EFFECTS OF FOOD-GRADE CROSS-LINKERS AND ADDITIVES ON THE ENZYMATIC PERFORMANCE OF MAGNETIC NANOPARTICLES CLEA-LIPASE OF *HEVEA BRASILIENSIS*

BY

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ABSTRACT

Skim latex from *Hevea brasiliensis* (rubber tree) is a major waste product from rubber factories which gives out odor and water pollution to the surrounding community. The management of skim latex waste is also very costly as it involves high effluent treatment before being released into the main waterways. However, skim latex contains various beneficial proteins and enzymes that can be potentially useful for industrial applications. Lipase from skim latex serum was recovered and immobilized by cross-linked enzyme aggregates (CLEA) technique, while supported by magnetic nanoparticles termed 'Magnetic Nanoparticles CLEA-lipase' (MNP-CLEA-lipase). Preparation of MNP-CLEA-lipase involves chemical cross-linking of enzyme aggregates with amino-functionalized magnetic nanoparticles. One of the main advantages of this method is, the biocatalyst can be easily recovered by a magnetic field for recycling. Normally, glutaraldehyde is used as the cross-linker, and BSA is used as the additive for CLEA immobilization. However, glutaraldehyde has a small molecular size which can easily penetrate the enzyme's active site. It is also toxic which makes it unsuitable for industrial applications. BSA is overall expensive and will increase the final processing cost of the biocatalyst. This research aims to replace toxic glutaraldehyde with green, non-toxic, and renewable macromolecular cross-linkers (dialdehydic dextran, chitosan, gum Arabic and pectin). It also aims to replace the expensive BSA with low cost, food-grade additives (egg protein, soy protein, and milk protein). The optimized preparation conditions for MNP-CLEA-lipase were determined by the OFAT method followed by FCCCD under RSM. Characterization was conducted in terms of its thermal and pH stabilities, optimum thermal and pH activities, storage stability, and reusability. FTIR and FESEM were conducted to analyze the structural features and the morphological appearances respectively, of the newly produced biocatalyst. Kinetic studies and its application as additives in detergent were conducted. Dialdehydic pectin and soy protein were shown to be the best substitutes for glutaraldehyde and BSA with optimum operating conditions at 180 mg/ml and 0.6% w/v respectively and with 80% saturation $(NH_4)_2SO_4$, giving a Residual Activity of 154%. The optimum temperature for MNP-CLEA-lipase was at 40^{o}C (127% RA) while the optimum pH was at 8 (127% RA). It also resulted in high stability in extreme temperature and pH conditions. It retained 20% Residual Activity after 100 days of storage and the reusability test showed that it maintained 7% RA after the 10th cycle. FTIR results showed that MNP-CLEA-lipase portrayed characteristics originating from silanized MNPs and free lipase. FESEM images showed that MNP-CLEA-lipase with dialdehydic pectin and soy protein was less structured with minimum clumping. MNP-CLEA-lipase achieved 4 times higher catalytic efficiency compared to its free lipase counterpart which means that MNP-CLEA-lipase is more efficient and required less substrate compared to free lipase. For application in commercial detergent, MNP-CLEA-lipase showed high stability in sodium carbonate and sodium bicarbonate mixture (pH 9) for alkalinity agent and Tween 20 for detergent component. It also showed impressive stain removal percentage. Overall, this research showed that value-added products can be recovered from the skim latex, thus minimizing waste management in the natural rubber industries. The production of MNP-CLEA-lipase with enhanced

properties demonstrated that the wasteful by-products can easily be converted to innovative biomaterial which has the potential to be used in many biotechnological applications. By alternating to use low-cost food-grade cross-linkers, such as dialdehydic pectin, and protein additives, such as soy protein, enhanced biocatalyst can be produced which can be safely used in industrial applications.

