OPTIMIZATION OF BIODIESEL PRODUCTION FROM THE USED COOKING OIL BY LIPASE FROM FISH VISCERA AND ULTRASONICATION

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บทคัดย่อ

ชื่อวิทยานิพนธ์ : สภาวะที่เหมาะสมในการผลิตไบโอดีเซลจากน้ำมันพืชใช้แล้วโดยใช้เอนไซม์ไลเปสจากเครื่องในสัตว์น้ำและวิธีอัลตราโซนิกส์

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จากการศึกษากิจกรรมของเอนไซม์ไลเปสจากเครื่องในปลาน้ำจืด 3 ชนิด คือ ปลาดุกบิ๊กอุย (*Clarias macrocephalus* × *Clarias gariepinus*) ปลาช่อน (*Channa striata*) และปลานิล (*Oreochromis niloticus*) พบว่าเอนไซม์ไลเปสจากปลาทั้ง 3 ชนิด มีกิจกรรมสูงสุดที่พีเอช 7.0-8.5 และอุณหภูมิ 40-45 องศาเซลเซียส โดยเครื่องในปลานิลมีกิจกรรมของเอนไซม์ไลเปสสูงสุดรองลงมา คือ ปลาช่อนและปลาดุกบิ๊กอุย เอนไซม์มีความคงตัวต่อความร้อนอุณหภูมิไม่เกิน 40 องศาเซลเซียส และมีความคงตัวต่อพีเอชในช่วงพีเอช 7.0-10.0 เป็นเวลา 30 นาที กิจกรรมของเอนไซม์จากเครื่องในปลาทั้ง 3 ชนิด สามารถกระตุ้นด้วย NaN_3 แต่ถูกยับยั้งด้วย $MgCl_2$ $MnCl_2$ $HgCl_2$ $AlCl_3$ $CuCl_2$ EDTA EDAC DEPC PMSF CTAB sodium percarbonate และ sodium polyacrylate เอนไซม์ไลเปสจากเครื่องในปลานิลมีความคงตัวต่อสารลดแรงตึงผิวสูงกว่าเอนไซม์ไลเปสจากปลาช่อนและปลาดุกบิ๊กอุย นอกจากนี้การศึกษาผลของสารสกัดต่อการเก็บเกี่ยวเอนไซม์ไลเปสจากเครื่องในปลานิลและความคงตัวของเอนไซม์กับสารซักล้างทางการค้า พบว่าการเก็บเกี่ยวเอนไซม์ไลเปสด้วยสารละลาย Tris-HCl ที่ระดับความเข้มข้น 50 มิลลิโมลาร์ พีเอช 7.0 ที่มีโซเดียมคลอไรด์ความเข้มข้น 1 โมลาร์ และ Brij 35 ความเข้มข้นร้อยละ 0.2 ให้ประสิทธิภาพในการเก็บเกี่ยวเอนไซม์ไลเปสสูงกว่าสารสกัดชนิดอื่น ๆ ($P<0.05$) และเอนไซม์ไลเปสสามารถทำงานและมีเสถียรภาพในสารซักล้างทางการค้าเป็นเวลา 15-30 นาที ดังนั้นจากคุณลักษณะของเอนไซม์ไลเปส สารสกัดจากเครื่องในปลานิลเป็นตัวเลือกที่มีศักยภาพสำหรับการประยุกต์ใช้ในอุตสาหกรรมสารซักล้าง

จากการศึกษาการแยกส่วนและการเก็บเกี่ยวเอนไซม์ไลเปสจากเครื่องในปลานิลโดยใช้ระบบสารละลายน้ำสองวัฏภาคร่วมกับความร้อน (Thermoseparating Aqueous Two-Phase System, T-ATPS) โดยศึกษาปัจจัยที่มีผลต่อการแยกส่วน ได้แก่ ชนิดและความเข้มข้นของเกลือ ความ

เข้มข้นของพอลิเมอร์ร่วมระหว่างเอทิลีนออกไซด์และโพรพิลีนออกไซด์ (Ethylene Oxide-Propylene Oxide Copolymers, EOPO) การเติมโซเดียมคลอไรด์ สัดส่วนระหว่าง EOPO ต่อน้ำกลั่น และอุณหภูมิ พบว่าระบบการแยกส่วนขั้นแรก (Primary ATPS) ซึ่งประกอบด้วย สารสกัดเอนไซม์ร้อยละ 20 (น้ำหนักต่อน้ำหนัก) EOPO (น้ำหนักโมเลกุล 3900) ที่ความเข้มข้นร้อยละ 40 (น้ำหนักต่อน้ำหนัก) เกลือแอมโมเนียมซัลเฟตร้อยละ 10 (น้ำหนักต่อน้ำหนัก) และโซเดียมคลอไรด์ร้อยละ 4 (น้ำหนักต่อน้ำหนัก) เป็นสภาวะที่ดีที่สุดในการแยกเอนไซม์ไลเปสไปยังเฟสของ EOPO ซึ่งเป็นเฟสบน ในระบบการแยกส่วนขั้นที่สอง (Secondary ATPS) อัตราส่วนที่เหมาะสมระหว่าง EOPO จากเฟสบนของระบบการแยกส่วนขั้นแรกและน้ำกลั่น เท่ากับ 1 ต่อ 1 (น้ำหนักต่อน้ำหนัก) และอุณหภูมิ 60 องศาเซลเซียส เป็นอุณหภูมิที่เหมาะสมสำหรับการเหนี่ยวนำให้เกิดการแยกเฟสภายหลังการแยกส่วนด้วยความร้อน เอนไซม์ไลเปสแยกไปยังเฟสบนซึ่งเป็นชั้นของน้ำและ EOPO แยกไปยังเฟสล่าง ภายใต้สภาวะที่เหมาะสมของการแยกส่วน ได้ผลผลิตร้อยละ 64.45 และความบริสุทธิ์เพิ่มขึ้น 6.30 เท่า นอกจากนี้จากการศึกษาขั้นตอนการรีไซเคิล เอนไซม์ไลเปสที่เหลืออยู่ในเฟสของเกลือซึ่งเป็นเฟสล่างจากระบบการแยกส่วนขั้นแรกสามารถเก็บเกี่ยวเพิ่มได้ไม่เกิน 3 ครั้ง ดังนั้นจากระบบ T-ATPS สามารถเก็บเกี่ยวผลผลิตทั้งหมดได้ร้อยละ 93.59 จากการศึกษาผลของอัลตราโซนิกส์ต่อกิจกรรมของเอนไซม์ไลเปสที่ผ่านการแยกส่วน พบว่าเอนไซม์ไลเปสที่ผ่านการแยกส่วนมีกิจกรรมสูงสุดเมื่อตัวอย่างผ่านอัลตราโซนิกส์ที่กำลัง 180 วัตต์ ความถี่ 24 กิโลเฮิร์ต เป็นระยะเวลา 20 นาที ที่อุณหภูมิ 40 องศาเซลเซียส ซึ่งภายใต้สภาวะดังกล่าวกิจกรรมของเอนไซม์ไลเปสเพิ่มขึ้นสูงกว่าชุดควบคุมร้อยละ 110.73 ดังนั้นการใช้ระบบ T-ATPS เป็นเทคนิคที่มีประสิทธิภาพสำหรับการเก็บเกี่ยวและทำบริสุทธิ์บางส่วนเอนไซม์ไลเปสจากเครื่องในปลานิล นอกจากนี้อัลตราโซนิกส์เป็นวิธีที่สามารถเพิ่มกิจกรรมของเอนไซม์

เพื่อขยายการใช้ประโยชน์เอนไซม์ไลเปสที่ผ่านการทำบริสุทธิ์บางส่วนจากเครื่องในปลานิลจึงใช้เป็นตัวเร่งปฏิกิริยาในการผลิตไบโอดีเซลจากน้ำมันพืชใช้แล้ว ซึ่งเป็นตัวเร่งปฏิกิริยาและสารตั้งต้นที่มีต้นทุนต่ำ พบว่าปัจจัยที่มีผลต่อการผลิต ได้แก่ สัดส่วนเชิงโมลของเมทานอลต่อน้ำมัน ความเข้มข้นของเอนไซม์ เวลาในการทำปฏิกิริยา ชนิดของแอลกอฮอล์ ปริมาณน้ำ และอุณหภูมิในการเกิดปฏิกิริยา มีผลต่อผลผลิตของไบโอดีเซล โดยสภาวะที่เหมาะสมในการผลิตไบโอดีเซล คือ การใช้เอนไซม์ไลเปสที่ระดับ 30 กิโลยูนิต สัดส่วนเชิงโมลของเมทานอลต่อน้ำมันอยู่ที่ 4 ต่อ 1 ปริมาณน้ำร้อยละ 3 ทำปฏิกิริยาที่อุณหภูมิ 45 องศาเซลเซียส เป็นระยะเวลา 28 ชั่วโมง ภายใต้สภาวะที่เหมาะสมสามารถผลิตไบโอดีเซลได้สูงสุดร้อยละ 96.50 เทคนิควิเคราะห์สารด้วยอินฟาเรด (ATR-FTIR) ถูกนำมาใช้ทดสอบเพื่อยืนยันการเปลี่ยนแปลงน้ำมันพืชใช้แล้วไปเป็นไบโอดีเซล คุณสมบัติของไบโอดีเซลที่ผลิตได้อยู่ในเกณฑ์มาตรฐานของไบโอดีเซลตามมาตรฐาน

EN 14214 และ ASTM D 6751 ดังนั้นจากผลการศึกษาสามารถลดต้นทุนการผลิตไบโอดีเซล โดยการใช้วัสดุเศษเหลือเป็นสารตั้งต้นและตัวเร่งปฏิกิริยา

เพื่อเพิ่มอัตราการเกิดปฏิกิริยาของเอนไซม์โดยการใช้อัลตราโซนิกส์จึงทำการศึกษาผลของอัลตราโซนิกส์ต่อการผลิตไบโอดีเซลจากน้ำมันพืชใช้แล้วร่วมกับเมทานอลโดยใช้เอนไซม์ไลเปสจากเครื่องในปลานิลเป็นตัวเร่งปฏิกิริยา จากการศึกษาปัจจัยที่มีผลต่อผลผลิตไบโอดีเซล ได้แก่ ความถี่ ระยะเวลาการทำปฏิกิริยา และอุณหภูมิในการเกิดปฏิกิริยา พบว่าการใช้อัลตราโซนิกส์สามารถลดระยะเวลาการเกิดปฏิกิริยาจาก 28 ชั่วโมง เหลือ 3 ชั่วโมง ภายใต้สภาวะการใช้อัลตราโซนิกส์ที่ความถี่ 16 กิโลเฮิร์ต สัดส่วนเชิงโมลของเมทานอลต่อน้ำมันอยู่ที่ 4 ต่อ 1 เอนไซม์ไลเปสที่ระดับ 30 กิโลยูนิต ทำปฏิกิริยาที่อุณหภูมิ 40 องศาเซลเซียส นอกจากนี้จากการศึกษาเปรียบเทียบการผลิตไบโอดีเซลภายใต้สภาวะที่เหมาะสมระหว่างการใช้วิธีอัลตราโซนิกส์และวิธีการกวน พบว่าการใช้อัลตราโซนิกส์ร่วมกับวิธีการกวนสามารถเพิ่มประสิทธิภาพการเกิดปฏิกิริยาทรานส์เอสเทอริฟิเคชันของน้ำมันพืชใช้แล้วร่วมกับเมทานอลโดยใช้เอนไซม์ไลเปสเป็นตัวเร่งปฏิกิริยา ซึ่งให้ผลผลิตสูงสุดร้อยละ 97.59 คุณสมบัติของไบโอดีเซลที่ผลิตได้อยู่ในเกณฑ์มาตรฐานของไบโอดีเซลตามมาตรฐาน EN 14214 และ ASTM D 6751 ดังนั้นการใช้อัลตราโซนิกส์ร่วมกับเอนไซม์ไลเปสในการเร่งปฏิกิริยาทรานส์เอสเทอริฟิเคชันของน้ำมันพืชใช้แล้วกับเมทานอลเป็นวิธีที่มีประสิทธิภาพ และสามารถใช้เป็นทางเลือกสำหรับการผลิตไบโอดีเซล

Abstract

Thesis Title : Optimization of Biodiesel Production from the Used Cooking Oil by Lipase from Fish Viscera and Ultrasonication

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Degree and Program : Master of Science in Biotechnology

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The study of lipolytic activities of viscera extract from three freshwater fish species, hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), striped snake-head fish (*Channa striata*), and Nile tilapia (*Oreochromis niloticus*), showed that optimal activity from all species was at pH 7.0-8.5 and 40-45°C. Among all species tested, Nile tilapia showed the highest activity, followed by striped snake-head fish and hybrid catfish. The enzymes were stable to heat treatment up to 40°C and a pH range of 7.0-10.0 for 30 minutes. The activities of enzymes of three fish species could be enhanced in the presence of NaN_3 but were inhibited by $MgCl_2$, $MnCl_2$, $HgCl_2$, $AlCl_3$, $CuCl_2$, EDTA, EDAC, DEPC, PMSF, CTAB, sodium percarbonate, and sodium polyacrylate. The Nile tilapia lipase was more stable against surfactants than both striped snake-head fish and hybrid catfish lipases. In addition, the study of extractants on lipase recovery effects from Nile tilapia viscera and its stability in the presence of different commercial dishwashing detergents presented that Nile tilapia viscera powder isolation with 50 mM Tris-HCl, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35 gave a higher lipase recovery than other extractants tested ($P<0.05$). The Nile tilapia lipase also exhibited substantial stability and compatibility with tested commercial dishwashing detergents for 15-30 minutes. Considering its characteristics, Nile tilapia crude enzyme containing lipase activity was considered a potential candidate for future application in detergent processing industries.

Lipase from Nile tilapia viscera were partitioned and recovered using a thermoseparating aqueous two-phase system (T-ATPS). Different phase partitioning parameters, including type and concentration of salts, concentration of EOPO, NaCl

addition, EOPO phase/distilled water ratio and temperature were optimized. In the primary ATPS, lipase was satisfactorily partitioned to the EOPO-rich top phase in the optimum system composed of 20% (w/w) crude enzyme, 40% (w/w) EOPO3900, 10% (w/w) $(NH_4)_2SO_4$, and 4% (w/w) NaCl. In the secondary ATPS, the optimum ratio between EOPO-rich top phase and distilled water was 1:1 (w/w), and the optimum temperature for inducing phase separation was 60°C. After thermoseparation, water solution containing lipase, and EOPO were formed in the top and bottom phase of this step, respectively. Under the optimal partitioning condition, the yield (64.45%) and purification fold (PF: 6.30-fold) were obtained. Additionally, the lipases retained in salt-rich bottom phase in the primary ATPS were successfully recovered from the recycling step no more than three times. The total yield of 93.59% was obtained from this separation system. An effect of ultrasound on the partitioned lipase activity was also studied. The highest lipase activity was achieved when the sample was treated with ultrasound at 180 W and 24 kHz for 20 min at 40°C, under which the activity was increased by 110.73% over the control. Therefore, the T-ATPS was found to be an attractive technique for the recovery and partial purification of lipase from Nile tilapia viscera. Moreover, ultrasound could obviously improve its activity.

To widen the application of lipase from viscera of Nile tilapia, partially purified lipase was prepared and further used for production of biodiesel. Biodiesel synthesis through transesterification of used cooking oil or frying oils using the viscera lipase from Nile tilapia as a low-cost feedstock and catalyst, respectively, was optimized. The influences of operating factors, including methanol/oil molar ratio, concentration of enzyme, reaction time, type of alcohol, water content and reaction temperature on the yield of biodiesel were investigated. The optimized conditions to achieve maximum biodiesel yield were obtained using a lipase concentration of 30 kUnit, a methanol to oil molar ratio of 4:1, a water content of 3%, a reaction temperature of 45°C, and a 28 h reaction time. Under these optimal operating conditions, the highest biodiesel yield observed was 96.50%. The attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR) was applied to ensure the conversion of used cooking oil into biodiesel. The biodiesel characteristics met the specifications set as prescribed by EN 14214 and ASTM D 6751. The results from

this investigation showed viability of economical biodiesel production using byproducts as both source and catalyst.

To improve the rate of enzymatic reaction using ultrasonic irradiation, the biodiesel production with used cooking oil and methanol through transesterification using lipase from the viscera of Nile tilapia under the effect of ultrasonic irradiation was also studies. The influence of experimental condition such as ultrasonic frequency, irradiation time and temperature on biodiesel yield were investigated. Research results showed that the use of ultrasound decreased reaction time from 28 h to 3 h with the use of ultrasonic frequency of 16 kHz, methanol to oil molar ratio of 4:1, lipase concentration of 30 kUnit, and 40°C reaction temperature. The efficacy of using ultrasound was also compared with conventional stirring under the optimum operating condition. Ultrasonic coupled with stirring explained further improvement transesterification of used cooking oil with methanol using lipase with the highest yield of 97.59% was obtained. The properties of obtained biodiesel satisfy the recommended biodiesel standards as prescribed by EN 14214 and ASTM D 6751. Therefore, ultrasound-assisted lipase-catalyzed transesterification of used cooking oil with methanol would be a promising alternative for conventional methods.

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Jaran Patchimpet

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CHAPTER 1

Introduction and Literature Review

Introduction

In Thale Noi, Phatthalung province, Thailand, the freshwater processing industries, especially fermentation and drying, are becoming increasingly important because they are one of the main income generators. Three freshwater fish species commercially used in the processing industries include hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), striped snake-head fish (*Channa striata*) and Nile tilapia (*Oreochromis niloticus*). Fish viscera, byproducts from processing industries, are generated. Fish internal organs constitute approximately 7.5% of body weight. However, they are also a potential source of lipases. Fish lipases exhibit some characteristics and properties that complement those of lipases from mammalian and microbial sources due to the evolutionary pathways, diets and habitats of fish (Kurtovic, Marshall, Zhao and Simpson. 2009 : 18). In general, fish lipases have cold-adapted properties, which are known to show more catalytic activity and stability than mammalian lipases, thereby making them suitable for applications in food processing at low temperatures (Sae-leaw and Benjakul. 2018 : 9).

Lipases (E.C. 3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of the water insoluble triglycerides into free fatty acids and glycerol and operate at the interface of the emulsified lipid substrate. Lipases have become increasingly important in the last two decades for industrial applications in the food, detergent, pharmaceutical, bioenergy and biodiesel industries (Aryee, Simpson and Villalonga. 2007 : 394). From this reason, there is a great potential for the extraction, characterization and use of lipases from fish sources, especially from the viscera. Different strategies for lipases purification methods in general are time-consuming, have low scalability and cost effectiveness, and sometimes may offer an unsatisfied purification yield of the desired product (Gupta, Gupta and Rathi. 2004 : 763).

Therefore, there exists a need for the development of an alternate method that facilitates an easier recovery of the product.

Thermoseparating aqueous two-phase system (T-ATPS) has been used successfully for the separation and purification of proteins or enzymes. T-ATPS using a co-polymer, poly(ethylene glycol-ran-propylene glycol), monobutyl ether (EOPO), and a salt (i.e., magnesium sulfate, sodium citrate, or potassium phosphate) has received special attention for several years due to advantages such as a short and economical process, low energy consumption, environmental friendliness, and relatively easy scaling-up. As well, phase composition can be recycled, and the protein/enzymes can be recovered in a water phase (Pereira, Wu, Venâncio and Teixeira. 2003 : 131; Show, Tan, Anuar, Ariff, Yusof, Chen and Ling. 2011 : 577; Ng, Tan, Mokhtar, Ibrahim, Ariff, Ooi and Ling. 2012 : 9). T-ATPS has been applied for partitioning and recovery of various enzymes such as protease (Ketnawa, Benjakul, Martínez-Alvarez and Rawdkuen. 2014 : 2158), and biodiesel, commonly called fatty acid methyl ester (FAME) and synthesized using transesterification of edible, non-edible, and waste oils, is a renewable alternative to diesel fuel (Park, Sato and Kojima. 2008 : 3130). The biodiesel synthesis is classified as chemical or enzymatic production according to the catalysts employed in the process. Nowadays, enzymatic production of biodiesel has attracted considerable interest since it is more efficient and highly selective, involves less energy consumption, and produces less side products or waste. Also, no complex operation is needed for the recovery of glycerol and for eliminating the catalyst and salt in comparison with chemical methods (You, Yin, Zhou and Zhang. 2013 : 202). The requirement of costly feedstock is the major problem for commercialization of biodiesel. Higher cost of refined vegetable oil and limitation of food security make refined vegetable oil an unfavorable starting raw material. Recently, used cooking oil has been explored as an attractive option to reduce the raw material cost for biodiesel production. From an economic point of view, the enzyme activity is low in lipase-catalyzed biodiesel production, compared with chemical catalysts; therefore, it is necessary to find a proper method to increase the reaction rate to promote the application of enzyme catalytic biodiesel production. Amongst the newer processes reported for improving the yield, ultrasound irradiation has been reported to be an efficient method, which

results in a significant degree of process transesterification due to the cavitation phenomenon (Rokhina, Lens and Virkutyte. 2009 : 298).

The overall rationale for this study was to discover a new source of lipases for potential use to meet the growing demands for enzymes in commercial applications. In this connection, lipase from viscera of freshwater fish including hybrid catfish, striped snake-head fish and Nile tilapia were selected for recovery, partitioning and characterization to ascertain whether or not they have unique properties that would make them better suited for some applications.

Literature Review

1. Nile Tilapia (*Oreochromis niloticus*)

The Nile tilapia *Oreochromis niloticus* is a deep-bodied fish with cycloid scales. Silver in color with olive/grey/black body bars, the Nile tilapia often flushes red during the breeding season (Picker and Griffiths. 2011) Figure 1. It grows to a maximum length of 62 cm, weighing 3.65 kg (at an estimated 9 years of age). The average size (total length) of *O. niloticus* is 20 cm (Bwanika, Makanga, Kizito, Chapman and Balirwa. 2004 : 93).

O. niloticus is a tropical freshwater and estuarune species. It prefers shallow, still waters on the edge of lakes and wide rivers with sufficient vegetation (Picker and Griffiths. 2011)**.** Nile tilapia are known to feed on phytoplankton, periphyton, aquatic plants, invertebrates, benthic fauna, detritus, bacterial films and even other fish and fish eggs. (Food and Agriculture Organization of the United Nations Retrieved Oct 22, 2019 from http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/)

Figure 1. Nile Tilapia
(Source : Montota-López, Moreno-Arias, Tarazona-Morales, Olivera-Angel and Betancur. 2019 : 194)

2. Striped Snake-Head Fish (*Channa striata*)

The striped snakehead has a long body characterized with dark black-brown on the upper section of its body, and bands of a white on its belly (Figure 2). The striped snakehead can reach lengths up to 90 cm and up to 3 kg - growth studies report they reach an average body mass is reported as 60 g by 12 weeks past the fingerling stage (Cagauan. 2007 : 48).

Channa striata occurs mainly in shallow freshwater, typicall at depths of 1-2 m, rarely below 10 meters. This species can be found in most types of slow-moving freshwater habitat, including rivers, lakes, ponds, canals, creeks, flooded rice paddies, irrigation reservoirs, and swamps. Compared to most freshwater fish it is quite tolerant of turbid conditions and low oxygen livels. In regions with rainy and dry seasons, these fish may migrate out from permanent lakes and strams into flooded areas during the rainy season, and then return to permanent waters as the fooded areas dry. *Channa striata* is a predacious, ambush feeding fish that has a carnivorous-specifically piscivorous-diet. It is a generalist species that preys on any available source of food that is attainable. As young fish, also known as a fry, the striped snakehead hunts in groups with their main source of food being zooplankton and small insects. Once the young fry become adults, they begin to hunt on their own, feeding on other adult fish, and the progeny of those fish. They can also feed on frogs, snakes, insects, earthworms, tadpoles, brids, small mammals, and crustaceans. When food sources become limited, adult snakeads may feed on young of their own species (Animal Diversity Web. Retrieved Oct 22, 2019 from http://animaldiversity.org/accounts/Channa_striata/).

Figure 2. Striped Snake-Head Fish

(Source : Fishing-Khaolak. Retrieved Oct 22, 2019 from http://fishing-khaolak.com/fish_species/freshwater_species/striped_snakehead.html)

3. Hybrid Catfish (*Clarias macrocephalus* x *C. gariepinus*)

The big-oui hybrid catfish (female *C. macrocephalus* x male *C. gariepinus*) was successfully achieved using artificial hybridization (Figure 3). This hybrid combines the superior taste of the *C. macrocephalus* with the faster growth rate and higher resistance to environmental conditions of the *C. gariepinus*. Many characteristics of the hybrids (e.g., shape of occipital process, body and head) are intermediate between the parental species. (Lawonyawut. 1995) The catfish naturally feeds on small fish and some aquatic animals, so it is considered as a carnivore.

Figure 3. Hybrid Catfish

(Source : Ponjarat, Singchat, Monkheang, Suntronpong, Tawichasri, Sillapaprayoon, Ogawa, Muangmai, Baicharoen, Peyachoknagul, Parhar and Na-Nakorn. 2019 : 84)

4. Lipase

4.1 Definition and Mechanism of Action

Lipases are enzymes that catalyze the hydrolysis of triacylglycerols (TAGs) at the oil-water interface to release glycerol and free fatty acids (FFA). Lipases bear the international name of triacylglycerol acylhydrolases (E.C. 3.1.1.3). The presence of water in lipase-catalyzed reactions facilitates the breaking of covalent bonds in the substrate and subsequent assimilation of these molecules in product formation (Aryee, Simpson and Villalonga. 2007 : 394). TAG-lipases hydrolyze TAGs in three steps (Figure 4); in the first step, one primary fatty acid (FA) is cleaved from the TAG molecule leaving a diacylglyceride (DAG) and FFA. In the second step, the remaining primary FA is removed leaving a monoacylglyceride (MAG) and FFA. Complete enzymatic hydrolysis can be carried out by a MAG-lipase. In this step the 2-MAG is split into a FA and a glycerol molecule, since many impure lipase preparations are contaminated with MAG-lipase, complete hydrolysis often occurs. Furthermore, in general, the hydrolysis sequence is much reliant on the specificity of the lipase. For instance, 1, 3-specific lipases catalyze the release of FA at the *sn*-1 and 3 positions of the TAG molecule to form 1,2 (2, 3)-DAG and 2-MAG, while non-specific lipases hydrolyze at the *sn*-l, 2 or 3 positions of the TAG molecule non-sequentially. Lipases can also be substrate specific; FA acyl chain length (long, short or medium chain lipases) and type of FA (saturated or unsaturated), as well as regio- or stereo-specific; based on the alcohol moiety of the TAG substrate.

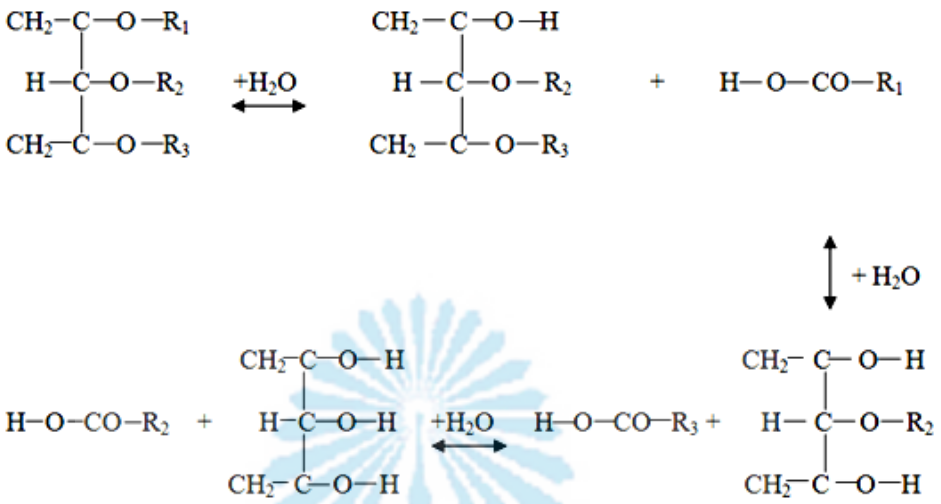


Figure 4. Lipase Hydrolyzes Triacylglycerols

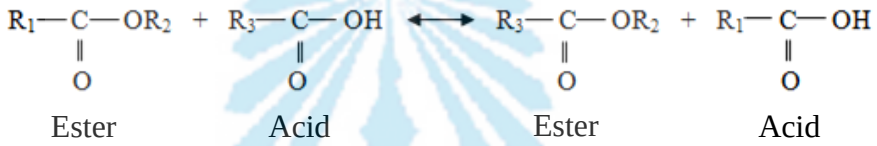
(Source : Björkling, Godtffredsen and Krik. 1991 : 360)

Although lipases are designed by nature for the hydrolytic cleavage of the ester bonds of TAGs (Quinlan and Moore. 1993 : 580), lipases can catalyze the reverse reaction; ester synthesis (Figure 4) in a low-water environment (Björkling, Godtffredsen and Krik. 1991 : 360). Hydrolysis and esterification can occur simultaneously by continual removal of water molecules via distillation or evaporation under reduced pressure (Yan, Bornscheuer and Schmid. 2002 : 31). It is also known that concurrent hydrolysis and esterification is not always practically possible, thus a lipase that is efficient in hydrolysis may not necessarily have esterification capabilities (Wu, Jääskeläinen and Linko. 1996 : 226).

Lipase-catalyzed ester synthesis has been exploited. Depending on the substrates, lipases can catalyze the resolution of racemic mixture in non-aqueous media with water-immiscible organic solvents, and transesterification reactions (Osório, Ferreira-Dias, Gusmão and Fonseca. 2001 : 677) including; acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol (polyhydric or monohydric such as ethanol), aminolysis (where an acyl moiety is displaced between an acyl glycerol and an amine), and interesterification (where two acyl moieties are exchanged between two acylglycerols at different

positions on the TAG backbone) to produce acid, alcohol, amine or ester respectively instead of water (in esterification) as by-products, and esters of different acyl group (Figure 5). Lipase-promoted acyl migration has been used to modify the physical and nutritional properties of TAGs by either randomly or specifically distributing FA residues on the glycerol backbone (Xu. 2000 : 287).

a) Acidolysis



b) Alcoholysis

c) Aminolysis

d) Interesterification

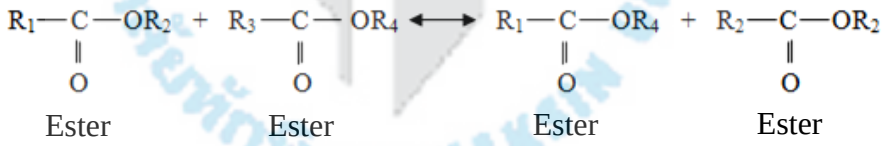


Figure 5. Lipase-Catalyzed Transesterification Reactions

(Source : Osório, et al. 2001 : 677)

4.2 Occurrence of Lipases

Lipases are ubiquitous enzymes (Beisson, Tiss, Rivière and Verger. 2000 : 133), and have been found in animals, plants, bacteria, yeast and fungi (Mukherjee and Hill. 1990 : 1; Jaeger, Ransac, Dijkstra, Colson, van Heuvel and

Misset. 1994 : 29). However, most industrial lipases are derived from fungi, and bacteria. Lipases from *Candida*, *Penicillium* and *Streptomyces* species have been extensively studied and these lipases differ from each other in their molecular and catalytic properties (Abramic, Lescic, Korica, Vitale, Saenger and Pgac. 1999 : 522). Microbial lipases have attracted considerable attention and are preferred for commercial applications due to its relative abundance, easy extraction procedures, good stability and many stereo-specific properties. Conversely, the source, physical and chemical conditions or properties of the enzyme have been said to determine the extraction efficiency, purification, stability and ultimately cost of the enzyme. Plants and animal sources were formally considered ideal for bulk enzyme production intended for the food industry, since they were assumed to be devoid of problems associated with toxicity or contamination, tedious extraction procedures and cost. However the growing demand for enzymes have overwhelmed plant or animal sources in addition to international politics, the wide seasonal variation in yields (for plant), and the mercy of the weather (which is unpredictable). The search for new enzyme sources is thus a key element to the growth and sustainability of the enzyme technology.

4.3 The Active Site of Lipase

The active site of lipases contains the catalytic triad in a highly conserved pentapeptide consensus; Gly-X-Ser-X-Gly sequence (where X = any amino acid) (Brad, Brzozowski, Derewenda, Dodson, Dodson, Tolley, Turkenburg, Christiansen, Huge-Jensen, Norskov, Thim and Menge. 1990 : 767; Jin, Broedl, Monajemi, Glick and Rader. 2002 : 268). The catalytic triad comprises the primary sequence of serine (Ser), histidine (His), and glutamate (Glu) or aspartate (Asp) residues. The Ser residue is responsible for the nucleophilic attack on the carbonyl group of the substrate (Ollis, Cheah, Cyler, Dijkstra, Frolow, Franken, Harel, Remington, Silman, Schrag, Sussman, Verschueren and Goldman. 1992 : 197), the hydrogen-bonded His residue functions as a general base to accept the proton from Ser while the negatively charged Asp residue stabilizes the positive charge that forms on the His residue. These results in increased nucleophilicity in the Ser residue by the His side chain and prevents the development of an unstable positive charge on the Ser

hydroxyl group. Consistent with the importance of Ser is its position in the "nucleophilic elbow"; a characteristic β-strand-turn-helix motif (Cygler, Schrag, Sussman, Harel, Silman, Gentry and Doctor. 1993 : 366). This pentapeptide sequence has been recognized as the lipid/interfacial-binding segment (Verger. 1984 : 83). The lack of sequence homology between lipases and other serine proteases like trypsin, papain or subtilisin is due to the order of arrangement of the amino acid sequence of the three active site residues; nucleophile (Ser), acid (Asp) and base (His). While the catalytic triad of lipases follow the order Ser-Asp-His, that of trypsin follows His-Asp-Ser, and this accounts for the ready distinction in their catalysis (Wang and Hartsuck. 1993 : 1).

However, there is contradicting evidence of this highly conserved pentapeptide sequence in lipases. Guidoni, Bendouka, De Caro and Rovery (1981 : 148) identified the Ser in the sequence as involved in interfacial binding and not the putative active site in porcine pancreatic lipase, Reddy, Maraganore, Meredith, Heinrikson and Kèzdy (1986 : 9678) identified Ser as the catalytic center in bovine milk lipoprotein lipase, this prediction was however later disproved by Wion, Kirchgessner, Lusis, Schotz and Lawan (1987 : 1638). Komaromy and Schotz (1987 : 1526) identified two Gly-X-Ser-X-Gly sequences in the cDNA of hepatic lipase. Hide, Chan and Li (1992 : 167) did not find a second Gly-X-Ser-X-Gly motif in pancreatic lipase and predicted a low probability of a second lipid-binding site in lipoprotein lipase and hepatic lipase. Fortunately this contention was settled with site-directed mutagenesis (Emmerich, Beg, Peterson, Previato, Brunzell, Brewer and Santamarina-Fojo. 1992 : 4161), which identified Ser as the putative active center of these lipases.

4.3.1 The Lid of Lipases

The super family members, i.e., lipase gene family, esterases, and thioesterases belong to the hydrolase fold family (Schrag and Cygler. 1997 : 85) of which serine protease is a member (Brenner. 1988 : 528). They are also characterized by the presence of two motifs; α/β hydrolase fold made of a central β-sheet and two α-helices. This fold provides a typical scaffold for the conserved residues in the pentapeptide sequence and the lid sequence adjoining the active site.

The 'lid' or flap is made of the two α-helices. The lid domain forms an amphiphatic helix that covers the catalytic pocket of the enzyme. It is most likely that these two motifs shared by lipases and serine esterases result from convergent rather than divergent evolution (Derewenda and Derewenda. 1991 : 842).

Among the characteristic structural features of lipases are the conformational changes in the lid upon substrate binding. These conformational changes in the lid have been implicated for the kinetic phenomenon of interfacial activation by several crystallographic studies (Derewenda, Swenson, Green, Wei, Yamaguchi, Joerger, Haas and Derewendra. 1994 : 551). The conformational changes in the lid also affect substrate binding; by aiding in the smooth gliding of the insoluble-substrate into the active site, as well as modulating substrate preference or specificity by discriminating enzyme activity against several different substrates (Dugi, Dichek and Santamarina-Fojo. 1995 : 25396). The lid is also a highly conserved feature of lipases. Although the residue surrounding the lid region is conserved in lipase H, the minimal homology sequence between lipase H and other members of the gene family in the lid region itself may account for their different substrate specificities (Jin, et al. 2002 : 268).

The lid domain, derived from the N-terminal extends outwards the C-terminal when in open conformation. In the inactive state, the lid covers and seals the active site of the enzyme so that the hydrophobic side of the amphililic lid is buried in the active site and the hydrophilic polar side is exposed to the solvent, and the oxyanion hole residue also orient incorrectly. At the interface upon activation, the lid opens by rotating around its hinges. The movement of the lid also affords interaction and the correct orientation of the oxyanion residue, which later stabilizes the lipase in the transition state (Derewenda, Brzozowski, Lawson and Derewenda. 1992 : 1532). The newly formed lipase conformation has been proposed to be stabilized by extensive hydrophobic and electrostatic interactions as well as other structural elements present in the enzyme (Winkler, d'Arcy and Hunziker. 1990 : 771). For instance, tryptophan (Trp) residues, which show affinity towards the carbonyl group of lipid molecules, have been found to be among the lid's surface residues. These features and observations of lipases partly explain the complexity of the mechanism of contact between the water-insoluble substrate, the water-soluble lipase

and catalysis; since specific interaction between the lid, lipid and the interface may be responsible for exposing the active site and contribute in stabilizing the opened and conformationally changed enzyme (Julita, Zhu, Patkar, Vind, Svendsen and Kinnunen. 2000 : 1634). The lid may also protect the lipase from proteolytic degradation (Brad, et al. 1990 : 767).

4.3.2 Interfacial Activation of Lipase

A unique physiochemical characteristic feature of lipase-mediated reaction is the presence of the lipid-water interface; involve in interfacial absorption and subsequent catalysis. In contrast to esterases, lipases display very little or no activity on water-soluble substrates. The catalysis of lipases is heterogeneous i.e., the enzyme, like esterase is water-soluble, but unlike esterase acts on water-insoluble substrates and activity is maximized at the interface (Verger. 1997 : 32). It is still not clearly understood how lipases and lipids interact at the interface and this has been a subject of immense investigation (Julita, et al. 2000 : 1634). This unique phenomenon whereby lipase activity is maximized when the enzyme is absorbed into the interface has been called interfacial activation (Gargouri, Julien, Sugihara, Verger and Srada. 1984 : 326). The interface occurs between the water-insoluble substrate-containing phase and water-soluble enzyme-containing phase. The heterogeneity of lipase activity therefore makes accurate quantification of both the amount of interface (specific phase) (Benzonana and Desnuelle. 1965 : 121) and interfacial parameters (such as tension, potential, viscosity) responsible for interfacial quality of the substrate impossible (Panaitov and Verger. 2000 : 8369). The lid or flap covering the active site and the orientation of the oxyanion stabilizing residues have been identified as the two main structural features involved in interfacial activation (Derewenda, et al. 1992 : 1532; van Tilbeurgh, Egloff, Martinez, Rugani, Verger and Cambillau. 1993 : 814).

The widely accepted enzyme and substrate models proposed by Verger and de Haas (1976 : 77) have been used to explain this phenomenon of interfacial activation. The enzyme model relates to the modification in the active site and increase in catalytic efficiency due to conformational changes in the enzyme at the interface where the lipid substrates have aggregated (Verger, Mieras and de Haas.

1973 : 4023). This model also makes assumption of an additional active site, topographically and functionally distinct from the main active site (Brockerhoff. 1973 : 215; Verger, et al. 1973 : 4023) and this has been called penetration site (Verger, et al. 1973 : 4023), super substrate binding site (Brockerhoff. 1973 : 215), and interface recognition site (Verger. 1980 : 340). The substrate model illustrates changes in the substrate itself in terms of concentration and orientation caused by the surface created as a result of the aggregation process. At low substrate concentration and in aqueous milieu, the enzymes are inactive, in free monomeric state the substrates plunge unreservedly in solution, and when substrate concentration is high enough to form micelles, there is increased hydrophobicity and interaction (Verger. 1980 : 340). The substrates of lipases consequently require surface-active amphiphiles such as other proteins, lipid, and salts for emulsification at the lipid-water interface, however the presence of non-specific inhibition of some lipases by proteins at the interface have been shown to occur (Gargouri, Pièroni, Rivière, Srada and Verger. 1986 : 1733).

