



PRODUCTION OF CROSS LINKED LIPASE FROM
COCOA POD HUSK

BY

SOOFIA KHANAHMADI

A dissertation submitted in fulfilment of the requirement for
the degree of Master of Science (Biotechnology
Engineering)

Kulliyyah of Engineering
International Islamic University Malaysia

FEBRUARY 2016

ABSTRACT

Cocoa pod husk (CPH) is a by-product obtained after removal of cocoa beans from the cocoa fruit which causes many environmental problems. The analysis of CPH has shown that this waste material contains high amounts of protein. Hydrolase is one of the products that can be extracted from the CPH protein. Compared to other enzymes, hydrolases are more frequently used industrially. However, enzymes are biocatalysts that are quite unstable. Hence, cross linked enzyme aggregate (CLEA) is a known powerful tool for improving enzyme performance, stability, selectivity and reusability. In this study, screening and extraction of lipase from cocoa pod husk was performed. The optimum condition of lipase extraction is used for preparation of cross-linked enzyme aggregate. The characterization of free and CLEA lipase regarding stability, reusability and kinetic was carried out and finally CLEA-lipase was applied for biodiesel production from *Jatropha curcas* oil. In all three stages the optimum condition was achieved using Face centered central composite design (FCCCD) with response surface methodology (RSM). From 20 runs the highest activity for extraction of lipase was 11.43 U/ml (around 2.5 fold increase in lipase production) under the condition of 50mM sodium phosphate buffer pH8 with the ratio of 7% (w/v) CPH. The highest activity for CLEA-lipase at the presence of using 20% saturated ammonium sulfate, 60 mM glutaraldehyde as cross-linker and 0.17 mM bovine serum albumin as feeder. The optimal reaction temperature and pH value in enzymatic reaction for both crude enzyme and immobilized were found to be 45°C at pH 8 and 60°C at pH 8.2, respectively. A systematic study of the stability of CLEA and crude enzyme was taken with regards to temperature (25-60 °C) and pH (5-10) value and in both factors, CLEA-lipase showed more stability than free lipase. The K_m value of CLEA was higher compared to free enzyme (0.55 mM vs. 0.08mM). The CLEA retained more than 60% of the initial activity after 6 cycles of reuse compared to free enzyme. Structural characterization of CLEA-lipase by Field Emission Scanning Electron Microscope (FE-SEM) revealed that CLEA from CPH have spherical appearance. Application of CLEA-lipase for biodiesel production was done and the optimum levels of oil-to-ethanol molar ratio, catalyst loading, reaction temperature, agitation and reaction time were found to be 1:6, 3 (w/w%), 45°C, 200 rpm and 3 h respectively with 93.86% conversion of Free fatty acid (FFA) in to biodiesel which is obtained from GC-MS. In conclusion, the development of this process would be an alternative source for immobilized lipase production in large scale and cost effective in terms of using a wasteful by-product to produce a recyclable biocatalyst that has a wide range of applications. This study can add for more information on the application of low cost oil (*J. Curcas* oil), ethanol and using low-cost catalyse (CLEA-lipase from CPH), for the production of renewable biodiesel.

