



**PRODUCTION OF CROSS- LINKED ENZYME
AGGREGATES (CLEA) AMYLASE FROM COCOA POD
HUSK (CPH) FOR DETERGENT APPLICATION**

BY

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ABSTRACT

In Malaysia, large quantities of Cocoa Pod Husk (CPH), a by-product of cocoa industry are produced and generally go to waste, leading to problem in waste management. CPH was known to contain several enzymes and amylase is one of them. Knowing the potential of amylases has in several industries, this research aims to optimally extract amylase from CPH, and this was followed by immobilization via Cross-linked Enzyme aggregate (CLEA) technology for further stabilization. It is pertinent to maximize the extraction yield and to optimize the extraction process. For that matter, One-Factor-At-a-Time (OFAT) strategy was first carried out to determine the maximum values of the process parameters, and this was followed by adopting a Face Centered Central Composite Design (FCCCD) strategy under the Response Surface Methodology (RSM). The effect of independent parameters, namely, the concentration of buffer, the pH of buffer and the concentration of CPH (w/v %) of the extraction process on amylase activity was studied. Further on, the most active CLEA-amylase was prepared using the same statistical strategy, that is, OFAT followed by FCCCD on three independent parameters, and they are the concentration of three moieties, namely, acetone (as precipitant), glutaraldehyde (as cross-linker) and bovine serum albumin, (BSA) (as additive). The characterization of CLEA-amylase was compared with free amylase in terms of pH and temperature optimum, pH and temperature stabilities and kinetics parameters. Reusability of CLEA-amylase was also conducted. CLEA-amylase was analyzed by FESEM for morphology, FTIR for structural identity and finally applied to detergent for the capability in stain removal. The results show that the highest amylase activity (11.48 U/ml) was observed when the extraction was conducted with sodium phosphate buffer pH 7, 150 mM buffer with 7% (w/v) CPH. The highest activity in CLEA-amylase, with 92.24% recovery based on free enzyme activity, was when the preparation was done using 50% acetone, 60mM glutaraldehyde and 1.2 mg/ml BSA. The optimum temperature of amylase increases from 45 to 65°C and the optimum pH changes from 7 to 9 when immobilized. A systematic study of the stability of CLEA and free enzyme was taken with regards to temperature (25-100°C) and pH (5-12), and for both factors, CLEA-amylase showed higher stability than free amylase. Assuming that the enzymatic reactions follow the Michaelis-Menten kinetic, the K_M of free amylase and CLEA-amylase are 0.675 and 1.0443 respectively, whereas the V_{max} for free and CLEA-amylase are 3.44 and 2.71 respectively. The prepared CLEA-amylase was found to be able to be recycled six times, after which it still retained 30.87 % of its former activity. For applying in detergent, CLEA-amylase showed high stability against several detergent components and alkalinity agents and its addition in enzyme-free commercial detergent had improved the stain removal. In short, this study could be a stepping stone for the production of immobilized amylase in large scale so that it could be used in wider industrial applications. Future studies should incorporate different types of precipitating agents, as well as, the cross-linker to further optimize the CLEA preparation.

ملخص البحث

في ماليزيا، تُنتج كميات كبيرة من قشور بذر الكاكاو (CPH) - وهو نتاج ثانوي لصناعة الكاكاو - تذهب جلها إلى النفايات، مما يفاقم مشكلة إدارة تلك النفايات. وكان من المعروف أن قشور بذر الكاكاو تحتوي على العديد من الإنزيمات، ومنها الأميليز. وبحكم معرفتنا بإمكانيات الأميليز في العديد من الصناعات، فإن هذا البحث يهدف إلى استخراج الأميليز على النحو الأمثل من قشور بذر الكاكاو، وثم تسكينه عن طريق تكنولوجيا التجميع عبر ربط الإنزيم (CLEA) لمزيد من الاستقرار.