The interfacial activation mechanism is believed to be more complicated and various studies have revealed different features and mechanisms for different lipolytic enzymes (Carvalho, Aires-Barros and Cabral. 1999 : 17). Nevertheless, not all lipolytic enzymes exhibit interfacial activation i.e., the commencement of conformational changes in the presence of lipid substrates is not unanimous. The catalytic Ser of *Fusarium solanipisi* cutinase is not buried under surface loops, and is accessible to both the substrate and solvent, and active on soluble as well as on emulsified TAG (Carvalho, Aires-Barros and Cabral. 1999 : 17) contradicting earlier reports (Brzozowski, Derewenda, Derewenda, Dodson, Lawson, Turkenburg, Bjorkling, Huge-Jensen, Patkar and Thim. 1991 : 491; Derewenda, et al. 1992 : 1532). The lack of interfacial activation can be attributed to both the absence of the lid and the presence of preformed oxyanion hole (Martinez, Nicholas, van Tibeurgh, Egloff, Cudrey, Verger and Cambillau. 1994 : 83). Cutinases therefore establish a bridge between esterases and lipases. The presence of more than one lid on the active site of *Candida rugosa* lipase has been reported (Grochulski, Li, Schrag, Bouthillier, Smith, Harrison, Rubin and Cygler. 1993 : 12843). It has therefore been suggested that interfacial activation should not the unique criterion to characterize and

define lipases (Chahinian, Nini, Boitard, Dubès, Sarda and Comeau. 2000 : 919; Nini, Sarda, Comeau, Boitard, Dubès and Chanhinian. 2001 : 34). Lipases can therefore expediently be said to be fat splitting ferments (Verger. 1997 : 32).

4.4 Fish Lipase

Digestive lipolysis is well studied in mammals, and is achieved by a concerted action of by two major enzymes secreted by the pancreas, i.e., the bile salt activated lipase (BSAL) and the pancreatic lipase with its colipase (Verger. 1984 : 83; Wang and Hartsuck. 1993 : 1). However less is known of digestive lipolysis in fishes whose main energy source is dietary lipids, since the availability and digestibility of carbohydrates is often low (Gjellesvik, Lombardo and Walther. 1992 : 123; Iijima, Tanaka and Ota. 1998 : 59). These dietary lipids also contain high proportions of polyunsaturated fatty acids. It is still debated as to whether enzymes responsible for digestive hydrolysis of TAGs in fishes are comparable to mammalian lipolytic enzymes (Gjellesvik, Raae and Walther. 1989 : 177).

Several studies involving; anchovy (*Engraulis mordax*, Patton, Nevenzel and Benson. 1975 : 575), leopard shark (*Triacus semifasciata*, Patton, Warner and Benson. 1977 : 322), rainbow trout (*Salmo gairdnerii*, Tocher and Sargent. 1984 : 561), cod (*Gadus morhua*, Lie and Lambersten. 1985 : 447), and dogfish (*Squalus acanthias*, Rasco and Hultin, 1988 : 671), have shown that TAG hydrolysis by lipases were non-specific in relation to the position of FAs on the glycerol molecule. The distribution of lipases among tissues, and species has been reported to differ considerably (Izquierdo, Socorro, Arantzamendi and Hernandèz-Cruz. 2000 : 97). Other investigators have reported other lipases and lipolytic activities involving pancreatic lipase, lipoprotein lipase and phospholipases in the digestive tract, liver, and adipose tissue of different fish species (Mukundan, Gopakumar and Nair. 1985 : 191; Borlangan. 1990 : 315; Michelsen, Harmon and Sheridan. 1994 : 509; Izquierdo, et al. 2000 : 97; Liang, Ogata and Oku. 2002 : 913). Nevertheless mammalian type pancreatic lipase isolated from the intercaecal pancreatic tissue of rainbow trout (*Salmo gairdnerii*) by Leger, Bauchart and Flanzy (1977 : 359), and the hepatopancreas of sardine (*Sardinella longiceps*) by Munkundan, et al. (1985 : 191) were not examined for their colipase dependency or

bile salt inhibition (Gjellesvik, Lombardo and Walther. 1992 : 123), thus leaving unresolved its presence or absence in those fishes. On the other hand activation of porcine pancreas lipase by dogfish colipase, and rainbow trout pancreatic lipase by porcine pancreas lipase has been reported (Iijima, Tanaka and Ota. 1998 : 59).

It has been suggested that BSAL is probably the most important digestive enzyme in teleostean carnivorous fishes (Patton, Warner and Benson. 1977 : 322; Gjellesvik, Lombardo and Walther. 1992 : 123; Murray, Gallant, Perez-Casanova, Johnson and Douglas. 2003 : 816) since dietary wax esters and TAGs high in polyunsaturated fatty acids the predominant lipid in marine habitat, are more resistant to mammalian-type pancreatic lipase hydrolysis (Chen, Wternby, Åkesson and Nilsson. 1990 : 111). Mammalian- BSAL have been isolated and purified from the pyloric ceaca of cod (*Gadus morhua*, Gjellesvik, Lombardo and Walther. 1992 : 123), and the hepatopancreas of red sea bream (*Pagrus major*, Iijima, Tanaka and Ota. 1998 : 59), while the cDNA of the pancreatic carboxylester lipase (BSAL) containing a predicted bile salt and a putative lipid-binding sites have been cloned from Atlantic salmon, winter flounder and haddock with ≥58% amino acid homology with mammalian BSAL (Gjellesvik, Lorens and Male. 1994 : 603; Murray,et al. 2003 : 816; Perez-Casanova, Murray, Gallant, Ross, Doudlas and Johnson. 2004 : 601). Despite the relative importance of lipases in marine fish digestion, very few studies with conclusive results have been carried out.

Lipid metabolism appears more important in homeostasis maintenance in fishes than in homeotherms, since most fishes utilize lipids as the major energy source in contrast to mammals, which mainly use carbohydrates (Watanabe. 1982 : 3). Studies have shown that many fish species have the capacity to digest wax esters (Patton, Nevenzel and Benson. 1975 : 575; Mankura, Kayama and Saito. 1984 : 2127; Rasco and Hultin. 1988 : 671); however the hydrolytic activity towards wax esters in the gut has been reported to be much lower than towards TAGs (Patton, Nevenzel and Benson. 1975 : 575; Olsen, Henderson and Pederson. 1991 : 59). This has raised some concerns that, wax esters may not be effectively utilized, particularly in juvenile fishes (Olsen, Henderson and Pederson. 1991 : 59).

In addition to the role of lipases in digestion is its undesirable role in spoilage. The role of lipases in lipid hydrolysis and quality deterioration is usually

underestimated. Deterioration is very rapid and is carried out mainly by enzymes in the digestive tract; microorganisms such as bacteria have also been implicated. Studies on fishes have linked the autolytic degradation of fish abdominal tissue to digestive enzymes (Heu, Kim and Pyeun. 1995 : 557) as well as oxidative deterioration of polyunsaturated fatty acids by lipid oxidation, and the accumulation of non-esterified free fatty acids (Frankel. 1981 : 1). The accumulation of these non-esterified free fatty acids have been shown to result in the development of off-flavors, destruction of amino acids, modification of texture and color (in fresh and frozen fishes) as well as the water holding capacity of the fish. The main components of lipid oxidation have been identified as carbonyl compounds (aldehydes and ketones); and these produce the undesirable aromas in the postmortem fish.

5. Lipase Purification

Conventional protocols for purification of proteins, such as adsorbing separation, ammonium sulfate precipitation and chromatography techniques, have been used in lipase purification (Rovery, Boudouard and Bianchetta. 1987 : 373; Garner and Smith. 1970 : 503; Genest and Borst. 2007 : 213). However, these protocols are time-consuming, expensive and difficult to use for industrial applications. An effective alternative method for the separation and purification of proteins is partitioning in a Thermoseparating aqueous two-phase system (T-ATPS)

5.1 Thermoseparating Aqueous Two Phase Partitioning (T-ATPS)

In T-ATPS, a conventional polymer (PEG) phase was replaced with a thermoseparating copolymer; composed of ethylene oxide and propylene oxide (EOPO) and a salt (i.e. sodium citrate, potassium hydrogen phosphate). In this system, enzyme purification is concerned with two step process. In a primary 1, 1, 3, 3-tetramethoxypropane, the target protein should preferentially enter the EOPO phase at the start. The secondary system is formed during the next step by a temperature-induced phase separation of the EOPO solution. Consequently, a water solution of the target protein and a concentrated EOPO solution are obtained (Johansson, Karlström, Tjerneld and Haynes. 1998 : 3). In this type of partitioning, the aim is to partition the target protein to the top polymer (EOPO) phase and leaving contaminants collected in

the bottom polymer or salt phase (Johansson, Karlström, Tjerneld and Haynes. 1998 : 3). It has previously been suggested that the EOPO copolymer could be recovered and reused to prepare the next primary ATPS (Persson, Johansson and Tjerneld. 1999 : 31). In T-ATPS, a primary two-phase system is first formed and the target protein is partitioned to the EOPO top phase. Next, the EOPO phase is withdrawn and heated above the cloud point of the polymers to induce thermoseparation and subsequently a new two-phase system consisting of a water top phase and a polymer bottom phase is observed. The target enzyme is harvested from the water phase and the EOPO polymer can be recovered from the EOPO bottom phase of the system (Ng, Tan, Mokhtar, Ibrahim, Ariff, Ooi and Ling. 2012 : 9). EOPO random copolymers (linear and non-ionic) can be separated from aqueous solution by heating the solution above the cloud point (also known as the lower critical solution temperature or LCST) at a low temperature of around 50ºC (Persson, Johansson and Tjerneld. 1999 : 31; Show, Tan, Anuar, Ariff, Yusof, Chen and Ling. 2012 : 74). In this case, the polymers can be recycled and the salt component can also be reused in subsequent ATPS extractions (Persson, Johansson and Tjerneld. 1999 : 31). During the thermoseparation, (in accordance with a model based on the Flory-Huggins theory of polymer solution), it was shown that the separation of enzymes from polymers by temperature-induced phase separation results from an excluded volume effect because the thermoseparated polymer rich phase is entropically unfavorable for proteins, compared to a water phase (Johansson, Karlstrom and Tjerneld. 1997: 315). The advantages and limitations of T-ATPS methods of enzyme partitioning were presented in Table 1. A schematic diagram depictes the recycling of phase components using EOPO as the thermoseparating polymer and salt in T-ATPS is illustrated in Figure 6.

Table 1 Advantages and Limitations of T-ATPS

Advantages	Limitations
- Polymer Easily Recovered - Phase Composition Can be Reused - Scalability - Cost Effectiveness - Environmental Benefits	- Denaturation of Biomolecules When High Temperature Used

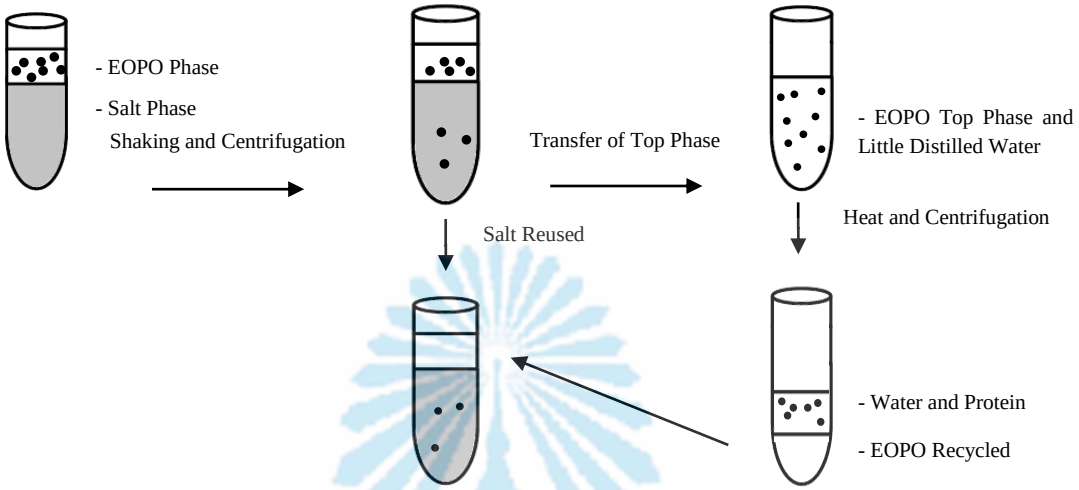


Figure 6. Purification of a Target Protein in an Aqueous Two-Phase System with Recycling of EOPO Copolymer by Thermoseparating.
(Source : Ng, et al. 2012 : 9 with a slight modificatioin)

6. Lipase Characterization

6.1 Molecular Weight of Lipases

The molecular weight of purified cod BSAL was reported as 60 kDa and 56 kDa as judged by SDS-PAGE and gel filtration, respectively (Gjellesvik, Lombardo and Walther. 1992 : 123), and Iijima, Tanaka and Ota (1998 : 59) reported a 64-kDa lipase from the hepatopancreas of red sea bream also by SDS-PAGE (Table 2). However in the presence of bile salts, BSALs induce the formation of multimers (Hyun, Kothari, Herm, Mertenson, Treadwell and Vahouny. 1969 : 1937; Gjellesvik, Lombardo and Walther. 1992 : 123). Other investigators have reported similar or higher molecular weight from pleopods of whiteleg shrimp, marine snails, bovine, rat and human pancreatic lipase activity in the range of 65-83 kDa (Rivera-Pérez, del Toro and García-Carreño. 2011 : 99; Zarai, Ali, Fendri, Louati, Mejdoub and Gargouri. 2012 : 2434; Camulli, Linke, Brockman and Hui. 1989 : 177; Kyger, Wiegand and Lange. 1989 : 1302), while the molecular weight of human milk BSAL was reported as 110-125 kDa (Wang. 1980 : 398; Bläckberg, Lombardo, Hernaell, Guy and Olivercrona. 1981 : 284). The striking discrepancy between the molecular weight of lipases from the pancreas and milk have been attributed to the different

degree of glycosylation and/or the different processes of degradation that occur during purification as well as the nature of the proline-rich repeating unit near the C-terminal (Rudd, Mizuno, and Brockman. 1987 : 106; Abouakil, Rogalska, Bonnicel and Lombardo. 1988 : 229; Wang and Hartsuck. 1993 : 1).

6.2 Factors Affecting Lipase Activity and Stability

The catalytic efficiency of enzymes invariably depends on the substrate, state of purity of the enzyme, buffers used, and methods of assay. The temperature, pH, ionic strength, activators, inhibitors, and substrate concentration among other factors, all affect the structural and the chemical nature of enzyme (Kuby. 1991 : 163; Whitaker. 1994 : 240). These properties determine the enzyme's biotechnological importance and there is an upsurge in finding novel enzyme sources with unique characteristics.

- pH

Enzymes are generally active in a narrow pH range and factors such as substrate type and concentration, temperature, reaction time, source and purity of the enzyme affect the pH profile of the enzyme (Kuby. 1991 : 163; Whitaker. 1994 : 240). pH studies can provide information and the identification of activators and other ionizable residues important for substrate fixation and metabolism (Kuby. 1991 : 163). Changes in the pH of the reaction medium modifies enzymatic action by changing the ionization state of the amino acid side chains involved in catalysis, changing the ionization state of the substrate, and the equilibrium of the reaction; when H^+ and OH^- are involved. Most characterized lipases have optimal pH of 7.5-10 (Gjellesvik, Lombardo and Walther. 1992 : 123; Iijima, Tanaka and Ota. 1998 : 59; López –López, Nolasco and Vega-Villasante. 2003 : 337; Aryee, Simpson and Villalonga. 2007 : 394; Zarai, Bacha, Horchani, Bezzine, Zouari, Gargouri and Mejdoub. 2010 : 121; Rivera-Pérez, del Toro and García-Carreño. 2011 : 99; Zarai, et al. 2012 : 2434; Smichi, Gargouri, Miled and Fendri. 2013 : 87) while pH stability of lipases varies considerably (Sztajer, Lünsdorf, Erdmann, Menge and Schmid. 1992 : 253; Yadav, Saxena, Gupta and Davidson. 1998 : 243; Saxena, Davidson, Sheoran and Giri. 2003 : 3239)

Table 2 Some Properties of Lipases from the Organs of Selected from Fish

Fish Species	Origin	Temperature (°C)	pH	Molecular weight (kDa)	Substarte	Reference
Leopard Shark (*Triakis semifasciata*)	Pancreas	36	-	-	Olive Oil	Patton, Warner and Benson. (1977 : 322)
Rainbow Trout (*Salmo gairdnerii*)	Intercaecal Pancreatic Tissue	--	-	-	-	Leger, Bauchart and Flanzy. (1977 : 359)
Sardine (*Sardinella longiceps*)	Hepatopancreas	37	8.0	~54-57	Tributyrine	Mukandan, et al. (1985 : 191)
Dogfish (*Squalus ancanthias*)	Pancreas	35	8.5	-	Olive Oil	Rasco and Hultin (1988 : 671)
Cod (*Gadus morhua*)	Pyroric Caecum	25	6.5-7.5	~60	*p*-Nitrophenyl Myristate	Gjellesvik, Lombardo and Walther. (1992 : 123)
Neon Flying Squid (*Ommastrephes bartramii*)	Hepatopancreas	25	7.0	-	*p*-Nitrophenyl Laurate	Takahashi, Hatano and Sakura (1996 : 515)
Red Sea Bream (*Pagrus major*)	Hepatopancreas	-	8-9	~64	-	Iijima, Tanaka and Ota. (1998 : 59)
Crayfish (*Cherax quadricarinatus*)	Digestive Gland	35-40	8.5	~43, 46, 63, 118	β-Naphthyl Caprylate	López –López, et al. (2003 : 337)
Grey Mullet (*Mugil cephalus*)	Viscera	50	8.0	-	*p*-Nitrophenyl Palmitate	Aryee, Simpson and Villalonga. (2007 : 394)
Crab (*Carcinus mediterraneus*)	Hepatopancreas	60	-	-	Olive Oil	Cherif and Gargouri (2009 : 82)
Sardine (*Sardinella aurita*)	Digestive Glands (Pyloric Caeca)	37	9.0	~43	Tributyrin	Smichi, Fendri, Chaâbouni, Rebah, Gargouri and Miled (2010 : 1483)
Shrimp (*Litopenaeus vannamei*)	Pleopods	30	10.0	~95.26, 63.36	Triolein	Rivera-Pèrez, et al. (2011 : 99)
Marine Snail (*Hexaplex trunculus*)	Hepatopancreas	50	8.5	~70	Tributyrin	Zarai, et al. (2012 : 2434)
Golden Grey Mullet (*Mugil auratus*)	Viscera	50	8.0	~35	Tributyrin	Smichi, et al. (2013 : 87)
Shrimp (*Litopenaeus vannamei*)	Hepatopancreas	55	8.0	~45, 34, 24	*p*-Nitrophenyl Palmitate	Kuepethkaew, Sangkharak, Benjakul and Klomklao. (2017: 3880)

- Temperature

Enzyme catalysis is greatly affected by temperature. Temperature affects the stability of the enzyme, the availability, and affinity of the substrate to the enzyme, cofactors, activators, inhibitors, and product formation (Whitaker. 1994 : 240). At higher temperatures the weak bonds in some substrates and enzymes are distorted and rearranged, resulting in alteration in their physiochemical properties. Thermal stability of lipases has long been regarded as important property for exploitation by industry (Herbert. 1992 : 395). Heat labile enzymes offer the prospect of being mildly inactivated for short periods of time upon completion of bioconversion without damaging the products (Simpson and Haard. 1984 : 613; Margesin and Schinner. 1994 : 1). Gjellesvik, Lombardo and Walther (1992 : 123) did not report significantly higher activity at the normal habitat temperature (0-8°C) of cod BSAL but significantly higher activity at 20-30°C, and a considerable lost of activity at higher temperatures (above 40°C) in comparison with human pancreatic bile salt lipase. It is general assumed that enzyme activity will continually increase with increasing temperature until the rate of denaturation becomes greater than the increase in activity.

- Activators and Inhibitors

Enzymes in general (75%) require the presence of metal ions; monovalent (Na^{+}, K^{+}), and divalent (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) to fully express their catalytic efficiency (Whitaker. 1994 : 240). These metal ions may inhibit other enzymes present in the reaction perturbing activity, or combine with naturally occurring inhibitor present; in that way relieve inhibition of the enzyme of interest. These metals may also act as scavengers of free fatty acids to minimizing product inhibition, and may accordingly impart stimulatory effect on enzyme activity. The binding of the metal ion may also alter the enzyme activity by stabilizing protein conformation. Enzyme inhibitors provide important tools for examining the physiological function of metabolic pathways, and the characterization of the enzyme. Inhibitors also provide information on the degree of interaction between the enzyme and other molecules present, the nature of the active site and strategies to control unwanted enzyme activity (Aryee, Simpson and Villalonga. 2007 : 394).

- Emulsifiers or Surfactants

Lipase reaction occurs at the lipid-water interface and is critically dependent upon the binding of the enzyme to the interface. The substrate may exist either as emulsion, micelles, monolayers or bilayers, and the rate of hydrolysis varies with the type and physical state of the substrate (Lowe. 1999 : 59). The concept of lipase interfacial activation stems from the finding that the efficiency of catalytic activity of most lipases depends on the aggregation state of their substrates (Verger. 1997 : 32; Panaitov and Verger. 2000 : 8369). It is considered that, activation involves the unmasking and restructuring of the enzyme's active site through conformational changes, and the presence of oil-in-water droplets. In this respect, all amphiphilic substances acting as emulsifiers are expected to influence the rate of the interfacial reaction. Reaction rate is also dependent on the amount of surface-active substance in the emulsion, thus the substrate can be fully available through better emulsification. The substrate may also contain, or the reaction may produce surface-active materials like soaps of free fatty acids that tend to accumulate at the interface and consequently reduce the effective surface area for catalysis (Aryee, Simpson and Villalonga. 2007 : 394).

These surface-active substances may alter the biochemical reaction through a variety of physiochemical mechanisms (Verger. 1997 : 32) including, modifying the molecular conformation in both native and transition state, lowering the surface tension when added to water in small amounts, altering the thermal stability of the enzyme; which affect the binding of substrate or product to and from the enzyme, and by retarding substrate diffusion through the medium to the enzyme, or product away from the enzyme (Panaitov and Verger. 2000 : 8369). Besides, as the surfactant concentration increases above the critical micelle concentration (CMC) the surfactants tend to be inhibitory by increasing repulsion between the enzyme and the interface and subsequently reduce enzyme penetration and catalysis. At surfactant concentrations below the CMC, the surfactant molecules are loosely integrated into the water structure, while in the region of the CMC, the surfactant-water structure is changed in such a way that the surfactant molecules begin to build up their own structures as monolayers at the surface and micelles in the interior.

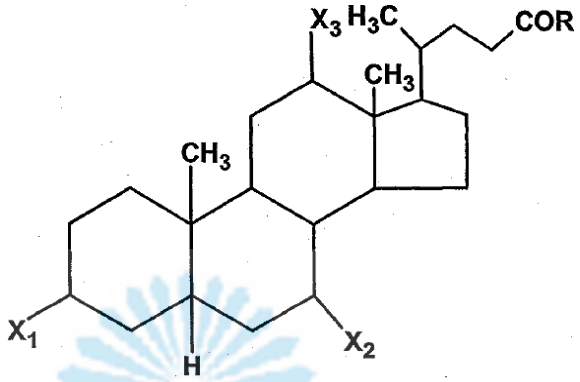
Lipid-water emulsions are dynamic systems with an inherent tendency to change during experimental procedure. It has been shown that surface-active amphiphiles such as bile salts, synthetic detergents, lipids, and proteins added to preformed emulsions of short or long chain TAG behave as strong non-specific inhibitors of lipolysis of certain lipases such as pancreatic lipases (Gargouri, et al. 1986 : 1733) or enhance the action of other lipases such as BSALs (Patton, et al. 1977 : 322; Gjellesvik, Lombardo and Walther. 1992 : 123; Iijima, Tanaka and Ota. 1998 : 59); by acting as activators/stimulators of lipolysis or fatty acid acceptors (Wang and Hartsuck. 1993 : 1). The inhibitory effects of bile salts on pancreatic lipase activity have been extensively studied with emulsified substrates of short-chain (Borgström and Edanson. 1973 : 60), as well as of long chain TAG (Rathelot, Julien, Canioni, Coeroli and Sarda. 1975 : 1117), though these effects are still poorly understood, and different opinions have been put forward regarding the possible mechanisms. Inhibition is thought to be due to desorption of the enzyme from the surface of the substrate. It is not yet known whether desorption results from direct interaction between the enzyme and amphiphilic molecules or from the modification of the properties of the substrate by the adsorbed amphiphile. Hence, it has also be suggested that lipases that hydrolyze substrates at the interface in the presence of emulsifiers possess a penetration power higher than those of pancreatic lipase, and·some microbial lipases, which allows them to hydrolyze water-insoluble compounds in the presence of these amphiphiles (such as bile salts). In fact, the rate of hydrolysis of vinyl esters solution by some lipases is relatively higher than emulsions (Chahinian, et al. 2000 : 919).

Bile acids and their corresponding salts are the most important physiological detergent-like molecules having properties which differ considerably from ordinary synthetic aliphatic detergents such as Triton X-100, Tween 80 or SDS. Bile acids have one hydrophilic and one lipophilic side on the steroid backbone, and an ionized tail, which increases its water solubility. The amphiphilic nature thus provides additional bile salts interaction with the enzyme, and other assay constituents as well as supplementary lipid binding capability (Gjellesvik, Lorens and Male. 1994 : 603; Perez-Casanova, et al. 2004 : 601). It has therefore been suggested that the

binding of some lipases such as BSAL with the substrate involve direct interaction between bile salts and the active site of the enzyme (Wang and Harstuck. 1993 : 1).

The activation effect of bile salts (Figure 7) is mediated through electrostatic interaction between the enzyme and emulsified substrate. The difference in the number, and position of the hydroxyl (OH) group on the cyclopentanoperhydrophenanthrene nucleus account for the disperse effect of bile salts on lipase activity, and the selective activation of lipases by bile salts with the 7α-OH^- group of have been reported (Verger. 1980 : 340; Wang. 1980 : 398; Iijima, Tanaka and Ota. 1998 : 59). The role of bile salts on the activation of some lipases has thus been said to be more precise and selective and not merely related to general detergent properties (Erlanson. 1975 : 401).

The grey mullet lipase was activated by the bile salts (sodium cholate and sodium taurocholate) of cholic acid and its conjugates however the enzyme was not appreciably activated by sodium deoxycholate (NaDC) at all the concentration tested, in fact NaDC was almost inhibitory (Aryee, Simpson and Villalonga. 2007 : 394). Bile salts have been known to play an important role in lipid digestion in *vivo*. Its amphiphilic nature may have modified the physical properties of *p*-nitrophynyl palmitate (*p*-NPP) by converting it to a more appropriate form for efficient interaction with the enzyme. It may also have changed the affinity of the substrate to the enzyme in the aqueous medium by causing a conformational change in the enzyme to provide access to the substrate to the active site (Wang and Lee. 1985 : 824; Wang and Hartsuck. 1993 : 1; Gargouri, et al. 1986 : 1733).



Bile Salt	X_1	X_2	X_3
Sodium Cholate	α-OH	α-OH	α-OH
Sodium Taurocholate	α-OH	α-OH	α-OH
Sodium Deoxycholate	α-OH	H	α-OH

Figure 7. Structure of Bile Salt
(Source : Aryee, Simpson and Villalonga. 2007 : 394)

- Organic Solvents

The predictability of the catalytic performance of enzymes in organic solvent is based on correlation with the physiochemical properties of the solvent (Pogorevc, Stecher and Faber. 2002 : 857). The log *P* value; defined as the log of the partition coefficient of a given organic solvent between water and 1-octanol, is the most widely used parameter in correlating enzyme activity and organic solvent. As defined by Laane, Boeren, Vos and Veeger. (1987 : 81) organic solvents with log *P* value < 2 are most inappropriate for enzymatic reaction dueto their relative high polarity, these solvents have a relatively greater aptitude to strip off the essential water molecules from the enzyme (Gorman and Dordiek. 1992 : 392; Klibanov. 1997 : 97). Such solvents are often toxic to the enzyme (Laane, et al. 1987 : 81). Pogorevc, Stecher and Faber (2002 : 857) suggested that the deactivation effect exerted by protic solvents such as methanol on enzyme stability and activity is usually underestimated. Solvents with log *P* value ($2 \geq \log P \leq 4$) are less polar and more hydrophobic, very erratic, and less likely to distort the water layer, resulting in exceptionally modest

enzyme-solvent interaction, and are thus best suitable for enzymatic reactions. Organic solvents with log $P > 4$ are more viscous and less favorable for catalysis (Laane, et al. 1987 : 81). It has also been shown that the stability, and activity of the enzyme are less predietable in medium polar solvents and may depend more on the enzyme itself than the nature of the organic solvent (Laane, et al. 1987 : 81). Pogorevc, Stecher and Faber (2002 : 857) since the little deactivation associated with these organic solvents could not necessarily be correlated to catalytic activity in such environment.

7. Used cooking oil

Used cooking oil normally undergoes repeated mixing with fresh oil for continued apply in local restaurants before being discarded. At homes, used cooking oil is not usually kept for mixing and is discarded onto local drainage systems. The oil disposal contributes to environmental pollution by causing local pollution of the water bodies in which the drainage systems end. Hence, conversion of the used cooking oil into biodiesel automatically eliminates these problems. Used cooking oil is abundantly available and can be easily obtained free of cost, if not at a minimal price. As a result, the processing cost of biodiesel gets decreased drastically (Dhawane, Kaemakar, Ghosh and Halder 2018 : 3971).

8. Biodiesel

Increased demand for energy, increasing price of crude oil, global warming due to emission of green house gases, environmental pollution, and fast diminishing supply of fossil fuels have led to the development of alternate fuels, especially biofuels like ethanol and biodiesel (Abbaszaadeh, Ghobadian, Omidkhah and Najafi. 2012 : 138; Lee, Posarac and Ellis. 2011 : 2826). Owing to its environmental advantages and the increase in petroleum price, a rapid increase in biodiesel production is observed. Until 2010, the supply of biodiesel was equivalent to its demand and it is estimated that in future, the demand would outweigh its production and increase in all regions of the world from 40.5 billion liters (2015) to 58.5 billion liters by 2020 (Figure 8) (Aarthy, Saravanan, Gowthaman, Rose and Kamini. 2014 : 1591).

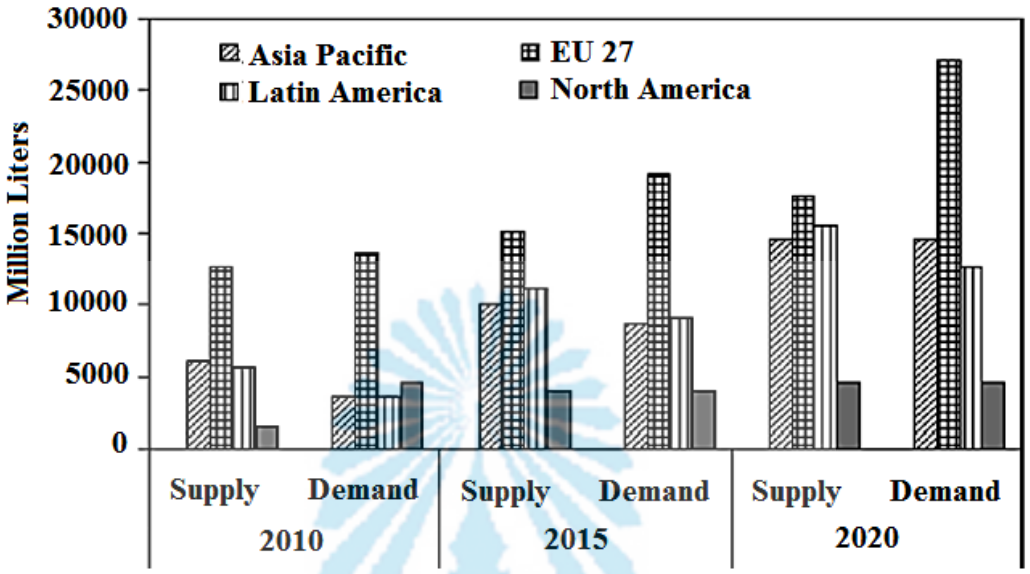


Figure 8. Supply and Demand of Biodiesel in Different Regions, 2010-2020

(Source : Aarthy, et al. 2014 : 1591)

8.1 Process or Method to Produce Biodiesel

Considerable efforts have been made to develop vegetable oil derivatives that approximate the properties and performance of hydrocarbons-based diesel fuels. The problem with substituting triglycerides for diesel fuel is mostly associated with high viscosity, low volatility and polyunsaturated characters. These can be changed in at least four ways: pyrolysis, microemulsion, dilution and transesterification (Aarthy, et al. 2014 : 1591).

8.1.1 Pyrolysis

Pyrolysis is a method of conversion of one substance into another by mean of heat or by heat with the aid of the catalyst in the absence of air or oxygen (Sonntag. 1979 : 1). The process is simple, wasteless, pollution free and effective compared with other cracking processes.

8.1.2 Microemulsion

A micro emulsion define as a colloidal equilibrium dispersion of optically isotropic fluid microstructure with dimensions generally into 1–150 range formed spontaneously from two normally immiscible liquids and one and more ionic or more ionic amphiphiles (Schwab, Dykstra, Selke, Sorenson and Pryde. 1988 : 1781). They can improve spray characteristics by explosive vaporization of the low boiling constituents in micelles (Pryde. 1984 : 1609; Ziejewski, Kaufman, Schwab and Pryde. 1984 : 1620). The engine performances were the same for a microemulsion of 53% sunflower oil and the 25% blend of sunflower oil in diesel (Ziejewski, Kaufman and Pratt. 1983 : 106). A microemulsion prepared by blending soybean oil, methanol, and 2-octanol and cetane improver in ratio of 52.7:13.3:33.3:1.0 also passed the 200 h EMA test (Aarthy, et al. 2014 : 1591).

8.1.3 Transesterification

Transesterification (also called alcoholysis) is the reaction of a fat or oil with an alcohol to form esters and glycerol. A catalyst is usually used to improve the reaction rate and yield (Table 3). Excess alcohol is used to shift the equilibrium toward the product because of reversible nature of reaction. For this purpose primary and secondary monohybrid aliphatic alcohols having 1-8 carbon atoms are used (Sprules and Price. 1950 : 366).

8.1.3.1 Alkali Catalyzed Transesterification

In transesterification method we can use different catalyst. The reaction mechanism for alkali catalyzed transesterification was formulated in three steps (Eckey. 1956 : 575). The first step is an attack on the carbonyl carbon atom of the triglycerides molecule by the anion of the alcohol (Methoxide ion) to form a tetrahedral intermediate reacts with an alcohol (Methanol) to regenerate the anion of alcohol (Methoxide ion). In the last step, rearrangement of tetrahedral intermediate results in the formation of a fatty acid ester and a diglyceride. When NaOH, KOH, K_2CO_3 or other similar catalysts were mixed with alcohol, the actual catalysts, alkoxide group is formed (Sridharan and Mathai. 1974 : 178). Kim, et al. (2004 : 315) have developed a process for the production of biodiesel from

vegetable oils using heterogeneous catalyst $Na/NaOH/Al_2O_3$. These catalysts showed almost the same activity under the optimized reaction conditions compared to conventional homogeneous NaOH catalyst. For an alkali catalyzed transesterification, the glyceride and alcohol must be substantially anhydrous (Wright, Segur, Clark, Coburn, Langdon and Dupuis. 1994 : 145) because water makes the reaction partially change to saponification, which produces soap.

A number of researchers have worked with feed stokes that have elevated free fatty acid levels (Cravotto, Binello, Merizzi and Avogadro. 2004 : 147; Mittelbach, Pokits and Silberholz. 1992 : 74; Mittelbach and Tritthart. 1988 : 1185). However, in most cases, alkaline catalysts have been used and the free fatty acids were removed from the process stream as soap and considered waste. Waste greases typically contain from 10% to 25% free fatty acids. This is far beyond the level that can be converted to biodiesel using an alkaline catalyst.

Table 3 Comparison of the Chemical and Enzymatic Method for Production of Biodiesel

Parameter	Chemical Method		Enzymatic Method
	Acid Process	Alkaline Process	
Biodiesel Yield	>90%	>96%	>96%
Free Fatty Acid Content in the Substrate	Converted to Biodiesel	Soap Formation	Converted to Biodiesel
Water in the Substrate	Interference with Reaction	Interference with Reaction	No Influence
Purification of Methyl Esters	Repeated Washing	Repeated Washing	None
Clycerol Recovery	Complex, Low Grade Glycerol	Complex, Low Grade Glycerol	Easy, High Grade Glycerol
Reaction Rate	Slow	High	Low
Reaction Temperature	>100^{o}C	60-80^{o}C	20-50^{o}C
Catalyst Recovery	Difficult, the Catalyst Ends up in the By-Products	Difficult	Easy
Catalyst Reuse	No Reusability	Partially Lost in Post-Processing Steps	Reusable
Cost of Catalyst	Low	Low	High
Waste Water Generation	High	High	Low

(Source : Gog, Roman, Tosa, Paizs and Irimie. 2012 : 10)

8.1.3.2 Acid Catalyst Transesterification

An alternative process is to use acid catalyst that some researchers have claimed are more tolerant of free fatty acids (Freedman and Pryde. 1982 : 17; Aksoy, Kahraman, Karaosmanoglu and Civelekoglu. 1988 : 936; Liu. 1994 : 1179). However, it can be extended to di- and triglycerides. The protonation of carbonyl group of the ester leads to the carbocation, which after a nucleophilic attack of the alcohol produces a tetrahedral intermediate. This intermediate eliminates glycerol to form a new ester and to regenerate the catalyst. We can use acid alkali and biocatalyst in transesterification method. If more water and free fatty acids are in triglycerides, acid catalyst can be used (Keim. 1945 : 383). Transmethylation occur approximately 4000 times faster in the presence of an alkali catalyst than those catalyzed by the same amount of acidic catalyst (Formo. 1945 : 548).

8.1.3.3 Lipase Catalyst Transesterification

This transesterification process is like alkali transesterification, only ratio of catalyst and solvent a stirring time different, and in this transesterification we have used lipase catalyst. The process is explained in the following Figure 9. Various studied have been conducted for transesterification reaction for different catalyst, alcohol and molar ratios at different temperature. The results and experimental conditions of some studies are summarized in Table 4.

