Production and Characterization of Lipase from *Aspergillus oryzae* ST11, Immobilization with Magnetic Nanoparticles and Nanofibers, and Application for Biodiesel Production

Pattarapon Paitaid

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การผลิตและการศึกษาสมบัติของไลเปสจาก *Aspergillus oryzae* ST11 การตรึงเอนไซม์กับอนุภาคนาโนแม่เหล็กและนาโนไฟเบอร์ และการประยุกต์ใช้ในการผลิตไบโอดีเซล

ผู้เขียน นายภัทรภณ ไผ่เทศ

สาขาวิชา เทคโนโลยีชีวภาพ

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บทคัดย่อ

เชื้อราจำนวน 7 ไอโซเลทถูกนำมาทดสอบ เพื่อศึกษาว่ามีไอโซเลทใดบ้างที่มีความสามารถในการผลิตไลเปส โดยเลี้ยงเชื้อราในอาหารเหลวที่ประกอบด้วย น้ำมันปาล์ม 1% เปปโตน 2% โซเดียมไนเตรท 0.2% ไดไฮโดรเจนโปแทสเซียมฟอสเฟตและ 1% แมกนีเซียมซัลเฟต 0.05% (น้ำหนัก/ปริมาตร) ปรับพีเอชเป็น 6 โดยเลี้ยงที่อุณหภูมิ 37 องศาเซลเซียสและเขย่าที่ความเร็ว 150 รอบต่อนาที เป็นเวลา 3 วัน พบว่ามีเพียงเชื้อราไอโซเลท ST11 เท่านั้นที่สามารถเจริญและผลิตเอนไซม์ไลเปสปล่อยออกนอกเซลล์โดยมีกิจกรรมของเอนไซม์ไลเปส 12.2 ยูนิตต่อมิลลิลิตร และเอนไซม์หยาบจากเชื้อดังกล่าวสามารถเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นได้ จึงนำเชื้อรา ST11 ไปศึกษาหาสภาวะที่เหมาะสมในการผลิตเอนไซม์ไลเปส พบว่าสภาวะที่เหมาะสมคือการใช้อาหารที่ประกอบด้วย น้ำมันมะกอก 1% น้ำตาลแลกโตส 1% เปปโตน 2% และโซเดียมไนเตรท 0.2% (น้ำหนัก/ปริมาตร) พีเอช 6 จำนวนสปอร์เชื้อราที่ใส่ลงไปในอาหาร 10^7 สปอร์ต่อมิลลิลิตรในปริมาตรอาหาร 100 มิลลิลิตร เลี้ยงเชื้อที่อุณหภูมิ 37 องศาเซลเซียสและเขย่าที่ 150 รอบต่อนาที เป็นเวลา 4 วัน เชื้อ ST11 ผลิตเอนไซม์ไลเปสได้ 31 ยูนิตต่อมิลลิลิตร

ทำการจัดจำแนกเชื้อรา ST11 ใช้ทั้งวิธีแบบการวิเคราะห์สัณฐานวิทยาของเชื้อและวิธีทางชีวโมเลกุลเข้าร่วม โดยวิธีทางโมเลกุลใช้ไพรเมอร์ ITS1 และ ITS4 เมื่อวิเคราะห์ส่วนของ ITS พบว่าเชื้อ ST11 อยู่ในในกลุ่มของ *Aspergillus* ซึ่งไม่สามารถระบุได้ว่าเป็นสปีชีส์ใดระหว่าง *A. flavus* กับ *A. oryzae* จึงศึกษาต่อโดยใช้วิธีทางสัณฐานวิทยา ทำการเพาะเลี้ยงบนอาหารแข็ง 2 ชนิด คือ malt extract agar และ Czapek yeast agar และสังเกตลักษณะของ conidia และ conidiophore ภายใต้กล้องจุลทรรศน์ พบว่าเชื้อรา ST11 มีลักษณะของ *Aspergillus oryzae* จึงกำหนดให้เป็น *A. oryzae* ST11 หลังจากนั้นนำเชื้อราดังกล่าวไปเลี้ยงในอาหารเหลวเพื่อผลิตและแยกเอนไซม์ให้บริสุทธิ์ โดยการตกตะกอนด้วยแอซีโทนแช่เย็น ผ่านคอลัมน์แบบแลกเปลี่ยนประจุลบ (Q-HiTrap) และคอลัมน์แบบไม่ชอบน้ำ (Butyl Toyopearl 650M) ได้ผลผลิตเอนไซม์ไลเปสสุทธิ 7.9% และมีความบริสุทธิ์เพิ่มขึ้น 13 เท่า เอนไซม์บริสุทธิ์ที่ได้มีขนาด 25 kDa จากนั้นจึงนำไปศึกษาคุณสมบัติ

เอนไซม์ พบว่าเอนไซม์ไลเปสบริสุทธิ์มีความเสถียรที่พีเอชระหว่าง 7-8 และมีพีเอชที่เหมาะสมต่อการทำงานที่พีเอช 7.5 มีอุณหภูมิที่เหมาะสมต่อการทำงานที่ 37 องศาเซลเซียสและเอนไซม์ทนต่ออุณหภูมิระหว่าง 30-37 องศาเซลเซียส แต่กิจกรรมเอนไซม์ไลเปสจะลดลงมากที่ 55 องศาเซลเซียส ในการทดสอบผลของไอออนโลหะในระบบพบว่า Ca^{2+}, K^{+} และ Mg^{2+} ที่ความเข้มข้น 1.0 mM ช่วยให้การทำงานของเอนไซม์ไลเปสเพิ่มขึ้น แต่การทำงานลดลงในระบบที่มี Hg^{2+} Zn^{2+} และ Cu^{2+} ในส่วนของสารลดแรงตึงผิวและตัวยับยั้งการทำงานของเอนไซม์ พบว่า Triton X-100, Tween-20, Tween-80, SDS และ Arabic gum ทำให้การทำงานของเอนไซม์ลดลง ขณะที่ EDTA, PMSF และ β-mercaptoethanol ไม่มีผลต่อการทำงานของเอนไซม์ สำหรับการศึกษาผลของตัวทำละลายอินทรีย์ต่อการทำงานของเอนไซม์ไลเปส พบว่าตัวทำละลายที่มีขั้วเช่นเอทานอลและเมทานอลมีผลยับยั้งการทำงานของเอนไซม์ไลเปสเมื่อเทียบกับตัวทำละลายที่ไม่มีขั้วเช่น ไอโซออกเทน เฮกเซน ในด้านความจำเพาะต่อซับสเตรท ในบรรดาน้ำมันพืชที่ใช้ทดสอบนั้น เอนไซม์มีความสามารถในการเร่งปฏิกิริยาสูงสุดเมื่อใช้น้ำมันมะกอกเป็นซับสเตรท

เมื่อนำเอนไซม์ไลเปสของ *A. oryzae* ST11 ในรูปของเอนไซม์เข้มข้นจากการตกตะกอนด้วยแอซีโทนเย็น มาตรึงบนวัสดุต่างๆ ด้วยวิธีที่แตกต่างกัน เพื่อนำไปใช้ในการผลิตไบโอดีเซล โดยวัสดุชนิดแรกคือ วัสดุผงแม่เหล็กขนาดนาโนโดยตรึงเอนไซม์ด้วยวิธี magnetic cross linked enzyme aggregate ที่มีการเติม bovine serum albumin (BSA) (6 มิลลิกรัม/มิลลิลิตร) และสารละลาย glutaraldehyde (20 mM) แล้วนำเอนไซม์ตรึงรูปไปใช้เร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชั่น จากการหาสภาวะที่เหมาะสมต่อการผลิตไบโอดีเซลพบว่า สามารถผลิตไบโอดีเซลได้สูงสุด 95 % ที่ 24 ชั่วโมง โดยการเติมเมทานอลครั้งละ 1 โมล จนครบ 3 โมล เอนไซม์ตรึงรูปนี้สามารถใช้งานซ้ำได้ 5 รอบ และยังสามารถเร่งปฏิกิริยาได้มากกว่า 60% วัสดุชนิดที่สองคือ เส้นใยที่ผลิตจากพอลิเมอร์ผสมระหว่าง polystyrene กับ trimethylolpropane tris [poly (propylene glycol) amine terminated] ether โดยวัสดุดังกล่าวสามารถตรึงเอนไซม์ไลเปสได้ 0.15 ยูนิตต่อมิลลิกรัมของวัสดุ เอนไซม์ตรึงรูปมีความสเถียรต่อความเป็นกรดด่างและอุณหภูมิในช่วงที่กว้างกว่าเอนไซม์อิสระ เมื่อนำมาใช้เร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นเพื่อผลิตไบโอดีเซล พบว่าผลิตไบโอดีเซลสูงสุดได้ 95% ภายใน 24 ชั่วโมง เมื่อมีการเติมเมทานอลครั้งละโมล จนครบ 3 โมล และยังสามารถนำกลับมาใช้ใหม่ได้ 10 รอบ โดยยังมีกิจกรรมเอนไซม์ที่ 81% วัสดุสุดท้ายที่นำมาใช้ตรึงเอนไซม์ไลเปสคือเส้นใยนาโนจากพอลิเมอร์ polyacrylonitrile (PAN) โดยในการทดลองนี้ทำการตกตะกอนเอนไซม์ไลเปสบนเส้นใยนาโนด้วยแอมโมเนียมซัลเฟตและมีการเติม BSA เพื่อช่วยการจับตัวเป็นกลุ่มของไลเปสหลังจากการเติมสารละลาย glutaraldehyde พบว่า สามารถตรึงไลเปสได้ถึง 87% ในขณะที่ไม่มีการเติม BSA ได้ 42% เมื่อนำเอนไซม์ตรึงรูปบนเส้นใยนาโน PAN ที่ได้มาใช้เร่งปฏิกิริยาเพื่อ

ผลิตไบโอดีเซลเปรียบเทียบกับเอนไซม์ตรึงรูปทางการค้า (Novozym 435) พบว่าเอนไซม์ตรึงรูปบนเส้นใยนาโน PAN ผลิตไบโอดีเซลได้สูงสุด 95% ในเวลา 18 ชั่วโมง ขณะที่ Novozym 435 ผลิตไบโอดีเซลได้ในปริมาณที่น้อยกว่ามากในเวลาที่เท่ากัน ในการทดลองนี้สามารถเติมเมทานอลเพียงครั้งเดียว (3.5 โมลของเมทานอลต่อ 1 โมล ของน้ำมัน) และสามารถนำเอนไซม์ตรึงรูปกลับมาใช้ได้ 10 ครั้ง โดยยังมีความสามารถในการเร่งปฏิกิริยาได้ถึง 83%

จากการศึกษาทั้งหมดที่กล่าวไป เอนไซม์ไลเปสจากเชื้อรา *Aspergillus oryzae* ST11 นั้นมีศักยภาพในการผลิตไบโอดีเซล เพื่อทดแทนการเร่งปฏิกิริยาโดยตัวเร่งปฏิกิริยาเคมี ที่ใช้สภาวะในการเร่งปฏิกิริยาที่มีความเป็นกรดด่างสูง รวมถึงอุณหภูมิของระบบที่สูง อีกทั้งยังสามารถลดผลกระทบต่อสิ่งแวดล้อมจากของเสียของกระบวนการผลิตไบโอดีเซล เอนไซม์ไลเปสที่ได้ยังสามารถนำไปตรึงบนวัสดุเพื่อให้สามารถนำกลับมาใช้ใหม่ได้ทำให้สามารถลดต้นทุนของการผลิตไบโอดีเซล

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ABSTRACT

Seven isolates of fungi were screened for the ability to produce an extracellular lipase. The tested isolates were cultivated in the submerged fermentation with the basal medium containing 1% (w/v) palm oil, 2% peptone, 0.2% $NaNO_3$, KH_2PO_4, 0.05% $MgSO_4$ and pH 6 at 37ºC and 150 rpm for 3 days. Only isolate ST11 grew and produced an extracellular lipase (12.2 U/ml). The ST11 was selected for optimization of the lipase production. The optimum conditions for lipase production was 1% olive oil, 1% lactose, 2% peptone, 1% $NaNO_3$ (w/v) at pH 6. The inoculum (10^7 spores/ml) was applied into 100 ml of the medium. The fermentation was carried out at 37ºC and 150 rpm for 4 days. The lipase unit after optimization was 31 U/ml

The molecular and morphological techniques were used to identify the selected fungus (ST11). In this study, the ITS1/ITS4 primers were used for amplifying the ITS region. It showed that the ST11 belonged to *Aspergillus* group. However, this technique could not differentiate the *A. flavus* from *A. oryzae.* So, the fungal morphology was studied. The morphological growth of *Aspergillus* could be differentiated on the malt extract agar and Czapek yeast agar. From the observation of conidia and conidiophore of the fungus ST11, it confirmed that this isolate was *Aspergillus oryzae*. Therefore, it was designated as *Aspergillus oryzae* ST11. After cultivating *A. oryze* ST11 in the optimized medium, the crude lipase was purified by chilled acetone precipitation, anion exchange column chromatography (Q-HiTrap) and hydrophobic column chromatography (Butyl Toyopearl 650M). The yield of the purified lipase was 7.9% with the 13 purification folds. The molecular mass of the purified lipase was 25 kDa. The purified lipase was stable between pH 7-8 with the optimum activity at pH 7.5, it showed a good stability in the temperature between 30-37ºC and the maximum activity was at 37ºC. The activity drastically dropped at 55ºC.

For the effect of metal ions on the lipase activity, it showed that in the presence of Ca^{2+}, K^{+} and Mg^{2+} promoted the lipase activity whereas in the presence of Hg^{2+}, Zn^{2+} and Cu^{2+} decreased the lipase activity. For the effect of surfactants and inhibitors on the lipase activity, it showed that all surfactants tested including Triton X-100, Tween-20, Tween-80, SDS and Arabic gum decreased the lipase activity. In contrast, the inhibitors (EDTA, PMSF and β-mercaptoethanol) did not decrease the lipase activity. Next study was the effect of solvents on the lipase stability. The hydrophilic solvents such as methanol and ethanol showed the lower in the activity compared to the hydrophobic solvents such as isooctane and hexane. For the substrate specificity, many natural oils were tested, it showed that the olive oil gave the highest activity.

The partially purified lipase was prepared by precipitation with chilled acetone. It was immobilized on different materials for biodiesel production. The first material was the magnetic nanoparticle. The lipase was immobilized via the cross-linked enzyme aggregate technique with bovine serum albumin (BSA) (6 mg/ml) and 20 mM glutaraldehyde. The immobilized lipase on magnetic nanoparticle produced the highest biodiesel conversion (95%) after 24 h of reaction with the stepwise addition of methanol. The immobilized lipase was reused for 5 times with 60% remained activity. The next material was the electrospun nanofibrous membrane prepared by co-solvent between polystyrene and trimethylolpropane tris [poly (propylene glycol) amine terminated] ether. The 0.15 U/mg-support was obtained at the optimum immobilization conditions. It was stable to the wide range of pH and temperature compared to the free lipase. The highest biodiesel conversion was 95% with stepwise addition of methanol. It retained the ability to produce biodiesel at 81% at the 10^{th} cycle. The last material used for immobilization was the electropun polyacrylonitrile (PAN) nanofibrous membrane. The lipase was precipitated on the surface of modified PAN nanofibrous membrane by ammonium sulfate with the addition of BSA. After glutaraldehyde cross linking, the highest immobilized activity was 87% compared to 42% of non-BSA addition. The immobilized lipase on PAN nanofibrous membrane was used for biodiesel production and compared with the commercially immobilized lipase (Novozym 435). The biodiesel conversion from immobilized lipase was 95% which was higher than that of Novozym 435 (52%) at 24 h. Moreover, the immobilized lipase on PAN nanofibrous membrane catalyzed the transesterification reaction with the one

step addition of methanol (3.5 mole of methanol per 1 mole of palm oil). The immobilized lipase retained 83% activity after 10 cycles of use.

Aspergillus oryzae ST11 lipase shows the potential for biodiesel production. It could be used to replace the chemical catalysts. The lipase could be immobilized on many different materials for reducing the cost of the biodiesel production process. The use of lipase also reduced the environment problem from the waste of biodiesel production process.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEWS

Introduction

The trend of fossil fuel consumption has been increased since many years ago. It leads to the problem of energy reserve as well as the environmental impact due to the releasing of greenhouse gases generated after combustion of fossil fuel. Biodiesel or fatty acid alkyl esters (FAAEs), has become a popular topic among many countries. Because, biodiesel is non-toxic, decomposable and used as the renewable fuel source. Moreover, it also releases a lower exhaust emission of particle and greenhouse gases compared to fossil fuel (Tan *et al*., 2010). The catalysts used in biodiesel synthesis can be divided into four types alkaline, acid, enzymatic and inorganic heterogeneous catalysts (Gog *et al*., 2012). The biodiesel production is occurred by the chemical catalyzation using acid or alkaline catalysts. However, there are some limitations for the chemical process such as high energy required in the system, the by-product (glycerol) separation from the biodiesel, the waste treatment, the interference of free fatty acids and water to the biodiesel production. The transesterification catalyzed by enzymatic approach could be used to solve the limitations from chemical catalysts (Jegannathan *et al*., 2010).

Lipases are enzymes that can catalyze the hydrolysis reaction as well as the synthetic reactions such as esterification, transesterification and aminolysis (Gog *et al*., 2012). Filamentous fungi are regarded as the best lipase producers and are currently used for producing extracellular lipases. The lipases are easily harvested from the culture medium using a filtration. Moreover, the stability, selectivity, and broad substrate specificity of fungal lipases are suitable for the industrial use. Their lipases have been studied in many applications due to some special characteristics. The lipases from *Aspergillus awamori* HB-03 and *A. carneus* can be active in the pH range of 7-11 and 6-12, respectively. They are also active in wide range of temperature. Some species of *Aspergillus* genera might have the distinct characteristic compared to others. *A. niger* NCIM 1207 lipase is acidic lipase, its optimum pH was 2.5 that mean it can be used in

acidic condition. Moreover, when fungal lipase is applied in biodiesel production, it showed a high ability to catalyze the transesterification. The lipases from *Mucor miehei* and *Candida antarctica* were used in biodiesel production by different kinds of oil in the solvent system (hexane). *M. miehei* was highly efficient to convert primary alcohols such as methanol, ethanol, propanol, and 1-butanol into the biodiesel with the high yields of 95% and 98%, while *C. antarctica* lipase was more suitable for converting the secondary alcohols such as isopropanol and 2-butanol and gave the biodiesel between 61% and 84% (Singh and Mukhopadhyay, 2012).

Using free lipase could enhance the reaction speed. However, it also has some disadvantages from the cost and instability of free enzyme. Enzyme immobilization can overcome the limitation of free enzyme use. The most common method for enzyme immobilization can be performed by physical adsorption and covalent binding. Moreover, the decision of method is depended on the desired properties such as microbial resistance, thermal stability, chemical durability, chemical functionality, enzyme loading capacity, hydrophobic/hydrophilic character, cost of the process and reusability of the materials (Ondul *et al*., 2012).

In this study, the lipase from potential fungal strain was selected and used for biodiesel production. To increase the feasibility for the process, the fungal lipase was immobilized on the nanoscale materials such as the electrospun nanofibrous membrane and the magnetic nanoparticle. The immobilized lipase from these materials was used as a biocatalyst for biodiesel synthesis.

Review of literature

1. Lipase

Lipases or triacylglycerol acyl hydrolases (EC 3.1.1.3) are the versatile enzymes which are important to many industrial applications (Salihu *et al*., 2012). They have been widely used in the diary industry, oil processing, productions of surfactants and pharmaceuticals (Gutarra *et al*., 2009).

1.1 Lipase-catalyzed reaction

Triglycerides are hydrolyzed to fatty acids and glycerol by lipase under certain conditions. In some cases, the reverse reaction could take place to synthesize glycerides from glycerol and fatty acids. Some lipases possess both transesterification and enantioselective hydrolysis reactions (Falony *et al*., 2006).

1.1.1 Mechanism of lipase-catalyzed reaction

The α/β-hydrolase fold is found in lipases showing a conserved catalytic triad (Ser, His, Asp/Glu) with Gly-x-Ser-x-Gly as a consensus sequence around the serine active site. This triad is covered completely below a short α-helical segment, which opens with an interface. The rearrangement of enzyme's structure during catalyzation, an electrophile region (oxyanion hole) is formed on every side of serine residue. The intermediate during transition state is stabilized by exposing the hydrophobic residues and burying the hydrophilic residues. The lid-like structure and an amphiphilic loop cover the active site of lipase, these features make them different from esterase (Salihu and Alam, 2012). The lid or flap composed of an amphiphilic a helixpeptide sequence is the unique structure of lipase. In the absence of solvent, the lid will be closed to prevent the accessibility of the substrate to the catalytic triad site. When the lid has opened, a large hydrophobic surface is established to bind the hydrophobic super substrate (usually the oil drop) (Figure 1). The study of inhibitors binding such as alkyl-, cycloalkyl phosphonates emulated the mechanism of acylation and deacylation of substrates (Schmid and Verger, 1998).

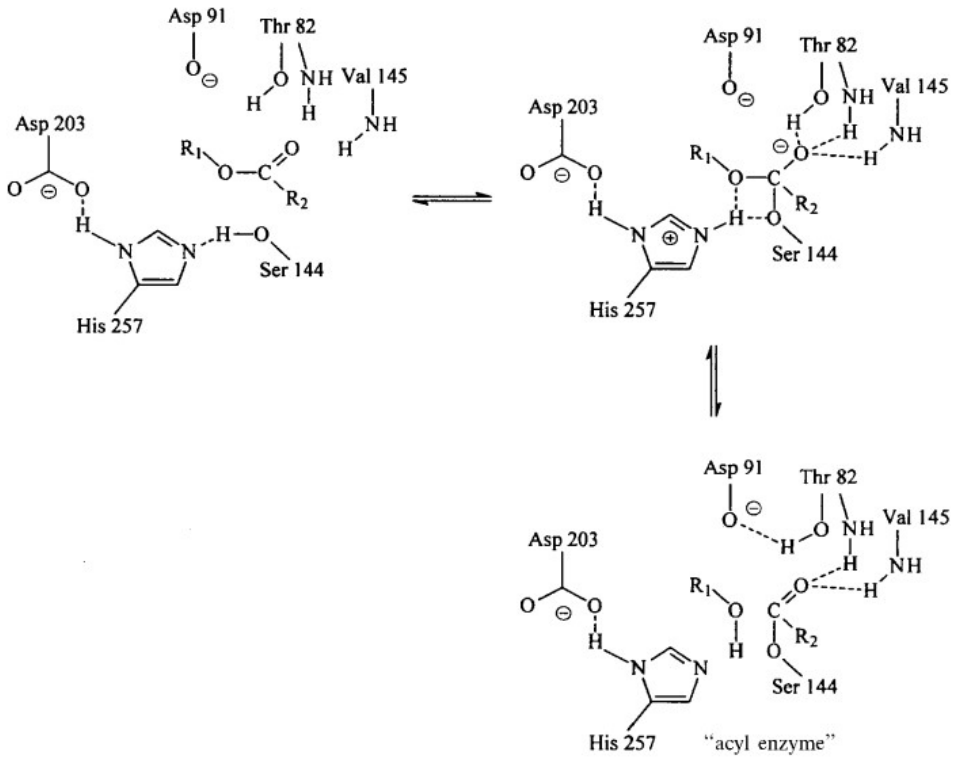


Figure 1. Mechanism of lipase catalysis based on a catalytic triad of serine, histidine and aspartate. The amino acid numbers are referred to lipase from *Rhizopus oryzae.*

Source: Schmid and Verger (1998)

The catalysis mechanisms of lipase are illustrated in Figure 2. The serine residue was activated by the deprotonation accompanied with the histidine and aspartate (Figure 2a). Subsequently, the nucleophilicity of hydroxyl group of serine is promoted, the activated hydroxyl attacks the carbonyl group of the substrate leading to the formation of acyl-enzyme intermediate (Figure 2b). The formation of an oxianion hole stimulates the charge spreading stability leading to the reduction of the ground state energy of the intermediate. The stage of diacylation is shown in Figure 1c. The electronegativity of the molecules is controlled populating the interface. After that, a nucleophile such as H_2O and monoglyceride attacks the acylated enzyme releasing product and regenerating the active site (Reis *et al*., 2009).

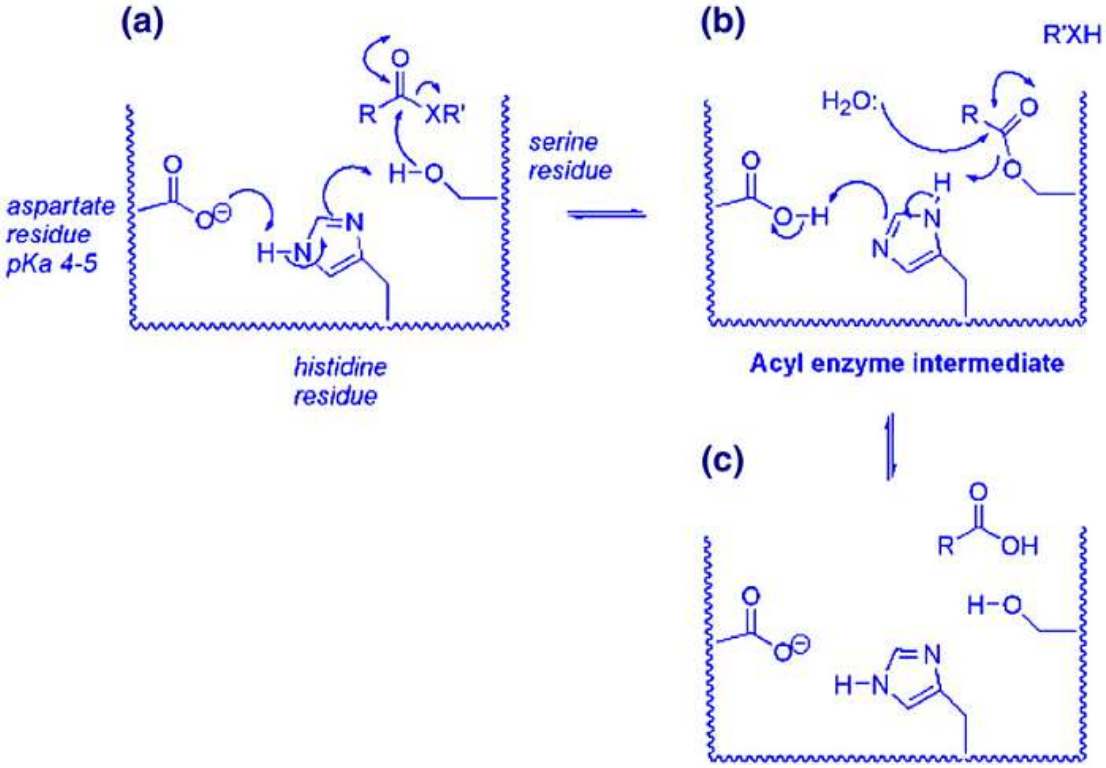


Figure 2. Mechanism of lipase catalysis.

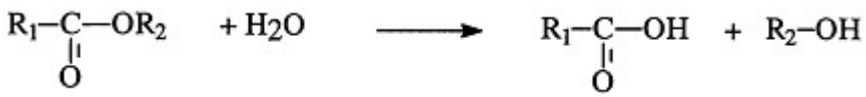
Source: Reis *et al*. (2009)

1.1.2 Type of the lipase-catalyzed reactions

The ester bonds from the long chain triglycerides are hydrolyzed by lipase to produce free fatty acids, di-, monoglycerides and glycerides. They are capable of catalyzing esterification and transesterification reactions. The equilibrium of the reactions between hydrolysis and synthesis is controlled by the water content in the system. (Villeneuve *et al*., 2000).

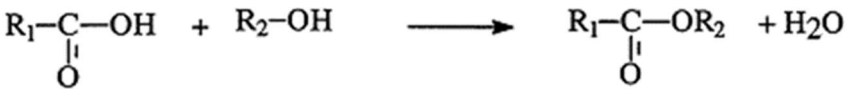
1.1.2.1 Hydrolysis

The triglyceride is hydrolyzed in the presence of excess water.



1.1.2.2 Esterification

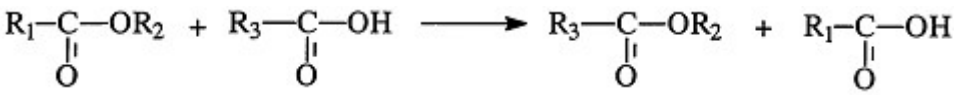
The polyhydric alcohols and free fatty acid are esterified by lipase in organic solvent conditions with low water activity or even solvent free systems.



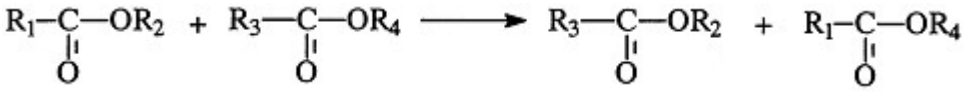
1.1.2.3 Transesterification

The exchange of acyl group occurs between the ester and acid for acidolysis, the ester and another ester for interesterification or the ester and alcohol for alcoholysis. All reactions are catalyzed by the lipase.

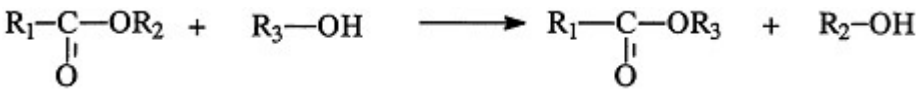
a. Acidolysis



b. Interesterification



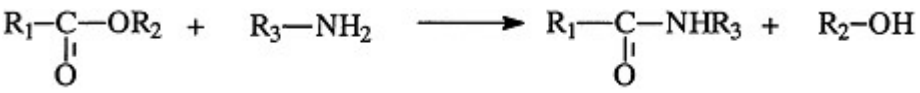
c. Alcoholysis



1.1.2.4 Catalysis on ‘‘unnatural’’ substrates

Lipase can utilize compounds which are not water and alcohols as nucleophiles with different reactions such as aminolysis, thiotransesterification, and oximolysis in organic solvents with selectivity.

a. Aminolysis



1.1.3 Specificity of lipases

The specificity of lipase could be divided into three main groups (Barros *et al*., 2010).

1.1.3.1 Substrate specificity

Lipases are capable of hydrolyzing triglycerides (TAGs), di- and monoglycerides and even phospholipids, in the case of phospholipases.

1.1.3.2 Regioselectivity

a. Non-specific lipase

Lipases hydrolyze triglycerides and randomly release fatty acids and glycerol. The products such as mon-, diglycerides are existed in the reaction as the intermediates (Figure 3).

b. Specific 1,3 lipases

The position 1 and 3 of triglycerides are hydrolyzed releasing fatty acids, 2-monoglycerides and 1,2-or 2,3-diglycerides. the intermediates (1,2 or 2,3 diglycerides) are unstable and migration of the acyl group producing 1,3-diglycerides and 1- or 3-monoglycerides is occurred (Figure 3).

c. Specific or selective type fatty acid

Lipase are capable of distinguishing enantiomers in a racemic mixture. The lipase specificity on enantiomer is different according to the substrate and this variation can be connected to the nature of the ester.

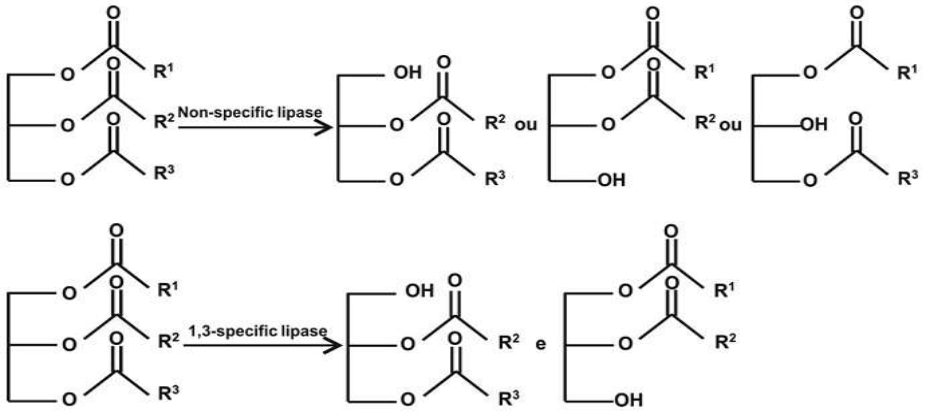


Figure 3. Reactions catalyzed by non-specific and 1,3 specific lipases.
Source: Barros *et al*. (2010)

1.2 Microbial lipase

Lipases are obtained from animals, plants, and microorganisms. Microbial lipases have gained special industrial attention because they have high stability, selectivity, and broad substrate specificity. Many microorganisms such as bacteria, yeast, and fungi are microbial potential producers of extracellular lipases (Treichel *et al*., 2010). Lipases have been intensively investigated for their multiplexity of catalysis with unique characteristics, which have been used in various applications such as oleochemistry, organic synthesis, detergent formulations and nutrition. Moreover, lipases show beneficial properties associated to their stability as organic solvent-tolerant and thermostable enzymes. Hence, microbial lipases have been studied and many of lipases have been identified, purified and characterized to date (Mala and Takeuchi, 2008). Microbial lipases are preferably used in biotechnological applications. They are the most potential source of lipases produced in large scale in both submerged and solid-state fermentation (Treichel *et al*., 2010).

1.3 Fungal lipase

Filamentous fungi are acknowledged as the most suitable lipase producers for producing extracellular lipases. The growth of fungi could facilitate the extraction of extracellular enzyme from fermentation media (Contesini *et al*., 2010).

Lipase producing fungi are found in many habitats, including an oil contaminated soil, vegetable oil wastes, dairy product factory, seeds, and deteriorated food. Moreover, the molecular development, as well as the efficiency of screening procedures have enhanced the use of lipase for organic synthesis. Nowadays, wild-type enzyme has been produced and used as a biocatalyst to many desired applications. The new lipolytic microorganism strains are used. The development of techniques is studied to obtain higher efficiency for catalyzation in each application making it more feasible to be used in industrial applications.

Fungal lipases have drawn the attention from industry because of their stability, selectivity, and wide range of substrate specificity. They possess the significant industrial potential and have some good characteristics for example high catalytic activity, mild condition for the operation, friendly to the environment, and have more specificity to chemical, enantio- and regioselectivity. However, there is some drawbacks of using lipase, i.e., enzyme has lower reaction rate compared to the chemical catalysts and has a high production cost. *Mucor*, *Rhizopus*, *Rhizomucor*, *Aspergillus*, *Candida*, and *Penicillium* are considered the most common strains used for lipase production. Some fungal strains are the thermophile such as *Mucor pusillus*, *Rhizopus homothallicus*, and *Aspergillus terreusare* which produce thermostable extracellular lipase. However, there is few fungi producing lipase which is stable at high alkaline condition and high temperature. (Singh and Mukhopadhyay, 2012).

1.4 Application of lipases

Lipases are widely used in the lipid, food, pharmaceuticals etc. The fatty waste and polyurethane removal are accelerated by lipase. The lipase applications are summarized in Table 1. The bacteria and fungi are considered as source for lipase used in the industrial processes (Table 2) (Sharma *et al*., 2001).

Table 1. The applications of microbial lipases.

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Elimination of lipid from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Enhancing the flavor in the diary products such as milk, cheese and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

Source: Sharma *et al*. (2001)

Table 2. Some commercially available microbial lipases.

Type	Source	Application	Producing company
Fungal	*C. rugosa*	Organic synthesis	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme,Sigma
	C. antarctica	Organic synthesis	Boehringer Mannheim, Novo Nordisk
	T. lanuginosus	Detergent additive	Boehringer Mannheim, Novo Nordisk
	R. miehei	Food processing	Novo Nordisk, Biocatalysts, Amano
Bacterial	*Burkholderia cepacia*	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	P. alcaligenes	Detergent additive	Genencor
	P. mendocina	Detergent additive	Genencor
	Ch. viscosum	Organic synthesis	Asahi, Biocatalysts

Source: Sharma *et al*. (2001)

1.5 Production, purification and characterization of fungal lipases

Submerged fermentation is the most suitable cultivation method used for producing lipases. The lipase derived from submerged fermentation is affected by the influence of pH, temperature, aeration and medium formula as well as the type and carbon and nitrogen concentrations that might affect the lipase production. Moreover, the lipase yield might be enhanced because of the existence of lipidic compounds and surfactants (Costas *et al*., 2004).

1.5.1 Effect of media and culture condition on lipase production

1.5.1.1 Effect of nitrogen sources

The organic and inorganic nitrogen sources show the important effect on the lipase synthesis. The inorganic nitrogen sources will be used very fast by cells. For organic nitrogen sources, they are used for cell growth and supplied for amino acids which required for cell metabolism and protein synthesis. Hence, the lipase fermentation processes require both organic and inorganic nitrogen sources (Tan *et al*., 2004). There is a report of nitrogen source affecting lipase production of *Penicillium aurantiogriseum*. The high nitrogen concentrations are required to produce lipases as dominant enzyme. The increase in concentrations of ammonium sulfate with the controlled olive oil concentration of 1, 2.5, 5 and 10 (C/N ratio). At the ratio 5, the highest lipase activity was obtained. The increase of ratio over than 10, the lipase production was low. The growth of fungus was poor in the medium containing the C/N ratio of 1 (Lima *et al.,* 2003*)*. Another experiment is the effect of nitrogen source on lipase synthesis of *Aspergillus* sp. (RBD-01). The increase in the concentration of both inorganic and organic nitrogen sources with 10% oil as a carbon source was studied. It was found that the increase of peptone enhanced the lipase activity and reached the maximum activity at 16.0 U/ml with 1.5% of peptone after 72 h of incubation. For inorganic nitrogen source, 0.5% of diammonium hydrogen orthophosphate promoted the activity of lipase (15.0 U/ml) while 0.5% of urea showed the negative effect on the lipase activity (Aulakh and Prakash, 2010).

1.5.1.2 Effect of carbon sources

The carbon is considered as the fundamental component of cells. Many organic compounds such as various triglycerides and ester-based detergents were acted as the lipase inducers for lipase production (Yang *et al*., 2005). The type and concentration of carbon source which are the important factor for producing lipase from *Aspergillus* sp. (RBD-01) were studied. The lipase activity was increased when concentration of cottonseed oil increased and it reached the maximum (21.8 U/ml) with the supplementation of cottonseed oil at 50% in the culture medium (Aulakh and Prakash, 2010). Mascena *et al*. (1999) investigated various types of oil and their effect

on the growth of *Fusarium solani* FS1. The medium containing sesame oil gave the highest lipase production at 0.88 U/ml while triolein only gave the lipase production at 0.78 U/ml. Other oils such as corn oil and olive oil showed a lower lipase activity compared to sesame oil and triolein. Lima *et al.* (2003) investigated the lipase production by *P. aurantiogriseum* using corn, soybean, sunflower and olive oils comparing to the medium that contained only glucose as a sole carbon. At 72 h of fermentation, the olive oil addition gave the highest lipase activity at 12.5 U/ml while sunflower oil, soybean oil and corn oil only gave the activity of lipase at 5 U/ml. Interestingly, no lipase activity was detected in the medium containing only glucose. It confirmed the role of inducers on the production of lipase by *P. aurantiogriseum*. Besides oils, the impact of carbohydrate addition on the lipase production was studied. Jin-lan *et al*. (2011) studied the effect of carbohydrate on the lipase activity of *Aspergillus awamori* HB-03 lipase. The different carbohydrate sources were tested at 0.5% in media supplemented with 1% olive oil. The addition of carbohydrate promoted the lipase production excepting the addition of glucose. The highest lipase production at 43.9 U/ml was obtained in the medium containing with sucrose. However, there was a report showed that *A. niger* did not promote the lipase production after the addition of sucrose. Adham and Ahmed (2009) reported that the addition of glucose as sole carbon inhibited lipase production of *A. niger* A20, *A. niger* NRRL 599 and *A. niger* NRRL3.

1.5.1.3 Effect of temperature and pH

The lipase production from microorganisms was affected by various factors It is known that each lipase producer needs specific fermentation condition and nutrient medium. Yang *et al*. (2005) determined the temperature effect (20–37ºC) on the growth of *R. arrhizus* and the production of lipase. Increasing temperature from 20-27ºC led to the increase of lipase activity. However, after the temperature was higher than 30ºC, it showed a lower lipase activity and inhibited the *R. arrhizus* growth. The effect of initial pH was varied from 5.0 to 8.0. At neutral and alkali initial pH, the *R. aarhizus* gave a high activity. They found that pH 7.18 was suitable initial pH for culture medium. Jin-lan *et al*. (2011) also mentioned the important role of initial pH for

cell growth and lipase production. The report revealed that at pH 7.0, the highest lipase activity of *A. awamori* strain HB-03 was obtained at 43.7 U/ml. It was similar with the lipase production of *Aspergillus* sp. RBD-01 that showed the maximum lipase activity at the neutral pH (Aulakh and Prakash, 2010). However, some *Penicillium* strains preferred the lower pH for lipase production (Wolski *et al*., 2009).

1.5.1.4 Effect of inoculum level

Inoculum level was also an important factor for the lipase production. The lower production of lipase might be affected by the insufficient fungal biomass due to low density of inoculum level, whereas a higher inoculum may produce too much biomass leading to the poor production of lipase. The highest lipase production (48 U/g substrate) from *Rhizopus oligosporous* was obtained with 1.0% inoculum size. Increase or decrease in the inoculum size from the optimum point did not stimulate enzyme activity (Ul-Haq *et al.*, 2002).

1.5.1.5 Effect of rotation speed and fermentation volume

Yang *et al*. (2005) studied the aeration effect on lipase production by *Rhizopus arrhizus*. The rotating speed and the amount of culture broth in the flask were investigated. The rotation speed and medium volume were controlled in the range 75–200 rpm and 30–120 ml. The rotation speed at 130 rpm with 100 ml of culture medium in 250 ml exhibited the highest lipase production. Therefore, the increase in the lipase production was related to the increase of aeration. However, the strong mechanic strength occurred at too high rotation speed might decrease the lipase production by damaging the mycelium growth.

1.5.1.6 Effect of incubation time

The association between the time of biomass and enzyme productions was very important to the process. The incubation time of lipase production from *Aspergillus fumigatus* MTCC 9657 was studied for 8 days. The activity was observed after 2 days and increased to the maximum point at 4 days of incubation. This observation was corresponded to the growth of fungus after reaching the log phase. The

lipase production of *A. fumigatus* MTCC 9657 was related to the growth of fungal biomass (Rajan and Nair, 2011).

1.5.2 Purification and characterization of fungal lipase

The lipases are necessarily purified for investigating their sequence of primary amino acid and the 3-dimensional structure of enzyme. The X-ray studies of pure lipases promotes the understanding of the association of protein structure and function and explains the mechanisms of lipase on hydrolysis and synthesis reactions. Besides, the purified lipase preparations are required in industries using the enzymes for the bioconversion of fine chemicals, medications and cosmetics which concerned for the contamination of the product (Saxena *et al*., 2003). In many different purification studies, the purpose is to purify the lipase to obtain the homogeneity and crystallized form. The most common techniques for enzyme purification consist of the protein precipitation, the ion exchange chromatography, the hydrophobic chromatography and gel filtration chromatography. Moreover, in some cases, the affinity chromatography is used for the specific binding of target protein to reduce the purification steps (Sharma *et al*., 2001).

A single step of chromatography is usually insufficient for enzyme purification to get the decent purity. Therefore, the serial steps of chromatography are required. The common purification step was an ion exchange chromatography. The diethylaminoethyl (DEAE) are the most frequent column used as an anion exchanger. In vice versa, the carboxymethyl (CM) is used as a cation exchanger. The strong ion exchangers such as triethylaminoethylgroups and Q-Sepharose have also been used in lipase purification. Gel filtration is the second most frequently technique used in purification. Hydrophobic interaction chromatography with the octyl or phenyl functional groups is also used in many purification studies. Adsorption chromatography is applied in the purification schemes and the adsorbent hydroxyapatite is used frequently. In some purification schemes, the affinity column chromatography has been introduced as one of the purification steps. (Sharma *et al*., 2001).

Different procedures used for lipase purification from various fungi have been summarized in Table 3. The purified enzyme from final purification step is

required for evaluating the characteristics such as the effect of pH, temperature, metal ions, solvents or even the kinetic study.

1.5.2.1 Effect of pH

The purified lipase from *A. awamori* HB-03 was studied for the pH stability. The tested lipase was incubated in the buffer pH 7.0 to 11.0 for 60 min. At pH between 8.0-9.0, lipase was stable at this range of pH with only 5% decrease while the stability drastically decreased at pH 10.0-11.0 (Jin-lan *et al*., 2011). the purified lipase of *Mucor* sp. was active at pH 5.0-9.0 and the optimum pH was 7.0. The recovered activity (pH 5.0-7.0) of lipase after 1 and 24 h of incubation at 30^{o}C retained the activity more than 87%. However, when the lipase was applied in the pH lower than 5 or higher than 7, the loss of activity was noticeably observed after 24 h at pH 3.0 and 9.0 (Abbas *et al*., 2002),

1.5.2.2 Effect of temperature

The lipase activity of *A. awamori* HB-03 was determined for the effect of temperature (Jin-lan *et al*., 2011). Their result showed that the optimal temperature for the activity was 40^{o}C and it still retained the activity at 100% after 60 min of incubation in the temperature range between 30-45^{o}C. Another case was the purified *Mucor* sp. lipase (Abbas *et al*., 2002), it had the optimum temperature at 35^{o}C and was active in between 30-45^{o}C which was similar with the result of *A. awamori* HB-03 strain.

1.5.2.3 Effect of metal ions

The metal ions were important to the activity of enzyme due to the association between ions and protein structure. Some ions like $Cu^{2+}$and Fe^{3+}strongly inhibited the lipase activity of *A. awamori* HB-03. However, the addition of Mn^{2+}, Ba^{2+}significantly enhanced the lipase activity to 122% and 135%, respectively. The interesting thing was the Zn^{2+}, at different concentration could affect differently on the activity of lipase. From the result, it showed that lipase activity was promoted at 1 mmol/l. However, it reduced the lipase activity at 10 mmol/L (Jin-lan *et al*., 2011).