خلاصة البحث

تعتبر قشور الكاكاو (CPH) من النواتج الثانوية لعملية إزالة حبوب الكاكاو من ثمرة الكاكاو ويؤدي رميها بدون معالجة إلى العديد من المشاكل البيئية. حيث أظهرت التحاليل أن نفايات قشور الكاكاو تحتوي على كميات عالية من البروتين. ومن أهم منتجاتها هو الهيدروليز المستخلص من بروتين قشور الكاكاو. بالمقارنة مع الإنزيمات الأخرى، فإن أنزيمات التحلل المائي (الهيدروليز) هي الأكثر استخداماً في مجال الصناعة. ومع ذلك فإن محفزات حيوية (الإنزيمات) غير مستقرة نوعاً ما. وبالتالي، يعتبر ربط المجموع الإنزيمية بواسطة العبور (CLEA) بمثابة أداة قوية معروفة لتحسين أداء الإنزيم واستقراره وانتقائيته وإعادة استخدامه. في هذه الدراسة تم فحص واستخراج انزيم الليباز من قشرة الكاكاو للمرة الأولى. حيث استخدمت الظروف المثلى لاستخراج انزيم الليباز لإعداد المجموعات الإنزيمية المترابطة بالعبور (CLEA). تم تحديد خصائص الانزيم بشكله الحر والمترابط فيما يتعلق بالاستقرار وإعادة الاستخدام والدراسة الحركية، وفي نهاية الدراسة استخدم الليباز المترابط بالعبور لإنتاج وقود الديزل الحيوي من زيت *Jatropha curcas*. وفي المراحل الثلاثة للدراسة تم حساب الظروف المثلى للعمليات الثلاثة باستخدام تصميم *Face centered central composite (FCCCD)* تحت منهجية استجابة السطح (RSM). حيث وجد أن أعلى نشاط لاستخراج الليباز كان في التجربة رقم 20 حيث تم الحصول على 11.43 وحدة / مل (حوالي 2.5 أضعاف لليباز المنتج) باستخدام الظروف المثلى وهي: 50 ملي مولار من فوسفات الصوديوم والرقم الهيدروجيني قيمته 8 و نسبة 7% (كتلة/حجم) من قشور الكاكاو. إن أعلى نشاط لليباز المترابط بالعبور تم الحصول عليها باستخدام 20% كبريتات الأمونيوم المشبعة و 60 ملي مولار للغلوتارالدهيد باعتباره عامل للعبور و 0.17 ملي مولار من بروتين BSA كمادة مغذية. ووجدت درجة الحرارة المثلى للتفاعل وقيمة الرقم الهيدروجيني لكل من الانزيم الحر والانزيم المترابط بقيمة 45 درجة مئوية في درجة حموضة 8، و 60 درجة مئوية في درجة حموضة 8.2، على التوالي. وتم دراسة استقرار الانزيم الخام والمترابط دراسة منهجية فيما يتعلق بدرجة الحرارة (25-60 درجة مئوية) و نسبة الحموضة (5-10)، حيث أظهر الليباز المترابط بالعبور مزيداً من الاستقرار مقارنة بالليباز الحر. وفي الدراسة الحركية، كانت قيمة K_m لليباز المترابط بالعبور أعلى من الانزيم الحر (0.55 و 0.08 ملي مولار). واحتفظ الليباز المترابط بالعبور بأكثر من 60% من نشاطه الأولي بعد 6 دورات من إعادة الاستخدام. حيث بين التوصيف الهيكلي لليباز المترابط بالعبور مظهره الكروي بواسطة FE-SEM. وقد تم تطبيق الليباز المترابط بالعبور لإنتاج وقود الديزل الحيوي وتم إيجاد المستويات المثلى وهي: 6:1 النسبة المولية للزيت-إلى-الإيثانول و (w/w%) 3 المحفز الحيوي ودرجة حرارة التفاعل بمقدار 45 درجة مئوية وسرعة الخلط 200 دورة في الدقيقة و وقت التفاعل 3 ساعات. مع نسبة تحويل 93.86% من FFA إلى وقود الديزل الحيوي الذي تم فحصه بواسطة GC-MS. ختاماً إن تطوير هذه العملية ستكون مصدراً بديلاً لإنتاج الليباز المثبط في نطاق واسع وبفعالية اقتصادية لإنتاج محفز حيوي من منتج ثانوي قابل لإعادة التدوير وله تطبيقات عديدة في مجالات مختلفة. و بإمكان هذه الدراسة إضافة المزيد من المعلومات حول عملية تحويل نفايات الزيت منخفضة التكلفة (زيت *J. Curcas*) والإيثانول واستخدام محفز منخفض التكلفة (CLEA-الليباز من CPH) في تطبيق إنتاج وقود الديزل الحيوي المتجدد.

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 Biotechnology Engineering

.....
Faridah Yusof
Supervisor

.....
Azura Amid
Co-Supervisor

I certify that I have 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 Biotechnology Engineering.

.....
Nassereldeen Ahmed Kabbashi
Internal Examiner

.....
Mohamed Elwathig Saeed Mirghani
Internal Examiner

This dissertation was submitted to the Department of Biotechnology Engineering and is accepted as a partial fulfillment of the requirements for the degree of Master of Biotechnology Engineering.

.....
Faridah Yusof
Head, Department of
Biotechnology Engineering

This dissertation was submitted to the Kulliyah of Engineering and is accepted as a partial fulfillment of the requirements for the degree of Master of Biotechnology Engineering.

.....
Md Noor Hj Salleh
Dean, Kulliyah of Engineering

DECLARATION

I hereby declare that this dissertation 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.

Soofia Khanahmadi

Signature.....

Date.....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

**DECLARATION OF COPYRIGHT AND AFFIRMATION
OF FAIR USE OF UNPUBLISHED RESEARCH**

Copyright © 2016 by International Islamic University Malaysia. All rights reserved.

PRODUCTION OF CROSS LINKED LIPASE FROM COCOA POD HUSK

I hereby affirm that the International Islamic University Malaysia (IIUM) holds all rights in the copyright of this Work and henceforth any reproduction or use in any form or by means whatsoever is prohibited without the written consent of IIUM. No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder.