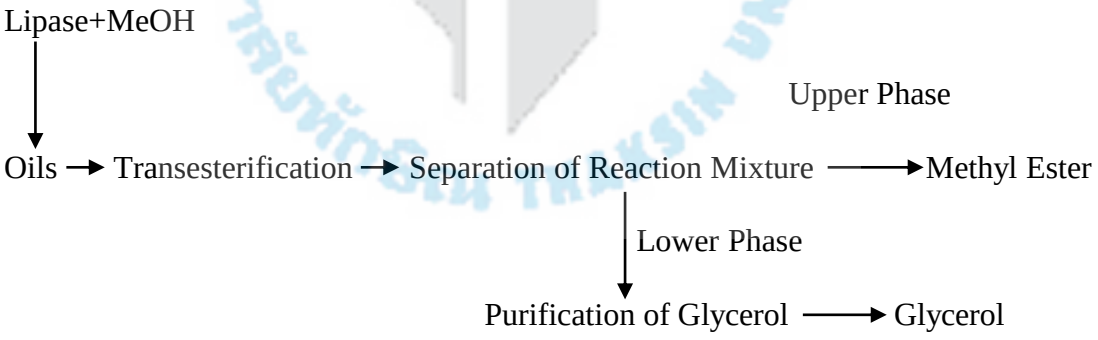


Figure 9. Flow Diagrams Comparing Biodiesel Production Using Lipase-Catalysis

(Source : Fukuda, Kondo and Noda. 2001 : 405)

Table 4 Biodiesel Production Catalyzed by Lipases Using Different Raw Materials

Source of Lipase	Source of Oil	Processing Conditions						Reference
		Alcohol	Temperature (°C)	Time (h)	Molar Ratio of Alcohol to Oil	Water Content	Yield (%)	
Novozym 435	Palm Oil	Methanol	55	24	10:1	-	90.5	Zhang, Sun, Xin, Sheng and Liu (2010 : 3960)
Burkholderia cepacia	Palm Oil	Methanol	30	72	7:1	1 g	100	Jegannathan, Yun-Lee, Chan and Ravindra (2010 : 2272)
Pseudomonas cepacia G63	*Sapium sebiferum* Oil	Methanol	41	12	4:1	7%	96.22	Li and Yan (2010 : 3148)
Burkholderia sp.	Olive Oil	Methanol	40	12	4:1	10%	70%	Liu, Huang, Wang, Lee and Chang (2012 : 41)
Burkholderia cepacia	*Jatropha curcas* L. Oil	Methanol	35	24	6.6:1	7%	94	You, et al. (2013 : 202)
Burkholderia cepacia	*Jatropha curcas* L. Oil	Ethanol	35	24	10:1	1 g	100	Abdulla and Ravindra (2013 : 8)
Candida sp. 99-125	*Cinnamonum camphora* Seed oil	Ethanol	40	24	3.5:1	10%	93.5	Liu, Deng, Wang, Nie, Liu, Tan and Wang (2014 : 1215)
Rhizopus oryzae	*Calophyllum inophyllum* Oil	Methanol	35	72	12:1	15%	92	Arumugm and Ponnusami (2014 : 276)
Thermomyces lanuginosus	Palm Oil	Methanol	50	24	4.7:1	3.4%	97.2	Raita, Arnthong, Champreda and Laosiripojana (2015 : 189)
Novozym 435	White Mahlab Seeds	Methanol	40	5	5:1	0.75%	97.80	Sbihi, Nehdi, Blidi, Rashid and Al-Resayes (2015 : 1049)
Thermomyces lanuginosus	Soybean Oil	Ethanol	40	7	4:1	2%	96%	Mukherjee and Gupta (2016 : 166)
Candida sp. 99-125	Waste Cooking Oil	Methanol	40	9	1.1:1	-	97.11	Zhang, Zheng, Liu, Liu and Tan (2016 : 446)
Aspergillus niger	*Scenedesmus obliquus* Lipids	Methanol	35	36	5:1	2.5%	90.82	Guldhe, Singh, Kumari, Rawat, Permaul and Bux (2016 : 1002)

8.2 Characterization of Biodiesel

After biodiesel conversion by the above mentioned processes, the characteristics of the biodiesels need to be investigated. The properties of biodiesel are characterized by physico-chemical properties. The properties of non-edible biodiesel depend on the type of feedstock, their chemical compositions as well as fatty acid compositions which cause conspicuous effects on engine performance and emissions (Atabani, Silitonga, Ong, Mahlia, Masjuki, Badruddin and Fayaz. 2013 : 211; Borugadda and Goud. 2012 : 4763; Jena, Raheman, Prasanna and Machavaram. 2010 : 1108; Hayyan, Alam, Mirghani, Kabbashi, Hakimi, Siran and Tahiruddin. 2011 : 920). The research is ongoing worldwide to meet the biodiesel standards. There are two international standards available, namely the American Society for Testing and Materials standard (ASTM D 6751-3) and the European Standard (EN 14214) for B100 biodiesel (Atabani, et al. 2013 : 211; Sanjid, Masjuki, Kalam, Rahman, Abedin and Palash. 2013 : 664; Atadashi, Aroua and Aziz. 2010 : 1999). However, many countries use their own standards for biodiesel such as Japan (JISK 2390), Germany (DIN 51606), China (GB/T20828), Austria (ONC1191), Malaysia (MS 2008), Indonesia (SNI Biodiesel no.04-7182), Thailand (DOEB), Vietnam (TCVN 7717), Republic of Korea (KS M) etc. (Atabani, et al. 2013 : 211; Lin, Cunshan, Vittayapadung, Xiangqian and Mingdng. 2011 : 1020; Atadashi, Aroua and Aziz. 2010 : 1999). The ASTM, European and DOEB standard biodiesel specifications are presented in Table 5.

Table 5 Standard Biodiesel Specification Both ASTM D 6751, EN 14214 and DOEB

Properties Specification	Unit	ASTM D 6751 Standard		EU Standard EN 14214		Thailand Standard (DOEB)	
		Limits	Test Method	Limits	Test Method	Limits	Test Method
Viscosity at 40°C	mm^2/s	1.9-6.0	ASTM D445	3.5-6.0	EN ISO 3104	3.5-5.0	ASTM D445
Flash Point	°C	Min. 130	ASTM D93	Min. 101	EN ISO 3676	Min. 120	ASTM D93
Sulfated Ash Content	% w/w	Max. 0.002	ASTM D874	Max. 0.020	EN ISO 3987	Max. 0.020	ASTM D874
Water and Sediment Moisture	wt%	Max. 0.005	ASTM D2709	500 mg/kg	EN 1412	Max. 0.20	ASTM D2709
Copper Strip Corrosion	3 h at 50°C	Max. No.3	ASTM D130	Class 1	EN ISO 2160	Max. No.1	ASTM D130
Acid Number	mg KOH/g	Max. 0.50	ASTM D664	Max. 0.50	EN 14104	Max. 0.50	ASTM D664
Phosphorous Content	% w/w	Max. 0.001	ASTM D4951	Max. 0.001	EN 14107	Max. 0.001	ASTM D4951
Oxidation Stability	110°C, Hours	-	-	Min. 3.0	EN 14112	Min. 6.0	EN 14112
Ester Content	% w/w	-	-	Min. 96.5	EN 14103	Min. 96.5	EN 14103
Water content	mg/kg	-	-	Max. 500	EN 1412	Max. 500	EN ISO12937
Carbon Residue	% w/w	Max. 0.050	ASTM D4530	Max. 0.3	EN ISO 10370	Max. 0.30	ASTM D4530
Total Contamination	mg/kg	24	ASTM D5452	24	EN 12662	Max. 24	EN 12662
Iodine Value	g Iodine/100 g	-	-	120	EN 14111	Max. 120	EN 14111
Linolenic Acid Methyl Ester	% w/w	-	-	Max. 12.0	EN 14103	Max. 12.0	EN 14103
Monoglyceride Content	% w/w	-	-	Max. 0.8	EN 14105	Max. 0.80	EN 14105
Diglyceride Content	% w/w	-	-	Max. 0.2	EN 14105	Max. 0.20	EN 14105
Triglyceride Content	% w/w	-	-	Max. 0.2	EN 14106	Max. 0.20	EN 14105
Free Glycerol	% w/w	Max. 0.02	ASTM D6584	Max. 0.02	EN 1405/14016	Max. 0.02	EN 14105
Total Glycerol	% w/w	0.24	ASTM D6548	0.25	EN 14105	Max. 0.25	EN 14105
Group I Metals (Na, K^+)	ppm	-	-	Max. 5.0	EN 14108-9	Max. 5.0	EN 14108
Group II Metals (Ca^+, Mg)	ppm	-	-	Max. 5.0	EN 14538	Max. 5.0	EN 14109
Density at 15°C	kg/m^3	870-890	D1298	860-900	EN ISO 3675-12185	860-900	ASTM D1298
Cloud Point	°C	Report[a]	ASTM 2500	Report[a]	-	Report[a]	ASTM 2500
Pour Point	°C	Report[a]	ASTM 97	-	-	Report[a]	ASTM 97

[a] Low temperature properties are not strictly specified, but should be agreed upon by the fuel supplier and purchaser.

(Source : Nakpong and Woothikanokkhan. 2010 : 1806; Hoekman, Broch, Robbins, Ceniceros and Natarajan. 2012 : 143; Azad, Rasul, Khan, Sharma, Mofijur and Bhuiya. 2016 : 302)

9. Ultrasound

Ultrasonics is the term used for sound waves having frequencies higher than the normal human hearing range (i.e., > 18 kHz) (Mason. 1988) Ultrasonic waves propagate in a medium as a series of alternate compression and rarefaction regions of pressure as detailed in Figure 10. The frequency of a sound wave is defined as the number of waves that pass through a single point at unit time (s). Wavelength is the peak to peak distance between two adjacent waves. Ultrasound can be divided into two categories:

1. High power ultrasound: These ultrasonic waves have high power and typically lower frequency. These waves, if applied to liquids, have the potential of producing physical and chemical changes in the liquids. They are used in industry for welding, cleaning, chemical reactions, etc. They typically have a frequency range of 20 kHz –100 kHz.

2. Low power ultrasound: These ultrasound waves typically have high frequency and low power. They do not cause and chemical physical changes. They are used to measure velocity and the absorption coefficient of waves in a medium, and thus are used in medical scanning, imaging, treatments of stains, dentistry, etc. High frequency ultrasonic waves have small wavelengths that enable detection and imaging of small areas with high definition. Frequencies of 1-10 MHz are used for this purpose.

Ultrasonic waves or ultrasound has been used in industry for many years. The first commercial application of ultrasound was in 1917, when it was used for estimating the depth of water through an echo-sounding technique. Ultrasound has found its application in many fields, including:

- Industry: Ultrasonic welding and ultrasonic cleaning are the most common applications of high power ultrasonics. It is also used for drilling, cutting, and grinding. Low power applications include non-destructive testing and flaw detection.

- Medicine: Ultrasound imaging (2-10 MHz) is used in obstetrics, cleaning, drilling of teeth, and muscle strain treatments (20-50 kHz).

Biology and biochemistry: High power ultrasound is used for cell disruption, filtration (e.g. reducing clogging of filters and by increasing filtration

rates), degassing of liquids, crystallization (by producing more uniform and smaller crystals in supersaturated solutions), and dispersion of solids. The use of ultrasonics in chemistry is known as sonochemistry. Research has shown that ultrasonics can accelerate the rate of reaction in many chemical reactions. This is also true for the experiments in this thesis.

Ultrasonics do not directly react with liquids in a chemical reaction but it induces several physical effects in the liquid that help in increasing the reaction rate; namely cavitation and streaming, which are detailed in the following sections (Chand. 2008).

9.1 Cavitation

Cavitation is the phenomenon of the generation of large numbers of microbubbles (cavities) in a liquid when a negative pressure is applied. When sound waves propagate through a liquid medium, they generate compression and rarefaction regions in the liquid. The intermolecular distances between the liquid molecules also expand and contract along these waves. At very low pressure in the rarefaction region, the intermolecular spaces exceed the critical molecular distance and the liquid tears apart to form void spaces or micro bubbles. These micro bubbles oscillate with the wave motion and grow in size by taking in vapor from the surrounding liquid medium and by aggregating with other micro bubbles. Within a few cycles they grow to an unstable size and collapse violently, releasing large amounts of energy and creating localized temperatures of up to 5,000°K (Flint and Suslick. 1991 : 1397). The growth and subsequent collapse of cavitation bubbles is shown in Figure 10. The bubble collapse produces high shear forces which mix the liquid vigorously and fracture nearby particles.

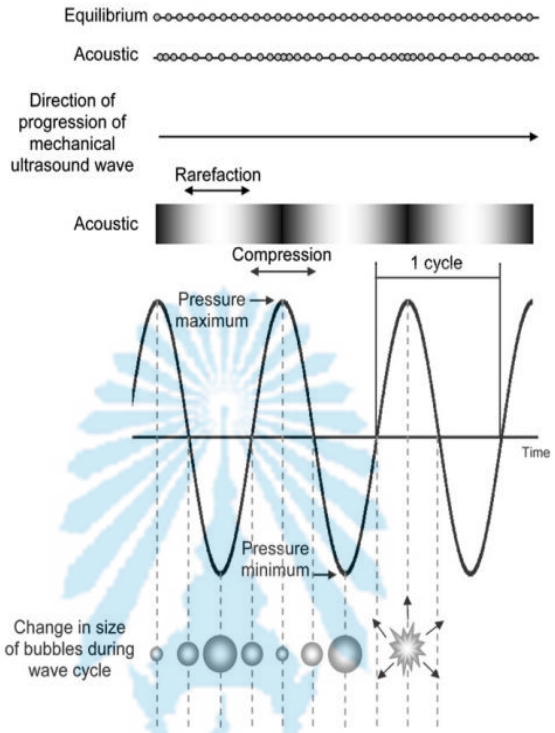


Figure 10. Ultrasonic Cavitation

(Source : Soria and Villamiel. 2010 : 324)

9.2 Acoustic Streaming

When ultrasonic waves are introduced into a liquid, movement of liquid opposite to the direction of ultrasonic waves is observed (Figure 11). The sound energy is converted into kinetic energy and this effect is independent of the cavitation effect. Acoustic streaming helps in heat and mass transfer in the liquid. It facilitates distribution of ultrasonic energy and dissipation of heat (Knothe, Gerpen and Krahl. 2005). In addition to cavitation and acoustic streaming, heat is produced in the liquid by shearing at interfaces such as the interface between the metal horn and liquid.

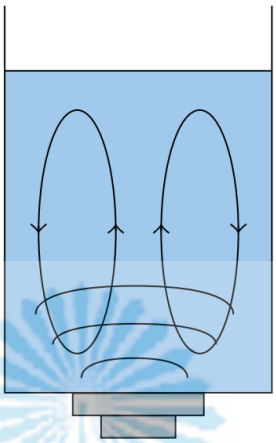


Figure 11. Acoustic Streaming
(Source : Legay, Gondrexon, Person, Boldo and Bontemps. 2011 : 1)

9.3 Ultrasonic Factors

There are many variables involved in the ultrasonic process. All of the main factors to consider in the process affecting the chemical reactions.

9.3.1 Frequency: At the same ultrasonic power input, the high frequencies acoustic streaming makes a weaker cavitation effect than that of low frequencies. As the frequency of sonication increases rapidly, the cavitation strength decreases.

9.3.2 Intensity: Intensity is an energy transmitted through a cross sectional area per time. When the distance between ultrasonic sources are increased, the intensity will be decreased exponentially.

9.3.3 External pressure: External pressure is press the cavity in the liquid medium during the cycle of compression and rarefaction. The cavitation may not reach to unstable point and not collapse.

9.3.4 Vapor pressure of liquid medium: The liquid medium with high vapor pressure tends to reduce the violence of implosion because the bubbles are filled with medium vapor and absorb some of cavitation energy. As the higher vapor pressure, the violence of cavitation effect will be lower.

9.3.5 Temperature: Higher operating temperature will increase the vapor pressure of liquid medium which reduce the violence of cavitation.

9.3.6 Viscosity of liquid medium: As the higher of viscosity, the harder to generate cavitation because viscous medium has higher cohesive force between the molecules.

9.3.7 Dissolved gas in liquid medium: Dissolved gas help to nucleate the cavity. Monoatomic gases such as helium, argon with higher heat capacity will provide the greater effect than diatomic gases such as nitrogen, oxygen (Poosumas. 2014)

9.4 Relation between Frequency of Ultrasonic Waves and Power

The frequency of ultrasonic wave propagation is inversely proportional to available power. In more detail high power is typically generated at lower frequencies of 20 kHz to 100 kHz. As the frequency increases, the size of the converter decreases and power density (power per unit volume) of the converter increases. Because converters are 95-96% efficient, a small amount of energy is dissipated as heat from the converter surface. At high frequencies, the smaller converter results in higher energy densities and promotes heating of the converter, which causes internal stresses in the transducers. To maintain proper balance, high frequency transducers are designed for lower power dissipation applications.

High frequency waves cannot effectively be used for producing cavitation effects. At higher frequency, the rarefaction or low pressure phase is very short in duration. It does not allow micro bubble formation and therefore cavitation does not effectively occur. Therefore, higher amplitude (or power) is required to produce the same cavitation effects as produced at lower frequencies. Because high power at high frequency cannot be obtained owing to the limitations discussed in last paragraph, cavitation cannot be produced at high frequencies.

9.5 Enhancing Transesterification Reaction Using Ultrasound

Transesterification reactions involve reactions between oil and alcohol in the presence of a catalyst. Oil and methyl alcohol are immiscible liquids and form separate layers when mixed together in a vessel. Traditional transesterification reaction requires mixing continuously for long periods of time to facilitate the reaction between oil and alcohol, because the reaction can take place

only in the interfacial region between the two liquids. When this mixture is sonicated, ultrasonic waves produce cavitation at these interfacial areas. As a result, an emulsion of oil and alcohol forms, providing large surface areas for reaction. It is observed that reaction time is reduced significantly (Chand. 2008).

9.6 Ultrasonic Assisted Reactor

The ultrasonic assisted reactor has been promised as one of the suitable technologies for the production of biodiesel. The research of Gogate and Kabadi (2009 : 60) shown that ultrasonic wave is induced cavity in the fluid with the repeated compression and expansion until reached the unstable state before collapse which generates the energy for mixing and stimulating the reactions. A mixing of immiscible is effective enhancing of mass transfer.

In addition from reducing restrictions on the exchange of material, there are many research results indicate that the use of ultrasonic assisted reactor increases the reaction rate in a shorter reaction time and also reduces the amount of catalyst and alcohol in the system.

Bhangu, Gupta and Askokkumar (2017 : 305) produced biodiesel from canola oil and methanol catalyzed by lipase from *Candida rugosa* under different ultrasonic experimental conditions using horn (20 kHz) and plate (22, 44, 98 and 300 kHz) transducers. The effects of experimental conditions such as horn tip diameter, ultrasonic power, ultrasonic frequency and enzyme concentrations on biodiesel yield were investigated. The results showed that the application of ultrasound decreased the reaction time from 22–24 h to 1.5 h with the use of 3.5 cm ultrasonic horn, an applied power of 40 W, methanol to oil molar ratio of 5:1 and enzyme concentration of 0.23 w/w% of oil. Low intensity ultrasound is efficient and a promising tool for the enzyme catalyzed biodiesel synthesis as higher intensities tend to inactivate the enzyme and reduce its efficiency.

Choedkiatsakul, Ngaosuwan, Cravotto and Assabumrungrat (2014 : 1585) studied the production of biodiesel from palm oil using a combined mechanical stirred and ultrasonic reactor (MS–US). The incorporation of mechanical stirring into the ultrasonic reactor explored the further improvement the transesterification of palm oil. Initial reaction rate values were 54.1, 142.9 and 164.2 mmol/L min for the

mechanical-stirred (MS), ultrasonic (US) and MS–US reactors, respectively. Suitable methanol to oil molar ratio and the catalyst loading values were found to be 6 and 1 of oil, respectively. The effect of ultrasonic operating parameters; i.e. frequency, location, and number of transducer, has been investigated. Based on the conversion yield at the reactor outlet after 1 h, the number of transducers showed a relevant role in the reaction rate. Frequency and transducer location would appear to have no significant effect. The properties of the obtained biodiesel (density, viscosity, pour point, and flash point) satisfy the ASTM standard. The combined MS–US reactors improved the reaction rate affording the methyl esters in higher yield.

Transesterification of used frying oil with methanol, in the presence of potassium hydroxide as a catalyst has been investigated using low frequency ultrasonic reactor (20 kHz). Effect of different operating parameters such as alcohol–oil molar ratio, catalyst concentration, temperature, power, pulse and horn position on the extent of conversion of oil have been investigated. The optimum conditions for the transesterification process have been obtained as molar ratio of alcohol to oil as 6:1, catalyst concentration of 1 w%, temperature as 45°C and ultrasound power as 200W with an irradiation time of 40 min. (Hingu, Gogate and Rathod. 2010 : 827)

The frequency of the ultrasonic wave and input power are usually in the range of 20-50 kHz and 10-1500 W, respectively. Hanh, Dong, Okitsu, Nishimura and Maeda (2009 : 766) conducted experiments to confirm that the transterification can occur at room temperature by using the ultrasonic assisted reactor at a frequency of 40 kHz with a power of 1200 W provided the 95% of oil conversion in the 25 min of reaction time. This experiment showed the effective of biodiesel production, but if considered in terms of energy input to the system through ultrasonic assisted reactor is not economic because, the energy input to the system via ultrasonic irradiation is higher than the energy needed to raise the temperature of the substance directly at the same biodiesel production effective. This is more likely due to some energy for ultrasonic assisted reactor is transformed in to other forms of energy such as heat, sound or absorb by a reactor material. Table 6 shows the research studies performance of ultrasonic assisted transterification reaction for biodiesel production.

Table 6 Research Studies of Ultrasonic Assisted Transesterification Reaction for Biodiesel Production

Freedstock	Catalyst (%wt)	Temperature (°C)	Alcohol/Oil molar ratio	Time (min)	Frequency (kHz)/Power (W)	Yield (%)	Reference
Palm Oil	3% CaO	65	Methanol 15:1	60	20/200	77.3	Mootabadi, Salamatinia, Bhatia, Abdullah. (2010 : 1818)
Palm Oil	3% BaO	65	Methanol 15:1	60	20/200	95.2	Mootabadi et al. 2010 : 1818
Palm Oil	3% SrO	65	Methanol 15:1	60	20/200	95.2	Mootabadi et al. 2010 : 1818
Purified Palm Oil	3% CaO	65	Methanol 6:1	60	40/160	80	Choedkiatsakul, Ngaosuwan and Assabumrungrat. (2013 :22)
Purified Palm Oil	3% K_3PO_4	65	Methanol 6:1	60	40/160	70.85	Choedkiatsakul et al. 2013 :22
Purified Palm Oil	6% Immobilized Novozym 435	40	Methanol 6:1	240	40/250	96	Yu, Tian, Wu, Wang, wang, Ma and Fang. (2010 : 519)
Soybean Oil	5% Immobilized Novozym 435	60	Ethanol 3:1	240	37/132	90	Batistella, Lerin, Brugnerotto, Danielli, Trentin, Popiolski, Treichel, Oliveira and Oliveira. (2012 : 452)
Waste Cooking Oil	Novozym 435	60	Dimethyl Carbonate 6:1	240	25/200	86.61	Gharat and Rathod. (2013 : 900)
Sunflower Oil	5% Lipozyme TL-IM	40	Methanol 3:1	120	45/120	96	Subhedar, Botelho, Ribeiro, Castro, Pereira, Gogate and Cavaco-Paulo. (2015 : 530)
Canola Oil	0.23% lipase from *Candida rugosa*	-	Methanol 5:1	90	22/40	95	Bhangu, Gupta and Askokkumar. (2017 : 305)

10. Applications of Lipase

Lipases form an integral part of the industries ranging from food, dairy, pharmaceuticals, agrochemical and detergents to oleo-chemicals, tea industries, cosmetics, leather and in several bioremediation processes (Table 7) (Verma, Thakur and Bhatt. 2012 : 88).

Table 7 Industrial Applications of Lipase

Industry	Action	Product of Application
Dairy Food	Hydrolysis of milk, fat, cheese ripening, modification of butter fat	Development of flavoring agent in milk cheese and butter
Bakery food	Flavor improvement	Shelf life prolongation
Food dressing	Quality improvement	Mayonnaise dressing and whippings.
Health food	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish product fat removal.
Laundry	Reducing biodegradable strains	Cleaning cloths
Cosmetics	Esterification	Skin and sun-tan creams, bath oil etc.
Surfactants	Replaces phosphoilpases in the production of lysophospholipids	Polyglycerol and carbohydrates fatty acid esters used as industrial detergents and as emulsifiers in food formulation such as sauces and ice creams.
Fuel industries	Transesterification	Biodiesel production
Pollution control	Hydrolysis and transesterification of oils and grease	To remove hard stains, and hydrolyze oil and greases.

(Source : Verma, Thakur and Bhatt. 2012 : 88)

Objective of the Study

1. To characterize the biochemical properties of lipase from fish viscera.
2. To study the effect of extraction media on recovery of lipase from fish viscera.
3. To investigate the use of T-ATPS for partitioning and recovery of lipase from fish viscera.
4. To study transesterification reaction using used cooking oil and partially purified lipase from fish viscera.
5. To study the effect of ultrasonic irradiation on lipase-catalyzed biodiesel production.

Significance of the Study

1. The information regarding the biochemical characteristics of lipase from fish viscera was obtained.
2. Lipase from fish viscera could be recovered using T-ATPS.
3. Biodiesel from used cooking oil was produced by lipase from fish viscera and ultrasound.

Definition of Terms

Viscera	:	The organs in the cavities of the body, especially those in the abdominal cavity.
Lipase	:	Enzyme catalyzes the hydrolysis of triacylglycerols at the oil-water interface to release glycerol and free fatty acids.
T-ATPS	:	A conventional polymer (polyethylene glycol; PEG) phase was replaced with a thermoseparating copolymer; composed of ethylene oxide and propylene oxide (EOPO) and salts. In this system, enzyme purification is concerned with two step process.
Ultrasound	:	Ultrasound is the term used for sound waves having frequencies higher than the normal human hearing range.
Biodiesel	:	Biodiesel is defined as the non-petroleum-based dieselfuel consisting of fatty acid alkyl esters, typically made by transesterification of vegetable oils or animal fats with alcohols, which could be used alone, or blended with routine petrodiesel in unchanged diesel-engine vehicles.

CHAPTER 2

Lipolytic Activity of Viscera Extract from Three Freshwater Fish Species in Phatthalung: Comparative Studies and Potential Use as Dishwashing Detergent Additive

1. Abstract

Lipolytic activities of viscera extract from three freshwater fish species including hybrid catfish, striped snake-head fish and Nile tilapia were studied. Optimal activity from all species was at pH 7.0-8.5 and 40-45^{o}C. Among all species tested, Nile tilapia showed the highest activity, followed by striped snake-head fish and hybrid catfish. The enzymes were stable to heat treatment up to 40^{o}C and over a pH range of 7.0-10.0 for 30 min. Lipases from the viscera of three fish species were enhanced in the presence of NaN_3. However, the lipase activities from all species were inhibited by $MgCl_2$, $MnCl_2$, $HgCl_2$, $AlCl_3$, $CuCl_2$, EDTA, EDAC, DEPC, PMSF, CTAB, sodium percarbonate and sodium polyacrylate. The Nile tilapia lipase was more stable against surfactants than both striped snake-head fish and hybrid catfish lipases. The effect of extractants on lipase recovery from Nile tilapia viscera was studied and its stability in the presence of different commercial dishwashing detergents was also investigated. Nile tilapia viscera powder isolation with 50 mM Tris-HCl, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35 gave a higher lipase recovery than other extractants tested ($P<0.05$). The Nile tilapia lipase exhibited substantial stability and compatibility with tested commercial dishwashing detergents for 15-30 min. Considering its characteristics, Nile tilapia crude enzyme containing lipase activity may be considered a potential candidate for future application in detergent processing industries.

2. Introduction

The growing consumer demand for healthy fish products has led to a thriving fish processing industry worldwide. Processing of fish includes: scaling, cutting, filleting, cooking, salting and canning. When fish fillets or cans are produced, a large fraction (30-80%) of fish (flesh, heads, bones, fins, skin, tails and viscera) are left to waste. Fish waste is usually disposed of in landfills or in the sea resulting in environmental problems which underscore the need for a proper utilization of fish wastes for the recovery of valuable products. Fish waste can be utilized as ingredients in animal feed and fertilizer (Zhao, Buge, Ghaly, Brooks and Dave. 2011: 2) or used for the recovery of valuable biomolecules, especially enzymes such as proteinase (Klomklao, Benjakul and Visessanguan. 2004 : 355) and lipase (Kuepethkaew, Sangkharak, Benjakul and Klomklao. 2017 : 769).

In Thale Noi, Phatthalung province, Thailand, the freshwater processing industries, especially fermentation and drying, are becoming increasingly important because they are one of the main income generators. Three freshwater fish species commercially used in the processing industries include hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), striped snake-head fish (*Channa striata*) and Nile tilapia (*Oreochromis niloticus*). Fish viscera, byproducts from processing industries, are generated. However, they are also a potential source of lipases. Fish lipases exhibit some characteristics and properties that complement those of lipases from mammalian and microbial sources due to the evolutionary pathways, diets and habitats of fish (Kurtovic, Marshall, Zhao and Simpson. 2009 : 17). In general, fish lipases have cold-adapted properties, which are known to show more catalytic activity and stability than mammalian lipases, thereby making them suitable for applications in food processing at low temperatures (Sae-leaw and Benjakul. 2018 : 9).

Lipases (E.C. 3.1.1.3) belong to a class of hydrolyzes and water soluble enzyme(s) which catalyzes the hydrolysis of insoluble triacylglycerols to generate free fatty acids, diacylglycerols, monoacylglycerolsand glycerol. Lipases catalyze a variety of reactions including hydrolysis, transesterification and interesterification of other esters and the ester synthesis, and exhibit a range of regio-, enantio- and stereoselective transformation properties (Geoffry and Achur. 2018 : 241). Lipases constitute the most important group of biocatalysts for biotechnological applications

(Benjamin and Pandey. 1998 : 1069). Based on their versatility of being activated only when adsorbed on to an oil–water interface, lipases have emerged as a stupendous enzyme to catalyze dynamic reactions with promising uses in organic chemical processing, synthesis of biosurfactants, the oleochemical industry, dairy industry, agrochemical industry, paper manufacturing, nutrition, cosmetics and pharmaceutical processing (Geoffry and Achur. 2018 : 241) as well as synthesis of biodiesel (Kimtun, Yunu, Paichid, Klomklao, Prasertsan and Sangkharak. 2017 :1). Lipases are also added to household and industrial laundry detergents and household dishwashers, where their function is in the removal of fatty residues and cleaning clogged drains. The cleaning power of lipase detergents had increased markedly (Hasan, Shah, Javed and Hameed. 2010 : 9). Recently, lipase from the hepatopancreas of Pacific white shrimp were used as detergent and its efficacy was comparable to commercial laundry detergents (Sae-leaw and Benjakul. 2018 : 9).

Compared with other hydrolytic enzymes (e.g. proteases and carbohydrases), lipases from fish are relatively less studied. Lipases from aquatic animals, especially freshwater fish, are even less well known versus their counterparts from mammalian, plant and microbial sources (Kuepethkaew, et al. 2017 : 769). This study aimed to characterize the biochemical properties of lipase from the viscera of three freshwater fish species, including hybrid catfish, striped snake-head fish and Nile tilapia commonly consumed and used for processing in ThaleNoi, Phatthalung province, Thailand. Their compatibility with various commercial dishwashing detergents was also investigated.

3. Materials and Methods

3.1 Chemicals

Acetone, tris (hydroxymethyl) aminomethane, Brij35, Triton X-100 and gum arabic were acquired from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) and *p*-nitrophenyl palmitate were purchased from Sigma Chemical Co. (St. Loius, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from Fluka (Buchs, Switzerland). The surfactants and other chemicals used were of analytical grade and were procured from Merck (Darmstadt, Germany).

3.2 Preparation and Isolation of Viscera Extract from Freshwater Fish

Whole viscera from three species of freshwater fish including hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), striped snake-head fish (*Channa striata*) and Nile tilapia (*Oreochromis niloticus*) were collected from a local market in Thale Noi, Phatthalung, Thailand. Samples were placed in polyethylene bags and imbedded in a polystyrene box containing ice with an ice/sample ratio of 2:1 (w/w) and transported to our laboratory at Thaksin University, Phatthalung campus within 30 min. Thereafter, the samples were cut and homogenized into powder in three volumes of acetone at -20^{o}C for 30 min according to the method of Kuepethkaew et al., (2017 : 769). The homogenate was filtered in vacuo on Whatman No.4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was left at room temperature until dried and free of acetone odor.

To prepare the viscera extract, the viscera powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g at 4°C for 30 min (Biofuge Stratos Bestell-Nr. 75005289, Germany). The supernatants were collected and referred to as "viscera extract". The viscera extracts from three species of freshwater fish were used for further study.

3.3 Enzyme Assay and Protein Determination

Lipase activity of viscera extract from each fish was determined spectrophotometrically using *p*-NPP as substrate according to the method of Kuepethkaew, et al. (2017 : 769). One volume of 8.0 mM substrate solution in isopropanol was mixed just before use with nine volumes of 50 mM Tris-HCl buffer pH 7.5 containing 0.4% (w/v) Triton X-100 and 0.1% gum arabic. This solution (0.9 mL) was equilibrated at 37°C and the reaction was started by the addition of 0.1 mL of the enzymatic solution. The variation of optical density at 410 nm was monitored against a blank without enzyme using a Shimadzu ultraviolet (UV)-1700 spectrophotometer (Kyoto, Japan).

The amount of liberated *p*-nitrophenol was determined at 410 nm during the first 5 min of reaction. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per minute under the assay conditions. Protein concentration was determined by the Biuret method (Robinson and Hodgen. 1940 : 707) using BSA as a standard.

3.4 pH and Temperature Profiles

Lipolytic activity was measured within the pH range of 6.0-10.0. Different buffers were used for different pH conditions: 0.2 M phosphate buffer for pH 6.0-7.5, 50 mM Tris-HCl for pH 7.5-9.0 and 0.1 M glycine-NaOH for pH 9.0-10.0. The activity was assayed at 37°C for 5 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 45, 50, 55, 60, 70 and 80°C) for 5 min at the optimal pH found for the respective segment.

3.5 Thermal and pH Stability

Viscera extract were incubated at various temperatures (0, 10, 20, 30, 40, 50, 60, 70 and 80°C) for 30 min, followed by cooling in iced water. The residual activities were assayed using *p*-NPP as a substrate and the activities were reported as the relative activities (%) compared with the initial enzyme activities. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activities after incubation at various pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) for 30 min at room temperature. The residual activities were assay with *p*-NPP as previously described.

3.6 Effect of Some Chemicals

Different chemicals ($CaCl_2$, $MgCl_2$, $MnCl_2$, $HgCl_2$, $AlCl_3$, $CuCl_2$, NaN_3, ethyldiaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), 1-(3-Dimethylaminopropyl)-3-ethlcarbodiimide) (EDAC), diethyl pyrocarbonate (DEPC), sodium percarbonate and sodium polyacrylate) were mixed with the enzyme solution to obtain the final concentration of 1 mM (Kuepethkaew et al. 2017 : 769). The control contained the enzyme without the tested chemical. The mixtures were

kept at room temperature for 30 min and the remaining activity was determined using *p*-NPP as a substrate.

3.7 Effect of Some Surfactants

Different surfactants (Tween 20, Tween 80, Triton X-100, gum arabic, sodium dodecyl sulfate (SDS), and cetyltrimethylammonium bromide (CTAB)) were added to the viscera extract to obtain a final concentration of 1 mM. The lipase activity was assayed after incubation for 30 min at room temperature. The residual activity was determined and reported as the relative activity compared with the original activity.

Fish viscera yielding the highest lipase activity were selected for further study.

3.8 Effect of Extraction Media on the Recovery of Lipase from the Viscera of Selected Freshwater Fish

3.8.1 Effect of Extractants

Different extraction media, (distilled water, 50 mM sodium phosphate buffer, pH 7.0, and 50 mM Tris-HCl, pH 7.0) were used to extract lipase from the viscera of selected freshwater fish. The medium was added into the defatted viscera powder at a ratio of 1:9 (w/v) and stirred continuously at 4^{o}C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g at 4^{o}C for 30 min. The lipase activity and protein content in the extracts were determined, and the yield and specific activity of the extracts obtained using different media were compared. The extraction media used for solubilizing the lipase, which was able to extract lipase with the highest yield, was selected for further steps.

3.8.2 Effect of NaCl Concentration

Defatted viscera powder was suspended in 50 mM Tris-HCl, pH 7.0 containing different NaCl concentration (0, 0.25, 0.5, 0.75, 1, 1.25 and 1.50 M) at a ratio of 1:9 (w/v) and stirred continuously at 4^{o}C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g at 4^{o}C for 30 min. The lipase activity and protein content in the extracts were measured. The extraction yield and

specific activity of the extracts were calculated. The extractant showing the highest yield was chosen for further steps.

3.8.3 Effect of Surfactant

Defatted viscera powder was suspended in 50 mM Tris-HCl, pH 7.0 containing 1 M NaCl and 0.2% (v/v) different surfactants (Brij 35, Tween 20, Tween 80, Triton X-100, and SDS) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000 × g at 4°C for 30 min. The lipase activity and protein content in the viscera extracts were measured. The extraction yield and specific activity of the extracts were calculated. The extractant rendering the highest yield was chosen for preparation of crude lipase extract.

3.9 Compatility and Stability with Commercial Dishwashing Detergents

The compatibility and stability with commercial dishwashing detergents of the samples were performed according to the method of Kuepethkaew, et al. (2017 : 769) with a slight modification. The detergents tested were Daiwa® (Standard Manufacturing, Thailand), Sunlight® (Uniliver, Thailand), Lipon F® (Lion, Thailand), Tesco® (Mit Mongkol Industry, Thailand) and Teepol® (Sherwood Corporation, Thailand). The lipases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65^{o}C prior to addition of the enzyme preparation. The viscera extract was incubated with different detergents for 15 and 30 min at room temperature, and then the remaining activities were determined under the standard assay conditions. The enzyme activity of the control without dishwashing detergent, incubated under the similar conditions, was taken as 100%.

3.10 Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie. 1980). For pair comparison, T-test was used. Statistical analysis was performed using a SPSS package (SPSS 11.5 for windows, SPSS Inc., Chicago, IL, USA).

4. Results and Discussion

4.1 pH and Temperature Profiles

Lipase activities of viscera extract from three freshwater fish species were investigated at different pH values (6.0-10.0). Results revealed that the lipase activity had an optimal pH of 7, 7.5 and 8.5 for hybrid catfish, striped snake-head fish and Nile tilapia, respectively (Figure 12a). These activities from all fish were reduced drastically above their optimal pH. A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules (Klomklao, Benjakul, Visessanguan, Kishimura and Simpson. 2006 : 5617). The optimum pH value for the three fish species lipase activity was close to those described for other fish species such as grey mullet viscera (*Mugil cephralus*; pH 8.0) (Aryee, Simpson and Villalonga. 2007 : 394), Pacific white shrimp hepatopancreas (*Litopenaeus vannamei*; pH 8.5) (Kuepethkaew, et al. 2017 : 769) and neon flying squid hepatopancreas (*Ommastrephes bartramii*; pH 7.0) (Takahashi, Hatano and Sakura. 1996 : 515).

The temperature profiles of viscera extract from all fish are displayed in Figure 12b. The lipase activities were measured at temperatures ranging from 20 to 80^{o}C using *p*-NPP as substrate at their optimum pHs. Results showed that the maximum activities of lipase for the hybrid catfish, striped snake-head fish and Nile tilapia were found at temperatures of 40, 40 and 45^{o}C, respectively. These results were similar to those reported for crayfish (40^{o}C) (López-López, Nolasco and Vega-Villasante. 2003 : 337), and whiteleg shrimp (30-40^{o}C) (Rivera-Pérez, del Toro and García-Carreño. 2011 : 99), which were slightly lower compared to marine snail (50^{o}C) (Zarai, et al. 2012 : 2434) and grey mullet (50^{o}C) (Aryee, Simpson and Villalonga. 2007 : 394). However, these results were different from those reported for lipase from neon flying squid and cod (25^{o}C), in which *p*-nitrophenyl laurate and *p*-nitrophenyl myristate were used as substrate, respectively (Aryee, Simpson and Villalonga. 2007 : 394; Takahashi, Hatano and Sakura. 1996 : 515). The difference in temperature optima might be due to several factors including the varying mechanical properties of the homologous lipases and the different substrate used for measurements, since different substrates do exhibit temperature activity differences with enzymes (Kuepethkaew, et al. 2017 : 769).

