# CROSS-LINKED ENZYME AGGREGATES OF RECOMBINANT XYLANASE FOR CONVERSION OF HEMICELLULOSES TO XYLOOLIGOSACCHARIDES

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy *(Bioprocess Engineering)*

Faculty of Chemical and Energy Engineering
Universiti Teknologi Malaysia

MARCH 2018

To my beloved husband (Muhamad Azraf bin Johana), mother (Hjh. Faridah Osman), brother (Prof. Dr. Sharifudin Md. Shaarani) and son (Haziq Naqiuddin).

## ACKNOWLEDGEMENTS

Alhamdulillah. First and foremost, I owe my deepest gratitude to my supervisor, Prof. Dr. Rosli Md. Illias, whose encouragement, assistance, guidance and advices from the initial to the final stage, has greatly facilitated me in developing a good understanding of the subject. I wish to express my appreciation to my two co-supervisors, Prof. Dr. Jamaliah Md. Jahim (Universiti Kebangsaan Malaysia) and Prof. Madya Dr. Roshanida A. Rahman for the valuable discussions, great opinions and continuous motivation given. Special thanks go to the helpful laboratory staff, Mr. Yaakob, Mr. Latfi, Mr. Jefri and Madam Zainab for their kind assistance throughout this work. Without their help, the labwork might not have been completed successfully.

I am also greatly indebted to all my laboratory colleagues who have been helping me in numerous ways, particularly Dr. Nur Izyan, Dr. Hasmaliana, Dr. Fatimah, Dr. Baidurah, Dr. Rohaida, Izawati, Ling How Lie, Amalina, Aishah, Ummu Qistina, Rabiatul, Intan, Joyce, Samson, Hakimi, and Abbas.

Sincerely, I am thankful to the Ministry of Higher Education (MOHE) of Malaysia and Universiti Malaysia Pahang for funding my doctoral study as well as Universiti Teknologi Malaysia (UTM) for providing the facilities for my research.

Last but not least, my utmost appreciation to my beloved husband (Muhamad Azraf Johana), my loving mother (Hjh. Faridah Osman), my understanding brother (Prof. Dr. Sharifudin Md Shaarani), my son (Haziq Naqiuddin) and my cherished family members for their patience, eternal support and undying love during my studies.

# ABSTRACT

Hemicelluloses are heterogeneous branched polymers of sugars that exist abundantly in nature. Enzymatic hydrolysis is envisioned as a highly potential method in converting hemicelluloses into fuels and value-added chemicals. However, the use of free enzyme is hampered by low operational stability, difficulty in recovery and non-reusability, which requires for enzyme immobilization. Carrier-bound immobilization leads to utilization of high cost matrices, clogging of filters during downstream processing and presence of large amounts of non-catalytic ballast. Therefore, cross-linked enzyme aggregates (CLEA), a carrier-free technology that combines purification (precipitation) and immobilization into a single operation and does not require purified enzymes, is the solution to these problems. In this study, a recombinant xylanase (Xyl) from *Trichoderma reesei* was immobilized using three approaches: Xyl-CLEA, Xyl-CLEA-BSA (bovine serum albumin) and Xyl-CLEA-silanized maghemite. The use of ethanol as precipitant (1:9 volume ratio of enzyme to precipitant), glutaraldehyde (0.2:1 of glutaraldehyde to enzyme of 100 mM concentration) as cross-linking agent and the introduction of (3-aminopropyl) triethoxysilane (APTES) silanized maghemite (0.0075:1 of silanized maghemite to enzyme) prevailed in forming xylanase CLEAs with good enzyme activity recovery (78 %), thermal stability (50 % retained activity) and reusability (50 % retained activity). The Xyl-CLEA-silanized maghemite enhanced the activity recovery 1.66- and 1.50-fold compared to Xyl-CLEA and Xyl-CLEA-BSA, respectively. At elevated temperature of 60 °C and pHs of 3.0 and 8.0, Xyl-CLEA-silanized maghemite achieved better stability compared to the other CLEAs and free enzyme. Xyl-CLEA-silanized maghemite also successfully retained more than 50 % of its activity after 6 cycles, whereas Xyl-CLEA only retained approximately 10 % after 5 cycles. Therefore, the performance of Xyl-CLEA-silanized maghemite was further investigated by xylan hydrolysis under optimised reaction conditions. Xylooligosaccharides yield was slightly improved by 1.26- fold compared to the free enzyme. Kinetic parameters confirmed that CLEA immobilization did affect the productivity of the designed biocatalyst.