Affirmed by Soofia Khanahmadi

.....
Signature

.....
Date

ACKNOWLEDGEMENTS

In the name of Allah the most Merciful and the most Compassionate

Alhamdulillah, all praise to Allah SWT for His blessings and mercy, for the successful completion of this research work.

I am highly appreciative of all the invaluable inputs of my supervisor Prof. Dr. Faridah Yusof for her patience, motivation, enthusiasm and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master study. I would like to thank Assoc. Prof. Dr. Azura Amid for her constructive suggestions, advice and guidance through the research period.

I appreciate all the support from the staff of Biotechnology Engineering Department for their valuable support during my research. I would like to express my deep gratitude to my colleagues Fatemeh, Jannah, Nafeesa, Safa, Vivi, Emy, Harmen and Johan for their continuous support, suggestions and advices. I would like to thank Dr. Alireza Rafieerad, who as a good friend was always willing to help and give his best suggestions.

Last but not least, I would like to express my deepest appreciation to my parents and my beloved sister, who have been a source of encouragement and inspiration to me throughout my life. My deepest gratitude and love for their dedication and the many years of support during my undergraduate and master's studies that provided the foundation for this work.

TABLE OF CONTENTS

Abstract	ii
خلاصة البحث	iii
Approval Page.....	iv
Declaration	v
Copyright Page.....	vi
Acknowledgments	vii
Table of Contents	viii
List of Tables	xii
List of Figures	xiv
List of Symbols	xvii
List of Abbreviations	xviii
CHAPTER ONE: INTRODUCTION	1
1.1 Background.....	1
1.2 Problem Statement.....	5
1.3 Scope of Research.....	6
1.4 Significance of Research	6
1.5 Research Objectives.....	8
1.6 Dissertation Organization	8
CHAPTER TWO: LITERATURE REVIEW	11
2.1 Introduction.....	11
2.2 Cocoa Pod Husk	12
2.3 Enzyme and Industrial Enzymes Applications	15
2.4 Hydrolases	19
2.4.1 Lipases and Their Characteristics	20
2.4.2 Structure of Lipase	21
2.4.3 pH Optima and Stability of Lipase	23
2.4.4 Application of Lipase in Industries.....	23
2.4.4.1 Lipases in Drug Industry	25
2.4.4.2 Lipases in Laundry Detergent.....	25
2.4.4.3 Lipases in Pulp and Paper Industry	26
2.4.4.4 Lipases in Oleochemical Industry	26
2.4.4.5 Lipases in Food Industry	27
2.4.5 Factors Influencing the Extraction Process of lipase	28
2.4.5.1 Temperature of Extraction Process.....	28
2.4.5.2 pH of Extraction Buffer	29
2.4.6 Centrifugation to Obtain Crude Enzyme Solution.....	31
2.5 Protein Content	31
2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	32
2.7 Immobilization Technique.....	33
2.7.1 Types of Enzyme Immobilization Techniques	35
2.7.1.1 Support Binding.....	35
2.7.1.2 Entrapment.....	36