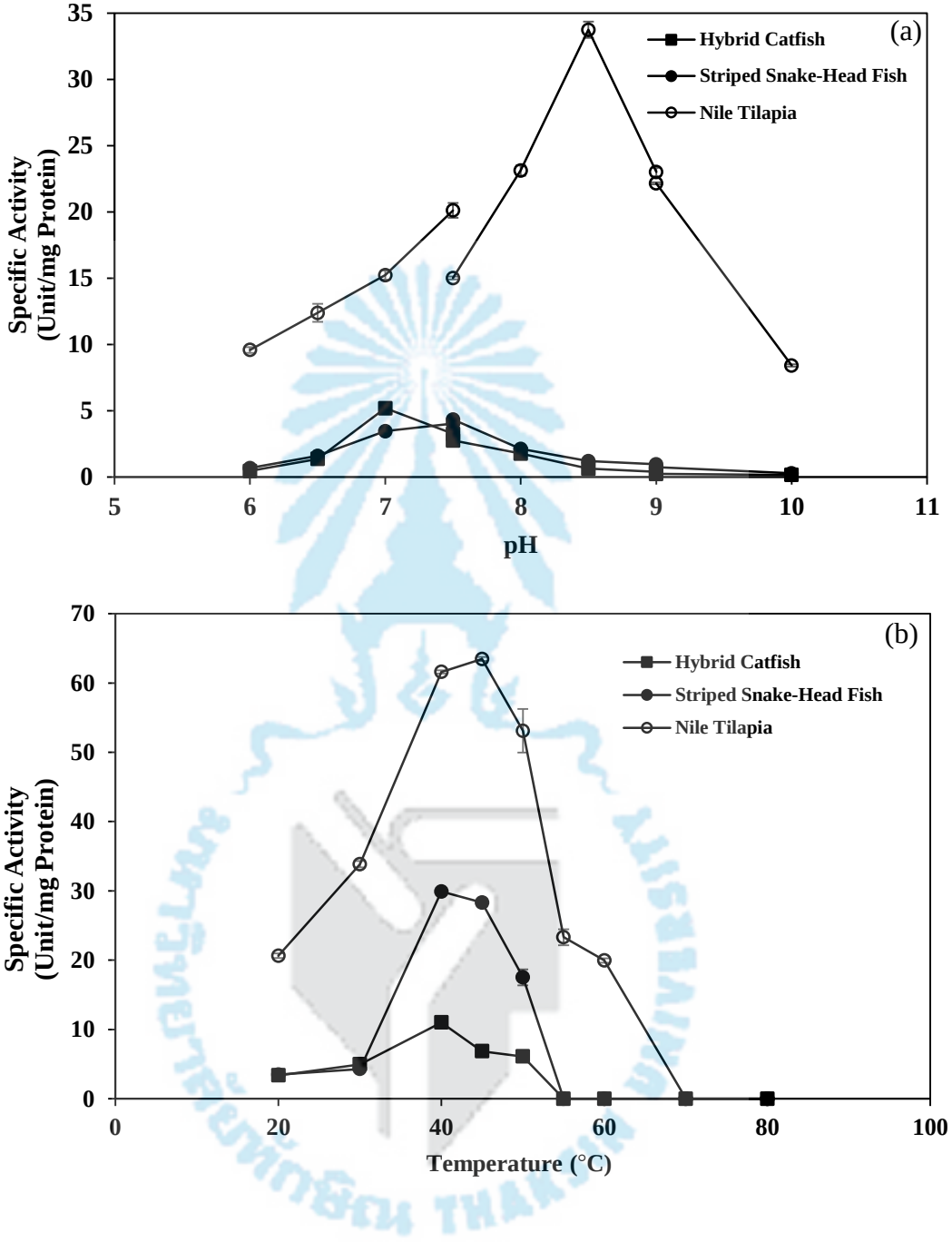


Figure 12. pH (a) and Temperature (b) Profiles of Lipases from the Viscera of Different Freshwater Fish Species. Bars Represent the Standard Deviation from Triplicate Determinations (⊕ = ± 1 SD).

4.2 Lipase Activity in Viscera from Different Fish Species

Viscera lipase activities of all fish species tested are displayed in Table 8. Under their optimal condition, viscera extract of Nile tilapia showed the highest specific activity when *p*-NPP was used as a substrate, followed by those of striped snake-head fish and hybrid catfish, respectively. It is speculated that differences in the level of viscera lipase activity among fish species might be related to growth and physiological change in fish such as metabolism, season, habitat temperature and depth, spawning, age, size and diet (Klomklao, Benjakul and Visessanguan. 2004 : 355).

Table 8 Activity of Viscera Lipases from Different Freshwater Fish Species*

Fish Species	Total Activity ($x10^3$ Unit)	Total Protein (mg)	Specific Activity (Unit/mg Protein)	Lipase Activity ($\times10^3$ Unit/g Tissue)***
Hybrid Catfish	2.72 ± 0.14^{a}	246.99 ± 3.50^{a}	11.01 ± 0.58^{a}	$5.43\pm0.29^{a**}$
Striped Snake-Head Fish	12.87 ± 0.11^{b}	430.24 ± 3.43^{b}	29.91 ± 0.25^{b}	25.81 ± 0.12^{b}
Nile Tilapia	32.21 ± 0.15^{c}	507.54 ± 1.61^{c}	63.46 ± 0.28^{c}	64.42 ± 0.29^{c}

* Activity was analyzed using *p*-NPP as a substrate under their optimal condition.

** The different letters in the same column denote significant differences ($P<0.05$).

*** Mean ± SD from triplicate determinations.

4.3 Thermal and pH Stability

The thermal stability of lipase from all fish viscera extracts are shown in Figure 13a. The enzymes were stable when incubated at temperatures up to 40^{o}C for 30 min. Nevertheless, a sharp decrease in activities was noticeable at temperatures above 40^{o}C. No activity remained at 80^{o}C, suggesting complete loss in activity caused by a thermal denaturation of the lipase. Kuepethkaew, et al. (2017 : 769) reported that lipase from the hepatopancreas of Pacific white shrimp was stable when incubated at temperatures up to 40^{o}C for 30-120 min. The lipase from grey mullet viscera was active within the temperature range of 20-60^{o}C (Aryee, Simpson and Villalonga. 2007 : 394). For pH stability, both hybrid catfish and Nile tilapia viscera extracts were found to be mostly stable in alkaline pH values ranging from 7.0 to 10.0 for 30 min. However, viscera extract striped snake-head fish was stable in the pH range of 7.0-8.0 with an exposure time of 30 min and the lipase stability slightly decreased at a pH value above pH 8.0 (Figure 13b). At a pH value below 7.0, the stability of the enzyme from all fish species sharply decreased. Aryee, Simpson and Villalonga (2007 : 394) reported that lipase was stable between pH 7.0 and 10.0 during incubation at 25^{o}C for 30 min. The lipase from the hepatopancreas of Pacific white shrimp was stable in the pH range of 7.0-10.0 with an exposure time of 30-120 min (Kuepethkaew, et al. 2017 : 769). Both lipase and phosphor lipase from grey mullet viscera were found to be mostly stable in an alkaline pH value ranging from 7.5 to 9.0 (Smichi, et al. 2013 : 87). The stability of the enzyme at a particular pH might be related to the net charge of the enzyme at that pH (Klomklao, et al. 2006 : 5617). At extreme pH levels, strong intramolecular electrostatic repulsion caused by high net charge, results in swelling and unfolding of the protein molecules (Klomklao, Benjakul and Kishimura. 2010 : 711). Since tolerance in the alkaline range of viscera extract from three freshwater fish was observed, this can be considered as a potential candidate for application in processes that are conducted in an alkaline range such as detergent applications (Balaji and Jayaraman. 2014 : 380).

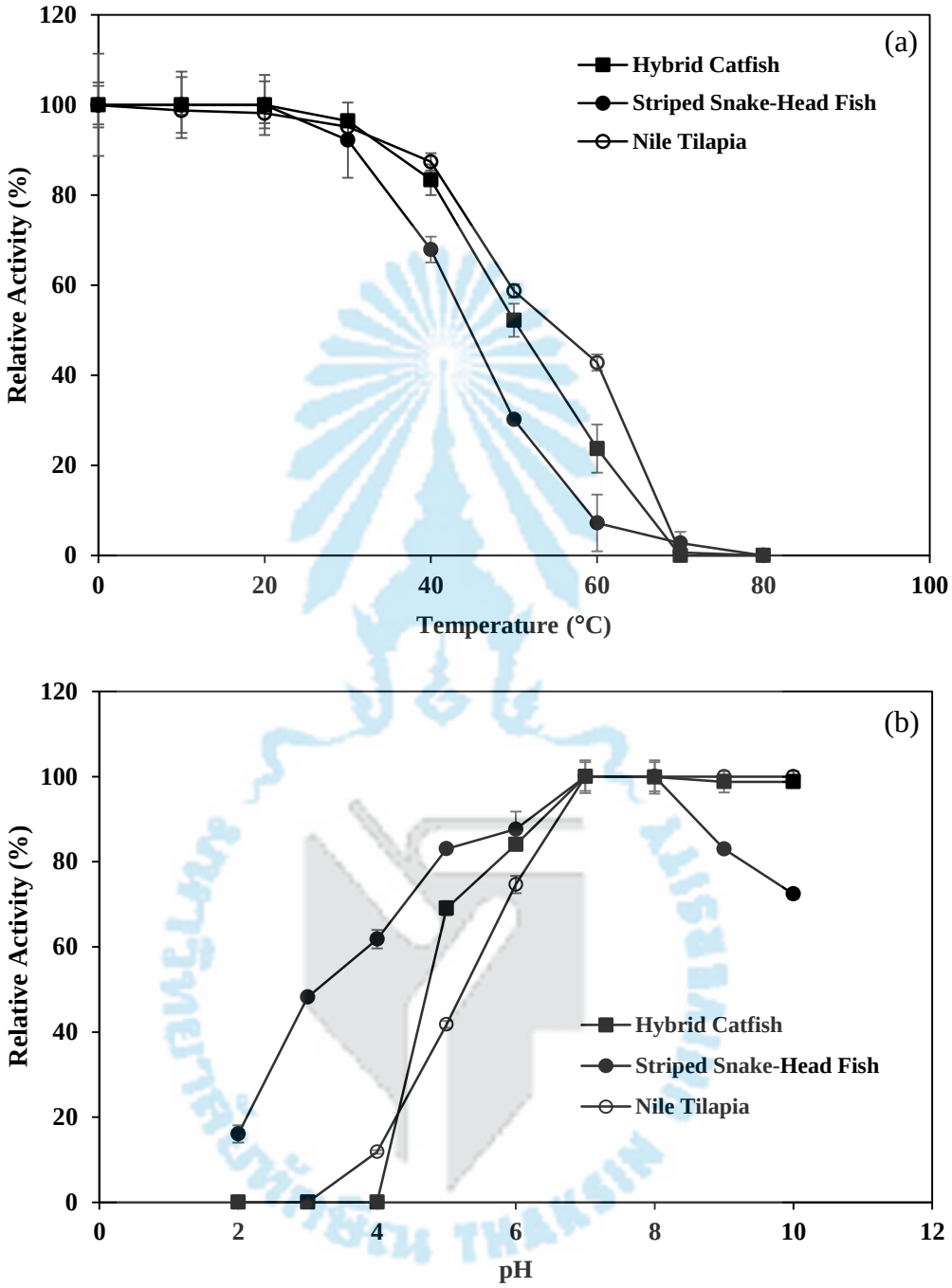


Figure 13. Thermal (a) and pH (b) Stability of Lipases from the Viscera of Different Freshwater Fish Species. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.4 Effect of Some Chemicals on the Lipase Activity

The viscera lipases from Nile tilapia, striped snake-head fish and hybrid catfish were incubated with various compounds and their relative activities were determined after incubation for 30 min at room temperature (Table 9). Generally, similar results were observed in all fish species. The lipase activities from the three fish species were found to increase slightly in the presence of NaN_3, while the lipase retained approximately 107-110% relative activity. Kuepethkaew, et al. (2017 : 769) reported that NaN_3 at concentrations of 1 and 10 mM increased lipase from Pacific white shrimp hepatopancreas by 2 and 17%, respectively. One and 10 mM NaN_3 activated grey mullet by 23 and 30%, respectively (Aryee, Simpson and Villalonga. 2007 : 394).

Various metal ions were also studied for their effects on the lipase activities from the viscera of three freshwater fish species. Treatment with Ca^{2+} at 1 mM inhibited the activity of viscera lipases from both striped snake-head fish and hybrid catfish. Aryee, Simpson and Villalonga (2007 : 394) found that 1 and 10 mM Ca^{2+} decreased grey mullet digestive lipase by 4% and 7%, respectively. However, the viscera lipase from Nile tilapia was slightly enhanced in the presence of Ca^{2+} (110% relative activity), which was in agreement with results obtained from the lipase from halotolerant *Bacillus* sp. VITL8 (Balaji and Jayaraman. 2014 : 67). Calcium ion has been reported to form complexes with ionized fatty acids, changing their solubility and behaviors at interfaces (Gulomova, et al. 1996 : 379). Also, the calcium-induced increase on lipase activity could be attributed to the complex action of calcium ion on the released fatty acids. Moreover, the enzyme structure was stabilized because of the binding of calcium ions to the lipase, bridging the active region to a second subdomain of the protein and, hence, stabilizing the enzyme tertiary structure (Kim, Kim, Lee, Park and Oh. 2000 : 280).

In general, lipases are strongly inhibited by Hg^{2+} (a thiol group inhibitor). This is likely due to the proximity of -SH group to the catalytic and interfacial binding site, but spatially remote from the catalytic site. This may have induced the marked loss of activity (Aryee, Simpson and Villalonga. 2007 : 394). The catalytic effect of lipases might be considered to be of Ser, His, and Glu or Asp residues. Thus, the bulky Hg^{2+} group might cause steric interference to the approach

of the substrate to the active site (Islam, Parveen, Hossain, Khatun, Karim, Kim, Absar and Haque. 2009 : 71). From the results, the viscera lipase of all fish species retained approximately 88%, 30% and 25% relative activity for Nile tilapia, striped snake-head fish and hybrid catfish, respectively. In the case of Mg^{2+}, Mn^{2+}, Al^{3+} and Cu^{2+}, there was a decrease in lipase activities. Other metal ions slightly inhibited lipase activity, which may be due to the fact that transition metal ions change the conformation of the protein to a less stable form (Joseph, Shrivastava and Ramteke. 2012 : 137). The inhibitory nature of metals has been thought to be due to the interaction of ions with charged side chain groups of surface amino acids, thus influencing the conformation and stability of the enzyme (Rahman, Baharum, Salleh and Basri. 2006 : 583).

EDTA at 1 mM had a strong inhibitory effect on the activity of hybrid catfish lipase with only 69% residual activity after incubation at room temperature for 30 min although 1 mM EDTA caused a slight decrease in the activities of viscera lipases from Nile tilapia and striped snake-head fish (13-20%). The effect of group specific reagents (DEPC, PMSF, EDAC) on lipases from the viscera of three freshwater fish was also investigated. Strong inhibition of viscera lipases from three fish species by DEPC, PMSF and EDAC was observed. The results indicated the respective involvement of histidine, serine and carboxylate for their catalytic activity (Ramakrishman, Goveas, Suralikerimath, Jampani, Halami and Narayan. 2016 : 19). Ramakrishnan, et al. (2016 : 19) reported that lipase activity from *Enterococcus faecium* MTCC5695 was inhibited by DEPC, PMSF, EDAC and NBS.

Lipase activities of viscera extracts from all species decreased with the incubation of a bleaching agent (sodium percarbonate) (Table 9). The bleaching agent released hydrogen peroxide, an oxidizing agent, when dissolved into water. This could easily lead to the oxidization of some amino acids and cause the inactivation of enzymes. For sodium polyacrylate, the only dispersing agent tested in this study, the activity of lipases from both striped snake-head fish and hybrid catfish were strongly affected by sodium polyacrylate, whereas Nile tilapia lipase showed more than 60% stability compared to the control, similar to the result of Naganthran, Masomian, Rahman, Ali and Nooh. (2017 : 1577). From the results, the differences in lipase activities inhibited/activated by some chemicals were found, the results probably

implied the differences in binding properties of those chemicals of the lipases in viscera extract of the three freshwater fish species.

Table 9 Effect of Some Chemicals on the Activity of Lipases from the Viscera of Different Freshwater Fish Species*

Substance	Relative Activity (%)***		
	Nile Tilapia	Striped Snake-Head Fish	Hybrid Catfish
Control	100.00±1.82^{h}**	100.00±0.98^{h}	100.00±0.91^{j}
$CaCl_2$	110.50±2.25^{i}	60.93±2.03^{f}	79.24±2.19^{i}
$MgCl_2$	91.21±2.78^{g}	77.00±0.78^{g}	55.78±2.85^{f}
$MnCl_2$	89.21±1.56^{g}	62.73±1.04^{f}	39.62±0.25^{e}
$HgCl_2$	88.71±0.61^{g}	30.52±1.35^{d}	25.84±2.02^{c}
$AlCl_3$	77.61±2.48^{f}	60.82±3.90^{f}	73.99±1.67^{h}
$CuCl_2$	64.40±1.68^{e}	9.21±0.69^{ab}	18.95±1.07^{b}
NaN_3	107.34±1.92^{i}	110.41±2.46^{i}	109.02±3.47^{k}
EDTA	87.42±1.76^{g}	80.46+±2.88^{g}	69.89±3.08^{g}
EDAC	29.31±1.94^{c}	49.88±2.39^{e}	9.27±1.14^{a}
DEPC	33.79±0.61^{c}	11.22±0.95^{ab}	30.19±2.94^{d}
PMSF	14.63±1.31^{a}	12.23±0.32^{b}	15.50±1.13^{b}
Sodium Percarbonate	21.44±1.91^{b}	7.41±0.90^{a}	29.53±0.25^{d}
Sodium Polyacrylate	53.88±2.74^{d}	16.97±1.46^{c}	28.47±2.33^{cd}

* Activity was analyzed using *p*-NPP as a substrate under their optimal condition.

** The different letters in the same column denote significant differences ($P<0.05$).

*** Mean ± SD from triplicate determinations.

4.5 Effect of Some Surfactants on Lipase Activity

Surfactants possess the common characteristic of lowering the surface tension when added in small amounts to water, and this could influence enzyme catalysis. (Aryee, Simpson and Villalonga. 2007 : 394; Ogino, Nakagawa, Shinya, Muto, Fujimura, Yasuda and Ishikawa. 2000 : 451). The effect of surfactants (1.0 mM) on the lipase activity from the viscera of three freshwater fish was investigated after a 30 min duration at room temperature (Table 10). Different surfactants had different influences on lipase activities. For non-ionic surfactants, the viscera lipase from Nile tilapia showed a high stability toward non-ionic surfactants such as Triton X-100, Tween 20 and Tween 80 with the residual activity higher than 83%. However, the viscera lipase from both hybrid catfish and striped snake-head fish were strongly inhibited by these non-ionic surfactants. The lipase from *Penicillium aurantiogriseum* was also inactivated in the presence of Triton X-100 (Lima, Krieger, Mitchell and Fontana. 2004 : 65). The lipase from the hepatopancreas of Pacific white shrimp was evaluated as highly stable against surfactants (Tween 20, Tween 80 and Triton X-100) (Kuepethkaew, et al. 2017 : 769). In this study, gum arabic had no appreciable effect on lipase activity from the viscera of either Nile tilapia or striped snake-head fish at the 1% level, although it caused a slight (26%) decrease in activity from hybrid catfish viscera. Peng, Lin, and Wei (2010 : 733) reported that 1.2% gum arabic increased the lipase activity of *Pseudomonas aeruginosa* CS-2 with a 130% relative activity. Gum arabic is most commonly used for hydrolysis of triacrylglycerol, giving emulsion which can be stored for several weeks. It has generally been assumed that gum arabic was simply an emulsifying agent stabilizing emulsions without interfering with the lipase assay itself (Kuepethkaew, et al. 2017 : 769). In addition, after 30 min of incubation of the viscera lipases of Nile tilapia, striped snake-head fish and hybrid catfish with 1% SDS as an anionic surfactant, the lipases exhibited 71, 38 and 29% of the initial activity, respectively. Hence, SDS destabilized the viscera lipases of all fish species tested in this study. Ungcharoenwiwat and H-Kittikun (2015 : 96) found that 1% SDS inactivated the lipase *Burkholderia* sp. EQ3 activity. The ionic interactions between enzyme and the SDS head group may cause the inactivation of globular protein (Naganthran, et al. 2017 : 1577). The cationic CTAB strongly destabilized lipases of all fish species. Consequently, the viscera lipase of all fish species might

have precipitated and lost its functionality. The stability and improvement of enzymes by surfactants therefore vary depending on the enzymes and their characteristics (Rahman, Rahman, Salleh and Basri. 2013 : 427). Wang, Yu and Xu (2009 : 94) reported that 1% CTAB slightly inhibited the enzyme activity of *B. cepacia* ATCC 2541.

The goal of the present investigation was to produce lipase, which could be suitable for using in dishwashing detergents. Therefore, it is relevant to test lipase stability in the presence of this additive. Overall, Nile tilapia viscera yielded the highest lipase activity (Table 10) and it was evaluated as highly stable against surfactants, delineating itself as a desirable additive for better detergent formulation. Hence, Nile tilapia viscera was selected for further investigation in lipase recovery and its application in dishwashing detergents.

Table 10 Effect of Surfactants on the Activity of Lipases from the Viscera of Different Freshwater Fish Species*

Surfactants	Relative Activity (%)***		
	Nile Tilapia	Striped Snake-Head Fish	Hybrid Catfish
Control	100.00±3.29^{d}**	100.00±7.48^{c}	100.00±3.91^{e}
Tween 20	86.61±2.50^{c}	53.55±1.42^{b}	40.41±2.36^{c}
Tween 80	83.15±1.08^{c}	47.32±1.70^{b}	29.25±2.04^{b}
Triton X-100	86.96±1.28^{c}	35.53±2.94^{a}	39.06±2.89^{c}
Gum Arabic	100.29±1.88^{d}	100.10±3.11^{c}	74.16±2.20^{d}
SDS	71.01±2.55^{b}	38.82±2.04^{a}	29.79±2.03^{b}
CTAB	66.57±0.75^{a}	39.29±0.97^{a}	9.54±1.56^{a}

* Activity was analyzed using *p*-NPP as a substrate under their optimal condition.
** The different letters in the same column denote significant differences (P<0.05).
*** Mean ± SD from triplicate determinations.

4.6 Effect of Extraction Media on the Lipase Recovery from the Viscera of Nile Tilapia

4.6.1 Effect of Extractants

The effect of extraction media on viscera lipase extraction from Nile tilapia is shown in Table 11. Viscera extract using 50 mM Tris-HCl, pH 7.0 showed a higher lipase activity than those extracted with distilled water and 50 mM sodium phosphate buffer, pH 7.0 when *p*-NPP was used as substrate ($P<0.05$). The results suggested that Tris-HCl buffer had a greater efficiency to isolate lipase than sodium phosphate and distilled water. Tris-HCl buffer might favor solubilization of lipase associated with the cell membrane by increasing the charge of enzyme and proteins (Klomklao, Benjakul and Kishimura. 2010 : 711). The repulsion between the enzyme and tissues might lead to the ease of the extraction of lipase from the viscera. Kuepethkaew, et al. (2017 : 769) reported that 50 mM Tris-HCl buffer, pH 7.0 was the optimum medium to dissolve Pacific white shrimp hepatopancreas lipase. From this result, 50 mM Tris-HCl, pH 7.0 was selected as the extractants for the recovery of Nile tilapia viscera lipase since the viscera extract had the maximum lipase activity.

Table 11 Effect of Extraction Media on the Recovery of Lipase from the Viscera of Nile Tilapia*

Extraction Media	Total Activity ($\times 10^3$Unit)	Total Protein (mg)	Specific Activity (Unit/mg Protein)***
Distilled Water	30.38±1.09^{a**}	488.36±5.07^{a}	62.21±2.22^{ab}
50 mM Na-Phosphate, pH 7.0	32.60±1.80^{a}	565.35±2.92^{c}	57.66±3.19^{a}
50 mM Tris-HCl, pH 7.0	35.92±1.22^{b}	536.79±2.03^{b}	66.92±2.28^{b}

*The defatted Nile tilapia viscera powder was extracted in different media at 4°C for 30 min and hydrolytic activity was analyzed using *p*-NPP as substrate at pH 8.5 and 37°C.

**The different letters in the same column denote the significant differences ($P< 0.05$).

***Mean ± SD from triplicate determinations.

4.6.2 Effect of NaCl Concentration

Tris-HCl buffer containing different NaCl concentrations were used to extract the lipase from Nile tilapia viscera (Table 12). The lipolytic activity apparently increased ($P< 0.05$) when the NaCl concentration was increased from 0 to 1 M ($P<0.05$). Addition of salt at low ionic strength can enhance solubility of a protein by neutralizing charges on the surface of the protein, reducing the ordered water around the protein and increasing entropy of the system (Poonsin, Sripokar, Benjakul, Simpson, Visessanguan and Klomklao. 2016 : 1). However, there was no further increase in the lipolytic activity with NaCl above 1 M. The decrease in lipase activity might be because of the enzyme denaturation caused by the salting out effect with increasing concentration of NaCl (Klomklao, Benjakul and Visessanguan. 2004 : 355).

Table 12 Effect of NaCl Concentrations on the Recovery of Lipase from the Viscera of Nile tilapia

NaCl Concentration (M)	Total Activity ($\times 10^3$ Unit)	Total Protein (mg)	Specific Activity (Unit/mg Protein)***
0	35.23±1.44^{b**}	531.23±4.23^{c}	66.32±2.72^{a}
0.25	35.95±1.01^{b}	572.01±2.01^{d}	62.85±1.76^{a}
0.5	36.23±1.27^{bc}	574.18±0.77^{d}	63.10±2.21^{a}
0.75	38.33±1.07^{c}	593.78±1.36^{e}	64.55±1.81^{a}
1	41.89±0.87^{d}	591.00±4.20^{e}	70.88±1.48^{b}
1.25	36.93±1.12^{bc}	499.22±2.81^{b}	73.98±2.24^{b}
1.5	32.67±1.04^{a}	448.82±6.21^{a}	72.79±2.32^{b}

*The defatted Nile tilapia viscera powder was extracted in 50 mM Tris-HCl, pH 7.0 containing different NaCl concentrations at 4°C for 30 min and hydrolytic activity was analyzed using *p*-NPP as substrate at pH 8.5 and 37°C.

**The different letters in the same column denote the significant differences ($P< 0.05$).

***Mean ± SD from triplicate determinations.

4.6.3 Effect of Surfactant

Table 13 shows the influence of some surfactants on the recovery of lipase from Nile tilapia viscera. Addition of SDS, Tween 20, Tween 80 and Triton X-100 in 50 mM Tris-HCl, pH 7.0 containing 1 M NaCl mainly decreased the yield. On the other hand, the highest yield or lipase activity and specific activity were obtained when the Nile tilapia viscera powder was extracted with 50 mM Tris-HCl, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35 ($P < 0.05$). The yield of lipase extracted with the aid of Brij 35 was approximately 1.08-fold higher than that of lipase extracted without Brij 35. Poonsin, et al. (2016 : 1) reported that Brij 35 addition to crude extract from albacore tuna spleen led to a small increase in trypsin activity. The lipase recovery from the hepatopancreas of Pacific white shrimp was strongly enhanced in the presence of Brij 35 (Kuepethkaew, et al. 2017 : 769). The Brij 35 was added to facilitate improved extraction of soluble cell material and to emulsify the small amount of lipid present in viscera extract to prevent lipid interference with the lipase activity (Klomklao, Benjakul and Kishimura. 2010 : 711). Therefore, 50 mM Tris-HCl, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35 was selected as the extraction medium for lipase in Nile tilapia viscera.

Table 13 Effect of some Surfactants on the Recovery of Lipase from the Viscera of Nile Tilapia*

Surfactants	Total Activity ($\times 10^3$ Unit)	Total Protein (mg)	Specific Activity (Unit/mg Protein)***
Control	41.59±0.60^{d**}	593.34±1.56^{e}	70.10±1.21^{b}
SDS	32.57±0.50^{a}	457.02±4.84^{a}	71.27±1.11^{b}
Tween 80	35.47±0.64^{b}	499.92±1.27^{b}	70.95±1.28^{b}
Triton X-100	37.58±0.86^{c}	568.54±11.51^{d}	66.10±1.52^{a}
Tween 20	38.20±0.79^{c}	514.52±5.65^{c}	74.24±1.54^{c}
Brij 35	42.31±0.54^{d}	504.29±4.35^{bc}	83.90±1.06^{d}

*The defatted Nile tilapia viscera powder was extracted in 50 mM Tris-HCl, pH 7.0 containing 1.0 M NaCl in different surfactants at 4°C for 30 min and hydrolytic activity was analyzed using *p*-NPP as substrate at pH 8.5 and 37°C.
**The different letters in the same column denote the significant differences ($P< 0.05$).
***Mean ± SD from triplicate determinations.

4.7 Stability of the Lipase with Commercial Dishwashing Detergents

Due to the high activity and stability of the viscera lipases from Nile tilapia in an alkaline pH range and their relative activity toward different surfactants, viscera lipases may eventually be used as a detergent additive. Hence, the compatibility of viscera lipase from Nile tilapia with some commercial dishwashing detergents (Daiwa®, Sunlight®, Lipon F®, Tesco® and Teepol®) was studied. The viscera lipase was pre-incubated in the presence of various commercial dishwashing detergents for 15 and 30 min at room temperature, and the results are summarized in Figure 14. High lipase resistance for 15 and 30 min was observed because retained activity was above 85%. The viscera lipase retained 98% of its activity in the presence of Daiwa®, Sunlight® and Lipon F® after 15 min incubation at room temperature. At 30 min incubation, the lipase was found more stable in Daiwa® and Sunlight® than those (Lipon F®, Tesco® and Teepol®) of all dishwashing detergents. However, more than 85% of its initial activity was observed in the presence of all dishwashing detergents after 30 min incubation at room temperature. The lipolytic activity varied with each dishwashing detergent, indicating that the enzyme performance in

detergents depends on a number of factors, including the compounds in dishwashing detergents. Hence, the lipase from viscera of Nile tilapia, which is stable and works at alkaline pH with suitable wash conditions for enzymated-detergent powders and liquids, has good potential for use in the dishwashing detergent industry (Hasan, et al. 2010 : 4836), especially for most formulations available in Thailand.

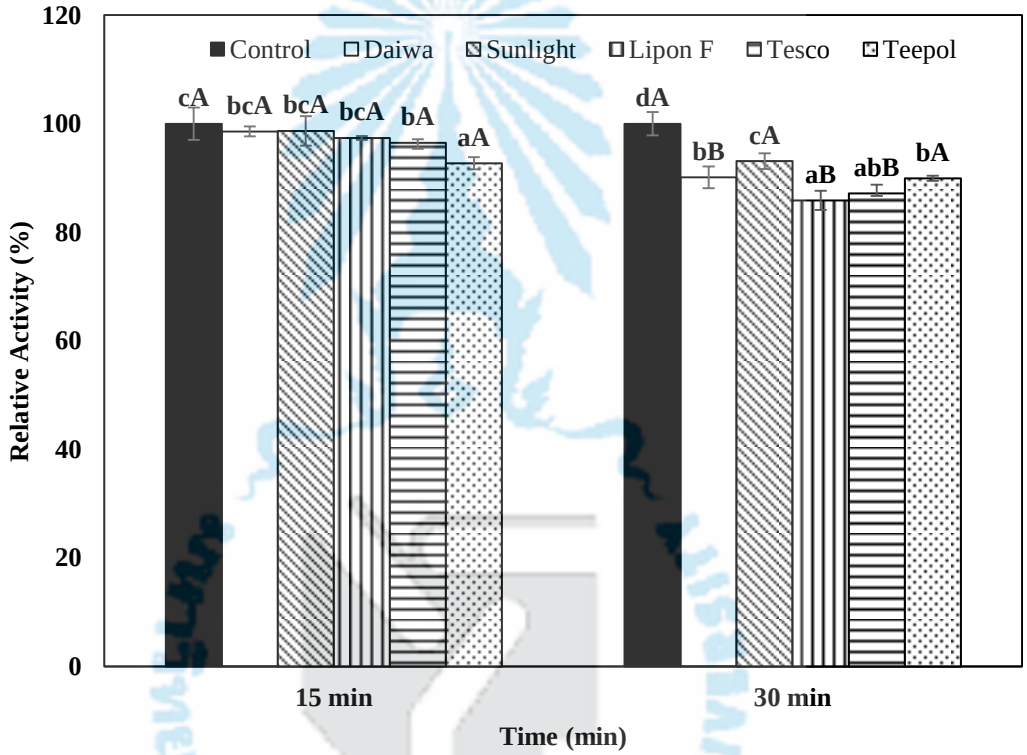


Figure 14. Stability of Lipase from the Viscera of Nile Tilapia in the Presence of Various Commercial Dishwashing Detergents. Bars Represent the Standard Deviation from Triplicate Determinations (⧳ = ± 1 SD). Different Lowercase Letters on the Bars within the Same Incubation Time Indicate Significant Differences ($P<0.05$). Different Uppercase Letters on the Bars within the Same Commercial Dishwashing Detergents Indicate Significant Differences ($P<0.05$).

5. Conclusion

Viscera extract from three freshwater fish species contained lipases with optimum pH and temperatures of 7.0-8.5 and 40-45^{o}C, respectively. Nile tilapia exhibited the highest lipase activity followed by striped snake-head fish and hybrid catfish. Lipase from Nile tilapia viscera can be successfully extracted using 50 mM Tris-HCl buffer, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35. The high stability of the lipase from the viscera of Nile tilapia towards surfactants and commercial dishwashing detergents makes it a novel lipase for further commercial utilization as a potential additive in dishwashing detergent formulations.

CHAPTER 3

Thermoseparating Aqueous Two-Phase System for Lipase Recovery and Partitioning from Nile Tilapia Viscera: Biochemical Properties and Effect of Ultrasound

1. Abstract

Lipase from Nile tilapia (*Oreochromis niloticus*) viscera were partitioned and recovered using a thermoseparating aqueous two-phase system (T-ATPS). Different phase partitioning parameters, including type and concentration of salts, concentration of EOPO, NaCl addition, the EOPO phase/distilled water ratio and temperature were optimized. In the primary ATPS, lipase was satisfactorily partitioned to the EOPO-rich top phase in the optimum system composed of 20% crude enzyme, 40% EOPO3900, 10% $(NH_4)_2SO_4$ and 4% NaCl. In the secondary ATPS, the optimum ratio between the EOPO-rich top phase and distilled water was 1:1 (w/w) and the optimum temperature for inducing phase separation was 60°C. After thermoseparation, water solution containing lipase and EOPO were formed in the top and bottom phase of this step, respectively. Under the optimal partitioning condition, the yield (64.45%) and purification fold (PF: 6.30-fold) were obtained. Additionally, the lipases retained in the salt-rich bottom phase in the primary ATPS were successfully recovered from the recycling step no more than three times. The total yield of 93.59% was obtained from this separation system. The partitioned enzyme exhibited optimal activity at pH 8.5 and 40°C and was stable at a temperature range of 0-40°C and a pH range of 8-10. It showed high tolerance in the presence of ethanol and methanol. The effect of ultrasound on the partitioned lipase activity was also studied. The highest lipase activity was achieved when the sample was treated with ultrasound at 180 W and 24 kHz for 20 min at 40°C, under which the activity was increased by 110.73% over the control. Therefore, the T-ATPS was found to be an attractive technique for the recovery and partial purification of lipase from Nile tilapia viscera. Also, ultrasound could obviously improve its activity.

2. Introduction

Nile tilapia (*Oreochromis niloticus*) is widely cultured in tropical and subtropical areas of the world (Lluyemi, Hanafi, Radziah and Kamarudin. 2010 : 502). In Thailand, it constitutes the largest group of freshwater fish production (Thongprajukaew, Kovitvadhi, Kovitvadhi and Preprame. 2017 : 292) It is predominantly herbivorous and able to produce high quality protein for human consumption (Bezerra, Lins, Alencar, Paiva, Chaves, Coelho and Carvalho. 2005 : 1829). However, the processing of fish generates a large amount of waste. Different uses in applying the wastes from this waste, have been developed to solve the pollution problems. The majority of fish by-product consists of viscera, which are a potential source of many digestive enzymes, such as trypsin, pepsin (Unajak, Meesawat, Paemanee, Areechon, Engkagul, Kovitvadhi, Kovititvadhi, Rungruangsak-Torrissen and Choowongkomon 2012 : 1533) and lipase (Kuepethkaew, et al. : 2017 : 769).

Thermoseparating aqueous two-phase systems (T-ATPS) using a co-polymer, poly(ethylene glycol-ran-propylene glycol), monobutyl ether (EOPO), and a salt (i.e., magnesium sulfate, sodium citrate, or potassium phosphate) have received special attention for several years due to their advantages such as a short and economical process, low energy consumption, environmental friendliness, and relatively easy scalability. As well, phase composition can be recycled, and the protein/enzymes can be recovered in a water phase (Pereira, et al. 2003 : 131; Show, et al. 2011 : 577; Ng, et al. 2012 : 9). In this system, enzyme recovery and purification are performed in a two-step process. In the primary step, the target protein should preferentially enter the EOPO phase. In the secondary step, the secondary system is formed by a temperature-induced phase separation of the EOPO solution. Consequently, a water solution of the target protein (top phase) and a concentrated EOPO solution (bottom phase) are obtained (Ketnawa, et al. 2014 : 2158). The use of EOPO copolymer instead of polyethylene glycol (PEG) in ATPS will result in two positive effects. First, EOPO random copolymers (linear and non-ionic) can be separated from an aqueous solution by heating the solution above a cloud point (or known as lower critical solution temperature, LCST), which is at low a temperature of around 50°C (Ng, et al. 2012 : 9; Show, Tan, Anuar, Ariff, Yusof, Chen and Ling. 2012 : 226). In this case,

the polymers could be recycled and the salt component could also be reused in a new ATPS, and the second effect is that the proteins can be recovered in a water phase (Persson, Johansson and Tjerneld. 1999 : 31). T-ATPS has been applied in extraction and partitioning of various enzymes such as endo-polygalacturonase (Pereira, et al. 2003 : 131), proteases (Ketnawa, et al. 2014 : 2158) and lipase (Show, et al. 2012 : 226).

Nowadays, application of ultrasonic technology in biological processing has widely attracted attention. Use of ultrasound produces cavitation phenomenon which mainly consists of formation, growth and violent implosion of microbubbles formed by the sound waves (Yachmenev, Condon, Klasson and Lambert. 2009 : 25). More recently, the interest of food technologists has turned to the use of power ultrasound in altering enzyme activities. Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of a number of enzymes (Kadkhodaee and Povey. 2008 : 133). However, in some cases, enzyme activities have been found to have increased activity following short exposures to ultrasound (Duan, Zhou, Qiao and Wei. 2011 : 4290).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are serine hydrolases that catalyze the hydrolysis of triacylglycerols (TAGs) into glycerol and fatty acids, the synthesis of esters from various alcohols and fatty acids, and transesterification and aminolysis reactions (Navvabi, Razzaghi, Fernandes, Karami and Homaei. 2018 : 61). Lipases have become increasingly important in the last two decades for industrial applications in the food, detergent, pharmaceutical, bioenergy and biodiesel industries. Different strategies for lipase purification methods in general are time-consuming with low scalability and cost effectiveness, and sometimes may offer an unsatisfied purification yield for the desired product (Kuepethkaew, et al. 2017 : 3880). Lipases are relatively costly enzymes, and a significant decrease in cost would be important for their commercial application. Also, it is an important way to enhance the lipase activity by molecular modification. Therefore, the aim of this study was to demonstrate the use of T-ATPS, for partitioning and recovery of lipase from the viscera of Nile tilapia. Its biochemical properties and the impacts of ultrasound pretreatment on the activity of partitioned lipase were also investigated.

3. Material and Methods

3.1 Materials

Acetone, tris (hydroxymethyl) aminomethane, Brij 35, Triton X-100, and gum arabic were obtained from Merck (Darmstadt, Germany). Poly (ethylene glycol-ran-propylene glycol) monobutyl ether (EOPO) with an average MW of 3900 g/mol, Bovine serum albumin (BSA), wide range molecular weight markers, Coomassie Brilliant Blue R-250 and *p*-nitrophenyl palmitate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was obtained from Fluka (Buchs, Switzerland). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals of analytical grade were procured from Merck (Darmstadt, Germany).

3.2 Preparation of Crude Extract

Fresh viscera of Nile tilapia were collected from a local market in Thale Noi, Phatthalung, Thailand. Samples were placed in polyethylene bags and imbedded in polystyrene boxes containing ice with an ice/sample ratio of 2:1 (w/w) during transportation for approximately 30 min. Upon arrival, the samples were homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao, Kishimura, Yabe and Benjakul (2007 : 682). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude extract, the defatted viscera powder was suspended in 50 mM Tris–HCl, pH 7.0 containing 1.0 M NaCl and 0.2% (v/v) Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000 × g to remove the tissue debris. The supernatant was collected and referred to as ‘‘crude extract, CE’’.