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# ABSTRAK

Hemiselulosa adalah polimer bercabang heterogen yang wujud dengan sangat banyak dalam alam semulajadi. Hidrolisis enzimatik dibayangkan sebagai kaedah yang berpotensi tinggi untuk menukarkan hemiselulosa menjadi bahan api dan bahan kimia bernilai tambah. Walau bagaimanapun, penggunaan enzim bebas terhad disebabkan oleh kestabilan operasinya yang rendah, kesukaran untuk pemulihan dan ketidakbolehan guna semula, yang menyebabkan keperluan kepada proses imobilisasi enzim. Imobilisasi pembawa melibatkan penggunaan matriks yang berkos tinggi, penyumbatan penapis semasa pemprosesan hiliran dan kehadiran sejumlah besar balast bukan pemangkin. Oleh itu, agregat enzim terpaut silang (CLEA), teknologi bebas pembawa yang menggabungkan pemurnian (pemendakan) dan imobilisasi ke dalam satu operasi dan tidak memerlukan enzim tulen, adalah penyelesaian kepada masalah ini. Dalam kajian ini, *xylanase* rekombinan (Xyl) dari *Trichoderma reesei* diimobilisasi menggunakan tiga pendekatan: Xyl-CLEA, Xyl-CLEA-BSA *(bovine serum albumin)* dan Xyl-CLEA-*silanized maghemite*. Penggunaan etanol sebagai pemendak (1:9 nisbah isipadu enzim kepada pemendak), glutaraldehid (0.2:1 glutaraldehid kepada enzim berkepekatan 100 mM) sebagai agen pemaut silang dan pengenalan *(3-aminopropyl) triethoxysilane* (APTES) *silanized maghemite* (0.0075: 1 *silanized maghemite* kepada enzim) berjaya membentuk *xylanase* CLEA dengan pemulihan aktiviti enzim yang baik (78 %), kestabilan terma (50 % aktiviti tersimpan) dan kebolehan guna semula (50 % aktiviti tersimpan). Xyl-CLEA-*silanized maghemite* meningkatkan pemulihan aktiviti 1.66- dan 1.50- kali ganda berbanding dengan Xyl-CLEA dan Xyl-CLEA-BSA, masing-masing. Pada suhu tinggi 60 °C dan pH 3.0 dan 8.0, Xyl-CLEA-*silanized maghemite* mencapai kestabilan yang lebih baik berbanding dengan CLEA yang lain dan enzim bebas. Xyl-CLEA-*silanized maghemite* juga berjaya mengekalkan lebih daripada 50 % aktiviti selepas 6 kitaran, manakala Xyl-CLEA hanya mengekalkan kira-kira 10 % selepas 5 kitaran. Oleh itu, prestasi Xyl-CLEA-*silanized maghemite* dikaji selanjutnya melalui hidrolisis xilan di bawah keadaan tindak balas yang dioptimumkan. Hasil xilooligosakarida meningkat sebanyak 1.26- kali ganda berbanding dengan enzim bebas. Parameter kinetik mengesahkan bahawa imobilisasi CLEA mempengaruhi produktiviti biomangkin yang direka.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| A | - | Absorbance |
| ANOVA | - | Analysis of variance |
| ATCC | - | American type culture collection |
| B | - | Blank / buffer only |
| BMGY | - | Buffered glycerol-complex medium |
| BMMY | - | Buffered methanol-complex medium |
| BSA | - | Bovine serum albumin |
| CCD | - | Central composite design |
| $dH_2O$ | - | Distilled water |
| DNS | - | 3, 5-Dinitrosalicylic acid |
| EC | - | Enzyme control |
| e.g. | - | “for example” |
| etc. | - | et cetera / “and so forth” |
| g | - | gram |
| g/L | - | gram per liter |
| h | - | hour |
| HCl | - | Hydrochloric acid |
| HPLC | - | High performance liquid chromatography |
| *i.e.* | - | “that is” |
| kDa | - | kilo Dalton |
| L | - | Liter |
| M | - | Molar |
| min | - | minute |
| mL | - | milliliter |
| mm | - | millimete |