2.7.1.3	Cross-linking of Enzyme Aggregates or Crystals	37
2.7.2	Overview of Cross-linked Enzyme Aggregate (CLEA)	38
2.7.3	Factors Influencing CLEA Preparation.....	42
2.7.3.1	Effect of Precipitant on CLEA Preparation	42
2.7.3.2	Effect of Cross-Linker on CLEA Preparation	44
2.7.3.3	Effect of Temperature on CLEA Preparation.....	47
2.7.3.4	Effect of Shaking Rate on CLEA Preparation.....	47
2.7.3.5	Effect of Cross-Linking Time on CLEA Preparation.....	47
2.7.3.6	Effect of pH on CLEA Preparation	48
2.7.3.7	Effect of Additive on CLEA Preparation	48
2.7.4	Enzyme Activity of Free-Enzyme and CLEA	50
2.7.5	Stabilities of CLEA	52
2.7.5.1	Thermal Stability	53
2.7.5.2	pH Stability	53
2.7.6	Optimum pH and Temperature of CLEA.....	54
2.7.6.1	Effects of pH on The Enzyme Activity	54
2.7.6.2	Effects of Temperature on the Enzyme Activity	55
2.7.7	Reusability of CLEA.....	56
2.8	Kinetic of Enzyme	56
2.8.1	Enzymes Catalyzing Kinetics Based on Michaelis-Menten Kinetics Models	57
2.8.2	Lineweaver-Burk Plot (Double-Reciprocal Plot)	60
2.8.3	Eadie-Hofstee Plot	60
2.8.4	Hanes-Woolf (Langmuir) Plot	61
2.9	Application of Software in Experimental Study.....	61
2.9.1	Design of Experiment	61
2.9.2	Response Surface Methodology.....	62
2.9.3	Hyperbolic Regression Software	63
2.10	Overview of Biodiesel	63
2.10.1	Source of Biodiesel	65
2.10.2	Composition of Biodiesel	66
2.10.3	Chemical Structure of Vegetable Oils.....	67
2.10.4	Transesterification Reaction (Alcoholysis).....	70
2.10.5	Use of Crude Jatropha Curcas L. Oil in Biodiesel Production ...	74
2.10.6	Ethanol Versus Methanol in Biodiesel Production.....	79
2.10.7	Enzyme-Catalyzed Transesterification	80
2.10.8	Plant Lipase Versus Microbial Lipase.....	83
2.10.9	Immobilization of Lipase in Biodiesel Industry.....	84
2.10.10	Parameters Influence on Biodiesel Production	86
2.10.10.1	Molar Ratio of Alcohol to Oil	86
2.10.10.2	Enzyme Loading.....	87
2.10.10.3	Reaction Temperature.....	87
2.10.10.4	Reaction Time.....	88
2.10.10.5	Agitation	89
2.10.11	Biodiesel Characterization and Standardization	89
2.11	Summary of Chapter Two	90

CHAPTER THREE: RESEARCH METHODOLOGY	91
3.1 General Overview	91
3.2 Flow Chart	92
3.3 Chemical and Reagents	93
3.4 Equipment and Instruments	93
3.5 Extraction of Crude Sample From Cocoa Pod Husk	93
3.6 Lipase Activity Assay	94
3.7 Protein Concentration	94
3.8 Experimental Design for the Optimum Extraction of CPH.....	96
3.9 Molecular Weight (SDS-PAGE)	98
3.10 CLEA Preparation	100
3.10.1 Screening of Various Additives	103
3.10.2 Experimental Design of Preparation of CLEA-lipase.....	103
3.11 Biochemical Characterization of CLEA-lipase	105
3.11.1 Determination of Kinetic Parameters.....	105
3.11.2 Optimum Activities.....	105
3.11.3 Stability Tests.....	106
3.11.3.1 Temperature Stability	106
3.11.3.2 pH Stability.....	106
3.11.4 Reusability of CLEA-Lipase	106
3.12 Structural Characterization by Field Emission Scanning Electron Microscopy (FE-SEM)	107
3.13 Applications of CLEA-lipase in Transesterification Reaction	107
3.13.1 Preparation of Sample for GC-MS.....	107
3.13.2 Fatty Acid Composition Determination.....	107
3.13.3 Study of Operating Condition of Transesterification Reaction (OFAT Analysis)	108
3.13.4 Experimental Design of Transesterification Reaction (FAEE %)	109
3.14 Summary of Chapter Three	111
 CHAPTER FOUR: RESULTS AND DISCUSSION	 112
4.1 Introduction.....	112
4.2 Screening and Extraction of Lipase From Cocoa Pod Husk	113
4.2.1 Lipase Activity Assay	114
4.2.2 Determined the Protein Concentration.....	114
4.2.3 Study of Operating Conditions of Extraction of lipase from CPH (OFAT Analysis)	115
4.2.4 Optimization of CPH Extraction.....	115
4.2.5 Estimation of Molecular Weight of Lipase.....	121
4.3 Preparation of CLEA-lipase	123
4.3.1 Screening of Additives for CLEA Preparation	123
4.3.2 Effect of Incubation Time on CLEA-lipase of CPH Activity.....	127
4.3.3 Study of Operating Conditions of Extraction of CLEA-lipase from CPH (OFAT Analysis).....	128
4.3.3.1 Effect of Ammonium Sulfate	129
4.3.3.2 Effect of Glutaraldehyde	130
4.3.3.3 Effect of BSA	131
4.3.4 Optimization of Preparation of CLEA-lipase	133