البحث خلاصة

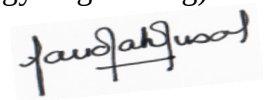
يحتوي اللاتكس المزلج skim latex المستخلص من شجرة *الهيفيا البرازيلية* *Hevea brasiliensis* (شجرة المطاط) على العديد من البروتينات والإنزيمات التي قد تكون مفيدة للتطبيقات الصناعية. نم يلاخلا طاطملا نإف ، كلذ عمو تايافنلل مهم جتنم وه مسدلا فلكم رمأ وهو ةيسيئرلا ةيئاملا تارمملا يف اهقلاطإ لبق ةلئاسلا تايافنلل ةيلاع ةجلاعم أضيأ مسدلا ةعوزنم سكتلالا .أضيأتتم استرداد اللاييز من مصل اللاتكس المزلج skim latex serum وابطاله من خلال تقنية تجميعات الإنزيمات المرتبطة ببعضها (CLEA)، بينما تم دعمه بنانو مغناطيسي يطلق عليه النانوية الممغنطة "CLEA-Lipase" (MNP-CLEA-Lipase)ويتضمن إعدادMNP-CLEA-Lipase الربط الكيميائي بين تجميعات الإنزيمات باستخدام الجزيئات النانوية المغناطيسية الوظيفية. ومن بين المزايا الرئيسية لهذه الطريقة، يمكن استعادة المحفز البيولوجي biocatalyst بسهولة عن طريق المجال المغناطيسي لإعادة التدوير. فبدلاً من استخدام الجلوتارالديهايد كرابط متقاطع، تم فحص الجزيئات (الكبيرة) الماكروية الخضراء وغير السامة والمتجددة للروابط (ديكستران، الكيتوزان، اللبان العربي، البكتين) واختيرت أفضل البدائل لمزيد من التحليل. ةلصوك ديهدلاراتولجلا مادختسا متي ، ةداع ةنكمي ريغصي ئيزجم مجحب ديهدلاراتولجلا نإف ، كلذ عمو .CLEA ةكرحل ةفاضم ةدامك BSA مدختسيو ةعطاقتم أفلكم BSA ربتعي .ةيعانصلا تاقيبطتلل ةبسانم ريغ اهلعجي امم ةماسلا اهنأ امك .ميزنلإا طشنلا عقوملا قارتخا ةلوهسب .يويحلا زفحملا ةيئاهنلا ةجلاعملا ةفلكت نم ديزيس امم ةريغص ةيمك لباقم. وينطبق نفس الشيء على المواد المضافة؛ فقد تم استبدال الاستخدام التقليدي BSA الغالي التكلفة، بإضافات منخفضة للتكلفة من فئة الأغذية (بروتين البيض، وبروتينات الصويا وبروتينات الحليب). وتم تحديد شروط التحضير المحسنة لـ MNP-CLEA-Lipase بواسطة طريقةواحد متغير في وقت واحد OFAT يتبعها طريقة سطح الاستجابة المركزية المركبة المتمركزة حول الوجه FCCCD-RSM. وقد تم إجراء التشخيص من حيث القدرة الحرارية وثبات درجة الحمضي والقاعدي، وأنشطة الحرارة القصوى ونشاط الرقم الهيدروجيني (pH)، واستقرار التخزين وإعادة الاستخدام. وقد أُجريت عمليتي تحويل فوريه للطيف بالأشعة تحت الحمراء(FTIR) والمجهر الإلكتروني للمسح الضوئي للانبعاثات الميدانية (FESEM) لتحليل السمات البنيوية والمظاهر المورفولوجية تباعًا للمحفزات البيولوجية المنتجة حديثًا. وأجريت دراسات حركية وتطبيقاتها كإضافات في المنظف. وقد تبين أن ثنائي ألداهايد البيكتين (Dialdehydic pectin) وبروتينات الصويا هما أفضل البدائل للجلوتارالديهايد وBSA في ظروف التشغيل المثلى عند 180 ملغ/مليلتر و0.6% w/v على التوالي و80% من كبريتات الامونيوم المشبعة saturated $(NH_4)_2SO_4$، مما يعطي النشاط المتبقي 154%. وكانت درجة الحرارة المثلى لـ MNP-CLEA-Lipase عند 40 درجة مئوية (127% RA) بينما كان الرقم الهيدروجيني الأمثل عند 8 (127% RA). كما أدى إلى ثبات مرتفع في درجات الحرارة القصوى وحالات درجة الحموضة. وقد احتفظت بنشاط متبقٍ بنسبة 20% بعد 100 يوم من التخزين وأظهر اختبار إعادة الاستخدام أنها حافظت على نسبة (7% RA) بعد الدورة العاشرة.أظهرت نتائج تقرير تحويل فوريه للطيف بالأشعة تحت الحمراء(FTIR) أن-MNP CLEA-Lipaseصوِّر الخصائص الناشئة من silanized MNPs واللاييز الحر. أظهرت صور FESEM أن-MNP CLEA-Lipaseمع البكتين وبروتينات الصويا كانت أقل تنظيمًا مع الحد الأدنى من التخثر. حقق -MNP-CLEA Lipase كفاءة حفاز أعلى بأربعة أضعاف مقارنة بنظيره من اللاييز الحر مما يعني أن MNP-CLEA-Lipase أكثر كفاءة ويتطلب مادة أقل مقارنة باللييز الحر. وبالنسبة إلى الاستخدام في المنظف التجاري، أظهر-MNP-CLEA Lipaseثباتًا عاليًا في كربونات الصوديوم ومزيج بيكربونات الصوديوم9 لعامل القلوية pH و20 لمكون المنظف، كما أظهر نسبة عالية من إزالة البقع .وفي الإجمال، أظهرت هذه البحوث أن منتجات القيمة المضافة من الممكن استردادها من اللاتكس المزلج، وبالتالي الحد من إدارة النفايات في صناعات المطاط الطبيعي. وقد أظهر إنتاج -MNP CLEA-Lipase مع خصائص محسنة أن المنتجات الثانوية المسرفة يمكن تحويلها بسهولة إلى مواد بيولوجية مبتكرة يمكن استخدامها في العديد من التطبيقات الحيوية. من خلال التبديل لاستخدام روابط متقاطعة ذات درجة غذائية منخفضة