| | | |
|---|---|---|
| mM | - | milimolar |
| MWCO | - | Molecular weight cut off |
| NaOH | - | Sodium hydroxide |
| nm | - | nanometer |
| OD | - | Optical density |
| OFAT | - | One-factor-at-a time |
| pI | - | Isoelectric point |
| PMSF | - | Phenylmethylsulfonyl fluoride |
| PEG | - | Polyethylene glycol |
| RBB | - | Remazol brilliant blue |
| RI | - | Refractive index |
| Rpm | - | revolutions per minute |
| RSM | - | Response surface methodology |
| SC | - | Substrate control |
| SD | - | Standard deviation |
| SDS PAGE | - | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| T | - | Temperature |
| TRS | - | Total reducing sugar |
| U | - | Unit enzyme |
| UV | - | Ultra violet |
| V | - | Velocity |
| v/v | - | Volume per volume |
| w/w | - | Weight per weight |
| w/v | - | Weight per volume |
| X1 | - | Xylose |
| X2 | - | Xylobiose |
| X3 | - | Xylotriose |
| X4 | - | Xylotetraose |
| X5 | - | Xylopentaose |
| X6 | - | Xylohexaose |
| XOS | - | Xylooligosaccharides |
| Xyn2 | - | Xylanase2 |
| YPD | - | Yeast extract peptone dextrose |

# LIST OF SYMBOLS

| | | |
|---|---|---|
| ˚C | - | Degree Celsius |
| °F | - | Degree Fahrenheit |
| % | - | Percentage |
| α | - | alpha |
| β | - | beta |
| $\gamma$ | - | gamma |
| µL | - | microlitre |
| 10X D | - | Ten times dilution |
| µg/mL | - | microgram per litre |
| 1X | - | One time |
| µmol | - | micromole |
| 5X | - | Five times |
| 3D | - | Three dimensions |
| ™ | - | Trademark symbol |
| ~ | - | Approximate value |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

The intensifying concern among the nations in the 21st century is the heavy dependence on fossil resources. Today the society is aware of the progressive depletion, and the demand to develop materials from renewable resources with low environmental impact is indeed crucial. Lignocellulose has emerged as a highly promising candidate to substitute fossil raw materials due to its abundant availability as forest and agriculture residues or in the form of waste streams of the paper industry. Moreover, it is of great economic importance that the application of lignocellulose as a feedstock does not compete with food production since it is non digestible by humans (Negahdar *et al.*, 2016). Being the most attractive renewable and sustainable resource, lignocellulose can be utilised in the biorefinery framework for the production of fuels, materials and chemicals (Silva-Fernandes *et al.*, 2015; Menon and Rao, 2012). Lignocellulosic biomass is primarily constituted by cellulose, hemicellulose and lignin (Zhang *et al.*, 2015). Different parts of the lignocellulose have already been exploited for a long time mostly for the production of pulp and energy, but the last decade has brought new inventions and applications based on hemicelluloses (Rissanen *et al.*, 2015). Key factors for the shift to hemicellulose based materials are availability in large quantities at a reasonable price and that the material manufactured from

hemicellulose has similar or superior properties than the present fossil fuel-based material (Börjesson and Westman, 2016). Recent innovations include various biopolymers, carbon fibers, fuels and platform chemicals based on hemicelluloses (Martin-Sampedro *et al.*, 2014a,b). Modifications are performed on the extracted hemicellulose using different functional groups which will later be employed for pharmaceutical and packaging applications such as polyelectrolyte layers (Rissanen *et al.*, 2015; Kisonen *et al.*, 2014). Another value-added product obtained during the hydrolysis of xylan, the major component of hemicelluloses is the Xylooligosaccharides (XOs) (Akpinar *et a*l., 2009). Xylooligosaccharides are sugar oligomers that have a variety of great physiological properties such as lowering cholesterol levels, improving gastrointestinal function and the biological availability of calcium, and reducing the risk of colon cancer (Lin *et al.*, 2017; Samanta *et al.*, 2015). They are extensively used as prebiotics and functional food, thus the demand of XOs has been greatly increased in recent years (Yang *et al.*, 2016; Uçkun Kiran *et al.*, 2013). XOs can be used as a source of xylose for the production of xylitol or for the preparation of ethers and esters which can be further utilised as thermoplastic compounds for water-soluble films, coatings, and capsules. The rapid growth of the functional food market and the growing number of other industrial applications force researchers to explore different sources and technologies for producing XOs in high yields (Moure *et al.*, 2006). Hydrolysis via enzymatic approach is therefore highly preferable because it does not produce undesirable by-products or high amount of monosaccharides (Uçkun Kiran *et al.*, 2013; Akpinar *et al.*, 2009).