4.4 Characterization of CLEA-lipase	141
4.4.1 Optimum Temperature and pH for Free and CLEA-lipase.....	141
4.4.2 Effect of Temperature Stability of Free and CLEA-lipase	143
4.4.3 Effect of pH Stability of Free and CLEA-lipase.....	143
4.4.4 Reusability of CLEA-lipase	145
4.4.5 Determination of Kinetic Parameters.....	146
4.4.6 Structural Characterization of CLEA-lipase From CPH by Field Emission Scanning Electron Microscopy (FE-SEM).....	148
4.5 Production of Biodiesel	150
4.5.1 Fatty Acid Composition of <i>Jatropha curcas</i> L oil.....	151
4.5.2 Study of Operating Conditions of Transesterification Reaction (OFAT Analysis)	153
4.5.2.1 Effect of the Ratio of Oil to Ethanol.....	153
4.5.2.2 Effect of Enzyme Loading.....	155
4.5.2.3 Effect of Agitation	156
4.5.2.4 Effect of Temperature.....	157
4.5.2.5 Effect of Reaction Time	159
4.5.3 Experimental Design of Transesterification	160
4.6 Summary of Chapter Four	169
CHAPTER FIVE: CONCLUSION AND RECOMMENDATION.....	170
5.1 Conclusion	170
5.2 Recommendations.....	172
REFERENCES.....	173
APPENDIX A.....	198
APPENDIX B.....	199
APPENDIX C.....	200
APPENDIX D.....	206
APPENDIX E.....	207

LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
2.1	Chemical composition of Cocoa pod husk (% w/w oven dried materials)	14
2.2	Enzymes used in different industrial part and their application	17
2.3	Recent studies about the different types of precipitants used in CLEA preparation	43
2.4	Summary of tested additives on the activity of CLEA in recent studies	50
2.5	Summary of related studies and the recovery activity obtained with different enzymes and different	51
2.6	Different method for biodiesel production	71
2.7	Fatty acid compositions of <i>J. curcas</i> oil	77
2.8	Comparison of biodiesel production Routes	78
2.9	Lipases used for the enzymatic production of biodiesel	82
3.1	Different BSA concentrations to prepare BSA standard curve	96
3.2	Experimental design for optimization of lipase extraction from CPH	97
3.3	Resolving and Stacking gels formulation	98
3.4	Experimental design for optimization of CLEA-lipase preparation	104
3.5	Experimental design for optimization of transesterification reaction	110
4.1	Experimental design using FCCCD of three independent variables with their actual and coded values and six centre points showing the experimental and predicted	117
4.2	Analysis of variance of quadratic model for lipase production	119
4.3	Experimental design using FCCCD of three independent variables with their actual and coded values and six centre points showing the experimental and predicted	135
4.4	Analysis of variance of quadratic model for CLEA-lipase activity	137

4.5	Validation of the experimental model	139
4.6	Fatty acid compositions of selected <i>Jatropha curcas</i> oil	152
4.7	Experimental design using FCCCD of three independent variables with their actual and coded values and six centre points showing the experimental and predicted	162
4.8	Analysis of variance of quadratic model for conversion of FAEE	164
4.9	Validation of the experimental model	167

LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
2.1	Cocoa fruit and cocoa pod husk	12
2.2	Theobroma cocoa tree	12
2.3	Lipase reaction (Macrae & Hammond, 1985)	22
2.4	General reaction scheme of hydrolysis, esterification, transesterification	24
2.5	Effect of temperature on reaction rate	29
2.6	Effect of pH on the reaction rate	30
2.7	Enzyme immobilization by solid support method	36
2.8	Immobilization of enzymes using entrapment method	37
2.9	Cross-linking enzyme immobilization technique	38
2.10	Possible structure of glutaraldehyde in aqueous solution (Barbosa et al., 2014)	46
2.11	Graphical determination of K_m and V_{max} (Berg & Jain, 2002)	57
2.12	Central composite design (JMP, 2005)	62
2.13	A. Synthesis of FAAE a Transesterification of TAG and short-chain alcohols leading to FAAE and glycerol b. Esterification of fattyacid and short-chain alcohol leading toFAAE and water R1–4 acyl residues, R' alcohol moiety (R'=CH ₃ for meth (Hoydonckx et al., 2004)	65
2.14	TG of oils and fats (R1, R2, R3 = C4-C22 chains)	67
2.15	Molecular structure of fatty acids	68
2.16	Molecular structure of FFA of oils and fats	69
2.17	Molecular structure of: (a) Methanol, (b) Ethanol, (c) Propanol, (d) Butanol	69
2.18	Transesterification reaction	73