وبما أنه من المناسب تحقيق أقصى قدر من استخراج العائد وتحسين عملية الاستخراج، لذلك، تم تنفيذ استراتيجية عامل واحد في كل وقت (OFAT) لتحديد القيم القصوى لمعاملات العملية. وأعقب ذلك اعتماد استراتيجية التصميم المركزي المركب (FCCCD) في إطار منهجية منهجية استجابة السطح الخارجي (RSM). تم دراسة تأثير المعاملات المستقلة، وهي تركيز الصائل، ودرجة الحموضة (pH) من الصائل وتركيز CPH (%w/v) من عملية الاستخراج على نشاط الأميليز. وعلاوة على ذلك، تم إعداد CLEA-amylase الأكثر نشاطاً باستخدام نفس الاستراتيجية الإحصائية، وهي OFAT تليها CCD، وذلك على ثلاثة معايير مستقلة، بتركيز الأنصاف الثلاثة، وهي الأستون (كمربسب)، غلوتارالدهايد (كرباط عابر) وزلال المصل البقري (BSA) (كمضاف). تم مقارنة توصيف CLEA-amylase مع الأميليز الحر من حيث درجة الحموضة (pH) ودرجة الحرارة المثلى، ودرجة الحموضة (pH) ودرجة الحرارة المستقرة ومعاملات الحركة. إعادة استخدام CLEA-amylase تمت كذلك. حيث تم تحليل CLEA-amylase بواسطة FESEM للتشكل، FTIR لتحديد الهوية الهيكلية وتطبيقها أخيراً على المنظفات للقدرة في إزالة البقع.

أظهرت النتائج أن أعلى نشاط للأميليز (11.48 U/ml) لوحظ عند إجراء الاستخراج مع فوسفات الصوديوم العازل PH7، 150mM العازل مع 7% (w/v) CPH. أعلى نشاط في CLEA-amylase، عند 92.24% استرجاع استناداً إلى نشاط الإنزيم الحر، كان عندما تم إعداده باستخدام 50% أستون، 60mM غلوتارالدهايد و 1.2 مغ/مل BSA. درجة الحرارة المثلى للأميليز ازدادت من 45 إلى 65 درجة مئوية، وتغيير درجة الحموضة pH المثلى من 7 إلى 9 عندما يجمد. أجريت دراسة منهجية لاستقرار CLEA والإنزيم الحر فيما يتعلق بدرجات حرارة بين (100-25 درجة مئوية) ودرجات حموضة pH عند (5-12) وفي كلا العاملين، أظهر CLEA-amylase استقراراً أعلى من الأميليز الحر. وعلى افتراض أن ردود الفعل الأنزيمية تتبع حركية مايكليس-منتن، كانت قيمة KM من الأميليز الحر و CLEA-amylase قدرت بـ 0.675 و 1.0443 على التوالي، في حين أن قيمة Vmax للأميليز الحر و CLEA-amylase هي 3.44 و 2.71 على التوالي. اتضح أن CLEA-amylase المعدة كانت قادرة على أن تكون معادة التدوير 6 مرات، وبعد ذلك لا تزال تحتفظ بـ 30.87% من نشاطها السابق. وفي تطبيقها في المنظفات، أظهرت CLEA-amylase استقراراً عالياً ضد العديد من عناصر المنظفات والمعاملات القلوية، وعند إضافتها للمنظفات التجارية لخالية من الإنزيم، فقد تحسنت إزالة البقع.

باختصار، يمكن لهذه الدراسة أن تكون نقطة انطلاق لإنتاج الأميليز المسكن على نطاق واسع بحيث يمكن استخدامها في التطبيقات الصناعية الكبرى. وينبغي أن تشمل الدراسات المستقبلية أنواع مختلفة من العوامل المترسبة، وكذلك الوصلات المتقاطعة لزيادة تحسين إعداد CLEA.

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 dissertation for the degree of Master of Science (Biotechnology Engineering).

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Erry Yulion Triblas Adesta
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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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DEDICATION

To Almighty Allah, the ever living, the self-subsisting,

To the ever-supportive role and trust of my parents,

To the efforts and tutelage of my supervisors and lecturers,

I dedicate this little achievement of mine

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variables
BSA	Bovine Serum Albumin
CLEA	Cross-Linked Enzyme Aggregates
CLEC	Cross-Linked Enzyme Crystal
CPH	Cocoa Pod Husk
ES	Enzyme-substrate complex
FCCCD	Face Centered Central Composite Design
FE-SEM	Field Emission Scanning Electron Microscopy
G	Gravity
GA	Glutaraldehyde
PBS	Phosphate Buffer Saline
RCF	Relative Centrifugal Force
RPM	Revolutions per Minute
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate

LIST OF SYMBOLS

k_1	Rate constant of the forward reaction of E+S
k_{-1}	Rate constant of the reverse reaction where ES falls apart to E+S
k_2	Rate constant of the forward reaction of ES forming E+P
K_m	Michaelis-Menten kinetic constant
v	Volumetric rate
R^2	Regression coefficient
S	Substrate concentration
U	Unit enzyme
V_{max}	Maximum rate of reaction

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

A challenging problem facing food, pharmaceutical, and other industries in the 21st century is to minimise and avoid the generation of waste and hazardous materials. Large amounts of these wastes create disposal problems. One solution is to dispose of such waste by manufacturing useful by-products effectively.

