PRODUCTION OF CROSS LINKED LIPASE FROM COCOA POD HUSK

BY

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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 قشرور الكاكاو (CPH) من النواتج الثانوية لعملية إزالة حبوب الكاكاو من ثمرة الكاكاو ويؤدي رميها بدون معالجة الى العديد من المشاكل البيئية. حيث أظهرت التحاليل أن نفايات قشور الكاكاو تحتوي على كميات عالية من البروتين. ومن احد أهم منتحاتها 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 (الانزيمات) غير مستقرة نوعا ما. وبالتالي، يعتبر ربط المجموع الانزيمية 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 الانزيمية المترابطة بالعبور (CLEA). تم تحديد خصائص الانزيم بشكليه الحر والمترابط فيما يتعلق بالاستقرار وإعادة الاستخدام و الدراسة الحركية، و في نهاية الدراسة استُخدم الليباز المترابط بالعبور لإنتاج وقود الديزل الحيوي من زيت *Jatropha curcas*. و في المراحل الثلاثة للدراسة تم حساب الظروف المثلى للعمليات الثلاثة باستخدام تصميم Face centered central composite (FCCCD) تحت منهجية استجابة السطح (RSM). حيث وُجد 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 و0.17 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. و تم 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 بيّن التوصيف الهيكلي لليبازالمترابط بالعبور مظهره الكروي بواسطة FE-SEM. وقد تم تطبيق الليبازالمترابط بالعبور لإنتاج وقود الديزل الحيوي وتم ايجاد 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 الى 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 محفزالحيوي من منتج ثانوي قابل لإعادة التدوير وله تطبيقات عديدة في مجالات مختلفة. و بإمكان هذه الدراسة اضافة المزيد من المعلومات حول عملية تحويل نفايات الزيت منخفضة التكلفة (زيت *J.Curcas*) والإيثانول واستخدام محفّز 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

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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.

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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
υ	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	*Candida Antarctica* 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
*p*NPP	*para* Nitophenyl 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 that those require for the precipitation of some contaminates (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 recycle.

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 release 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 concern 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 none 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