2.19	Jatropha curcas plant	74
3.1	Flow chart showing the main steps carried out in this research	92
3.2	Position of the immobilization sample inside the shaker	100
3.3	Preparation of CLEA lipase inside the incubator for 17 hours	101
3.4	CLEA lipase from CPH is washed and centrifuged three times with distilled water after 17 hours incubation time	102
3.5	The termination of reaction after addition of 1 ml NaOH	102
4.1	Extraction of crude sample from CPH. The picture shown the orange colour extracted sample after centrifugation	113
4.2	Effect of different ratio of CPH (1-10% w/v) on lipase activity	115
4.3	SDS-PAGE of lipase extracted from CPH. Lane 1: molecular weight of marker; Lane 2: molecular weight of lipase	122
4.4	The effect of different additives on the activity of CLEA-lipase	123
4.5	Schematic diagram of preparation of CLEA with addition of BSA as additive	125
4.6	Effect of surfactants such as SDS on enzymes	126
4.7	Effect of polar solvent such as heptane on enzymes (Nelson & Cox, 2004)	127
4.8	Effect of cross-linking time on CLEA-lipase activity. Reaction condition: 0.5 ml crude sample, 30%w/v (NH ₄) ₂ SO ₄ , 70 mM glutaraldehyde and 0.23 mM BSA at room temperature	128
4.9	Effect of different concentration of ammonium sulphate (10-50% w/v) on CLEA-lipase activity	130
4.10	Effect of different concentration of glutaraldehyde (40-80 mM) on CLEA- lipase activity from CPH	131
4.11	Effect of different concentration of BSA (0-0.37 mM) on CLEA-lipase activity from CPH	132
4.12	3D response surface curves of the combined effect of concentration of ammonium sulfate and concentration of glutaraldehyde on CLEA-lipase enzyme activity from CPH	138
4.13	Optimum temperature for free and CLEA-lipase from CPH.	

	Reaction condition: 0.5ml crude enzyme in different range of temperature (25-65 °C) with tris-HCl buffer (100mM, pH 8.2). incubation time: 30 min	142
4.14	Optimum pH for free and CLEA-lipase from CPH. Reaction condition: 0.5ml crude enzyme in different range of pH (100mM, pH 5-10) at optimum temperature incubation time : 30min	142
4.15	The stabilities of CLEA and free enzyme in aqueous solution. (a) Effect of temperature; (b)Effect of buffer pH. Reaction condition: enzyme solutions were incubated in buffe (100 mM, pH 5-10) at (25-60 °C) at 60 min incubation time	145
4.16	Reuse of CLEA-lipase from CPH in water. Reaction condition: 0.5 ml crude enzyme with 20(%w/v) (NH ₄) ₂ SO ₄ , 60 mM glutaraldehyde, 0.17 mM BSA in 4 ml water. At 37°C and 200 rpm	147
4.17	FE-SEM picture of CLEA-lipase from CPH	150
4.18	Separation of two layers after centrifugation and removal the CLEA-lipase. The upper layer is oil (biodiesel) and the bottom layer is glycerol (by-product of transesterification)	168
4.19	Effects of oil-ethanol molar ratio. (The process conditions: 45°C, 200 rpm agitation speed and 4 h reaction time, 3 (w/w %) catalyzer loading	155
4.20	Effects of enzyme loading. (The process conditions: 45° C, 200 rpm agitation speed, 4 h reaction time and 1:6 ratio of oil to ethanol)	156
4.21	Effect of agitation. (The process conditions: 35° C, 5 h reaction time, 1:6 ratio of 175 oil to ethanol and 3 (w/w%) enzyme loading)	157
4.22	Effect of temperature. (The process conditions: 200 rpm agitation speed, 5 h reaction time, 1:6 ratio of oil to ethanol and 3 (w/w%) enzyme loading)	159
4.23	Effect of time (The process conditions: 45°C, 1:6 ratio of oil to ethanol, 200 agitation rate and 3 (w/w%) enzyme loading)	160
4.24	3D contour plot showing the interactions of operating parameters between (A). catalyst loading and reaction time, (B). catalyst loading and ratio, (C). ratio and reaction time	166