Cocoa husk (*Theobroma cacao* L.) is an important crop in many tropical countries. In chocolate manufacturing, cosmetic and pharmaceutical preparation, cocoa beans are used and have a massive value (Kalvatchev et al., 1998). Malaysia is among the top ten chocolate industries in the world and the largest producer in South East Asia after Indonesia for extracting the beans from mature cocoa fruit and using it in the production of chocolate. However, the cocoa husk is discharged as waste. Since the increased use of cocoa beans generates increasing wastes, there is a need to find beneficial solutions for this by-product (Redgwell et al., 2003; Serra Bonvehì & Escolà Jordà, 1998). Using cocoa pod husks as a low-cost unconventional feed ingredient for livestock nutrition could reduce feed costs by replacing some of the expensive conventional feed ingredients used in ration formulation (Atuahene et al., 1984; Sobamiwa, 1998).

The cocoa pod husk has been studied for its compositions as a potential source of amylase and other enzymes (Khanahmadi, 2015). Amylase is an enzyme that can catalyse the hydrolysis of starch into sugars. They are widely distributed in animals, plants and microbes. Due to its prime importance, various microbial amylases have been introduced commercially and use of amylases as biocatalysts has almost overcome

chemical catalysts in starch processing. The 3-D structure which enables substrate binding and supports the cleavage of the glycoside links through the action of unique catalytic groups can be manipulated. Amylase plays important roles in various areas, especially in food manufacturing, beverages, brewing, textile and detergent industries (Souza & de Oliveira Magalhaes, 2010; R. Gupta et al., 2003).

Bioprocess technology uses biocatalysts. In many sectors, biocatalyst is welcomed for their easy production, specificity, and harmlessness to the environment. However, research is needed to commercialise these bio-derived catalysts and reduce costs. In doing so, it is necessary to maintain the structural stability. Therefore, despite the cost of the process, immobilised enzymes have functional efficiency and upgrade reproducibility. Enzymes or the whole cells can be immobilised biocatalysts (Kawaguti et al., 2006). Researches has been carried out to produce immobilized enzymes with good performance, and one way to do this is to study the optimum process conditions on the effective method of immobilized enzymes preparation.

A cross-linked enzyme aggregate (CLEAs) is a novel, versatile and effective methodology for enzyme immobilisation. The mechanism is an easy concept that involves precipitation of the enzyme from the aqueous buffer by cross-linking of the resulting physical aggregates of enzyme molecules. It can be applied in a large category of enzymes, including recyclable catalysts with high confinement activity, cofactor-dependent oxidoreductases and lyases, and allow good stability, sometimes higher than the free enzyme derived thereof. The enzyme no needs to be purified. As such, any method needs to combine the purification and immobilisation in one step to make it practical (Scheldon, 2009). Immobilising the enzymes using CLEA can be applied to most sources of protein.

For this study, to obtain aggregates, the enzyme is precipitated. For the following step, the enzyme is cross-linked using glutaraldehyde (Sheldon, 2011). However, the chemistry is not fully understood. Cross-linking appears after the rebound of amino groups of lysine residues on the neighbouring enzyme molecules, with polymers or oligomers of glutaraldehyde resulting from inter- and intramolecular aldol condensations. Cross-linking can associate both Michael-type 1, 4-addition to α,β -unsaturated aldehyde moieties and Schiff's base formation. The cross-linking mode is also pH dependent (Walt & Agayn, 1994; Migneault et al., 2004).

Due to environmental concerns, the source of enzyme can be extracted from the large amount of natural waste meant for disposal. The largest applications of industrial enzymes are being used in the detergent in terms of volume or value due to the various benefits, such as the efficient removal of stains. Furthermore, the improvements of engineered version of the traditional detergent industry in particular the compatibility of the enzyme with the composition of the detergent, the ability to function in low temperatures, the stability properties have been reported. To this end, CLEA-amylase has been investigated for its performance and ability to remove stains to save energy and cost in laundry industry and dishwasher.

1.2 PROBLEM STATEMENT

Cocoa (*Theobroma cacao* L.) fruits are valued in Malaysia due to the value of its beans and seeds to synthesise highly demanded products such as chocolate made from cocoa powder and butter. The cocoa fruits produce a large quantity of cocoa pod husks which are rejected as trash. This organic trash is dumped in a landfill. Large amounts of waste pose the problem of disposal and environmental pollution. In recent years, owing to the drive to prevent pollution of the environment as well as for economic motives, novel

strategies for trash processing and treatment have emerged for the recovery, bioconversion and utilisation of valuable constituents from fruit processing wastes. In many cases, these can be transformed into raw materials (Laufenberg, 2003).