Another study was the metal ion effect on the activity of *Penicillium* sp. DS-39 (DSM 23773) lipase (Dheeman *et al*., 2011). The lipase was enhanced and stable in the system containing Ca^{2+} or Mn^{2+}. In contrast, $Hg^{2+}$and Zn^{2+}drastically hindered the activity by 73.2% and 45.6%, respectively which had similar effect to other *Penicillium* including *P. aurantiogriseum*, *P. abeanum* and *P. simplicissimum*. There was a report showed that Hg^{2+} strongly inhibited the lipase from *P. expansum.*

1.5.2.4 Effect of solvents

Dheeman *et al*. (2011) investigated the effect of solvent on lipase from *Penicillium* sp. DS-39 (DSM 23773). The purified lipase was incubated in different solvents for 24 h. The result showed that the lipase was still active in the non-polar solvents such as toluene, n-xylene, n-hexane, n-heptane, isooctane and dodecane. However, the activity was decreased when lipase was incubated in polar solvents such as dimethylsulfoxide, dimethylformamide, methanol, ethanol, 2-propanol and n-butanol. The result was similar with the study of Saxena *et al*. (2003) which indicated that the lipase was stable in non-polar solvent and almost lost in the activity in polar solvent. This deactivation of lipase might be caused by striping-off of water which is necessary to stabilize the enzyme structure.

1.5.2.5 Substrate specificity

Many kinds of lipase have the different specificity towards different kinds of oil. Lipase from *A. carneus* expressed the high activity in the natural substrates such as karanja oil, linseed oil, neem oil, castor oil, soybean oil, corn oil and sunflower oil. It indicated that the lipase was the most effective towards the triglycerides containing fatty acid of lauric acid (C 12:0). However, it was still active in triglycerides with C4-18 (Saxena *et al*., 2003). Lipase from *P. candidum* preferred the triglycerides to mono- or diglycerides such as monocaprin, monolaurin, monoolein, dicaprin and diolein. However, these substrates were partially hydrolyzed compared to tributyrin (Ruiz *et al*., 2001).

Table 3. Purification procedures for lipase from various fungi.

Type of fungi	Purification steps	Recovery (%) and purification factor	Molecular mass (kDa)	References
Aspergillus niger NCIM 1207	ammonium sulfate precipitation, Phenyl-sepharose column, Sephacryl S-100 column	54%/149	32.2	Mhetras *et al*.,2009
Aspergillus carneus	ammonium sulfate precipitation, octyl Sepharose CL-4B	38.4%/24	27±1	Saxena *et al*., 2003
Aspergillus niger MYA 135	concentrated with PEG 20,000, DEAE-sepharose CL-6B column	47%/8.4	65	Romero *et al*., 2012
Penicillium expansum PED-03	DEAE-Sepharose, sephacryl S-200	19.8%/81.8	28	Lianghua *et al*., 2007
Penicillium sp. DS-39 (DSM 23773*)*	DEAE-cellulose column, Sephacryl® 100-HR column, Q Sepharose®HP column	129	43	Dheeman *et al*., 2011
Mucor hiemalis f. corticola	ammonium sulfate precipitation, Sephadex G75 column, Q-Sepharose fastflow	27.7%/12.63	46	Ülker and Karaoglu, 2012
Mucor hiemalis f. hiemalis	Ultrafiltration, ammonium sulfate fractionation, Sephadex G75, Q-Sepharose, Sephacryl S-200	18.1%/2200	49	Hiol *et al*., 1999
Rhizopus oryzae	ammonium sulfate precipitation, sulphopropyl-Sepharose, Sephadex G75	22%/1200	32	Hiol *et al*., 2000

Source**:** Saxena *et al*. (2003)

2. Enzyme immobilization

The biodiesel production using enzymatic process catalyzed by immobilized lipase has drawn attention from industry because the glycerol which is a byproduct could be easily separated from biodiesel (Yucel *et al.*, 2011). In the past, there were some limitations of using free enzymes for biodiesel production such as unreliable process, impossible to recovery and reuse. These reasons increased the process cost, Moreover, the mixing of free enzyme led to impurity of final product. The immobilization is used to overcome these limitations by facilitating the reuse of immobilized enzyme for many times. This reduce costs and further improve the product quality. The immobilization procedures could be done with different techniques such as physical adsorption, covalent bonding, enzyme entrapment, enzyme encapsulation, and cross-linking. The enzyme activity might be affected by the types of immobilization techniques. The interaction between enzyme and carrier might cause the structural change of protein. As well as, the use of immobilized lipase in the actual medium that might leads to the loss of activity during transesterification reaction due to leaching of enzyme from the carrier. In the most successful procedure, the increase of contact surface might raise the mass transfer and increase the efficiency of the enzyme as a biocatalyst (Ribeiro *et al*., 2011). Immobilization also promotes the potential of economic aspect. It is capable of reuse in the continuous and fixed-bed operation. Moreover, it enhances the lipase stability and a long-time storage (Sheldon, 2007).

In the case of lipase immobilization, the covalent bonding is expected to irreversibly bind the lipase to the solid matrix. There was a study of lipase immobilization from *Thermomyces lanuginosus.* The polyglutaraldehyde activated styrene-divinylbenzene (STY-DVB) copolymer was used for immobilizing lipase for synthesizing the biodiesel from canola oil. It showed that the maximum yield of biodiesel at 97% at 50ºC after 24 h of reaction. Moreover, it could be reused for 10 times of use (Jegannathan and Abang, 2008). A comparison of these different immobilized methods is shown in Table 4 (Tan *et al*., 2010).

Table 4. Comparison of different lipase immobilized methods.

Methods	Advantages	Disadvantages
Adsorption	Preparing conditions are mild and easy with low cost. The carrier can be regenerated for repeated use.	The interaction between the lipase and the carrier is weak, so the immobilized lipase was sensitive to pH, ionic strength and temperature etc. The adsorption capacity is low and the protein might be stripped off from the carrier.
Covalent bond	The immobilized lipase is rather stable because of the strong forces between the protein and the carrier.	The preparation conditions are rigorous, so the lipase might lose its activity during the immobilized process. Some coupling reagents are toxic.
Cross-linking	The interaction between the lipase and the carrier is strong and the immobilized lipase is stable.	The cross-linking conditions are intense and the mechanical strength of the immobilized lipase is low.
Entrapment	The entrapment conditions are moderate, and the immobilized method is applicable to a wide range of carrier and lipases.	This immobilized lipase always has the mass transfer restriction during the catalytic process, so the lipase is only effective for low molecular weight substrates.

Source: Tan *et al*. (2010)

2.1 Nanofiber

The industrial process for organic synthesis requires a lot of enzymes. Some enzymes are sufficiently cheap to use in washing powders and other bulk

applications. However, there are many processes requiring the expensive enzymes, immobilization using nanofiber as a carrier is generally considered favorable since it allows for continuous processes. The International Standards Organization (ISO) considers nanomaterials to be materials that are typically but not exclusively below 100 nm in at least one dimension. However, if the diameter of nanofiber is smaller than 100 nm. It could be accepted as a nanoscale material. The nanofiber could be synthesized via many techniques but the most technique is the electrospinning. The synthesized nanofiber shows the advantage for many applications because it has a large area compared to the volume of material which facilitate the interaction of nanofibers with the targeted substrates. Therefore, the nanofibers could be promising material to be used in many applications. (Nguyen *et al*., 2012; Tran and Balkus, 2012).

2.1.1 Electrospinning

The nanofibers are fabricated by using electrostatic forces. The droplet of polymer solution is extended when a high voltage is applied leading to occurrence of the electrostatic repulsion. After voltage reaches to the critical point, the extension of solution is erupted from the surface. The elongation of solution occurred leading to the formation of continuous fibers. From the principal, the apparatus system has been designed to synthesize nanofibrous membrane (Nguyen *et al*., 2012). Two types of the electrospinning have been set up; vertical and horizontal (Figure 4). From this basic method, many researchers have invented more sophisticated systems to fabricate the complex structure of nanofibers (Bhardwaj and Kundu, 2010). The electrospinning equipment consist of necessary parts such as a power supply, a collector and a syringe pump. Figure 5 shows the setup of the electrospinning equipment. A polymer solution filled in the syringe is charged and the syringe pump feeds a solution at a persistent rate. One electrode is connected to the needle and a high voltage (10–25 kV) is applied to a polymer solution causing the formation of polymer jet due to the overcoming of a repulsively electrostatic forces over surface tension.

The selection of polymer type is very important for forming fibers and to be interacted with the enzyme. There are many kinds of polymers that could be used for electrospinning. However, there is some limitation for some polymers to be

fabricated. One of the polymers in this case is nylon, it must be dissolved in formic acid which is not friendly to the enzyme. Other commercial polymers such as polystyrene, polyacrylonitrile, polymethymethacrylate, polylsulfone and polyurethane have been chosen because they are not soluble in water. Some polymers are blended with other polymers to synthesize the desired properties for enzyme immobilization (Tran and Balkus, 2012). Since the development of nanofiber production, more than 200 polymers have been used for synthesizing nanofibrous membrane for various applications. Different electrospun polymers and their applications are summarized in Table 5 (Bhardwaj and Kundu, 2010).

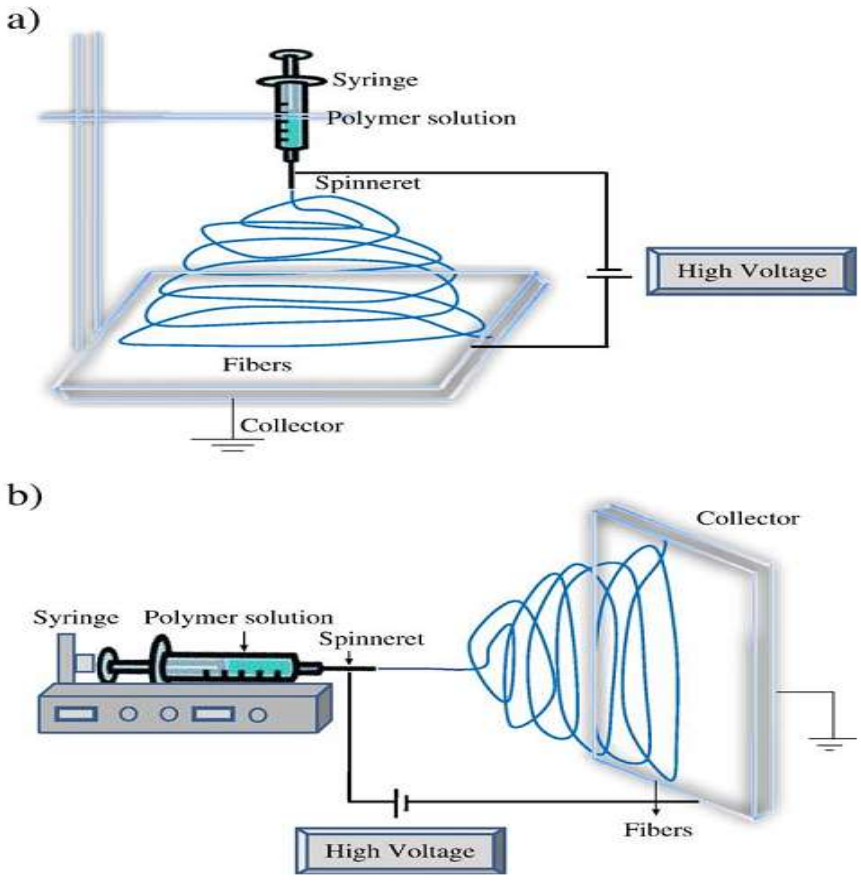


Figure 4. The electrospinning equipment (a) typical vertical arrangement and (b) horizontal arrangement of electrospinning module.

Source: Bhardwaj and Kundu (2010)

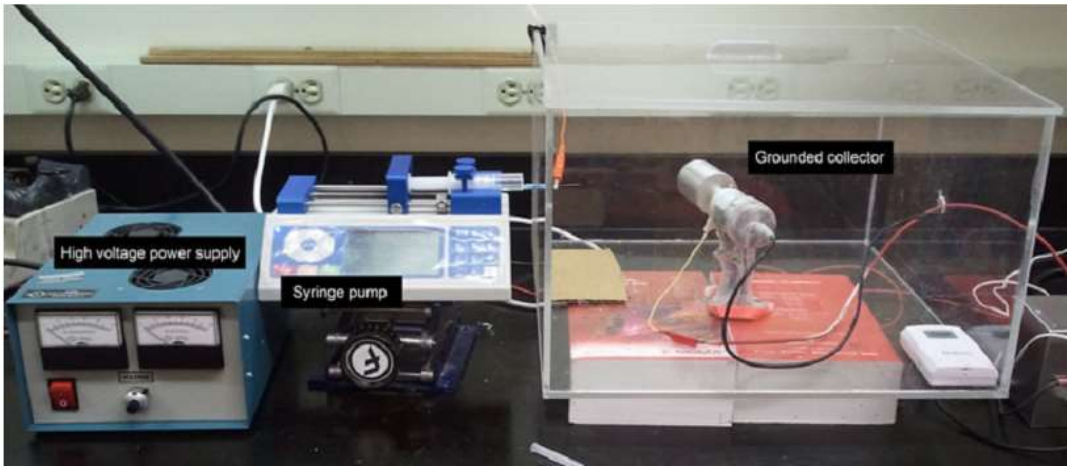


Figure 5. A high voltage power supply, syringe pump and collector are the main components for the electrospinning equipment. The feeding needle and collector are placed inside the humidity and temperature controllable housing.

Source: Tran and Balkus (2012)

2.1.2 Applications of enzyme immobilized with nanofibers

Li *et al*. (2007) investigated the immobilization of *Candida rugosa* lipase onto electrospun polyacrylonitrile nanofibers (PAN) by amidination reaction. After immersion in solution for 5 min and mixed with enzyme solution for 60 min, the 81% of free enzyme was recovered. For storage, the immobilized lipase retained >95% when kept in buffer at 30°C for 20 days, in contrast, free lipase lost 80% of its initial activity. The immobilized lipase was reused up to 10 cycles without losing activity significantly. This result also indicated the best potential compared to various immobilization methods using the same source of lipase and substrate. Sakai *et al*. (2010) also studied the immobilization of *Pseudomonas cepacia* lipase onto the PAN by using physical adsorption technique to convert a (S)-glycidol with vinyl n-butyrate to glycidyl n-butyrate in isooctane. The rate of reaction with the adsorbed lipase was 23-fold higher than the initial material. After 10 cycles of use, the rate of reaction was 80% of the initial batch.

Table 5. Different polymers used in electrospinning, characterization methods and their applications.

Polymers	**Applications**	**Characterizations**
Poly(glycolide) (PGA)	Nonwoven TE[a] scaffolds	SEM, TEM, *in vitro* rat cardiac fibroblast culture, *in vivo* rat model
Poly(lactide-co-glycolide)(PLGA)	Biomedical applications, wound healing	SEM, WAXD, SAXS, degradation analysis
Poly(ε-caprolactone) (PCL)	Bone tissue engineering	SEM, *in vitro* rat mesenchymal stem cell culture
Poly(l-lactide) (PLLA)	3D cell substrate	SEM, in vitro human chondrocyte culture
Polyurethane (PU)	Nonwoven tissue template wound healing	SEM, in vivo guinea pig model
Poly(ethylene-co-vinyl alcohol) (PEVA)	Nonwoven tissue engineering scaffold	SEM, in vitro human aortic smooth muscle cell and dermalfibroblast cultures
Polystyrene (PS)	Skin tissue engineering	SEM, in vitro humanfibroblast, keratinocyte, and endothelial single or cocultures
Syndiotactic 1,2-polybutadiene	Tissue engineering applications	ESEM, XRD, FTIR
Fibrinogen	Wound healing	SEM, TEM, mechanical Evaluation
Poly (vinyl alcohal)/cellulose acetate (PVA/CA)	Biomaterials	SEM, FTIR, WAXD, mechanical evaluation
Cellulose acetate	Adsorptive membranes/felts	SEM, FTIR
Poly(vinyl alcohol)	Wound dressings	SEM, EDX
Silk fibroin, silk/PEO[j]	Nanofibrous TE scaffold	SEM, FTIR, XPS
Silk	Biomedical Applications	SEM, TEM, WAXD

Source: Bhardwaj and Kundu (2010)

Table 5. Different polymers used in electrospinning, characterization methods and their applications (Continued).

Polymers	Applications	Characterizations
Silkfibroin	Nanofibrous scaffolds for wound healing	SEM, ATR-IR, C CP/MAS NMR, WAXD, NMR, in vitro human keratinocyte culture
Chitosan/PEO	TE scaffold, drug delivery, wound healing	SEM, XPS, FTIR, DSC
Gelatin	Scaffold for wound healing	SEM, mechanical evaluation
Hyaluronic acid, (HA)	Medical implant	SEM
Cellulose	Affinity membrane	SEM, DSC, ATR-FTIR^{o}
Gelatin/polyaniline	Tissue engineering scaffolds	SEM, DSC, conductivity measurement, tensile testing
Collagen/chitosan	Biomaterials	SEM, FTIR

Source: Bhardwaj and Kundu (2010)

Wang and Hsieh (2008) synthesized the nanofibrous membrane containing the *Candida rugosa* lipase via one step of electrospinning of lipase solution and polyvinyl alcohol (PVA). It was found that the loading enzyme increased from 25 to 50% showed the same catalytic activity as the crude enzyme. Moreover, there was no sign of negative effects from either electric charges or PVA on the structure or functions of the enzyme. It also showed the good characteristics towards the high temperature and humidity. Sawada *et al*. (2012) studied the synthesis of polyvinyl alcohol (PVA) nanofiber prepared in the co-solvent between water and dimethylformamide (DM) aiming to encapsulate *Burkholderia cepacia* lipase within the fibers. The lipase-encapsulated PVA fibers (10 wt% DMF) displayed the improved activity with a 1.5-fold increase in initial reaction rate to convert the (S)-glycidol and vinyl butyrate to (S)-glycidyl butyrate compared to the encapsulated lipase in the PVA fibers prepared in the water only. The result support the idea of improvement of the electrospun PVA fibrous matrices for numerous applications including the use as carriers carrying lipase for bioconversion in organic solvents.

2.1.3 Efficiency of enzyme immobilized on nanofiber comparing with other supports

Li *et al*. (2011) studied the protein loading and specificity of immobilized *P. cepacia* lipase with the electrospun PAN. The protein loading and specific activity of immobilized lipase were 43.4 ± 4.0 mg/g-matrix and 8.9 ± 1.8 U/mg, respectively, which meant 79 % activity retention of free lipase was recovered. Comparing to the basic immobilization technique of hydrogel entrapment (Betigeri and Neau, 2002). The *Candida rugosa* lipase was entrapped in chitosan and alginate beads. Both alginate and chitosan were formed spherical beads. Its efficiency was 44-50% in both types of beads. The styrene-divinylbenzene (STY-DVB) was used for immobilization of Novozym 388 and Lipozyme TL-100L by adsorption, covalent binding with glutaraldehyde (STY-DVB-GA) and polyglutaraldehyde (STY-DVB-PGA). The results showed that the highest efficiency of immobilization was as 35 % from Lipozyme TL-100L and 37 % from Novozym 388 on the micro porous STY-

DVB-PGA copolymer by covalent binding. In contrast, the yields of immobilized lipase from both commercial lipases using adsorption method were 24 and 12%, respectively.

For reusability of immobilized lipase. the reusability is considered as one of the important characteristics for industrial applications. Li *et al*. (2011) investigated the effect of reuse on the activity of immobilized *P. cepacia* lipase. After 10 cycles, the immobilized lipase retained about 98 % of its initial activity. Comparing to the retained activity of lipase immobilized on the macro-porous resin (NWZT2), silica-PEG gel and PAN nanofibrous membrane. The results showed that after 10 cycles of use, they retained about 62.1, 74.4 and 80 %, respectively. These results apparently showed that the immobilized *P. cepacia* lipase on the PAN nanofibrous membrane retained a high enzyme activity as well as a good reusability. The comparison was summarized in Table 6.

Table 6. The comparison of lipase immobilized with nanofibers and other supports.

Support	Immobilization technique	Type of lipase	Protein loading (mg/g support)	Immobilization yield (%)	Activity retention (%) at 10th batch	References
-Nanofiber						
Polyacrylonitrile (PAN)	Covalent bonding	*Pseudomonas cepacia* lipase	43.4 ± 4.0	-	98	Li *et al*. (2011)
Polycaprolactone (PCL)	Encapsulation	*Burkholderia cepacia* lipase	-	5-20	50	Song *et al.* (2012)
-Other supports						
Hydrogel beads	Entrapment	*Candida rugosa* lipase	-	48.4±4.0	50	Betigeri and Neau (2002)
Styrene-divinylbenzene (STY-DVB) copolymer	Adsorption	Lipozyme TL-100L	4.79	28.70	0	Yucel *et al.* (2011)
Styrene-divinylbenzene (STY-DVB) copolymer	Adsorption	Novozym 388	0.99	11.65	0	Yucel *et al.* (2011)
Divinylbenzene-polyglutaraldehyde (STY-DVB-PGA)	Covalent bonding	Lipozyme TL-100L	5.81	34.66	90	Yucel *et al.* (2011)
Divinylbenzene-polyglutaraldehyde (STY-DVB-PGA)	Covalent bonding	Novozym 388	4.19	36.99	90	Yucel *et al.* (2011)
Accurel EP1000	Adsorption	*Burkholderia. multivorans* PSU-AH130 lipase	-	94.2	-	Chaiyaso *et al.* (2012)

2.3 Magnetic nanoparticle

Magnetic particle has offered the potential use in many biotechnological applications which the size could be varied from a few nanometers to the size of tens of nanometers. In the biological approach for magnetic use, the surface of the material is modified to carry the target bioreactive molecules. This immobilized magnetic are usually used in medical use, and biosensors (Roque *et al*., 2009). The use of this material shows the promising strategy in the step of immobilization of enzymes or proteins by easy separation using an external magnetic. This could be used to recover the immobilized enzyme from the reaction mixture making the use of this material more feasible in the industrial process (Vinoth *et al.*, 2014). The magnetic is easily synthesized by co-precipitation of Fe^{2+} and Fe^{3+} in the ammonia solution under hydrothermal conditions (Xie and Ma, 2010).

The magnetic particle has been used as a core material for carrying the biomolecule and applied in many biotechnological applications including medical approach and biosynthesis. In the medical approach, the magnetic will be used as a carrier to deliver the drug to the target organs. This material is used as a biosensor by conjugating antibody (Roque *et al*., 2009). For biosynthesis approach, the enzymes will be immobilized on the surface of magnetic via many surface modification methods. The immobilized enzymes will be applied in the reaction mixture by aiming to be separated at the end of the process by the external magnetic. The immobilized enzyme could be used for many times showing the promising characteristic for an industrial application. Gupta *et al*. (2013) reported the immobilization of amyloglucosidase of *Aspergillus niger* on the magnetic nanoparticle. The recovered activity at the optimum conditions was 93%. The immobilized amyloglucosidase showed the enhanced thermal stability, affinity efficiency to the substrate and the reusability. Xiao *et al*. (2006) studied the immobilization of laccase on amine-terminated magnetic nano-composite by glutaraldehyde crosslinking method. The laccase was immobilized on copper tetraaminophthalocyanine (CuTAPc)-Fe_3O_4. The activity of immobilized laccase reached the highest at 1430 U/g-nano composite. This immobilized enzyme showed the promising reusability by retaining the activity at 80% after 5 consecutive operations. Jia *et al*. (2016) immobilized ω-transaminase on magnetic PVA-Fe_3O_4 nanoparticles.

The immobilized ω-transaminase could be operated in wider range of pH (6-8) compared to the free enzyme. It also showed a better thermal stability at 60ºC. And it could be reused for 13 times. For the immobilization of lipase on the magnetic nanoparticle. Mukherjee and Gupta (2016) used the cluster technique to coat lipase on iron oxide nanoparticles for biodiesel synthesis in a solvent free system. They immobilized *Thermomyces lanuginosus* lipase by precipitation on the cluster of magnetic nanoparticles. The immobilized lipase gave the highest conversion of biodiesel at 96% in 7 h with 4 moles of methanol per 1 mole of soybean oil. López *et al*. (2014) studied the magnetic biocatalysts and the application to obtain biodiesel and biosurfactants. The lipase from *Candida antarctica* B (CALB) was immobilized on the magnetic nanoparticle conjugating with 3-Aminopropyltriexysilane. The immobilized lipase was used for producing biodiesel. The different non-edible vegetable oils were converted to biodiesel by magnetic biocatalysts of CALB. It showed that bioconversion reached over 90% in 24 h at 30ºC.

3. Biodiesel

Biodiesel or fatty acid alkyl ester (FAAE) is derived from the diverse triglycerides such as vegetable oils and animal fats used as fuel for the engines. Its properties are similar with petroleum diesel. Hence, biodiesel has become a source of fuels to replace the diesel (Vieira *et al*., 2006). The transformation of oil into biodiesel is catalyzed by transesterification reaction (Figure 6). The biodiesel production can be performed by using different catalytic methods or in supercritical conditions (Gog *et al*., 2012). Biodiesel is a kind of renewable energies that is friendly to the environment. The drawbacks of fossil fuels such as the market price and the impact on the environmental issue have drawn the attention from the researcher to solve these problems with an alternative and renewable energy source. Various feedstocks have been acknowledged as potential raw materials for biodiesel production such as rapeseed oil, soybean oil, palm oil and jatropha oil. Recently, the use of palm oil and jatropha oil for biodiesel production has been widely studied as raw materials for renewable energy with high potential for the future (Mekhilef *et al*., 2011).

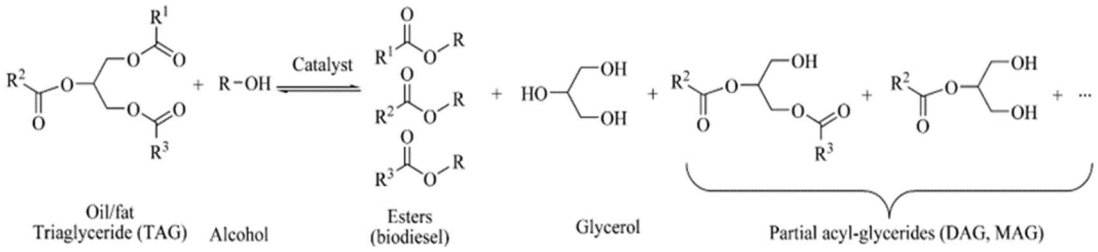


Figure 6. The bioconversion of oil to biodiesel by transesterification reaction. The sequence reactions, the triglyceride is started to be converted to diglycerides, monoglyceride and glycerol, respectively.

Source: Gog *et al.* (2012)

3.1 Biodiesel synthesis

Biodiesel can be synthesized from many triglycerides including fat, lard, tallow, and vegetable oils. These combinations of fatty acids (FFA) and triglycerides (TAG) must to be chemically transformed to fatty acid alkyl esters (FAAE) to be beneficial as biodiesel fuel for currently used diesel engines. Catalysts examined for transesterification could be acids, bases, free or immobilized enzymes (Fjerbaek *et al*., 2009).

3.1.1 Acid and alkali catalysis

The transestrification reaction is catalyzed by either acids or bases which triglycerides are converted first to diglycerides and monoglyceride and finally to glycerin. In each step, one ester is formed. In the overall reaction using methanol as alcohol, 3 mol of methyl esters are produced for each mole of triglyceride *(*Macario *et al*., 2010). Most of the industrial process for transesterification reaction is alkaline catalysis, the transesterification reaction needs a catalyst to increase the reaction rate for the biodiesel production. At present, most biodiesel is produced using alkaline catalysts from the methoxides and hydroxides form of sodium and potassium. However, the NaOH and KOH are frequently chosen for the production due to low cost.

However, the base-catalyzed process still has some limitations. The main problem is the amount of FFAs from the feedstock which should not exceed 0.5 wt%. Otherwise, the formation of soap will arise and hamper the production efficiency of biodiesel. Soap formation is generated when the metal hydroxide of catalyst reacts with free fatty acids from the raw material. The soap increases the formation of gel, viscosity, and the cost of product separation. The presence of water in the alcohol and catalyst is crucial for the production process. The excess amount of water will promote hydrolysis of the alkyl esters to FFAs leading to the soap formation (Lotero *et al*., 2005).

3.1.2 Enzymatic transesterification

Biodiesel production may be produced by using enzymatic catalysts. Lipases can catalyze the hydrolysis and synthetic reactions (esterification, transesterification and aminolysis). Lipases are also stable in non-aqueous system and still retain its specificity, regioselectivity and enantioselectivity which is important to many applications in organic system. The advantages and disadvantages of using lipases as biocatalysts are compared to chemical catalysts in Table 7. Several free commercial lipases used for biodiesel production are listed in Table 8. Free lipases have some disadvantages, the enzyme recovery after production is quite impossible. This is the main reason that make the cost of enzymatic process higher than that of chemical processes (Gog *et al*., 2012).

Table 7. Comparison of enzymatic technology versus chemical (alkaline and acid) technology for biodiesel production.

Parameter	Enzymatic process	Chemical process	
		Alkaline process	Acid process
FFA content in the raw material	FFA are converted to biodiesel	Soaps formation	FFA are converted to biodiesel
Water content in the raw material	It is not deleterious for lipase	Soaps formation. Oil hydrolysis resulting more soaps	Catalyst deactivation
Biodiesel yield	High, usually around 90%	High, usually>96%	High yields (>90%) only for high alcohol to oil molar ratio, high catalyst concentration and high temperature
Reaction rate	Low	High	Slower than for alkaline process
Glycerol recovery	Easy, high grade glycerol	Complex, low grade glycerol	Complex, low grade glycerol
Catalyst recovery and reuse	Easy Reusability proved but not sufficiently studied.	Difficult; neutralized by an acid Partially lost in post-processing steps	Difficult, the catalyst ends up in the by products No reusable catalyst
Energy costs	Low Temperature: 20-50ºC.	Medium Temperature: 60-80 ºC	High Temperature:>100 ºC
Catalyst cost	High	Low	Low High cost of equipment due to acid corrosion
Environmental impact	Low; wastewater treatment not needed.	High; wastewater treatment needed.	High; wastewater treatment needed

Source: Gog *et al*. (2012)

Table 8. Commercial lipase powders used for biodiesel production.

Lipase origin	Substrate	Acyl acceptor	Yield (%)
Rhizopus oryzae	Soybean oil	Methanol	80-90
Pseudomonas cepacia	Soybean oil	Methanol	80
Candida cylindracea	Pam oil	Methanol	78
Chromobacterium viscosum	Jatropha oil	Ethanol	73
Mucor miehei	Butyric acid	Methanol	Kinetic study
Mucor miehei	Pam oil	Methanol	45.4
Pseudomonas cepacia	Jatropha oil	Ethanol	70
Pseudomonas cepacia	Mahua oil	Ethanol	98
Aspergillus oryzae	Oleic acid	Methanol	95
Thermomyces lanuginosus	Soybean oil	Ethanol	96
Pseudomonas cepacia	Palm kernel oil	Ethanol	72

Source: Gog *et al*. (2012)

3.1.3 Factors affecting lipase catalyzed biodiesel synthesis

The transesterification reaction is affected by many factors including selection of alcohol, solvents, molar ratio between alcohol and oil, water content in the system and temperature of the system (Ghaly *et al*., 2010).

3.1.3.1 Selection of alcohol

There are many different acyl acceptors used for transesterification reaction such as primary, secondary, straight chained and branched alcohols The alcohols with the longer chain can also be used. However, the yield of biodiesel is lower than that of methanol. The frequently used alcohols are: methanol, ethanol, propanol, iso-propanol, 2-propanol, n-butanol and iso-butanol. Even though methanol and ethanol (short chain alcohols) are concerned to be more feasible in the biodiesel production, the deactivation of lipase due to the insoluble methanol occurred as drops in the oil was reported. Moreover, the glycerol will be absorbed onto the surface of the immobilized lipase leading to the inactivation of lipase activity due to the increase of the medium polarity and the removal of the water molecule from enzymes. Therefore, the stepwise

addition of alcohol can maintain the stability of lipase by preventing the accumulation of alcohol on the surface of enzyme. It also enhances the efficiency of biodiesel production with the increase in biodiesel yield (Ribeiro *et al*., 2011).

3.1.3.2 Use of solvents

The insolubility of short chain alcohols has inhibited the enzyme activity. Using solvents can overcome the enzyme deactivation by increasing the solubility of alcohol in the reaction system. Moreover, solvents can dissolve a glycerol coated on the surface of enzyme molecule. The use of a solvent reduces inhibition of enzymes and make a reaction mixture homogeneously. The solvent stabilizes the enzyme and decreases the viscosity of the reaction mixture eliminating the mass transfer problem around the enzyme (Fjerbaek *et al*., 2009). Moreover, the use of solvents increases the reaction rate of the enzyme compared to solvent free systems (Ghaly *et al*., 2010).

The hydrophobic solvents are commonly used in the transesterification reaction such as hexane, isooctane, n-heptane, petroleum ether, cyclohexane, 2-butanol and tert-butanol. In the case of hexane, lipases from *Mucor miehei* had a good efficiency to convert methanol, ethanol, propanol, and 1-butanol (primary alcohol) and produced the high biodiesel yields between 95 and 98%, whereas *C. antarctica* lipase prefers the isopropanol and 2-butanol (secondary alcohol) producing the biodiesel yields between 61 and 84%. In the solvent free system, the biodiesel yields from methanol and ethanol decreased, especially the use of methanol. Another study was the effect of petroleum ether on the biodiesel production from sunflower oil catalyzed by immobilized *Pseudomonas fluorescens* lipase. The biodiesel yield was obtained at 99% methanol, ethanol, or 1-butanol were used in the presence of petroleum ether. In contrast, yields were reduced to 3%, 70, and 76%, respectively in the absence of petroleum ether (Ribeiro *et al*., 2011).

3.1.3.3 Alcohol to substrate molar ratio

An extra mole of alcohol on the mole of oil is required to guarantee the transesterification reaction. Mostly, a higher molar ratio between alcohol to oil can

accelerate the faster reaction. Nevertheless, if the amount of alcohol is higher than the solubility point, it will lead to droplet formation which coat the enzyme causing the deactivation (Ghaly *et al*., 2010). The transesterification activity of most lipases would be suppressed by an excessive amount of methanol. Liu *et al*. (2012) reported that stepwise addition of methanol is the best way to prevent the alcohol inhibition during the transesterification reaction. However, the stepwise addition of alcohol requires a longer time to achieve the same yield of biodiesel compared to the one step addition of alcohol. Hsu *et al*. (2002) reported biodiesel production from grease, as in the ethanolysis reactions, which showed that a single addition of methanol to solvent-free alcoholysis reactions catalyzed by SP435 lipase gaving a low conversion of methyl esters. This was a cause of inactivation of the enzyme with the one-step addition of methanol to the reaction mixture.

3.1.3.4 Water content of the system

The water in the system is important for enzymatic transesterification involving on the reaction rate and biodiesel yield. Water is a crucial molecule to stabilize the 3-dimensional structure of the protein (Antczak *et al*., 2009). At the interface between the organic and aqueous phases was the area that the lipase catalyzes the reaction. Its capability depends on the available interfacial area. Addition of water forms the oil–water droplets, this increase the interfacial area. However, an excess amount of water could force the forward reaction of hydrolysis. The optimum content of water in the system is needed to minimize the hydrolysis activity and maximize the enzyme activity for the transesterification reaction (Li and Yan, 2010).

Kumari *et al*. (2009) studied the effect of initial water concentration on the biodiesel production by adding water ranging from 5 to 20% (v/v) of the total amount of reaction mixture. The highest yield of biodiesel was obtained when there was no additional water in the reaction mixture. Although water is necessary for maintenance of enzymes' structure, an excess water decreases enzyme activity.

3.1.3.5 Reaction temperature

The temperature does not affect much on the transesterification as long as the operation temperature is controlled in the range between 20 and 70°C. However, most lipases have optimum temperature between 30 and 60°C. And the optimum temperature would normally increase in the case of immobilized enzyme (Fjerbaek *et al*., 2009). The increase in temperature would usually increase the reaction rate. However, at higher temperature, the enzyme could be denatured leading to the lower activity or loss of activity (Li *et al*., 2011).

Wang *et al*. (2010) investigated the effect of temperature on the lipase activities of the free and the immobilized enzyme in the bioconversion of biodiesel from soybean oil using methanol. It showed that the immobilized lipase had a wider temperature operation compared with the free lipase in temperature between 30 and 50ºC. Even though, the optimum temperature for both free and immobilized lipases were narrowly different, at 35ºC for the free lipase and 37ºC for the immobilized one but the thermal stability at high temperature was seemingly unalike. The activity of free lipase was retained at 10% when the transesterification reaction was carried out at 50ºC for 60 h. On the other hand, the retained activity of the immobilized lipase was higher at 77% of its activity under the same conditions. Therefore, the immobilization of enzyme improves the thermal stability of lipase at high temperature.

Dizge and Keskinler (2008) studied the effect of temperature on the catalytic activity of the immobilized lipase *Thermomyces lanuginosus* in the biodiesel production of canola oil with methanol in the temperature ranging from 30 to 70ºC. When temperature was higher than 50ºC, the activity of enzyme was reduced dramatically, and at 40ºC was the optimal temperature for biodiesel production. The maximum biodiesel yield (86%) catalyzed by *T. lanuginosus* lipase was achieved at 40ºC with methanol. At the temperature higher than 40ºC, a decrease in the biodiesel production and an increase in the free fatty acids were observed. This result indicated that a high temperature will stimulate the hydrolysis reactions.

4. Objectives

1. To screen and select lipase producing fungi obtained from the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University.
2. To study the lipase production using submerged fermentation and to purify and characterize the fungal lipase.
3. To immobilize the lipase on the nanofiber support.
4. To immobilize the lipase on the magnetic nanoparticle.
5. To study the biodiesel production from palm oil using the immobilized lipases on nanofibrous membrane and magnetic nanoparticle.

CHAPTER 2

OPTIMIZATION OF MEDIUM COMPOSITION AND PHYSICAL CONDITION FOR LIPASE PRODUCTION FROM *ASPERGILLUS* SP. ST11

Abstract

Lipases are natural catalysts that catalyze hydrolytic reaction as well as transesterification reaction. Now, they become more attractive due to the desire characteristics compared to chemical catalysts. This work was focused on the selection of lipase producing fungus and optimizing medium and condition for lipase production. Seven isolates of fungi were cultivated in the liquid medium (1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6). The isolate *Aspergillus* sp. ST11 showed the highest hydrolysis and transesterification activities. Therefore, *Aspergillus* sp. ST11 was chosen to optimize the lipase production. The optimized medium contained 1% olive oil, 2% peptone, 1% lactose, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v), and pH 6. The cultivation was incubated at 37^{o}C and shaken at 150 rpm using 10^7 spores/ml of inoculum. *Aspergillus* sp. ST11 produced the maximum activity with 31 U/ml compared to 12.2 U/ml before optimization.

Introduction

Currently, the awareness of releasing green-house gases from fossil fuels has been much concerned and the use of biodiesel has been drawn attention from many countries. Biodiesel is a renewable energy source releasing the low amount of green-house gases. Moreover, it is able to be degraded in the environment (Tan *et al*., 2010). Biodiesel can be synthesized from oil and alcohol via transesterfication reaction or esterification of free fatty acid and alcohol. The transesterification reaction will transform triglycerides into fatty acid alkyl esters, in the presence of an alcohol, such as methanol or ethanol and a catalyst (Vasudevan and Briggs, 2008).

The catalysts used for biodiesel production can be classified as alkaline, acid, enzymatic and inorganic heterogeneous catalysts (Gog *et al*., 2012). Biodiesel is commercially produced by chemical catalysts for higher conversion in a short time.

However, this process also has some disadvantages such as high energy process, difficulty in glycerol separation, the contamination of catalysts in the product, water treatment for alkaline catalysts, interference of free fatty acids and water to the reaction (Jegannathan *et al*., 2010). The drawbacks from using acid or alkali catalysis has driven the study of enzymatic process, because this process is very simple, lower energy consumption, high quality of glycerol as a by-product and no soup formation in the biodiesel production process (Christopher *et al*., 2014).

Lipases (E.C. 3.1.1.3) are ubiquitous enzymes discovered in most organisms. They are important enzymes in nature for utilizing lipids. Lipases can catalyze not only hydrolysis but also esterification and transesterification reactions (Reis *et al*., 2009). Lipases are enzymes with noteworthy industrial use since they display advantages such as high catalytic activity, operation under mild reaction conditions, eco-friendly, and delicate chemical, enantio- and regioselectivity (Contesini *et al*., 2010). Filamentous fungi are recognized as the best lipase producers since they produce extracellular lipases, facilitating the extraction from fermentation media. Fungal lipases have drawn the attention for the industrial sector due to their stability, selectivity and wide substrate specificity. Some species of *Aspergillus* genera might possess the distinct characteristics compared to other. For example, *Aspergillus niger* NCIM 1207 lipase was acidic lipase, its optimum pH was 2.5 that means it can be used in acidic condition. Moreover, when fungal lipase is applied in biodiesel production, it showed the good possibility to catalyze the transesterification. *Aspergillus* species have been widely studied for biodiesel production. Most studies showed the use of *Aspergillus* lipase as a whole-cell catalyst to catalyze the transesterification such as the biodiesel production in the packed-bed reactor using immobilized *Aspergillus niger* mycelium. At the optimized conditions, *A. niger* biocatalyst produced biodiesel at 23.1% (Almyasheva *et al*., 2018). Another work was the biodiesel production from microalgal lipid catalyzed by the immobilized whole cell lipase from *A. niger* giving the highest biodiesel conversion at 80.9% (Guldhe *et al*., 2016). There were some studies working with the extracellular lipase produced by *Aspergillus* species for biodiesel production. Romero *et al*. (2012) reported the production of biodiesel using long-chain fatty acids (C18) catalyzed by the extracellular lipase from *A. niger* MYA entrapped inside polyacrylamide gel. The immobilized lipase still retained the activity

more than 60% after 1 cycle of use. There were some reports of using lipase from *Mucor miehei* and *Candida antarctica* in the transesterification of many oils using hexane. It showed that the lipase from *M. miehei* is more effective in the conversion of primary alcohols such as methanol, ethanol, propanol, and 1-butanol with biodiesel yields between 95% and 98%. While *C. antarctica* lipase is more suitable for the conversion of secondary alcohols such as isopropanol and 2-butanol with biodiesel yields between 61% and 84% (Singh and Mukhopadhyay, 2012).

The lipase producing fungi could be found in many inhabitants including the waste from oil factory, the dairy products, soil contaminated with oils, spoiled food etc. The habitant conditions of isolated fungi were related to the difference in temperature and pH stability and the specificity onto the substrates (Griebeler *et al*., 2011). After the isolation of lipase producing fungi. The strains were inoculated in the medium with different compositions and cultivation conditions aiming to increase the lipase production. Essamri *et al*. (1998) studied the optimization of *Rhizopus oryzae* lipase production isolated from Cameroonian palm fruit. The highest lipase activity was achieved in the medium with 3% rape oil, 7% corn steep liquor, pH 5 at 25ºC, and 120 h of incubation. Colin *et al*. (2010) optimized the extracellular lipase production from *Aspergillus niger* MYA 135. The lipase activity was enhanced in the presence of olive oil (3.5%) and the addition of 0.5 g/l $CaCl_2$ and 1.0 g/l $FeCl_3$ increased the lipase activity. Aulakh and Prakash (2010) studied the optimization of medium and process parameters for the lipase production from *Aspergillus* sp. (RBD-01), the maximum activity (21.8 U/ml) was obtained with 50% (v/v) of cotton seed oil and 0.5% (w/v) of peptone, pH 7.5 at 35ºC and 120 h of incubation.

In this study, we aimed to screen for the extracellular lipase producing fungus which had the ability to produce biodiesel. The selected fungus was optimized for both medium compositions and cultivation conditions for lipase production. The obtained lipase was immobilized and used for biodiesel production as well as the purification study to understand the characteristics of the purified lipase from the selected isolate.

Materials and methods

1. Materials

Palm oil, sunflower oil, olive oil and fish oil were purchased from the local supermarket. Crude palm oil and molasses were obtained from Faculty of Engineering, Prince of Songkla University. Lactose, glucose, maltose, and fructose were purchased from Univar® Analytical Reagent, New Zealand. Beef extract, tryptone, yeast extract and peptone were purchased from Himedia, India.

2. Methods

2.1 Screening of lipase producing fungus

Seven isolates of fungi obtained from the stock culture collection of the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University were investigated in submerged fermentation for lipase production. One ml of each isolate ($1x10^7$ spores/ml) was transferred into 250 mL Erlenmeyer flask containing 100 mL of liquid medium (Talukder *et al*. 2013) (1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and adjusted pH 6) incubated at 37°C and 150 rpm for 3 days. After fermentation finished, the mycelium in each flask was filtered by Whatman paper no.1 and the filtrate was collected as a crude enzyme for investigating hydrolysis activity and transesterification by TLC-plate.

2.2 Hydrolysis activity assay

The 0.1 ml of crude enzyme was added to 0.5 ml of 10% w/w palm oil in isooctane. After that, the mixture was incubated by Thermo mixer at 37°C 1200 rpm for 30 min. The reaction was eventually stopped by adding 0.15 ml of 6 N HCl. The 0.1 ml of upper organic phase was withdrawn and mixed with 0.9 ml of iso-octane. The solution was then mixed thoroughly with 0.2 ml of cupric acetate solution. The upper phase was measured for the absorbance at 715 nm and using isooctane as a blank (Kwon and Rhee, 1986).

2.3 Dry cell weight

The mycelium after filtration was placed in hot air oven at 60°C overnight. After that, the weight of dried mycelium was measured.

2.4 Transesterification assay

For the transesterification, the 100µl of crude lipase or 10 mg of filtered mycelium were added to the mixture containing 0.2 g of palm oil and 31µl of methanol (1:3 mol:mol) and shaken by Thermomixer at 37°C and 1200 rpm for 72 h. After incubation, 10µl of upper phase was withdrawn and mixed with 90 µl of chloroform. The diluted sample was spotted on the TLC-plate and run with 2 solvent systems (1^{st} solvent; n-heptane:diethyl ether:acetic acid [55:45:1 v/v/v], and 2^{nd} solvent; n-hexane:benzene [1:1 v/v]) (Diaz et al., 2006). The sample was visualized by copper sulfate reagent and compared the sample to the standard products (methyl ester, triglyceride, diglyceride and free fatty acid). The isolate that showed the activity of both hydrolysis and transesterification was selected for lipase production.