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

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variables
BSA	Bovine Serum Albumin
CLEA	Cross-Linked Enzyme Aggregates
CALB	<i>Candida Antarctica</i> Lipase B
CLEC	Cross-Linked Enzyme Crystal
CPH	Cocoa Pod Husk
DAG	Diacylglycerol
EIC	Extracted-ion Chromatogram
ES	Enzyme-substrate complex
FFA	Free Fatty Acids
FAEE	Fatty Acid Ethyl Ester
FCCCD	Face Centered Central Composite Design
FE-SEM	Field Emission Scanning Electron Microscopy
G	Gravity
GA	Glutaraldehyde
GC/MS	Gas Chromatography/Mass Spectrometry
MAG	Monoacyleglycerol
PBS	Phosphate Buffer Saline
<i>p</i> NPP	<i>para</i> Nitrophenyl palmitate
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Malaysia is currently one of the top ten major cocoa (*Theobroma cacao*) producers in the world and become the second largest producers in South East Asia after Indonesia. Cocoa Pod Husk (CPH) is an agro based by-product obtained after the removal of cocoa beans from the cocoa fruit material which in turn resulted in landfill and environmental problems (Bello, Ahmad, & Siang, 2011). It may be a significant source of disease inocula, such as black pod rot (Barazarte, Sangronis, & Unai, 2008; Donkoh, Atuahene, Wilson, & Adomako, 1991; Figuiera, Janick, & BeMiller, 1993). The burden of cocoa pod husk waste has caused a serious challenge for waste Management (Vriesmann, Teófilo, & Lúcia de Oliveira Petkowicz, 2012). In the cocoa crop, only the beans (around 10% fresh weight of cocoa fruit) are commercially valuable (Vriesmann, Teófilo, & Petkowicz, 2011). For each ton of dry beans produced, ten tons of cocoa pod husks are generated, which represents a serious challenge for waste management (Figuiera et al., 1993). This CPH can be a source of phytochemicals that has potential for development into various nutraceuticals and pharmaceutical products. Up to date none of these phytochemicals have been harvested from CPH, mostly because of lack of research and development in this area. Recovery of these nutraceuticals and pharmaceuticals can contribute further towards value adding efforts in the Malaysian cocoa industry.

The chemical analysis of CPH showed crude protein between 70-90 g/kg (Donkoh et al., 1991; Vriesmann et al., 2012). High protein content of this waste

material can be manipulated to produce something that can benefit the cocoa industry itself and reduce some of the environmental problem caused by CPH. Hydrolase enzyme is one of the products that can be extracted from the CPH protein. Types of hydrolases that can be recovered from CPH are lipases, cellulases, amylases, proteases, etc. these enzymes provide very useful industrial enzyme for food, flavor, detergents, biocatalytic resolution of pharmaceuticals as well as preparation of fine chemicals.

Lipases (glycerol ester hydrolysis) are the most relevant enzyme in organic chemistry and enzyme technology. The high stability of this enzyme is caused to remain active even under undesirable conditions. They can be extracted from animals, plants, and natural or recombinant microorganisms. The physiological role of lipases (water-soluble triacylglycerol acylhydrolases, EC 3.1.1.3) is the catalytic conversion of tri-glycerides into di-, or monoglycerides and fatty acids (Stergiou et al., 2013). They have a wide range of application in industry from energy to food industries, and pharmaceutical chemistry (Gotor-Fernández, Brieva, & Gotor, 2006). Moreover, they have wide application in hydrolysis of oils and fats, surfactant and biofuel and they can produce intermediate for organic synthesis (Christensen, Andersen, Kirk, & Holm, 2001).

However, enzymes are biocatalysts that are quite unstable. Enzymes are often facile and denatured entities in vitro milieu. Short catalytic lifespan hampers their usefulness and increase the cost of the enzyme based applications. Therefore, immobilization of enzyme is a known powerful tool for improving enzyme performance, stability, activity and selectivity. Immobilization of lipase is a popular technique for most large-scale industrial applications due to the ease in

biocatalyst, recycling, continuous operation and product purification (Balcão, Paiva, & Xavier Malcata, 1996; Chen et al., 2012).

Cross linked enzyme aggregate (CLEA), is a known technique for immobilization of enzyme. Recently, this new enzyme immobilization method has gained popularity, because it is a simple method and has many advantages (Sheldon, 2007; Sheldon, 2011). CLEA for the first time is developed by Cao, 2000 (Cao, Rantwijk, & Sheldon, 2000). CLEA uses the aggregate proteins to be cross-linked, generating a solid biocatalyst (Valdés, Soto, & Arcaya, 2011). This method is to overcome the disadvantages of carrier-bound immobilized enzyme systems, usually associated with large amount of non-catalytic mass and expensive carrier beads.

The immobilization of enzyme using CLEA is compatible with most contaminant proteins. Purification of protein may be obtained in CLEA technology if the full precipitation conditions for the target protein are milder than those required for the precipitation of some contaminants (Anbu, Gopinath, Hilda, & Annadurai, 2005). However, very high level of enzyme purification should not be expected (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). The enzyme loading in this method is very high with low loss of activity (Mateo, Palomo, Van Langen, Van Rantwijk, & Sheldon, 2004).