Enzymes have become a significant factor in several industries due to their valuable properties such as rapid and efficient action at low concentrations under mild temperatures and pH values, high substrate specificity, low toxicity, and ease of termination of activity (Rodrigues *et al.*, 2014; Sanchez and Demain, 2011). The use of enzymes in industrial processes is commonly related to a minimised consumption of energy as well as chemicals and therefore beneficial for the environment (Demarche *et al.*, 2011). Successful application of enzymatic processes is governed by the cost competitiveness with the existing and well established chemical processes. The development of industrial enzymes has strongly relied on the use of microbial sources for the past two decades as they are more stable than their corresponding plant and

animal counterparts, and their production is more convenient and safer (Kartal and Kilinc, 2012). The ability to be produced economically in cheap media and short fermentation cycles make them more practical to be used. Enzymes have thrived in penetrating various industries including food, textiles, detergents, pulp and paper as well as diagnostics and therapy. The global market for industrial enzymes was estimated approximately $4.2 billion in 2014 and predicted to develop at a compound annual growth rate (CAGR) of about 7% over five years (2015 to 2020) to reach nearly $6.2 billion (Singh *et al.*, 2016). In addition to this, over half percentage of the enzyme market has been provided by recombinant enzymes. The production levels of enzymes are majorly affected by the advanced recombinant DNA technology which enables genes encoding enzymes to be cloned from microbes and expressed at levels multifolds higher than those naturally produced. It does not only increase the production levels tremendously but also moved the enzyme productions from strains not suited for industry into industrial strains such as species of *Bacillus, Aspergillus, Trichoderma* and *Kluyveromyces* (Sanchez and Demain, 2011).

Despite having many benefits, there are bottlenecks that hamper the use of enzymes. Enzymes are highly sensitive molecules with unique three-dimensional structures that are responsible for their activities. However, exposure to extreme operating conditions such as elevated temperature and pH will cause the enzyme structures to unfold (denature) and consequently lose their activities. Other than that, enzymes are usually utilised in aqueous solution and the soluble state makes it cumbersome for recovery and reuse. It can jeopardize the purity level of the product and hence result in product contamination (Sheldon, 2011). To overcome these limitations, several enzyme stabilization techniques have been explored by worldwide researchers to improve a biocatalyst process for optimal product yield. The methods include screening for enzymes from extremophiles and their isolation, production of stable enzymes in genetically manipulated organisms and stabilizing unstable enzymes by protein engineering, chemical modification, use of additives and immobilization. Although every approach has its own advantages, immobilization thrives in terms of convenient handling, ease of separation and reuse as well as being economically viable (Ansari and Husain, 2012).

Therefore, immobilization offers the best solution to overcome the obstacles previously mentioned. Immobilization can realize the reuse of expensive enzymes and improve the enzyme properties (operating and storage stability) by limiting the conformational change induced by distorting agents, preventing activity loss due to dissociation, generating convenient (hydrophilic or hydrophobic) environment and altering an active site or the conformational flexibility of the enzyme (Kartal and Kilinc, 2012). Moreover, immobilized enzymes, in contrast to free enzymes which can penetrate the skin, are hypoallergenic (Sheldon, 2011). Numerous books and comprehensive reviews have been published on enzyme immobilization thus reporting thousands of protocols. Immobilization strategies generally consist of adsorption, entrapment, covalence, affinity or cross-linking and combination of several methods. For instance, a pre-immobilized enzyme which is adsorbed on beads can be further entrapped in a porous polymer (Sassolas *et al.*, 2012). Immobilization typically involves binding the enzyme to or encapsulate in a support or carrier. The presence of a large proportion of non-catalytic ballast (about 90-99% of total mass) causes dilution of their volumetric activity. On the contrary, cross-linked enzyme aggregates (CLEA) is a 'carrier-free' immobilization strategy that has attracted increasing attentions due to its simplicity in preparation and robustness in industrial applications (Cui *et al.*, 2014; Kartal and Kilinc, 2012; Sheldon, 2011). CLEAs held several prominent advantages including highly concentrated catalytic activity, high stability against extreme operating conditions, low production cost due to exclusion of carriers, ease of synthesis, facile recovery and reusability as well as the fact that no extensive purification of enzymes is needed. The synthesis implies two main procedures which are precipitation of enzymes by aggregating agents such as salts, water miscible organic solvents or non-ionic polymers, followed by subsequent cross-linking of the precipitated enzymes by bifunctional reagent like glutaraldehyde.