3.3 Thermoseparating Aqueous Two-Phase System (T-ATPS)

3.3.1 Effect of Salts on the Partitioning of Viscera Lipase

In this investigation, the phase systems were prepared in 15 mL centrifuge tubes following the method of Ketnawa, et al. (2014 : 2158) with a slight modification. To study the influence of salt types and concentrations on the lipase partitioning, different salts including disodium hydrogen phosphate (Na_2HPO_4), dipotassium hydrogen phosphate (K_2HPO_4), trisodium citrate ($Na_3C_6H_5O_7$) and ammonium sulphate ($(NH_4)_2SO_4$), at different concentrations (5, 10 and 15%, w/w) were added to 40% (w/w) EOPO copolymer and 20% (w/w) CE. Distilled water was used to adjust the system to obtain a final mass of 10 g. The mixtures were mixed continuously for 3 min by a vortex mixer (G506E, Scientific Industries, USA) and then centrifuged for 15 min at 5,000 × g to induce phase separation. This system was referred to as the "primary ATPS."

After centrifugation, the EOPO-rich top phase was collected from the primary ATPS and diluted with distilled water in a ratio of 1:1 (w/w). The samples were then placed in a water bath at 60°C for 15 min to induce thermo-separation. Next, the samples were centrifuged at 5,000 × g for 15 min to ensure complete thermo-induced phase separation and the secondary ATPS was formed. Once the phase separation of the diluted top phase sample was attained, the lipase containing water-rich top phase and EOPO-rich bottom phase were withdrawn separately. Volumes of the separated phase, top and bottom phases, were determined. Aliquots from each phase were taken for lipase assay and protein determination. Total activity (TA), total protein (TP), lipase partition coefficient (K_E), protein partition coefficient (K_P), specific activity (SA), purification fold (PF) and yield were then calculated by the following equations:

$K_E = A_T/A_B$

where A_T and A_B are the total lipase activities (in Unit) in the top and bottom phases, respectively.

$K_P = P_T/P_B$

where P_T and P_B are the total protein content (in mg) in the top and bottom phases, respectively.

SA (Unit/mg protein) = lipase activity / protein concentration

$PF = SA_T / SA_I$

where SA_T is the SA of the lipase rich phase and SA_I is the initial SA of the crude extract or fraction before being partitioned.

Yield (%) = $(A_T/A_I) \times 100$

where A_T is total lipase activity in the lipase rich phase and A_I is the initial lipase activity of the crude extract. The system providing the highest lipase recovery and purity was selected for further investigation.

3.3.2 Effect of Concentration of EOPO on the Partitioning of Viscera Lipase

Influence of EOPO at different concentrations (30, 35, 40, 45 and 50%, w/w) on partitioning of viscera lipase was studied in the presence of salt with the type and concentration exhibiting the highest yield and purity. Partitioning was carried out as previously described. T-ATPS giving the most effective partitioning of lipase was selected for further study.

3.3.3 Effect of NaCl Concentration on the Partitioning of Viscera Lipase

To investigate the effect of NaCl concentration on the lipase partitioning, the primary ATPS that provided the highest lipase recovery from the previous step was selected. The method was performed by NaCl (solid form) addition into the system in a final concentration of 0, 2, 4, 6, and 8% (w/w). Partitioning from this step was carried out and determined as previously described. The phase composition that provided the maximum lipase recovery and purity was chosen for the next step of the study.

3.3.4 Effect of Phase Composition Ratio used in the Step of Secondary ATPS

To study the influence of the phase composition ratio used in the step of secondary ATPS on lipase partitioning, the EOPO-rich top phase from the primary ATPS were mixed with distilled water in a different ratio (0.5:1.5, 1:1 and 1.5:0.5 (w/w)). Partitioning was made as previously described. The ratio of EOPO-

rich top phase from the primary ATPS and distilled water yielding the maximum enzyme recovery and purity was chosen.

3.3.5 Effect of Temperature on the Partitioning of Viscera Lipase

The influence of temperature on lipase partitioning in the secondary ATPS was investigated. Temperatures of 55°C, 60°C, and 65°C were used. The mixture was incubated for 15 min at each setting temperature before separation into two fractions. Collection and testing were made as mentioned before. The T-ATPS fraction with the highest purity and yield was used for the characterization study.

3.3.6 Recycling of Phase Component

The process of recycling phase components using EOPO as the thermoseparating polymer in an ATPS was studies. From the primary ATPS, the EOPO-rich top phase was collected using a pipette and diluted twice with distilled water in a new centrifuge tube. Then, the new tube was placed in a water bath to induce thermoseparation. After heating for 15 min at 60°C, the secondary ATPS was formed. The bottom phase consisted of a concentrated EOPO solution, and the top phase was a lipase/water solution. The concentrated EOPO solution recovered from the secondary ATPS was mixed with the ammonium sulfate solution recovered from the primary ATPS to create an identical composition as that used during the first extraction. The recycling steps were repeated until the partition of the lipase in the EOPO-ammonium sulfate ATPS was no longer at the optimized condition. After that, each phase was collected and the protein content and lipase activity were further determined.

3.4 Lipase Activity Assay and Protein Determination

Lipase activity was measured by the *p*-NPP (*p*-nitrophenyl palmitate) method as described previously by Patchimpet, Sangkharak and Klomklao (2019a) and Kuepethkaew, et al. (2017 : 3880). The substrate solution was prepared by dissolving 30 mg *p*-NPP in 10 mL of propane-2-ol. The lipase assay was performed at 37°C by direct an appropriately diluted enzyme in a 100 µL cuvette with 810 µL of a

preincubated 50 mM Tris-HCl buffer (pH 8.5) and 90 µL of substrate solution. The amount of liberated *p*-nitrophenol was measured at 410 nm during the first 5 min of the reaction. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per min under the assay conditions.

Protein concentration was measured by the Bradford method using BSA as a protein standard (Bradford. 1976 : 248).

3.5 Characterization of Recovered Lipases

3.5.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of fractionated lipases was carried out according to the method of Laemmli (1970 : 680). CE and T-ATPS fraction were mixed at a 1:1 (v/v) ratio with the SDS-PAGE sample treatment buffers (0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protein II Cell apparatus. After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% method and 10% acetic acid.

3.5.2 pH and Temperature Profiles

The activity of lipase from Nile tilapia viscera after the T-ATPS process was determined within the pH range of 6.0-10.0. Different buffers were used for different pH conditions: 0.2 M phosphate buffer for pH 6.0-7.5, 50 mM Tris-HCl for pH 7.5-9.0 and 0.1 M glycine-NaOH for pH 9.0-10.0. The activity was assayed at 37°C for 5 min.

For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 45, 50, 55, 60, 70 and 80°C) for 5 min at pH 8.5.

3.5.3 Thermal and pH Stability

T-ATPS fraction was incubated at different temperatures (0, 4, 10, 20, 30, 40, 50, 60, 70 and 80°C) for 30, 60 and 120 min, followed by cooling in

iced water. The residual activity was assayed using *p*-NPP as a substrate and reported as the relative activity (%) compared with the original activity.

The effect of pH on lipase stability was evaluated by measuring the residual enzyme activity after incubation at various pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) for 30, 60 and 120 min at room temperature. The residual activity was assay with *p*-NPP as previously described.

3.5.4 Effect of Organic Solvents

The influence of methanol and ethanol at a concentration of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50% (v/v) on the lipase activity was studied by incubating the fractionated lipase for 30, 60 and 120 min at room temperature. The residual enzyme activity was determined by the spectrophotometric method using *p*-NPP as substrate.

3.5.5 Effect of Bile Salts

The effect of varying concentrations of sodium deoxycholate (NaDC), sodium taurocholate (NaTC), and sodium cholate (NaC) on the activity of lipase was studied by incubating the T-ATPS fraction with the bile salts at room temperature for 30 min. The residual activity was assay with *p*-NPP.

3.5.6 Effect of Ultrasound on the Activity of Fractionated Lipase.

Fractionated lipase was sonicated in an ultrasonic bath (Zealway Inc., Model S06H, China). The instrument can deliver a maximum frequency of 40 kHz and temperature of 60°C. Fractionated lipase (2 mL containing 28.6 Unit of lipase and 200 µg/mL of protein content) was used in this study. The first set of experiments were used to investigate the influence of ultrasound at different ultrasonic times (0, 5, 10, 15, 20, 25, 30 min) at 180 W, 24 kHz and 30°C. The second set of experiments were used to study the effect of ultrasound at different frequencies (16, 20, 24, 28, 32 kHz) for 20 min at 180 W and 30°C. The third set of experiments were used to investigate the effect of ultrasound at different temperatures (20, 30, 35, 40, 45, 50, 60°C) for 20 min at 180 W and 24 kHz.

3.6 Statistical Analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie. 1980). Statistical analysis was performed using a SPSS package (SPSS 11.5 for windows, SPSS Inc., Chicago, IL, USA).

4. Results and Disscussion

4.1 Use of T-ATPS for Partitioning of Viscera Lipase from Nile Tilapia

4.1.1 Effect of Salts with Different Types and Concentrations

Salts are frequently used in an aqueous two-phase system to direct partitioning of target molecules between the phases (Persson, Johansson and Tjerneld. 1999 : 31). As different ions have different affinity for the two phases there will be a driving force toward uneven partitioning of the ions between the phases, although electroneutrality must be retained in each phase. The electrochemical driving force in partitioning has been explained by the formation of an electrostatic potential difference over the interface (Schluck, Maurrer and Kula. 1995 : 443; Johansson, Karlström, Tjerneld and Haynes. 1998 : 3; Pfennig, Schwerin and Gaube. 1998 : 45). This potential difference is created by the different affinities of the ions for the two phases. The electrostatic potential difference will affect the partitioning of proteins or other charged molecules present in the phase system (Persson, Johansson and Tjerneld. 1999 : 31). In order to identify a suitable salt for the lipase partitioning from Nile tilapia viscera, CE was performed using several biphasis systems of 40% EOPO 3900 with various salts, Na_2HPO_4, K_2HPO_4, $Na_3C_6H_5O_7$ and $(NH_4)_2SO_4$ at different concentrations (5, 10 and 15%) (w/w). Table 14 showed that TA, TP, K_E, K_P, SA, PF and the yield of lipase obtained from EOPO 3900-salt systems depended on the types and concentrations of salts used. For all T-ATPS studied, systems were shown to be able to partition the lipase from the viscera of Nile tilapia. From the results, the K_E were one or higher, which means that the partition of the lipase was favored to the water-rich top phase in the secondary ATPS. The phase systems containing $(NH_4)_2SO_4$ generally showed the superior partitioning efficiency to those containing

other salts. A phase system containing 40% EOPO3900 and 10% $(NH_4)_2SO_4$ showed the highest TA (2002.09 Unit), TP (1167.65 µg), SA (1.72 Unit/µg protein), PF (2.10-fold) and yield (65.75%). Zhao, Budge, Ghaly, Brooks and Dave (2012) reported that 15% $(NH_4)_2SO_4$-18% PEG 1500 was the optimal ATPS combination and presented the best partition of pepsinogen from the stomach of red perch (*Sebastes marinus*).

The system containing K_2HPO_4 or Na_2HPO_4 showed a lower lipase recovery than that of the system with $(NH_4)_2SO_4$ and the lowest recovery was obtained when $Na_3C_6H_5O_7$ was incorporated in the system (Table 14). Ketnawa, et al. (2014 : 2158) reported that the influence of salts in T-ATPS on alkaline proteases partitioning is dependent on the types and amounts of salt used. The protein partitioning is influenced by the presence of salts and this effect is enhanced when the net charge of the protein increases (Johansson. 1988). Huddleston, Veide, Köhler, Flanagan, Enfors and Lyddiatt (1991 : 381) reported that the efficiency of the salts in promoting phase separation reflects the lyotropic series. Their effectiveness is mainly measured by the nature of the anion. Multi-charged anions being the most effective are ordered of SO_4^{2-}>HPO_4^{2-}>acetate >Cl^-, whereas the order of cation is usually given as NH_4^+>K^+>Na^+ >Li^+ >Mg^{2+} > Ca^{2+} (Senphan and Benjakul. 2014 : 57). Therefore, the relative effectiveness of the salts tested in promoting phase separation follows the Hofmeiter series, which is a classification of ions based or the salting-out ability. The contribution of the anion is more important than that of the cation in determining the effectiveness of a particular salt. The multivalent anions like HPO_4^{2-} and SO_4^{2-} are most effective in inducing phase separation with polymers (Ketnawa, et al. 2014 : 2158). Accordingly, the highest specific activity, purification fold and yield were observed in the EOPO/$(NH_4)_2SO_4$ system with 40%/10% (w/w); hence, this combination was selected for further experiments.

Table 14 T-ATPS for Partitioning of Lipase from Viscera of Nile Tilapia as Influenced by Salts with Different Types and Concentrations

Phase Composition*	**TA (Unit)****	**TP (μg)**	**K_E**	**K_P**	**SA (Unit/μg)**	**PF (fold)**	**Yield (%)**
Crude Extract	3044.80±14.55	3828.23±14.61			0.80±0.01	1	100
Secondary ATPS							
Water-Rich Top Phase							
40%EOPO-5%K_2HPO_4	1468.94±17.17^{c}	1813.73±36.78^{i}	1.27±0.19^{a}	1.73±0.05^{h}	0.81±0.01^{a}	1.01±0.01^{a}	48.25±0.56^{c}
40%EOPO-10%K_2HPO_4	1652.22±50.37^{d}	2022.73±16.60^{j}	4.27±0.25^{d}	1.92±0.09^{i}	0.82±0.02^{a}	1.02±0.03^{a}	54.26±1.65^{d}
40%EOPO-15%K_2HPO_4	1292.86±10.78^{b}	1589.45±24.16^{h}	4.23±0.16^{d}	1.62±0.05^{g}	0.81±0.01^{a}	1.02±0.01^{a}	42.46±0.35^{b}
40%EOPO-5%$(NH_4)_2SO_4$	1632.67±26.02^{d}	1078.33±14.95^{e}	10.02±1.02^{f}	0.82±0.04^{a}	1.51±0.02^{c}	1.89±0.03^{c}	53.62±3.00^{d}
40%EOPO-10%$(NH_4)_2SO_4$	2002.09±77.52^{e}	1167.65±13.07^{g}	8.82±1.05^{e}	0.81±0.02^{a}	1.72±0.07^{d}	2.14±0.08^{d}	65.75±2.55^{e}
40%EOPO-15%$(NH_4)_2SO_4$	1567.06±47.60^{cd}	1011.84±11.18^{cd}	8.07±0.28^{e}	0.90±0.02^{ab}	1.55±0.04^{c}	1.94±0.06^{c}	51.47±1.56^{cd}
40%EOPO-5%$Na_3C_6H_5O_7$	991.17±46.42^{a}	1021.80±23.71^{d}	2.96±0.11^{bc}	1.15±0.05^{f}	0.97±0.05^{b}	1.21±0.06^{b}	32.55±1.52^{a}
40%EOPO-10%$Na_3C_6H_5O_7$	1195.20±80.15^{b}	1130.90±74.91^{fg}	2.01±0.15^{ab}	1.12±0.09^{ef}	1.06±0.07^{b}	1.32±0.09^{b}	39.25±2.63^{b}
40%EOPO-15%$Na_3C_6H_5O_7$	961.34±87.03^{a}	962.30±32.21^{b}	1.87±0.13^{a}	0.93±0.03^{bc}	1.00±0.09^{b}	1.25±0.11^{b}	31.57±2.86^{a}
40%EOPO-5%NaH_2PO_4	1475.04±4.75^{c}	969.87±19.10^{b}	4.00±0.18^{cd}	1.01±0.06^{cd}	1.52±0.01^{c}	1.91±0.01^{c}	48.44±0.16^{c}
40%EOPO-10%NaH_2PO_4	1689.42±105.58^{d}	1100.13±12.74^{ef}	4.02±0.06^{cd}	1.10±0.06^{def}	1.53±0.09^{c}	1.92±0.12^{c}	55.49±0.47^{d}
40%EOPO-15%NaH_2PO_4	1288.88±86.80^{b}	892.82±2.71^{a}	3.28±0.01^{cd}	1.05±0.07^{de}	1.44±0.10^{c}	1.81±0.12^{c}	42.33±2.86^{b}

*The secondary ATPS derived from water rich-top phase of primary ATPS (40%EOPO-20%crude extract and salts with different types and concentrations) mixed with distilled water (1:1, w/w) and induced phase separation at 60°C.

**TA, total activity; TP, total protein; K_E, lipase partition coefficient; K_P, protein partition coefficient; SA, specific activity; PF, purification fold.

Mean ± SD from triplicate determinations. The different letters in the same column denote the significant differences ($P< 0.05$).

4.1.2 Effect of Concentration of EOPO

In order to investigate the influence of the concentration of EOPO3900 on lipase partitioning of lipase, the copolymer concentration was varied over a range of 30-50% (w/w), whereas the salt concentration was kept constant (10%, w/w). The results are shown in Table 15. K_P decreased with increasing EOPO concentration ($P< 0.05$). The decrease in the partition coefficient of the total protein could be attributed to the influence of volume exclusion, which increases with increasing copolymer concentration (Babu, Rastogi and Raghavarao. 2008 : 83). However, an increase in the concentration of copolymer from 30% to 40% (w/w) resulted in an increase in TA (from 1302.77 to 2044.65 Unit), TP (from 844.42 to 1139.97 µg), K_E (from 9.82 to 10.78), SA (from 1.54 to 1.79 Unit/µg), PF (from 1.93 to 2.24) and yield (42.19 to 66.67%) and the TA, TP, K_E, SA, PF and yield decreased at an EOPO concentration above 40% (w/w). Similar observations were reported for the partition of pepsinogen from the stomach of red perch (*sebastes marinus*) (Zhao, Budge, Ghaly, Brooks and Dave. 2013). At low EOPO concentrations (30-40%, w/w), the electrostatic interaction is enhanced, which favors the partition of lipase into the EOPO-rich top phase. However, high EOPO concentrations lead to higher viscosities, and make the partition difficult. In addition, high EOPO concentrations can result in denaturation and possible precipitation of lipase (Zhao, et al. 2013). Priyanka, Rastogi, Raghavarao and Thakur (2012 : 1358) have reported that the increase in concentration of polymer resulted in a decrease in the volume of the bottom phase resulting in an increased concentration of salt in the bottom phase, leading to the precipitation of enzyme at the interface probably due to the "salting out effect". From the results, the EOPO/$(NH_4)_2SO_4$ system (40%/10%, w/w) ($P<0.05$) exhibited the highest specific activity, PF and yield. Therefore, this combination was chosen for further experiments.

Table 15 T-ATPS for Partitioning of Lipase from Viscera of Nile Tilapia as Influenced by EOPO with Different Concentrations

Phase Composition*	**TA (Unit)****	**TP (μg)**	**K_E**	**K_P**	**SA (Unit/μg)**	**PF (fold)**	**Yield (%)**
Crude Extract	3066.13±10.71	3835.03±13.42			0.80±0.01	1	100
Secondary ATPS							
Water-Rich Top Phase							
30%EOPO-10%$(NH_4)_2SO_4$	1302.77±88.31^{a}	844.42±10.02^{b}	9.82±0.33^{b}	1.17±0.05^{d}	1.54±0.10^{a}	1.93±0.13^{a}	42.19±2.88^{a}
35%EOPO-10%$(NH_4)_2SO_4$	1697.79±74.32^{b}	1079.41±9.15^{cd}	10.18±0.92^{b}	1.02±0.09^{c}	1.57±0.07^{a}	1.96±0.09^{a}	55.37±2.42^{b}
40%EOPO-10%$(NH_4)_2SO_4$	2044.65±65.36^{c}	1139.97±3.28^{d}	10.78±0.23^{b}	0.83±0.01^{b}	1.79±0.06^{c}	2.24±0.07^{c}	66.67±2.10^{c}
45%EOPO-10%$(NH_4)_2SO_4$	1799.06±27.70^{b}	1029.95±8.99^{c}	9.41±0.46^{b}	0.68±0.02^{a}	1.75±0.03^{bc}	2.19±0.04^{bc}	58.68±0.90^{b}
50%EOPO-10%$(NH_4)_2SO_4$	1229.12±81.44^{a}	747.34±12.71^{a}	5.16±0.09^{a}	0.64±0.02^{a}	1.64±0.11^{ab}	2.06±0.13^{ab}	40.08±2.66^{a}

*The secondary ATPS derived from water rich-top phase of primary ATPS (20%crude-10%$(NH_4)_2SO_4$ and EOPO at different concentrations) mixed with distilled water (1:1, w/w) and induced phase separation at 60°C.

**TA, total activity; TP, total protein; K_E, lipase partition coefficient; K_P, protein partition coefficient; SA, specific activity; PF, purification fold.

Mean ± SD from triplicate determinations. The different letters in the same column denote the significant differences ($P< 0.05$)

4.1.3 Effect of NaCl

The effect of NaCl addition (0, 2, 4, 6 and 8%, w/w) on lipase partitioning of Nile tilapia in the selected phase system (40% EOPO 3900 and 15% $(NH_4)_2SO_4$) is presented in Table 16. The addition of NaCl to the ATPS results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases (Goja, Yang, Cui and Li. 2013), thus affecting the protein partitioning in ATPS (Barbosa, Souza, Fricks, Zanin, Soares and Lime. 2011 : 3853). An increase in the hydrophobicity will decrease the amount of bound water, which keeps the final composition of the systems constantly. Moreover, it increases the ionic strength and enhances the migration of low molecular weight compounds towards the polymer phase. From the results, the concentration of NaCl at 2-8% (w/w) in the EOPO-$(NH_4)_2SO_4$ system had an adverse effect on the partition of Nile tilapia lipase. The results showed that TA, TP and yield decreased with the addition of NaCl. However, SA and PF increased with NaCl addition up to 4% (w/w). The decreases in SA and PF were observed when NaCl concentrations at 6 and 8% (w/w) were added. Kuepethkaew, et al. (2017 : 3880) reported that the recovery yield of Pacific white shrimp lipase has practically decreased with increasing NaCl concentration. Occurrence of this effect may be due to a decrease in protein solubility as a result of the NaCl addition (Naganagouda and Mulimani. 2008 :1293). Also, the addition of high concentration of NaCl may cause denaturation of proteins existing in the system (Goja, et al. 2013). The highest PF (6.17-fold) was obtained when 4% (w/w) NaCl was added. So keeping both the enzyme recovery and purity in view, the T-ATPS experiment with NaCl addition of 4% (w/w) was is chosen for next investigation.

Table 16 T-ATPS for Partitioning of Lipase from Viscera of Nile Tilapia as Influenced by NaCl with Different Concentrations

NaCl Concentration*	**TA (Unit)****	**TP (µg)**	**K_E**	**K_P**	**SA (Unit/µg)**	**PF (fold)**	**Yield (%)**
Crude Extract	3048.80±66.00	3840.48±3.68			0.79±0.02	1	100
Secondary ATPS							
Water-Rich Top Phase							
NaCl 0 %	1985.61±52.18^{d}	1073.83±21.70^{c}	7.30±0.42^{bc}	1.03±0.01^{b}	1.85±0.05^{a}	2.34±0.06^{a}	65.13±1.71^{d}
NaCl 2 %	1920.77±37.85^{cd}	781.97±6.27^{b}	6.47±0.17^{a}	0.99±0.02^{b}	2.46±0.05^{b}	3.11±0.06^{b}	63.00±1.25^{cd}
NaCl 4 %	1863.75±7.42^{c}	382.14±9.08^{a}	7.79±0.04^{c}	0.49±0.04^{a}	4.88±0.02^{e}	6.17±0.21^{e}	61.13±0.24^{c}
NaCl 6 %	1370.13±3.43^{b}	370.25±23.88^{a}	6.66±0.35^{ab}	0.50±0.04^{a}	3.70±0.01^{d}	4.68±0.01^{d}	44.94±0.11^{b}
NaCl 8 %	1200.60±24.61^{a}	364.22±6.26^{a}	6.53±0.28^{a}	0.53±0.02^{a}	3.30±0.06^{c}	4.17±0.08^{c}	39.38±0.81^{a}

*The secondary ATPS derived from water rich-top phase of primary ATPS (40%EOPO-20%crude extract-10%$(NH_4)_2SO_4$ and NaCl at different concentrations) mixed with distilled water (1:1, w/w) and induced phase separation at 60°C.

**TA, total activity; TP, total protein; K_E, lipase partition coefficient; K_P, protein partition coefficient; SA, specific activity; PF, purification fold.

Mean ± SD from triplicate determinations. The different letters in the same column denote the significant differences ($P< 0.05$).

4.1.4 Effect of Phase Composition Ratio Used in Secondary ATPS Step

Dilution of the EOPO-rich top phase from the primary ATPS with water caused two distinct phases to form during thermoseparation. The dilution was performed to decrease the solubility of the polymer. Pilot experiments showed that omission of the dilution step resulted in three phases with indistinct boundaries (Kepka, Collet, Persson, Ståhl, Lagerstedt, Terneld and Veide. 2003 : 165). In a secondary ATPS step, the ratio of the EOPO-rich top phase from the primary ATPS and distilled water was investigated at relations of 0.5:1.5, 1.0:1.0 and 1.5:0.5 (w/w). The ratio of 1.0:1.0 provided the maximum recovery (63.56%) with PF 6.27-fold, TA 1922.64 Unit, TP 383.84 µg, K_E 11.52, K_P 0.72 and SA 5.01 Unit/µg (Table 17). In contrast, the lowest recovery (40.33%), with PF 4.00-fold, TA 1246.08 Unit, TP 389.13 µg, K_E 9.69, K_P 0.80 and SA 3.20 U/µg were obtained from the ratio of 1.5:0.5 (w/w).

Table 17 T-ATPS for Partitioning of Lipase from Viscera of Nile Tilapia as Influenced by Phase Composition Ratio Used in the Secondary ATPS

Phase Composition Ratio*	**TA (Unit)****	**TP (µg)**	**K_E**	**K_P**	**SA (Unit/µg)**	**PF (fold)**	**Yield (%)**
Crude Extract	3089.73±37.83	3875.10±10.54			0.80±0.01	1	100
Secondary ATPS							
Water-Rich Top Phase							
1.5:0.5	1246.08±70.80^{a}	389.13±10.67^{a}	9.69±0.43^{b}	0.80±0.05^{a}	3.20±0.18^{a}	4.00±0.22^{a}	40.33±2.29^{a}
1.0:1.0	1922.64±67.02^{c}	383.84±7.15^{a}	11.52±1.30^{c}	0.72±0.01^{a}	5.01±0.18^{c}	6.27±0.22^{b}	63.56±2.22^{c}
0.5:1.5	1660.47±95.39^{b}	462.72±27.72^{b}	7.38±0.43^{a}	0.96±0.08^{b}	3.59±0.21^{b}	4.48±0.26^{a}	53.73±3.09$_{b}$

*The secondary ATPS derived from water rich-top phase of primary ATPS (40%EOPO-20%crude extract-10%$(NH_4)_2SO_4$ and 4%NaCl) mixed with distilled water at different ratio and induced phase separation at 60°C.

**TA, total activity; TP, total protein; K_E, lipase partition coefficient; K_P, protein partition coefficient; SA, specific activity; PF, purification fold.

Mean ± SD from triplicate determinations. The different letters in the same column denote the significant differences ($P< 0.05$).

4.1.5 Effect of Temperature

Temperature is one of the limiting factors in T-ATPS. Generally, the water solution of a thermo-separating copolymer will, upon heating, form a turbid solution. At the cloud-point, the copolymer will form macroscopic aqueous droplets which are dispersed in the water phase and at higher temperature the copolymer droplets will aggregate and form on top the aqueous copolymer phase in equilibrium with the bottom water phase (Fero and Jahim. 2010 : 2596). For example, low temperature could not cause phase separation since EOPO random copolymers are not separated from the aqueous solution below a critical temperature or cloud point. However, at extremely high temperature, enzymes usually thermally denaturize. Temperatures above 50°C will cause thermoseparating because the cloud point (LCST) for EOPO3900 is 50°C at 10% (w/w) (Ketnawa, et al. 2014 : 2158). From Table 18, an increment of temperature (55, 60 and 65°C) induced a phase separation in the secondary ATPS on partitioning parameters. No phase separation was found when temperature below 55°C was used (data not shown). Increasing temperature up to 60°C in the thermoseparating step showed the highest TA (1991.33 Unit), K_E (11.86), SA (5.04 Unit/µg), PF (6.30-fold) and recovery yield (64.45%). While thermoseparation at 65°C gave the lower yield (42.00%). Ketnawa, et al. (2014 : 2158) reported that the optimum system contained 40% EOPO3900-10% $MgSO_4$ with 17% NaCl, induced phase separation at 55°C, and provided the highest recovery and purity of alkaline proteases from Giant catfish (*Pangasianodon gigas*) viscera. According to the results obtained, 60°C was an optimum temperature for inducing phase separation.

Table 18 T-ATPS for Partitioning of Lipase from Viscera of Nile Tilapia as Influenced by Temperature Induced Phase Separation

Temperature*	**TA (Unit)****	**TP (µg)**	**K_E**	**K_P**	**SA (Unit/µg)**	**PF (fold)**	**Yield (%)**
Crude Extract	3024.80±70.69	3798.81±36.75			0.80±0.02	1	100
Secondary ATPS							
Water-Rich Top Phase							
55°C	1584.00±79.73^{b}	958.14±16.47^{c}	10.86±0.20^{b}	1.52±0.14^{b}	1.65±0.08^{a}	2.07±0.11^{a}	52.37±2.64^{b}
60°C	1991.33±78.67^{c}	395.18±10.39^{b}	11.86±0.44^{b}	0.75±0.01^{a}	5.04±0.20^{c}	6.30±0.25^{c}	64.45±2.55^{c}
65°C	1270.43±49.85^{a}	352.07±12.22^{a}	4.35±0.10^{a}	0.66±0.22^{a}	3.61±0.14^{b}	4.51±0.18^{b}	42.00±1.65^{a}

*The secondary ATPS derived from water rich-top phase of primary ATPS (40%EOPO-20%crude extract-10%$(NH_4)_2SO_4$ and 4%NaCl) mixed with distilled water (1:1, w/w) and induced phase separation at different temperatures.

**TA, total activity; TP, total protein; K_E, lipase partition coefficient; K_P, protein partition coefficient; SA, specific activity; PF, purification fold.

Mean ± SD from triplicate determinations. The different letters in the same column denote the significant differences ($P< 0.05$).

4.1.6 Effect of Recycling of Phase Component on Lipase Recovery in the T-ATPS

Some target enzymes were still retained in the salt-rich bottom in the primary ATPS and the EOPO-rich bottom phase from the secondary ATPS. Therefore, the effect of the recycling step of the phase component on lipases recovery (%) was investigated. The lipase recovery and PF in each ATPS using recycled copolymer and ammonium sulfate salt decreased with increasing numbers of the recycling cycle (Figure 15). The lipase recovery obtained in the recycling step might be due to the target enzyme that was left in the phases from the first extraction. However, the EOPO copolymer from the secondary ATPS and salt from the primary ATPS must not be recycled and reused more than three times. These recycling procedures would give an advantage in harvesting target enzymes without requiring either back extraction in to a salt-rich phase, ultrafiltration, or ion-exchange chromatography. Besides, recycling procedures could reduce cost and processing time and minimize environmental pollution (Ketnawa, et al. 2014 : 2158).

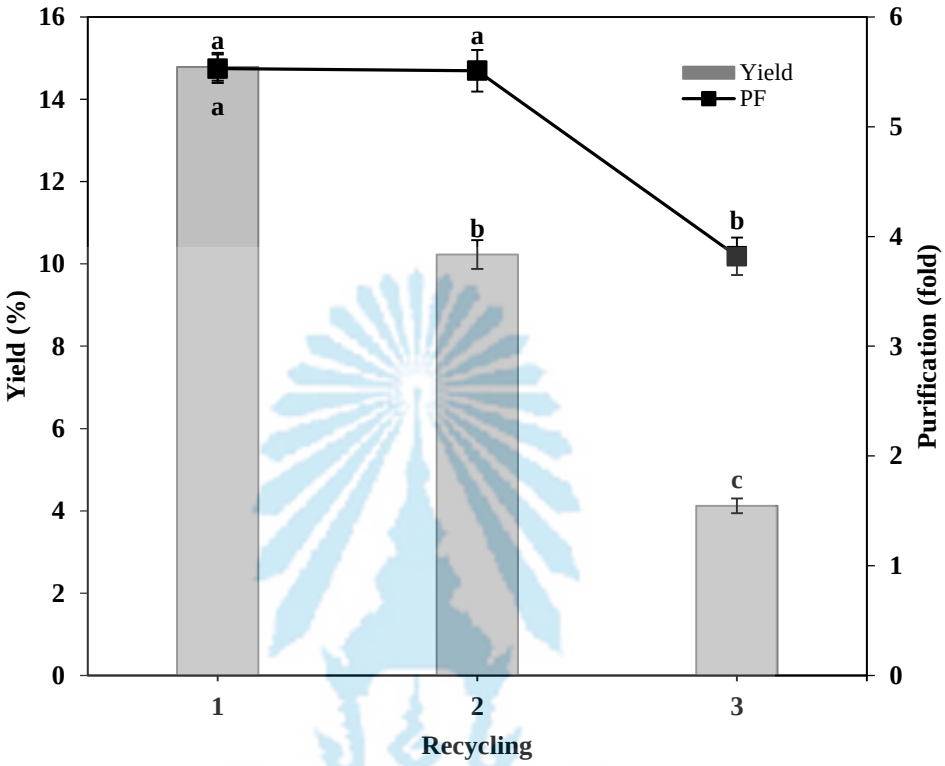


Figure 15. Effect of Recycling Step on the Yield (%) and Purification (Fold) of Lipase Remained in the Salt-Rich Bottom Phase from the Primary ATPS and the EOPO-Rich Bottom Phase from the Secondary ATPS. Different Letters Within the Same Parameter Indicate the Significant Difference ($P<0.05$). Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2 Characterization of Fractionated Lipase

4.2.1 Protein Patten of Lipase from Nile Tilapia Partitioned with T-ATPS

Figure 16 shows the SDS-PAGE pattern of Nile tilapia lipase recovered from the system of 40% EOPO-10% $(NH_4)_2SO_4$ and 4% NaCl thermoseparation. CE contained multiple bands, representing contaminant protein present in the original crude extract. A large number of contaminating proteins were removed after partitioning with T-ATPS, particularly proteins with higher or lower molecular weights. Hence, a higher purity of interested lipase was obtained. When the

proteins or enzymes to be separated differ significantly in their structural properties from others, partitioning can be performed successfully (Rosa, Azevedo, Sommerfeld, Mutter and Aires-Barros. 2009 : 306). The realization of recovery of lipase from crude extract in a partial form was confirmed by SDS-PAGE analyses with molecules weights of about 64, 33, 28, 27 and 26 kDa. The SDS-PAGE analysis revealed a substantial level of purification of lipase from Nile tilapia viscera. Therefore, T-ATPS in this investigation could be used as a partial purification step of the viscera lipase from Nile tilapia.

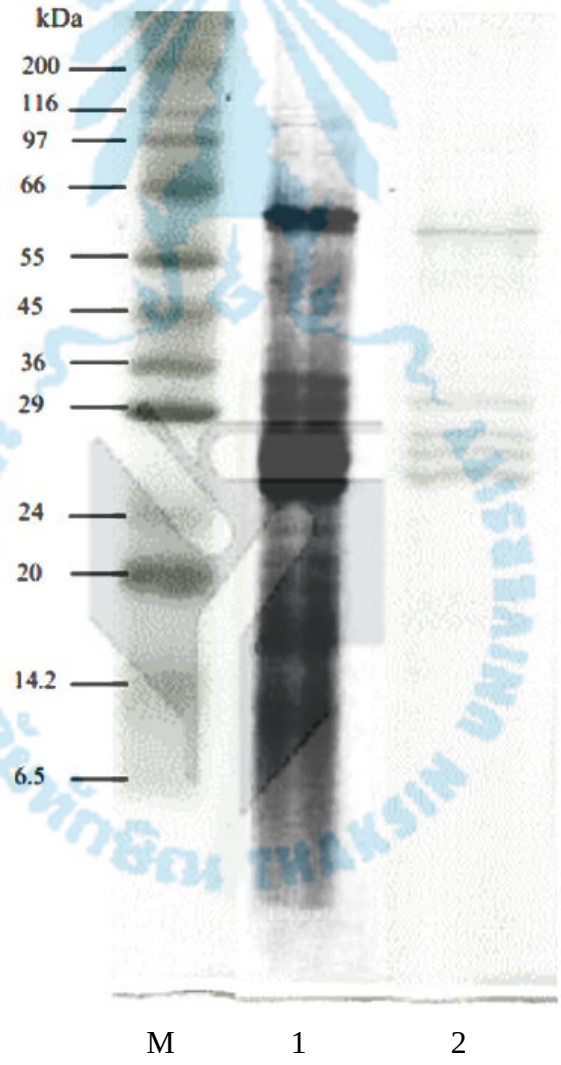


Figure 16. SDS-PAGE Patterns of CE and T-ATPS Fractions from Viscera of Nile Tilapia. M: Molecular Weight Standard, Lane 1: CE, Lane 2: T-ATPS Fraction.

4.2.2 Effect of Temperature and pH on the Lipase Activity and Stability

The optimum temperature of fractionated lipase from viscera of Nile tilapia was 40°C when *p*-NPP was used as a substrate (Figure 17a). For optimum pH, the enzyme exhibited the maximal activity at pH 8.5 (Figure 17b). The enzyme displayed acidic lability at pH below 7.0, probably owing to the denaturation of the enzyme. The activity was also decreased at the alkaline pH (pH 9). The lowered activity at very acidic and alkaline pH was plausible because of the conformational changes of the enzyme under harsh conditions (Sriket, Benjakul, Visessanguan, Hara, Yoshida and Liang. 2012 : 351). The optimum temperature and pH of fractionated lipase were in agreement with that of lipase from Pacific white shrimp hepatopancreas (*Litopenaeus vannamei*) (Kuepetkaew, et al. 2017 : 769) and juvenile redclaw crayfish digestive gland (*Cherax quadricarinatus*) (López-López, Nolasco and Vega-Villasante. 2003 : 337).

The thermal and pH stability of fractionated lipase from Nile tilapia viscera are shown in Figure 17c and d. It was stable below 40°C, but the activity sharply decreased above 50°C (Figure 17c). The enzyme was almost completely inactivated at 80°C. At high temperatures, enzymes most likely underwent denaturation and lost their activity. Fractionated lipase from Nile tilapia viscera exhibited a similar thermal stability to those of other fish species (Kuepetkaew, et al. 2017 : 3880; Aryee, Simpson and Villalonga. 2007 : 394). For pH stability, the fractionated lipase was stable in pH ranging from 8.0 to 10.0 with an exposure time of 30-120 min (Figure 17d). However, fractionated lipase was unstable at pH below 7.0. The stability of lipase at a particular pH may be relevant to the net charge of the enzyme at that pH. From the result, it is suggested that the enzyme might undergo denaturation under acidic conditions, where the conformational change took place and the enzyme could not bind to the substrate properly (Klomklao, et al 2007 : 682). A similar pH effect on activity has been reported for lipase from several fish species (Kuepetkaew, et al. 2017 : 3880; Aryee, Simpson and Villalonga. 2007 : 394). Since tolerance in the alkaline range was found, this can be considered as a potential candidate for use in processes that are conducted in the alkaline range such as detergent applications (Zarai, et al. 2012 : 2434).