In this method, enzyme is precipitated to obtain aggregates. In the next step, the aggregate is cross linked with different reagents. The best known reagent for preparation of CLEA is glutaraldehyde (Sheldon, 2011). Glutaraldehyde is a bi-functional reagent with the capacity to polymerize (Migneault, Dartiguenave, Bertrand, & Waldron, 2004). During the immobilization, the internal structure of the enzyme can be infiltrated by glutaraldehyde and the aldehyde group reacts with the

amino group of the protein, although it may eventually react with other groups (thiols, phenols, and imidazoles) (Habeeb & Hiramoto, 1968; Migneault et al., 2004; Wine, Cohen-Hadar, Freeman, & Frolow, 2007). This could be critical for enzyme catalytic activity (Sheldon, 2011). The addition of glutaraldehyde to a protein solution may produce a chemical aggregation of the enzyme, causing protein molecules to react among themselves, and can directly yield a “solid biocatalysts” (Barbosa et al., 2014; Caballero Valdés et al., 2011). However, when the amine content of enzyme is low, the cross-linking might not be very effective. To overcome this issue, the aggregation can be prepared in the presence of certain additives such as Bovine Serum Albumin which has a large number of amine groups (Dong, Zhao, Huang, & Tan, 2010). The use of BSA can enhance the activity and stability of CLEA (Shah, Sharma, & Gupta, 2006).

Biodiesel is a clean-burning fuel that is now getting higher attention to solve the problem of climate change and reduce the dependency on fossil fuels which are facing many issues such as unstable escalation of price, depleting reserve and higher air pollutants. It can be produced from different sources such as vegetable oil, animal oil or waste cooking oil (Ganesan, Rajendran, & Thangavelu, 2009; Raja, 2011; Röttig, Wenning, Bröker, & Steinbüchel, 2010)

There are different methods for production of biodiesel. Out of which enzymatic transesterification using lipase technique is better than the other ways. The advantages of lipases in biodiesel production included: high efficiency, complete conversion of free fatty acids (FFA) to methyl or ethyl esters, less energy consumption, more selective mild reaction condition, low temperature and low formation of side products and waste (Jegannathan, Jun-Yee, Chan, & Ravindra, 2010). The main disadvantage

in using lipase for biodiesel production is its high cost. This issue can be overcome with the immobilization of lipase with different methods which can easily be recycled.

1.2 PROBLEM STATEMENT

Cocoa pod husk (CPH) is a waste product and may cause many environmental problems when dumped around the processing plants. As the cocoa industries and demand for products related to cocoa improved in recent years, a large amount of this waste produced and released to the environment.

In the recent years, owing to the increasing necessity to take into consideration aspects aimed at preventing pollution of the environment as well as for economic motives, and the need to conserve energy and new materials, new methods for waste handling and treatment have been introduced in the recovery, bioconversion and utilization of valuable constituents from fruit processing wastes. In many cases these wastes might have the potential for conversion into useful raw materials (Laufenberg, Kunz, & Nystroem, 2003).

Use of free enzyme as biochemical catalysis has some disadvantages such as high cost, low stability and non-reusability. In aqueous solutions enzymes are relatively unstable and their recovery could be difficult due to their water solubility. To overcome these problems immobilized form of the enzyme has been used as a potential tool in industrial processes.

CLEA is a new method of carrier-free immobilized enzyme for biocatalysts as a replacement for carrier-bound immobilized enzyme. This new enzymatic technology can overcome the problem in carrier-bound systems usually associated with large amount of non-catalytic mass and expensive carrier beads and it is attractive in its

simplicity and robustness.

1.3 SCOPE OF RESEARCH

In this research lipase is extracted from cocoa pod husk and then immobilized using Cross-linked enzyme aggregates method. Immobilizations of enzymes with CLEA include simple precipitation followed by cross-linking. Experiments will be designed to optimally immobilize lipase in aqueous media. Using different amount of precipitants, cross linker and additives. The optimum parameters in extraction of crude lipase and producing CLEA-lipase were studied using Design Expert version 6.0.8. Soluble and immobilized enzyme was carried out in lab scale and was evaluated according to their activities, kinetic parameters, pH stability, thermostability and reusability. Lastly, the performance of the produced CLEA was applied in esterification and transesterification reactions.

1.4 SIGNIFICANCE OF RESEARCH

The significances of this research work are multiple. The production of important industrial enzyme; lipase, from CPH the wasteful agro-based by-product, would be of interest and might provide an additional source of revenue to the cocoa industry and at the same time saving the environment from pollution. This high active and stable lipase has various applications in industries. Extracting an enzyme from a waste by-product compare to microorganisms is less expensive and simpler.

The effort to produce enzymes from known halal sources is a 'Fard Kifayah' to satisfy the Islamic requirements in food and consumer industries. As most of the globally marketed enzymes are known to have originated from the non-halal or dubious sources, this research attempted to contribute to the effort of solving this