التكلفة، مثل ثنائي ألداهايد البكتين والإضافات البروتينية، مثل بروتين الصويا، يمكن إنتاج المحفزات المعززة التي يمكن استخدامها بأمان في صناعات المشروبات والأغذية.

Dr. Nassereldeen Ahmed Kabbashi 14/12/2020

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology Engineering).



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Firstly, it is my utmost pleasure to dedicate this work to my dear parents and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal. Thank you for your support and patience.

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LIST OF SYMBOLS

A_w	Absorbance of red colored acetone after washing
B_w	Absorbance of red colored acetone before washing
K_m	Michaelis constant
s	Substrate concentration
U	Units
v	Reaction rate
V_{max}	Maximum rate of reaction

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CLEA	Crossed-linked Enzyme Aggregates
CPH	Cocoa pod husk
DMSO	Dimethyl Sulfoxide
DNPH	Dinitrophenyl hydrazine
DRC	Dry rubber content
FCCCD	Face Centered Central Composite Design
LAH	Lipid Acyl Hydrolase
MNP	Magnetic nanoparticles
MNP-CLEA-lipase	Magnetic CLEA-lipase
OFAT	One Factor at a Time
pNPP	p-Nitrophenyl palmitate
pNPB	p-Nitrophenyl butyrate
RSM	Response Surface Methodology

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Natural rubber (cis-1,4-polyisoprene) of *Hevea brasiliensis* is highly used in the latex-based industry to produce tires, latex gloves, latex threads, and rubber catheters (Mohammadi et al., 2010; Tahir & Misran, 2019). Fresh field latex is the main raw material for rubber processing, and it undergoes certain procedures during manufacture to dry rubber or high concentrated latex. The procedure for fresh latex processing involves centrifugation, creaming, and evaporation (Nguyen & Luong, 2012; Ochigbo et al., 2011; Veerasamy & Ismail, 2012). Some of the processes involve chemical treatment which might have negative impacts on the environment (Nguyen & Luong, 2012).

High concentrated latex preparation involves the addition of ammonia to the natural rubber followed by centrifugation to separate the concentrated rubber from the aqueous part. This aqueous part is known as skim latex which is one of the most contaminating wastes from rubber factories (Mohamed & Yusof, 2014). Skim latex is basically pretreated in the effluent treatment oxidation pond before being released into the main water system. Manufacturing skim latex into valuable substances would become an alternative to minimize the discharge of effluent into the main waterways, thus making latex processing more environmentally friendly (Mohammadi et al., 2010; Tahir & Misran, 2019).

Lipid acyl hydrolase (LAH) enzyme is one type of lipase and a useful protein that can be obtained from the skim latex serum (Mohamed & Yusof, 2014). From a study conducted by Mohamed and Yusof (2014), the highest total activity of LAH in skim latex serum is 61.152 U/ml. Lipase enzyme is immobilized by cross-linked enzyme aggregates (CLEA) technology which is an enzyme immobilization technique for biocatalysis without a carrier. Traditionally, CLEA preparation involves protein precipitation using ammonium sulphate and cross-linking by a cross-linker such as glutaraldehyde.