2.5 Optimization of physical condition and medium composition for lipase production.

2.5.1 Optimization of physical condition

Aspergillus sp. ST11 was cultivated in 100 ml of the liquid medium (1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and adjusted pH 6). The incubation time was varied at 24, 48, 72, 96, 120, 144 and 168 h. At each period, the culture broth was filtered from the liquid medium and the filtrate was used as a crude enzyme. The incubation time showing the highest hydrolysis activity was selected for further study. The initial pH of culture medium was varied at 5.0, 5.5, 6.0, 6.5 and 7.0. The inoculum level (1 ml) was added to the culture medium with different amount, $1x10^6$, $1x10^7$ and $1x10^8$ spores/ml. The last parameter for optimizing physical condition was rotation speed. The rotation speed was set at 100, 150 and 200 rpm.

2.5.2 Optimization of medium composition

To study the effect of different oils on lipase production, palm oil, crude palm oil, olive oil, sunflower oil and fish oil were added to the basal medium as a carbon source at 1.0 % (w/v). The oil showing the high lipase activity was selected and varied for different concentration of 0, 0.5, 1.0, 1.5 and 2.0 % (w/v). In addition, the carbohydrate source was also investigated for lipase production. The lactose, glucose,

maltose, fructose and molasses were added to the basal medium at the concentration of 1.0 % (w/v). In the case of nitrogen source, both organic and inorganic sources were tested. Beef extract, tryptone, yeast extract and peptone were investigated at the concentration of 2.0 % (w/v). After the suitable organic nitrogen was obtained, the concentration was varied at 0, 0.5, 1.0, 2.0, and 4.0 % (w/v). For inorganic nitrogen, sodium nitrate, urea, ammonium nitrate and ammonium sulfate were added to the basal medium at 0.2 % (w/v).

2.6 Statistical analysis

Each study was conducted with triplicate. The lipase activity under different condition was analyzed by Analysis of Variance (ANOVA) and all treatment means were compared using Turkey's studentized range test for comparing mean at 5% level of significance. All statistical analysis was done using SPSS software version 14.

Results and discussion

1. Screening of an extracellular producing fungus

Seven fungal isolates were screened for the extracellular lipase production. To evaluate the potential of lipase production, the hydrolysis activity and transesterification were introduced for screening lipase producing fungi. The fungal isolates were inoculated to the liquid basal medium as described earlier. The extracellular lipases were collected from the culture broth by filtration and tested for hydrolysis and transesterification activities by TLC-plate. Among 7 isolates, *Aspergillus* sp. ST11 expressed the highest hydrolysis activity at 12.2 U/ml while other isolates just showed lower hydrolysis activity as shown in Figure 7. For the fungal biomass, the isolate ST11 showed the highest biomass at 1.25 g/100ml of medium. In the case of biodiesel production potential, the crude lipases as well as dried fungal cells (obtained from filtration was washed with chilled acetone) were added in the mixture containing palm oil and methanol for transesterification. The samples were withdrawn and analyzed by TLC-plate technique. Only crude lipase of *Aspergillus* sp. ST11 could catalyze transesterification and produced biodiesel. In the case of cell bound lipase, only the dried cells of *A. nomius* produced biodiesel (Figure 8). In this study,

Aspergillus sp. ST11 was chosen for the further study due to its capability to produce the extracellular lipase. The produced lipase was aimed to use for immobilization on the synthetic materials for biodiesel production.

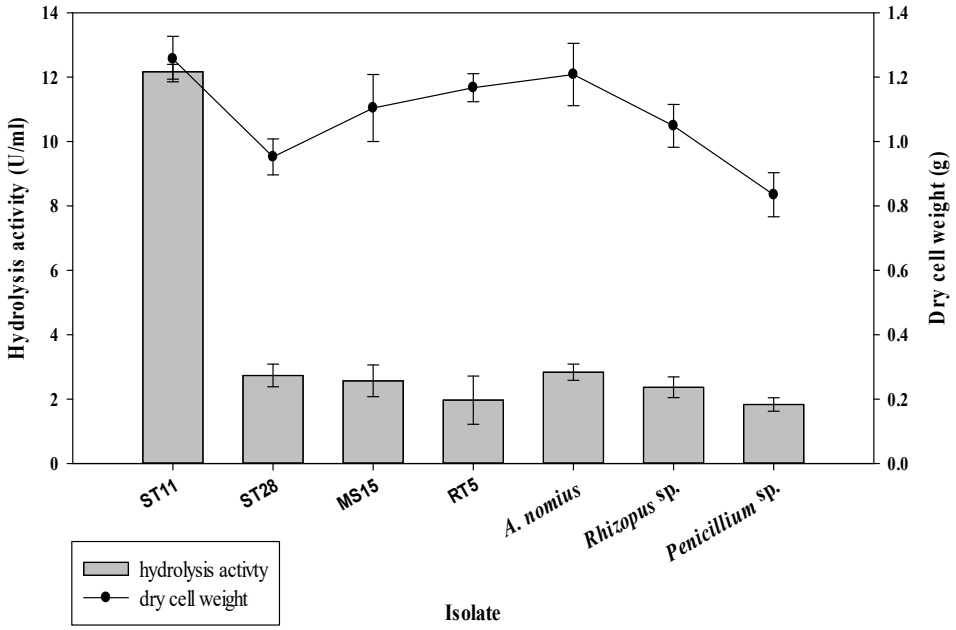


Figure 7. Hydrolysis activity of extracellular lipase and dry cell weight of 7 fungal isolates in the basal medium after 3 days.

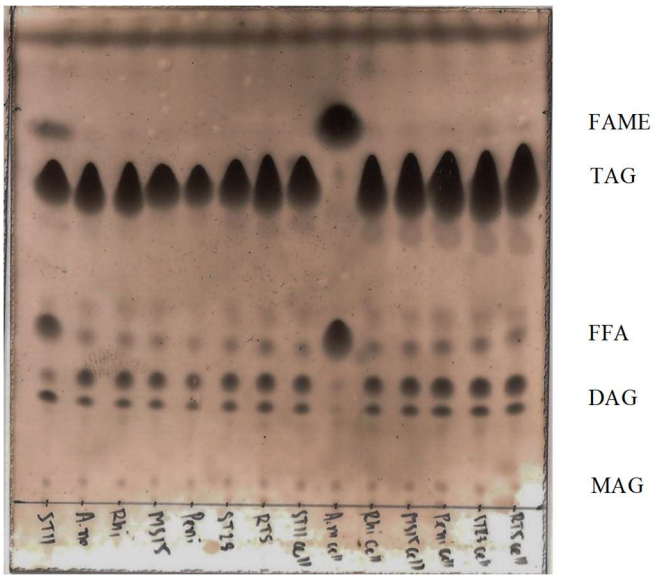


Figure 8. The TLC-Plate shows products of transesterification; lane 1-7 shows products catalyzed by crude lipase and lane 8-14 shows products catalyzed by dried fungal cells of *Aspergillus* sp. ST11, *A. nomius*, *Rhizopus* sp, MS15, *Penicillium* sp, ST28 and RT5.

2. Optimization of physical condition for lipase production

The conventional one-factor-at-a-time method was used to optimize the production of extracellular lipase of *Aspergillus* sp. ST11. The effects of physical parameters (e.g. cultivation time, initial pH, temperature, inoculum level and rotation speed) on lipase production were studied. The spores of *Aspergillus* sp. ST11 were inoculated into the basal medium. The time for incubating microorganisms is quite important on the production of extracellular lipase. The inoculated medium was shaken at 150 rpm 37ºC for 168 h. From the result, the hydrolysis activity increased and reached the maximum activity (16.9 U/ml) at 96 h and then gradually decreased (Figure. 9). The increase in the hydrolysis activity was associated with the cell growth. After reaching the stationary phase, the lipase activity decreased which might be due to the effect of depletion of nutrients, accumulation of end product that could inhibit the enzyme as well as the change in pH of the medium (Imandi *et al*., 2013). Several researchers have reported different incubation periods for optimal lipase production. *Rhizopus* sp. BTNT-2 produced lipase which increased up to 48 h and later showed the decline in lipase activity (Bapiraju *et al*., 2005). The lipase production from *A.*

fumigatus MTCC 9657 had the maximum lipase activity could be observed at 96 h and started to decrease at 120 h due to the consumption of nutrients (Rajan and Nair, 2011).

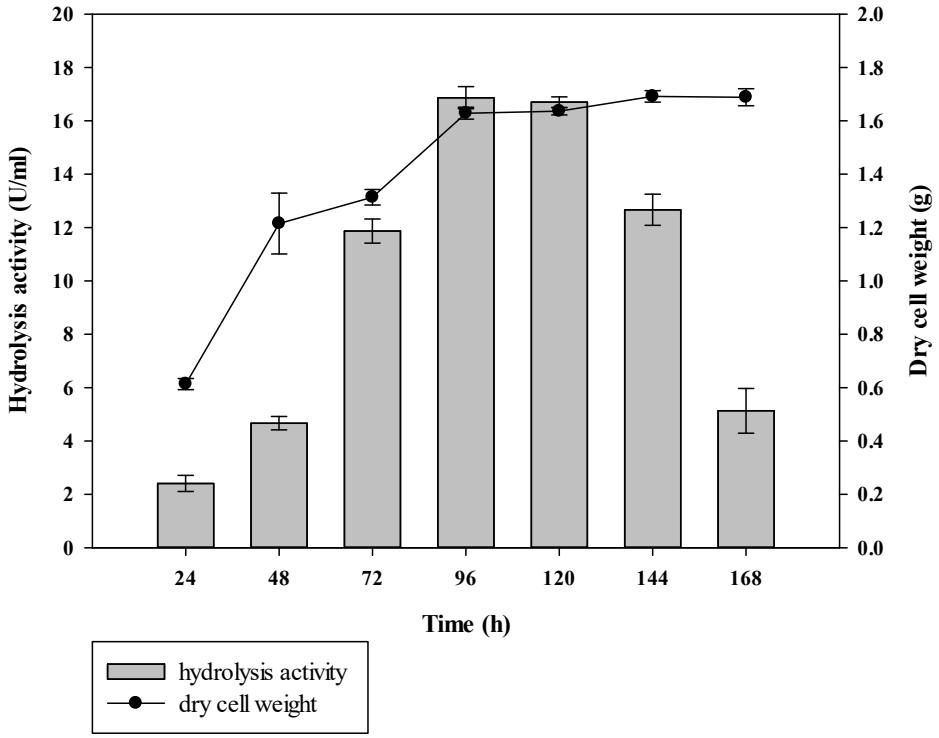


Figure 9. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 during cultivation in the basal medium. (The medium compositions were 1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37ºC and shaken at 150 rpm).

The pH of medium played the important role by inducing morphological change in the microorganism and in enzyme secretion (Bapiraju *et al*., 2005) The effect of pH on the lipase production was studied by varying the initial pH of medium (5.0, 5.5, 6.0, 6.5 and 7.0). The maximum hydrolysis activity was observed when the initial pH was pH 6.0. (Figure 10) which was slightly acidic. It is similar to the result of *Rhizopus* sp. BTNT-2 lipase that showed the maximum activity and biomass at pH 5.5 which was slightly acidic condition. The sharp decrease in the lipase activity when the initial pH changed was reported by (Cho *et al*., 2015). They studied the effect of initial pH on the lipase production from *Penicillium cyclopium*. The optimum initial pH for

lipase production was 6.0 (68 U/ml) while the changes of pH to 5.5 and 6.5 led to a lower lipase production (<20 U/ml).

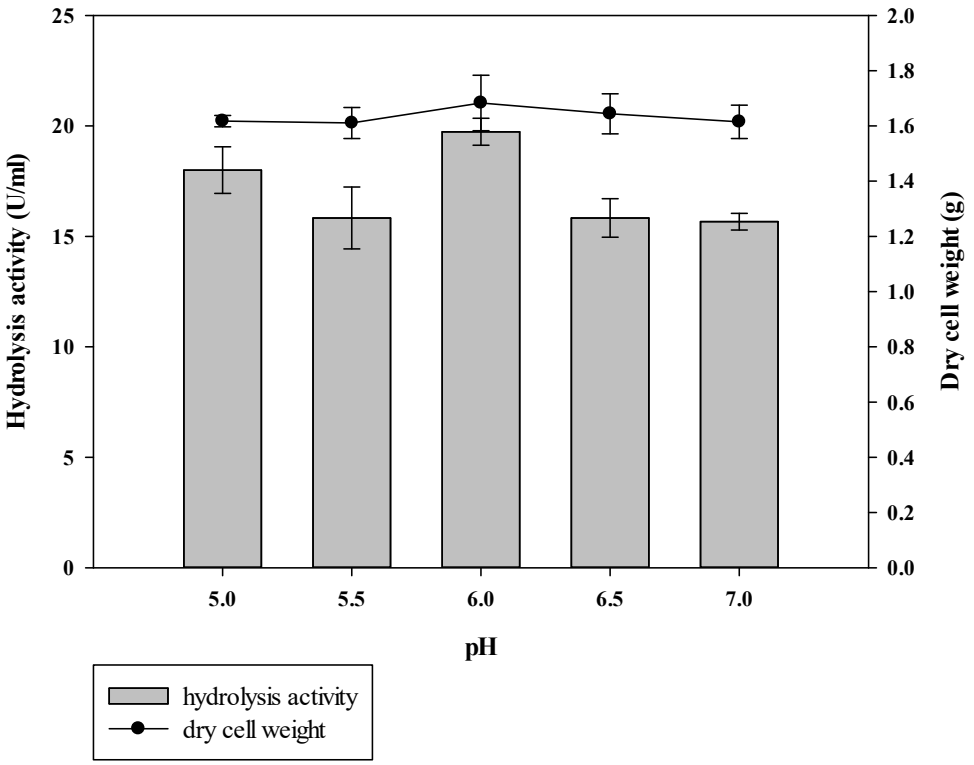


Figure 10. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different pH. (The medium compositions were 1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v). The cultivation was incubated at 37°C and shaken at 150 rpm for 4 days).

For inoculum level, the inoculum size higher or lower than the optimal level might show the low activity of lipase. The lower inoculum size might be insufficient for forming the biomass leading to reduce product formation. The higher inoculum size might produce too much biomass causing a poor formation of the product. Moreover, different inoculum levels affected the mycelial morphology. Teng *et al*. (2009) studied the lipase production by *R chinensis* and reported that when the inoculum with 10^8 spores/ml was applied to the medium, the morphology of mycelium was changed to pulp like form and produced the lipase at 109 U/g while the lower inoculums (10^6 and 10^7 spores/ml) showing a fully entangled filaments produced higher

lipase activities at 650 and 691 U/g, respectively. Bapiraju *et al*. (2005) studied the inoculum levels (2-20% v/v) of *Rhizopus* sp. BTNT-2. The highest activity and biomass were 46.5 U/ml and 8.22 mg/ml at 8 % (v/v) of inoculum. In this experiment, three levels of inoculum were tested. From the result 10^7 spores/ml of *Aspergillus* sp. ST11 showed the maximum lipase activity at 18.4 U/ml (Figure 11). The lower lipase activity in the medium with a high inoculum level in this experiment was similar with the study by Teng *et al*. (2009). The high inoculum level led to the formation of dispersed mycelia structure, this morphology of mycelia showed a lower catalytic efficiency by reached the highest point (300 U/g) at 36 h of the incubation and then the activity drastically decreased to 60 U/g after 95 h of incubation which differed from the biomass production that gradually increased when the incubation time was increased. Comparing to a lower inoculum size that formed an entangled structure, its activity reached 600 U/g at 60 h, and still maintained the activity at 650 U/g when fully entangled filaments were formed.

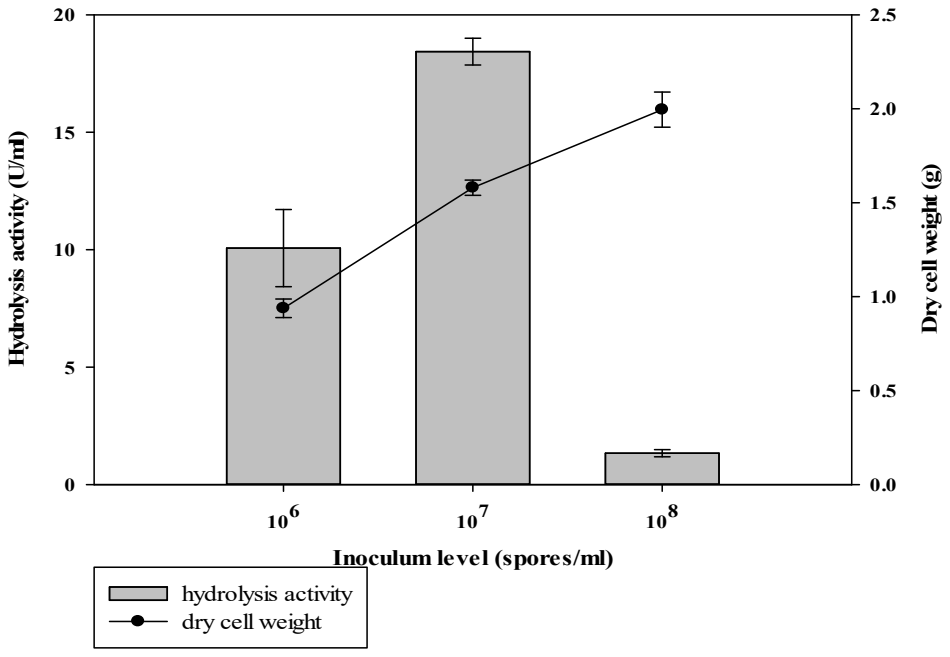


Figure 11. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different inoculum level. (The medium compositions were 1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37ºC and shaken at 150 rpm for 4 days).

The agitation of the culture could enhance aeration to the medium and also the dispersion of the nutrients throughout the medium. The inoculated flasks were shaken with different rotation speed for lipase production by *Aspergillus* sp. ST11 in the basal medium with the palm oil as the carbon source. The result indicated that 150 and 200 rpm gave the highest activity (18.1 and 17.5 U/ml) with no significant difference (Figure 12). This result was different from the result of *Rhizopus* sp. BTNT-2 that the optimum speed was 120 rpm and the lipase activity drastically decreased when the rotation speed as increased more than 120 rpm. (Bapiraju *et al*., 2005).

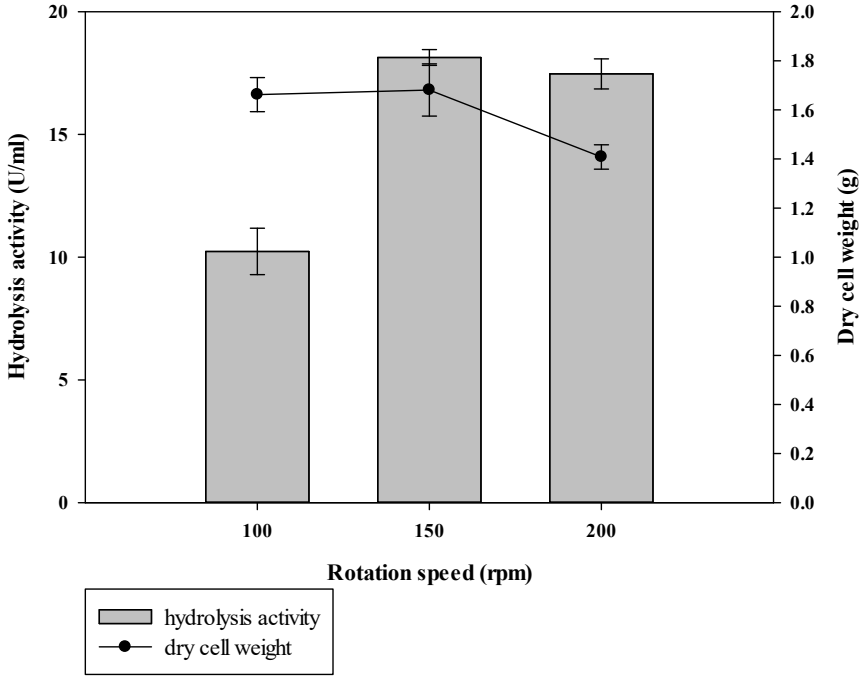


Figure 12. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different rotation speed. (The medium compositions were 1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37ºC for 4 days).

3. Optimization of medium composition for lipase production

Many studies investigated the lipase production and have claimed that both sugars and oils improved the production of extracellular lipase (Maia *et al*., 2001). In this experiment, many kinds of oil were used as a carbon source (palm oil, crude palm oil, sunflower oil, olive oil and fish oil) in the basal medium for lipase production

by *Aspergillus* sp. ST11 compared to the control set (without oil). The result indicated that when palm oil in the basal medium was changed to olive oil, the lipase activity was enhanced significantly from 18.4 U/ml to 26.1 U/ml (Figure 13). When the fungal spores were inoculated into the medium with no oil, the activity of lipase decreased drastically (1.1 U/ml). In the medium with fish oil, *Aspergillus* sp. ST11 grew well but it produced very low lipase activity. The effect of olive oil on lipase activity in this experiment was similar to the result of lipase production from *Rhizopus homothallicus* which the olive oil gave the highest lipase activity among other 5 oils (nut oil, corn oil, peanut oil, grapeseed oil and sunflower oil) (Rodriguez *et al*., 2006). Lakshmi *et al*. (1999) reported that the lipase production increased in the same way as the increase of C18:n fatty acids which existed in large amount in olive oil. For the concentration of olive oil in the medium, it was found that 1% w/v of olive oil was optimum for lipase production by *Aspergillus* sp. ST11 (Figure 14). The higher concentration of oil might inhibit the lipase production according to substrate inhibition (Noor *et al*., 2003).

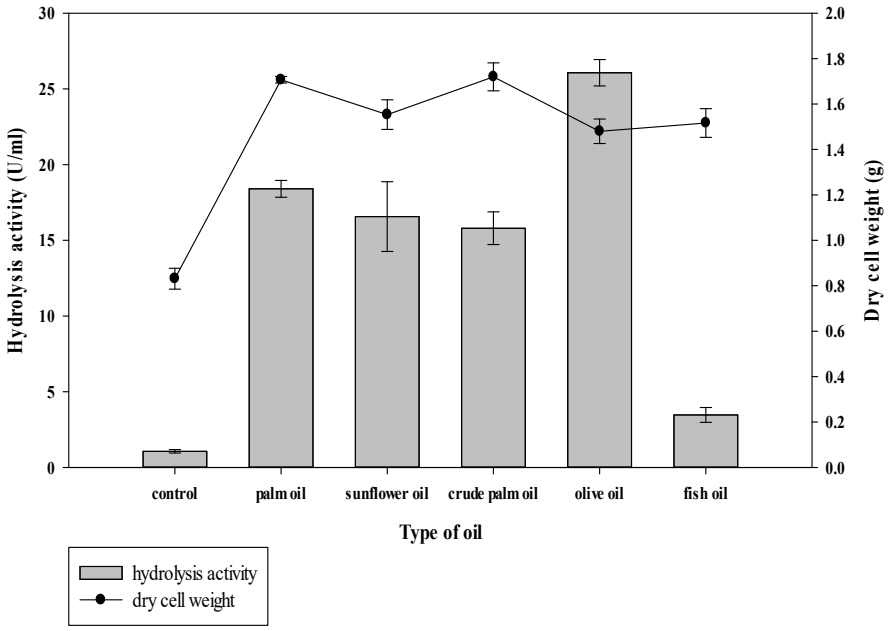


Figure 13. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different oils. (The medium compositions were 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37ºC and shaken at 150 rpm for 4 days).

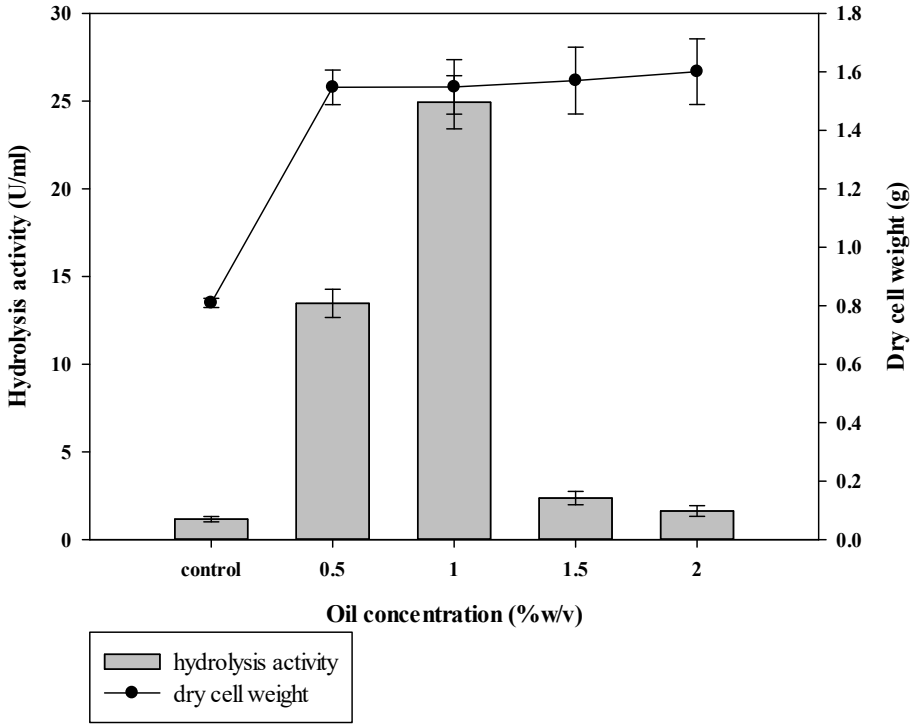


Figure 14. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different concentrations of olive oil comparing with the medium without oil. (The medium compositions were 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm for 4 days).

For carbohydrate source, five carbohydrate sources (glucose, fructose, lactose, maltose and molasses) were added into the basal medium with olive oil at 1.0% w/v compared to the set of experiment with no co-carbon source. From the result, most carbohydrate added to the medium had the negative effect on lipase production by *Aspergillus* sp. ST11 excepted lactose which enhanced the lipase production (Figure 15). The addition of lactose promoted the significant production of lipase. (Rout et al., 2012) studied the addition of various sugars to the medium, the existence of lactose in the medium enhanced the highest lipase activity from *A. terreus* whereas the addition of other carbohydrates such as fructose and mannose did not enhance the lipase activity. The result in this experiment was similar to the result of lipase production from *A. niger* that sugars might effect on biomass of microorganism instead of producing lipase (Falony *et al*., 2006) as well as the work of Lima *et al*. (2003) that cultivated *P.*

aurantiogriseum for lipase production and found that there was no lipase activity detected when glucose was used.

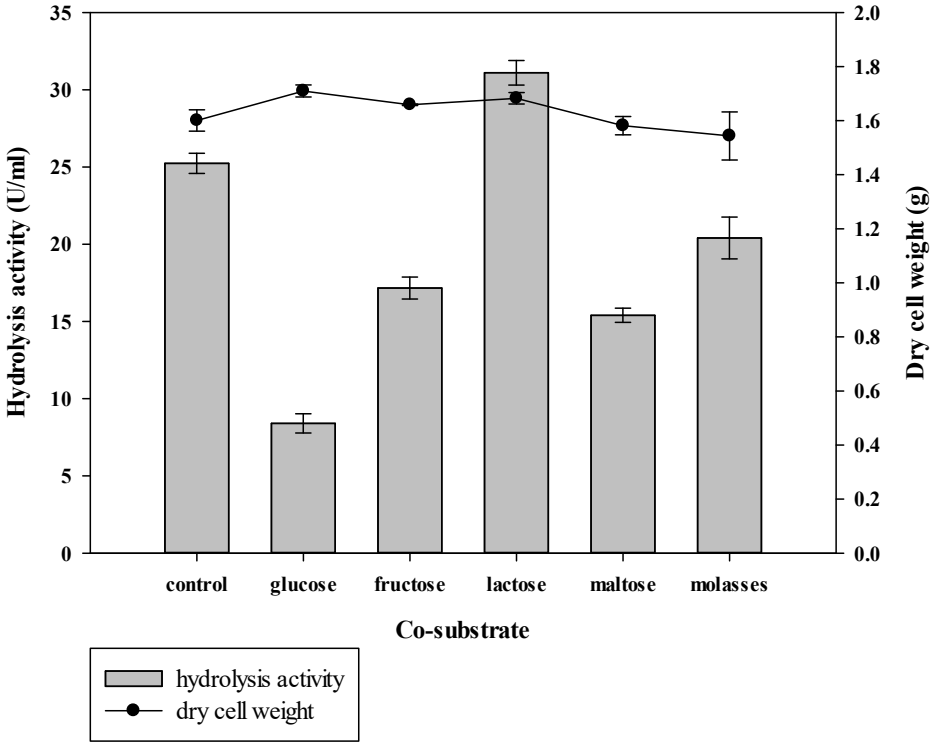


Figure 15. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different co-substrates comparing to medium without co-substrate. (The medium compositions were 1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37ºC and shaken at 150 rpm for 4 days).

Nitrogen source is also important for lipase production. The production of extracellular lipase could increase by increasing the concentration of nitrogen (Lima *et al*., 2003). Various kinds of organic nitrogen such as beef extract, tryptone, yeast extract and peptone at 2.0 % (w/v) were used for lipase production. Peptone expressed the highest lipase activity compared to other organic nitrogen sources and was greatly different to the set of experiment without nitrogen source (Figure 16). After that, peptone was varied in different concentration (0.5, 1.0, 2.0 and 4.0% w/v) and it was found that the medium with peptone as 2.0% provided the highest lipase activity (Figure 16). The lipase production from *A. niger* NRRL3 showed the optimum lipase activity

at 3% peptone (Ahmed and Ahmed, 2009). For co-nitrogen source, four kinds of co-nitrogen sources (sodium nitrate, ammonium nitrate, ammonium sulfate and urea) were used in this study. It was found that sodium nitrate showed the highest lipase activity among other co-nitrogen sources (Figure 18). Rajeshkumar and Ilyas (2011) reported that the medium containing the inorganic nitrogen could enhance lipase production. Their study showed that the addition of sodium nitrate produced the highest lipase activity compared to lower lipase activities obtained from the addition of ammonium sulfate, potassium nitrate, and urea. However, Rodriguez *et al.* (2006) revealed the negative effect of sodium nitrate on the lipase production of *R. homothallicus*.

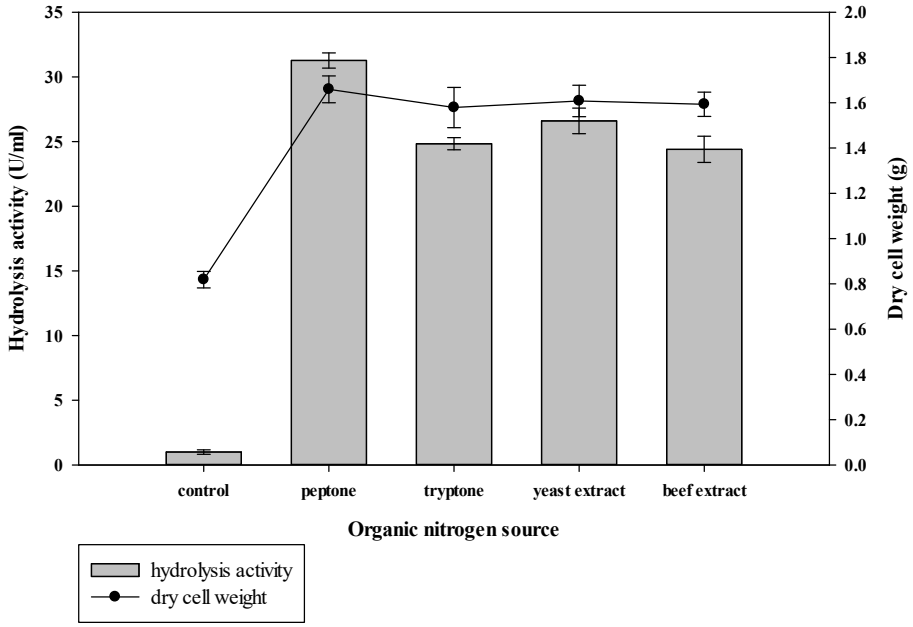


Figure 16. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different organic nitrogen sources comparing to medium without organic nitrogen. (The medium compositions were 1% palm oil, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm for 4 days).

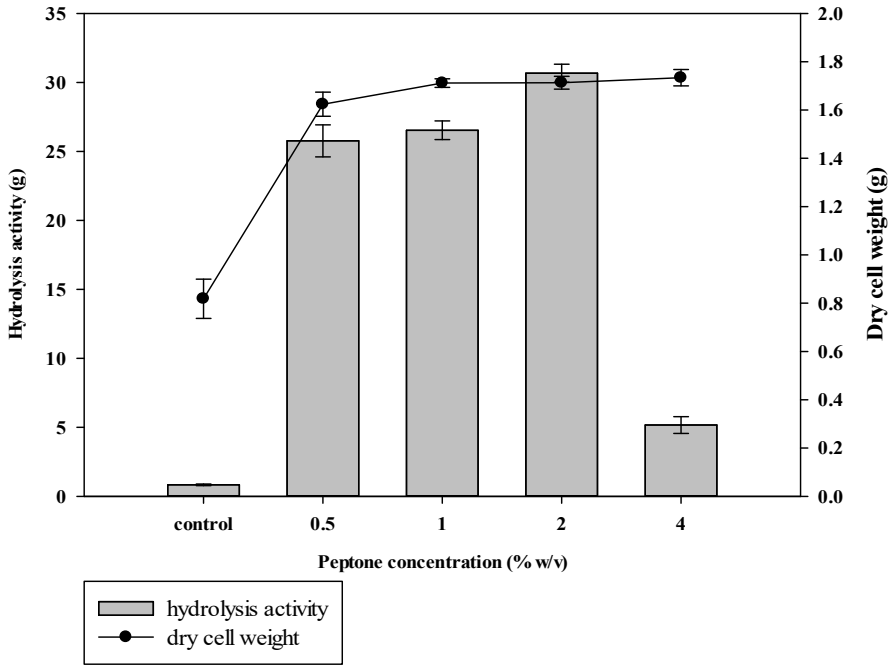


Figure 17. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different concentrations of peptone comparing with the medium without peptone. (The medium compositions were 1% palm oil, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm for 4 days).

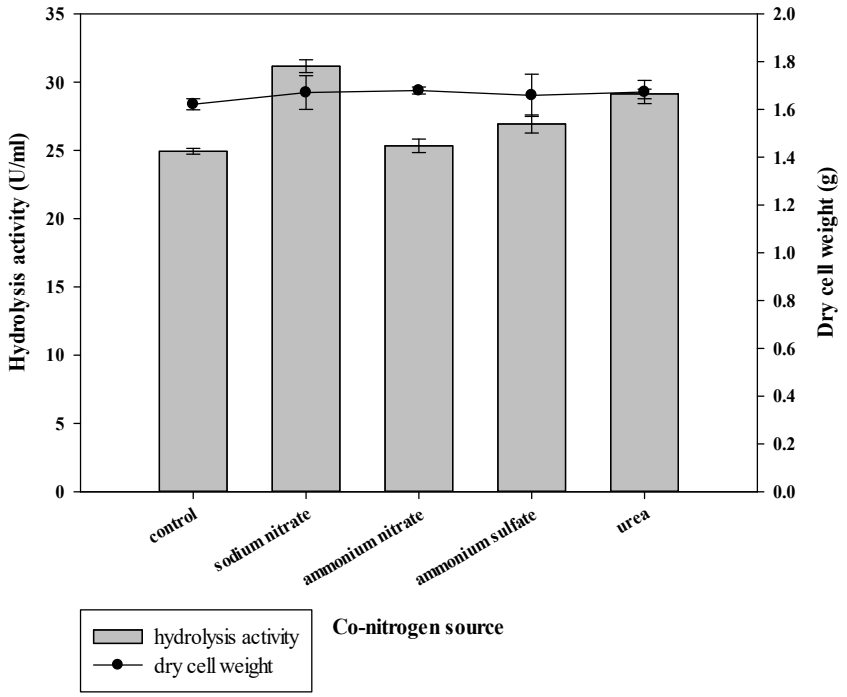


Figure 18. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 in the basal medium with different co-nitrogen sources comparing with the medium without co-nitrogen. (The medium compositions were 1% palm oil, 2% peptone, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v) and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm for 4 days).

The production of extracellular lipase by *Aspergillus* sp. ST11 was investigated using optimum conditions. The hydrolysis activity and dry cell weight were monitored during the cultivation for 7 days. The optimized medium was composed of 1% olive oil, 2% peptone, 1% lactose, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v), and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm. It showed the hydrolysis activity increased rapidly and reached the maximum activity at 31 U/ml after 96 h of incubation. After 168 h, the hydrolysis activity dropped to 25 U/ml. The increase in the incubation time also showed the increase in the biomass. The dry cell weight increased in the same direction as hydrolysis activity, this indicated the growth-dependent character of lipase (Figure 19).

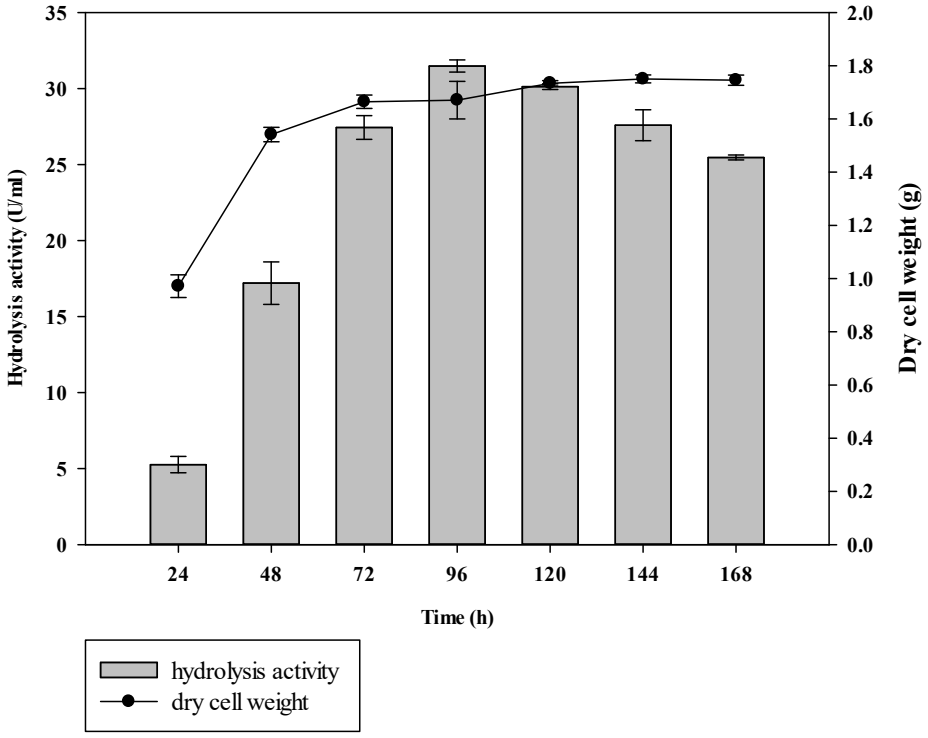


Figure 19. Cell growth and hydrolysis activity of *Aspergillus* sp. ST11 during cultivation in the optimized medium. (The medium compositions were 1% olive oil, 2% peptone, 1% lactose, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (w/v), and pH 6. The cultivation was incubated at 37°C and shaken at 150 rpm).

Conclusion

Among the seven isolates of fungi, *Aspergillus* sp. ST11 was the only one isolate which was capable to produce extracellular lipase and could catalyze transesterification reaction. The production of lipase by selected *Aspergillus* sp. ST11 was optimized by both physical condition and medium composition (1% palm oil, 2% peptone, 1% lactose, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and pH 6). Interestingly, adding olive oil and lactose enhanced the lipase activity. The lipase activity after the optimization was 31 U/ml which was higher than that of before optimization for 2.6 time. The unit number of a lipase after optimization indicated the feasibility for using this extracellular lipase for biodiesel production due to a high production of lipase by submerged fermentation. Moreover, the extracellular lipase

would be easier recovered and applied to the step of purification as well as the flexibility of enzymatic uses in the form of both free and immobilized lipase for bioconversion.

CHAPTER 3

IDENTIFICATION OF *ASPERGILLUS ORYZAE* ST11, PURIFICATION AND CHARACTERIZATION OF ITS LIPASE

Abstract

The lipase producing *Aspergillus* sp. ST11 was identified by the molecular and morphological method. The primers ITS1/ITS4 were used for amplifying the ITS region. It showed that the strain was grouped with *Aspergillus oryzae* and *Aspergillus flavus* (98% bootstrap value). The colony morphology of *Aspergillus* sp. ST11 on the malt extract agar (MEA) and Czapek yeast agar (CYA) showed a characteristic of *A. oryzae*. So, it was identified as *Aspergillus oryzae* ST11. The lipase produced by the strain was purified and characterized. The purification steps involved precipitation with chilled acetone and separation by column chromatography with HiTrap® Q HP and Toyopearl Butyl-650M, respectively. After purification, the lipase activity was increased 13-fold and 7.9 % yield. Its molecular mass was 25 kDa. The purified lipase was stable at the pH between 5.0-8.0 and had the optimum activity at pH 7.5. It was stable at 30ºC and had the optimum activity at 37ºC. Its activity was promoted in the presence of Mg^{2+} while greatly decreased in the presence of Co^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+}. The surfactants (Triton X-100, Tween-80, Tween-20, Arabic gum and sodium dodecyl sulfate) showed the negative effect on the lipase activity while inhibitors (PMSF, EDTA and β-mercaptoethanol) did not reduce the activity significantly. The polar solvent such as methanol and ethanol had much negative effect on the lipase activity compared to non-polar solvents such as hexane and isooctane.

Introduction

Triacylglycerol hydrolases or Lipases (E.C. 3.1.1.3) are the group of hydrolytic enzymes that break down the molecule of triglycerides and release free fatty acids and glycerol. They are also able to synthesize ester compounds. Lipases are obtained from various sources of living organisms including plants, animals or even microorganisms (Chaiyaso *et al*., 2012; Ungcharoenwiwat and H-Kittikun, 2015). Most of the lipases used in biotechnological application are from bacteria and fungi which

are cultivated in submerged and solid-state fermentations (Treichel *et al*., 2010; Marini *et al*., 2011; Fehrholz *et al*., 2010). The prominent points of using microbial lipase for industrial application are the stability, substrate specificity and production cost. They are more stable compared to the lipases derived from animals or plants. Moreover, microbial lipases could be produced in large-scale process which are very important to many industries such as pharmaceutical, detergent and food (Contesini *et al*., 2010; Geoffry and Achur, 2018; Bajaj *et al*., 2010). Among lipase producing microorganisms, filamentous fungi have become the best source for producing lipases. They can produce an extracellular enzyme which is easy for harvest and purification compared to bacterial lipases (Geoffry and Achur, 2018).

The development of polymerase chain reaction (PCR), DNA sequencing and molecular techniques has become the standard discipline to identify the unknow organisms (Chen *et al*., 2011). The identification of fungal strain is very important for strain selection. The safety of fungal use has been concerned for many applications. Some fungi produce mycotoxin which affect the health of both human and animal. *Aspergillus* spp. such as *A. niger*, *A. flavus*, *A. fumigatus* and *A. terreus* are known as mycotoxin producers. Hence, the identification of fungal strains to the species level with morphological and molecular techniques are required. The internal transcribed spacer (ITS) is amplified by using ITS1 and ITS4 primers (Zulkifli and Zakaria, 2017). Kantak *et al.* (2011) identified the isolate JK-1 using ITS1 and ITS4 primers and found that this isolate belonged to *Rhizopus oryzae.* Durairaj *et al.* (2018) isolated the pathogenic fungal strains from ginseng root rot. The ITS1 and ITS2 primers were used for amplifying ITS region and showing the pathogenic strains such as *Cladosporium*, *Ilyonectria liliigena*, *Neonectria* sp. and *Fusarium* sp. However, some species of *Aspergillus* could not be distinguished by using only molecular technique. The morphological study is required to reliably identify the species level of *Aspergillus* (Zulkifli and Zakaria, 2017).

Lipases produced by living organisms have been extensively studied by the purification scheme to understand their characteristics. Generally, the common steps of a lipase purification consist of ion-exchange, hydrophobic and gel filtration chromatographies, respectively. For the purification of lipase from *Aspergillus* sp., it was normally started from an ammonium sulfate precipitation of a crude lipase

followed by applying to an ion exchange chromatography (Basheer *et al*. 2011). However, some studies had a slightly different scheme for a lipase purification by using a hydrophobic interaction column after an ammonium sulfate precipitation *(*Saxena *et al*., 2003; Mehta *et al*., 2018)

In this study, the selected strain of lipase producing fungus was identified and the production of lipase of selected strain was performed by cultivating in the submerged fermentation. The produced extracellular lipase was then purified and characterized to study the nature of lipase produced from this strain.

Materials and methods

1. Substrates and chemicals

Palm oil, rice bran oil, coconut oil, sunflower oil, soybean oil, corn oil and olive oil were purchased from a local market. The metal ions; $FeCl_3$, $CoCl_3$, $CuCl_2$, $MgCl_2$, $HgCl_2$, $CaCl_2$, $ZnCl_2$, KCl, NaCl, $AlCl_3$ and the surfactants; Triton X-100, Tween 80, Tween 20, Gum arabic and Sodium dodecyl sulfate (SDS) were purchased from Analytical Univar Reagent (Auckland, New Zealand). The enzyme inhibitors; ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, USA).

2. Analytical methods

2.1 Lipase activity

The hydrolytic activity of lipase was measured in a two-phase system according to the modified colorimetric method (Lee and Rhee, 1993) using palm oil as a substrate in isooctane. The 0.1 ml of crude enzyme was added to 0.5 ml of 10% w/v palm oil in isooctane. After that, the mixture was incubated by Thermo mixer at 37ºC 1200 rpm for 30 min. The reaction was eventually stopped by adding 0.15 ml of 6 N HCl. The 0.1 ml of upper organic phase was withdrawn and mixed with 0.9 ml of iso-octane. The solution was then mixed thoroughly with 0.2 mL of cupric acetate solution. The upper phase was measured for the absorbance at 715 nm and using isooctane as a blank (Ungcharoenwiwat *et al*., 2016).

2.2 Protein determination

The protein of the samples in the purification steps was determined using Folin-Ciocalteu reagent according to Lowry's method (Lowry *et al*., 1951).