The past two decades have shown tremendous development in the design of cross-linked enzyme aggregates. Despite being a straightforward immobilization strategy, the preparation of CLEAs remains challenging for enzymes with few lysine residues (Asco-Lozano *et al.*, 2014). Uneffective cross-linking occurs to enzymes with low amine content, resulting in CLEAs with low mechanical stability, and thus enabling the release of enzyme molecules into the reaction media (Hormigo *et al.*,

2012). Common solutions proposed are by introducing proteic feeder such as bovine serum albumin (Hormigo *et al.*, 2012; Dong *et al.*, 2010; Shah *et al.*, 2006 ), polylysine (Yamaguchi *et al.*, 2011) or polyionic polymers containing abundant amine groups such as polyethylenimine (PEI) (Asco-Lozano *et al.*, 2014; López-Gallego *et al.*, 2005) to increase the number of amino groups and facilitate intermolecular cross-linking, which increase the stability of final CLEAs. In other cases, the particle size of CLEAs which is usually small (below 10 μm) or being too soft greatly hinders the process of recovery (Cui *et al.*, 2014). These CLEAs are not mechanically resistant and may require physical support to increase rigidity for some industrial applications (Garcia-Galan *et al.*, 2011).

The rapid development of nanostructured materials has stimulated strong interest in using magnetic nanoparticles to improve the quality of the immobilized enzyme. Recently, magnetic CLEAs have been receiving considerable attention as they can be simply recovered using an external magnetic field and recycled for iterative uses (Kumar *et al.*, 2014; Talekar *et al.*, 2012). Enhanced stability is possible for repeated usage in continuous bioseparations along with enabling greater control over the catalytic process (Cui *et al.*, 2014). Moreover, appropriate surface functionalization provides these magnetic nanoparticles with recognition ability, enables controlled interaction between the magnetic cores with targeted biological species, and offers better aqueous dispersion and biocompatibility (Li *et al.*, 2010). Previous studies on magnetic CLEAs commonly employed magnetite as their magnetic support with amino group 3-aminopropyl triethoxy silane (APTES) being the most favoured functional groups for bonding various bioactive molecules to the nanoparticles (Bhattacharya and Pletschke, 2014; Gunda *et al.*, 2014; Talekar *et al.*, 2012).

Although magnetite has been extensively used, Kang *et al.*, (2007) in their study of human lung cancer A549 discovered that maghemite nanoparticles ($\gamma$- $Fe_2O_3$) had a greater binding specificity compared to magnetite nanoparticles ($Fe_3O_4$). Maghemite was possibly a better adsorbent than magnetite due to its larger active surface area (Netto *et al*., 2013). Maghemite also exhibits a strong magnetic behavior

which has been used practically in various biomedical and biological applications including magnetic resonance imaging (MRI) contrast enhancement, biomagnetic separations and magnetic drug targeting. Such wide applications of maghemite nanoparticles originate from their nontoxicity, biocompatibility, biodegradability, low particle dimension, large surface area and suitable magnetic properties (Kluchova *et al.*, 2009). To fully exploit the advantages of maghemite, it is essential to investigate its potential in stabilizing the cross-linked enzyme aggregates. This work, to our knowledge is the first report of CLEA preparation by introducing silanized maghemite nanoparticles into the free enzyme solution to produce stable and renewable biocatalyst for effective hydrolysis of hemicellulosic material. The results presented here suggest that Xyl-CLEA-silanized maghemite is a promising method for converting hemicelluloses into xylooligosaccharides.

## 1.2 Objectives of Study

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

a) To develop and characterize cross-linked enzyme aggregates (CLEA) of recombinant xylanase with high activity recovery.

b) To determine the best developed cross-linked enzyme aggregates for the hydrolysis of hemicellulose.

c) To investigate the performance of the developed cross-linked enzyme aggregates (Xyl-CLEA-silanized maghemite) in the hydrolysis of hemicellulose to xylooligosaccharides (XOS).

