRP	-	Horseradish Peroxidase
i.e	-	“that is”
IPTG	-	Isopropyl-β-D-1-Thiogalactopyranoside
kDa	-	Kilo Dalton
L	-	Liter
LB	-	Lysogeny Broth
M	-	Molar
min	-	minute
mL	-	mililiter
mm	-	milimeter
mM	-	milimolar
MD	-	Molecular Dynamic
MgSO4	-	Magnesium Sulphate
MNPs	-	Magnetite Nanoparticles
MWCO	-	Molecular Weight Cut-Off
Nm	-	nanometer
NaOH	-	Sodium hydroxide
NSM	-	Nano size magnetite
OD	-	Optical density
OFAT	-	One-factor-at-a-time
OE-PCR	-	Over Extension Polymerase Chain Reaction
OPEFB	-	Oil palm empty fruit bunches
PAA	-	Peracetic acid
PBC	-	Periodic boundary conditions
PDB	-	Protein database
PEI	-	Polyethylene imine
PEG	-	Polyethylene glycol
PES	-	Polyether sulfone
PGA	-	Penicilin G acylase
PME	-	Particle mesh ewald
Rs	-	Reducing sugar
Rsmax	-	Maximum reducing sugar
rpm	-	Revolution per minute

RGO	-	Reduced graphene oxide
RMSD	-	Root mean square deviation
RMSF	-	Root mean square fluctuation
SDS-PAGE	-	Sodium dodecyl polyacrylamide gel electrophoresis
SEM	-	Scanning electron microscope
SPC	-	Simple point charge
SC	-	Substrate control
TRS	-	Total reducing sugar
U	-	Unit enzyme
UV	-	Ultraviolet
V	-	Velocity
υ	-	Kinetic rate
v/v	-	Volume per volume
w/w	-	Weight per weight
w/v	-	Weight per volyme
X1	-	Xylose
X2	-	Xylobiose
X3	-	Xylotriose
X4	-	Xylotetraose
X5	-	Xylopentaose
X6	-	Xylohexaose
XOS	-	Xylooligosaccharide

LIST OF SYMBOLS

°C	-	Degree Celsius
K	-	Kelvin
%	-	Percentage
α	-	Alpha
β	-	Beta
µL	-	microliter
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The depletion of conventional energy, such as fossil fuel, has become a significant concern in recent years. The scarcity of this energy has resulted in increasing demand for alternative energy sources, parallel with the world moving into an era of renewable energy and energy efficiency. Lignocellulose biomass is a sustainable resource that can produce various bioproducts and biofuels (Billion-Ton, 2016). It is also a promising candidate to replace heavy dependence on fossil materials due to its abundant availability as agriculture and forest residues or in the form of a waste stream of any agriculture mills. In general, Lignocellulosic biomass consists of 40-50% of cellulose, 25-30% of hemicellulose, and 15-20% of lignin as the main constituents, with a small amount of pectin, extractive, protein, and ash (Menon and Rao, 2012; Jørgensen, Kristensen and Felby, 2007). While lignin and cellulose have been traditionally explored for decades with so many applications and inventions, surprisingly, hemicellulose has not yet been extensively studied. To date, there are so many innovations and materials produced from hemicelluloses (Martin-Sampedro *et al.*, 2014), including xylooligosaccharide (XOS), a high-value prebiotic which can produced via enzymatic hydrolysis.

The enzyme is a remarkable biocatalyst due to its high catalytic activity, able to operate under mild conditions, and lack of undesirable side reactions. For decades, they have been exploited in an increasing scale of pharmaceutical, food, and chemical industries (Rodrigues *et al.*, 2013). However, the industrial application of biocatalysts is generally hampered by insufficient stability or activity under harsh conditions. Researchers have paid a lot of attention to the robust immobilized catalysts that are highly stable, cost-effective, and allow multiple reuses of the enzyme to overcome these limitations. For example, a study was conducted to immobilize horseradish peroxidase (HRP) on a 60 nm reduced graphene oxide (RGO) as a carrier through

covalent immobilization using glutaraldehyde as the crosslinker (Besharati Vineh *et al.*, 2018). After 120 minutes at 40 °C, the immobilized HRP retained 90% of the initial activity while the soluble enzyme only retained 60% initial activity. Similarly, a study conducted by Zhang and co-workers (Zhang *et al.*, 2015) also found a stable immobilized HRP on RGO up to 7-fold after comparing with graphene and graphene oxide (GO). Nowadays, there are plenty of carriers that have been used and successfully produced a highly stable enzyme. For example, agarose beads (de Oliveira *et al.*, 2018), magnetic nanoparticles (Meng *et al.*, 2014), carbon nanotubes (Ahmad and Khare, 2018), and so much more. However, usage of carriers will often lead to the dilution of enzyme activity, which subsequently decreased productivity (Tischer and Kasche, 1999; Truppo *et al.*, 2012), and some of the physical adsorptions will not be strong enough to keep the enzyme intact to the carrier, especially when the process undergoes rigorous industrial conditions (Sheldon and van Pelt, 2013).