3. Molecular and morphological identifications

Aspergillus sp. Isolate ST11 was obtained from stock culture from the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. Hat Yai, Thailand. The internal transcribed spacer (ITS) region was amplified using primers ITS1/ITS4 (Innis *et al*., 1990). The ClutalWin Bioedit was used for aligning the consensus sequence amplified by ITS1/ITS4 primers (Hall, 1999). The sequence was then compared (BLAST) against GenBank database. Phylogenetic analysis was performed using MEGA software version 6.0 (Tamura *et al*., 2013) by applying 62llipsoi joining (NJ) method on ITS sequence.

For morphological identification, the isolate ST11 was cultured on malt extract agar (MEA) and Czapek yeast agar (CYA) and incubated at 25°C and 37°C for 7 days. The colony color and microscopic morphology were observed (Zulkifli and Zakaria, 2017).

4. **Production and purification of lipase**

The fungal spore was inoculated on the potato dextrose agar and incubated at 37ºC for 5 days. The spores were collected and adjusted to $1x10^7$ spore/ml using haemacytometer and 1 ml of inoculum was inoculated into the optimized medium (100 ml) containing 1% olive oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4$ and 0.5% lactose (w/v) and adjusted pH to 6.0. The culture medium was shaken at 150 rpm and 37ºC for 4 days. The purification of the lipase was carried out with the steps of a solvent precipitation (chilled acetone), an anion-exchange column chromatography (HiTrap Q HP 5 ml) and a hydrophobic column chromatography (Toyopearl Butyl-650M).

The culture broth was separated from the mycelium by filtration with Whatman no.1 filter paper. The crude enzyme was precipitated from the culture broth by adding 3 volumes of cold acetone per 1 volume of the culture broth and kept at 4ºC overnight. The solution was centrifuged (8000 rpm at 4ºC for 10 min) and the precipitate was dissolved with small amount of 50 mM Tris-HCl buffer pH 7.5 and transferred to the dialysis bag (3,500 Da). The sample was dialyzed against the same buffer at 4ºC overnight. The expanded volume of sample was reduced using the adsorption of carboxymethyl cellulose powder (CMC) The concentrated lipase was collected for the purification and characterization.

The lipase solution (2 ml) was applied to a HiTrap Q HP column by using ÄKTA Prime chromatography system (Amersham Pharmacia Biotechnique Group, Sweden). The process was carried out with the 2 buffer systems (50 mM Tris-HCl buffer pH 7.5) that contained no NaCl and with 1 N NaCl. The concentration of NaCl was increased from 0-5, 5-15, 15-100% NaCl, respectively. All fractions were measured by using UV absorbance at 230 nm. The absorbance corresponding fractions were collected and measured for lipase activity. The active fractions were combined together and used for next purification step.

The enzyme obtained from an anion exchange chromatography step was applied to the Toyopearl Butyl -650M. The column was equilibrated with 50 mM Tris-HCl buffer pH 7.5 with 50% ammonium sulfate. After applying enzyme solution to the column, the column was washed by equilibrate buffer and gradually decreased the concentration of salt from 50 to 0%. The fractions showing the absorbance (A_{280}) were collected and measured the lipase activity. The active fractions were pooled and dialyzed against the same buffer without ammonium sulfate at 4ºC overnight and concentrated by carboxymethyl cellulose (CMC). The concentrated enzyme solution was evaluated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE study was carried out by using 10% separating gel and 5% stacking gel. The molecular weight of purified ST11 lipase was compared to the standard marker (Pink Plus Prestained Protein ladder with sizes range from 10-175kDa).

5. Characterization of purified lipase from *Aspergillus oryzae* ST11

5.1 Effect of pH and pH stability

The optimum pH for lipase ST11 activity was studied, the lipase was prepared in different buffers with the same concentration (50 mM) and determined for the activity at 37ºC. The buffers used in the study were acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.0-9.0). In the case of pH stability, the purified lipase was mixed with the above buffers (1:3, v/v) and the sample was incubated at 30ºC for 2 h. The residual activity of the purified lipase ST11 was determined at pH 7.5.

5.2 Effect of temperature and thermostability

The purified lipase was studied for the optimum temperature in the temperatures ranging from 30-65ºC in 50 mM Tris-HCl buffer pH 7.5. For effect of temperature on lipase stability, the purified lipase was incubated at different temperatures from 30-65ºC for 2 h.

5.3 Effect of metal ions

Different metal ions (Fe^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Na^{+} and Al^{3+}) were used for testing the effect on the activity of the purified ST11 lipase. The enzyme was added into the reaction mixture containing 50 mM Tris-HCl buffer pH 7.5 and metal ions at 1.0 and 10.0 mM. The reaction was carried out at 30ºC for 30 min. The lipase activity was assayed and compared to the control without the addition of metal ions.

5.4 Effect of inhibitors and surfactants

Different inhibitors and surfactants were determined for the effect on the lipase ST11 activity. The purified ST11 lipase was incubated for 30 min at 30ºC in 50 mM Tris-HCl buffer pH 7.5 containing different inhibitors (1.0 mM): β-mercaptoethanol, phenyl methyl sulphonyl fluoride (PMSF) ethylene diamine tetra-acetic acid (EDTA) and surfactants (1.0%, w/v): Triton X-100, Arabic gum, Tween 20,

Tween 80 and sodium dodecyl sulfate (SDS). The lipase activity was assayed and compared to the control without the addition of inhibitors or surfactants.

5.5 Effect of organic solvents

Various organic solvents were determined for the lipase stability. The organic solvent (0.15 ml) was added to purified lipase solution (0.45 ml) in a microcentrifuge tube. The mixture was incubated in Thermomixer shaker (TAITEC, Japan) at 30ºC and 500 rpm for 1 h. The lipase activity was then measured and compared to the control.

5.6 Substrate specificity

The substrate specificity of the purified ST11 lipase was studied by using the natural oils; palm oil, rice bran oil, coconut oil, sunflower oil, soybean oil, corn oil and olive oil. Each kind of oils was prepared to replace the palm oil in the reaction mixture for lipase activity determination using cupric acetate method.

Results and discussion

1. Molecular and morphological identifications

According to neighbor joining (NJ) phylogenetic tree analysis of ITS sequence, the *Aspergillus* sp. ST11 was grouped with *A. flavus* and *A. oryzae* (98% bootstrap value). This strain showed sequence similarity to *A. flavus* UOA/HCPF 10017 (FJ878656) and *A. oryzae* A-4 (GU120193) with 100% (Figure 20). Molecular identification using ITS sequence cannot be used to differentiate *A. flavus* and *A. oryzae*. Therefore, morphology of strain ST11 on malt extract agar (MEA) and Czapek yeast agar (CYA) were important for identification.

For morphological identification, the strain ST11 grew well on CYA and MEA. The colony on CYA is yellow green colony (Figure 21 a-d). The colony on MEA agar is olive green colony (Figure 22 a-d). Conidiophores are in length 0.08-0.09 µm, colorless and roughened (Figure 23 a,b). Conidia are 3.2-4.1 µm in diameter, globose to 65llipsoidal and smooth wall (Figure 23 c). The results from this study related to the morphology study of *A. oryzae* colony on CYA and MEA (Zulkifli and Zakaria, 2017).

Therefore, the isolate ST11 was designated as *A. oryzae* ST11 using molecular identification based on ITS regions and morphological characters.

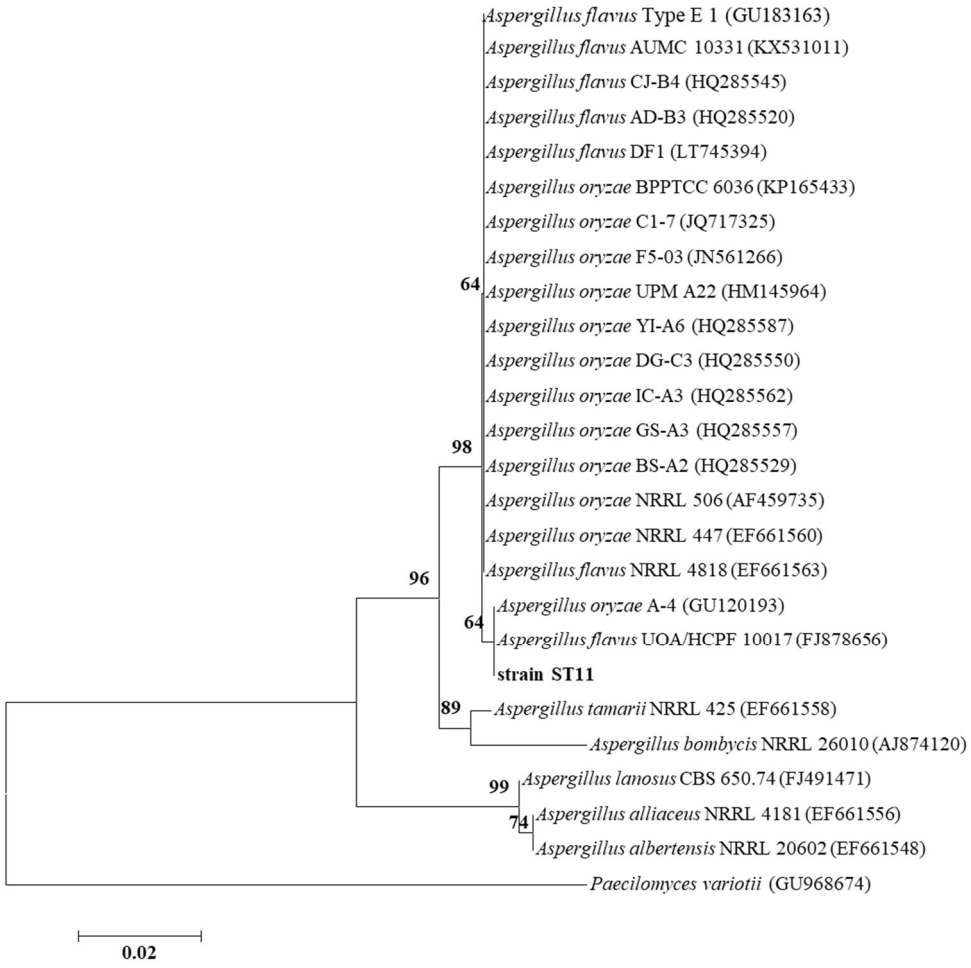


Figure 20. The Neighbor-joining phylogenetic tree obtained from ITS rDNA sequence analysis based on the Tamura-Nei model calculated using MEGA 6.0. The scale bar denotes 0.02 substitutions per position. *Paecilimyces variotii* was used as the root of tree.

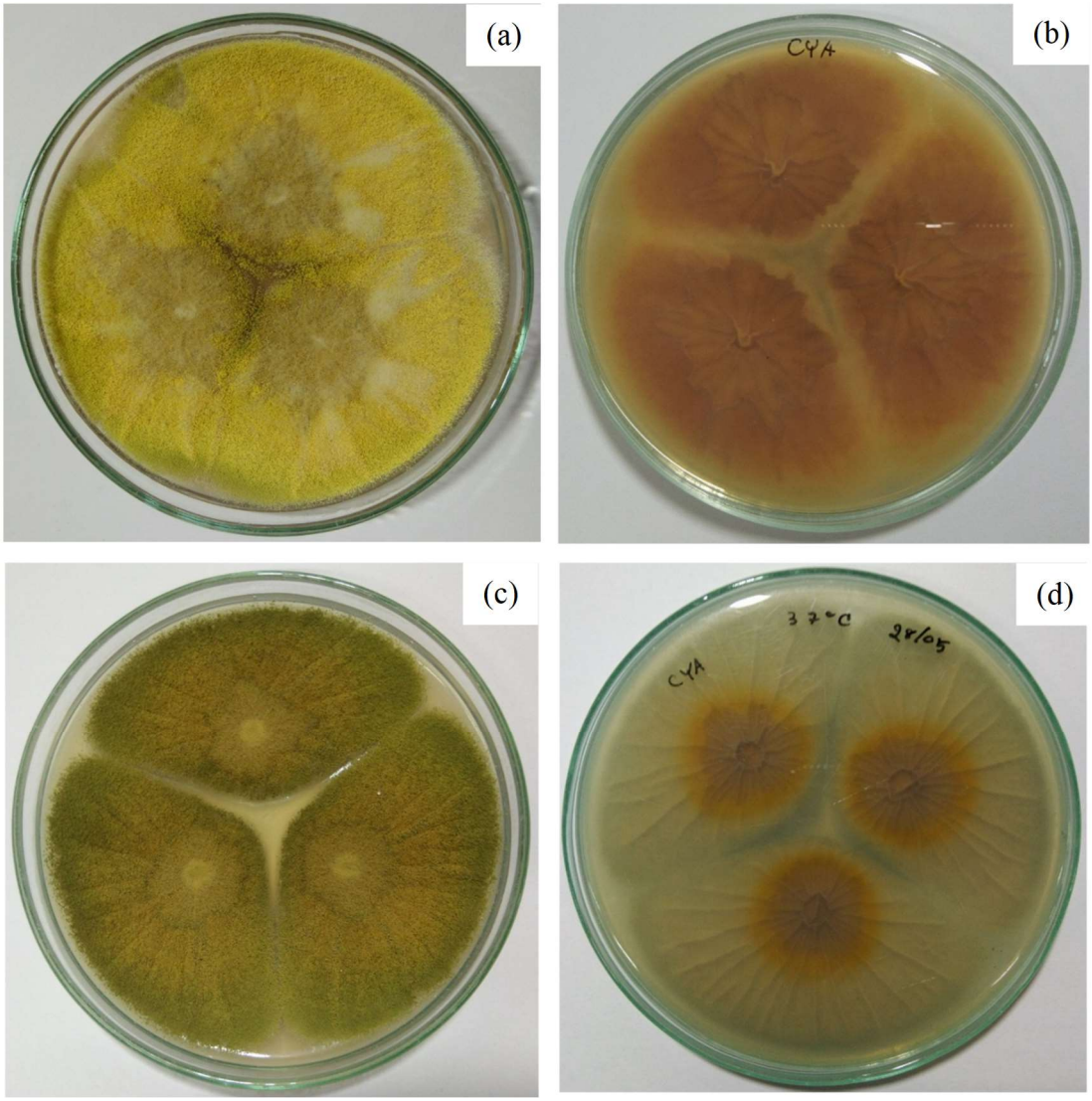


Figure 21. Colony morphology of *Aspergillus* sp. ST11 on Czapek's yeast agar at 25°C (a,b) and 37°C (c,d) for 7 days.

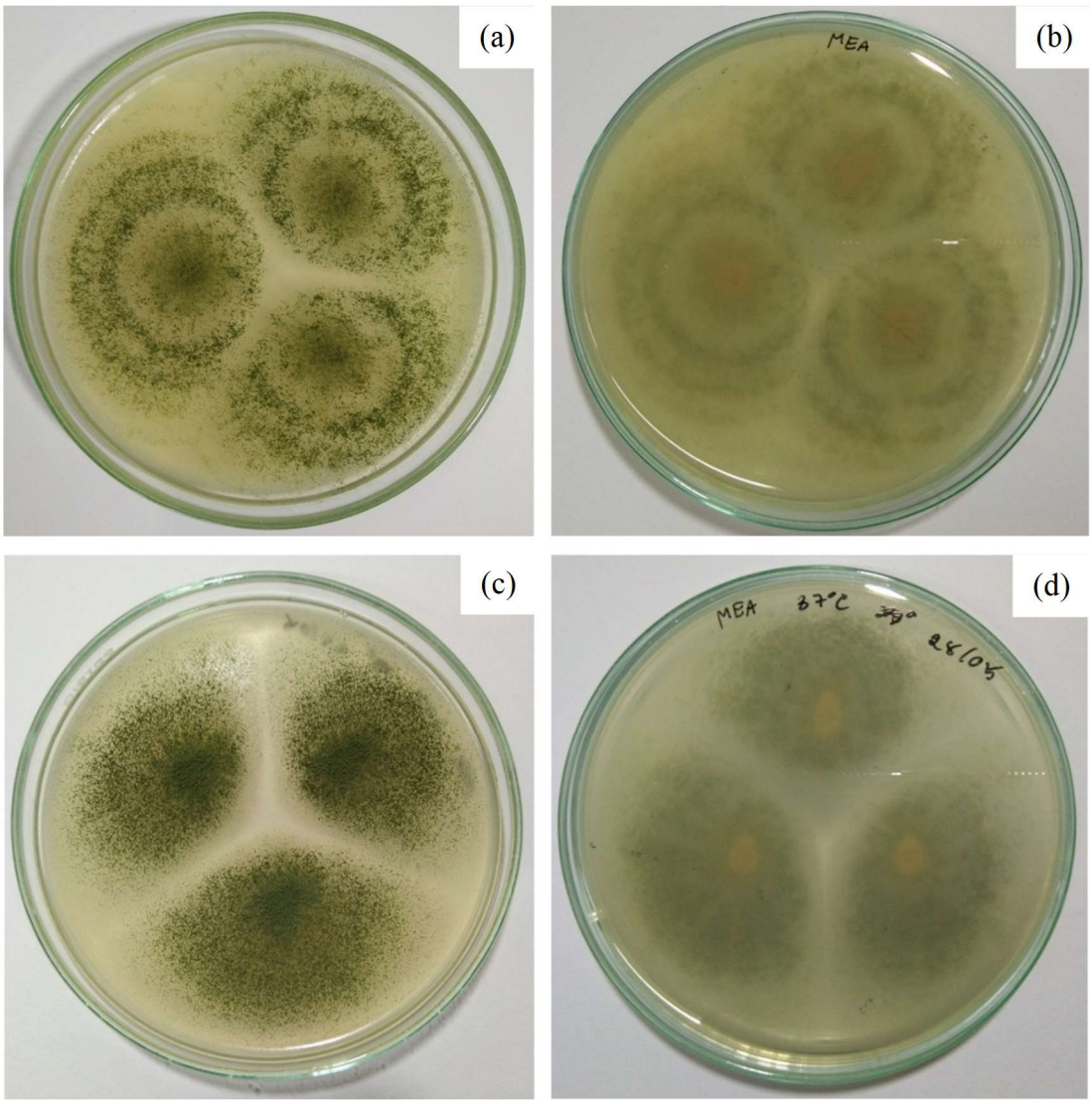


Figure 22. Colony morphology of *Aspergillus* sp. ST11 on malt extract agar at 25°C (a,b) and 37°C (c,d) for 7 days.

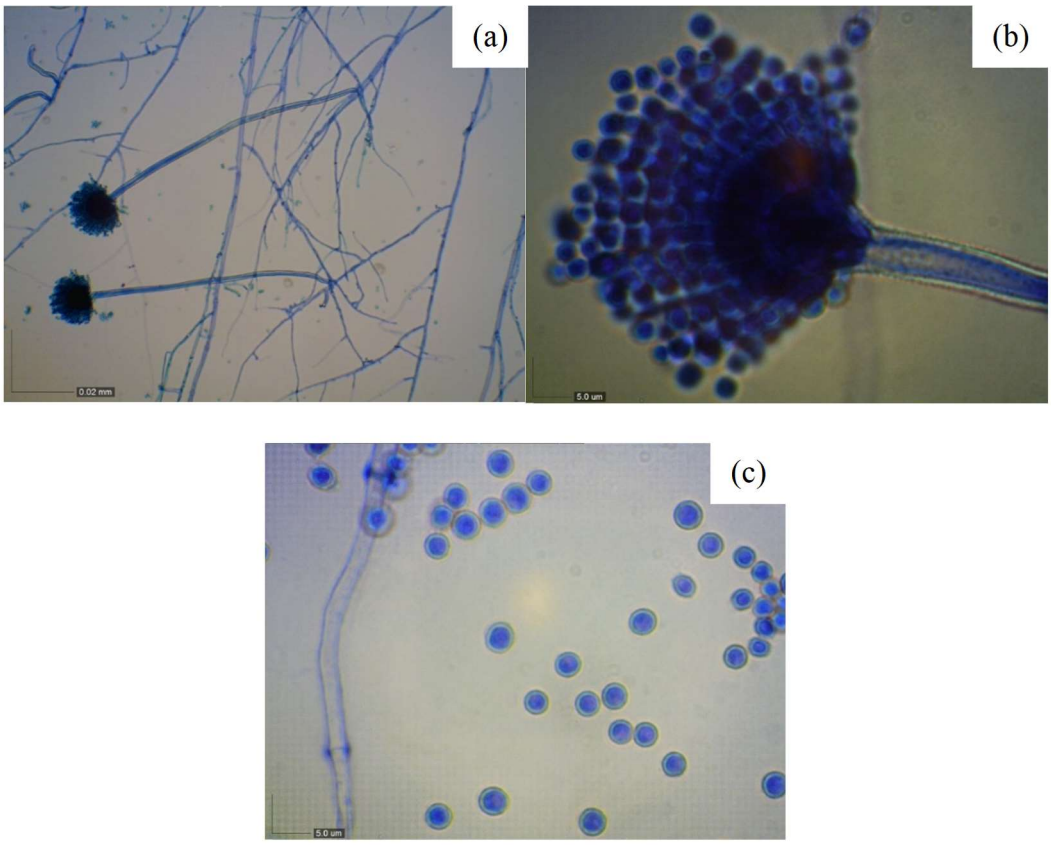


Figure 23. Conidiophores at 100x (a) and 400x (b), and conidia at 400x (c) of *Aspergillus* sp. ST11.

2. Purification of lipase

The three purification procedures consisting of chilled acetone precipitation, HiTrap Q HP chromatography and Toyopearl Butyl-650M chromatography were used for purification of *A. oryzae* ST11 lipase. The purification table was shown in Table 9. After fungal cultivation, the culture broth was filtered by Whatman paper no.1. The filtrate was precipitated with chilled acetone and dissolved again with small volume of 50 mM Tris-HCl buffer pH 7.5. The sample was dialyzed against the same buffer at 4°C overnight. The yield of lipase at this step of purification was 56% and 7.7-fold increase of activity.

The lipase was partially purified from the step of precipitation and dialysis. After the acetone precipitation, the enzyme was applied to anion exchange chromatography by using HiTrap Q HP column which connected with the ÄKTA Prime

chromatography system. It was found that 15% NaCl could elute the target enzyme from the column (Figure 24). At this step, 28.9% of lipase yield and 10.2-fold increase of activity were obtained. The enzyme from previous column was applied to Toyopearl Butyl-650M column chromatography. The concentration of ammonium sulfate was decreased by stepwise elution. The active fractions were obtained after 50 mM Tris-HCl buffer pH 7.5 with 0.4 % ammonium sulfate was applied to the column (Figure 25). The yield of lipase at this step was 7.9 % and the purification fold was 13.0 (Table 9). The decrease of the lipase yield after the lipase concentration step was similar to the study of Toida *et al.* (1995) that reported the decrease of lipase yield to 54% after ammonium sulfate precipitation and gave the yield only 5.5% after the final purification step. Mehta *et al*. (2018) studied the purification of lipase from *A. fumigatus* using Octyl Sepharose column chromatography (hydrophobic column). The result showed that the lipase yield was 11.03% and the purification fold was 6.96.

The molecular mass of the purified lipase was determined by using SDS-PAGE technique. The purity of the *A. oryzae* ST11 lipase was examined and confirmed with the single band of protein which was equivalent at 25 kDa of relative molecular mass compared with the size of standard marker from Pink Plus Prestained Protein ladder (Figure 26). The molecular mass of the lipase from *A. oryzae* ST11 in this study was similar with the size (27 kDa) of the lipase from *A. oryzae* isolated from waste cooking oily soil (Zhou *et al*., 2012). However, the study of Toida *et al*. (1995) showed that the molecular mass of the lipase from *A. oryzae* was 41 kDa.

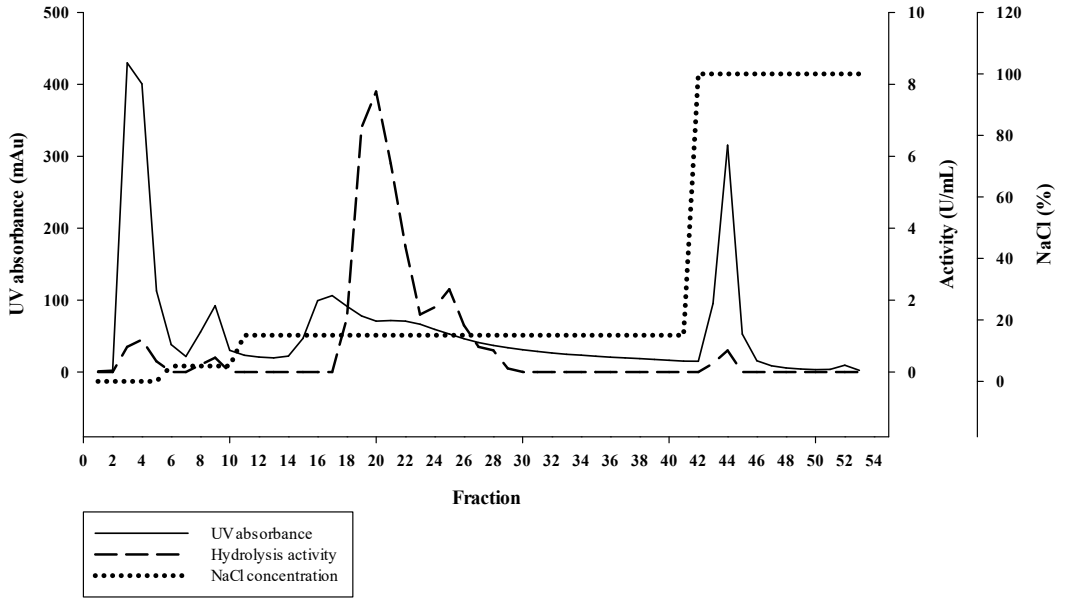


Figure 24. Purification of *Aspergillus oryzae* ST11 lipase carried out by ion exchange chromatography (Hitrap Q HP chromatography).

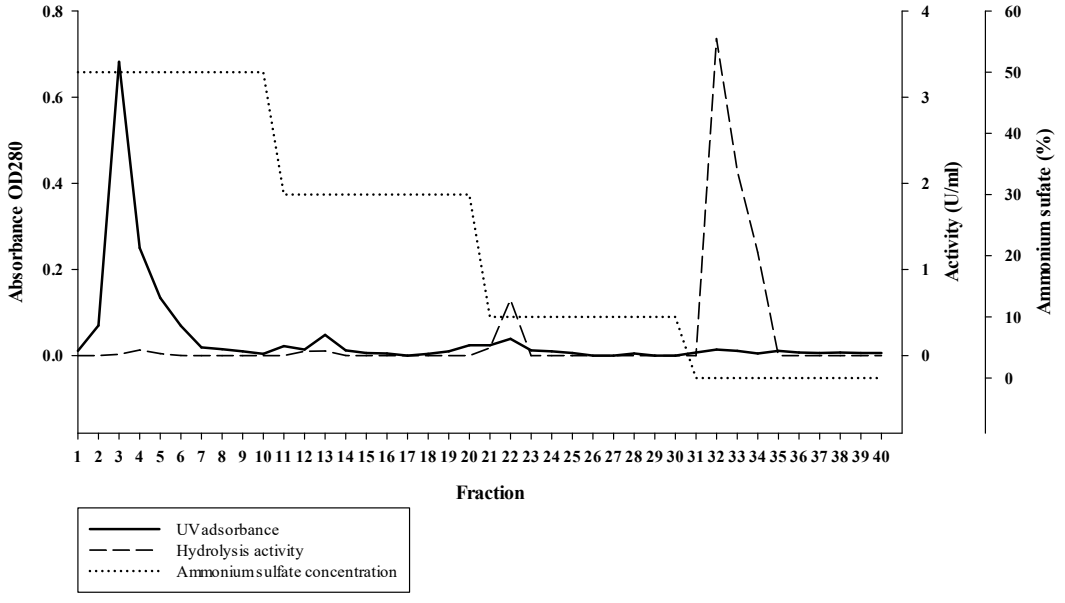


Figure 25. Purification of *Aspergillus oryzae* ST11 lipase carried out by hydrophobic chromatography (Toyopearl Butyl-650M).

Table 9. Purification table of lipase from *Aspergillus oryzae* ST11.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Yield (%)	Purification fold (fold)
Supernatant	1,300	16,000	12.3	100	1.0
Acetone precipitation	95	8,960	94.3	56	7.7
Q-HP column chromatography	37	4,635	125.3	28.9	10.2
Butyl-Toyo pearl column chromatography	8	1,278	159.8	7.9	13.0

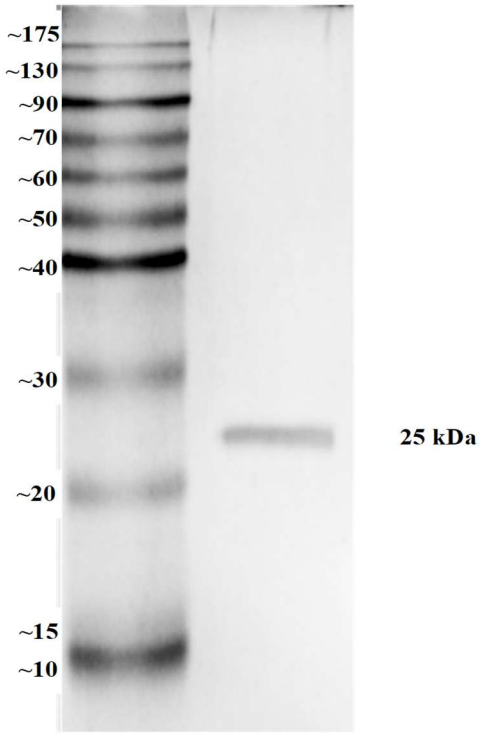


Figure 26. SDS-PAGE of the purified lipase from *Aspergillus oryzae* ST11.

3. Characterization of purified lipase from *Aspergillus oryzae* ST11.

3.1 Effect of pH on activity and stability.

The purified *A. oryzae* ST11 lipase had the optimum pH for lipase activity at pH 7.5 (Figure 26). It still gave the lipase activity more than 80% in the pH range between 6.5-8.0. The activity of the purified lipase was increased when the pH of the system increased to the optimum pH at 7.5 and the activity was decreased when the pH was higher than pH 7.5. The result showed that at pH 9.0, the lipase activity was 58 %. The optimum pH for the activity of the purified lipase of *A. oryzae* studied by Toida *et al*. (1995) was at pH 7.0. However, the optimum pH for the activity of the lipase from *A. oryzae* CJLU-31 was pH 4.0 which was considered as acidic lipase (Zhou *et al*., 2012). For other *Aspergillus* species, the *A. fumigatus* lipase had the optimum pH at 9.0 (Mehta et al., 2018) and *A. carneus* lipase had the optimum pH at 8.5 (Saxena *et al*., 2003). Whereas, *A. niger* lipase had the optimum pH at 7.0 (Pokorny et al., 1997). It could be concluded that the lipase produced by *Aspergillus* species had a wide range of optimum pH.

The pH stability was the desired characteristics of enzyme application. After incubation of the purified lipase in a wide range of pH buffer (Figure 27), the purified *A. oryzae* ST11 lipase retained the activity more than 80% in the buffers between pH 5.0-8.0. The relative activity of purified lipase was greatly dropped after incubation in the buffer lower than pH 5.0 and higher than pH 8.0. In comparison, the activity of *A. oryzae* lipase was low at acidic pH and was stable in a pH range from 6.0-9.0 at 25ºC (Toida *et al*., 1995). Another report showed that the purified lipase from *A. niger* NCIM 1207 was stable at pH 8.0-11.0 after 5 days of incubation but retained the activity at 10% in acidic pH (Mhetras *et al*., 2009).

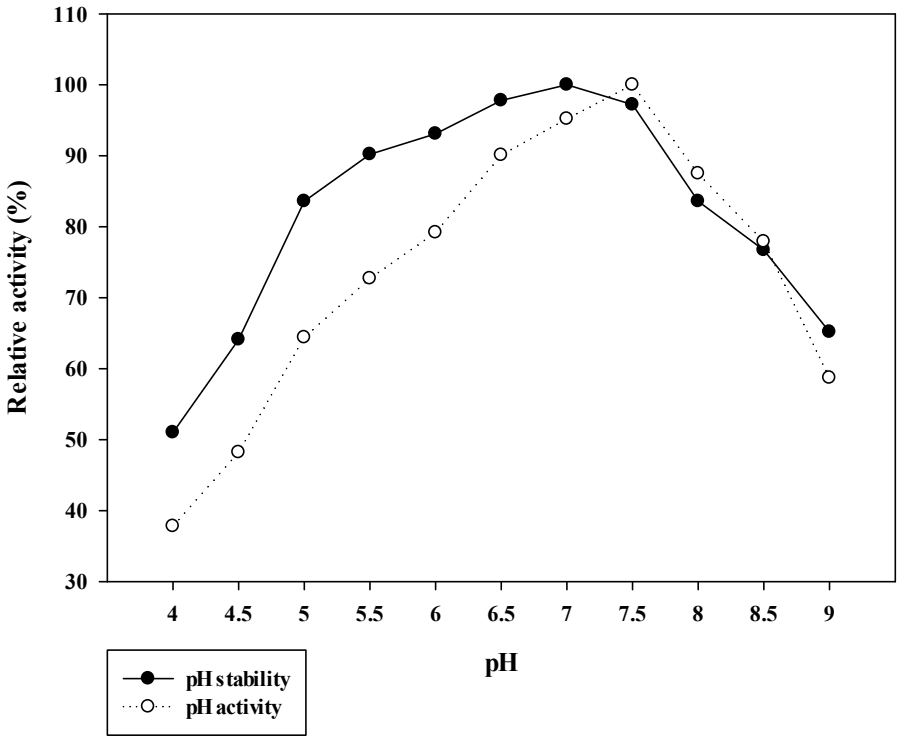


Figure 27. Effect of pH on the activity and stability of the purified lipase from *Aspergillus oryzae* ST11.

3.2 Effect of temperature on activity and stability

Figure 28 showed the effect of temperature on the activity and stability of *A. oryzae* ST11 lipase. The purified lipase from *A. oryzae* ST11 showed the optimum temperature for lipase activity at 37ºC. The result showed that it still had high activity in the range of temperature between 30 and 45ºC with slightly decrease in the activity compared to 37ºC. However, when the temperature of the system was higher than 55ºC, the activity was dropped drastically and retained only 23% at 65ºC. For the thermostability, after incubation of the purified lipase from *A. oryzae* ST11 for 2 h, the remaining activity of lipase at 30 and 37ºC were higher than 90%. The trend of the thermal stability was decreased rapidly when the samples were incubated in the temperature higher than 45ºC. At 65ºC, the activity was decreased to 32%. However, its stability was different from the *A. oryzae* studied by Toida *et al*. (1995). Their lipase was stable at 30ºC after 3 h of incubation and greatly reduced when the temperature increased to 40ºC. Comparing to the result of temperature on the activity in this

experiment, it indicated that the purified lipase from *A. oryzae* ST11 was more stable than their lipase.

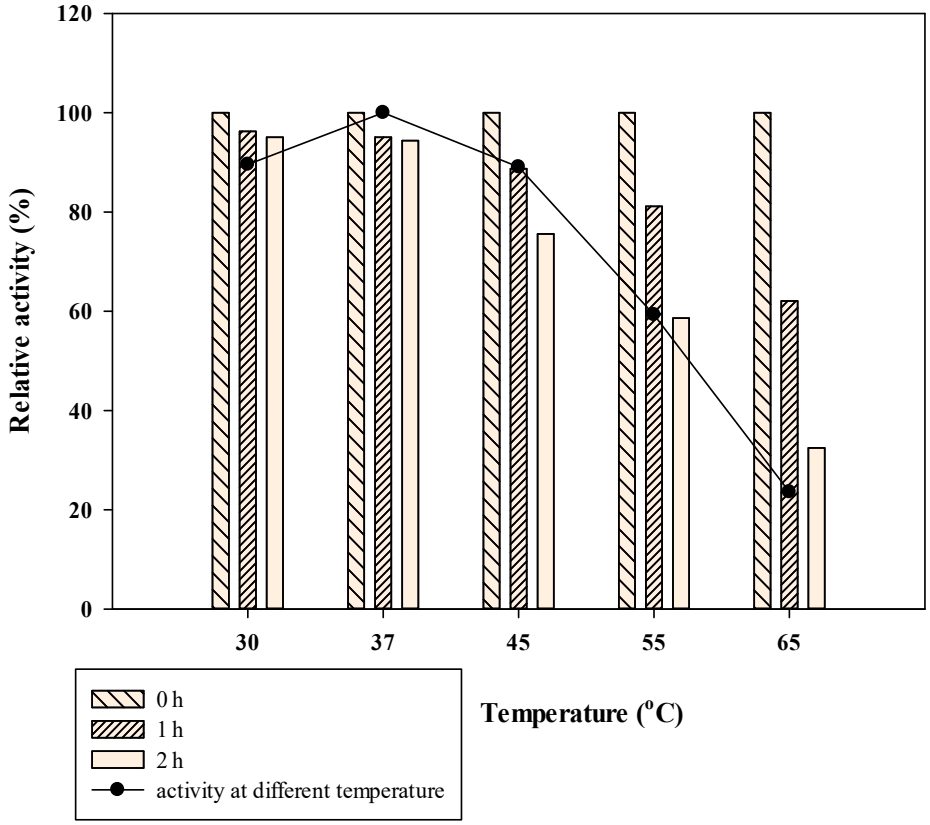


Figure 28. Effect of temperature on the activity and stability of the purified lipase from *Aspergillus oryzae* ST11.

3.3 Effect of metal ions

Different metal ions were used to study the effect on the activity of the purified *Aspergillus oryzae* ST11 lipase with the concentration at 1.0 and 10.0 mM (Table 10). The result showed that the activity of lipase was slightly enhanced when Ca^{2+}, K^{+} and Mg^{2+} (1.0 mM) were present in the reaction mixture. However, when the concentration of metal ions was increased to 10.0 mM, only the addition of Mg^{2+} showed the significant enhancement of activity compared to other ions. The increase of lipase activity in the existence of Mg^{2+} was also supported by the report of Adham and

Ahmed (2009) with the lipase from *Aspergillus niger* NRRL3 and *A. terreus* var. africanus (CBS 130.55). At 10.0 mM of Mg^{2+}, the lipase activity of *A. terreus* var. africanus (CBS 130.55) was stimulated to 133.9% (Hamdy and Abo-Tahon, 2012). However, the report of Toida *et al*., (1995) showed the opposite effect, Mg^{2+} (10.0 mM) slightly decreased the lipase activity of *A. oryzae*. The lipase activity was drastically suppressed by Hg^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} at both 1.0 and 10.0 mM of concentrations. The negative effect of Hg^{2+}, Zn^{2+} and Cu^{2+} was also reported in the study of the purified *A. oryzae* lipase (Toida *et al*., 1995; Hamdy and Abo-Tahon, 2012). The negative effect of Zn^{2+} and Cu^{2+} on lipase activity might due to the interaction of those ions on the enzyme surface charge. It made the change of enzyme conformation leading to be less stable. Hg^{2+} had the inhibitory effect on the participation of SH group in the enzyme structure (Hamdy and Abo-Tahon, 2012).

Table 10. Effect of metal ions on the stability of the purified *Aspergillus oryzae* ST11 lipase.

Effect of metal ions	Relative activity (%)	
	1.0 mM	10.0 mM
Control	100.0	100
$FeCl_3$	102.5 ± 6.6^{a}	83.5 ± 8.0^{b}
$CoCl_3$	82.8 ± 2.6^{b}	14.3 ± 0.9^{f}
$CuCl_2$	32.5 ± 5.4^{c}	33.5 ± 4.4^{de}
$MgCl_2$	103.9 ± 2.3^{a}	122.7 ± 1.4^{a}
$HgCl_2$	14.6 ± 2.6^{d}	14.1 ± 1.4^{f}
$CaCl_2$	109.3 ± 1.2^{a}	66.7 ± 1.4^{c}
$ZnCl_2$	18.7 ± 0.3^{d}	19.4 ± 1.6^{ef}
KCl	106.3 ± 1.9^{a}	84.1 ± 6.2^{b}
NaCl	101.9 ± 1.3^{b}	88.9 ± 9.0^{b}
$AlCl_3$	87.2 ± 5.9^{a}	48.2 ± 5.1^{d}

3.4 Effect of inhibitors and surfactants

Many surfactants and inhibitors were investigated for the effect on lipase activity. The surfactants were used to enhance the catalysis of lipase by increasing the lipid water interfacial area (Ungcharoenwiwat and H-Kittikun, 2015). However, in the study with the effect of surfactants on the activity of *A. oryzae* ST11 lipase showed that most surfactants used in this experiment showed the negative effect on the lipase activity (Table 11). The Triton X-100 severely reduced the activity of the purified lipase and the activity was retained only 6%. The negative effect of surfactants could be observed from Sodium dodecyl sulfate (SDS), Tween-80, Tween-20 and Arabic gum at 21.1, 21.6, 23.7 and 72.1, respectively. The reduction of lipase activity after the addition of surfactants also occurred in the study of Zhou *et al*. (2012). The activity of purified lipase from *A. oryzae* CJLU-31 was dropped in the presence of Tween-40, Tween-80 and SDS and retained the activity at 20.0, 11.0 and 22.0, respectively (Zhou *et al*., 2012). This phenomenon was supported by the study of Saxena *et al*., (2003) that mentioned about the negative effect of anionic detergent such as SDS on the lipase activity.

The inhibitors (EDTA, PMSF and β-mercaptoethanol) were used for studying the effect on the activity of at 1.0 mM and 10.0 mM of inhibitors. There was no significant loss of the lipase activity (Table 11). The presence of β-mercaptoethanol showed slight effect on the lipase activity. This meant there was no disulfide bond in the structure of lipase. In the case of EDTA addition, the lipase activity of *A. oryzae* ST11 lipase was slightly higher than that of control condition. It also implied that purified *A. oryzae* ST11 lipase was a non-metallo enzyme (Ungcharoenwiwat and H-Kittikun, 2015). The PMSF which was used as serine inhibitors did not reduce the *A. oryzae* ST11 lipase activity. The similar result was observed from the study of Toida *et al*. (1995) which revealed the non-significant reduction of *A. oryzae* lipase activity after incubation with PMSF. Although, PMSF affected negatively to the activity of purified *A. carneus* lipase (Saxena *et al*., 2003).

Table 11. Effect of surfactants and inhibitors on the stability of the purified *Aspergillus oryzae* ST11 lipase.

Effect of surfactants and inhibitors	Relative activity (%)
Control	100
Surfactants	
Triton X-100 (1%)	6.0 ± 0.5^{c}
Tween-80 (1%)	21.6 ± 9.8^{b}
Tween-20 (1%)	23.7 ± 1.0^{b}
Gum Arabic (1%)	72.1 ± 8.5^{a}
SDS (1%)	21.1 ± 4.7^{b}
Inhibitors	
EDTA (1.0 mM)	101.9 ± 5.2^{a}
EDTA (10.0 mM)	92.3 ± 1.6^{b}
β-mercaptoethanol (1.0 mM)	92.7 ± 2.3^{b}
β-mercaptoethanol (10.0 mM)	92.5 ± 2.9^{b}
PMSF (1.0 mM)	97.1 ± 4.1^{ab}

3.5 Effect of organic solvents

The hydrolysis reaction catalyzed by lipases of water-insoluble substrates is taken place at the interface. The use of organic solvents can encourage the hydrolysis reaction of water-insoluble substrates by the lipase (Chaiyaso *et al*., 2012). Hence, the high stability and the activity of lipase in the solvent system are concerned for the application of the enzyme. The investigation of organic solvent effect on the stability of the purified *A. oryzae* ST11 lipase was conducted. The different polar and non-polar organic solvents (polarity index 0.0-5.2) were studied (Table 12). The activity of purified lipase was decreased after 1 h of incubation in all organic solvents compared to the control condition containing 50 mM Tris-HCl buffer pH 7.5. The highest relative activity was obtained in the presence of isooctane and hexane. This observed result was similar to the test for lipase activity of *A. carneus* and *A. oryzae* CJLU-31 (Saxena *et al*., 2003; Zhou *et al*., 2012). Moreover, the hydrophilic solvents

such as methanol and ethanol showed very low enzyme stability compared to non-polar solvents. This indicated the potential of lipase application in solvent system. The common application of using solvent system was to produce biodiesel. It was shown that the direct mixing of methanol or ethanol with oil in a solvent-free condition tended to result in low conversions due to the poor solubility of methanol or ethanol in oils. The addition of organic solvents could facilitate the mixing of substrates and led to higher conversion and remove the inhibitory of glycerol by-product (Jeong and Park, 2008; Nasaruddin *et al*., 2014).

Table 12. Effect of organic solvents on the lipase activity of the purified *Aspergillus oryzae* ST11 lipase.

Organic solvents (25%)	Polarity index	Relative activity (%)
Ethanol	5.2	3.7 ± 0.4^{h}
Methanol	5.1	2.6 ± 1.1^{h}
Acetone	5.1	26.3 ± 2.8^{e}
Ethyl acetate	4.4	48.0 ± 2.1^{d}
Isopropanol	3.9	56.0 ± 2.9^{c}
Xylene	2.5	13.3 ± 1.1^{g}
Toluene	2.4	19.0 ± 1.4^{f}
Iso-Octane	0.1	70.0 ± 1.8^{a}
Hexane	0.1	62.0 ± 2.8^{b}
50 mM Tris-HCl buffer pH 7.5	-	100

3.6 Substrate specificity

The different natural oils were tested to determine the substrate specificity of the purified *A. oryzae* ST11 lipase. The result is shown in Figure 29. The hydrolysis reaction using palm oil was controlled as the base value at 100% relative activity and measured at pH 7.5 and 37^{o}C. Among nine natural oils, it was found that olive oil showed the highest activity at 110% relative activity higher than that of palm oil. The rice bran oil and coconut oil had the relative activity of lipase more than 70% followed by linseed oil that the activity was around 50%. In contrast, sunflower oil,

soybean oil, canola oil, and corn oil showed a lower activity below 40%. Considering to the fatty acid compositions in each type of oil, the substrate specificity of the lipase was enhanced when the content of C18:n in the oil increased corresponding to oleic acid (C18:1) content existing largely in olive oil (Lakshmi *et al*. 1999). Shu *et al*. (2007) also studied the substrate specificity of natural oils on the activity of *A. niger* F044 lipase. The result showed that the *A. niger* F044 lipase had a broad range utilization of oil and the olive oil gave the highest relative activity while the lowest relative activity was obtained from castor oil.