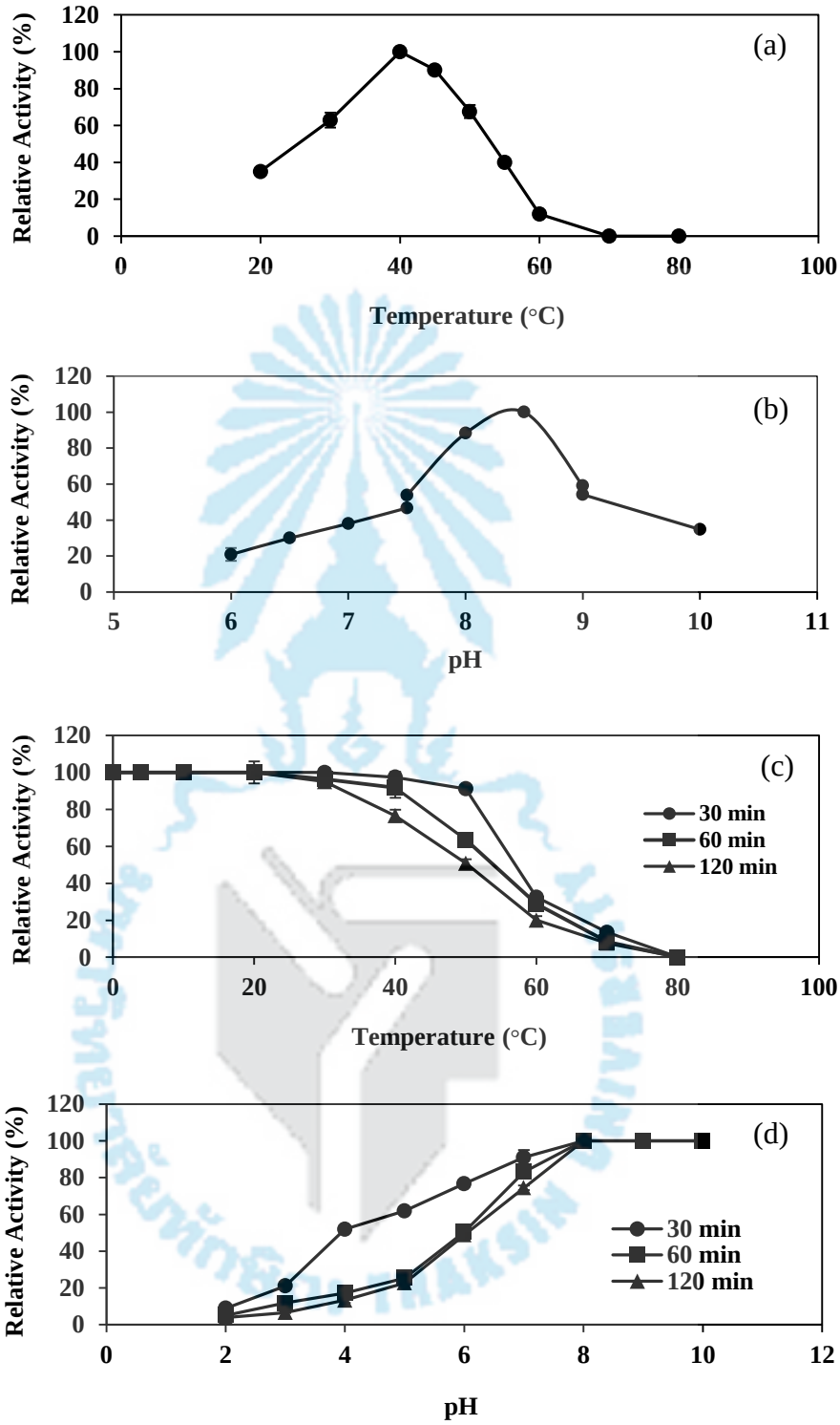


Figure 17. Temperature (a) and pH (b) Profiles and Thermal (c) and pH (d) Stability of Fractionated Lipase from Viscera of Nile Tilapia. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.3 Effect of Organic Solvents on Enzyme Activity

Lipase catalyzes the hydrolysis reaction of water-insoluble substrates as the reaction must occur at an interface. The use of a mixture of water and organic solvents can facilitate the hydrolysis reaction of water-insoluble substrates by the lipase. The high activity and stability of lipases in organic solvents are desirable for biotransformation. The influence of organic solvents (ethanol and methanol) with various concentrations on lipase activity from Nile tilapia viscera was investigated. The fractionated lipase exhibited high stability in the presence of ethanol and methanol, remaining more than 80% of original activity at 40% methanol and ethanol after incubation for 30 min at room temperature (Figure 18a and b). With an extended incubation time, lipase activity was lost to a greater extent. At the same organic solvent level, methanol displayed relative lipase activity compared to ethanol. According to the polarity index value, highly polar organic solvents, such as various alcohols, inhibit the lipase activity by reducing the water activity around the protein molecules and then promote structural denaturation (Takeda, Aono and Doukyu. 2006 : 269). This suggests that the enzyme stability in the presence of organic solvents depends on both the native enzyme and the native of the organic solvent being tested. Biocatalytic activity of enzymes is greatly influenced by the nature of organic solvents. The organic solvent may change the parent conformation of the lipase by disturbing the hydrogen bonding and hydrophobic interactions, which leads to influencing the activity and stability of the enzymes (Karra-Châabouni, Bouaziz, Boufi, do Rego and Gargouri. 2008 : 168). Kuepethkaew, et al. (2017 : 3880) reported that the lipase from Pacific white shrimp hepatopancreas showed high tolerance in the presence of ethanol and methanol. The purified *Mortierella echinosphaera* CBS 575.75 lipase was highly stable in 15% (v/v) methanol and ethanol presenting 97% and 73% relative activities, respectively (Kotogán, Zambrano, Kecskeméti, Varga, Szekeres, Papp, Vágvölgyi and Takó. 2018 : 1129).

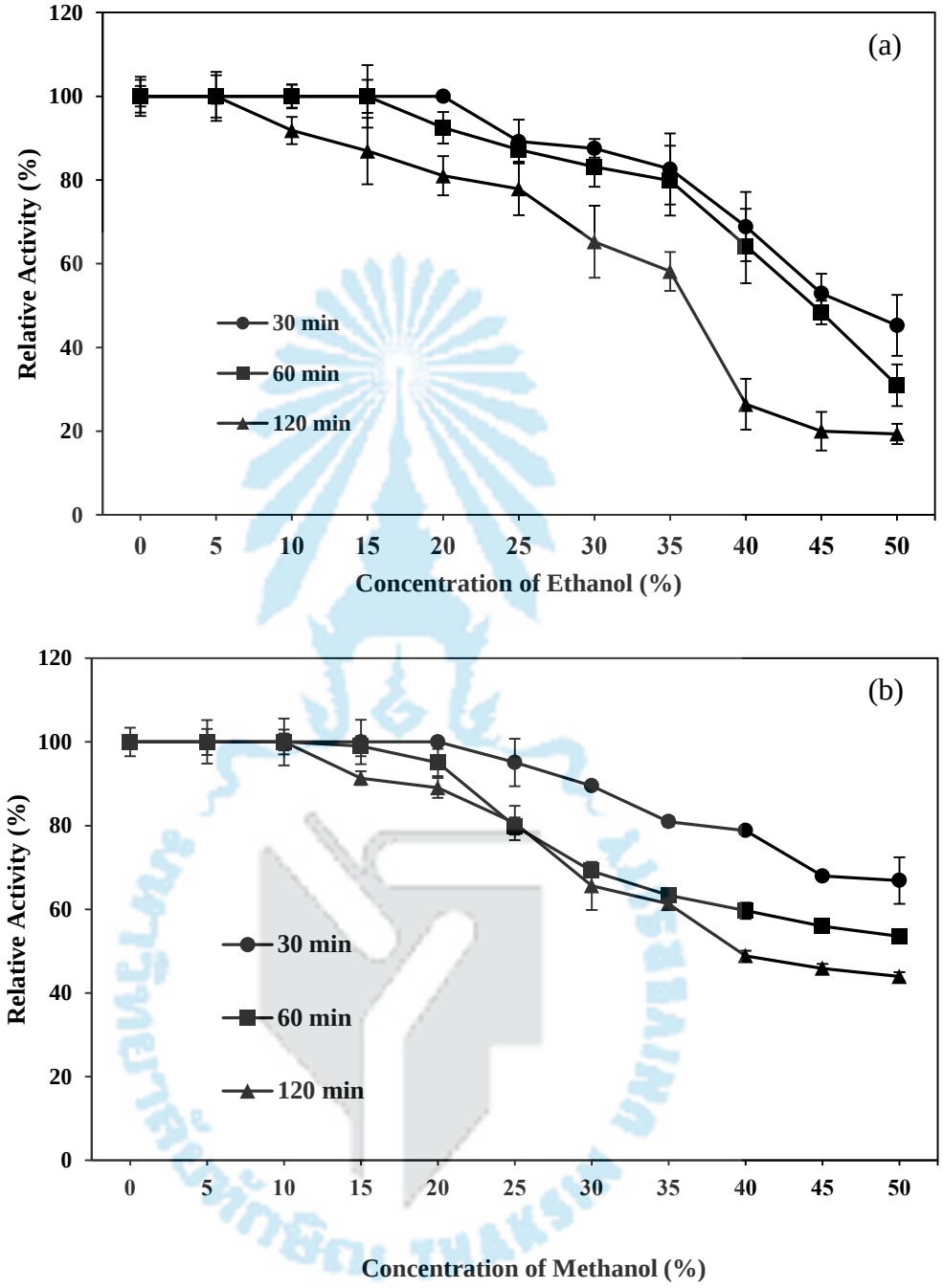


Figure 18. Effect of Ethanol (a) and Methanol (b) on Stability of Fractionated Lipase from Viscera of Nile Tilapia. Bars Represent the Standard Deviation from Triplicate Determinations (⧳ = ± 1 SD).

4.2.4 Effect of Bile Salts on Enzyme Activity

The effect of bile salts including NaDC, NaTC and NaC on the activity of the fractionated lipase from Nile tilapia viscera was measured by incubating the enzyme with bile salts at different concentrations (0-10 mM) for 30 min at room temperature. The fractionated lipase was activated by lower concentrations of bile salts NaC and NaTC (1 mM), but at higher concentrations of NaC and NaTC (≥ 7 mM), there were less pronounced or even no increase in activation of fractionated lipase. The increases in activity at lower bile salt concentrations found with fractionated lipase in this investigation may be because of the influence of the additional lipid binding capability of bile salts on the emulsified substrate (Aryee, Simpson and Villalonga. 2007 : 394). For NaDC, it was relatively unaffected at all the concentration used (Figure 19). Bile salts may also facilitate the absorption of free fatty acids by forming mixed micelle with the products to enhance lipase activity (Wang, et al. 1985 :824). Similar observations have been made with lipase from grey mullet and red sea bream (Aryee, Simpson and Villalonga. 2007 : 394; Iijima, Tanaka and Ota. 1998 : 59) .

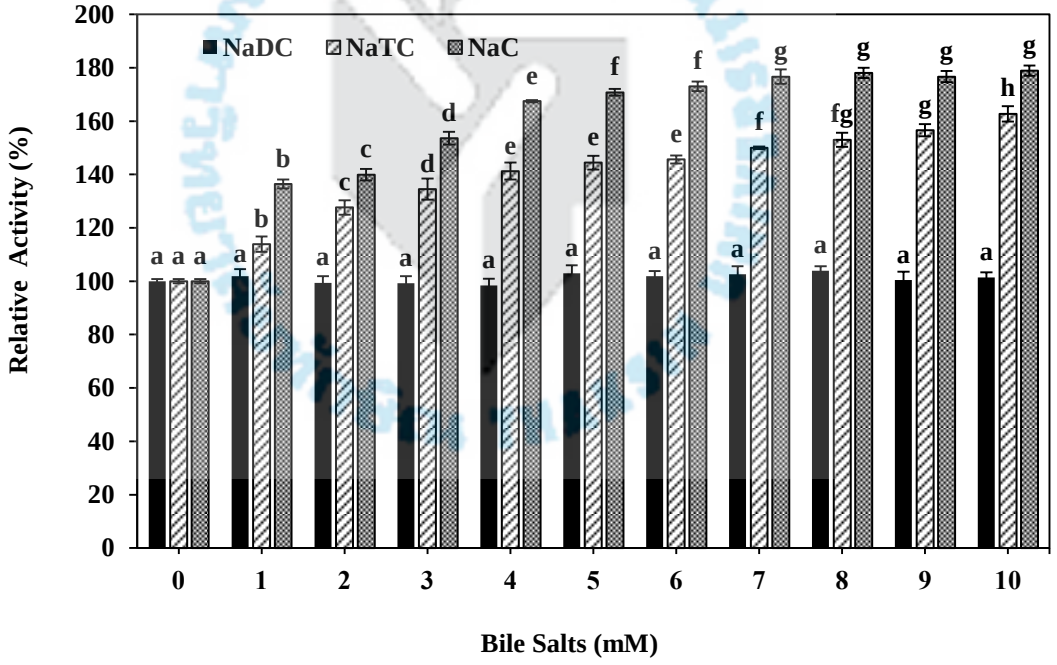


Figure 19. Effect of Bile Salts on the Activity of Fractionated Lipase from Viscera of Nile Tilapia. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.3 Effect of Ultrasound on Fractionated Lipase Activity

The influence of ultrasonic time on the activity of fractionated lipase from Nile tilapia viscera is shown in Figure 20a. The graph shows that an increase in ultrasonic treatment time significantly increased the lipase activity till 20 min of irradiation time and there was no significant difference on lipase activity after ultrasonic treatment for 25 min ($P>0.05$). However, when the ultrasonic treatment duration exceeded 25 min, fractionated lipase activity was decreased. By using ultrasound, a 51.05% increase in enzyme activity of fractionated lipase was obtained at optimum treatment time of 20 min. Application of ultrasound generates the conditions of fluctuations in local velocity and pressure in adjacent fluid resulting in varied turbulence (Sutkar and Gogate. 2009 : 26; Ashokkumar, Lee, Kentish and Grieser. 2007 : 470). It can be stated that when ultrasonic treatment was conducted for an optimum time, most of enzyme molecules in the solution underwent conformational change, which results in the enhancement in the lipase activity. Also, the mass transfer resistances were eliminated giving favourable results for the activity. It is important to note here that further increases in treatment time caused harmful effects, as continuous exposure to cavitating conditions for prolonged time led to degradation of the amino acid residues which contributed to the substrate binding domain or catalytic domain of the enzyme molecules resulting in a decrease in enzyme stability (Basto, Silva, Gubitzb and Cavaco-Paulo. 2007 : 355).

Figure 20b shows the influence of the ultrasonic frequency on the activity of fractionated lipase from Nile tilapia viscera. The maximum activity of fractionated lipase was achieved at an ultrasonic frequency of 24 kHz. When ultrasonic frequency exceeded 24 kHz, the activity of fractionated lipase decreased gradually with the increasing of ultrasonic frequency. The cavitation phenomenon occurring during ultrasonication was responsible for the observed behaviour of change in fractionated lipase activity with varying frequency. Ultrasound can rupture the weak linkages like hydrogen bonds or Van der Waals interactions and bring conformational changes in protein structure (Gebicka and Gekicki. 1997 : 133). Low frequency ultrasound irradiation in liquids causes the stable cavitation (Subhedar and Gogate. 2014 : 108). The forces induced by oscillation of stable cavitation bubbles changes the spatial conformation of enzymes, and thus enhances the activity of

enzymes (Wang, Huang, Huang, Wang and Huang. 2007 : 1121). On the other hand, high frequency ultrasound enhances the cavitation effects that cause significant shear in the liquid medium (Entezari and Petier. 2004 : 257).

The influence of ultrasonic temperature on the activity of fractionated lipase from Nile tilapia viscera was also investigated and can be observed in Figure 20c. The results demonstrated that maximum activity of fractionated lipase was observed for ultrasonic temperature of 40°C. When ultrasonic temperature exceeded 40°C, the activity of fractionated lipase decreased gradually with increasing in the ultrasonic temperature. Ultrasonic irradiation, under these extreme conditions, could cause great damage to polypeptide chains, leading to inactivation of the enzyme (Ozbek and Ulgen. 2000 : 1037). Additionally, extreme increases in localized pressure and temperature at higher intensity also leads to the generation of free hydroxyl and hydrogen radicals. These free radicals react with the enzyme causing its inactivation (Entezari and Petier. 2004 : 257). The fractionated lipase activity was also tested at temperatures ranging from 20 to 60°C without ultrasound. After incubation, the fractionated lipase without ultrasonic showed lower activity as compared with that of ultrasonic temperature (data not shown). From the results, ultrasound had an effect on the activity of fractionated lipase from Nile tilapia viscera and low frequency ultrasound was demonstrated to have a positive effect on fractionated lipase activity.

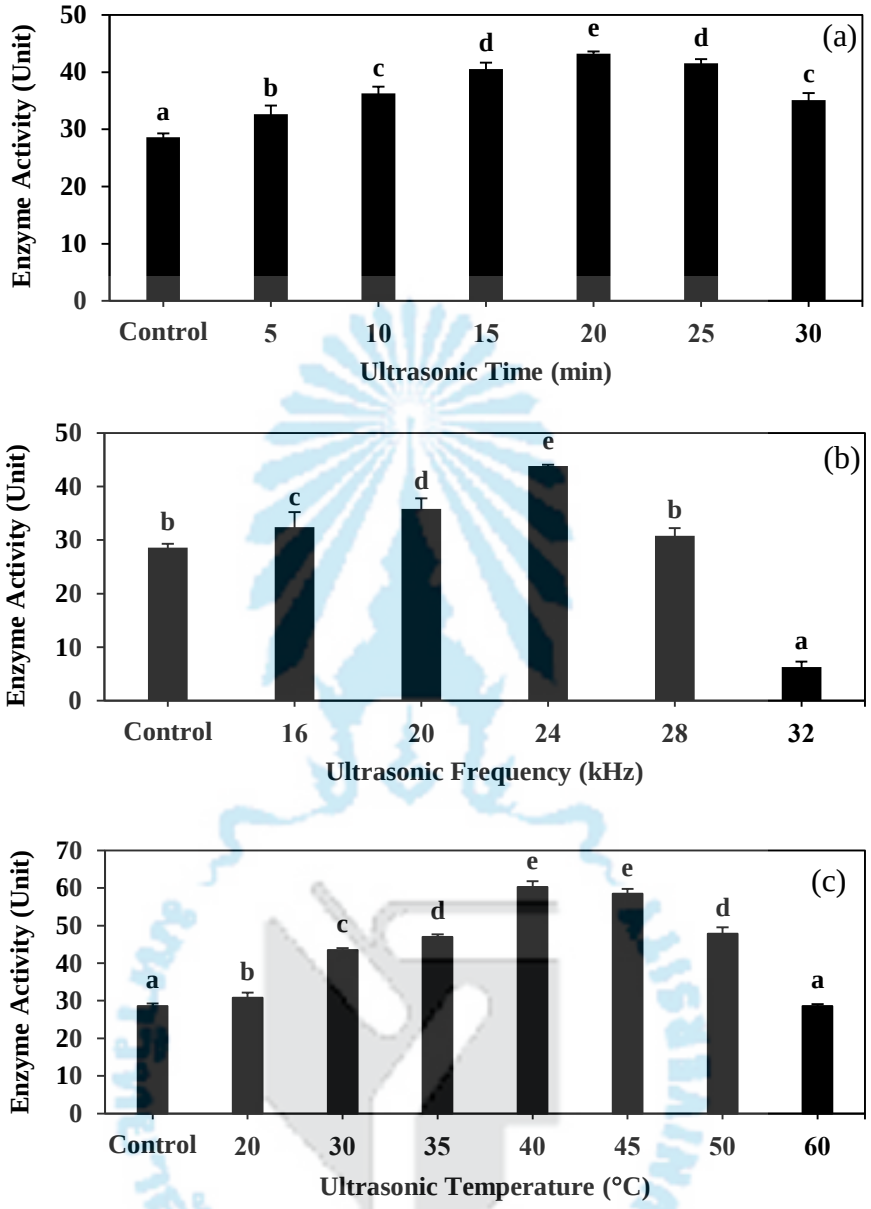


Figure 20. Effect of Ultrasonic Time (a) Ultrasonic Frequency (b) and Ultrasonic Temperature (c) on the Activity of Fractionated Lipase from Viscera of Nile Tilapia. Conditions: (a) Ultrasound was at 24 kHz, 180 W and 30°C; (b) Ultrasound was at 180 W and 30°C for 20 Min; (c) Ultrasound was at 24 kHz and 180 W for 20 Min; Control Represents Enzyme without Ultrasound. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

5. Conclusions

Nile tilapia viscera lipase was successfully purified by T-ATPS. The type and concentration of salt, concentration of EOPO, concentration of NaCl, temperature, and the ratio between EOPO/distilled water could influence lipase partitioning. The purification of Nile tilapia viscera lipase using thermoseparating phase components may be suitable for development as a primary recovery operation for lipase derived from fish viscera. Based on the biochemical characterizations, partitionated lipase has the potential to be applied for various applications. Moreover, the present work has clearly established that low frequency ultrasound irradiation could obviously improve the activity of partitioned lipase from Nile tilapia viscera.

CHAPTER 4

Optimization of Process Variable for the Production of Biodiesel by Transesterification of Used Cooking Oil Using Lipase From Nile Tilapia Viscera

1. Abstract

Biodiesel synthesis through transesterification of used cooking oil or frying oils using the viscera lipase from Nile tilapia (*Oreochromis niloticus*) as a low-cost feedstock and catalyst, respectively, was optimized. The influences of operating factors, including methanol/oil molar ratio, concentration of enzyme, reaction time, type of alcohol, water content and reaction temperature on the yield of biodiesel were investigated. The optimized conditions to achieve maximum biodiesel yield were obtained using a lipase concentration of 30 kUnit, a methanol to oil molar ratio of 4:1, a water content of 3%, a reaction temperature of 45°C and a 28 h reaction time. Under these optimal operating conditions, the highest biodiesel yield observed was 96.5%. The attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was applied to ensure the conversion of used cooking oil into biodiesel. The biodiesel characteristics met the specifications set as prescribed by EN 14214 and ASTM D 6751. The results from this investigation show the viability of economical biodiesel production using byproducts as both source and catalyst.

2. Introduction

Exhaustion of fossil fuel resources and their environmental effects are the main reasons for studying renewable fuels. Biodiesel is a renewable, non-petroleum based biofuel that has received increasing attention in recent years to apply in compression-ignition engines (Guldhe, Singh, Mutanda, Permaul and Bux. 2015 : 1447). Biodiesel is made of alkyl esters resulting from transesterification of triglycerides (TGs) or esterification of free fatty acids (FFAs) with short chain alcohols (Zhang, Dube, McLean and Kates. 2003 : 229). Biodiesel is now mainly produced from soybean oil (Cao, Han and Zhang. 2005 : 347), sunflower oil (Granados, Proves, Alonso, Mariscal, Galisteo, Tost, Santamaría and Fierro. 2007 : 317), palm oil (Kuepethkaew, Sangkharak, Benjakul and Klomklao. 2017 : 139), cotton seed oil (Onukwuli, Emembolu, Ude, Aliozo and Menkiti. 2017 : 103), and rapeseed oil (Georgogianni, Katsoulidis, Pomonis, Manos and Kontominas. 2009 : 1016). However, the cost of raw material and limited availability of vegetable oil feedstocks are always critical issues for the production of biodiesel. The high cost of vegetable oils, which could be up to 75% of the total processing cost, has led to the costs of biodiesel production becoming approximately 1.5 times higher than that for diesel (Cao, Han and Zhang. 2005 : 347). In order to overcome this limitation, biodiesel manufacturers are focusing their attention on using a low-cost feedstock such as used cooking oil in order to ensure economic viability in the production of biodiesel (Narkhede, Brahmkhatri and Patel. 2014 : 253).

Large amounts of used cooking oil are produced annually throughout the world. Used cooking oil normally undergoes repeated mixing with fresh oil for continued application in local restaurants before being discarded. At homes, used cooking oil is not usually kept for mixing and is discarded into local drainage systems. The oil disposal contributes to environmental pollution by causing local pollution of the water bodies in which the drainage systems end. Hence, conversion of used cooking oil into biodiesel automatically eliminates these problems. Used cooking oil is abundantly available and can be easily obtained free of cost, if not at a minimal price. As a result, the processing cost of biodiesel gets decreased drastically (Dhawane, et al. 2018 : 3971). The amount of these low quality oils are over 15 million tons per year, which, if converted to biodiesel, satisfy the main part of the

world demand (Lopresto, Naccarato, Albo, De Paola, Chakraborty, Curcio and Calabrò. 2015 : 229). Nevertheless, the use of waste cooking oils as feedstock is rather challenging as it basically contains high content of free fatty acids (FFAs) and water concentrations which makes them unsuitable for the homogeneous alkaline-catalyzed transesterification as used in a conventional biodiesel production process. Although a conventional chemical method using both acid and base chemical catalysts has been used to produce biodiesel, difficulties in recovery of glycerol from biodiesel and removal of inorganic salts, high temperature and undesirable side reactions along with the transesterification reaction are the certain limitations that restrict the use of chemical catalysts (Ranganathan, Narasimhan and Muthukumar. 2008 : 3975). On the other hand, utilization of lipases as a catalyst for the production of biodiesel fuel has great potential compared with chemical technology (Mehrasbi, Mohammadi and Peyda. 2017 : 593). However, using lipases for commercial biodiesel production has a high cost. To reduce the cost of commercial lipase applied for the production of biodiesel, cheap lipases from seafood processing wastes, especially from the viscera, could be an alternative.

Nile tilapia (*Oreochromis niloticus* L.) is one of the most important farmed fish in Thailand. Nowadays, Nile tilapia is used as a raw material for fermentation and drying (Santos, Malveira, Cruz and Fernandes. 2010 : 275). The growth of the Nile tilapia manufacturing industry has resulted in the production of large quantities of fish wastes, especially viscera. However, they are also a potential source of lipases. Lipase from Nile tilapia viscera show high activity and stability in high temperature (Patchimpet, Sangkharak and Klomklao. 2019a). Based on our previous study, a process for lipase recovery using thermoseparating aqueous two-phase system (T-ATPS) was developed successfully. However, to the best of our knowledge, there is no previous work on the use of partially purified lipase from Nile tilapia viscera as biocatalyst for the production of biodiesel. The aim of this investigation was to study the transesterification reaction using used cooking oil and lipase from Nile tilapia viscera. The parameters considered in this study for optimization are methanol/oil molar ratio, concentration of enzyme, reaction time, type of alcohol, water content and reaction temperature. The physicochemical parameters of the obtained biodiesel were investigated to determine key parameters

such as FAMEs content, viscosity, acid value, water content, iodine value, density, pour point and cloud point. These parameters were measured and compared with standard biodiesel parameters to identify the obtained biodiesel quality.

3. Material and Methods

3.1 Materials

Used cooking oil was obtained from the Sea Wealth Frozen Food Co., Ltd. (Songkhla, Thailand). The different characteristics of used cooking oil were determined. *p*-nitrophenyl palmitate (*p*-NPP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The standard methyl esters used for preparing calibration curves and the heptadecanoic acidmethyl ester used as an internal standard were also obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents used in the experimental study were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Nile tilapia (*Oreochromis niloticus*) viscera was collected from a local market in Thale Noi, Phatthalung, Thailand. Samples were placed in polyethylene bags and imbedded in a polystyrene box containing ice with an ice/sample ratio of 2:1 (w/w) and transported to our laboratory at Thaksin University, Phatthalung campus within 30 min. Viscera of Nile tilapia was collected, immediately frozen and stored at -20°C until needed.

3.2 Enzyme Production

3.2.1 Lipase Purification

Defatted powder of Nile tilapia (*Oreochromis niloticus*) viscera was prepared by the same method as Patchimpet, Sangkharak and Klomklao (2019b) and Kuepethkaew, et al. (2017 : 769). Lipase was extracted by stirring from the defatted powder in 50 vol 50mM Tris-HCl buffer (pH 7.0) containing 1.0 M NaCl and 0.2% (v/v) Brij 35. The extract was centrifuged at 5,000 × g at 4°C for 30 min, and then the supernatant was collected and referred to as “crude extract”.

Lipase from the viscera of Nile tilapia was purified using the thermoseparating aqueous two-phase system (T-ATPS) by the method of Patchimpet, Sangkharak and Klomklao (2019b). T-ATPS was prepared in 15 ml centrifuge tubes

by adding 20% crude enzyme, 40% EOPO3900, 10% $(NH_4)_2SO_4$ and 4% NaCl. Distilled water was used to adjust the system to obtain a final mass of 10 g. The mixtures were mixed continuously for 3 min by a vortex mixer and then centrifuged for 15 min at 5,000 × g to induce phase separation. After centrifugation, the EOPO-rich top phase was collected from the primary ATPS and diluted with distilled water in a ratio of 1:1 (w/w). The samples were then placed in a water bath at 60°C for 15 min to induce thermo-separation. After that, the samples were centrifuged at 5,000 × g for 15 min and the secondary two-phase separation was formed. The top phase was collected and freeze-dried using a Dura-Top™ mp freeze-dryer (FTS Systems Inc., Stone Ridge, NY, USA). The powder obtained was stored at -20°C until used and named as partially purified lipase.

3.2.2 Lipase Activity Assay

Lipase activity was measured spectrophotometrically using *p*-NPP as substrate according to the method of Patchimpet, Sangkharak and Klomklao (2019a). One volume of 8.0 mM substrate solution in isopropanol was mixed just before use with nine volumes of 50mM Tris-HCl buffer pH 7.5 containing 0.4% (w/v) Triton X-100 and 0.1% gum arabic. This solution (0.9 mL) was equilibrated at 37°C and the reaction was started by the addition of 0.1 mL of the enzymatic solution. The variation of optical density at 410 nm was monitored against a blank without enzyme using a Shimadzu ultraviolet (UV) 1700 spectrophotometer (Kyoto, Japan). The amount of liberated *p*-nitrophenol was determined at 410 nm during the first 5 min of reaction. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute under the assay conditions.

3.3 Biodiesel Optimization Studies

The enzymatic transesterification reactions were performed in a 50 mL closed flask with constant shaking at 200 rpm (Kuepethkaew, et al. 2017 : 139). The reaction mixture consisted of used cooking oil, enzyme, alcohol and deionized water. The effect of parameters such as effects of enzyme loading (10-60 kUnit), molar ratio methanol/oil (from 1:1 to 7:1), reaction time (4-36 h), type of alcohol (methanol, ethanol, 2-propanol and *t*-butanol), water content (1-9%, based on oil

weight) and reaction temperature (from 30°C to 55°C) on the biodiesel yield was investigated. Samples were centrifuged at 5,000 × g for 10 min to obtain the upper layer. The upper layer of samples were collected and referred to as "lipase-catalyzed biodiesel, LCBD". Heptadecanoic acid methyl ester which served as the internal standard and an aliquot of the upper layer were precisely determined and mixed thoroughly to GC analysis to measure the contents of FAMEs.

3.4 GC Analysis

The methyl ester contents in the reaction mixture were quantified using a GC7890. A gas chromatograph (Agilent Corp., California, America) was connected to a PEG-20M capillary column (0.32 mm × 30 m × 0.25 mm) and a flame ionizing detector (FID). The injector and detector temperatures were adjusted to 250 and 260°C, respectively. Nitrogen was used as the carrier gas. The column temperature was maintained at 180°C for 2 min, and then increased to 230°C at a rate of 5°C/min, and maintained at 230°C for 20 min. Percent biodiesel yield was defined as fatty acid methyl esters amount produced divided by the initial amount of used cooking oil.

3.5 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR was used to determine the lipid conversion by chemical group shift as it is a rapid and easy detection method (Kuepethkaew, et al. 2017 : 139). The ATR-FTIR spectra of LCBD, commercial biodiesel and diesel were taken using Agilent Cary 630 FTIR with a specified range of 800-4000 cm^{-1}.

3.6 Biodiesel Properties

Biodiesel produced in this study was analyzed for fuel characteristics such as FAMEs content, viscosity, acid value, water content, iodine value, density, pour point and cloud point using international standard methods and their values were compared with those of international standards, European norms, the European Committee for Standardization (EN 14214) and the American Society for Testing and Materials (ASTM D 6571) standards.

3.7 Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (Kuepethkaew, et al. 2017 : 139). Statistical analysis was performed using a SPSS package (SPSS 11.5for windows, SPSS Inc., Chicago, IL, USA).

4. Results and Discussion

4.1 Characterization of Used Cooking Oil

Properties of the used cooking oil used in this investigation are shown in Table 19. This used cooking oil showed the required characteristics for a basic transesterification reaction.

Table 19 Properties of Used Cooking Oil

Properties	Method	Used Cooking Oil[a]
Saponification Value (mg KOH/g of oil)	AOAC (1990)	202.46 ± 3.54
Acid Value (mg KOH/g)	ASTM D 664-01	0.74 ± 0.04
Viscosity at 40°C (mm^2/s)	ASTM D 445-06	59.90 ± 0.10
Density (15°C) (kg/m^3)	ASTM D 4052-96	891.39 ± 1.97

[a] Mean ± SD from triplicate determinations.

4.2 Biodiesel Optimization Parameters

4.2.1 Effect of Enzyme Loading

The amount of partially purified lipase from Nile tilapia viscera is a crucial factor that affects the biodiesel production. The influences of enzyme loading on transesterification of used cooking oil were studied in the range of 10-60 kUnit with constant used cooking oil to methanol ratio (4:1) and water concentration (1%). The total reaction time was 24 h and the reaction temperature was 45°C. The biodiesel yield increased as the amount of Nile tilapia lipase increased (Figure 21). The biodiesel yield peaked at approximately 88.89% with 30 kUnit Nile tilapia lipase.

However, with further increases in enzyme loading, the final yield slightly decreased. Su, Li, Fan and Yan (2015 : 298) reported that the biodiesel yield improved when the enzyme loading range was from 1% to 6% (based on the oil weight) with the highest yield of 93.40% at 6% lipase loading. With further increases in enzyme loading, the final yield did not result in any significant enhancement. Suhedar, et al. (2015 : 530) also found that the transesterification of sunflower oil using immobilized lipase from *Thermomyces lanuginosus* increased when the enzyme loading increased from 3% to 7% and the biodiesel yield increased from 19.25% to 93.30%. But further increase in enzyme loading did not show any significant increase in biodiesel yield. For higher enzyme loading, rapid formation of enzyme-substrate complex explains the higher conversion. However, excess enzyme leads to problems of mixing by increasing viscosity and ultimately limits the masstransfer (Gharat and Rathod. 2013 : 900). In view of this result, 30 kUnit of viscera lipase from Nile tilapia was chosed as the optimum enzyme loading used to the subsequent methanolysis of used cooking oil.

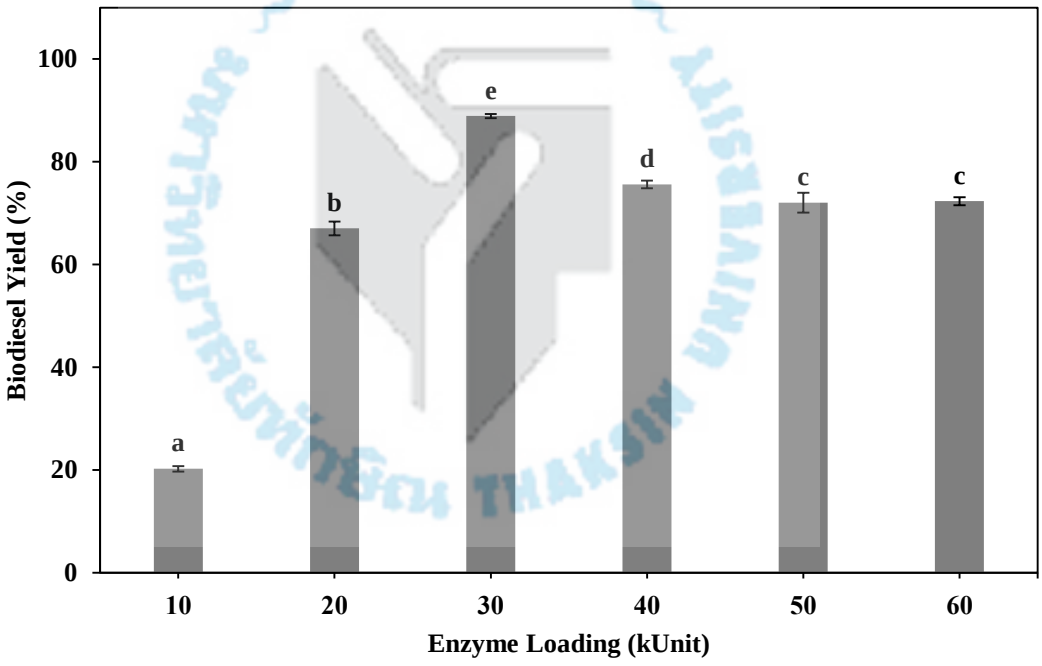


Figure 21. Effect of Enzyme Loading on Transesterification of Used Cooking oil. Process Parameter: 4:1 Methanol to Oil Molar Ratio, 1% Water Content, 45°C and 24 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.2 Effect of Methanol to Oil Molar Ratio

The molar ratio of alcohol to triglyceride is one of the most contributing factors in biodiesel production yield. To see the influence of the ratio of methanol to oil, the transesterification reaction was carried out by varying the methanol/oil ratio from 1:1 to 7:1 using 30 kUnit of the Nile tilapia lipase as a catalyst and water concentration of 1% for 24 h at 45°C. It was observed from Figure 22 that the conversion increased with increasing the ratio of methanol to oil and reached to 89.71% at the methanol/oil ratio of 4:1. However, a further increase in the ratio of methanol to oil beyond 4:1 resulted in a small decrease in the oil conversion ($P< 0.05$). Kuepethkaew, et al. (2017 : 139) studied the transesterification reaction of methanol with palm oil catalyzed by partially purified lipase from the hepatopancreas of Pacific white shrimp, and reported a 97.01% yield at a methanol to oil molar ratio of 4:1. Yang, He, Xu, Zhang and Yan (2016 : 76) also reported a 92.24% methyl ester yield for the transesterification reaction of soybean oil performed at 45°C and a methanol to oil molar ratio of 4:1 for 42 h reaction time using *Burkholderia cepacia* lipase. In stoichiometry ratio of transesterification, three alcohol moles and one triglyceride mole are required to produce three ester alkyl moles of fatty acid and one glycerol molecule (Reyero, Arazamendi, Zabala and Gandía. 2015 : 147). This is an equilibrium reaction and a great amount of alcohol is required to push the equilibrium toward the product. Being a reversible reaction, excess methanol is used to shift the reaction to the right (Keera, El Sabagh and Taman. 2018 : 979). However, methanol serves as reaction substrate for the production of biodiesel, and it is harmful to proteins in excessive proportions in particular when it is soluble in the reaction mixture (it forms an emulsion, and the size of the droplets depends on the intensity of stirring) (Yang, et al. 2016 : 76). The excess methanol dilutes the oil in the reaction system, decreasing the collision frequency of oil and catalyst (Zhang, et al. 2010 : 3960). In addition, excess alcohol levels may inhibit the enzyme activity and there by decrease its catalytic activity toward the tranesterification reaction (Noureddini , Gao and Philkana. 2005 : 769). Therefore, a methanol/oil molar ratio 4:1 was used for further experiments.

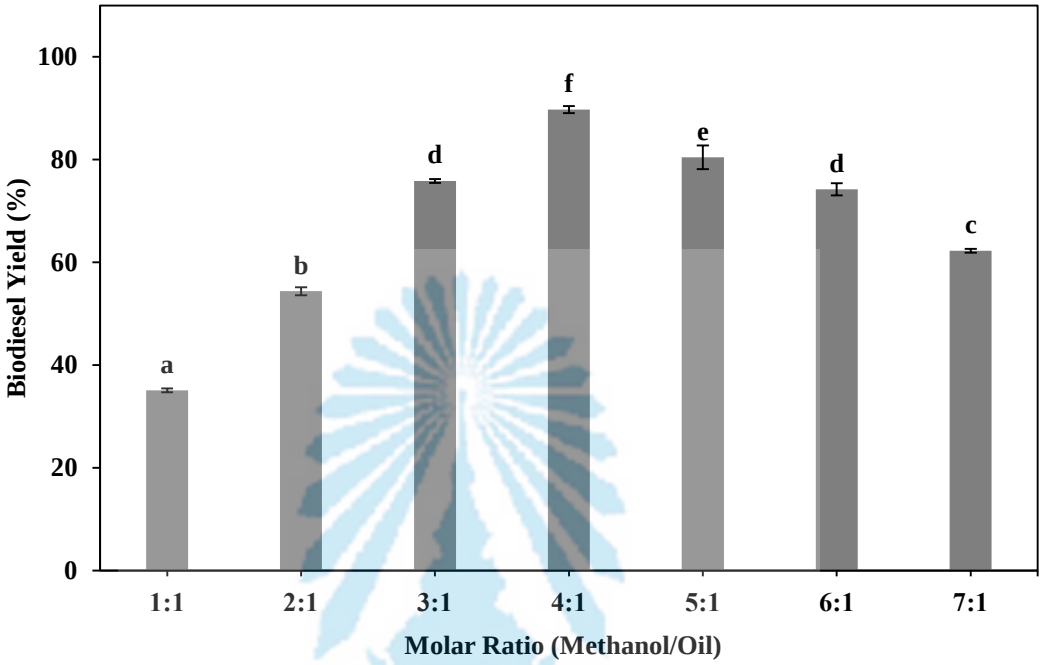


Figure 22. Effect of Methanol/Oil Molar Ratio on Lipase Catalyzed Transesterification of Used Cooking Oil. Process Parameter: 30 kUnit of Nile Tilapia Lipase, 1% Water Content, 45°C and 24 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.3 Effect of Reaction Time

Reaction time is known to be one of the most important variables affecting the yield of biodiesel. Effect of reaction time on the transesterification rate of used cooking oil with methanol using partially purified lipase from Nile tilapia viscera at levels of 30 kUnit was studied in the range of 4 and 36 h with an interval of 4 h. As observed from Figure 23, when the reaction time was extended to 28 h, the conversion increased to 93.24%, as an equilibrium conversion. However, no significant increase in the conversion was found with prolongation of the reaction beyond 28 h ($P > 0.05$). It may be because of a lack of substrate for the enzyme after 28 h of reaction time. Gharat and Rathod (2013 : 36) found that the maximum conversion of fatty acid methyl ester for used cooking oil was obtained in 24 h. In the other work, the optimum time for the highest conversion of fatty acid and the biodiesel yield from waste cooking oil was found to be 35 h with immobilized

Burkholderia cepacia lipase (Karimi. 2012 : 182). From this results, the reaction time of 28 h was selected to be studied later.