Furthermore, immobilization of enzymes can also be performed by encapsulation or entrapment. The enzymes are trapped inside a gel, fibres, or lattice structure of a material or polymer membrane. There are a few reports which showed that the method could develop a stable immobilized enzyme. Du and co-workers (Du *et al.*, 2017) fabricated catalase using a combination of enzyme nanocapsules and metal-organic framework (MOF). The same group (Du *et al.*, 2019) also developed a facile and controllable strategy of constructing metal-organic framework-based (MOF-based) hollow composites via a protein-induced soft-templating pathway. Both of these approaches resulted in an immobilized biocatalyst with significant improvement of thermal stability, storage stability and reusability. Nonetheless, the method has a crucial drawback especially when large molecule of substrates was used as it will face difficulty to penetrate into the enzyme. Therefore, carrier-free immobilized enzymes such as cross-linked enzyme aggregates (CLEAs) is the most promising and it has gained much attention due to its robustness, simplicity in preparation and also product separation in industrial application (Shaarani *et al.*, 2016). These approaches are advantageous over carrier-bound enzymes as the final preparation have higher volumetric activity and are highly stable under unnatural conditions (Cao *et al.*, 2003; Gao *et al.*, 2015).

The preparation of CLEAs is a straightforward immobilization strategy that involves two main steps, which are the precipitation of crude enzyme by aggregating with precipitants such as organic solvents, non-ionic polymer, or salts followed by subsequent cross-linking of the precipitated enzymes using a bifunctional reagent such as glutaraldehyde, aldehyde-dextran or L-lysine (Sinirlioglu *et al.*, 2013; Fuentes *et al.*, 2004; Ayhan *et al.*, 2012). Between these two steps, enzyme precipitation is crucial part because the chosen precipitating agent will determine the appearance of CLEA formed. For instance, using 2-propanol will produce a sticky CLEA, causing a problem during CLEA separation. The problem was disclosed by Jung and co-workers (Jung *et al.*, 2013) in the one-pot bioconversion of sucrose to trehalose. Recently, quite a number of CLEA preparations have been developed, making this method promising and has many aspects to explore further. For instance, CLEA can be prepared by using more than one enzyme. This innovation is called multi or combi-CLEA. Goetze and co-workers (Goetze *et al.*, 2017) prepared a combi-CLEA exhibiting pectinase, polygalacturonase, pectin lyase and pectin methyl esterase for grape juice clarification using feather meal (FM) and bovine serum albumin (BSA) as a proteic feeder. Another development in CLEA preparation is a carrier-bound CLEA which utilized carrier-free and carrier-bound concepts using a single enzyme (Gao *et al.*, 2015) and the development of porous-CLEA which utilized pore-making agent such as starch to minimize the mass transfer limitation (Jiang *et al.*, 2014; Wang *et al.*, 2011; Talekar *et al.*, 2012). The latest development in CLEA preparation is magnetic CLEA, which focused on CLEA separation. (Purohit *et al.*, 2017; Nadar and Rathod, 2016). The approach has gained much interest due to the ease of CLEA recovery, thus, making the downstream processing a lot easier.

However, apart from the CLEA development mentioned here, the cross-linking mechanism might not be as effective as expected, especially for enzymes with low free amino residue contents. High content of free amino, especially on the surface of the enzyme, is crucial because it will create strong intermolecular bonds between free amino and polymers or oligomers resulting from the aldol condensation of glutaraldehyde (Sheldon, 2011). It was reported that, xylanase has significant low amount of lysine residue from the total amino acid (Shaarani *et al.*, 2016; Manrich *et al.*, 2010). One way of compensating this is by the addition of BSA, which forms co-

aggregates with an enzyme containing low lysine residue content. This approach can form CLEA with high activity and improved mechanical properties (Shah *et al.*, 2006; Tükel *et al.*, 2013). However, when BSA was added, the finding of a previous study that used laccase has shown a reduction in laccase stability by more than 50% compared to the free enzyme (Matijošyte *et al.*, 2010).

Another approach to increase lysine content is via protein engineering. By designing the enzyme with few amino acid mutations into lysine at several regions or by introducing specific groups or tags at the targeted region of the enzyme, the cross-linking rate and chemical reactivity can be improved (Rodrigues *et al.*, 2014). Simultaneously, the enzyme becomes more rigid and stable. For instance, the introduction of three lysine residues on the surface region of penicillin G acylase (PGA) that was already rich in lysine showed a tremendous stabilization factor (Abian *et al.*, 2004). The observation showed the importance of lysine in enzymes' immobilization even though the number of lysines introduced was low. On the other hand, a study by Ryan and Ó'Fágáin (Ryan and Ó'Fágáin, 2007) reported that a directional and oriented immobilization of horseradish peroxidase (rHRP) onto a modified polyethersulfone (PES) membrane showed a negative effect on stability (free and immobilized state) when four constructed mutants containing two and three external arginines were replaced by lysine on the surface of the enzyme. The mutants' instability was caused by the reduction of hydrogen bonds as lysine could not form as many hydrogen bonds as arginine. Research on the modification of protein structure and amino acids substitution using site directed mutagenesis to improve intermolecular interaction of enzyme molecules by the crosslinker in CLEA has not yet been explored (Rodrigues *et al.*, 2014). There is a knowledge gap in using protein engineering to modify xylanase's molecular structure and the interaction with the crosslinker, enabling us to understand the effect on enzyme reaction mechanism and kinetics.