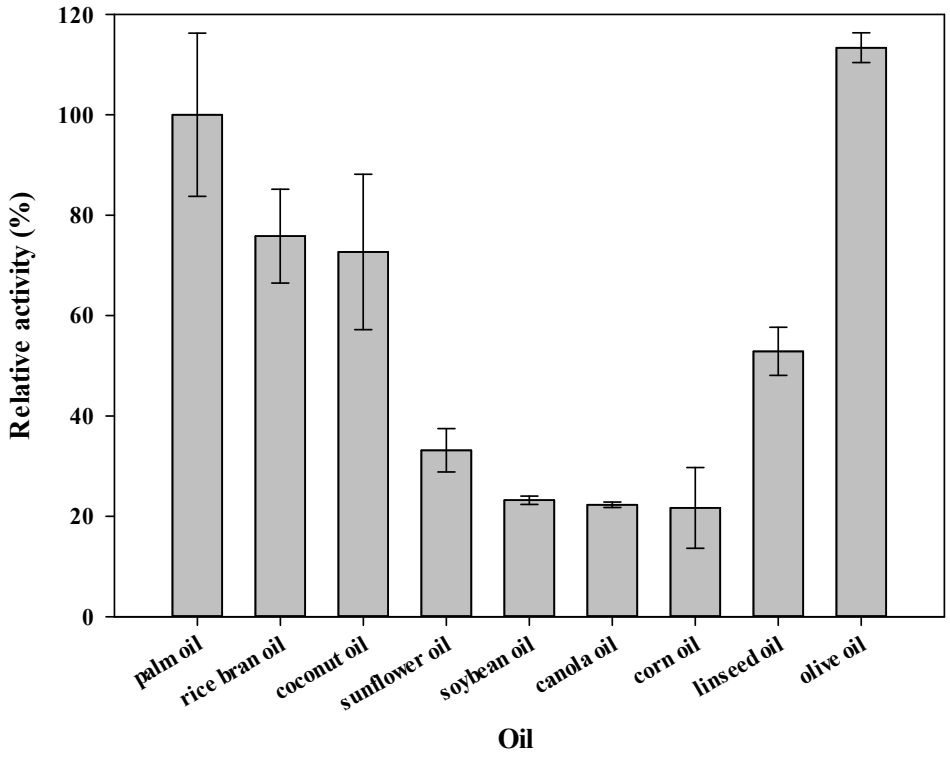


Figure 29. Effect of oil types on the activity of the purified lipase from *Aspergillus oryzae* ST11.

Conclusion

Aspergillus oryzae ST11 was identified by morphological and molecular methods (ITS1/ITS4). It was used for lipase production and purification. After purification of lipase by chilled acetone precipitation, HiTrap Q-HP and Toyopearl Butyl-650M column chromatographies, respectively. The activity was increased for 13-folds and 7.9% yield was achieved. Its molecular mass was 25kDa. The purified lipase

was stable at the pH between 5.0-8.0 with the relative activity higher than 80% and had the optimum pH for catalysis at pH 7.5. For thermal stability, it was stable at 30ºC after 2 h of incubation with slightly decrease in activity (95%). Its activity was also promoted in the presence of Mg^{2+} while greatly decrease in the presence of Hg^{2+}. For the effect of surfactants and inhibitors on lipase activity, it was found that most of surfactants tested showed the negative effect on the lipase activity while inhibitors did not reduce the activity significantly. Organic solvents were investigated for the lipase stability. The result indicated that the polar solvent such as methanol and ethanol had negative effect on the lipase compared to non-polar solvents.

CHAPTER 4

MAGNETIC CROSS-LINKED ENZYME AGGREGATES OF *ASPERGILLUS ORYZAE* ST11 LIPASE USING POLYACRYLONITRILE COATED MAGNETIC NANOPARTICLES FOR BIODIESEL PRODUCTION

Abstract

The biodiesel production by enzymatic catalysis is the environmentally friendly production process. In this work, the polyacrylonitrile coated magnetic nanoparticles were prepared and used as a core supporter for producing magnetic cross-linked enzyme aggregates (mCLEAs) from *Aspergillus oryzae* ST11 lipase with the co-feeding of bovine serum albumin (BSA). The highest immobilized lipase activity was 0.09 U/mg-support or 81.7% of recovered activity. The resulting mCLEAs exhibited the desired characteristics, it had improved the stabilities for both pH and temperature comparing to free enzyme. Moreover, when this biocatalyst was used for biodiesel production at the optimal conditions (30% w/w of mCLEAs, 30% w/w of water content and stepwise addition of methanol) the biodiesel conversion was 95 % within 24 h of reaction while one-step addition of methanol produced 81% conversion. The mCLEAs could be reused for 5 cycles and retained the biodiesel conversion higher than 60%.

Introduction

The increasing demand of energy consumption has greatly affected the development of most countries which are heavily relied on fossil fuel which is expected to be depleted in the near future. Hence, the renewable energy source has emerged to be the alternative way to overcome the problem of energy shortage. One of the renewable energy sources is biodiesel which is non-toxic and release low amount of green-house gases. Biodiesel can be produced from various kinds of vegetable oils by both chemical and enzymatic processes. For the chemical process, the reaction can be catalyzed by acid or base. Although, the chemical process can give the high yield of biodiesel it also shows many disadvantages including high energy consumption,

reaction hindrance from high free fatty acids or even the difficulty in biodiesel separation (Bajaj *et al*., 2010). In contrast, the enzymatic process has high specificity to the reaction, the glycerol which is a by-product can be easily separated from the main product. The substrate with high free fatty acid content can be used by the enzyme (Kumar and Dharmendra, 2013).

The important enzyme for biodiesel production is lipase. Lipase is classified as a hydrolytic enzyme, but it can also catalyze esterification and transesterification reactions. This enzyme has been drawn attention due to their properties such as high substrate specificity and high stability in solvent (Treichel *et al*., 2010; Menoncin *et al*., 2010). However, the use of biocatalyst still has some drawbacks such as a high cost of enzyme and its instability at a certain condition. Hence, immobilization will be the most effective way to overcome these limitations. Cross-linked enzyme aggregates (CLEAs) is one of the easiest method for immobilizing enzyme (Schoevaart *et al*., 2004). The enzyme was precipitated from the solution and aggregated by a cross-linker reagent (Sheldon *et al*., 2005). The magnetic particles have become the interesting material for immobilization of protein or enzyme to be used for medical applications (Moriyama *et al*., 2011). This material also has the advantages such as high specific surface for carrying protein and the magnetic particle can be easily separated by external magnet (Zhang *et al*., 2015).

In this study, the polyacrylonitrile coated magnetic nanoparticles were prepared and modified. Then, the magnetic nanoparticles were used for covalent immobilization of *Aspergillus oryzae* ST11 lipase by cross-linking enzyme aggregates (CLEAs) technique. The immobilized lipase was further applied as a biocatalyst for biodiesel production.

Materials and methods

1. Materials

Polyacrylonitrile (PAN) (MW 150,000), *N,N*-dimethyformamide and glutaraldehyde solution (50% in H_2O), lipid standards (methyl hexadecanoate, oleic acid, triolein, diolein and monoolein) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). Ethylenediamine (EDA) was purchased from

Lobachemie (Mumbai, India). All HPLC grade solvents were purchased from J.T. Baker (Pennsylvania, USA). Palm oil was purchased from the local company in Thailand.

2. Analytical methods

2.1 Determination of lipase activity

The hydrolytic activity of lipase was measured in a two-phase system according to the modified colorimetric method (Lee and Rhee, 1993) using palm olein as a substrate in isooctane. The reaction was performed in 20 ml glass vial capped with rubber stopper. The reaction was incubated at 37^{o}C by shaking at 250 rpm for 30 min. The hydrolytic activity was determined by measuring the amount of fatty acids released compared to standard curve of palmitic acid. One unit of lipase was defined as the lipase required to release 1 μmol of palmitic acid per minute at the specified condition (Ungcharoenwiwat and H-Kittikun, 2015).

2.2 Determination of biodiesel

The products of transesterification were analyzed by HPLC using Poroshell 120 HILIC column (150 mm x 3 mm, particle size of 2.7 μm, pores of 120 Å). The analytes were measured from HPLC (Agilent 1100, Agilent Technologies, USA) system connected with the evaporative light scattering detector PL-ELS 1000 (Polymer Laboratories Ltd., USA). The detector parameters were set as 50^{o}C for nebulization, 40^{o}C for evaporation and the compressed air flow of 1.5 mL/min. The results from analytes were compared to the retention time of standard lipids. The yield of biodiesel was calculated based on the area of biodiesel peak compared to area of all peaks (Fedosov *et al*., 2014).

3. Experimental methods

3.1 Lipase production

The lipase producing *Aspergillus oryzae* ST11 was selected from stock culture in the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of

Agro-Industry, Prince of Songkla University. The fungal spore was inoculated on the potato dextrose agar and incubated at 37^{O}C for 5 days. The spores were collected and adjusted to 1x10^{7} spore/ml using haemacytometer and inoculated into the optimized medium (100 ml) containing 1% olive oil, 0.5% lactose, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, and 0.05% $MgSO_4$ (w/v) and adjusted pH to 6.0. The culture medium was shaken at 150 rpm and 37ºC for 4 days. The culture broth was separated from the mycelium by filtration with Whatman no.1 filter paper. The crude enzyme was precipitated from the culture broth by adding 3 volumes of cold acetone per 1 volume of the culture broth and kept at 4ºC overnight. The solution was centrifuged (8000 rpm at 4ºC for 10 min) and the precipitate was dissolved with small amount of 50mM Tris-HCl buffer pH 7.0 and transferred to the dialysis bag (3,500 Da). The sample was dialyzed against the same buffer at 4ºC overnight and concentrated by absorption with carboxymethyl cellulose. The concentrated lipase was collected for immobilization.

3.2 Synthesis of magnetic nanoparticles

The magnetic nanoparticles (MNPs) were synthesized by co-precipitation method (Jadhav *et al.*, 2013). Ten ml of 0.25M $FeSO_4.7H_2O$ and 20 ml of 0.25M $FeCl_3.6H_2O$ were mixed. Then 30 ml of 12M NH_4OH were gradually dropped to the mixture and stirred for 1 h. The MNPs were separated from NH_4OH solution by using an external magnet and extensively washed with deionized water to get rid of NH_4OH. The MNPs were dried in hot air oven at 105ºC overnight and were ground into a fine powder by using a mortar and pestle.

3.3 Coating of magnetic nanoparticles

The MNPs were coated with polyacrylonitrile polymer (PAN). The MNPs (500mg) were dispersed in 2 ml of 4% (v/v) polyacrylonitrile solution dissolved in *N,N*-dimethylformamide and stirred at 50 rpm and 30ºC for 24 h. The PAN coated MNPs were separated by an external magnet. They were dried in a hot air oven (105ºC) overnight and then ground into a fine powder.

3.4 Surface modification of PAN coated MNPs

The nitrile group of PAN coated MNPs were modified according to the method of Godjevargova *et al*. (2005). The coated MNPs (500mg) were immersed in 5 ml of 15% w/v NaOH solution and kept in water bath (60ºC) for 1 h. The MNPs were separated from NaOH solution by an external magnet and washed thoroughly with deionized water. After that, the MNPs were mixed with 5 ml of 1N HCl solution and shaken for 2 h. The MNPs were separated and washed again until the pH of washing water reached to pH 7.0. The MNPs were then mixed with 5 ml of 10% solution of EDA at 50 rpm for 1 h. The MNPs were separated and washed with deionized water to remove excess EDA. The surface modified MNPs were finally washed twice with acetone and dried at 30ºC overnight.

3.5 Immobilization of *Aspergillus oryzae* ST11 lipase on modified surface MNPs

The modified MNPs (5-45 mg) were mixed with 1 ml of the lipase solution (3 U/ml) and sonicated in a sonicator bath (Elma, Germany) for 10 min to disperse the MNPs to the lipase solution. After that, $(NH_4)_2SO_4$ (700 µl, 500 mg/mL) was added to the mixture and gently shaken at 50 rpm and 4ºC for 1 h. The glutaraldehyde solution was added to the mixture (until getting 25 mM of concentration) and incubated at 4ºC for 3 h to form covalent crosslinking between amine group of carrier and enzyme. The BSA was added to the mixture to enhance the crosslinking due to high amount of lysine residual (Torres *et al*., 2014). For the optimal condition to produce the magnetic cross-linked enzyme aggregates (mCLEAs) of *A. oryzae* ST11 lipase, the effects of the amount of MNPs, glutaraldehyde solution and BSA as well as immobilization time were studied. The normal cross-linking enzyme aggregates of *A. oryzae* ST11 lipase without MNPs was also studied for comparison. The mCLEAs and CLEAs were lyophilized 6 h before use. The recovered activity of the immobilized lipase was calculated using the following equation.

$$\text{Recovered activity (\%)} = \frac{\text{activity of immobilized lipase x 100}}{\text{activity of lipase for immobilization}}$$

3.6 Morphology and IR Spectra of immobilized lipase

The morphology of magnetic nanoparticles before and after immobilization was observed by using the scanning electron microscope (SEM Quanta). The functional group of magnetic nanoparticle, modified magnetic nanoparticle and mCLEAs of *A. oryzae* ST11 lipase were analyzed by using the attenuated total reflection-Fourier transform infrared (ATR-FTIR, Bruker Vertex 70) spectra (Bhattacharya and Pletschke, 2014).

3.7 Temperature and pH stabilities of lipase

Both free and immobilized lipase were tested for temperature stability by using hydrolytic assay (pH was controlled at 7.0). The free lipase and the immobilized lipase were mixed with 50mM Tris-HCl buffer pH 7.0 and kept in the water bath at 20-70^{o}C for 1 h. The highest activity was normalized for relative activity. For pH stability, the free lipase was mixed with the buffer pH 4 - 9 after 1 h of incubation the pH was neutralized to 7.0 by adding HCl or NaOH solution and measured for hydrolytic activity. For immobilized lipase. the MNPs were separated from the buffer by external magnet and washed twice by 50 mM Tris HCl buffer pH 7.0 and measured for lipase activity.

3.8 Biodiesel production

The 25 mg of mCLEAs were mixed with 20 µl of 50 mM Tris-HCl buffer pH 7.0 (10% w/w compared to weight of palm oil) after that, 2 ml of 10% (w/w) palm oil in isooctane was added to the mixture. The methanol was added to the reaction mixture at the ratio of 3 moles of methanol to 1 mole of palm oil. The reaction was carried out by shaking at 250 rpm and 37^{o}C for 24 h. The organic phase of reaction mixture was withdrawn (10µl) and mixed with 990 µl of isooctane. The sample was analyzed for biodiesel production by HPLC system (Fedosov *et al*., 2014). The effects of physical factors on transesterification were investigated such as biocatalyst amount, the water content in the system, molar ratio between palm olein and methanol and the stepwise addition of methanol on the yield of biodiesel.

3.9 Reusability

After the transesterification reaction, the immobilized lipase was separated from the mixture by an external magnet and washed thoroughly with fresh isooctane before adding new batch of substrate. The immobilized lipase was reused for 5 times.

Results and discussion

1. Characteristics of magnetic nanoparticles (MNPs), modified MNPs and magnetic cross-linked enzyme aggregates (mCLEAs) of *Aspergillus oryzae* ST11 lipase

The nitrile group of coated MNPs (500 mg) was modified using 5 ml of 15% (v/v) NaOH solution. The NaOH hydrolyzed nitrile group of PAN and gave amide group and carboxylic group. The EDA addition reacted with the carboxylic and the amine groups. The amine groups of modified MNPs were used for covalent binding with amine groups of the lipase to form magnetic cross-linking enzyme aggregates (mCLEAs) with the help of ammonium sulfate as a precipitant and glutaraldehyde solution as a coupling reagent (Torres *et al*., 2014). The schematic of chemical modification of PAN coated MNPs and immobilization of *Aspergillus oryzae* ST11 lipase was illustrated in the Figure 30. The analysis of chemical bond was evaluated by using FT-IR. The adsorption peak at range 588 cm^{-1} was identified as stretching vibrations of Fe-O (Raita *et al*., 2015). The adsorption peak at 3423 cm^{-1} was identified as O-H stretching vibration of Fe_3O_4 (Figure 31a). Figure 31b showed the strong peak at 2923, 2242 and 1451 cm^{-1} which were identified as stretching vibration band of methylene, stretching vibration band of nitriles and bending vibration band of methylene, respectively. This activation of coated polymer generated the dominant signal at the 1563 cm^{-1} showing the appearance of carboxylic group generated from the hydrolysis treatment by NaOH. After the immobilization, the spectra were shown in Figure 31c. The presence of peaks was apparently observed at 1622 cm^{-1} which was considered as the amide group. This confirmed the success of immobilization of *Aspergillus oryzae* ST11 lipase. The characteristic spectra of our study were similar to

the results of the study with immobilized lysozyme on polyacrylonitrile nanofiber by Liu *et al*. (2015). Physical morphology of magnetic nanoparticles, modified MNPs and mCLEAs was observed by SEM and surface of materials were shown in Figure 32. The mCLEAs showed the rough surface due to the coating of enzyme compared to MNPs which showed the finer surface. The morphology of mCLEAs of *Candida antarctica* lipase B (CALB) expressed more fuzzy the slurred and diffuse edges compared to normal MNPs (López *et al*., 2014).

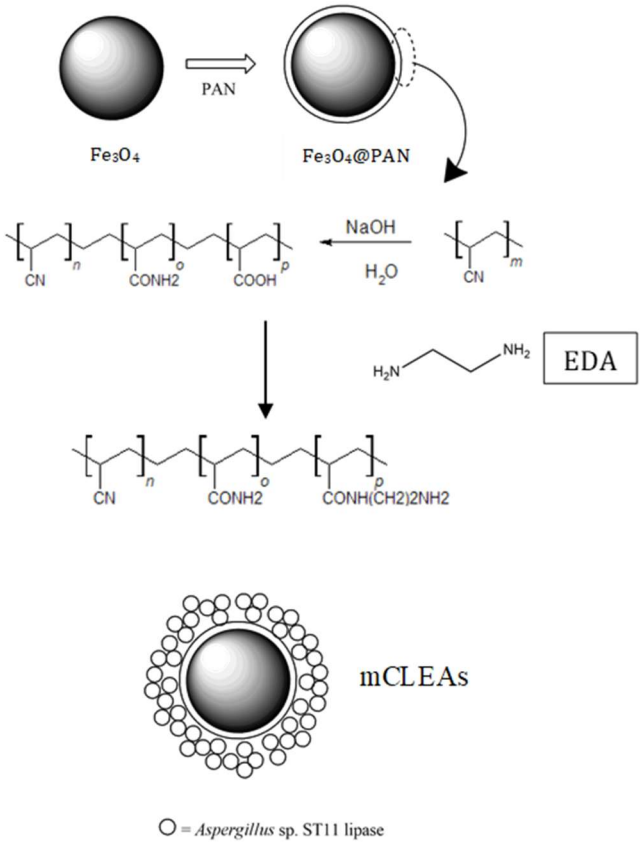


Figure 30. The mechanism of PAN coating and modification of nitrile group for lipase immobilization and the image presenting the mCLEAs formation of lipase from *Aspergillus oryzae* ST11.

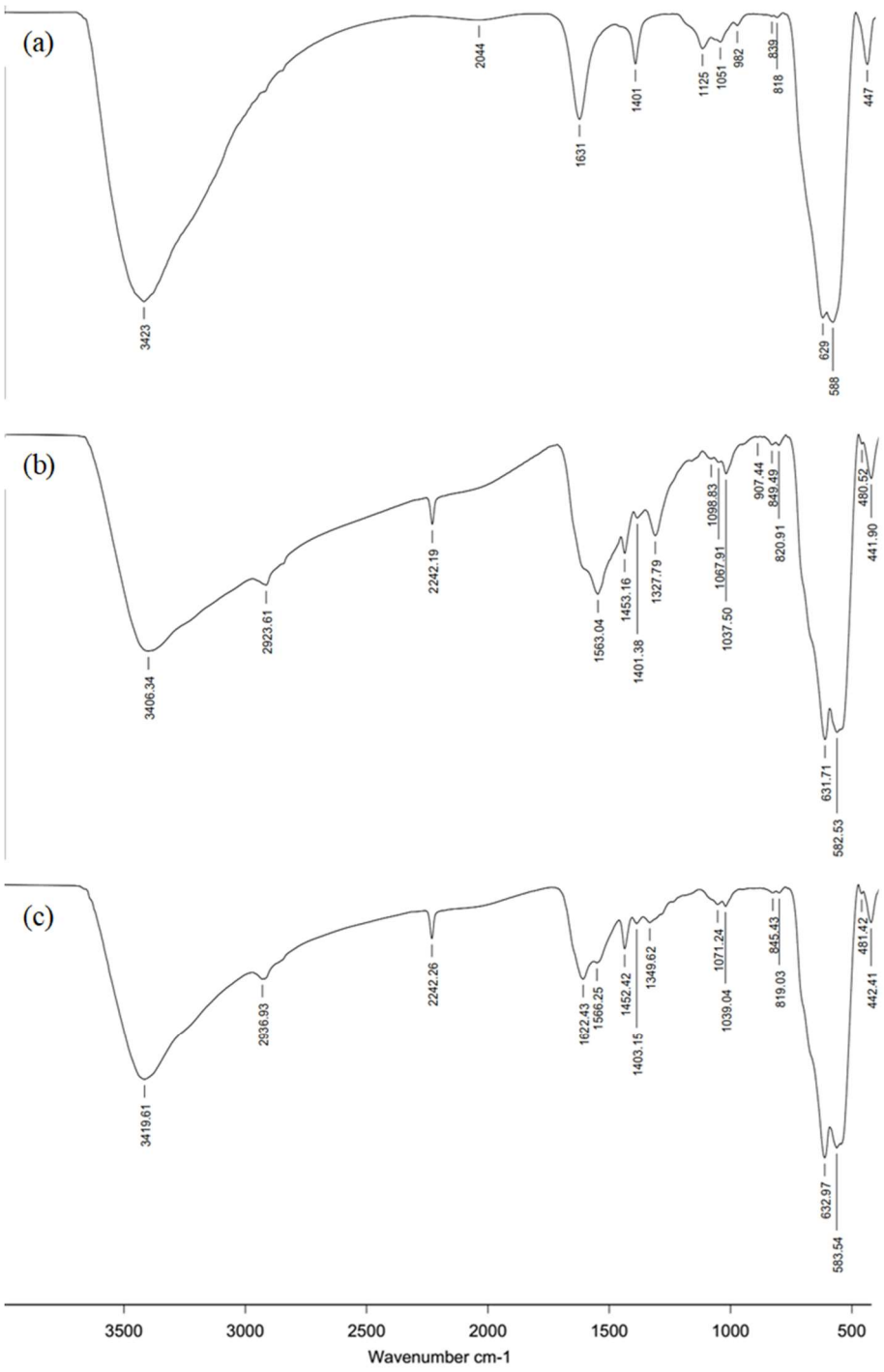


Figure 31. IR spectra of magnetic nanoparticle (a), modified magnetic nanoparticle (b) and mCLEAs of *Aspergillus oryzae* ST11 lipase (c).

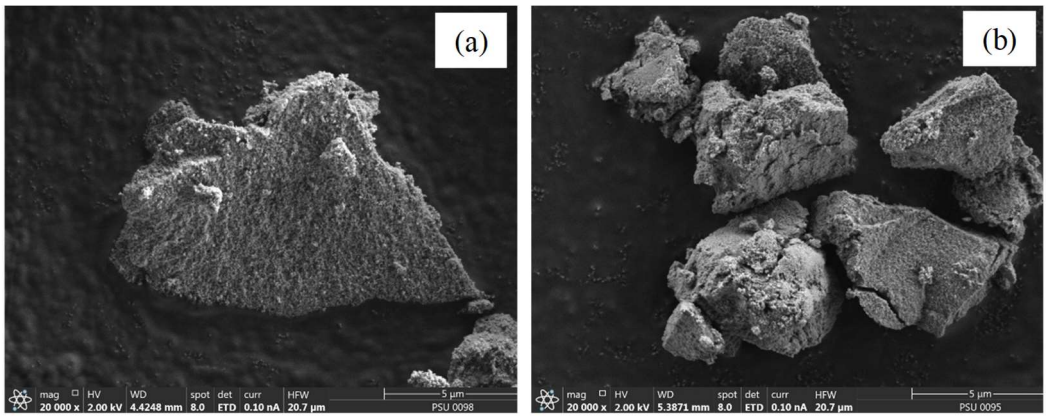


Figure 32. Morphology of magnetic nanoparticles (a) and magnetic cross-linked enzyme aggregates of *Aspergillus oryzae* ST11 lipase (b).

2. Optimization of *Aspergillus oryzae* ST11 lipase immobilization on modified MNPs

The cross-linking between *Aspergillus oryzae* ST11 lipase and modified MNPs was achieved by using CLEAs technique (Sheldon *et al*., 2005). To produce mCLEAs, many factors affecting the cross-linking were studied. The amount of modified MNPs was varied and the lipase solution was controlled (3 U/ml). The activity of mCLEAs was increased in relation to the amount of the modified MNPs. The highest recovered activity was at 25 mg of the modified MNPs with the immobilized lipase at 0.05 U/mg-support with 42% recovery activity (Figure 33). However, when the amount of modified MNPs was higher than 25 mg the recovered activity slightly decreased. The higher amount of the modified MNPs might cause low immobilization efficiency due to the imbalance of enzyme and support.

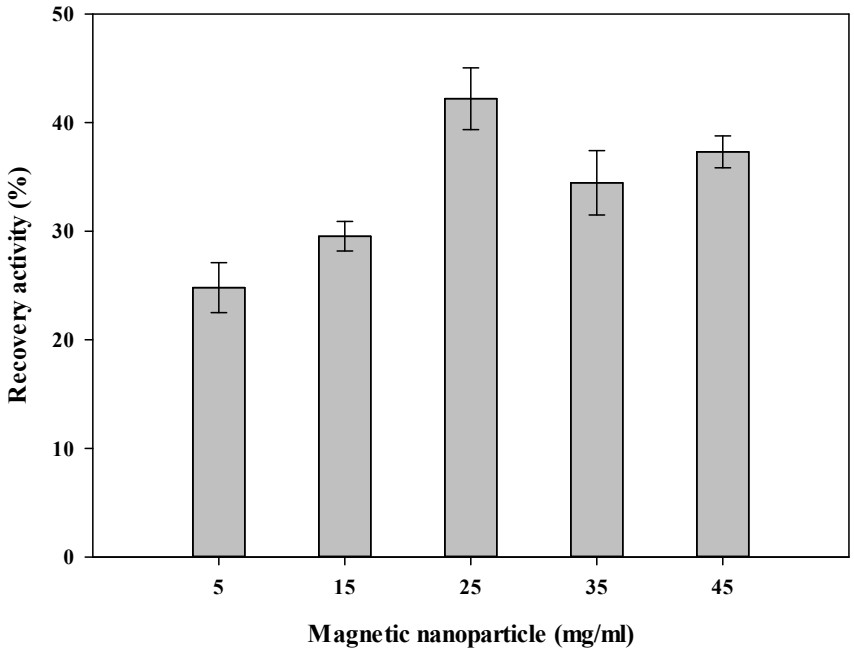


Figure 33. Effect of the amount of magnetic nanoparticles on the recovered activity of *Aspergillus oryzae* ST11 lipase for magnetic cross linked enzyme aggregates (mCLEAs).

Glutaraldehyde is one of the important reagents for CLEAs formation. It is the most effective protein cross-linker which can rapidly react to the amine residual (Migneault *et al*., 2004). The effect of different concentration of glutaraldehyde on the recovered activity of mCLEAs was investigated. The glutaraldehyde solution (50% v/v in H_2O, 5.6 M) was added to the enzyme solution containing 25 mg of modified MNPs. When the concentration of glutaraldehyde was 20mM, the maximum recovered activity of lipase (44%) was obtained (Figure 34) which was not significantly different when the concentration reached 30 mM. However, when the glutaraldehyde concentration was higher than 30 mM, the recovered activity was dropped gradually. In the case of CLEAs preparation without MNPs, the highest lipase activity (30%) was achieved at 30 mM of glutaraldehyde concentration. This result was similar to the mCLEAs production of CALB which the recovered activity increased when the concentration of glutaraldehyde increased. However, the recovered activity was achieved at a very high concentration of glutaraldehyde (400 mM) (Cruz-Izquierdo *et al*., 2014). The other study of the mCLEAs of glucoamylase showed that the increasing concentration of

glutaraldehyde enhanced the recovered activity at 95% when 1.5% v/v of glutaraldehyde was applied (Nadar and Rathod, 2016).

Bovine serum albumin (BSA) was added to enhance the immobilization efficiency. In the normal preparation of CLEAs, adding glutaraldehyde would modify some essential amino groups that needed for biological activity. BSA was used as a proteic feeder that enhanced the formation of CLEAs. It was useful in the case that the target protein had low protein concentration and also stabilized enzyme activity (Shah *et al*., 2006). In our study, BSA (1-14 mg) was added to the enzyme solution in order to increase the recovered activity of lipase. In the case of mCLEAs, adding BSA in the enzyme solution, the recovered activity increased to 80% at 6 mg of BSA but when 10 mg of BSA was applied the activity decreased. In contrast to the set of CLEAs, adding BSA up to 2 mg enhanced the recovered activity 43% and gradually dropped when the amount of BSA increased to 14 mg the recovered activity was 32 % (Figure 35). The decrease in the activity of lipase beyond the optimum concentration of BSA was caused by the competition between free amino acids of lipase and BSA leading to the blockage of the active site. The study of adding BSA as a co-feeder for forming CLEAs of CALB caused the hyperactivation of lipase at 188% and also stabilized the lipase activity for long time storage (Torres *et al*., 2014).

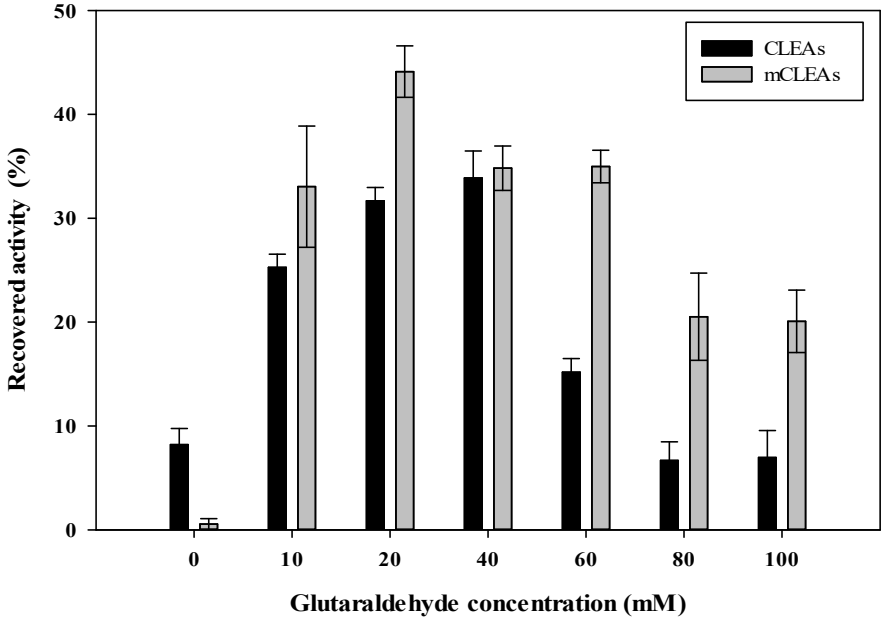


Figure 34. Effect of glutaraldehyde concentration on the recovered activity of CLEAs and mCLEAs of *Aspergillus oryzae* ST11 lipase (25 mg of magnetic nanoparticles, 3 U/ml of enzyme shaken 50 rpm at 4ºC for 3 h).

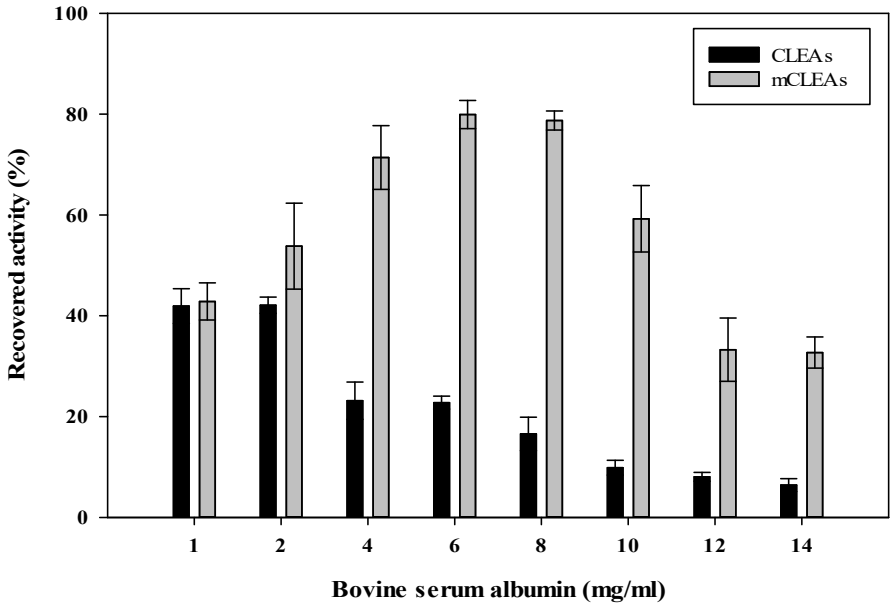


Figure 35. Effect of bovine serum albumin addition on the recovered activity of CLEAs and mCLEAs of *Aspergillus oryzae* ST11 lipase (25 mg of magnetic nanoparticles, 3 U/ml of enzyme, 20 mM glutaraldehyde solution, and shaken 50 rpm at 4ºC for 3 h).

The effect of immobilization time was also studied in the mixture containing 25 mg of MNPs, 20 mM of glutaraldehyde and 6 mg of BSA. The immobilization time was varied from 1–7 h. The result shows that the mCLEAs had the maximum recovered activity (80%) at 3 h (Figure 36). The extension of immobilization time longer than 3 h had the negative effect on the recovered activity by altering the enzyme's structure due to a long-time exposure to the glutaraldehyde. On the contrary, there was a report showing that the recovered activity of CLEAs from CALB increased from 65% to almost 100% when the immobilization time was prolonged from 5 to 24 h (Cruz-Izquierdo *et al*., 2014).

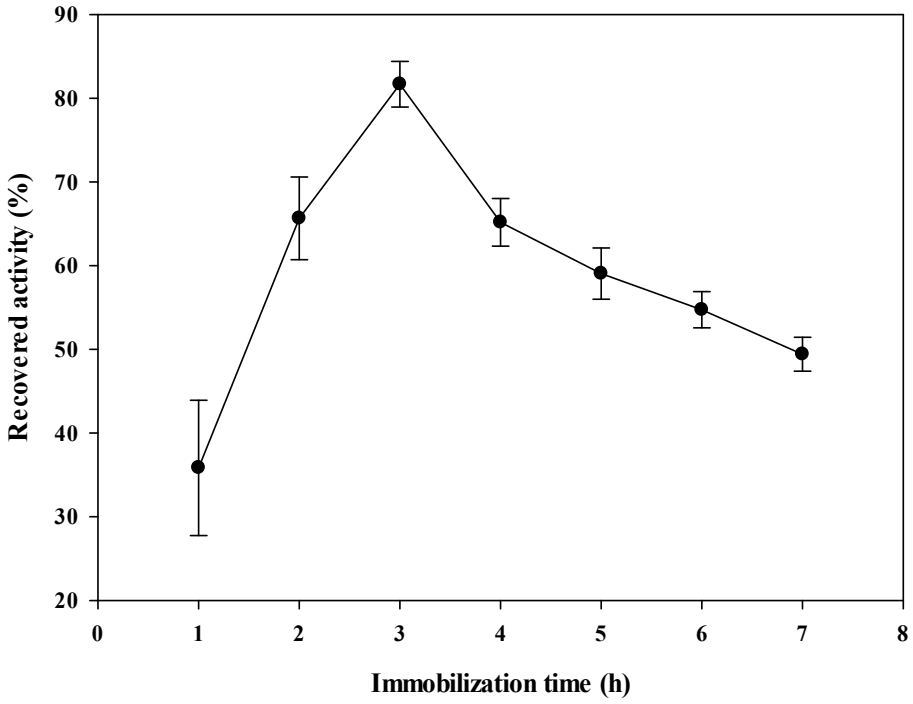


Figure 36. Effect of time on the activity of mCLEAs of *Aspergillus oryzae* ST11 lipase. (25 mg of magnetic nanoparticles, 3 U/ml of enzyme, 20 mM glutaraldehyde solution, and shaken 50 rpm at 4°C).

3. Stability of immobilized lipase

After getting the optimal conditions for lipase immobilization, the immobilized lipase was investigated for pH and temperature stabilities and compared to the free lipase from *Aspergillus oryzae* ST11. The mCLEAs showed the highest stability at pH 7.5 (Figure 37). Increasing or decreasing pH from the optimal pH led to

the decrease in activity. The mCLEAs retained 63% of the activity at pH 4.0 and 80% at pH 9.0. The free lipase showed the highest stability at pH 6.5-7.0 and retained the activity only 30% at pH 4.0 and 66% at pH 9.0. These pH stability results also confirmed the improved stability of the mCLEAs on the changing of pH higher or lower than optimal pH. Effect of temperature on the enzyme stability is shown in Figure 38. When the temperature was increased from 20 to 40ºC the lipase activities of both free and immobilized lipase are stable. When the temperature was higher than 40ºC the lipase activities of both free lipase and mCLEAs were decreased. The activity of mCLEAs was around 47% compared to 39% of free lipase at 70ºC. The change in the pH and thermal stabilities of the immobilized lipase compared to free lipase can be confirmed by the study of (Mehrasbi *et al*., 2017). They reported that the soluble and immobilized CALB lipase retained almost the same activity at 45ºC. However, when the temperature was increased, the activity of free CALB lipase decreased sharply compared to the activity of the immobilized lipase. At 65ºC, the immobilized CLAB lipase still retained 51% activity while the free lipase lost its activity completely at 60ºC. For pH stability, both soluble and immobilized CALB lipase still had the optimum pH at 7.5. However, at pH 9.5, the immobilized CALB lipase still retained the activity around 80% compared to 40% from the free CALB.

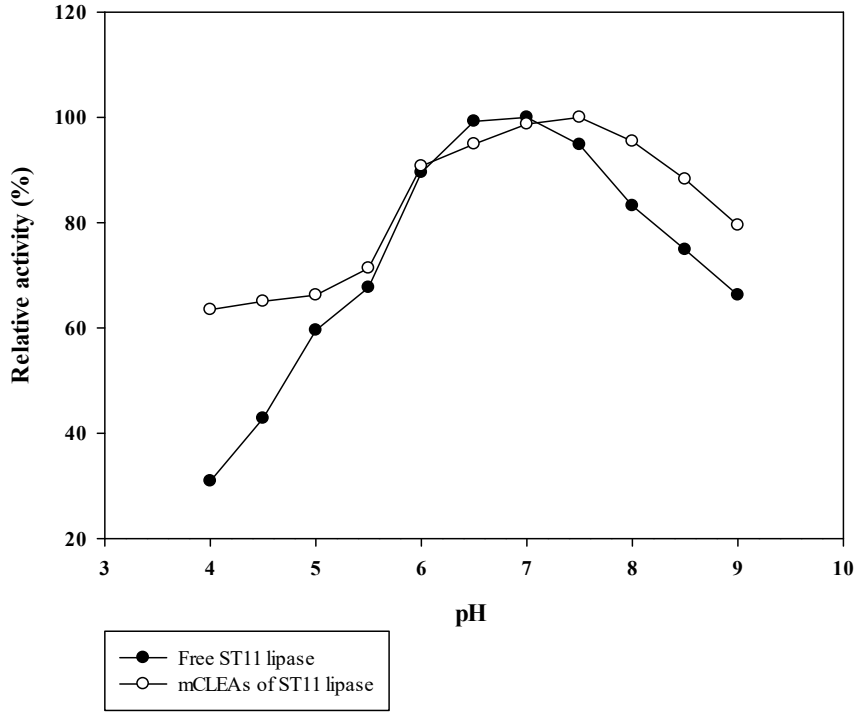


Figure 37. Effect of pH on the stability of free and mCLEAs of *Aspergillus oryzae* ST11 lipase.

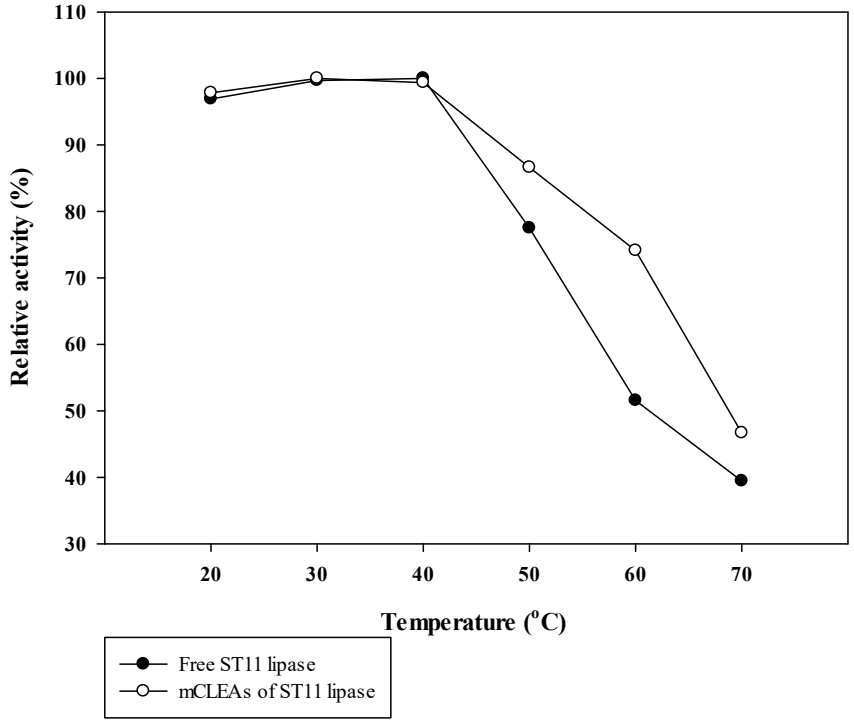


Figure 38. Effect of temperature on the stability of free and mCLEAs of *Aspergillus oryzae* ST11 lipase.

4. Factors affecting biodiesel production

Effect of the amount of biocatalyst on biodiesel production was studied by increasing the amount of mCLEAs from 10-50% w/w based on the weight of palm olein and the results are shown in Figure 39. When the amount of the biocatalyst was 30%, the biodiesel conversion was the highest (26% conversion) and decreased when the amount of the biocatalyst was higher than 30%. The excess amount of catalysts could hinder the mixing of reaction mixture and lead to the lower efficiency of catalysis.

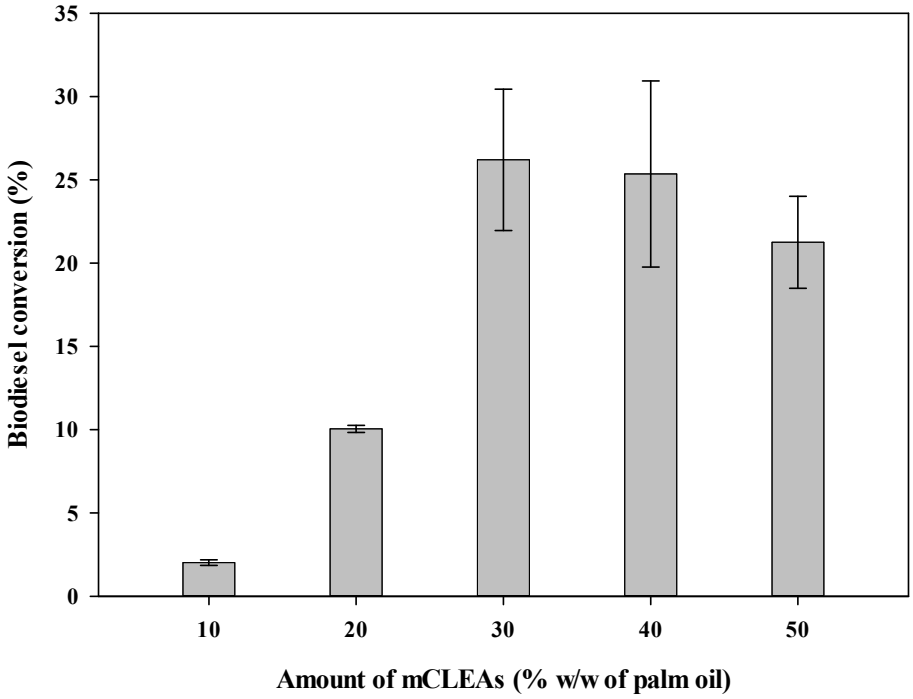


Figure 39. Effect of mCLEAs amount on biodiesel production (2 ml of 10% w/v palm oil in isooctane, mole ratio of palm oil:methanol (1:3), 10% water content, 250 rpm and 37°C for 24 h).

Water content was an important factor for enzyme catalysis. It stabilizes the 3-dimension structure of a protein and also promotes the water-oil interface. The activity of lipase also depends on this interface (Xiao *et al*., 2011). In this study, 50mM Tris HCl buffer pH 7.0 was added to the reaction mixture with the different amount based on the weight of oil in the system. The maximum biodiesel conversion reached 81% at 30% water content in the reaction mixture (Figure 40). The addition of water to

the system more than 30% might drive the reaction backward and lead to lower biodiesel conversion.