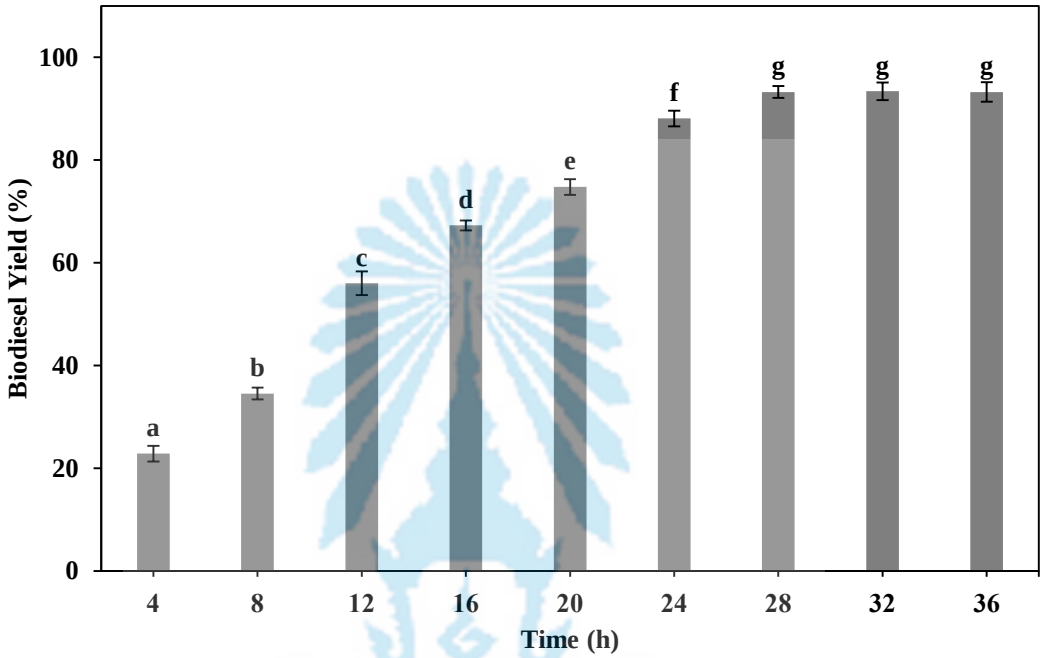


Figure 23. Effect of Time Course on Lipase Catalyzed Transesterification of Used Cooking Oil. Process Parameter: 30 kUnit of Nile Tilapia Lipase, 4:1 Methanol to Oil Molar Ratio, 1% Water Content and 45°C. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.4 Effect of Type of Alcohol

Once the best enzymatic preparation and some optimal operative conditions, such as enzyme loading, stoichiometric ratio and reaction time were measured, attention was focused on the different alcohols used for the reaction. The results obtained are displayed in Figure 24. The maximum biodiesel yield achieved by partially purified lipase from Nile tilapia viscera was 93.08% with methanol as the organic solvent. When ethanol, 2-propanol, and *t*-butanol were used as the organic solvent, the biodiesel yields were 69.59, 44.15 and 38.80%, respectively. Karimi (2012 : 182) reported the optimal solvent for transesterification reaction of waste cooking oil catalyzed by immobilized *Burkholderia cepacia* lipase was methanol. Similarly, Ali, Qureshi, Mbadinga, Liu, Yang and Mu (2017) reported

a methyl ester yield of 86% for the transesterification reaction of waste cooking oil with methanol as the optimal organic solvent using lipase from *Pseudomonas aeruginosa* FW_SH-1. Methanol is widely used as an acyl acceptor for the production of methyl esters and is often preferred over other alcohols because of its low cost, in particular. Furthermore, the rate of lipase-catalyzed transesterification reaction usually increase with decreasing the length of hydrocarbon chain of alcohol (Meneghetri Meneghetti, Wolf, Lima, de Lira Silva, Serra, Cauduro and de Oliveira. 2006 : 2262). In our experiment, methanol was chosen for the biodiesel process.

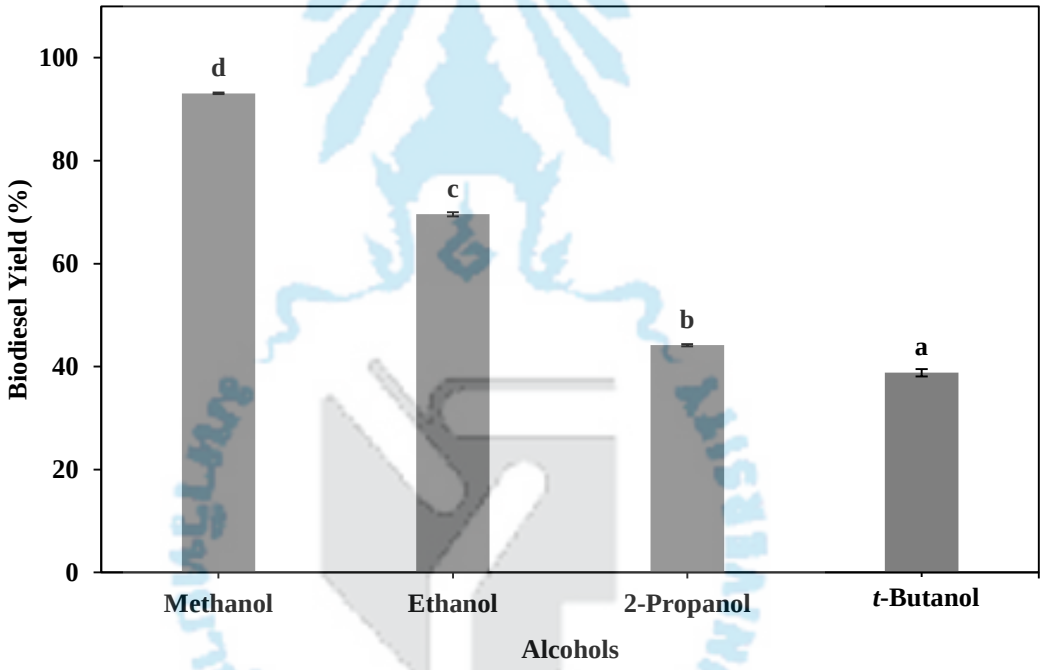


Figure 24. Effect of Type of Alcohol on Lipase Catalyzed Transesterification of Used Cooking Oil. Process Parameter: 30 kUnit of Nile Tilapia Lipase, 1% Water Content, 45°C and 28 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.5 Effect of Water Content

Water content in a reaction mixture is an essential factor for measuring the yield of enzymatic biodiesel synthesis (Antczak, Kubiak, Antczak and Bielecki. 2009 : 1185). Generally, some essential water is required to keep the enzyme active in organic solvents. On the contrary, water might take part in the transesterification, thus affecting the equilibrium (Kaieda, Samukawa, Matsumoto, Ban, Kondo, Shimada, Noda, Nomoto, Ohtsuka, Izumoto and Fukuda. 1999 : 627). Lipase possesses the unique feature of acting at the interface between an aqueous and an organic phase, so the lipase activity generally depends on the interfacial area. Water facilitates an increase in the available interfacial area and hence helps to maintain lipase activity. However, excess water might make the lipase more flexible and lead to some unintended side-reactions such as hydrolysis, especially in the transesterification process (Tan, Lu, Nie, Deng and Wang. 2010 : 628). In the present study, influence of water content was investigated by carrying out reactions at varying concentrations of 1-9%. Maximum yield was obtained with 3% of water (Figure 25). Higher water concentration resulted in lower biodiesel yields ($P < 0.05$). Kuepethkaew, et al. (2017 : 139) and Rakkan, Suwanno, Paichid, Yunu, Klomklao and Sangkharak (2017 : 309) reported the effect of water on the transesterificaion reaction by partially purified lipase from Pacific white shrimp hepatopancreas. In their study, the optimal water content for partially purified lipase was 3%. With high water content, the reaction slows down because of decreased contact between the lipase and the oil (Kuepethkaew, et al. 2017 : 139). In addition, a larger quantity of water might affect the mass transfer of oil and methyl esters through the aqueous phase; hence, lowering the production (Arumugam and Ponnusami. 2014 : 276). In our study, 3% water content was selected for biodiesel preparation.

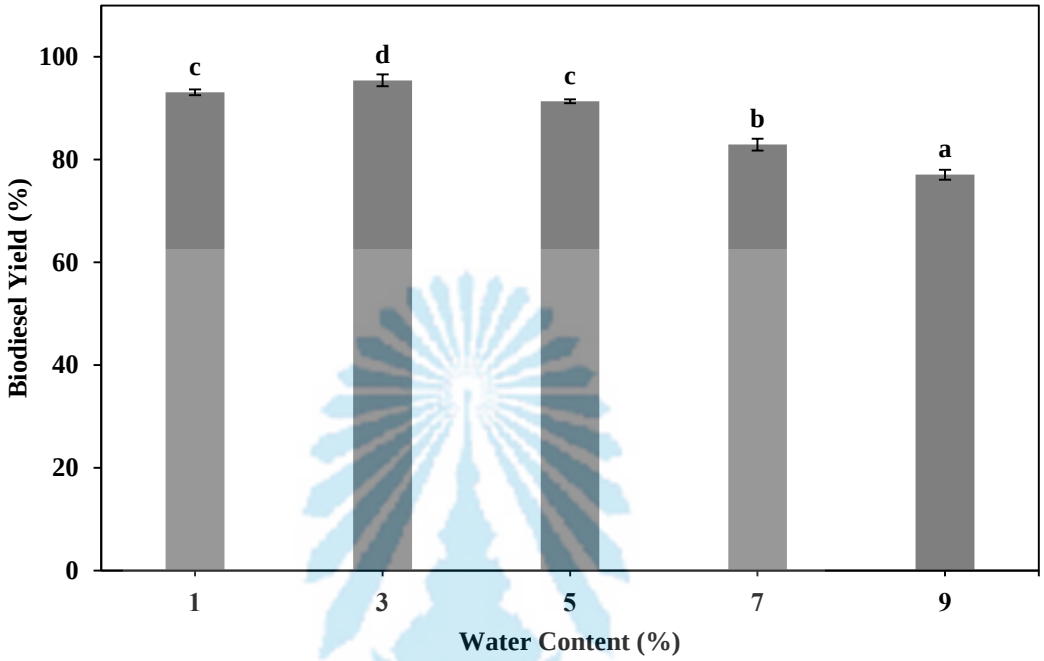


Figure 25. Effect of the Water Content on Lipase Catalyzed Transesterification of Used Cooking Oil. Process Parameter: 30 kUnit of Nile Tilapia a Lipase, 4:1 Methanol to Oil Molar Ratio, 45°C and 28 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2.6 Effect of Reaction Temperature

Temperature is specific to each enzyme and its functions. It is to be noted that biodiesel yield is proportional to the temperature involved; at a low temperature, a slow activity is evinced, and as the temperature increased the reaction rate also increased with an effective increase in the production of biodiesel (Rahimi, Aghel, Alitabar, Sepahvand and Ghasempour. 2014 : 599). However, enzymes are temperature-dependent and easily deactivated at high temperature. In order to investigate the effect of temperature on the enzymatic biodiesel process, the range studied was between 30-55°C with an interval of 5°C. As illustrated in Figure 26, the biodiesel conversion and production rate increased when the temperature was increased from 30°C to 45°C, but decreased when the temperature was further increased to 50°C ($P < 0.05$). The maximum biodiesel yield was obtained at a reaction

temperature of 45°C. This trend was found in almost all studies about the influences of temperature on the biodiesel production as the lipases catalysis (Kuepethkaew, et al. 2017 : 139; Su, et al. 2015 : 298; Ali, et al. 2017 : 93). As the reaction temperature elevated, the collision chance between the enzyme and substrate molecules increased, which might help to form enzyme-substrate complexes, and then led to an increase in biodiesel yield. As for the decrease in biodiesel yield with further temperature increases above 45°C, it was mostly likely due to the denaturation (alteration) of the protein structure that resulted from heat-induced destruction of noncovalent interactions (the breakdown of the weak ionic and hydrogen bonding that stabilized the three dimensional structure of the enzyme) (Yadav and Lathi. 2006 : 814). A temperature of 45°C seems to be the best compromise. Therefore, the other experiments were performed at this temperature.

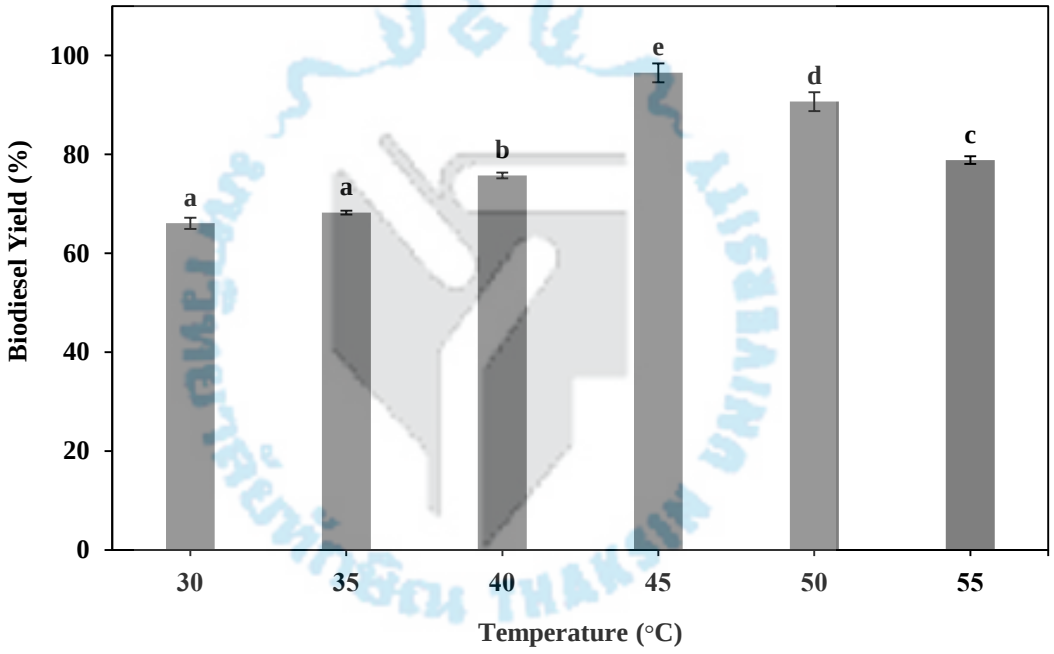


Figure 26. Effect of the Reaction Temperature on Lipase Catalyzed Transesteri fication of Used Cooking Oil. Process Parameter: 30 kUnit of Nile Tilapia Lipase, 4:1 Methanol to Oil Molar Ratio, 3% Water Content and 28 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (⧳ = ± 1 SD).

4.3 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR spectra for a sample of the LCBD, commercial biodiesel and conventional diesel are presented in Figure 27.The function groups in ATR-FTIR adsorption were assigned as: 2924 cm^{-1}(asymmetrical stretching of $–CH_2$), 2855 cm^{-1} (symmetrical stretching of $–CH_2$), 1743 and 1744 cm^{-1}(-C=O stretching), 1460 cm^{-1} ($-CH_2$ scissoring), and 722 cm^{-1} ($-(CH_2)_n$-rocking) (Kuepethkaew, et al. 2017 : 139; Fereidooni and Mehrpooya. 2017 : 145). A strong signal in Figure 27a and b at 1744.12 and 1743.05 cm^{-1} indicates the stretching of functional group (-C=O) of biodiesel. Absorption bands at 1363.32 cm^{-1} is because of the methyl group ($-CH_3$-). A cluster of peak at 1245.16, 1196.19 and 1169.81 cm^{-1} denotes C-O stretching (Figure 27b.). The ATR-FTIR spectra of LCBD with conventional diesel (Figure 27a, c) at 1744.12 cm^{-1} was not found in diesel and the strong adsorption bands at 2923.99 and 2855.17 cm^{-1} clearly divulges the stretching of aliphatic hydrocarbon (C-H) in diesel. The ATR-FTIR analysis in the present study was in accordance with Kuepethkaew, et al. (2017 : 139) and Mathimani, Uma and Prabaharan (2015 : 523). The results indicate that the transesterification process is a successful means to alter the fuel characteristics of oil and transfer it into a more valuable fuel.

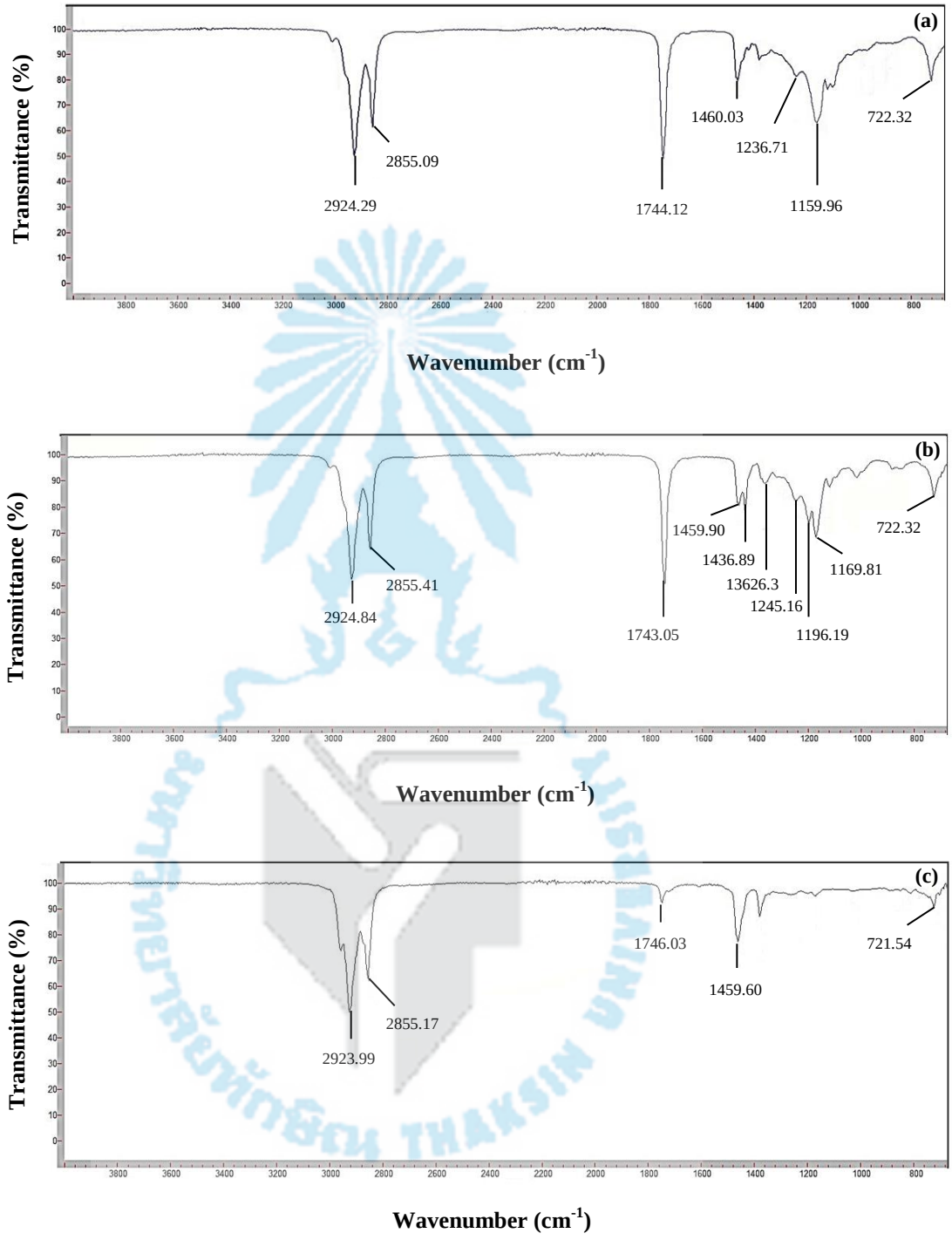


Figure 27. ATR-FTIR Spectra of (a) LCBD, (b) Commercial Biodiesel, (c) Diesel Showing Functional Groups. Signal at ~1740 cm^{-1} Indicates the Presence of Carbonyl Group in Biodiesel.

4.4 Physico-Chemical Characteristics of Biodiesel

Standard biodiesel specifications are set by agencies like ASTM (American Society for Testing and Materials) and EN (European norms, European Committee for standardization) for their suitability to use in compression ignition engines (Singh, Guldhe, Rawat and Bux. 2014 : 216). The fuel properties of LCBD synthesized by transesterification are listed in Table 20. The results were compared with international biodiesel standard values. In general, the LCBD meets the international standard requirements.

The drop formation of biodiesel and quality of fuel air mixture combustion are dependent on the viscosity of fuel. Viscosity (very low/high) is undesirable for the proper functioning of an engine. Low viscosity leads to low penetration, which results in black smoke emission because of low combustion. A very viscous fuel may penetrate the opposite wall of the injector, result in a cold cylinder surface and lead to a low combustion of fuel (Sahar, Sadaf, Iqbal, Ullah, Bhatti, Nouren, ur-Rehman, Nisar and Iqbal. 2018 : 220). The viscosity of LCBD was found to be 4.52 mm^2/s which is within the biodiesel standard (ASTM 445-06). For density of LCBD, it was 879.12 kg/m^3 which meets the biodiesel standards. High density of biodiesel fuel leads to poor atomization, carbon deposits, ring sticking and fuel pump failure (Ramadhas, Jayaraj and Muraleedharan. 2005 : 795). The acid value obtained was 0.41 mg KOH/g, which is well within the specified limits of ASTM (≤0.5 mg KOH/g) and EN (≤0.5 mg KOH/g) standards. The water content of the LCBD (155.2 mg/kg) was within the specific limit of 500 mg/kg.

Iodine value determines the amount of iodine in g absorbed by 100 g oil. Iodine value is a measure of degree of unsaturation of biodiesel; hence, it is helpful to study oil stability. High degree of unsaturation results in polymerization of fuel due to epoxide formation due to addition of oxygen in double bonds. The iodine value of LCBD was 36.80 g I_2 /100 g oil which was also within standard range of biodiesel.

The two important parameters for low temperature uses of a fuel are cloud point and pour point. The cloud point is the temperature at which wax first becomes visible when the fuel is cooled. The pour point is the temperature at which the amount of wax out of solution is sufficient to gel the fuel, thus it is the lowest

temperature at which the fuel can flow (Keera, El Sabagh and Taman. 2018 : 979). Generally, biodiesel has a higher cloud point and pour point compared to conventional diesel. However, the cloud point and pour point of non-edible biodiesel vary significantly with the feed stock depending on fatty acid compositions (Bhuiya, Rasul, Khan, Ashwath, Azad and Hazrat. 2016 : 1129). From these results, the cloud point and pour point for LCBD were 2.0 and 12.17°C, respectively. Kuepethkaew, et al. (2017 : 139) showed that the cloud point and pour point values of the biodiesel from palm oil were 1.0 and 8.5°C, respectively. Therefore, the fuel properties offered by the produced biodiesel comply with the specified EN 14214 and ASTM D 6751 and can be used as an alternative fuel or can be mixed with petro diesel.

Table 20 Properties of LCBD in Comparison with EN 14214 and ASTM D 6751.

Properties	Method	LCBD[a]	Biodiesel Specification	
			EN 14214	ASTM D6751
FAMEs Content (% w/w)	EN 14103	96.50 ± 1.02	Min. 96.5	-
Viscosity at 40°C (mm^2/s)	ASTM D 445-06	4.54 ± 0.03	3.5 – 5.0	1.9 – 6.0
Acid Value (mg KOH/g)	ASTM D 664-01	0.41 ± 0.01	Max. 0.5	Max. 0.5
Water Content (mg/kg)	EN ISO 12937	155.20 ± 0.38	Max. 500	-
Iodine Value (g I_2/100 g)	EN 14111	36.80 ± 0.37	Max. 120	-
Density (15°C) (kg/m^3)	ASTM D 4052-96	879.12 ± 2.20	860 - 900	870 - 890
Pour Point (°C)	ASTM D 5950-02	2.00 ± 0.3	-	Report
Cloud Point (°C)	ASTM D 2500	12.17 ± 1.0	-	Report

[a] Mean ± SD from triplicate determinations.

5. Conclusions

Biodiesel production from used cooking oil was investigated using partially purified lipase from Nile tilapia viscera as a biocatalyst. The maximum biodiesel yield (96.50%) was achieved under optimum conditions containing enzyme loading of 30 kUnit, a methanol/oil molar ratio of 4:1, a water content of 3%, a reaction temperature of 45°C and a reaction time of 28 h. The fuel produced from the lipase based transesterification process was found compatible with ASTM biodiesel standards (D 6751) and European biodiesel standards (EN 14214). The proposed process may lower the production cost of biodiesel and facilitate the disposal of wastes; both used cooking oil and Nile tilapia viscera. Therefore, the production of biodiesel from used cooking oil and partially purified lipase from Nile tilapia viscera has great potential to be utilized as an alternative substrate and biocatalyst.

CHAPTER 5

Ultrasonic Enhancement of Lipase-Catalyzed Transesterification for Biodiesel Production from Used Cooking Oil

1. Abstract

This work investigated the biodiesel production with used cooking oil and methanol through transesterification using lipase from the viscera of Nile tilapia (*Oreochromis niloticus*) under the effect of ultrasonic irradiation. The influence of experimental conditions such as ultrasonic frequency, irradiation time and temperature on biodiesel yield were investigated. The results showed that the use of ultrasound decreased the reaction time from 28 h to 3 h with the use of an ultrasonic frequency of 16 kHz, a methanol to oil molar ratio of 4:1, a lipase concentration of 30 kUnit and a 40°C reaction temperature. The efficacy of using ultrasound was also compared with conventional stirring under the optimum operating conditions. Ultrasonic coupled with stirring explained the further improvement in the transesterification of used cooking oil with methanol using lipase with the highest yield of 97.59%. The properties of obtained biodiesel satisfy the recommended biodiesel standards as prescribed by EN 14214 and ASTM D 6751. Therefore, ultrasound-assisted lipase-catalyzed transesterification of used cooking oil with methanol would be a promising alternative for conventional methods.

2. Introduction

The application of biodiesel to diesel engines for daily activities is advantageous for its environmental friendliness over petro-diesel fuel. The main advantages of using biodiesel is that it is biodegradable, can be used without modifying existing engines, and produces less harmful gas emissions such as sulfur oxide (Atadashi, Aroua and Aziz. 2010 : 1999). Biodiesel is synthesized mostly through the transesterification of vegetable oils and animal fats with alcohol, usually methanol in presence of a catalyst. Although the conventional transesterification by alkaline/acidic catalysts has been used for the production of biodiesel, there are several well-known drawbacks to this approach (Ma and Hanna. 1999 : 1; Fukuda, Kondo and Noda. 2001 : 405). In order to overcome the drawbacks of chemical-catalyzed processes, the enzymatic catalysis has attracted much attention, due to several advantages such as its eco-friendly process, the absence of byproducts, easy product recovery, and the use of lower reaction temperatures. Besides, research efforts have been made nowadays towards developing a cost-effective system using enzyme catalysts for the production of biodiesel (Yu, et al. 2010 : 519; Kuepethkaew, et al. 2017 : 139). Also, another major drawback in commercialization of biodiesel produced from pure vegetable oil is its cost. Substantially higher costs of vegetable oil makes use of refined vegetable oil an unfavorable starting raw material. Hence, there is a need to find ways to minimize the production cost of biodiesel in terms of the costs of raw materials as well as energy consumption. In recent days, used cooking oil or frying oil has been explored to be an attractive option to reduce the raw material cost for biodiesel production (Sonare and Rathod. 2010 : 142). Based on our previous study, optimized synthesis of biodiesel from used cooking oil with methanol through transesterification using lipase from Nile tilapia viscera was successfully carried out and a the higher yield was obtained (Patchimpet, Sangkharak and Klomklao. 2019c). However, the rates of triglycerides conversion to fatty acids alkyl esters were very slow. The optimum reaction time for biodiesel production was 28 h. Therefore, it is necessary to find a proper method to increase the reaction rate to promote the application of enzymatic catalytic biodiesel production.

Amongst the newer processes reported for enhancing yield, ultrasonic irradiation has been reported to be an efficient method, which results in a significant

degree of process intensification (Gharat and Rathod. 2013 : 900). For enzyme-based uses, ultrasound has been reported to increase the enzyme stability and catalytic activity, as well as the longevity of enzyme biocatalysts. (Rokhina, Lens and Virkutyte. 2009 : 298). Ultrasonic irradiation causes cavitation of bubbles near the phase boundary between the oil and alcohol phases and, therefore, may lead to a raise in temperature at the phase boundary, enhancing the transesterification reaction, which means that neither agitation nor heating are generally needed to produce biodiesel using ultrasound application (Batistella, et al. 2012 : 452). Little work has been done to date on the uses of ultrasonic irradiation in lipase-catalyzed biodiesel production. Therefore, to the best of our knowledge, this is the first report on the use of ultrasound for biodiesel production from used cooking oil with methanol as the catalysis of partially purified lipase from Nile tilapia viscera. Furthermore, the influences of key flow ultrasonic reactor operating parameters, such as frequency, time and temperature, have been optimized and a comparison has also been made with the use of conventional agitation as a source of mixing.

3. Material and Methods

3.1 Materials

Used cooking oil was obtained from the Sea Wealth Frozen Food Co., Ltd. (Songkhla, Thailand). Analysis of the used cooking oil showed the required properties for a basic transesterification reaction including saponification value (202.46 mg KOH/g of oil) , acid value (0.74 mg KOH/g), viscosity (59.90 mm^2/s) and density (891.39 kg/m^3) (Patchimpet, Sangkharak and Klomklao. 2019c).

p-nitrophenyl palmitate (*p*-NPP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The standard methyl esters used for preparing calibration curves and the heptadecanoic acid methyl ester used as an internal standard were also obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents used in the experimental study were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Nile tilapia (*Oreochromis niloticus*) viscera was collected from a local market in Thale Noi, Phatthalung, Thailand. The viscera were stored in ice with an ice/sample ratio of 2:1 (w/w) and transported to a laboratory at Thaksin University,

Phatthalung campus within 30 min of purchase. The powder was defatted with acetone and used for lipase extraction (Patchimpet, Sangkharak and Klomklao. 2019a; Klomklao, Benjakul and Kishimura. 2010 : 711; Klomklao and Benjakul. 2018 : 1864).

3.2 Enzyme Production

3.2.1 Lipase Purfication

Lipase from the viscera of Nile tilapia was purified using the thermoseparating aqueous two-phase system (T-ATPS) by the procedure of Patchimpet, Sangkharak and Klomklao (2019b). T-ATPS was prepared in 15 ml centrifuge tubes by adding 20% crude enzyme, 40% EOPO3900, 10% $(NH_4)_2SO_4$ and 4% NaCl. Distilled water was used to adjust the system to obtain a final mass of 10 g. The mixtures were mixed continuously for 3 min by a vortex mixer and then centrifuged for 15 min at 5,000 × g to induce phase separation. After centrifugation, the EOPO-rich top phase was collected from the primary ATPS and diluted with distilled water in a ratio of 1:1 (w/w). The samples were then placed in a water bath at 60°C for 15 min to induce thermo-separation. After that, the samples were centrifuged at 5,000 × g for 15 min and the secondary two-phase separation was formed. The top phase was collected and freeze-dried using a Dura-Top™ mp freeze-dryer (FTS Systems Inc., Stone Ridge, NY, USA). The powder obtained was stored at -20°C until used and named as partially purified lipase.

3.2.2 Lipase Activity Assay

The activity of both partially purified lipase and porcine lipase (Sigma, St. Louis, MO, USA) was determined spectrophotometrically using *p*-NPP as substrate according to the procedure of Patchimpet, Sangkharak and Klomklao (2019a). The amount of liberated *p*-nitrophenol was determined at 410 nm during the first 5 min of reaction. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute under the assay conditions.

3.3 Transesterification Reaction

Enzymatic transesterification reaction was carried out in an Erlenmeyer flask using a mixture of used cooking oil, methanol (molar ratio of methanol to oil was 4:1), 30 kUnit of lipase and water content of 3% (based on oil weight). Inaddition, the stirring speed in the flask was about 200 rpm using a mechanical stirrer that corresponded to the best conditions as reported by Patchimpet, Sangkharak and Klomklao (2019c). For the ultrasonic reaction, it was performed using an ultrasonic water bath (Zealway Inc., Model S06H, China) in which the flask was immersed and maintained at a height of 3.5 in. from the bottom of the ultrasonic water bath throughout of the experiments. The agitation was carried out by means of an electric motor that had a provision for speed control. Different operating conditions studied in the present work were a variation of ultrasonic frequencies (14, 16, 20, 24, 28 and 32 kHz at sonication power of 180 W), reaction time (1-28 h) and reaction temperature (from 30°C to 55°C).

Experiments were also carried out using a conventional stirring approach under an optimized set of operating parameters to compare the efficacy of the three modes of mixing. The experiments of the three modes included a mechanical stirred mode using a horizontal stirring rod inside the flask with a maximum speed of 200 rpm, ultrasonic irradiation and the integration of mechanical stirring and ultrasonic irradiation.

For biodiesel production using porcine lipase, the transesterification was conducted using the enzyme at the same level under the optimized operating conditions.

After the desired reaction time, samples were centrifuged at 5,000 × g for 10 min to obtain the upper layer. The upper layer of samples was collected and referred to as “biodiesel”. Heptadecanoic acid methyl esters which served as the internal standard and an aliquot of the upper layer were precisely determined and mixed thoroughly to GC analysis to measure the contents of FAMEs.

3.4 GC Analysis

The methyl ester contents in the reaction mixture were quantified using a GC7890. A gas chromatograph (Agilent Corp., California, USA) was

connected to a PEG-20M capillary column (0.32 mm × 30 m × 0.25 mm) and a flame ionizing detector (FID). The injector and detector temperatures were adjusted to 250 and 260°C, respectively. Nitrogen was used as the carrier gas. The column temperature was maintained at 180°C for 2 min, and then increased to 230°C at a rate of 5°C/min, and maintained at 230°C for 20 min. Percent biodiesel yield was defined as fatty acid methyl esters amount produced divided by the initial amount of used cooking oil.

3.5 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR was used to determine the lipid conversion by chemical group shift as it is a rapid and easy detection method (Kuepethkaew, et al. 2017 : 139). The ATR-FTIR spectra of biodiesel, commercial biodiesel and diesel were taken using Agilent Cary 630 FTIR with a specified range of 800-4000 cm^{-1}.

3.6 Biodiesel Properties

Biodiesel produced in this investigation was analyzed for fuel properties such as FAME content, viscosity, acid value, water content, iodine value, density, pour point and cloud point using international standard methods and their values were compared with those of international standards, European norms, the European Committee for Standardization (EN 14214) and the American Society for Testing and Materials (ASTM D 6571) standards.

3.7 Statistical Analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (Kuepethkaew, et al. 2017 : 139). For pair comparison, a T-test was used to compare the means between the types of enzymes. Statistical analysis was performed using a SPSS package (SPSS 11.5 for windows, SPSS Inc., Chicago, IL, USA).

4. Results and Discussion

4.1 Biodiesel Optimization Parameters

4.1.1 Effect of Reaction Time and Ultrasound on Transesterification Reaction

The time duration of the reaction is known to be the one of the critical parameters for the production of biodiesel. The influence of reaction time on transesterification of used cooking oil and methanol using lipase from Nile tilapia viscera with a stirrer in the absence and presence of ultrasound was studied and the results are shown in Figure 28. The reaction temperature was maintained at a constant temperature of 45°C and the mixture was stirred at 200 rpm. The reaction without ultrasound transesterification increased with increasing the reaction time resulting in an increase in the methyl ester yield. However, the rate of reaction without ultrasound was low at the start and transesterification requires 28 h to achieve the maximum yield (Figure 28). At 3 h of reaction, a low yield (25.82%) was observed and after 28 h, 96.5% biodiesel yield was obtained. The slow reaction and low yield of the biodiesel can be attributed to poor mixing of the alcohol and oil as they are not completely miscible with one another. Hence, poor mass transfer is a major issue for such enzyme catalyzed transesterification reactions (Bhangu, Gupta and Askokkumar. 2017 : 305).

Meanwhile, the reaction performed with an ultrasound (Figure 28) using a 24 kHz ultrasonic bath and ultrasonic power of 180 W provided a 61.03% yield in 1 h and a maximum biodiesel yield (96.23%) was observed in 3 h. Moreover, there was no significant difference for the yield at a shorter reaction time of 3 h compared with a longer reaction time at 12 h in the presence of ultrasound ($P>0.05$). Sivaramakrishnan and Incharoensakdi (2017 : 363) reported that the maximum methyl esters yield was achieved at 4 h and 36 h when direct transesterification was carried out with and without ultrasound, respectively. Bhangu, Gupta and Askokkumar (2017 : 305) reported that lipase from *Candida rugosa* catalyst could give complete conversion of biodiesel yield in 90 min and higher yield was achieved at a much shorter reaction time when ultrasonic energy was used. Without the use of lipase from Nile tilapia even 3 h of ultrasound didn’t provide any conversion of used cooking oil to biodiesel (data not shown), which suggests that just physical mixing of the liquid

alone is not enough for a transesterification reaction. Therefore, the application of ultrasound in used cooking oil transesterification with lipase catalysts has been shown to reduce the reaction time with high yield as compared to non-ultrasound transesterification. The mechanism of a sonication reaction involves the formation of micro-bubbles (4–300 μm in diameter) in which the unsymmetrical collapse of bubbles at an interface of solid and solvent (>200 μm) produces micro jets at high speed (>100 m/s) toward solid surfaces. The immediate collapse of bubbles is also responsible for strong shock waves that might be up to 10^3 MPa. This violent movement of cavitational bubbles is defined as micro-convection and results in extremely strong shear forces (Sivaramakrishnan and Incharoensakdi. 2017 : 363) This affects the activity of substances present in the reaction leading to an increase in product formation in a short duration. Physical effects of ultrasound help the formation of fine emulsion between immiscible fluids resulting in an increase in the transesterification reaction (Veljković, Avramović and Stamenković. 2012 : 1193; Adewuyi. 2001 : 4681). The physical and chemical effects of the ultrasonic irradiation on heterogeneous reactions are because of the phenomenon called acoustic cavitation. Acoustic cavitation leads to an implosive violent bubble collapse that produces intense local heating, high pressures, acoustic microstreaming, microturbulence and formation of small eddies that increase the mass and heat transfer in the surrounding liquid, and cause velocity gradients that result in shear stresses (Adewuyi. 2001 : 4681). Enhanced mass transfer as a result of cavitation bubble collapse boosts the rate of transesterification reactions by increasing the collision frequency between reactants (Bhangu, Gupta and Askokkumar. 2017 : 305). From the results, the reaction time for biodiesel production from used cooking oil and methanol using lipase catalyst in the presence of ultrasound was kept fixed at 3 h in the subsequent steps of this study.