In this study, surface modification of xylanase is expected to improve the binding efficiency of cross-linking. To my knowledge, no surface modification of enzyme has been demonstrated for CLEA apart from site-directed covalent immobilization on a carrier. Homology modelling and energy minimization will be performed to obtain an optimized three-dimensional (3D) model for xylanase. By

analysing and mutating a few residues on xylanase's surface, the cross-linking efficiency and the stability and reusability of xylanase are expected to improve. Surface modification of xylanase by protein engineering is proposed as it is the most promising and efficient tool to achieve oriented enzyme immobilization. Thus, this study provided a significant opportunity to advance the understanding of lysine substitution at specific residues on xylanase's surface. Furthermore, the developed xylanase-CLEAs can be further utilized in the production of xylooligosaccharides (XOS).

1.2 Problem Statement

Xylanase has been widely used in numerous application but it remains open for various discoveries and research. The xylanase used in this study was isolated from *Aspergillus fumigatus* RT-1 which has been studied previously in its soluble form (Abdul Wahab *et al.*, 2016). Simply put, this xylanase is a mesophilic enzyme and its thermostability has been improved via random mutagenesis. However, in soluble form, the xylanase was unstable for a longer period of time and its activity was rapidly decreased. Due to this, CLEA has been introduced to further improve its stability. As explained in the sub-section 1.1, key important in CLEA is numerous number of lysine or free amino residue at the surface. Having a great number of lysine will introduce more intermolecular and intramolecular bond with cross-linker. However, this xylanase lack number of lysine at the surface which could potentially does not improve in enzyme's stability once cross-linked and furthermore, the enzyme itself could possibly be easily leach out. Surface modification using protein engineering was proposed to increase the number of lysine on the surface.

1.3 Research Goal

1.3.1 Research Objectives

There are three main objectives to be achieved in this study. The objectives are:

a) To construct mutant xylanase with higher cross-linking efficiency based on data from molecular dynamic simulation
b) To develop and characterize CLEA aggregates of recombinant and mutant xylanase with high enzyme recovery activity
c) To evaluate the performance of developed CLEAs using pretreated oil palm empty fruit bunches (OPEFB)

1.3.2 Research Scopes

This study emphasizes the surface engineering of xylanase, the design preparation of developed CLEAs, and hydrolysis of the developed CLEAs activity on oil palm empty fruit bunches (OPEFB) and its reaction process in xylooligosaccharides production. Therefore, the following scopes were outlined to achieve the objectives.

(a) Computational design, analysis and molecular dynamic simulation of xylanase model using Modeller v9.13 and protein ligand complex simulation
(b) Expression, partial purification and characterization of recombinant xylanase from *Aspergillus fumigatus* RT-1
(c) Study on the effect of preparation parameters on the activity recovery in CLEA; types and concentration of precipitant, concentration of cross-linker, cross-linking time and concentration of BSA.
(d) Characterization in terms of biochemical, structural morphology and thermal inactivation kinetics of developed CLEAs (rXyn-CLEA, rXyn-CLEA-BSA, mXyn-CLEA and mXyn-CLEA-BSA)
(e) Screening the effect of several reaction conditions (substrate loading, enzyme loading, temperature, pH, agitation rate, incubation time and particle size) of the best developed CLEA on the hydrolysis of pretreated OPEFB
(f) Quantification of XOS produced using the best developed CLEA on the hydrolysis of pretreated OPEFB
(g) Evaluation of enzyme kinetics of xylanse on the pretreated OPEFB using modified Prout-Tompkins equation.

1.4 Novelties of The Study

There are two novelties of this study. The novelties are:

a) The improvement of cross-linking efficiency of CLEA-xylanase using computational analysis, including 3D model development, 3D structure assessment, and surface analysis, has not been carried out yet by previous researchers.
b) This is the first report on enzymatic hydrolysis of pretreated OPEFB using CLEA technology to the best of my knowledge. Furthermore, this is the first report utilizing xylanase from *Aspergillus fumigatus* RT-1 for hydrolysis of pretreated OPEFB

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LIST OF PUBLICATIONS

Journal with Impact Factor

1. **Abdul Wahab, M.K.H. bin**, Jonet, M.A. bin and Illias, R.M. (2016). Thermostability enhancement of xylanase Aspergillus fumigatus RT-1. *Journal of Molecular Catalysis B: Enzymatic*. 134, 154–163. https://doi.org/10.1016/j.molcatb.2016.09.020 **(Q3)**

2. **Abdul Wahab, M.K.H**., El-Enshasy, H.A., Bakar, F.D.A., Murad, A.M.A., Jahim, J.M. and Illias, R.M. (2019). Improvement of cross-linking and stability on cross-linked enzyme aggregate (CLEA)-xylanase by protein surface engineering. *Process Biochemistry*. 86, 40–49. https://doi.org/10.1016/j.procbio.2019.07.017 **(Q2, IF: 2.952)**