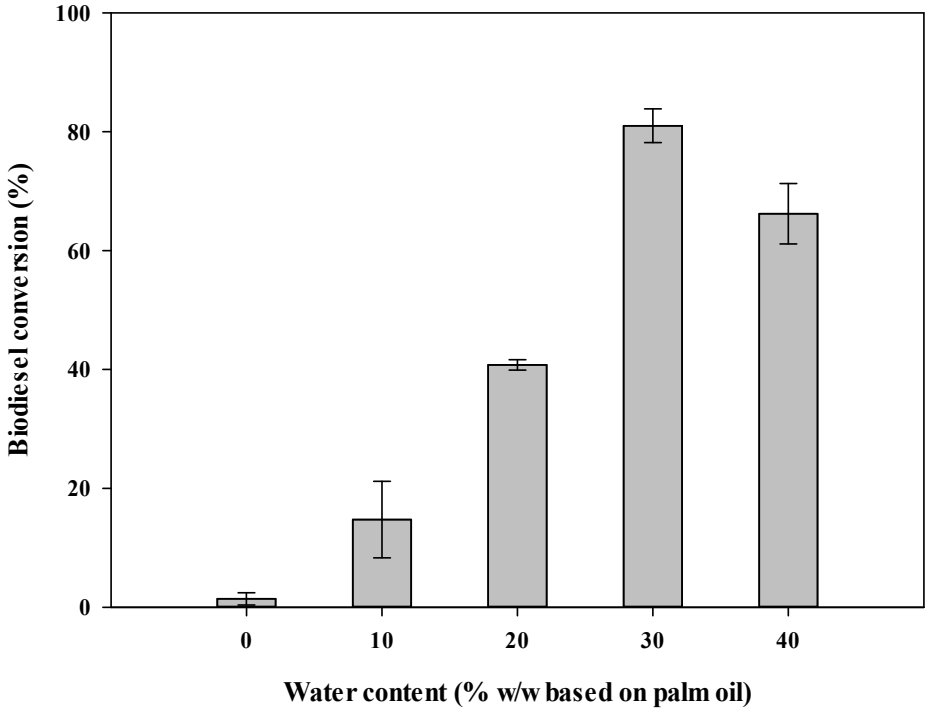


Figure 40. Effect of water content on biodiesel production by the mCLEAs of *Aspergillus oryzae* ST11 lipase. (30% w/v mCLEAs, 3 moles of methanol per mole palm oil, 250 rpm and 37°C for 24 h).

According to the ideal equation for biodiesel production from palm oil and methanol, 3 moles of methanol could react with 1 mole of palm olein to produce 3 moles of fatty acid methyl esters. The addition of methanol higher than 3 moles could force the forward reaction for transesterification and an excess amount of methanol might cause the deactivation of lipase. Figure 41 shows the biodiesel conversion at different molar ratio between palm olein and methanol. The biodiesel conversion was increased when the mole of methanol increased and it was achieved 81% with 3 moles of methanol per 1 mole of palm olein. Adding methanol higher than 3:1 led to the lower conversion of biodiesel. The stepwise addition of methanol would enhance the yield of biodiesel by elimination of methanol deactivation of enzyme. The comparison of biodiesel production by using one step addition and stepwise addition by adding 1 mole of methanol every 3 h is shown in Figure 42. The biodiesel conversion was improved

when stepwise addition of methanol was introduced. In the reaction with 3 moles of methanol per mole of oil after 24 h of reaction, the stepwise addition of methanol gave the biodiesel conversion 94.7% compared to 81% from one step addition. The productivity was used to determine the efficiency of immobilized lipase. From the result in Figure 43, the productivity of immobilized lipase with three-step addition of methanol increased until 6-9 h of reaction producing the biodiesel at 8.1 % biodiesel per hour. In contrast, the one step addition of methanol only gave the production rate at 5.4 % biodiesel per hour. Even though, at the early stage of the reaction, the one-step addition of methanol showed a higher production rate compared to that of three-step addition of methanol. This might be affected by the inhibition of methanol on the lipase.

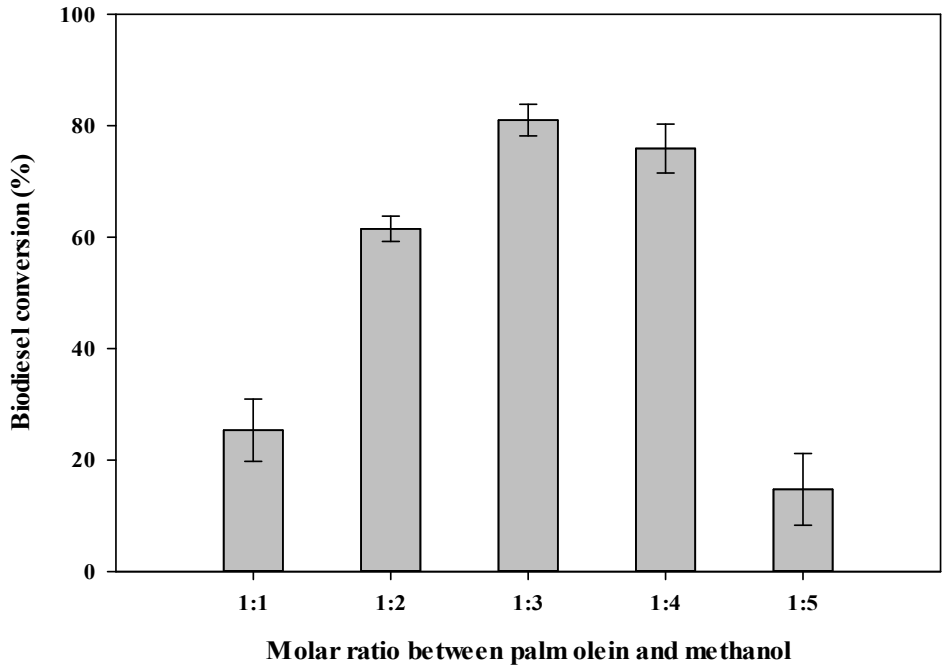


Figure 41. Effect of molar ratio between palm olein and methanol on biodiesel production by mCLEAs of *Aspergillus oryzae* ST11 lipase. (30% w/v mCLEAs, 30% water content, 250 rpm and 37ºC for 24 h).

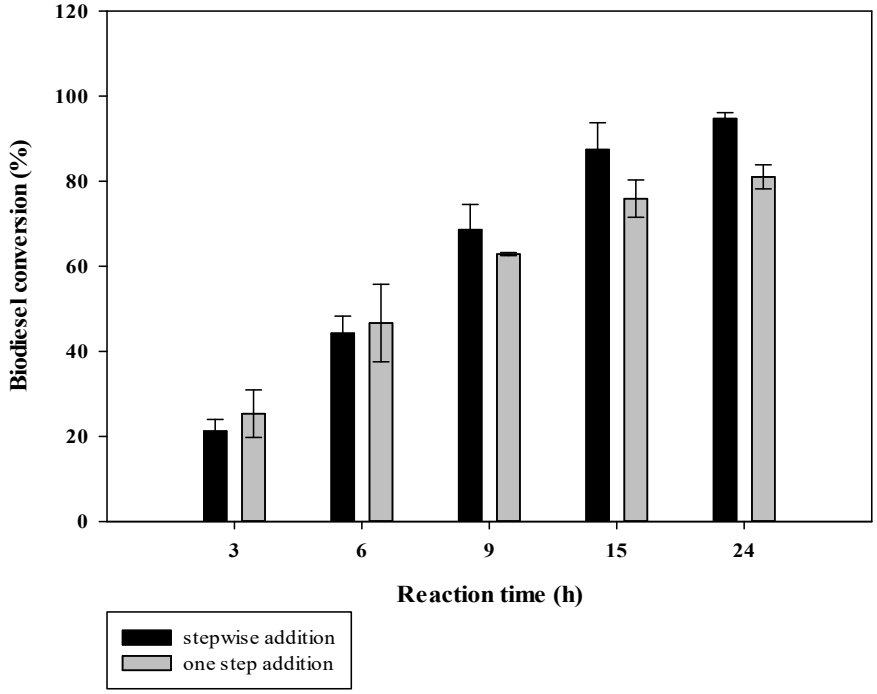


Figure 42. Effect of one step and stepwise addition of methanol (1 mole of palm oil per 3 moles of methanol by adding 1 mole of methanol every 3 h) and time on biodiesel production by mCLEAs of *Aspergillus oryzae* ST11 lipase. (30% w/v mCLEAs, 30% water content, 250 rpm and 37ºC for 24 h).

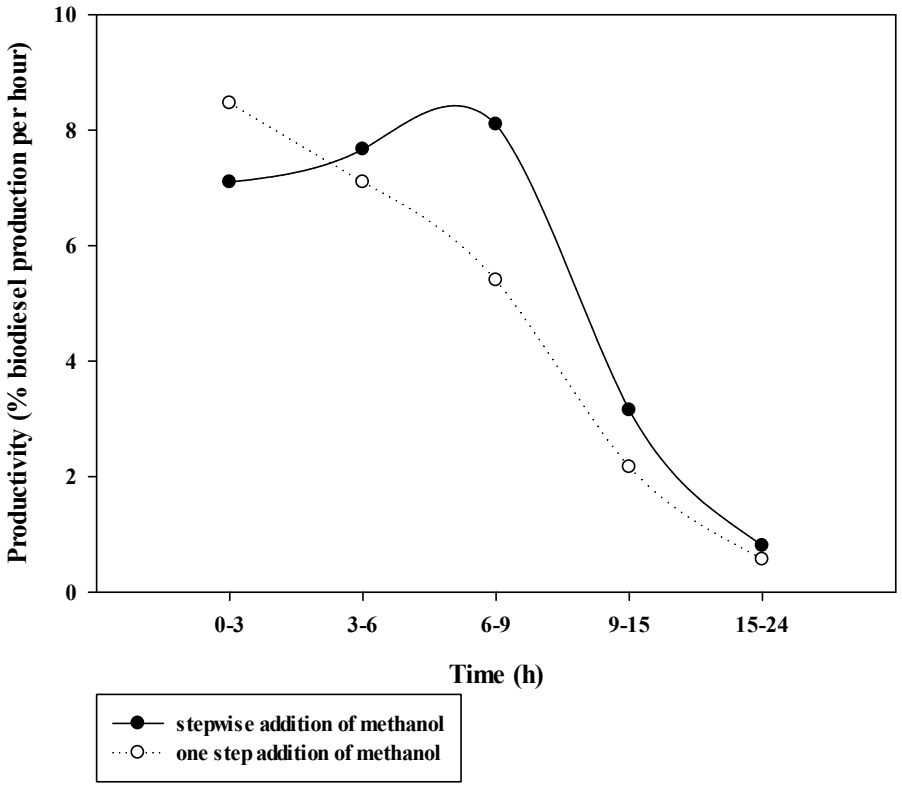


Figure 43. Productivity of biodiesel production with one step and stepwise addition of methanol catalyzed by mCLEAs of *Aspergillus oryzae* ST11 lipase. (1 mole of palm oil per 3 moles of methanol by adding 1 mole of methanol every 3 h for stepwise addition of methanol).

5. Reusability of mCLEAs for biodiesel production

Recycling the biocatalyst is one of the most important parameters for the application of the enzyme immobilization due to process cost for continuous production. The transesterification reaction was conducted to investigate the reusability of mCLEAs from *Aspergillus oryzae* ST11 lipase for 5 cycles. From Fig. 44, the conversion of biodiesel was dropped from 100% at the first cycle of biodiesel production to 65% at the 5th batch. The decrease of biodiesel production might due to the denaturation of lipase during long reaction time as well as the loss of biocatalyst in each cycle. Comparing the result of transesterification of soybean oil by immobilized *Thermomyces lanuginose* lipase on magnetic nanoparticles. It showed that biodiesel yield was dropped to 45% after 5 cycles of use (Xie and Ma, 2010). Zhang *et al*. (2015)

also studied the cross-linked enzyme aggregates of *Thermomyces lanuginose* lipase on the magnetic nanoparticles by using Tween-80 activation. After 10 cycles of use, mCLEAs still catalyzed the transesterification reaction with indifferent yield of biodiesel compared to initial cycle. However, according to the effect of surfactants on the lipase activity from the chapter 3. The results showed that the use of surfactant decreased the lipase activity from *Aspergillus oryzae* ST11. The negative effect of surfactants was reported by Santoro *et al*. (2014) showing the drastic decrease in the activity of lipase from *A. japonicus* LAB01 after the addition of Triton X-100, Triton X-114, Tween-80 and SDS. This might be affected by the competition between a substrate and surfactant to interact with an enzyme leading to the distortion of enzyme structure.

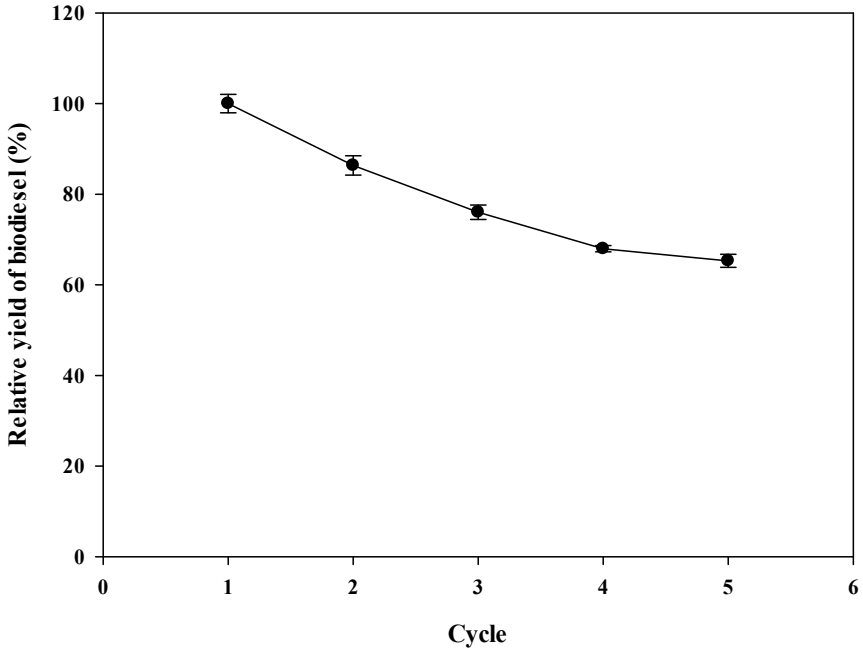


Figure 44. Reusability of mCLEAs of *Aspergillus oryzae* ST11 lipase for biodiesel production.

Conclusion

In this experiment, the production of mCLEAs from *Aspergillus oryzae* ST11 lipase was achieved by using glutaraldehyde and the recovered activity of mCLEAs was enhanced by adding bovine serum albumin. The prepared mCLEAs

exhibited the potential for biodiesel production which gave the high biodiesel conversion at the optimal conditions. In addition, the magnetic biocatalyst is easy to separate from the reaction mixture. From the point of reusability, the mCLEAs could be reused for 5 times with 65% relative activity for biodiesel production.

CHAPTER 5

COVALENT IMMOBILIZATION OF *ASPERGILLUS ORYZAE* ST11 LIPASE ON THE ELECTROSPUN PS-CO-TMP NANOFIBROUS MEMBRANE FOR BIODIESEL PRODUCTION FROM PALM OIL

Abstract

The lipase from *Aspergillus oryzae* ST11was immobilized on the electrospun polystyrene and trimethylolpropane tris [poly (propylene glycol) amine terminated] ether nanofibrous membrane (PS-co-TMP) using glutaraldehyde activation. The most successful way to immobilize lipase was to activate the nanofibrous membrane with glutaraldehyde before adding lipase, which could immobilize 54 mg-protein/g-nanofiber (0.15 unit/mg-support). The immobilized lipase was used for transesterification of palm oil and methanol in isooctane to produce biodiesel. The immobilized lipase was more stable than free lipase at pH 4 and 9 and conserved high biodiesel conversion around 70%. In contrast, the conversion by free lipase was drastically dropped when pH was lower than 7. For temperature stability, the biodiesel conversion of both immobilized and free lipases dropped when the temperature was increased from 30 to 70^{o}C. However, the immobilized lipase still retained higher activity (45%) than the free lipase (5%). The biodiesel production from palm oil was achieved at 96% conversion by using three-step addition of methanol (15% w/v immobilized lipase, 40% w/v water and shaken at 37^{o}C and 250 rpm for 18 h), while one step addition obtained 84% conversion. The immobilized lipase could be reused 10 times and still retained the activity 81%.

Introduction

Biodiesel or fatty acid alkyl ester is a new fuel source which is synthesized from triglycerides and alcohols (Kaieda *et al*., 1999). It has the desired properties for using in an engine such as high flash point, good lubricity and so on. Moreover, it also reduces emission of CO_2 and particulate matter to atmosphere. It

contained a small amount of sulfur, aromatic hydrocarbons and metals (Vasudevan and Briggs, 2008). It is produced by alkaline catalysis (KOH, NaOH, methoxide, etc.) or acid catalysis (Fedosov *et al*., 2013). However, the chemical methods still have some limitations for example, if the feedstock containing high amount of free fatty acids or water it will promote the formation of soap which leads to low yield of biodiesel as well as the difficulty of product separation during downstream process. Moreover, the chemical catalysts are also hazardous to the environment and water treatment are required for this process before releasing to the environment (Diego *et al*., 2011). Recently, transesterification reaction catalyzed by enzymatic process has been considered for producing biodiesel. There are many advantages such as biodiesel can be separated from glycerol easily. The oil with low quality contains high free fatty acid and water can be used for biodiesel synthesis (Dizge and Keskinler, 2008).

Lipase (EC 3.1.1.3) is one of hydrolytic enzymes catalyzing hydrolysis, esterification and transesterification reactions. It is stable in non-aqueous system as well as has high specificity, regioselectivity and enantioselectivity (Gog *et al*., 2012). Lipase has been immobilized by many ways including physical adsorption, covalent bonding, ionic interaction and entrapment to overcome the limitation due to the enzyme cost (Vaidya *et al*., 2008). Recently, the nanomaterials have been considered as the new materials for enzyme immobilization. The electrospun nanofiber also has the desired properties for immobilization application. It shows the large surface area to volume ratio due to the small diameter of fiber. This kind of material also get rid of mass transfer problem due to high porous among nanofibers (El-Aassar, 2013). Li *et al*. (2011) studied the immobilization of *Pseudomonas cepacia* lipase on the electrospun polyacrylonitrile nanofibrous membrane using amidination reaction for biodiesel production from soybean oil. The biodiesel conversion was achieved 90% after 24 h of reaction. Sakai *et al*. (2010) also immobilized *P. cepacia* lipase on the polyacrylonitrile nanofibers by physical adsorption for transesterification of *(S)*- glycidol with vinyl n-butyrate to glycidyl n-butyrate in isooctane. The result showed that the rate of reaction was 23-fold higher than free lipase and still retained 80% conversion after 10 cycles of use.

In this research, the lipase from *Aspergillus oryzae* ST11 was immobilized on the nanofibrous membrane of co-polymers between polystyrene (PS)

and trimethylolpropane tris [poly (propylene glycol), amine terminated] ether (TMP) by activation with glutaraldehyde. The immobilized lipase was used for biodiesel production from palm oil.

Materials and methods

1. Materials

Polystyrene (PS) (MW 280,000), trimethylolpropane tris [poly (propylene glycol) amine terminated] ether (TMP) (MW 440), *N,N*-dimethylformamide (DMF) and 25% glutaraldehyde solution (grade I) were purchased from Sigma-Aldrich (St. Louis, USA). Ethylenediamine was purchased from Loba Chemie (Mumbai, India). Peptone was purchased from Himedia (Mumbai, India). Sodium nitrate, potassium dihydrogen phosphate, magnesium sulfate and lactose were purchased from Analytical Univar Reagent (Auckland, New Zealand). Palm oil and olive oil were purchased from the local market.

2. Lipase production by *Aspergillus oryzae* ST11

The lipase producing *Aspergillus oryzae* ST11 was selected from the stock culture in the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. The fungal spore was inoculated on potato dextrose agar and incubated for 5 days. The spores were suspended in water and adjusted to $1x10^7$ spore/mL and inoculated (1 ml) into the optimized medium (100 ml) containing 1% olive oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4$ and 0.5% lactose (w/v) and adjusted pH to 6.0. The culture medium was shaken at 150 rpm and 37ºC for 4 days. The culture broth was separated from the mycelium by filtration with Whatman no.1 filter paper. The crude enzyme was precipitated from the culture broth by adding 3 volumes of chilled acetone per 1 volume of culture broth and kept at 4ºC overnight. The solution was centrifuged (8000 rpm at 4ºC) and the precipitate was dissolved with a small amount of 50mM Tris-HCl buffer pH 7.5 and transferred to the dialysis bag (3,500 Da). The sample was dialyzed against the same buffer at 4ºC overnight and concentrated by absorption with carboxymethyl cellulose powder (CMC).

3. Preparation of electrospun PS-co-TMP nanofibrous membrane

The polymer solution was prepared by dissolving PS pellets and TMP (10:1 w/w) for 3 g in *N,N*-DMF (7 g). The mixture was stirred at room temperature overnight to get the homogeneous solution. Electrospinning was initiated by injecting the polymer solution filled in a plastic syringe equipped with stainless steel needle (0.6 mm inner diameter) connected to the pump (model KDS 101, KD Scientific), at 1.9 ml/h with the applied voltage at +8.0 kV (Sakai *et al*., 2010). The obtained fibrous membrane was dried in fume hood overnight at room temperature to get rid of residual solvent and was cut into small pieces (1.0x1.0 cm).

4. Immobilization of *Aspergillus oryzae* ST11 lipase on electrospun PS-co-TMP nanofibrous membrane

4.1 Method A: Physical adsorption

The electrospun PS-co-TMP nanofibrous membrane (10 mg) was mixed with 1 ml of the *Aspergillus oryzae* ST11 lipase solution (0.7 mg protein/ml, 2 units/ml). The mixture was gently shaken at 4ºC for 7 h. The immobilized fibers were filtered through Whatman paper no.1 with vacuum pump and washed with 50 mM Tris-HCl buffer pH 7.0 until no protein was detected in the solution.

4.2 Method B: Mixing of glutaraldehyde solution together with nanofibrous membrane and enzyme

This method was performed according to the method A, but 0.5% (w/v) glutaraldehyde solution was added into the lipase solution containing 10 mg of nanofibrous membrane. The mixture was gently shaken at 4ºC for 7 h.

4.3 Method C: Addition of glutaraldehyde solution before adding enzyme

The nanofibrous membrane was mixed with 10% (w/v) glutaraldehyde solution and shaken at 4ºC for 3 h. After that, the activated nanofibers were retrieved and washed and transferred to the enzyme solution. The mixture was gently shaken at 4ºC for 7 h.

4.4 Method D: Ethylenediamine crosslinking after glutaraldehyde activation

The nanofibrous membrane was mixed with 10% (w/v) glutaraldehyde and shaken at 4ºC for 3 h. The nanofibrous membrane was retrieved and washed with distilled water. The fibers were transferred to 10% (w/v) ethylenediamine and shaken for 3 h and washed with distilled water again. The activated fibers were filtered and dried in the desiccator. The *Aspergillus oryzae* ST11 lipase was immobilized on the surface of material by adding 0.5% (w/v) glutaraldehyde solution. The mixture was gently shaken at 4ºC for 7 h. The immobilized fibers were filtered and washed with 50 mM Tris-HCl buffer pH 7.0 until no protein was detected in the solution.

The protein in washing buffer was determined by Lowry's method (Lowry *et al*., 1951). The immobilized protein and activity efficiency were calculated with the following formulas. Total protein in the lipase solution before and after immobilization was represented as (P_b) and (P_a), respectively.

$$\text{Immobilized protein (mg protein/g-support)} = \frac{(Pb-Pa)}{\text{g-support}}$$

$$\text{Immobilized activity (U/10 mg-support)} = \frac{\text{immobilized activity (U)}}{\text{10 mg-support}}$$

5. Determination of lipase activity

The immobilized lipase on nanofibrous membrane was tested for hydrolytic activity by immersing 10 mg of immobilized nanofibers to the reaction mixture containing 1 ml of 10% (w/v) palm oil in isooctane and 1 ml of 50 mM Tris-HCl buffer pH 7.0. The reaction mixture was shaken at 37ºC and 300 rpm for 30 min. The upper phase was withdrawn for hydrolysis determination by measuring the amount of fatty acids liberated as palmitic acid (Lee and Rhee, 1993). For the soluble lipase activity, the lipase solution was diluted with 50mM Tris-HCl buffer pH 7.0 and the reaction was carried out as same as the activity determination of immobilized lipase. One unit of enzyme activity was defined as the enzyme necessary to release 1 µmol of palmitic acid per minute at the specified conditions. The appearance of the nanofibrous membrane before and after immobilization with the chosen immobilization method was observed by scanning electron microscope (SEM) (Moon *et al*., 2008).

6. pH and temperature stabilities of immobilized and free lipases

The immobilized lipase and free lipase were evaluated for pH and temperature stabilities. For pH stability, 30 mg (~4.5 U) of immobilized lipase (0.15 U/mg-support) was placed in a 20 ml glass vial containing 5 ml of different buffer ranging from pH 4.0 to 9.0 and incubated at 30ºC for 2 h. After that, the nanofibrous membrane was washed with 50 mM Tris-HCl buffer pH 7.0. In the case of free lipase, the lipase solution was mixed with different buffer and adjusted to neutral pH by adding HCl or NaOH before using in the transesterification reaction. The effect of temperature was also determined by incubating the immobilized lipase and the free lipase at different temperature ranging from 30 to 70ºC for 2 h before using in the transesterification reaction.

7. Biodiesel production by the immobilized lipase

7.1 Enzymatic transesterification and determination of biodiesel production

The immobilized lipase (5-20% w/w based on palm oil weight) was transferred to the reaction mixture containing 2 ml of 10% (w/v) palm oil in isooctane and methanol (1:3 mol/mol) and 10% (w/v) of 50 mM Tris-HCl buffer pH 7.0 based on weight of oil. The reaction was carried out at 37ºC and 250 rpm for 24 h. After the reaction finished, 10 µl of organic phase was withdrawn and mixed with 990 µl of isooctane. The sample was filtered through 0.45 µm membrane filter and analyzed by high performance liquid chromatography (HPLC Agilent 1100) connected with evaporative light scattering detector (ELSD) by gradient system. Samples were detected on the evaporative light scattering detector PL-ELS 2100 (Polymer Laboratories Ltd., USA). The configuration of ELSD detector were 50º°C for nebulization, 40º°C for evaporation and the 1.5 ml/min for the compressed air flow (Fedosov *et al*., 2014). The optimization of biodiesel production was investigated by varying parameters for transesterification reaction.

7.2 Effect of various parameters on biodiesel production

Various parameters were investigated to find the suitable condition for transesterification of palm oil and methanol for biodiesel production. The parameters were the amount of the immobilized lipase and the water in the system, type of the solvents that was used for dissolving palm oil as well as the temperature and the molar ratio of palm oil and methanol. The parameter exhibited the highest biodiesel conversion was chosen for studying next parameter.

8. Reusability of immobilized lipase

After the immobilized lipase of *Aspergillus oryzae* ST11 was used in the transesterification reaction, the immobilized lipase was separated from the reaction mixture by filtration with Whatman no.1 filter paper and washed with fresh isooctane before using in the new batch. The immobilized enzyme was reused 10 times.

Results and discussion

1. Effect of different ways of lipase immobilization on electrospun PS-co-TMP nanofibrous membrane

Different methods were used to immobilize the lipase of *Aspergillus oryzae* ST11 on electrospun PS-co-TMP nanofibrous membrane as shown in Figure 45. Method A was the direct adsorption of enzyme and nanofibrous membrane. Method B and C were covalent bonding of the enzyme with nanofibrous membrane by adding glutaraldehyde at a different stage. Method D was prepared by covalent binding of nanofibrous membrane with glutaraldehyde and followed by ethylenediamine. After that the enzyme and glutaraldehyde were mixed with the activated fibers. From the results of percent immobilization (Figure 46a), all techniques showed the increasing trend of immobilization when time increased. However, physical adsorption (method A) and addition of glutaraldehyde (method B) techniques reached the equilibrium stage at 45 and 60 min, respectively. The amount of immobilized protein was slightly decreased in the case of adding glutaraldehyde (method B). In the case of the method D (ethylenediamine crosslinking), this technique showed the lowest potential of immobilization compared to the other three methods. This result might be due to the effect of two side binding of ethylenediamine on the activated fibers and led to less

available functional group for cross-linking with enzyme. The method C (glutaraldehyde activation after adding enzyme) showed the highest immobilized protein (54.0 mg protein/g-support) as well as immobilized activity (1.49U/10mg-support) (Figure 46a). This technique provided one side of glutaraldehyde to bind with amine of protein while in the method B, glutaraldehyde was added at the first step to react to both amine on the surface of nanofiber as well as amine group of protein molecules which might deactivate the functional group of protein. This could be explained by the process of forming cross linking enzyme aggregates. The increasing in concentration of glutaraldehyde could form the covalent bonding of lysine residuals in the molecule of enzyme itself leads to locking of active site or making the insoluble molecule (Nadar and Rathod, 2015). The residual activity after immobilization was also important. Increasing glutaraldehyde concentration and immobilization time might cause negative effect on the enzyme activity. The result of the method C indicated that the trend of immobilized protein was the same as the trend of immobilized activity which reached higher than 75% of an initial enzyme (Figure 46b). When the time of immobilization increased, the amount of immobilized protein on a support was increased also as well as the lipase activity.

Figure 47 shows the morphology of electrospun PS-co-TMP before and after immobilization by the method C. The surface of nanofiber was quite smooth before immobilization. After immobilization, there was the appearance of rough surface due to coating of enzyme was observed. The morphology of the immobilized lipase in this study was similar to that of the immobilized *Candida rugosa* lipase on the surface of polyacrylonitrile nanofiber (Li *et al.*, 2011).

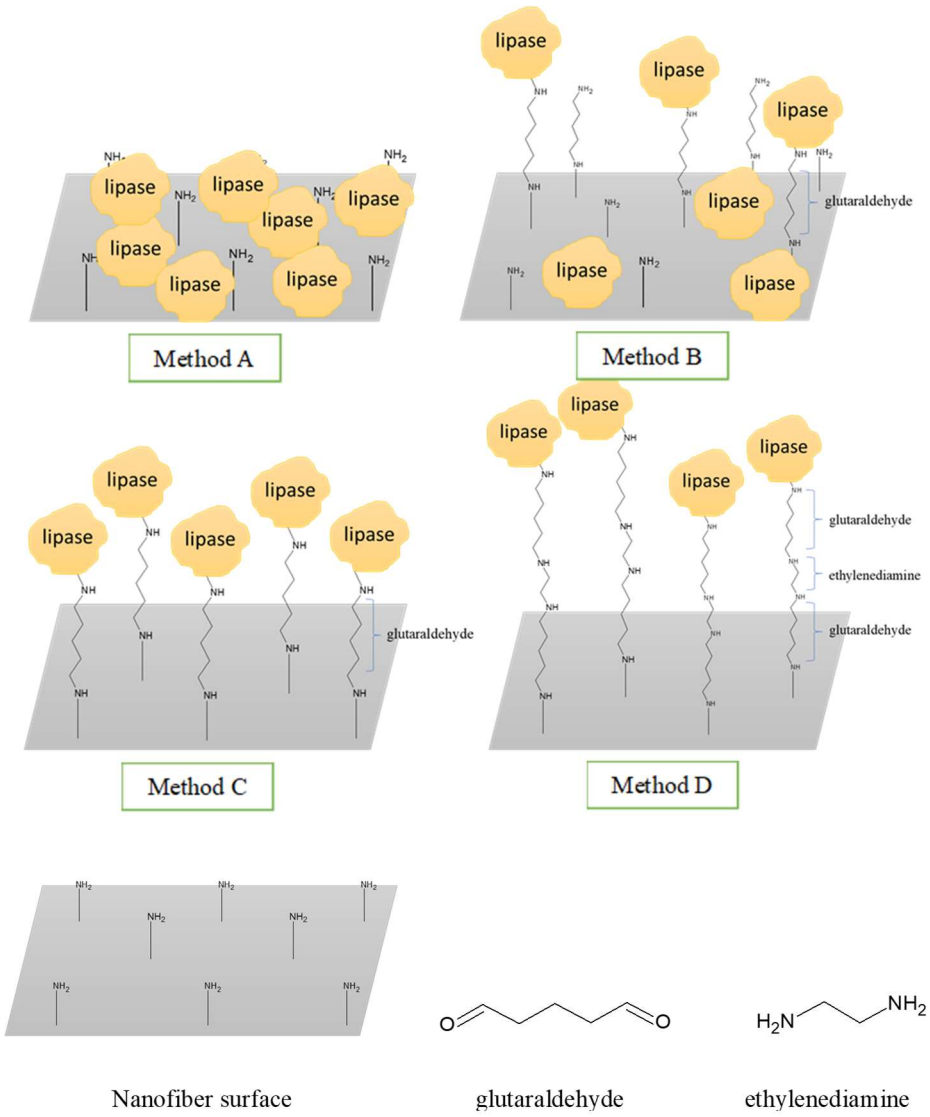


Figure 45. The different ways of immobilization of *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane. Physical adsorption (A), Mixing of glutaraldehyde solution together with nanofibrous membrane and enzyme (B), Addition of glutaraldehyde solution before adding enzyme (C) and ethylenediamine crosslinking after glutaraldehyde activation (D).

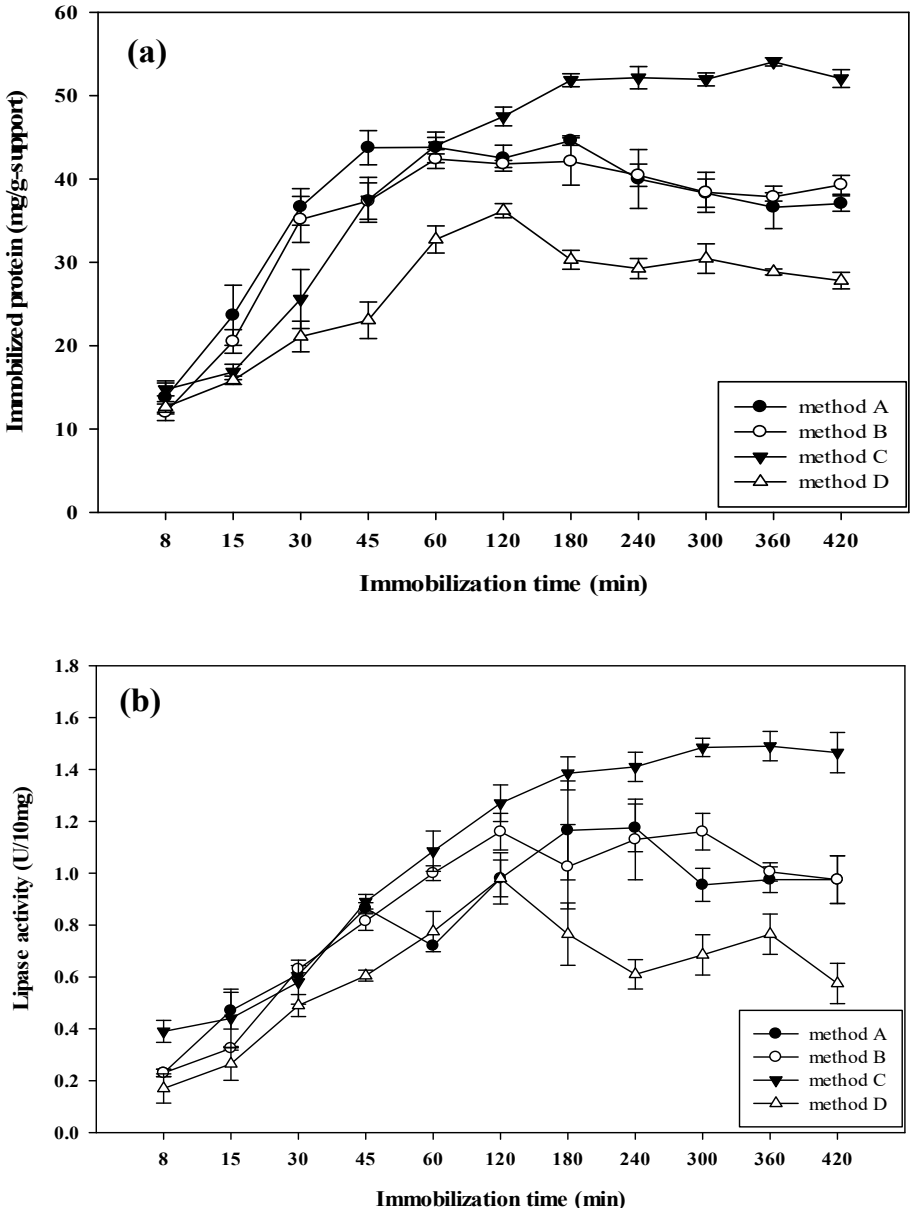


Figure 46. Effect of immobilization methods on immobilized protein (a) and activity (b) of *Aspergillus oryzae* ST11 lipase; physical adsorption (method A), addition of glutaraldehyde solution with enzyme (method B), addition of glutaraldehyde solution before adding enzyme (method C) and enzyme crosslinked with ethylenediamine-glutaraldehyde modified surface (method D).

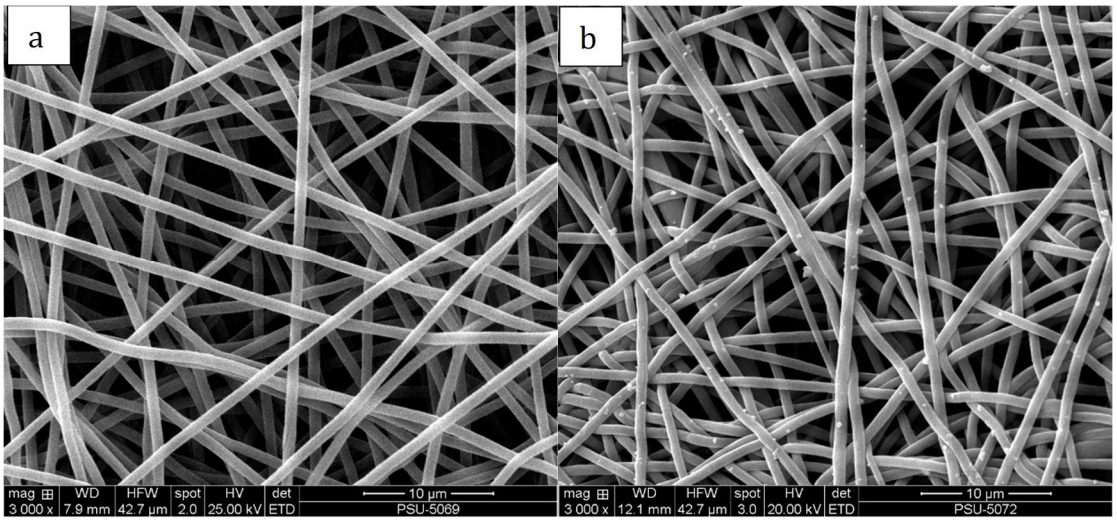


Figure 47. The SEM morphology of PS-co-TMP nanofiber (3,000X). The nanofiber before immobilization (a), and after immobilization with method C (b).

2. pH and temperature stabilities of immobilized and free lipase

The immobilized lipase of *A. oryzae* ST11 on the electrospun PS-co-TMP nanofibrous membrane and free lipase were tested for pH and temperature stabilities. The results of pH stability are shown in Figure 48. Both free and immobilized lipase showed the highest activity at pH 7.0. The results show that the immobilized lipase was stable at a broad pH range and the yield of biodiesel was still higher than 70% at pH 4.0 and 9.0. However, when pH was lower than 7.0 the biodiesel conversion catalyzed by the free lipase decreased and retained 3.9% at pH 4.0

The thermal stability of the enzyme was also concerned in the biosynthesis. In this experiment, both free and immobilized lipase were incubated in buffer pH 7.0 for 2 h in the water bath at the temperature ranging from 30 to 70^{o}C. The activity of the enzyme was determined at 37^{o}C. The results are shown in Figure 49. The immobilized lipase showed the maximum yield of biodiesel at 30^{o}C and the yield gradually decreased when the temperature was increased. It was noted that at 70^{o}C, the immobilized lipase still retained 47% conversion. The enhancement of enzyme stability was due to the multipoint bond cross-linking between the molecule of enzyme and the surface of support. In contrast, the biodiesel conversion of the free lipase drastically

decreased when the temperature was increased, and the enzyme almost lost the ability to produce biodiesel at 70ºC. The decreasing of the lipase activity while increasing temperature was similar with the work with *Pseudomonas cepacia* lipase immobilized onto the electrospun PAN nanofibrous membrane for transesterification. In that experiment, the activity of the free lipase was dropped greatly when the temperature was higher than 60ºC and only exhibited 5% activity. In contrast, the immobilized lipase still retained 90% activity at 60ºC (Li *et al*., 2011).

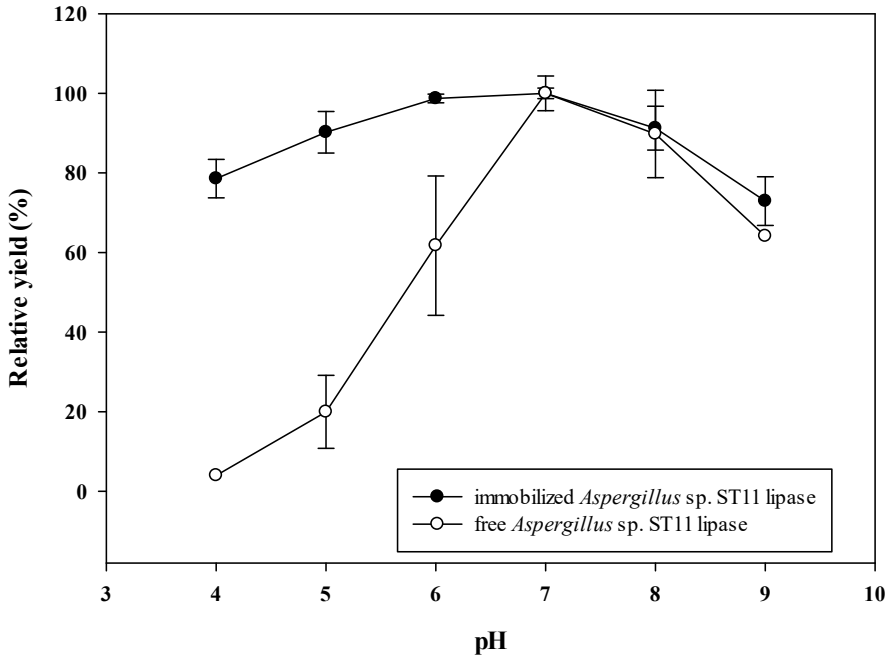


Figure 48. pH stability of free and immobilized lipases from *Aspergillus oryzae* ST11.

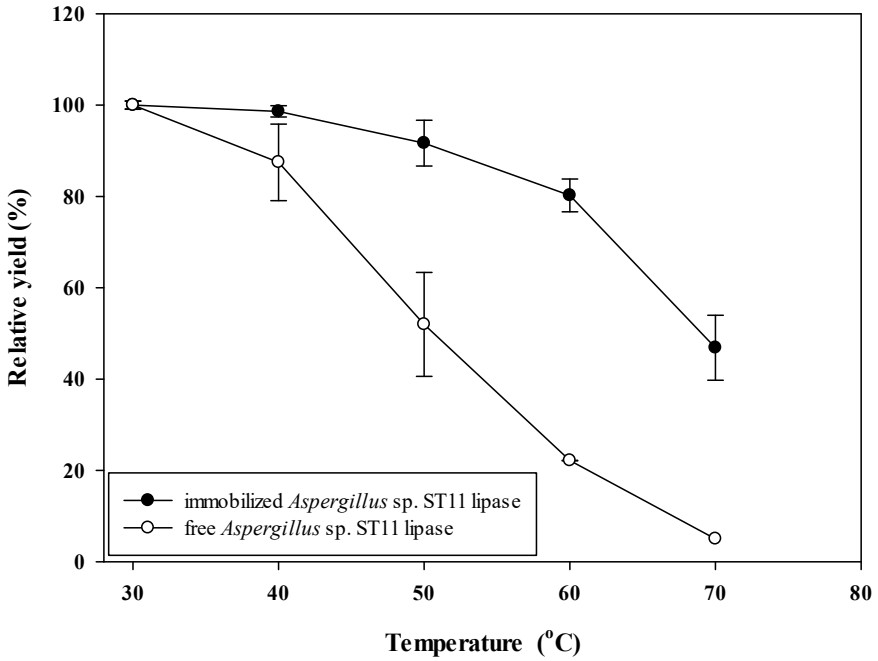


Figure 49. Temperature stability of free and immobilized lipases from *Aspergillus oryzae* ST11.

3. Effect of various factors on biodiesel production

The immobilized lipase from the method C (0.15 U/mg-support) was chosen for the study of the effect of various parameters on biodiesel production from palm oil. The first factor was the amount of immobilized lipase which was varied from 5 to 20% w/w based on weight of palm oil. The reaction was performed at 37ºC and 250 rpm for 24 h. The result shows that the percent conversion of biodiesel increased when the amount of immobilized lipase increased (Figure 50). However, the percent conversion was not significantly different when the amount of the immobilized lipase increased from 15 to 20 % w/w and achieved the maximum percent conversion at 80%. Adding the immobilized lipase more than 20% (w/w) could not enhance the biodiesel production due to the limitation of mass transfer from the large volume of catalyst in the reaction mixture leaded to a low interaction between enzyme and substrate. The effect of the biocatalyst amount in this study was similar to the previous report that studied the immobilization of *Pseudomonas cepacia* lipase on protein-coated microcrystals (Raita *et al*., 2010). The increase of the enzyme loading from 20 to 30% (w/w) did not show the significant differences of biodiesel conversion and they

achieved almost 90% of conversion within 12 h. The amount of biocatalyst used for catalyzing the reaction could be reduced if a purer form of lipase was used for immobilization due to higher specificity of an enzyme. That meant a higher unit of lipase could be immobilized on the supportive material. However, this procedure would require a further step of purification and also the time for the process. Hence, it would increase the cost of the process.

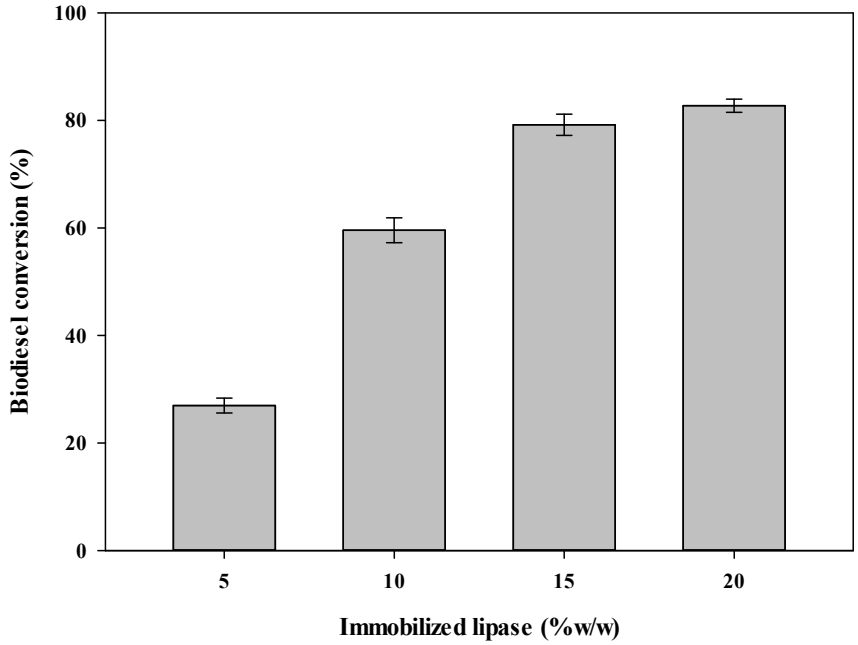


Figure 50. Effect of amount of immobilized enzyme on the biodiesel synthesis by the *Aspergillus oryzae* ST11 lipase immobilized on the electrospun PS-co-TMP nanofibrous membrane. (The reaction mixture contained 5-20% w/v (10-40 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 37^{o}C, 250 rpm, for 24 h).