Purification and immobilization processes are combined into a single operation in CLEA to produce highly stable and recyclable catalysts with high catalytic efficiency (Sheldon et al., 2019). However, CLEA works when the enzyme has enough lysine (amine residues) which links the enzyme and the cross-linker. Poor cross-linking might lead to leaching of enzyme into the reaction media and low stability (Mehde et al., 2018; Sousa et al., 2020). CLEA usually involves separation by centrifugation or filtration which would end up with clump formation due to low compression resistance. This might inhibit the internal mass transport of substrate and reduce catalytic activity (Talekar et al., 2012).

Magnetic cross-linked enzyme aggregates (magnetic CLEAs) is a new technique in CLEA technology which has been proven to improve the catalytic activity of CLEA-immobilized enzymes by increasing its cross-linking ability which leads to improved stability and catalytic activity (Gupta et al., 2013; Reza et al., 2010; Talekar et al., 2012). For the preparation of magnetic CLEAs, amino-functionalized magnetic nanoparticles are added to the enzyme solution for immobilization. The MNPs add more amine residues to generate more cross-linking of the enzyme aggregates which increases the mechanical stability of CLEAs and produces non-leachable CLEAs. Magnetic CLEAs have magnetic

properties that helps in the separation of MNP-CLEA-lipase from the reaction mixture using a magnet (Reza et al., 2010; Talekar et al., 2012; Mehde et al., 2018; Sousa et al., 2020). This solves the clump formation problems caused by centrifugation or filtration and makes recycling the immobilized enzyme much easier.

Cross-linking agents play an important role in locking the enzyme aggregates which form the solid biocatalyst. Glutaraldehyde is the common type of cross-linker used for CLEA technology due to its low price and availability in commercial quantities (Sheldon, 2019). However, glutaraldehyde is not compatible with certain enzymes such as nitrilases which leads to low activity retention. Also, glutaraldehyde's small-sized molecule and high reactivity allow it to leach into the enzyme's active site which would lead to low activity retention (Nadar et al., 2015; Sheldon, 2019). Nadar et al. (2015) and Gupta et al. (2013) discovered that macromolecular cross-linkers such as pectin, dextran, chitosan, and gum Arabic can replace glutaraldehyde as cross-linkers in CLEA-immobilization while increasing the immobilized enzyme's activity. They are also non-toxic and would not harm the environment. Therefore, other cross-linking agents namely chitosan, dextran, pectin, and gum Arabic were screened to choose the most suitable cross-linking agent that can replace toxic glutaraldehyde.

Furthermore, additives are used in CLEA immobilization to stabilize enzyme activity and to cater extra lysine to react with the cross-linker (Roessl et al., 2010). Additives can also prevent the leaching of cross-linking agents into the enzyme's active site. However, common additives such as bovine serum albumin (BSA) are expensive and can increase the final cost of the biocatalyst (Torres-Salas et al., 2011; Mafra et al., 2018). Karimpil et al. (2011), Mafra et al. (2018), and Veteikyte et al. (2017) discovered cheaper

food-grade additives such as soy protein, milk protein, and egg white protein that can replace BSA as an additive in CLEA-immobilization. These additives are also rich in lysine residues which can facilitate cross-linking of enzymes. Therefore, alternatives for BSA which are low cost, easily available, and non-toxic such as soy protein, milk protein, and egg protein were screened. The best additive that can replace BSA is chosen for further analysis. The use of macromolecular cross-linker and food-grade additives could be a discovery in producing eco-friendlier and more cost-effective MNP-CLEA immobilized enzymes with enhanced stability.

1.2 PROBLEM STATEMENT

Skim latex is one of the major sources of waste from rubber manufacturing factories together with other waste products such as uncoagulated latex and washings from certain processing stages (Mohammadi et al., 2010; Tahir & Misran, 2019). Besides that, skim latex is a by-product of high concentrated latex processing originated from field latex which has been treated with ammonia. This waste releases a disturbing ammoniated smell. Skim latex is pretreated in oxidation ponds for effluent treatment before it is discarded into the main water system. However, the process incurred in treating skim latex is quite expensive (Mohamed & Yusof, 2014; Tahir & Misran, 2019). Therefore, utilizing skim latex for research and developing it into value-added products could help lessen the pollution created by skim latex waste.

In CLEA technology, glutaraldehyde is the most frequently used cross-linker due to its low cost, easy to handle and it can form covalent bonds with various enzymes (Nadar et