Free enzymes as biochemical catalysis are disadvantageous in terms of the cost, effectiveness, stability and reusability, especially if they are to be used in the industrial settings. However, an amelioration of the problem can be carried out by enzyme immobilizations. By immobilization, these enzymes are cross-linked upon precipitation to construct particles of about 50-100 nm diameter (Lopez-Serrano et al., 2002; Kaul et al., 2007).

Recently, the proliferation of untapped enzyme immobilisation provides novel technologies. The immobilisation of enzymes aims to improve the cost of biocatalytic procedures. Immobilisation permits the reuse of the enzyme for a prolonged period and makes it easier to partition the catalyst from the product (Urszula Guzik, 2014).

Previous studies have been done for CLEA-lipase and protease extracted from cocoa pod husk for potential in biodiesel industry. In this study, we focus on CLEA-amylase for future use in detergent industry. For better performance, the preparation of CLEA-amylase is optimised and stabilised for effective application.

1.3 SCOPE OF THE STUDY

In this project, enzymatic proteins were extracted from cocoa pod husks (CPH). The extraction method of the targeted enzyme is optimised to get the highest enzyme activity. The enzyme is then immobilised using the CLEA approach, and the preparation is optimised. The performances of CLEA enzyme is determined based on their stabilities (broad pH and temperature), optimum activities (pH and temperature), kinetic

parameters (K_M and V_{max}) and reusability. All the optimum and characterised conditions of preparing CLEA-amylase are applied for stain removal in detergents.

1.4 RESEARCH OBJECTIVES

The objectives of this research are as follows:

- i. To screen and optimise the extraction process condition of crude amylase from cocoa pod husks.
- ii. To screen and optimise the process conditions of CLEA-amylase production in the conical flask as a batch reactor.
- iii. To characterise the produced conditions of CLEA-amylase through kinetic studies, optimum pH and temperature, stability and reusability.
- iv. To evaluate the performance of CLEA-amylases produced for detergents.

1.5 ORGANIZATION OF THE DISSERTATION

This dissertation consists of five chapters. Chapter one commences with a brief background about the research including the importance of choosing cocoa pod husks as a source of the enzymes for its richness of highly active enzymes and to avoid dumping this waste into the environment. The chapter details the benefits of the immobilisation technique. Furthermore, the problem statement, research objectives, scope and significance of the study are described.

Chapter two reviews the studies conducted in the field of extraction of enzymes, different types of immobilisation methods, characterisations and applications. A brief introduction explains the reason for choosing cocoa pod husk as a source of enzymes and the significant effect of reducing the discharge of this wasteful by-product into land or water. In addition, amylase enzymes extracted from cocoa pod husk are reviewed

with its importance and applications. The primary target of this study is to immobilise the enzymes by cross-linking enzyme aggregates. Hence, a brief background is given on the history of immobilisation and the different methods of immobilisation that lead at the end to cross-linking. The influence of the process parameters are discussed, and importance and uses of the cross-linked enzyme aggregates (CLEA) are detailed. CLEA is characterised in terms of pH and, thermal stabilities, determining the optimum pH and the optimum temperature, and the reusability of CLEA which is considered as one of the most important characteristics of CLEA. Chapter two also reviews the different analytical software used to analyse the optimisation of CLEA preparation, as well as the hyperbolic regression software used to determine the kinetic parameters (V_{\max} and K_m) using four different models including hyperbolic regression. The last part of the chapter covers the applications of cross-linked enzyme aggregates CLEA reported in previous studies and their application in different fields.

Chapter three details the materials, chemicals, equipment, and apparatus used in this study. It also describes the procedure followed in this research starting from extraction, screening of hydrolases, optimisation of immobilisation, characterisation, kinetic studies and finally the application.

The results and discussion of this research are described in chapter four beginning with the preparation of crude enzyme solution from cocoa pod husk and the protein content from crude extracted. The activity of amylase enzyme is calculated using the assay by focusing on the screening of optimum condition of the extraction of amylase from CPH. SDS-PAGE helped in characterising this enzyme by molecular weight determination. Lab scale optimisation is carried out using response surface methodology. Four design experiments are conducted for amylase enzyme. Results are analysed using ANOVA, and the design is validated. The optimised products are