The amount of water was important to the three-dimensional structure of lipase (Yücel *et al*., 2013) as well as the presence of oil-water interface is vital to the activation of lipase (Wang *et al*., 2010). For this experiment, the amount of water in the reaction mixture was varied from 10 to 50% (w/w) based on weight of palm oil. The result showed that the bioconversion of biodiesel increased when the water content was

increased. However, it was not significantly different when the water content was increased from 40 to 50% w/w which the biodiesel conversion reached 85% (Figure 51). Similar result of high water content on biodiesel production was reported by (Wang *et al*., 2010). They studied the biodiesel production by immobilized recombinant *Rhizopus oryzae* lipase. The water was added to the system in the range of 10-120% based on weight of oil. It showed that the highest biodiesel production (>80%) was obtained at 60% (w/w) of water content. Another study was from (Chen *et al*., 2006) reporting the biodiesel production from waste cooking oil catalyzed by immobilized *R. oryzae* lipase. The highest biodiesel conversion (>80%) was obtained at 50% (w/w) of water content whereas the biodiesel conversion greatly dropped to 60% after 75% w/w of water content was applied to the system after 24 h of the reaction.

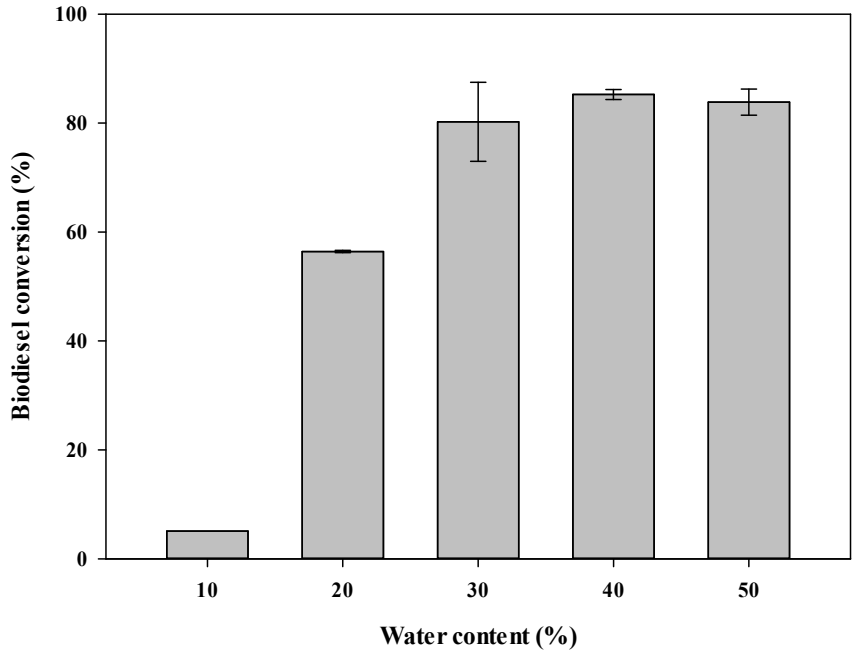


Figure 51. Effect of water on the biodiesel synthesis by *Aspergillus oryzae* ST11 lipase immobilized on the electrospun PS-co-TMP nanofibrous membrane. (The reaction mixture contained 15% w/v (30 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 10-40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 37ºC, 250 rpm, for 24 h)..

The effect of solvents on biodiesel production by the immobilized enzyme was also studied. The two-phase system was used for biodiesel production. According to the catalyzation of lipase, most lipases would catalyze the reaction at the oil-water interface promoting the open form of lid-like structure. However, the lipase would show a lower activity due to the closed form of lid in the system containing only organic solvents. That could be explained by the absence of water-oil interface. Hence, the use of the two-phase condition would induce the lid to open and enhance the catalyzation of enzyme (Maruyama *et al*., 2000). In our study, palm oil was diluted with different organic solvents (isooctane, heptane, hexane, toluene, petroleum ether, tert-butyl ether and tert-butanol). The hydrophobic organic solvents with higher log P (>2) promoted the activity of lipase (Li *et al*., 2006). Moreover, adding organic solvent can solve the problem of low solubility of short chain alcohols (methanol, ethanol) which normally inactivated enzyme activity and decreased yield of ester (Royon *et al*., 2007). The result showed that isooctane gave the maximum conversion (86%) while toluene gave the lowest conversion (23.5%) (Figure 52). The effect of isooctane on the activity of lipase was reported by Hiol *et al*. (2000) which the purified lipase from thermophilic *Rhizopus oryzae*. had the highest activity in isooctane and higher than those of hexane and heptane. Another case of the isooctane effect was the study of biodiesel production catalyzed by *Pichia partoris* whole cells inserted the gene from *Rhizomucor miehi* (Huang *et al*., 2012). The system containing isooctane gave the highest methyl esters yield at 54% at 72 h which was higher than the yields obtained from the hexane and heptane and polar solvents like tert-butyl alcohol and acetone. The effectiveness of isooctane used for lipase catalyzation was reported by Mukataka *et al*. (1989). The addition of isooctane would enhance the hydrolysis of lipids like palm oil and beef tallow by providing very large interfacial area between the lipid and aqueous phases. Moreover, Wang *et al*. (2007) reported the use of isooctane as a pretreatment of cell-bound lipase from *R. chinensis* could be suitable to promote the synthetic reaction.

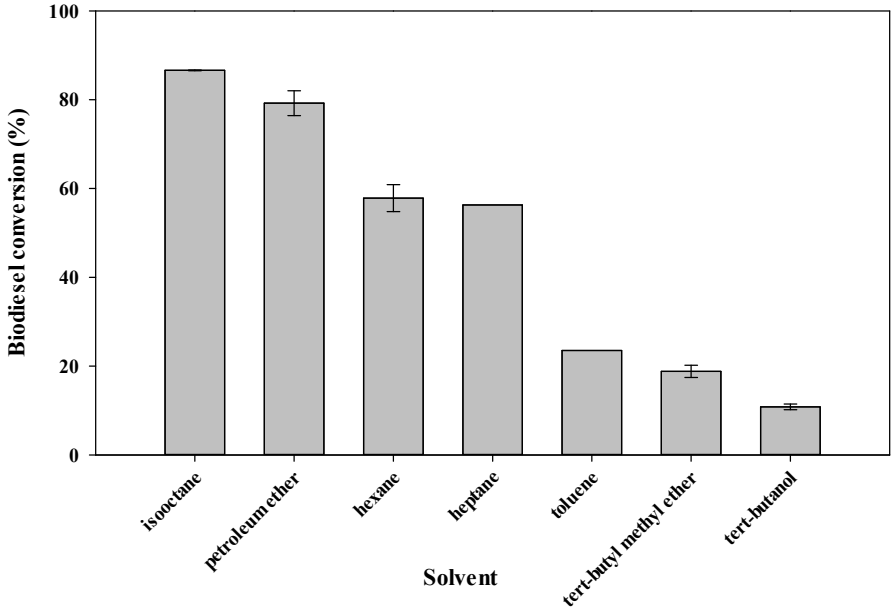


Figure 52. Effect of different solvents on the biodiesel synthesis by the immobilized *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane. (The 200 mg of palm oil was diluted in 2 ml of different solvents. The prepared reaction mixtures were used for transesterification reaction. The reaction was carried out with 30 mg of immobilized lipase, 40 % water, 250 rpm and 37ºC for 24 h)

The temperature also affected on the rate of biodiesel production. In this study, the reactions were performed at different temperature (30, 37, 45 and 55ºC). The results show that the highest conversion (83%) was obtained at 37ºC for 24 h. When the reaction was performed at 55ºC the conversion was drastically dropped to 5% which might be due to the denaturation of the enzyme at high temperature (Figure 53). There was the research studying the immobilization of *Pseudomonas cepacia* lipase on modified attapulgite. It showed that the biodiesel yield was lower (63%) when the temperature increased to 50ºC while 94% of biodiesel that was achieved at 35ºC after 36 h (You *et al*., 2013). Hence, the higher temperature leads to the deactivation of protein structure and affect the function of enzyme.

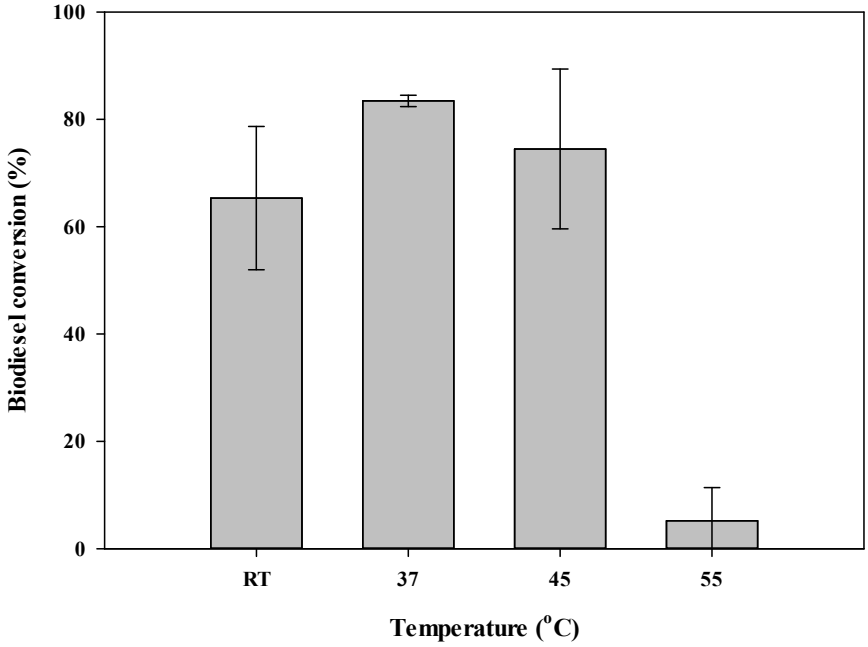


Figure 53. Effect of temperature on the biodiesel synthesis by the immobilized *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane. (The reaction mixture contained 15% w/v (30 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 250 rpm, for 24 h).

The effect of molar ratio of palm oil and methanol on the biodiesel production by immobilized *A. oryzae* ST11 lipase was studied. The report of biodiesel production from *Streptomyces* sp. W007 lipase showed that the biodiesel yield was increased when the ratio of oil/methanol increased from 1:1 to 1:3. At 1:3, the yield of biodiesel was 88%. When increasing of methanol beyond 3 moles led to the lower yield of biodiesel (40%) (Wang *et al*., 2017). Another experiment was the study using Novozym 435 to catalyze the transesterification of rapeseed oil to produce biodiesel. The ratios of oil/methanol were varied from 1:1 to 1:6, the yield of biodiesel increased in linear when the ratio was increased from 1:1 to 1:2 (40 to 70%). The yield of biodiesel was not significantly different at the ratios were 1:2 to 1:5 and slightly lower at 1:6 (68%) (Jeong and Park, 2008).

The stepwise addition of methanol could overcome this limitation by preventing the accumulation of methanol that lead to the denaturation of the enzyme. Xie and Ma (2010) reported that the biodiesel yield increased from 8 to 48% when the molar ratio (oil:methanol) increased from 1:0.5 to 1:1.5. Addition of methanol higher than 1.5 mole also decreased the biodiesel conversion (8%). They used stepwise addition of methanol with molar ratio between oil and methanol (1:1) at each step and achieved the highest conversion of biodiesel at 94%. In this work, the molar ratios of palm oil and methanol were varied from 1:2 to 1:5. Stepwise additions of methanol were also evaluated by adding 1 mole of methanol every 3 h to get the molar ratio of 1:3, 1:4 and 1:5. The results show that three steps addition of methanol could increase the biodiesel conversion to 96% compared to 87% of one step addition (Figure 54). The yield of biodiesel was not increased significantly when methanol was added more than 4 moles. Figure 55 shows the time course of biodiesel production with stepwise addition of 1 mole of methanol at every 6 h which enhanced the biodiesel production and the biodiesel conversion reached the highest biodiesel conversion (96%) at 18 h. Interestingly, the time course indicated that the pattern of catalysis was a combination of hydrolysis and esterification. At the early stage of reaction, the triglyceride gradually decreased whereas the free fatty acid increased due to the hydrolysis reaction of lipase. This was contrast to the pattern of transesterification reaction that show the direct conversion of triglyceride to biodiesel without generating a high portion of free fatty acid (Faria *et al*., 2009). The productivity of biodiesel production was determined in Figure 56. The production rate increased rapidly at the reaction time between 9-12 h giving 7.8 % biodiesel production per hour. After that, the reaction rate gradually decreased which was corresponded with the equilibrium stage of biodiesel production at 18 h. Even increasing the reaction, it did not increase the biodiesel production.

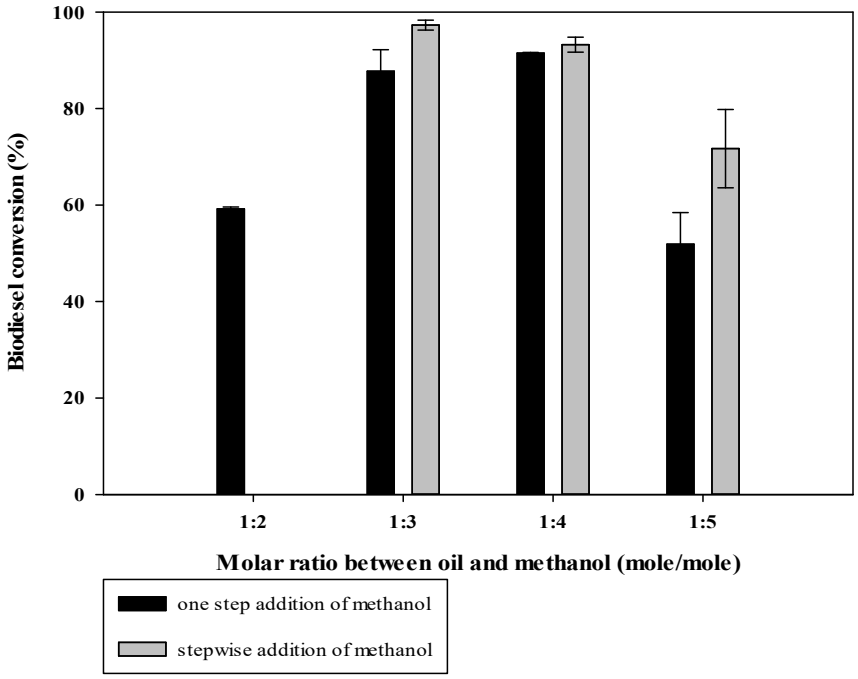


Figure 54. Effect of molar ratio of palm oil and methanol on the biodiesel synthesis by the immobilized *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane. using one step and stepwise addition of methanol (1 mole of methanol per 3 h). (The reaction mixture contained 15% w/v (30 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 37ºC, 250 rpm, for 24 h).

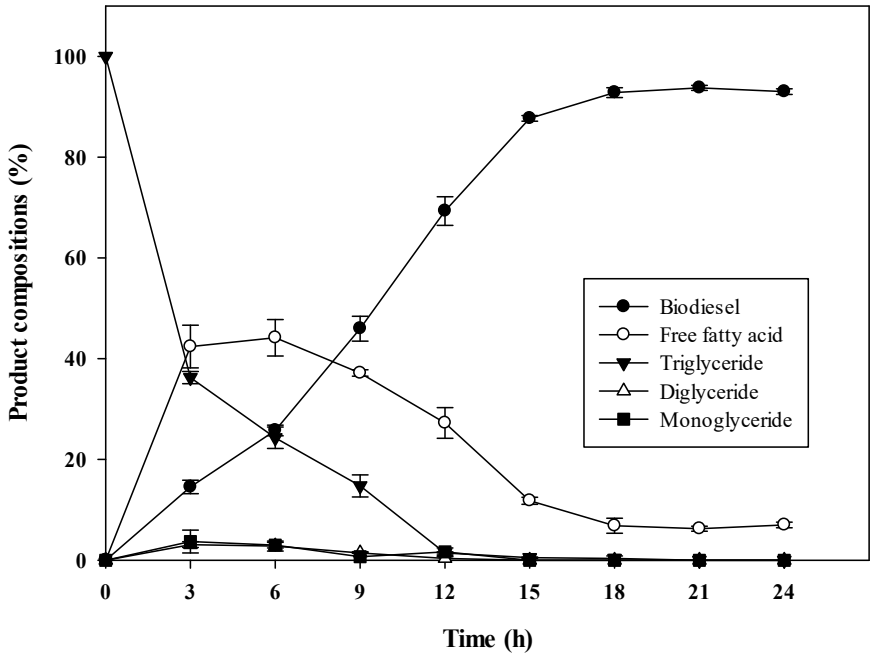


Figure 55. Time course of biodiesel synthesis catalyzed by the immobilized *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane with 3 moles of methanol per 1 mole of palm oil by using three-step addition of methanol (1 mole at every 6 h). (The reaction mixture contained 15% w/v (30 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 37^{o}C, 250 rpm, for 24 h).

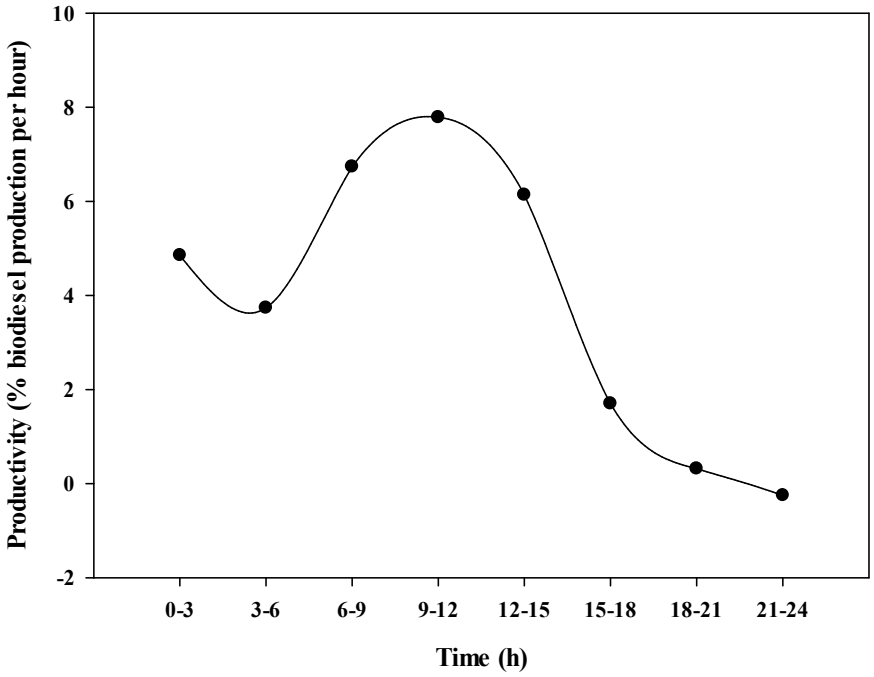


Figure 56. The productivity of biodiesel synthesis catalyzed by the immobilized *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane with 3 moles of methanol per 1 mole of palm oil by using three-step addition of methanol (1 mole at every 6 h). (The reaction mixture contained 15% w/v (30 mg) of immobilized lipase, 2 mL of 10 % (w/v) palm oil in isooctane, 3 moles of methanol)/mole of palm oil based on weight of palm oil and 40 % w/w of Tris-HCl buffer based on weight of oil and catalyzed at 37ºC, 250 rpm, for 24 h).

4. Reusability of immobilized lipase

Reusability was one of the desired characteristics of the biocatalyst for an enzymatic synthesis. Many cycles of use could represent the potential of using in industrial scale to overcome the limitation of enzyme cost. In this study, the immobilized lipase was used to synthesize biodiesel for 10 cycles. In each cycle, the immobilized lipase was retrieved and washed with fresh isooctane before applying to the fresh substrate. The result shows that the percent conversion was constant for 5 cycles. After that, the percent conversion was slightly decreased due to the folding of nanofibrous membrane during catalyzation. At the 10th cycle, the bioconversion

retained at 81% (Figure 57). This result of reusability was comparable to the study of physical adsorption of *P. cepacia* lipase on polyacrylonitrile (PAN) nanofibers which retained the activity at 81% after 10 cycles (Sakai *et al*., 2010). However, the covalent binding of *P. cepacia* lipase onto the electrospun PAN nanofibrous membranes could be used up to 10 cycles without losing the transesterification efficiency (Li *et al*., 2011).

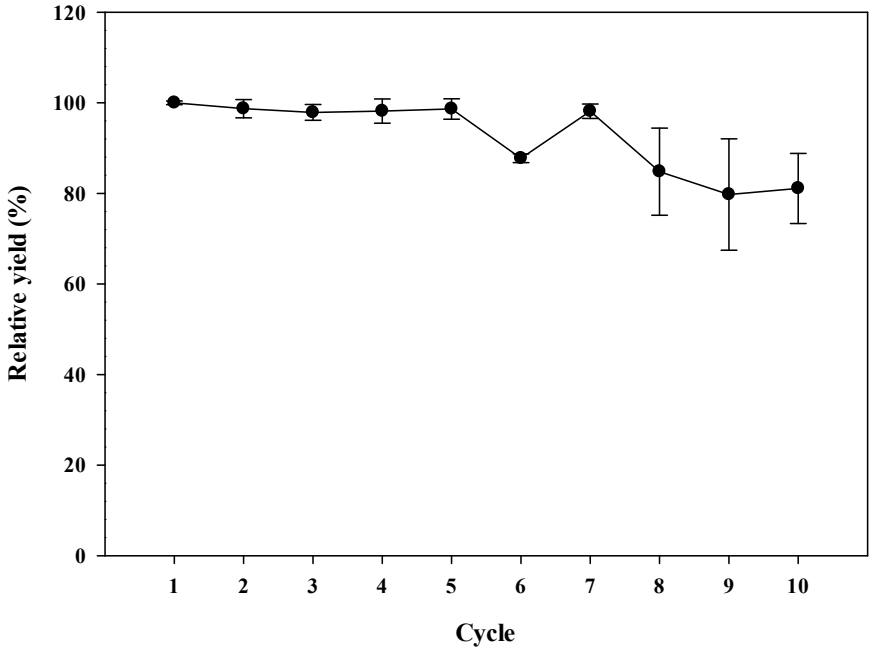


Figure 57. Reusability of immobilized lipase from *Aspergillus oryzae* ST11 on PS-co-TMP nanofibrous membrane.

Conclusion

The immobilization of *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane was achieved by using the activation of glutaraldehyde to modify the surface of the material before mixing with the enzyme and more than 75% of immobilization efficiency could be achieved. The immobilized lipase was stable in the wide range of pH and temperature compared to free lipase. Many factors were investigated for biodiesel synthesis. The increasing of immobilized lipase to 15% (w/w) also increased the biodiesel conversion. Water was also important for the reaction that indicated the lower content had negatively affected on biodiesel conversion which could be solved when water was increased. Isooctane was suitable for diluting palm oil which gave the highest biodiesel. Stepwise addition of methanol

was the way to prevent enzyme denaturation and showed the improvement of biodiesel conversion. The immobilized lipase on electrospun PS-co-TMP nanofibrous membrane could be reused for 10 cycles and still retained 81%.

CHAPTER 6

ENHANCING IMMOBILIZATION OF *ASPERGILLUS ORYZAE* ST11 LIPASE ON POLYACRYLONITRILE NANOFIBROUS MEMBRANE BY BOVINE SERUM ALBUMIN AND ITS APPLICATION FOR BIODIESEL PRODUCTION

Abstract

The polyacrylonitrile (PAN) nanofibrous membrane was prepared by the electrospinning method. This material was used as a carrier to immobilize the lipase from *Aspergillus oryzae* ST11. The immobilization efficiency was enhanced significantly after the addition of 6 mg/ml of bovine serum albumin (BSA) and 40 mM glutaraldehyde solution giving 86.9% of recovered activity compared to 42.0% in the absence of BSA addition. The immobilized PAN nanofibrous membrane had the lipase activity at 0.43 U/mg-support. The immobilized lipase was used in the biodiesel production. The optimum conditions for biodiesel production were 15% w/w of immobilized lipase, 40% w/w of Tris-HCl buffer, 3.5 moles of methanol per mole of palm oil (0.2 g palm oil). This immobilized lipase was used in comparison with the Novozym 435 to produce biodiesel. The result showed that the immobilized *A. oryzae* ST11 lipase produced a higher biodiesel conversion (95%) compared to commercial lipase (52%). Moreover, it was reused with 82.9% of activity retained at the 10^{th} cycle. These results indicated the potential use of immobilization of *A. oryzae* ST11 lipase for biodiesel production.

Introduction

In recent years, the synthetic materials in the nanoscale have been studied and used in many applications due to the promising property of large surface area. The larger space of nanomaterials could offer the use in tissue engineering, membrane and etc. (Yordem *et al*., 2008). One of the nanomaterials that become a candidate for many applications is the nanofibrous membrane. The nonwoven membranes consisting of many individual nanofibers is fabricated by electrospinning

method. This material owns the desired characteristic for biotechnology applications due to high surface to volume ratio and provides the space between the individual fibers to help the lower diffusion resistance. The nanofibrous membrane is chosen for immobilizing many biological substances such as proteins and enzymes (Li *et al*., 2011). One of the polymers widely studied is polyacrylonitrile (PAN). The nanofibrous membrane obtained from this polymer shows the good solvent tolerance as well as chemical, mechanical and thermal stabilities (Wang *et al*., 2007; Liu *et al*., 2015).

Lipases or triacylglycerol acyl hydrolases (EC 3.1.1.3) are the enzymes that can catalyze the hydrolysis of triacylglycerols at the interface between oil and water. Moreover, these enzymes can catalyze the synthetic reactions such as acidolysis, alcoholysis, aminolysis, esterification and interesterification in the specific conditions. Lipases have been interested in many industrial applications due to its variety of the reactions. Lipases are the ubiquitous enzymes found in many living organisms including animals, plants and microorganisms. However, microbial lipases show the feasibility of industrial use due to their wide range of stability, substrate specificity and low production cost. Among all kind of microorganisms, filamentous fungi are considered the suitable lipase producing microorganisms. They can produce the extracellular lipase enhancing the extraction and separation of the enzyme from the fermentation media (Contesini *et al*., 2010; Singh and Mukhopadhyay, 2012). The lipases are used as biocatalysts for biodiesel production. They have many advantages over chemical processes such as high purity of products, less wastewater, mild condition operation. However, the use of lipase still has some drawbacks such as the cost of enzyme and the low reaction rate (Guldhe *et al*., 2015; Aguieiras *et al*., 2017). The ideas of immobilization of enzymes are introduced for overcoming the limitations with the prolonged and continuous uses of enzyme (Soler *et al.*, 2015).

In the present work, the electrospun polyacrylonitrile nanofibrous membrane was used for immobilizing lipase from *Aspergillus oryzae* ST11. The lipase was precipitated on the modified surface of polyacrylonitrile nanofibrous membrane. It was shown that the addition of bovine serum albumin could enhance the recovered activity and increased the enzyme loading on the fibers. The biocatalysts were used in biodiesel production using one-step addition of methanol.

Materials and methods

1. Materials

Polyacrylonitrile (PAN) (MW 150,000), *N,N*-dimethyformamide and glutaraldehyde solution (50% in H_2O), lipid standards (methyl hexadecanoate, oleic acid, triolein, diolein and monoolein) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). Ethylenediamine (EDA) was purchased from Lobachemie (Mumbai, India). All HPLC grade solvents were purchased from J.T. Baker (Pennsylvania, USA). Palm oil was purchased from local company (Morakot) in Thailand.

2. Analytical methods

2.1 Determination of lipase activity

The hydrolytic activity of lipase was measured in a two-phase system according to the modified colorimetric method using palm olein as a substrate in isooctane (Kwon and Rhee, 1986). The reaction was performed in 20 ml glass vial capped with rubber stopper. The reaction was incubated at 37ºC by shaking at 250 rpm for 30 min. The hydrolytic activity was determined by measuring the amount of fatty acids released compared to standard curve of palmitic acid. One unit of lipase was defined as the lipase required to release 1 μmol of palmitic acid per minute at the specified condition (Ungcharoenwiwat *et al*., 2016).

2.2 Determination of biodiesel

The products of transesterification were analyzed by HPLC using Poroshell 120 HILIC column (150 mm x 3 mm, particle size of 2.7 μm, pores of 120 Å). The analytes were measured from HPLC (Agilent 1100, Agilent Technologies, USA) system connected with the evaporative light scattering detector PL-ELS 1000 (Polymer Laboratories Ltd., USA). The detector parameters were set as 50ºC for nebulization, 40ºC for evaporation and the compressed air flow of 1.5 mL/min. The results from analytes were compared to the retention time of standard lipids. The yield

of biodiesel was calculated based on the area of biodiesel peak compared to area of all peaks (Fedosov *et al.*, 2014).

2.3 Lipase production

The lipase producing *Aspergillus oryzae* ST11 was selected from stock culture in the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. The fungal spore was inoculated on the potato dextrose agar and incubated at 37^{O}C for 5 days. The spores were collected and adjusted to 1x10^{7} spore/ml using haemacytometer and inoculated into the optimized medium (100 ml) containing 1% olive oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4$ and 0.5% lactose (w/v) and adjusted pH to 6.0. The culture medium was shaken at 150 rpm and 37ºC for 4 days. The culture broth was separated from the mycelium by filtration with Whatman no.1 filter paper. The crude enzyme was precipitated from the culture broth by adding 3 volumes of cold acetone per 1 volume of the culture broth and kept at 4ºC overnight. The solution was centrifuged and the precipitate was dissolved with small amount of 50mM Tris-HCl buffer pH 7.0 and transferred to the dialysis bag (3,500 Da). The sample was dialyzed against the same buffer at 4ºC overnight and concentrated by absorption with carboxymethyl cellulose. The concentrated lipase was collected for immobilization.

2.4 Production of polyacrylonitrile nanofibrous membrane

The polyacrylonitrile polymer powder was dissolved in the dimethylformamide (DMF) for preparing the polymer solution. The electrospinning machine was set up by connecting a glass syringe containing polymer solution, a stainless-needle, a syringe pump (KD Scientific Corp., Massachusetts, USA), a high voltage power supply (Glassman, EL40P1, USA) and the collector covered with the aluminum foil. The parameters for electrospinning were 14 % (w/w) of polymer solution, 20.0 kV for applied voltage, 1.5 ml/h for feeding rate and 20 cm of the distance between the tip of needle and the collector. After the injection of polymer, the solvent was evaporated from the polymer. The nonwoven mats of nanofiber were formed at the

collector and dried under vacuum overnight to completely evaporate the residue solvent left after the process.

2.5 Surface modification of polyacrylonitrile nanofibrous membrane

The sheet of polyacrylonitrile nanofibrous membrane (1 g) was immersed in 15% (w/v) NaOH solution (50 ml) at 50ºC for 60 min. The color of membrane was changed from white to orange-red. The membrane was retrieved and washed extensively with deionized water. After that, the membrane was transferred into 10% v/v HCl solution (50 ml) for 2 h at room temperature. The membrane was separated and washed extensively again with deionized water until the pH of washing water became neutral. After that, the membrane was immersed into 10% w/v ethylenediamine solution (50 ml) for 1 h at room temperature. The membrane was finally separated from the solution and washed again with deionized water. The modified membrane was dried before use (Liu *et al*., 2015; Godjevargova *et al.*, 2005). The schematic of this step is shown in Figure 58.

2.6 Immobilization of *Aspergillus oryzae* ST11 lipase on modified membrane

The modified membrane (10 mg) was transferred into the lipase solution (5 U/ml). After that, the sample was added with 700 µl of $(NH_4)_2SO_4$ (500 mg/mL) and shaken at 4ºC and 50 rpm for 1 h. The glutaraldehyde solution was added into the mixture to get 25 mM of concentration. The sample was shaken at 4ºC and 50 rpm for different times ranging from 15 to 420 min. The bovine serum albumin (BSA) was added into the mixture as a co-feeder to enhance the recovered activity of immobilized lipase as well as the study of glutaraldehyde concentration. The effect of BSA and glutaraldehyde was studied to find out the best condition for the highest recovered activity (Torres *et al.*, 2014). The recovered activity of the immobilized lipase was calculated using the following equation.

$$\text{Recovered activity (\%)} = \frac{\text{activity of immobilized lipase x 100}}{\text{activity of lipase for immobilization}}$$

2.7 Morphology of the polyacrylonitrile nanofibrous membranes

The native membrane, the activated membrane with ethylenediamine and the membrane with immobilized enzyme were observed by using the scanning electron microscope (FEI Quanta 400) . The functional and chemical bonds were analyzed by the attenuated total reflection- Fourier transform infrared (ATR-FTIR, Bruker Vertex 70 FTIR spectrometer) (Liu *et al*., 2015).

2.8 Biodiesel production

The effect of the amount of biocatalysts and water in the system on transesterification reaction was investigated with the fixed molar ratio between methanol and oil at 3:1 (mole/mole). After that, the one-step addition of methanol with different molar ratio between methanol and oil from 2:1 to 5:1 (mole/mole) was determined. The best condition was chosen and used for studying the time course compared to the biodiesel production from Novozym 435 (commercial immobilized lipase). For the basic conditions for transesterification reaction was 50 mM Tris-HCl buffer pH 7.0 (10% w/w compared to weight of palm oil), 2 ml of 10% (w/w) palm oil in isooctane. The reaction was carried out by shaking at 250 rpm and 37ºC for 24 h. The organic phase of reaction was withdrawn (10µl) and mixed with 990 µl of isooctane. The sample was analyzed for biodiesel production by HPLC system (Fedosov *et al.*, 2014).

2.9 Reusability

In each batch of use, the immobilized lipase was taken out from the mixture by filtration and washed thoroughly by fresh isooctane before starting the new cycle of use. The immobilized lipase was reused up to 10 cycles.

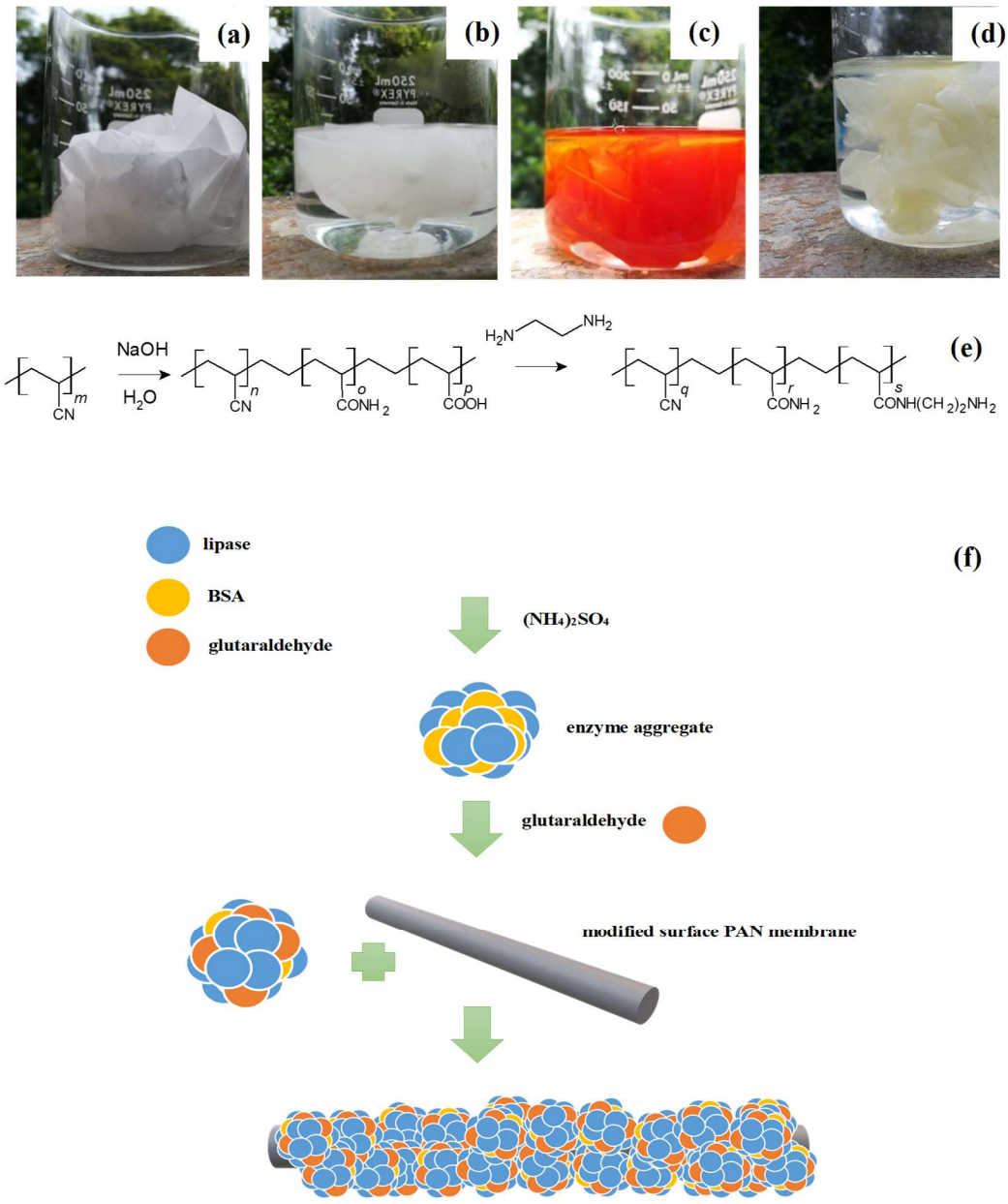


Figure 58. Surface modification of polyacrylonitrile nanofibrous membrane. Membrane before modification (a), Membrane immersed in NaOH solution (b), Membrane in NaOH solution after heating (c), After addition of ethylenediamine solution (d), The schematic reaction for converting nitrile to amine (e) and the step of immobilization of lipase with precipitation technique on modified membrane (f).

Results and discussion

1. Immobilization of *Aspergillus oryzae* ST11 lipase on modified membrane

The polyacrylonitrile nanofibrous membrane was activated by NaOH, HCl, and functionalized with ethylenediamine to provide the amine group for enzyme immobilization. The lipase and bovine serum albumin were precipitated by saturated ammonium sulfate on the surface of nanofibrous membrane. The lipase and BSA were cross-linked to nanofibrous membrane by glutaraldehyde solution. The immobilization of *Aspergillus oryzae* ST11 lipase was performed with the fixed amount of $(NH_4)_2SO_4$ (700 µl, 500 mg/ml) and 25 mM of glutaraldehyde solution. The different immobilization time was determined between 15 to 420 min. Figure 59 showed the activity (%) of the immobilized lipase. The activity increased and reached the highest point (44.2%; 0.22 U/mg-support) at 180 min of immobilization and gradually decreased after reaching that point. Prolonging the time of immobilization longer than the optimum point led to the decrease in the recovered activity. Ondul *et al.* (2012) showed the decrease in the activity of lipases from *Thermomyces lanuginosus* and *Candida antarctica* A aggregated by polyethyleneimine. The enzyme could not form an aggregate by increasing immobilization time due to the short time of enzyme cluster formation. Hence, increase in immobilization time longer than specific point could not increase the efficiency of lipase.

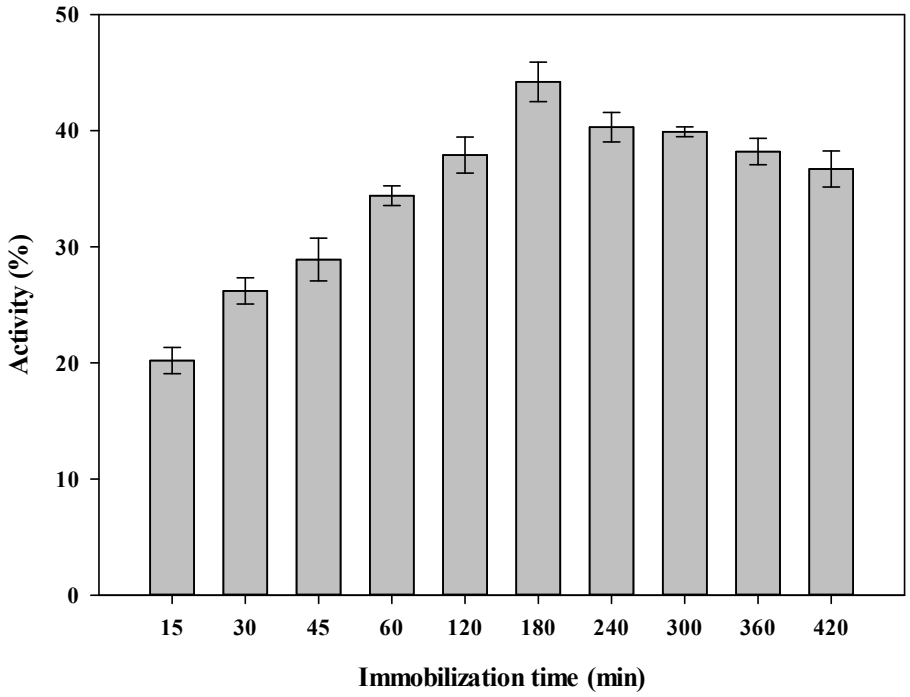


Figure 59. Effect of immobilization time on the activity of *Aspergillus oryzae* ST11 lipase immobilized on polyacrylonitrile nanofibrous membrane.

After optimum immobilization time was obtained, the effects of glutaraldehyde concentration and bovine serum albumin were determined. The relationship between glutaraldehyde (0-60 mM) and BSA (0-8 mg/ml) was investigated. The use of glutaraldehyde was important to the immobilization of protein by forming the stable crosslink between enzymes and support materials and enhanced the intermolecular bonding among the molecule of enzymes. This prevented the leakage of proteins from the carrier (Rodrigues *et al*., 2016). The BSA was used as a co-feeder to facilitate the cross-linking enzyme aggregate's formation in the case of a low concentration of enzyme and improved the stability of enzyme in the preparation step of immobilization (Torres *et al.*, 2014). From the result (Figure 60), the 40 mM of glutaraldehyde concentration and 6 mg/ml of BSA gave the highest activity of immobilized lipase at 86.9% (0.43 U/mg-support). The excess concentration of glutaraldehyde gave the lower recovered activity leading to the multi-point chemical bonds between the molecule of protein and the surface of the membrane at a high concentration of glutaraldehyde (Zhu and Sun, 2012). In the case of BSA addition, the

increase in the amount of BSA from 6.0 to 8.0 mg/ml did not enhance the recovered activity of the lipase. This may be caused by the reduction of mass transfer due to the poor accessibility of substrate to lipase entrapped inside the cross-linked structure of the protein (Torres *et al*., 2013).

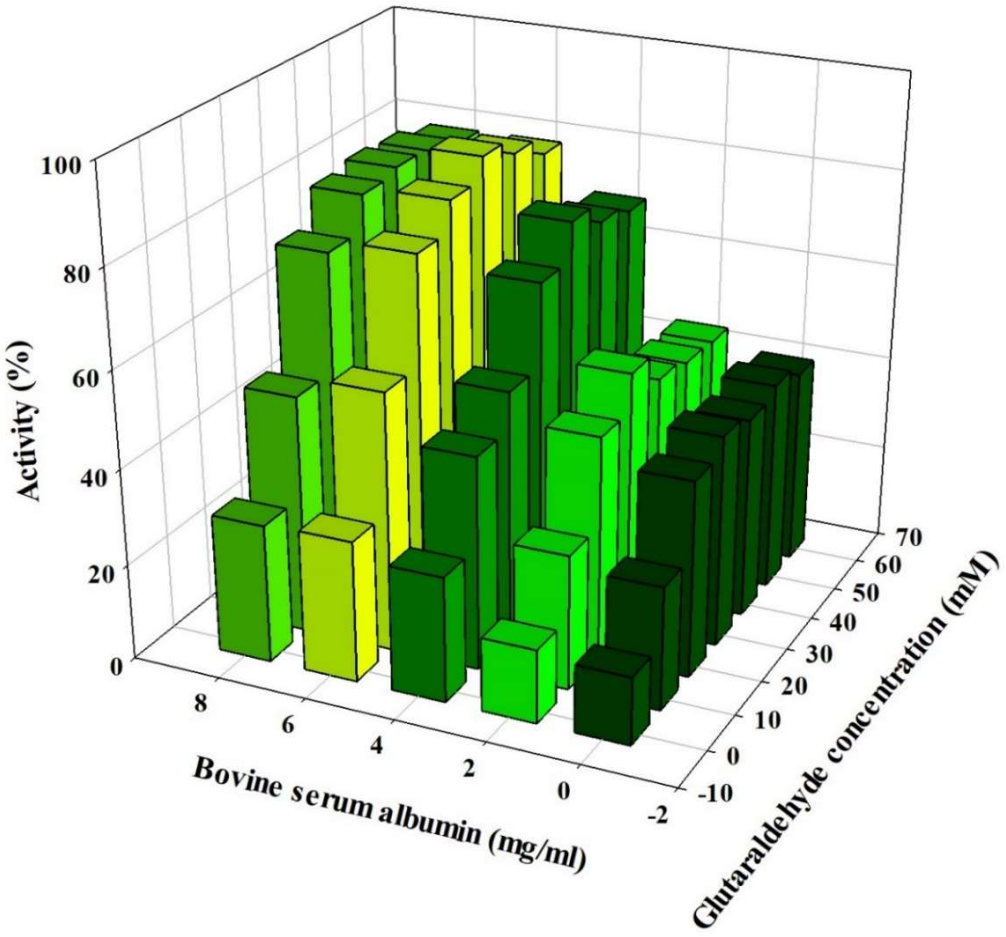


Figure 60. Effect of glutaraldehyde concentration and bovine serum albumin on the recovered activity of *Aspergillus oryzae* ST11 lipase immobilized on polyacrylonitrile nanofibrous membrane.