## 1.3 Scopes of Study

This study emphasizes on the preparation design and improvement of stability and hydrolysis of xylanase-cross-linked enzyme aggregates (CLEA) activity on hemicellulose and its reaction process for XOS production. Therefore, the following scopes were outlined to achieve the objectives.

a) Expression, partial purification and characterization of recombinant xylanase (Xyn2) from *Trichoderma reesei* ATCC 58350 in *Pichia pastoris* expression system.

b) Study on the effect of preparation parameters on the activity recovery in CLEA; enzyme to precipitant ratio, types of precipitants, concentration of cross-linker, cross-linker to enzyme ratio, cross-linking time and types of additives.

c) Effect of the most stable and reusable cross-linked enzyme aggregates by introducing bovine serum albumin (Xyl-CLEA-BSA) and co-immobilization with maghemite (Xyl-CLEA-silanized maghemite).

d) Characterizations in terms of biochemical and structure of the developed CLEAs (Xyl-CLEA, Xyl-CLEA-BSA and Xyl-CLEA-silanized maghemite).

e) Screening the effects of several reaction conditions (reaction volume, reaction time, temperature, pH, enzyme loading and substrate concentration) of the best developed CLEA on xylan hydrolysis using one factor at one time method (OFAT).

f) Study the significant factors of reaction conditions (reaction time, temperature, pH, enzyme loading and substrate concentration) of the best developed CLEA on xylan hydrolysis using Two Level Factorial Design.

g) Optimization of the reaction conditions (reaction time, temperature and substrate concentration) of the best developed CLEA on xylan hydrolysis by central composite design (CCD) towards the achievement of highest XOS yield.

h) Kinetic study of the best developed CLEA on xylan hydrolysis.

## 1.4 Rationale and Significance of the Study

Over the years, numerous efforts have been devoted to the development of enzymes for various applications. However, only in very few cases that a biocatalytic transformation on a technical scale works perfectly at the first attempt due to limitations related to process parameters or the enzyme, such as moderate stability (Garcia-Galan *et al.*, 2011) substrate- and product inhibitions, inefficient recycling and high production cost (Cui and Jia, 2015). Enzyme immobilization has been regarded as a promising technology to overcome those limitations. In the past decade, CLEA technology has been increasingly exploited to an extensive selection of hydrolases, lyases and oxidoreductases, for which the CLEAs exhibit superior operational stability, volumetric productivities and recoverability (Sheldon, 2011). The CLEA method is attractive in its simplicity and robustness. Nevertheless, the immobilization of an enzyme may produce different effects on enzyme activity (Garcia-Galan *et al.*, 2011). In the cases of CLEA, the active centre may be blocked by the cross-linking agent or additives used, and the cross-linking procedure may promote diffusion problems. The increased size of CLEAs clusters causes internal mass-transfer

limitations that hamper the catalytic efficiency (Cui and Jia, 2015). The preparation of CLEAs is even more challenging when the surface lysine content which is responsible for effective cross-linking, is initially low. Low cross-linking efficiency leads to major activity loss.

This report is the first in demonstrating the development of three different CLEAs using recombinant xylanase from *Trichoderma reesei*. The conventional CLEAs (Xyl-CLEA) was compared against Xyl-CLEA-BSA and Xyl-CLEA-silanized maghemite in terms of activity recovery, thermal and pH stability as well as reusability. The initially low lysine content in the recombinant xylanase propels the search for stabilization of the CLEAs. Although several other researchers suggested the use of proteic feeder such as BSA to enhance the cross-linking and thus the stability, this study proved otherwise. The present study showed that co-aggregating the xylanase with silanized maghemite (Xyl-CLEA-silanized maghemite) is superior in achieving high activity recovery, enhanced stability and reusability when compared to the others. The use of maghemite ($\gamma$-$Fe_2O_3$) is also unique as common nanoparticles used in CLEA formation is magnetite ($Fe_3O_4$). The findings demonstrated that maghemite is a potential element that could be exploited for the production of stable and reusable CLEAs. The application of CLEAs in hemicellulosic bioconversion still remains elusive as other works use different raw substrates. However, the structural complexity of these xylans of raw sources limits its usefulness for detailed mechanistic studies that are targeted at understanding the mode of action of the enzymes. Therefore, in this report, an established soluble substrate which is beechwood xylan is exploited to gain a better understanding of reaction mechanism of the developed CLEAs. The findings proved that Xyl-CLEA-silanized maghemite is a promising biocatalyst in the conversion of hemicelluloses to xylooligosaccharides.

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