The changes in the chemical structure of PAN nanofibrous membrane before and after immobilization of lipase were analyzed by ATR-FTIR (Bruker Vertex 70 FTIR spectrometer) shown in Figure 61. The spectra in this result was almost identical to the result of Liu *et al*. (2015), the native membrane (Figure 61a) showed

the strong peak at 2930, 2243 and 1451 cm^{-1} which were identified as stretching vibration band of methylene, stretching vibration band of nitriles and bending vibration band of methylene, respectively. For the activated membrane (Figure 61b), after the hydrolysis treatment of NaOH followed by HCl and ethylenediamine additions, the lower signal of peak at 2243 cm^{-1} was detected due to the hydrolysis of nitrile group compared to the native membrane. This activation of membrane generated the conspicuous peaks at the 1654 and 1551 cm^{-1}. There two peaks presented the existence of carboxylic group generated from the hydrolysis treatment by NaOH. After the immobilization, the spectra of membrane were shown in Figure 61c. The presence of peaks was apparently observed at 1644 cm^{-1} which was considered as the amide group. This confirmed the success of immobilization of *Aspergillus oryzae* ST11 lipase.

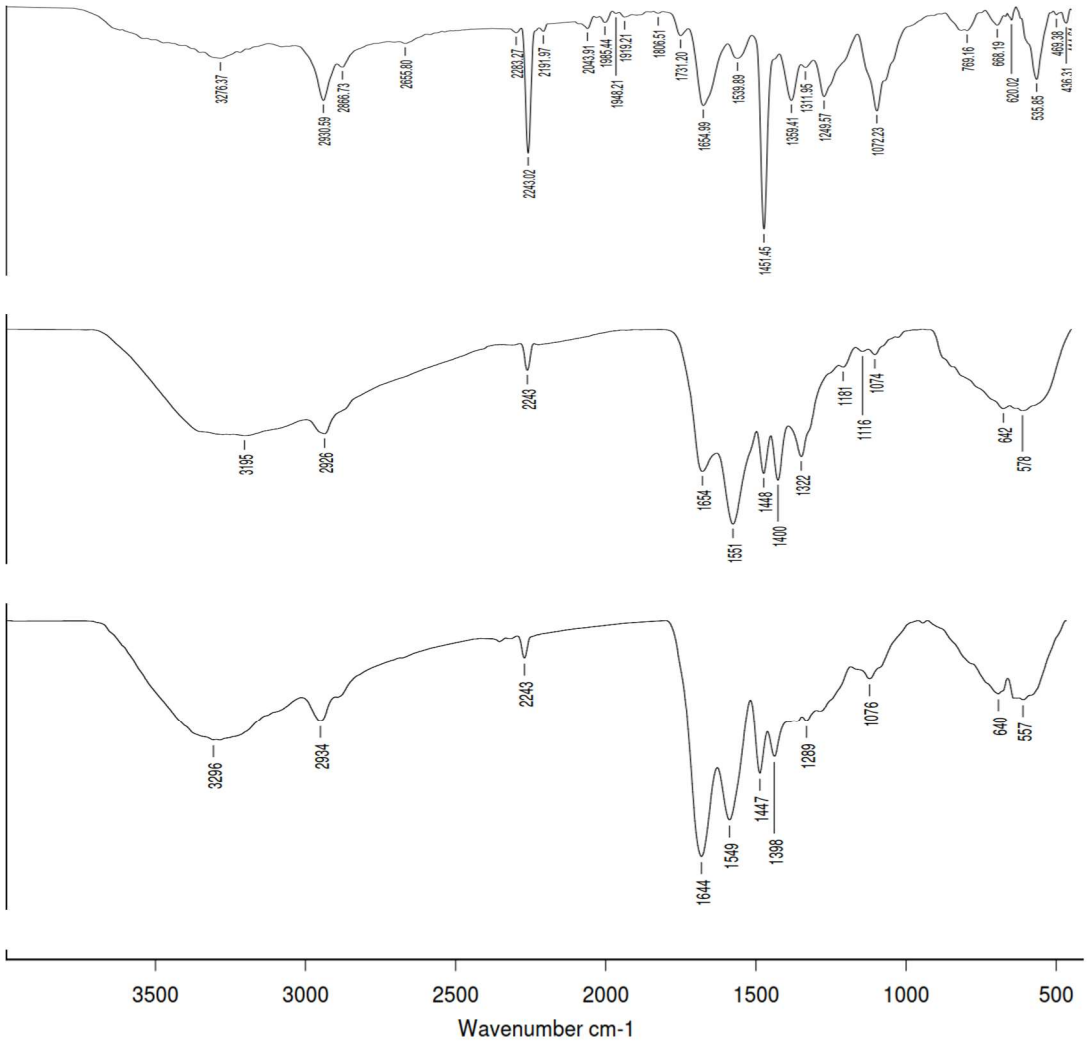


Figure 61. FT-IR spectra of native membrane (a), activated membrane (b) and immobilized membrane (c) in the frequency range between 4000-400 cm-1 using Bruker Vertex 70 FTIR spectrometer.

The morphology of PAN nanofibrous membrane was observed by scanning electron microscopic technique (FEI Quanta 400). The results are shown in Figure 62. The native PAN nanofibrous membrane showed the smooth surface and still retain the space among individual fiber (Figure 62a). After the activation process finished, the morphology of nanofibrous membrane was changed. The surface of the fiber became rougher and the swelling of fibers could be observed noticeably compared to the native membrane (Figure 62b). The last SEM image showed the pack of proteins

on the surface of fibers. This confirmed the success in immobilization of *Aspergillus oryzae* ST11 lipase (Figure 62c).

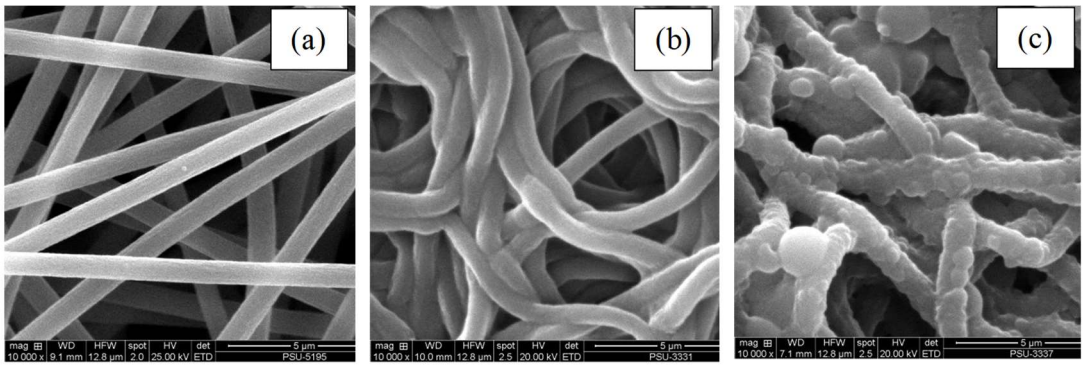


Figure 62. The SEM images (10,000x) of a native PAN membrane (a), the activated PAN membrane (b) and the PAN membrane with immobilized *Aspergillus oryzae* ST11 lipase (c).

2. Biodiesel production

The use of an electrospun nanofibrous membrane has drawn the attention in many applications due to its characters for giving the large surface-to-volume ratios (Sakai *et al*., 2010). The large area of materials encourages the researcher to immobilize many biomolecules on the surface such as enzymes, proteins or other bioactive compounds. In this study, the lipase from *Aspergillus oryzae* ST11 was aimed for being immobilized on the polyacrylonitrile nanofibrous membranes and used in biodiesel production.

After the immobilization step, the immobilized lipase was used to study for many parameters affecting the biodiesel production. From preliminary work. The main parameters that affect the bioconversion of biodiesel were the amount of biocatalyst and water content. The different initial amount of biocatalyst affected the biodiesel production. Shah and Gupta (2007) reported the effect of different amounts of biocatalyst on biodiesel production from Jatropha oil. The amounts of lipase PS were varied to convert Jatropha oil (0.5 g) with 4 moles of ethanol. The result showed that the increase in biocatalyst from 12.5 to 75 mg could increase the yield of biodiesel.

However, the increase of biocatalyst to100 mg gave the lower yield due to poor mass transfer.

Talukder *et al*. (2013) studied the whole-cell *A. nomius* for biodiesel production. The increase in lipase load from 5 to 10% enhanced the biodiesel yield to 95.3% after 40 h. The addition of excess amount of biocatalysts did not promote the increase of biodiesel yield due to the increase in the viscosity of mixture from matrix and lipase (Fatimah *et al.*, 2008). For the effect of water content on the biodiesel production. Water molecule was crucial for enzyme stability. The presence of water could promoted the open of lid and revealed the active site of lipases (Babaki *et al*., 2015). The absence of water after reuse of lipase showed some negative effect on the lipase activity. There was an observation of the activity loss due to rehydration of protein structure after reuse of an enzyme (Rodrigues *et al*., 2016). In this study (Figure 63), the amount of water was varied from 0-50% (w/w) based on the weight of palm oil and 3 moles of methanol were added to the mixture at the beginning of the reaction. As the importance of amount of biocatalyst and water, the result showed that the water was very important to the catalysis. The treatment of water between 0-10% showed the low biodiesel conversion even adding more biocatalysts did not enhance the biodiesel production. However, the biodiesel conversion was enhanced apparently when the water content was added to the mixture at 20 % w/w and the increase in the biodiesel conversion was observed after the increase of water content in the system. However, addition of water over 40% w/w gave a poor conversion of biodiesel. In the aspect of biocatalyst's addition (Figure 63), the increase in biocatalyst from 2.5-20 % w/w based on the weight of palm oil in the system containing water content lower than 20% w/w did not increase the biodiesel production. The effect of the amount of biocatalyst was noticed significantly after the higher amount of water was added to the system. The highest biodiesel conversion (91.8%) was obtained in the condition of 15% w/w of biocatalyst, 40% w/w of water content at 37^{o}C for 24 h.

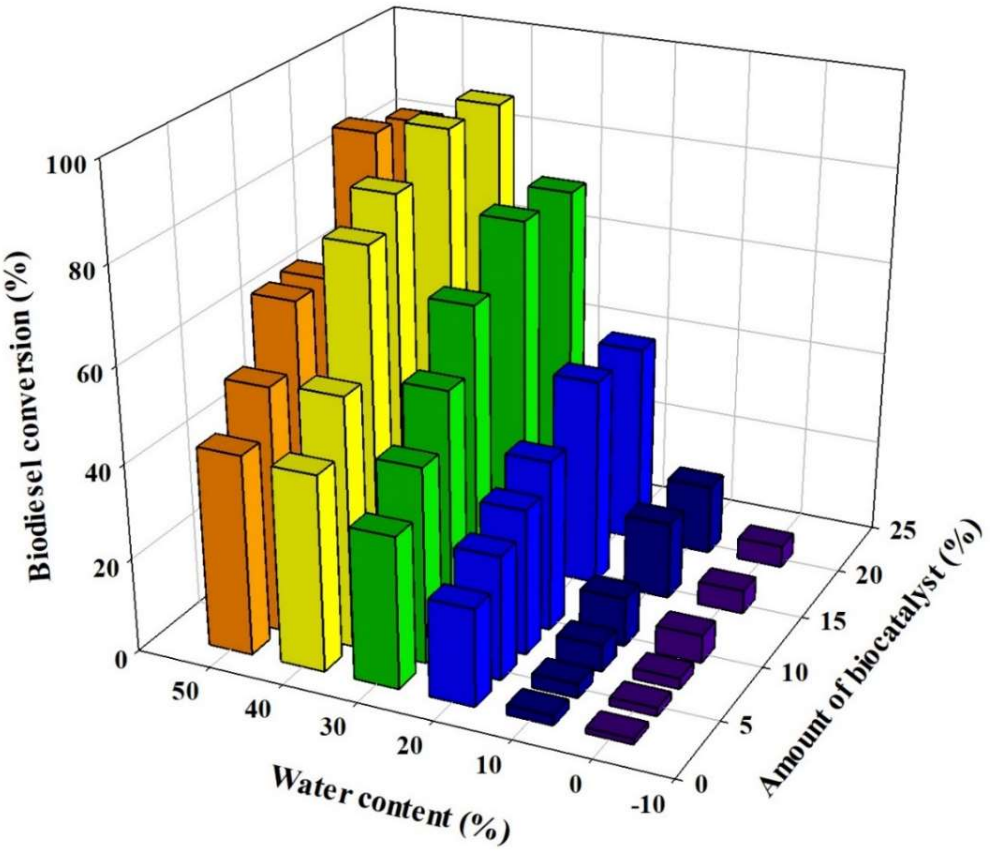


Figure 63. Effect of water content and amount of biocatalyst on biodiesel production. (The reaction mixture contained catalyzed in the mixture containing 3 moles of methanol, shaken at 250 rpm and at 37°C for 24 h.)

Methanol was a short chain alcohol that commonly used as an acyl acceptor. It was quite cheap to be used in the process compared to other alcohols. However, the methanol was insoluble in an oil and it may lead to the accumulation of alcohol due to poor mixing of substrate. The accumulation of alcohol affected negatively on the stability of lipases. Many methods were invented to prevent this obstacle such as stepwise addition of methanol or the dissolving of oil in an organic solvent to decrease the viscosity (Yücel *et al.*, 2013; Lotti *et al*., 2015; Su *et al*., 2015). In this study, according to the theoretical mole of methanol (3 moles), the biodiesel conversion was achieved higher than 90% using one-step addition of methanol due to a high enzyme loading on the carrier (Figure 64). The high unit number can overcome

the limitation from a high mole of methanol by increasing the rate of the reaction. After that, the addition of methanol higher than 3 moles was studied. It was found that the ratio of methanol and oil at 3.5:1 promoted the biodiesel production at 95.8%. However, the increase of methanol beyond this ratio did not enhance the biodiesel production.

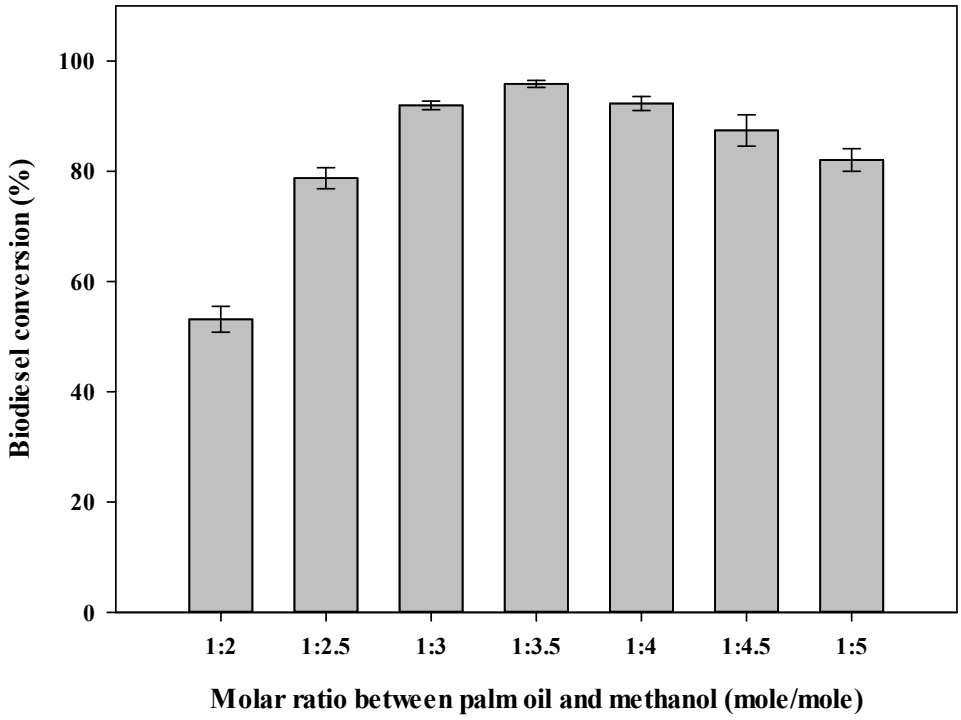


Figure 64. Effect of molar ratio between palm oil and methanol (mole/mole) on biodiesel production. (The reaction mixture contained 40 % w/w of water content, 15% w/w immobilized lipase (13U) and shaken at 250 rpm and 37^{o}C for 24 h).

After the study of transesterification reaction finished, the time course of biodiesel conversion was studied. The transesterification reaction was carried out in the comparison between the immobilized *Aspergillus oryzae* ST11 lipase and Novozym 435 (both immobilized lipases contained the equal unit at 13U). The 3.5 moles of methanol were added at the beginning of the reaction for both immobilized lipases. The reactions were carried out for 24 h. The samples were collected interval at every 3 h and analyzed by HPLC (Fedosov *et al.*, 2014) to determine the biodiesel conversion.

The results showed that the biodiesel conversion catalyzed by immobilized *A. oryzae* ST11 lipase was higher than that of Novozym 435 after 24 h of the reaction (Figure 65). The biodiesel conversion of immobilized *A. oryzae* ST11 lipase reached to the maximum (95%) at 18 h whereas Novozym 435 gave only 52% at 24 h. The biodiesel production of Novozym 435 showed the lower bioconversion. According to the study of biodiesel production catalyzed by Novozym 435, this commercial immobilized lipase required a longer time to complete the biodiesel production. Su *et al*. (2015) reported that the process needed almost 60 h to reach its equilibrium with the biodiesel yield at 90.3%. Another result was observed by the study of Talukder *et al*. (2009). Their study showed the biodiesel yield (~90%) catalyzed by Novozym 435 reached to the equilibrium at 24 h. However, a lower in biodiesel conversion catalyzed by Novozym 435 in this study might be caused by different optimal conditions. It was reported that Novozym 435 could catalyze the reaction more efficiently in a higher temperature (>40^{o}C). As well as tert-butanol which was preferably used in a solvent system (Talukder *et al*., 2009; Jeong and Park, 2008; Chen *et al*., 2011). The result of biodiesel production catalyzed by *A. oryzae* ST11 lipase indicated the potential use for biodiesel production with the shorter reaction time and the higher conversion compared to the commercial immobilized lipase (Novozym 435). The productivity of both immobilized *A. oryzae* lipase and Novozym 435 were studied in Figure 66. The production rate catalyzed by Novozym 435 was lower than that of immobilized *A. oryzae* lipase. However, it seemed that Novozym 435 took a longer time to increase the biodiesel production at 2.8% biodiesel per hour (15-18 h) whereas the immobilized *A. oryzae* lipase reached the highest production rate at 11.9 % biodiesel per hour at 6-9 h of reaction.

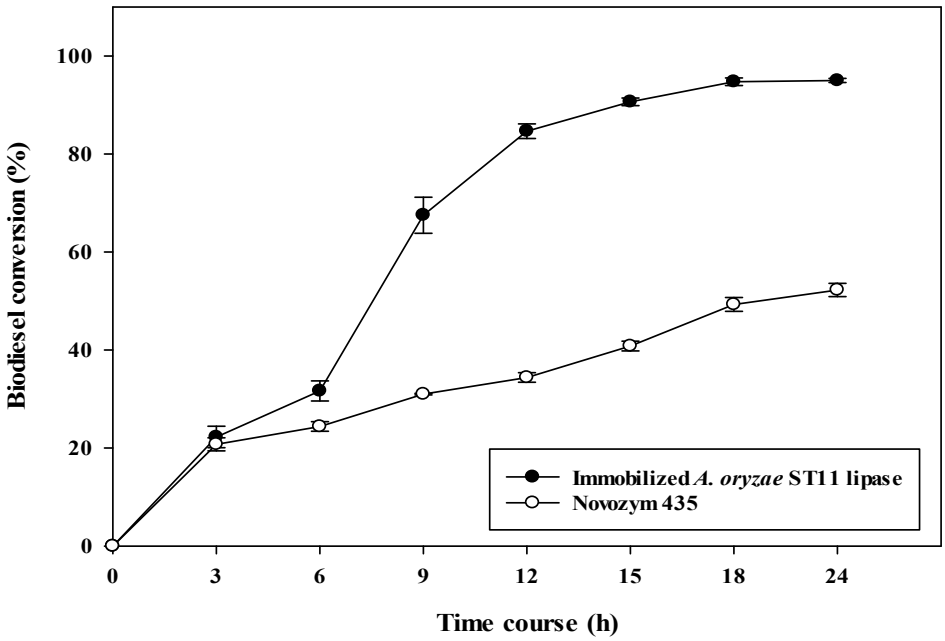


Figure 65. Biodiesel conversion (%) catalyzed by immobilized *A. oryzae* ST11 lipase and Novozym 435. (Reaction conditions: 13U of immobilized lipase, 40% w/w of water content, 3.5 moles of methanol per mole of palm oil, 250 rpm, 37ºC for 24 h).

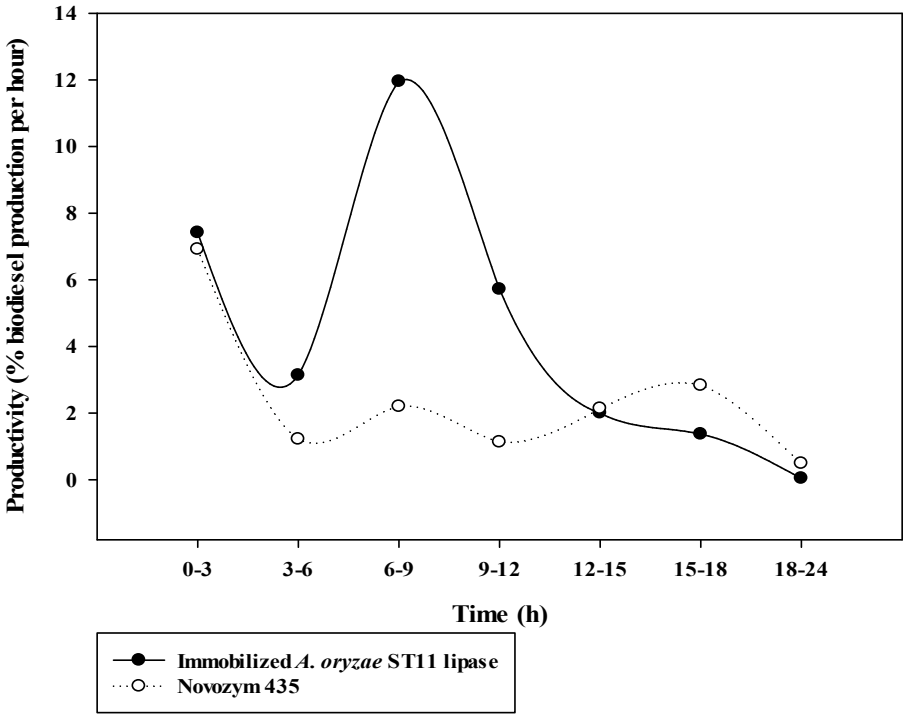


Figure 66. The productivity of biodiesel production catalyzed by immobilized *A. oryzae* ST11 lipase and Novozym 435. (Reaction conditions: 13U of immobilized lipase, 40% w/w of water content, 3.5 moles of methanol per mole of palm oil, 250 rpm, 37°C for 24 h).

3. Reusability

Reusability was considered as the important factor for an enzymatic use to reduce the process cost. The repeated use of immobilized lipase with a high degree of retained activity was the purpose of enzyme immobilization making the enzymatic process more feasible in the real operation (You *et al*., 2013). In this study, the immobilized lipase from *A. oryzae* ST11 was reused for 10 cycles (Figure 67). Before applying the immobilized lipase into the new batch of biodiesel production, the membrane was washed by fresh isooctane to remove the retained substrate and product from the previous batch. From the result, the relative yield of biodiesel production was conserved at 83% after 10 cycles. This confirmed the potential of immobilized lipase for applying in the process of biodiesel production.

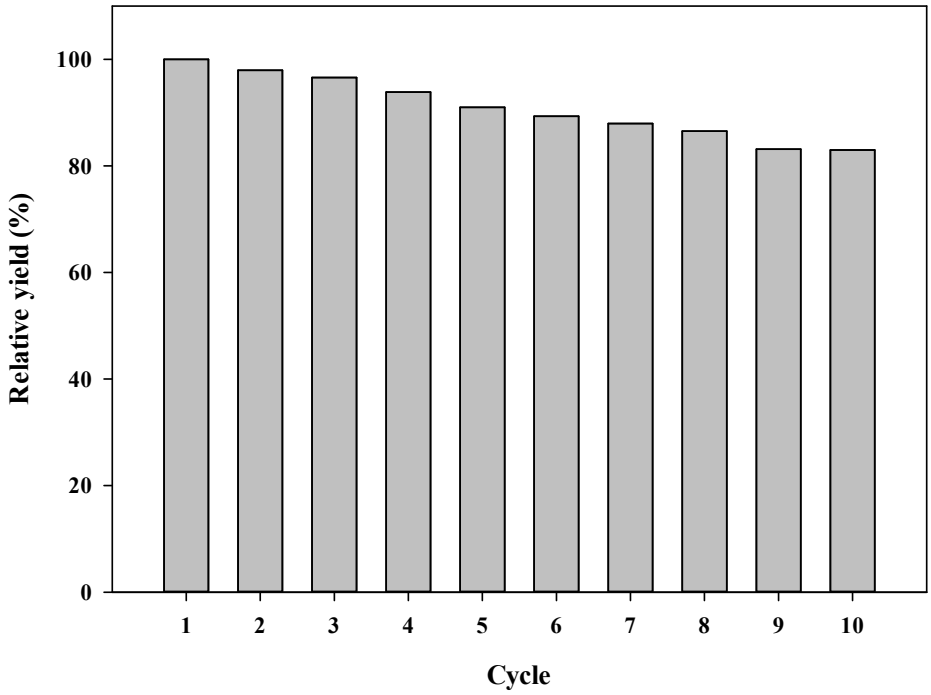


Figure 67. Reusability of immobilized *A. oryzae* ST11 lipase on polyacrylonitrile nanofibrous membrane. (Reaction condition: 15% w/w of immobilized lipase, 40% w/w of water content, 3.5 moles of methanol per mole of palm oil, 250 rpm, 37ºC for 24 h).

Conclusion

The lipase from *A. oryzae* ST11 was immobilized on the surface of modified polyacrylonitrile nanofibrous membrane facilitated by addition of bovine serum albumin. The highest recovered activity of immobilized lipase was 86.9%. Furthermore, the immobilized lipase showed the potential for biodiesel production. It catalyzed the transesterification reaction with one-step addition of methanol and gave the high bioconversion. In comparison with commercial immobilized lipase (Novozym 435), the immobilized lipase in this study displayed a better performance in the production process by using a shorter reaction time and gave a higher bioconversion of biodiesel. Moreover, this immobilized lipase could be reused for 10 cycles and retained the catalysis efficiency (82.9%) at cycle 10. These results indicated the potential of this immobilized lipase in the industrial application.

CHAPTER 7

CONCLUSION AND SUGGESTION

Conclusion

Seven fungal isolates were screened for the lipase production. Only the ST11 was capable to produce extracellular lipase and could catalyze transesterification reaction. The production of lipase by the selected ST11 isolate was optimized by both physical condition and medium composition (1% palm oil, 2% peptone, 0.2% $NaNO_3$, 1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and pH 6). The addition of olive oil and lactose into the basal medium showed the significant increase in the lipase activity (31 U/ml).

Aspergillus sp. ST11 was identified by morphological and molecular methods (ITS1/ITS4). It was identified as *Aspergillus oryzae* ST11. Its lipase was produced and purified. The activity was increased 13-folds and 7.9% of yield was achieved by chilled acetone precipitation, HiTrap Q-HP and Toyopearl Butyl-650M column chromatographies, respectively. The molecular weight of *Aspergillus oryzae* ST11 lipase was 25 kDa. The purified lipase was stable at the pH between 5.0-8.0 with the relative activity higher than 80% and had the optimum activity at pH 7.5. For optimum temperature and thermal stability, the optimum temperature of the purified lipase was 37ºC. Its activity was greatly decreased in the presence of Hg^{2+}, Zn^{2+} and Cu^{2+}. For the effect of surfactants and inhibitors on lipase activity, most surfactants including Tween-20, Tween-80, Triton X-100, SDS and gum arabic showed the negative effect on the lipase activity while inhibitors such as EDTA, PMSF and β-mercaptoethanol did not reduce the activity significantly. Organic solvents were investigated for the lipase stability. The result indicated that the polar solvent such as methanol and ethanol had negative effect on the lipase compared to non-polar solvents.

In this experiment, the lipase was immobilized on different materials and used for biodiesel production. The first immobilization technique was the magnetic cross-linked enzyme aggregates (mCLEAs). The production of mCLEAs from *Aspergillus oryzae* ST11 lipase was achieved by using glutaraldehyde and the recovered activity of mCLEAs was enhanced by adding bovine serum albumin. The prepared mCLEAs exhibited the potential for biodiesel production which gave the high biodiesel

conversion at the optimal conditions produced (95% after 24 h of reaction). From the point of reusability, the mCLEAs could be reused for 5 times with 65% relative activity for biodiesel production.

The second material used to immobilize the enzyme was the electrospun polystyrene-co- trimethylolpropane tris [poly (propylene glycol) amine terminated] ether (PS-so-TMP). The immobilization of *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane was achieved by using the activation of glutaraldehyde to modify the surface of the material. The highest recovered activity was achieved at 75%. The immobilized lipase was stable in the wide range of pH and temperature compared to free lipase. Many factors were investigated for biodiesel synthesis. The increase in immobilized lipase to 15% (w/w) increased the biodiesel conversion. Adding more biocatalyst did not enhance the biodiesel production significantly. Water was also important for the reaction that indicated the lower content had negatively effect on biodiesel conversion which could be solved when water was increased to 40% w/w of palm oil. Isooctane was suitable for dissolving palm oil which gave the highest biodiesel production. Stepwise addition of methanol was the way to prevent enzyme denaturation and showed the improvement of biodiesel conversion. The immobilized lipase on electrospun PS-co-TMP nanofibrous membrane could be reused for 10 cycles and still retained 81%.

The last material for lipase immobilization was polyacrylonitrile (PAN) nanofibrous membrane. The lipase from *A. oryzae* ST11 was immobilized on the surface of modified polyacrylonitrile nanofibrous membrane facilitated by addition of bovine serum albumin. The highest recovered activity of immobilized lipase was 87%. Furthermore, the immobilized lipase showed the potential for biodiesel production. With the higher in immobilized lipase, it catalyzed the transesterification reaction with one-step addition of methanol and gave the high bioconversion. In comparison with commercial immobilized lipase (Novozym 435), the immobilized lipase in this study displayed a better performance in the production process by using a shorter reaction time and gave a higher bioconversion of biodiesel. Moreover, this immobilized lipase could be reused for 10 cycles and retained the catalysis efficiency (83%) at the 10^{th} cycle. These results indicated that this immobilized lipase has a potential application for biodiesel production in the industrial level.

Efficiency of the three materials used for lipase immobilization from *Aspergillus oryzae* ST11 was summarized in table 13. From the results of productivity, it showed that all three materials gave a desirable characteristic for bioconversion. Moreover, they showed the highest production rate for biodiesel at 9-12 h of reaction. Polyacrylonitrile coated magnetic nanoparticle and polyacrylonitrile nanofibrous membrane showed the highest productivity at 8.1 and 11.9 % biodiesel per hour between 6-9 h of reaction time while polystyrene reached to the highest point at 7.8 % biodiesel per hour between 9-12 h of reaction which was slower than those of other two materials.

Table 13. Comparison of three materials used for lipase immobilization from *Aspergillus oryzae* ST11.

Material	Immobilization method	Reaction condition	Biodiesel (%)	Highest productivity (%biodiesel/h)
1. Polyacrylonitrile coated magnetic nanoparticle	Cross-linking enzyme aggregate with BSA addition	stepwise addition of methanol	95	8.1
2. Polystyrene-co-TMP nanofibrous membrane	Covalent bonding with glutaraldehyde	stepwise addition of methanol	96	7.8
3. Polyacrylonitrile nanofibrous membrane	Cross-linking enzyme aggregate with BSA addition	one step addition of methanol	95	11.9

Suggestions

For the study of lipase production from *Aspergillus oryzae* ST11 and the immobilization on the different materials for biodiesel production, the suggestions for the further work are

1. To use a lipase from a higher step of purification for immobilization aiming to get a higher enzyme loading on the material for a faster catalysis reaction.
2. To adapt the immobilized lipase in the process to produce a high value product.

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APPENDICES

APPENDIX A

1. Hydrolysis activity of lipase by cupric acetate method (Lee and Rhee, 1993)

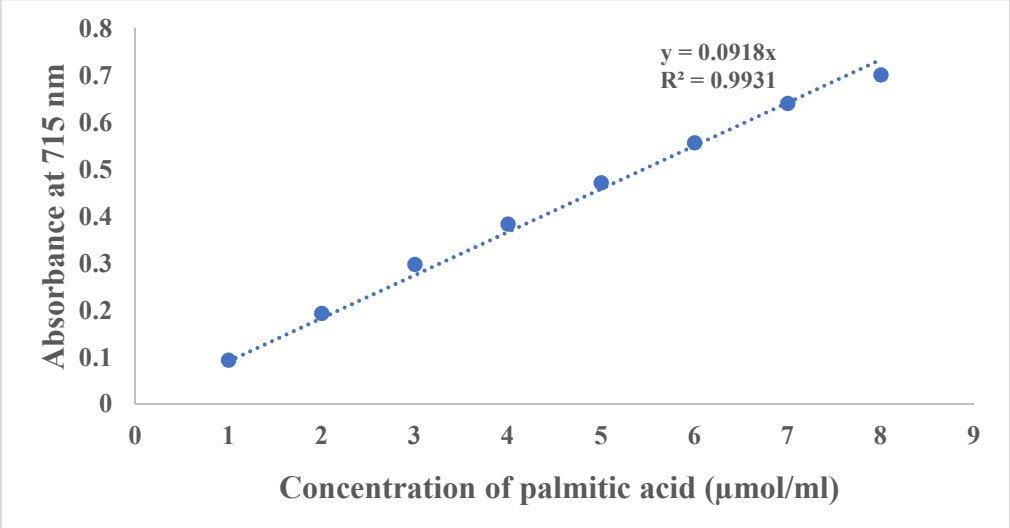


Figure 68. Calibration curve of palmitic acid.

2. Protein determination by Lowry's method (Lowry *et al*., 1951)

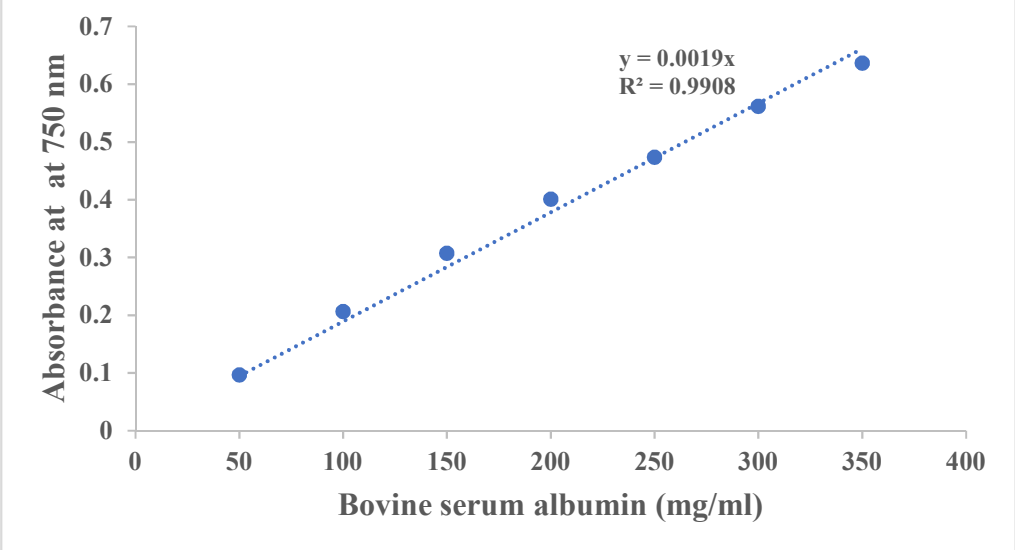


Figure 69. Calibration curve of bovine serum albumin.

3. Determination of molecular mass of protein by SDS-PAGE

The molecular mass of the protein sample was measured by comparing the R_f of the sample with the curve plotted between the R_f of standard protein against the log value of different standard masses under SDS-PAGE as below.

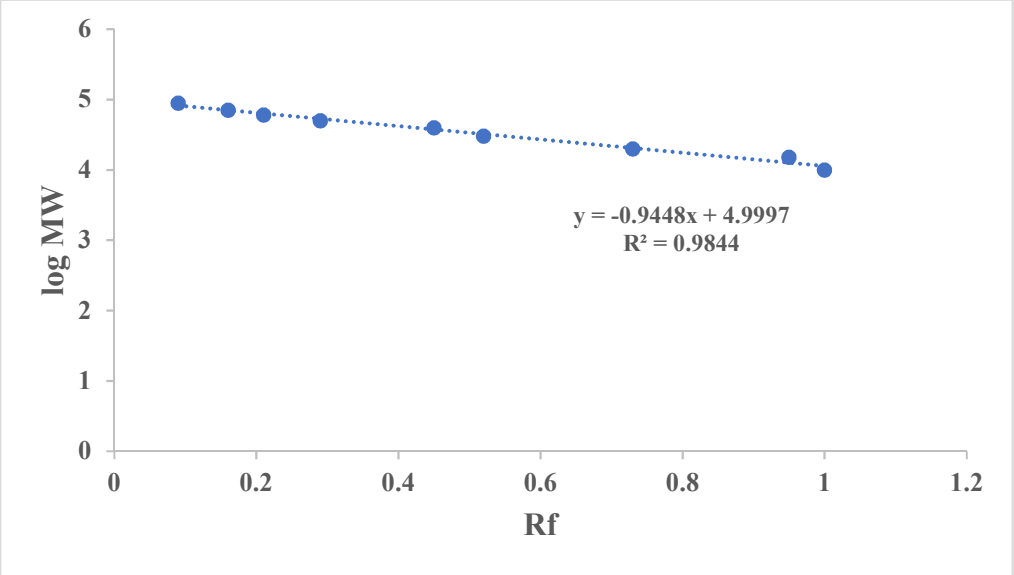
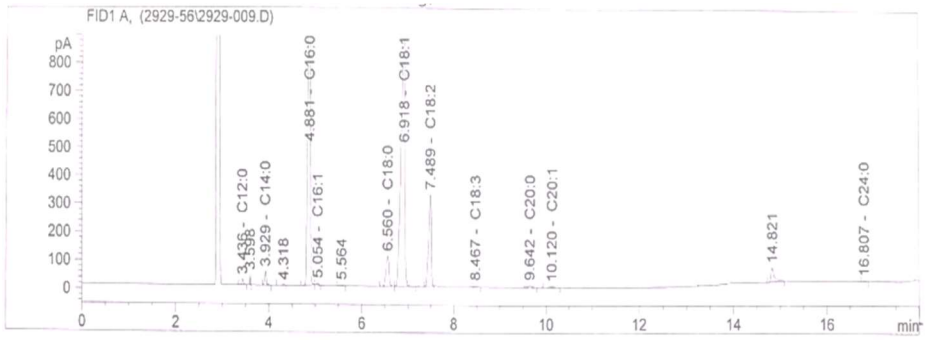


Figure 70. Calibration curve of log molecular weight of standard protein and R_f under SDS-PAGE.

4. Determination of fatty acid compositions by GC analysis

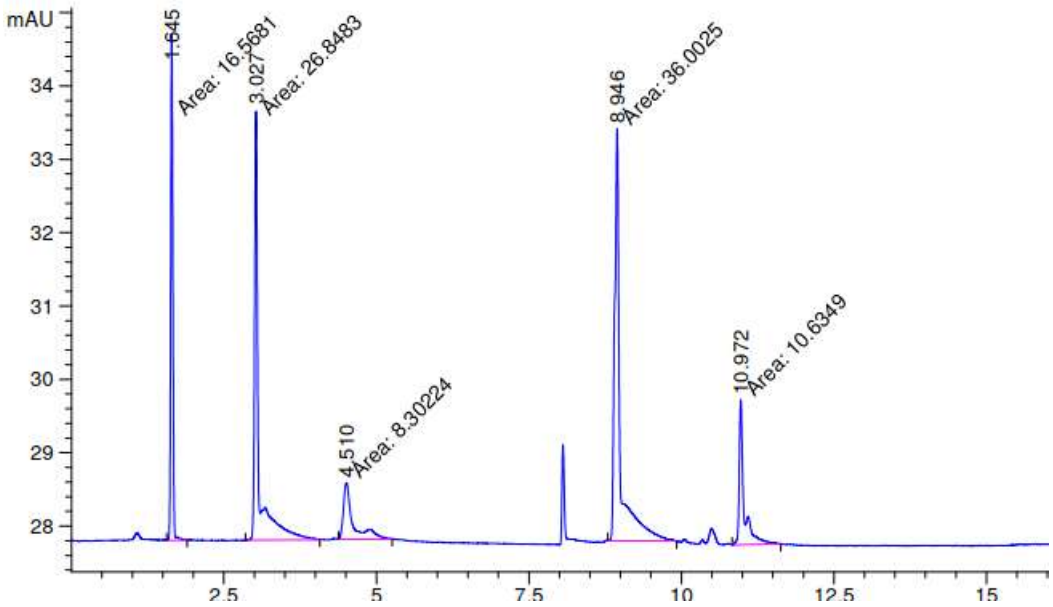


Peak #	RetTime [min]	Type		Width [min]	Area [pA*s]	Area %	Name
1	3.048			0.0000	0.00000	0.00000	C8:0
2	3.190			0.0000	0.00000	0.00000	C10:0
3	3.436	BB		0.0334	43.93206	0.35026	C12:0
4	3.929	PB		0.0362	111.77237	0.89114	C14:0
5	4.881	PB	+	0.0462	4884.16162	38.94031	C16:0
6	5.054	BB		0.0417	19.83597	0.15815	C16:1
7	6.560	BB		0.0694	480.30725	3.82938	C18:0
8	6.918	BB		0.0740	5316.49219	42.38718	C18:1
9	7.489	PB		0.0617	1313.81909	10.47478	C18:2
10	8.467	BB		0.0689	23.65814	0.18862	C18:3
11	9.642	BBA		0.0799	38.83247	0.30960	C20:0
12	10.120	BBA		0.0875	18.82600	0.15010	C20:1
13	13.947			0.0000	0.00000	0.00000	C22:0
14	14.245			0.0000	0.00000	0.00000	C22:1
15	16.807	BB		0.0539	8.36539	0.06670	C24:0
16	17.211			0.0000	0.00000	0.00000	C24:1
Totals :					1.22600e4	97.7462	

Equipment	Gas Chromatography, 6890, Agilent Technologies, USA
Inlet temperature	290°C, Split ratio 50:1, Carrier (He) flow 1.0 ml/min
Oven temperature	Initial temperature 210°C hold 12 min
	Ramp to 250°C at 20°C/min hold 8 min
Column	Select for FAME, 30 m x 0.32 mm ID. X film thickness 0.25 µm
Detector temperature	300°C
Hydrogen flow	30.0 ml/min
Make up flow	25.0 ml/min
Air flow	300.0 ml/min

Figure 71. Retention time of fatty acid methyl esters analyzed by GC.

5. HPLC chromatogram of transesterification products



peak no.	compounds	retention time (min)	peak area	area %
1	Fatty acid methyl esters	1.645	16.5681	16.84502548
2	Free fatty acids	3.027	26.8483	27.29705263
3	Triglycerides	4.510	8.30224	8.441006775
4	Diglycerides	8.946	36.0025	36.60425938
5	Monoglycerides	10.972	10.6349	10.81265574
			98.35604	100

Figure 72. HPLC chromatogram of standard oil compositions and fatty acid methyl esters.

APPENDIX B

1. Nucleotide sequence of 5.8S ribosomal RNA gene of *Aspergillus oryzae* ST11

Aspergillus flavus isolate FF-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

Sequence ID: MH064167.1Length: 592Number of Matches: 1

Range 1: 8 to 583 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
1040 bits(1152)	0.0	576/576(100%)	0/576(0%)	Plus/Plus

```
Query  1    AGGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8    AGGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCC  67

Query  61   ACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCGTGGCCGCCGGGGGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  68   ACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCGTGGCCGCCGGGGGC  127

Query  121  TCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128  TCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGT  187

Query  181  CTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188  CTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCA  247

Query  241  TCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  TCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCAT  307

Query  301  CGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  CGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGT  367

Query  361  CATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCggggggAC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  CATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGAC  427

Query  421  GGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428  GGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACC  487

Query  481  CGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488  CGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCT  547

Query  541  CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA  576
            ||||||||||||||||||||||||||||||||||||
Sbjct  548  CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA  583
```

Aspergillus oryzae isolate NJP18 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

Sequence ID: HQ710546.1Length: 589Number of Matches: 1

Figure 73. Alignment of nucleotide sequence of ITS region from 5.8S ribosomal RNA gene by ITS1 and ITS4 primers.

VITAE

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Student ID 5411030007

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Microbiology) (Second Class Honors)	Chiangmai University	2011

Scholarship Awards during Enrolment

- The Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0321/2552)
- The Graduate School, Prince of Songkla University

List of Publications and Presentations

Publications

Paitaid, P. and H-Kittikun, A. Synthesis of magnetic cross-linked enzyme aggregates from *Aspergillus oryzae* ST11 lipase using polyacrylonitrile coated magnetic nanoparticles for biodiesel production. (Preparing manuscript).

Paitaid, P. and H-Kittikun, A. Covalent immobilization of *Aspergillus oryzae* ST11 lipase on the electrospun PS-co-TMP nanofibrous membrane for biodiesel production from palm oil. (Preparing manuscript)

Paitaid, P. and H-Kittikun, A. Enhancing immobilization of *Aspergillus oryzae* ST11 lipase on polyacrylonitrile nanofibrous membrane by bovine serum albumin and its application for biodiesel production. (Preparing manuscript)

Presentations

Paitaid, P. and H-Kittikun, A. Optimization of medium composition and physical condition for lipase production by *Aspergillus* sp. ST11 for biodiesel application. RGJ-Ph.D. Congress 17 “Driving for stronger research excellence and innovation”. Jomtien Palm Beach Hotel & Resort, Pattaya, Chonburi, Thailand. June 8-11, 2016 S7-P7.