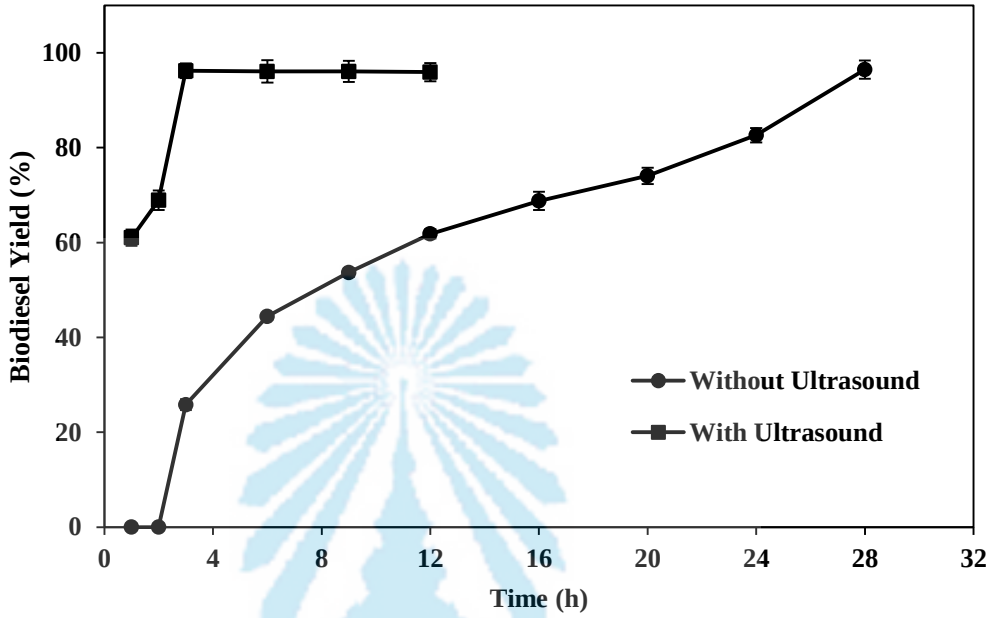


Figure 28. Effect of Reaction Time with Ultrasound (20 kHz) and without Ultrasound on Biodiesel Yield. Conditions: 30 kUnit of Nile Tilapia Lipase, 4:1 Methanol to Oil Molar Ratio, 3% Water Content, 45°C Reaction Temperature and 200 rpm Agitation Speed. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.1.2 Effect of Ultrasonic Frequency

The effects of ultrasonic frequency varying within a range of 12-32 kHz by intervals of 4 kHz were also investigated. Ultrasonic frequency can influence the reaction rate and biodiesel yield as it is directly related with the frequency of ultrasound. Figure 29 displays the variation of biodiesel yield with varied frequency of ultrasound. It was clear that there was a moderate increase in the biodiesel yield with an increase in the frequency. The biodiesel yields were not significantly different when 16 and 20 kHz ultrasonic frequencies were applied ($P>0.05$). The highest yields of 96.50% and 96.23% were observed using 16 and 20 kHz, respectively. With increasing ultrasonic frequency, the acoustic cavitation bubble size decreases which in turns reduces the collapse intensity and shear forces generated by the cavitation collapse. Shear generated by a bubble collapse helps to create an emulsion between the oil and methanol layer; thus, a higher interfacial

surface area for mass transfer and a higher biodiesel yield (Mahamuni and Adewuyi. 2010 : 2120). However, further increases in ultrasonic frequency i.e. 24 kHz to 32 kHz give lower conversion. At very high frequency, where the rarefaction cycles are very short, the finite time required for the rarefaction cycle is too small to permit a bubble to grow to a size sufficient to cause disruption of the liquid (Sonare and Rathod. 2010 : 142). Bhangu, Gupta and Askokkumar (2017 : 305) and Tomke and Rathod (2015 : 241) have reported similar results for the dependency on the frequency input into the system for the case of biodiesel synthesis.

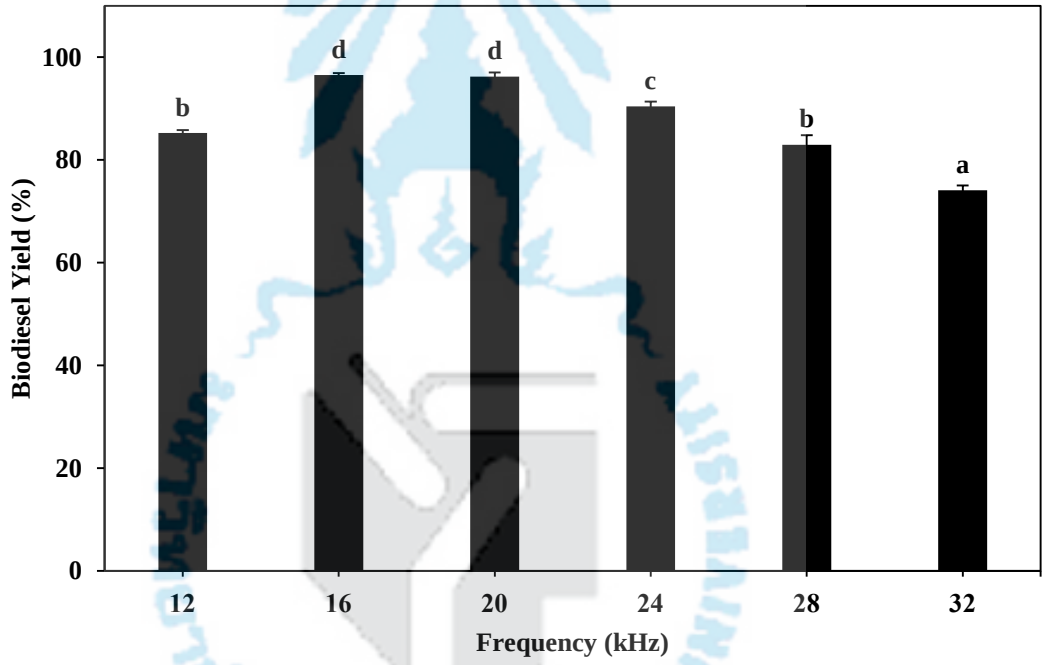


Figure 29. Effect of Ultrasonic Frequency on Biodiesel Yield. Conditions: 30 kUnit of Nile Tilapia Lipase, 4:1 Methanol to Oil Molar Ratio, 3% Water Content, 45°C Reaction Temperature, 200 rpm Agitation Speed and 3 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.1.3 Effect of Temperature

Temperature is an important parameter for enzyme catalyst synthesis both in the presence and absence of ultrasound. To investigate the influence of reaction temperature on the transesterification, a reaction was performed under ultrasonic irradiation at different operating temperatures ranging from 35 to 55°C. The other parameters i.e. reactant molar ratio of 4:1 methanol to used cooking oil, agitation of 200 rpm, enzyme loading of 30 kUnit and water content of 3% were kept constant. As displayed in Figure 30, as the temperature increased from 35°C to 40°C, the biodiesel yield also increased and reached its maximum yield (97.45%) at 40°C. As the reaction temperature was raised, the collision frequency between the substrate and enzyme molecules increased, which helped to form enzyme-substrate complexes at higher rates and improved the yield of methyl esters (Yu, et al. 2010 : 519). Apart from the collision frequency, raising the temperature also decreased the viscosity of the reaction mixture and increased the mass transfer (Sim, Kamaruddin and Bhatia. 2010 : 1027). However, a further increase in temperature gradually decreased the biodiesel yield. The decrease in conversion was because of the inactivation of catalytic activity of the enzyme at higher temperatures as an increase in temperature caused the conformational changes in the enzyme structure (Waghmare and Rathod. 2016 : 60). Also, there might be an effect of ultrasonic cavitation on the decreasing trend at higher temperature as it was reported that at higher temperature the cavitation effect was less intense compared to the effect at lower temperature. At lower temperatures, fewer numbers of bubbles are formed but collision intensity is relatively high, on other hand during high temperatures, the number of bubbles formed increase but collision intensity is very low (Charpe and Rathod. 2012 : 37). At higher temperature, surface tension decreases affecting bubble formation and collision intensity, which relatively decreases mass transfer (Alissandrakis, Daferera, Tarantilis, Polissiou and Harizanis. 2005 : 575). Sivaramakrishnan and Incharoensakdi (2017 : 363) have reported similar effects of temperature in the ultrasound assisted transesterification reactions. From the results, considering all above parameters, temperature of 40°C was chosen as optimum.

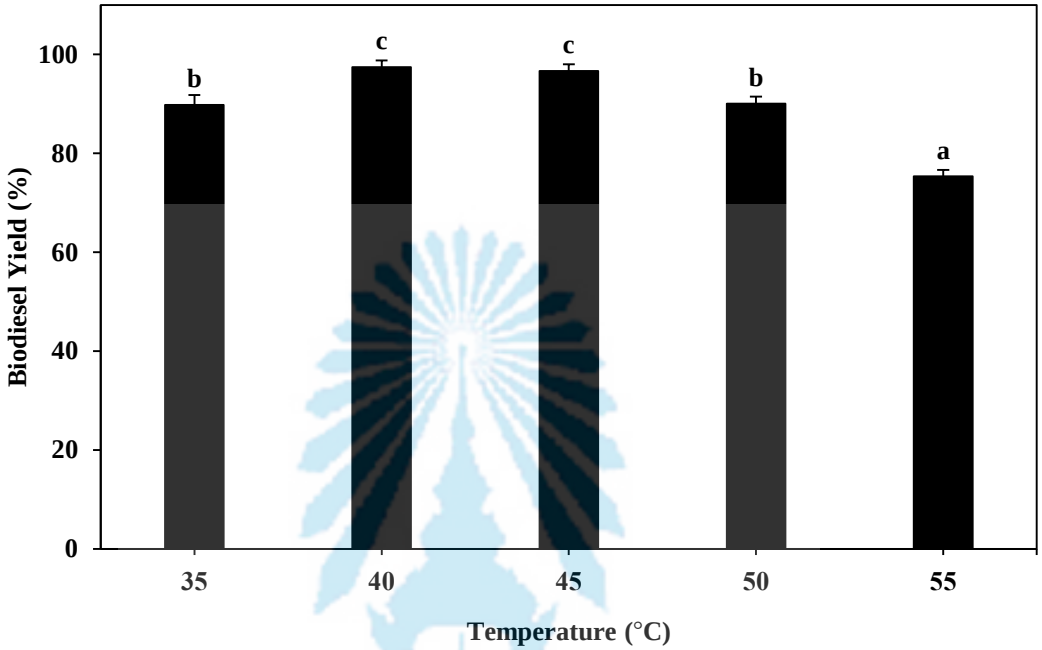


Figure 30. Effect of Temperature With Ultrasound (16 kHz) on Biodiesel Yield. Conditions: 30 kUnit of Nile Tilapia Lipase, 4:1 Methanol to Oil Molar Ratio, 3% Water Content, 200 rpm Agitation Speed and 3 h Reaction Time. Bars Represent the Standard Deviation from Triplicate Determinations (= ± 1 SD).

4.2 Comparison of Ultrasonic Irradiation with that of Conventional Stirring Method

Experiments were conducted at optimized conditions to compare the conversion obtained for enzyme catalyzed transesterification under three different conditions i.e. ultrasonic irradiation, ultrasonic irradiation coupled with mechanical stirring and conventional stirring method. The results obtained are tabulated in Table 21 by considering the biodiesel yield of used cooking oil after 3 h. It was found that under ultrasonic irradiation coupled with mechanical stirring, biodiesel yield of 97.59% was obtained whereas using simple mechanical stirring only 27.59% biodiesel yield was achieved. While using only ultrasonic irradiation without mechanical stirring the biodiesel yield obtained was 55.18%. Therefore, ultrasonication coupled with mechanical stirring gives higher conversion as compared with simple mechanical

stirring alone and ultrasonication without stirring. This can be attributed to the fact that as the reaction is mass transfer controlled, the micro level turbulence generated because of cavitational bubbles results in a higher availability of the interfacial area and, hence, higher conversion. The agitation intensity appears to be of particular importance for the alcoholysis process. The mass transfer of triglycerides from the oil phase towards the methanol–oil interface could be a rate limiting step and, hence, poor mass transfer between the two phases results in a slow reaction rate (Noureddini, and Zhu. 1997 : 1457). Ultrasonic field induced an effective emulsification and mass transfer and, therefore, the rate of reaction under ultrasonic irradiation condition was higher than that in the stirring condition (Hanh, Dong, Okitsu, Nishimura and Maeda. 2009 : 780). Enzyme are proteins consisted of a very complex structure of α and β helical strands bound together by hydrogen bonding that give them some conformational flexibility, which helps them to properly reorder their catalytic active domain with the substrate molecules (Waghmare and Rathod. 2016 : 60). Gharat and Rathod (2013 : 900) reported that the biodiesel produced by ultrasonic irradiation coupled with stirringhad a conversion of 86.61% was obtained in 4 h, whereas with the conventional stirring method, a lower conversion of about 38.69% was obtained in 4 h. Also, ultrasonication alone (absence of stirring) could yield 57.68% in 4 h. Choedkiatsakul, et al. (2014 : 1585) reported that initial reaction rate values were 54.1, 142.9 and 164.2 mmol/L min for mechanical stirring, ultrasonic and a combined mechanical stirred and ultrasonic reactors, respectively.

Table 21 Comparison of Ultrasonic Irradiation with Conventional Stirring Method After 3 h*.

Treatment	**Biodiesel Yield (%)****
Mechanical Stirring	27.59 ± 1.35^{a}***
Ultrasonic Irradiation Without Stirring	55.18 ± 0.77^{b}
Ultrasonic Coupled With Stirring	97.59 ± 0.38^{c}

* Conditions: Reactions were carried out in 30 kUnit of Nile tilapia lipase, 4:1 methanol to oil molar ratio, 3% water content, 40°C reaction temperature.

** Mean ± SD from triplicate determinations.

*** The different letters in the same column denote significant differences ($P < 0.05$)

4.3 Comparison of Biodiesel Yield Between Different Enzymes

The biodiesel production from used cooking oil and methanol with stirring in the presence of ultrasound was compared between using lipase from Nile tilapia viscera and porcine pancreas lipase as catalyst. At the same level of enzyme added (30 kUnit), both sources of lipase were effective in aiding the production of biodiesel, compared to the control (without lipase addition, data not shown) and partially purified lipase resulted in 97.59% conversion whereas the porcine pancreas lipase catalyzed resulted in a much lower extent of conversion (83.86%) over a similar 3 h time of operation (Table 22). Ebrahimi, Najafpour and Ardestani (2017 : 203) reported a methyl ester yield of 75% for the transesterification reaction of waste cooking sunflower oil catalyzed by porcine pancreas lipase. Hence, the partially purified lipase from Nile tilapia viscera has good potential for use in biodiesel production.

Table 22 Comparison of Nile Tilapia Viscera Lipase and Porcine Pancreas Lipase as Catalyst Aid for Biodiesel Production from Used Cooking Oil After 3 h*.

Enzyme	**Biodiesel Yield (%)****
Nile Tilapia Viscera Lipase	97.59 ± 0.38[a]***
Porcine Pancreas Lipase	83.86 ± 0.58[b]

* Conditions: Reactions were carried out in 30 kUnit of enzyme, 4:1 methanol to oil molar ratio, 3% water content, 40°C reaction temperature and 200 rpm agitation speed under ultrasonic frequency 16 kHz.

** Mean ± SD from triplicate determinations.

*** The different letters in the same column denote significant differences ($P < 0.05$)

4.4 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

As displayed in Figure 31, the present investigation used ATR-FTIR to ascertain the functional group and its shift in the ultrasound-assisted lipase-catalyzed biodiesel, commercial biodiesel and conventional diesel and their spectra. Absorption bands at 2924 and 2855 cm^{-1} were because of the stretching vibration of -CH_2- group. The presence of a broad band at about 1459 cm^{-1}, corresponded to the vibration of -CH_2 scissoring. An absorption peak at 722 cm^{-1} corresponded to -$(CH_2)_n$- rocking (Kuepethkaew, et al. 2017 : 139; Patchimpet, Sangkharak and Klomklao. 2019c; Fereidooni and Mehrpooya. 2017 : 145). A strong signal in Figure 31a and b at 1744.02 and 1743.01 cm^{-1} indicates the stretching of functional group (-C=O) of biodiesel. Absorption bands at 1362.91 cm^{-1} were because of the methyl group (-CH_3-). A cluster of peak at 1245.02, 1196.85 and 1170.20 cm^{-1} denotes C-O stretching (Figure 31b.). The ATR-FTIR spectra of ultrasound-assisted lipase-catalyzed biodiesel with conventional diesel (Figure 31a, c) at 1744.02 cm^{-1} was not found in diesel and the strong adsorption bands at 2923.89 and 2855.20 cm^{-1} clearly divulged the stretching of aliphatic hydrocarbon (C-H) in diesel. The ATR-FTIR analysis in the present investigation was in accordance with Kuepethkaew, et al. (2017 : 139) and Patchimpet, Sangkharak and Klomklao (2019c).

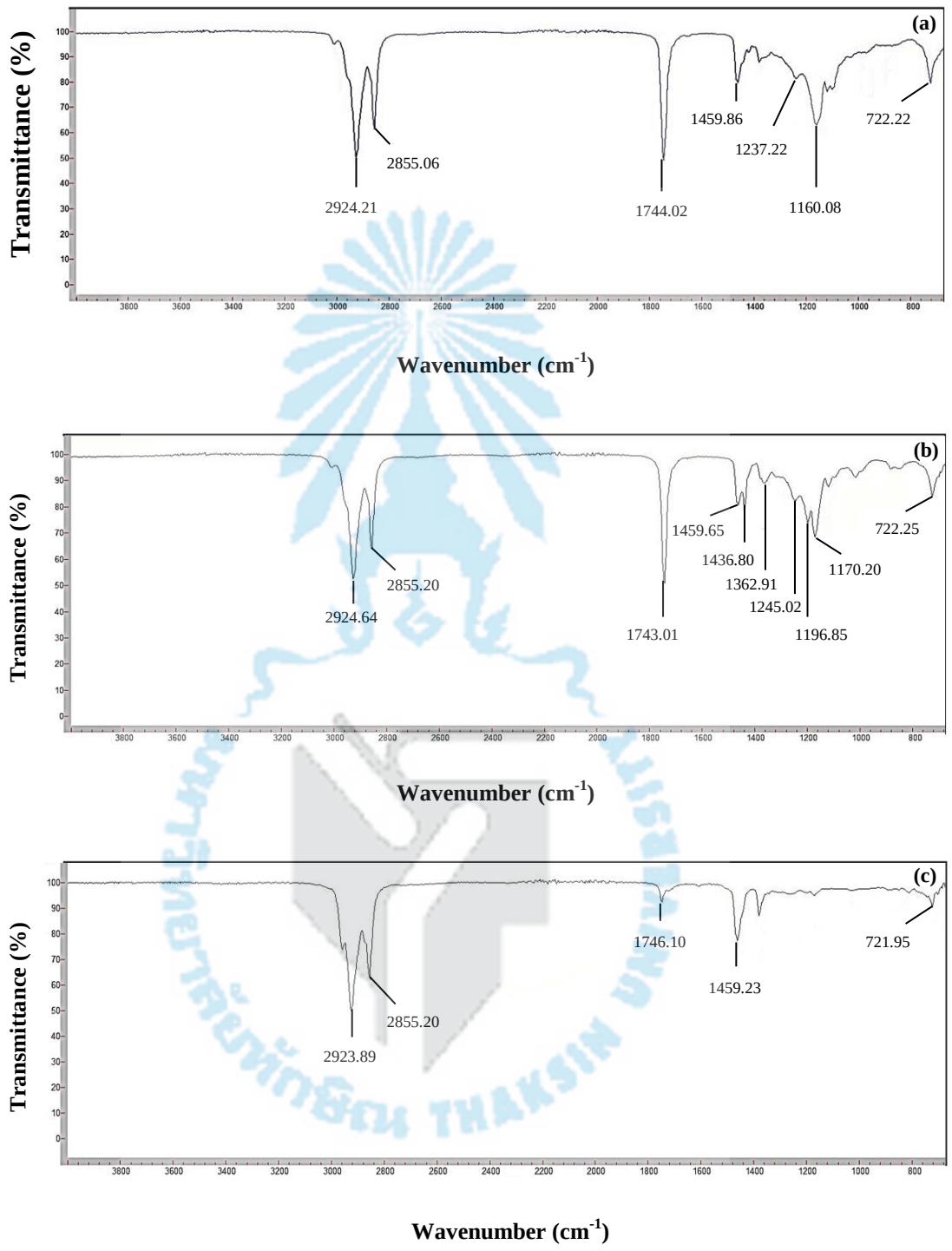


Figure 31. ATR-FTIR Spectra of (a) Ultrasound-Assisted Lipase-Catalyzed Biodiesel, (b) Commercial Biodiesel, (c) Diesel Showing Functional Groups. Signal at ~1740 cm^{-1} Indicates the Presence of Carbonyl Group in Biodiesel.

4.5 Evaluation for Biodiesel Characteristics

A fuel properties test of ultrasound-assisted lipase-catalyzed biodiesel was performed and compared with international biodiesel standard values that are summarized in Table 23. The FAME content, viscosity, acid value, water content, iodine value, density, pour point and cloud point of ultrasound-assisted lipase-catalyzed biodiesel were all estimated from EN 14103, ASTM D 445-06, ASTM D 664-01, EN ISO 12937, EN 14111, ASTM D 4052-96, ASTM D 5950-02 and ASTM D 25000, respectively. The properties of the obtained biodiesel satisfy the ASTM and EN standards. Therefore, it can be used in its pure form or mixed with diesel fuel in certain proportions.

Table 23 Properties of Ultrasound-Assisted Lipase-Catalyzed Biodiesel in Comparison with EN 14214 and ASTM D 6751

Properties	Method	Ultrasound-Assisted Lipase-Catalyzed Biodiesel	Biodiesel Specification	
			EN 14214	ASTM D 6751
FAMEs Content (% w/w)	EN 14103	97.59 ± 0.58	Min. 96.5	-
Viscosity at 40°C (mm^2/s)	ASTM D 445-06	4.23 ± 0.14	3.5 – 5.0	1.9 – 6.0
Acid Value (mg KOH/g)	ASTM D 664-01	0.44 ± 0.01	Max. 0.5	Max. 0.5
Water Content (mg/kg)	EN ISO 12937	152.98 ± 3.38	Max. 500	-
Iodine Value (g I_2/100 g)	EN 14111	37.76 ± 1.57	Max. 120	-
Density (15°C) (kg/m^3)	ASTM D 4052-96	873.63 ± 3.50	860 - 900	870 - 890
Pour Point (°C)	ASTM D 5950-02	2.00 ± 0.3	-	Report
Cloud Point (°C)	ASTM D 2500	9.67 ± 1.0	-	Report

* Mean ± SD from triplicate determinations.

5. Conclusion

In the present investigation, ultrasound coupled with stirring was proved to be an efficient method for enzymatic transesterification of the used cooking oil with methanol using partially purified lipase from Nile tilapia viscera as catalyst. Under the optimum conditions (16 kHz frequency, 180 W irradiation power supply, 200 rpm stirrer speed, methanol:oil molar ratio of 4:1, 30 kUnit of Nile tilapia lipase and 40°C temperature), a 97.59% yield of fatty acid methyl ester could be achieved, in a relative short reaction time (3 h). Also, biodiesel properties met some international standards, e.g. EN 14214 and ASTM D 6751.

CHAPTER 6

Conclusion and Recommendation

1. Conclusion

Based on the *p*-NPP hydrolysis, viscera of three fish species including hybrid catfish, striped snake-head fish and Nile tilapia is a good source of lipase. Nile tilapia displayed the highest activity, followed by striped snake-head fish and hybrid catfish. In addition, considering the high activity and stability in high alkaline pH, relative stability in the presence of surfactants and several commercial dishwashing detergents, Nile tilapia viscera lipase may find application in laundry detergents.

Nile tilapia viscera lipase was successfully purified by T-ATPS. The type and concentration of salt, concentration of EOPO, concentration of NaCl, temperature, the ratio between EOPO/distilled water could influence lipase partitioning. The purification of Nile tilapia viscera lipase using thermoseparating phase components may be suitable for development as a primary recovery operation for lipase derived from fish viscera. Biochemical properties of the enzyme have shown that the enzyme has the potential to be used for various applications. Moreover, the present work has clearly established that, low frequency ultrasound irradiation could obviously improve the activity of partitioned lipase from Nile tilapia viscera.

Lipase from Nile tilapia viscera was purified by T-ATPS. Later, this biocatalyst was employed for biodiesel production from used cooking oil. By validating, biodiesel yield 96.50% was obtained under the optimal condition: methanol/oil molar ratio 4:1, viscera lipase 30 kUnit, at 45°C, 3% water content and 28 h for reaction time. The proposed process may lower the production cost of biodiesel and facilitate the disposal of wastes; both used cooking oil and Nile tilapia viscera. Therefore, this purified lipase catalyzed process has the potential to replace the chemical catalyzed method for biodiesel production.

The transesterification of used cooking oil with methanol under the influence of the ultrasound using partially purified lipase from Nile tilapia viscera as

catalyst was also investigated. Results showed significant process intensification and illustrated promising outlook to the applicability of ultrasound for biodiesel synthesis from used cooking oil under mild operating conditions. Ultrasound coupled with stirring further enhanced biodiesel production. Under the optimum conditions (16 kHz frequency, 180 W irradiation power supply, 200 rpm stirrer speed, methanol:oil molar ratio of 4:1, 30 kUnit of Nile tilapia lipase and 40°C temperature), a 97.59% yield of biodiesel could be achieved, in a relative short reaction time (3 h). Therefore, ultrasound is a promising and efficient technique for biodiesel synthesis.

2. Recommendation

- Suggestions for Future Research

2.1 This study has laid the groundwork and provided additional information on some fundamental biological properties of viscera lipase, and it will be expected that future work will completely purification and characterization this lipase.

2.2 Kinetic and thermodynamic properties of purified lipase should be studied.

2.3 Viscera lipase from Nile tilapia recovered by T-ATPS should be prepared in dried form in which the appropriate drying technology and stabilization should be investigated.

2.4 Based on biochemical characteristics, the application of fish lipase in various food products should be more intensively investigated.

BIBLIOGRAPHY

Bibliography

Aarthy, M., Saravanan, P., Gowthaman, M. K., Rose, G. and Kamini, N. R. (2014). "Enzymatic transesterification for production of biodiesel using yeast lipases: An review," Chemical Engineering Researh and Design. 92, 1591-1601.

Abbaszaadeh, A., Ghobadian, B., Omidkhah, M. R. and Najafi, G. (2012). "Current biodiesel production technologies: A comparative review," Energy Conversion and Management. 63,138-148.

Abdulla, R. and Ravindra, P. (2013). "Immobilized *Burkholderia cepacia* lipase for biodiesel production from crude *Jatropha curcas* L. oil," Biomass and Bioenergy. 56, 8-13.

Abouakil, N., Rogalska, E., Bonnicel, J. and Lombardo, D. (1988). "Purification of pancreatic carboxylic-ester hydrolase by immunoaffinity and its application to human bile-salt stimulated lipase," Biochimica et Biophysica Acta (BBA). 961, 229-308.

Abramic, M., Lescic, I., Korica, T., Vitale, L., Saenger, W. and Pgac, J. (1999). "Purification and properties of extracellular lipase from *Streptomyces rimosus*," Enzyme and Microbial Technology. 7, 522-529.

Adewuyi, Y. G. (2001). "Sonochemistry: environmental science and engineering applications," Industrial & Engineering Chemistry Research. 40, 4681-4715.

Aksoy, H. A., Kahraman, I., Karaosmanoglu, F. and Civelekoglu, H. (1988). "Evaluation of Turkish sulphur olive oil as an alternative diesel fuel," Journal of the American Oil Chemists' Society. 65, 936-938.

Ali, C. H., Qureshi, A. S., Mbadinga, S. M., Liu, J. F., Yang, S. Z. and Mu, B. Z. (2017). "Biodiesel production from waste cooking oil using on site produced purified lipase from *Pseudomonas aeruginosa* FW_SH-1: Central composite design approach," Renewable Energy. 109, 93-100.

Alissandrakis, E., Daferera, D., Tarantilis, P., Polissiou, M. and Harizanis, P. (2005). "Evaluation of four isolation techniques for honey aroma compounds," Journal of the Science of Food and Agriculture. 8, 575-582.

Animal Diversity Web. Retrieved Oct 22, 2019 from http://animaldiversity.org/accounts/Channa_striata/

Antczak, M. S., Kubiak, A., Antczak, T. and Bielecki, S. (2009). "Enzymatic biodiesel synthesis-key factors affecting efficiency of the process," Renewable Energy. 34, 1185-1194.

Arumugm, A. and Ponnusami. V. (2014). "Biodiesel production from *Calophyllum inophyllum* oil using lipase producing *Rhizopus oryzae* cells immobilized within reticulated foams," Renewable Energy. 64, 276-282.

Aryee, N. A., Simpson, B. K. and Villalonga, R. (2007). "Lipase fraction from the viscera of grey mullet (*Mugil cephalus*): Isolation, partial purification and some biochemical characteristics," Enzyme and Microbial Technology. 40, 394-402.

Ashokkumar, M., Lee, J., Kentish, S. and Grieser, F. (2007). "Bubbles in an acoustic field: An overview," Ultrasonics Sonochemistry. 14, 470-475.

Atabani, A. E., Silitonga, A. S., Ong, H. C., Mahlia, T. M. I., Masjuki, H. H., Badruddin, I. A. and Fayaz, H. (2013). "Non-edible vegetable oils: Acriticale valuation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production," Renewable and Sustainable Energy Reviews. 18, 211-245.

Atadashi, I. M., Aroua, M. K. and Aziz, A. A. (2010). "High quality biodiesel and its diesel engine application: A review," Renewable and Sustainable Energy Reviews. 14, 1999-2008.

Azad, A. K., Rasul, M. G., Khan, M. M. K., Sharma, S. C., Mofijur, M. and Bhuiya, M. M. K. (2016). "Prospects, feedstocks and challenges of biodiesel production from beauty leaf oil and castor oil: A nonedible oil sources in Australia," Renewable and Sustainable Energy Reviews. 61, 302-318.

Babu, R. B., Rastogi, N. K. and Raghavarao, K. S. M. S. (2008). "Liquid-liquid extraction of bromelain and polyphenoloxidase using aqueous two-phase system," Chemical Engineering and Processing. 47, 83-89.

Balaji, L. and Jayaraman, G. (2014). "Metal ion activated lipase from halotolerant *Bacillus* sp. VITL8 displays broader operational range," International Journal of Biological Macromolecules. 67, 380-386.

Barbosa, J. M. P., Souza, R. L., Fricks, A., Zanin, G. M., Soares, C. M. F. and Lime, Á. S. (2011). "Purification of lipase produced by a new source of *Bacillus* in submerged fermentation using an aqueous two-phase system," Journal of Chromatography B. 879, 3853-3858.

Basto, C., Silva, C. J., Gubitzb, G. and Cavaco-Paulo, A. (2007). "Stability and decolourization ability of *Trametes villosa* laccase in liquid ultrasonic fields," Ultrasonics Sonochemistry. 14, 355-362.

Batistella, L., Lerin, L. A., Brugnerotto, P., Danielli, A. J., Trentin, C. M., Popiolski, A., Treichel, H., Oliveira J. V. and de Oliveira, D. (2012). "Ultrasound-assisted lipase-catalyzed transesterification of soybean oil in organic solvent system," Ultrasonic Sonochemistry. 19, 452-458.

Beisson, F., Tiss, A., Rivière, C. and Verger, R. (2000). "Methods of lipase detection and assay: A critical review," European Journal of Lipid Science and Technology. 102, 133-153.

Benjamin, S. and Pandey, A. (1998). "*Candida rugosa* lipases: molecular biology and versatility inbiotechnology," Yeast. 14, 1069-1087.

Benzonana, G. and Desnuelle, P. (1965). "Etude cinétique de l'action de la lipase pancreátique sur des triglycerides en emulsion. Esso d'une enzymologies en milieu heterogéne," Biochimica et Biophysica Acta (BBA). 105, 121-136.

Bezerra, R. S., Lins, E. J. F., Alencar, R. B., Paiva, P. M. G., Chaves, M. E. C., Coelho L. C. B. B. and Carvalho L. B. (2005). "Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*)," Process Biochemistry. 40, 1829-1834.

Bhangu, S. K., Gupta, S. and Askokkumar, M. (2017). "Ultrasonic enhancement of lipase-catalysed transesterification for biodiesel synthesis," Ultrasonics Sonochemistry. 34, 305-309.

Bhuiya, M. M. K., Rasul, M. G., Khan, M. M. K., Ashwath, N., Azad, A. K. and Hazrat, M. A. (2016). "Prospect of 2nd generation of biodiesel as a sustainable fuel-Part 2: properties, performance and emission characteristics," Renewable & Sustainable Energy Reviews. 55, 1129-1146.

Björkling, S., Godtffredsen, E. and Krik, O. (1991). "The future impact of industrial lipases," Trends in Biotechnology. 9, 360-363.

Bläckberg, L., Lombardo, D., Hernaell, O.,Guy, O. and Olivercrona, T. (1981). "Bile salt-stimulated lipase in human milk and carboxyl ester hydrolase in pancreatic juice," FEBS Letters. 136, 284-288.

Borgström, B. and Edanson, C. (1973). "Pancreatic lipase and colipase. Interactions and effects of bile salts and other detergents," European Journal of Lipid Science and Technology. 37, 60-68.

Borlangan, I. G. (1990). "Studies on the digestive lipases of milkfish, *Chanos chanos*," Aquaculture. 89, 315-325.

Borugadda, V. B. and Goud, V. V. (2012). "Biodisesl production from renewable feedstocks: Status and opportunities," Renewable and Sustainable Energy Reviews. 16, 4763-4784.

Brad, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L. and Menge, U. (1990). "A serine protease triad forms the catalytic centre of the triacylglycerol lipase," Nature. 343, 767-770.

Bradford, M. (1976). "A rapid and sensitive method for the quantition of quantities of protein utilizing the principle of protein dye binding," Analytical Biotechnology. 72, 248-254.

Brenner, S. (1988). "The molecular evolution of genes and properties: A tale of two proteins," Nature (London). 334, 528-530.

Brockerhoff, H. R. (1973). "A model of pancreatic lipase and the orientation of enzymes at interfaces," Chemistry and Physics of Lipids. 10, 215-222.

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, P., Huge-Jensen, B., Patkar,S. A. and Thim, L. (1991). "A model of interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex," Nature. 351, 491-494.

Bwanika, G. N., Makanga, B., Kizito, Y., Chapman, L. J. and Balirwa, J. (2004). "Observations on the biology of Nile tilapia, *Oreochromis niloticus*, L., in two Ugandan Crater lakes," African Journal of Ecology. 42, 93-101.

Cagauan, A. (2007) "Exotic aquatic species introduction in the Philippines for aquaculture-A threat to biodiversity or a boon to the economy," Journal of Environmental Science and Management. 10, 48-62.

Camulli, E. D., Linke, M., Brockman, H. L. and Hui, D. Y. (1989). "Identity of a cytosolic neutral choresterol esterase in rat liver with the bile salt-stimulated cholesterol esterase in pancreas," Biochimica et Biophysica Acta (BBA). 1005, 177-182.

Cao, W., Han, H. and Zhang, J. (2005). "Preparation of biodiesel from soybean oil using supercritical methanol and co-solvent," Fuel. 84, 347-351.

Carvalho, C. M., Aires-Barros, M. and Cabral, M. S. (1999). "Cutinase: From molecular level to bioprocess development," Biotechnology and Bioengineering. 66, 17-34.

Chahinian, H., Nini, L., Boitard, E., Dubés, J. P., Sarda, L. and Comeau, L. (2000). "Kinetic properties of *Penicillium cyclopium* lipase studied with vinyl ester," Lipids. 35, 919-926.

Chand, P. (2008) Enhancing biodiesel production from soybean oil using ultrasonics. Master's thesis Master of Science. Ames : Iowa state University.

Charpe, T. and Rathod, V. (2012). "Extraction of glycyrrhizic acid from licorice root using ultrasound: process intensification studies," Chemical Engineering and Processing: Process Intensification. 54 , 37-41.

Chen, Q., Wternby, B., Åkesson, B. and Nilsson, Å. (1990). "Effect of human pancreatic lipase-colipase and carboxyl ester lipase on eicosapentanoic and arachidonic acid ester bonds of triacylglycerols rich in fish oil fatty acids," Biochimica et Biophysica Acta (BBA). 1044, 111-117.

Cherif, S. and Gargouri, Y. (2009). "Thermoactivity and effects of organic solvents on digestive lipase from hepatopancreas of the green crab," Food Chemistry. 116, 82-86.

Choedkiatsakul, I., Ngaosuwan, K. and Assabumrungrat, S. (2013). "Application of heterogeneous catalysts for transesterification of refined palm oil in ultrasound-assisted reactor," Fuel Processing Technology. 111, 22-28.

Choedkiatsakul, I., Ngaosuwan, K., Cravotto, G. and Assabumrungrat, S. (2014). "Biodiesel production from palm oil using combined mechanical stirred and ultrasonic reactor," Ultrasonics Sonochemistry. 21, 1585-1591.

Cravotto, G., Binello, A., Merizzi, G. and Avogadro, M. (2004). "Improving solvent-free extraction of policosanol from rice bran by high intensity ultrasound treatment," European Journal of Lipid Science and Technology. 106, 147-151.

Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry,M. K. and Doctor, B. P. (1993). "Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins," Protein Science. 2, 366-382.

Derewenda, U., Brzozowski, A. M., Lawson, D. M. and Derewenda, Z. S. (1992). "Catalysis at the interface: The anatomy of a conformational change in a triglyceride lipase," Biochemistry. 31, 1532-1541.

Derewenda, Z. S. and Derewenda, U. (1991). "Relationships among serine hydrolases: Evidence for a common structural motif in triacyglyceride lipases and esterases," Biochemistry and Cell Biology. 69, 842-851.

Derewenda, Z. S., Swenson, L., Green, R., Wei, Y., Yamaguchi, S., Joerger, R., Haas, M. J. and Derewendra, Z. S. (1994). "Current progress in crystal of raphic studies of new lipases from filamentous fungi," Protein Engineering, Design and Selection (PEDS). 7, 551-557.

Dhawane, S. H., Kaemakar, B., Ghosh, S. and Halder, G. (2018). "Parametric optimization of biodiesel synthesis from waste cooking oil via Taguchi approach," Journal of Environmental Chemical Engineering. 6, 3971-3980.

Duan, X., Zhou, J., Qiao, S. and Wei, H. (2011). "Application of low intensity ultrasound to enhance the activity of anammox microbial consortium for nitrogen removal," Bioresource Technology. 102, 4290-4293.

Dugi, K. A., Dichek, H. L. and Santamarina-Fojo, S. (1995). "Human hepatic and lipoprotein lipases: The loop covering the catalytic site mediates lipase substrate specificity," Journal of Biological Chemistry. 270, 25396-25401.

Ebrahimi, S., Najafpour, G. D. and Ardestani, F. (2017). "Transesterification of waste cooking sunflower oil by porcine pancreas lipase using response surface

methodology for biodiesel production," Applied Food Biotechnology. 4, 203-210.

Eckey, E. C. (1956). "Esterification and interesterification," Journal of the American Oil Chemists' Society. 33, 575-579.

Emmerich, J., Beg, O. U., Peterson, J., Previato, L., Brunzell, J. D., Brewer, H. B. Jr. and Santamarina-Fojo, S. (1992). "Human lipoprotein lipase: Analysis of the catalytic triad by site directed mutagenesis of ser-132, asp-156, and his-241," Journal of Biological Chemistry. 267, 4161-4165.

Entezari, J. M. H. and Petier, C. P. (2004). "A combination of ultrasound and oxidative enzyme: Sono-biodegradation of phenol," Applied Catalysis B: Environmental. 53, 257-263.

Erlanson, C. (1975). "Purification, properties, and substrate specificity of a carboxylesterase in pancreatic juice," Scandinavian Journal of Gastroenterology. 10, 401-408.

Fereidooni, L. and Mehrpooya, M. (2017). "Experimental assessment of electrolysis method in production of biodiesel from waste cooking oil using zeolite/chitosan catalyst with a focus on waste biorefinery," Energy Conversion and Management. 147, 145-154.

Fero, A. B. J. and Jahim, J. Md. (2010). "Application of the aqueous two-phase thermoseparating system of dehypon®LS 54-the waxy maize starch for protein extraction," The Journal of Applied Science. 10, 2596-2601.

Fishing-Khaolak. Retrieved Oct 22, 2019 from http://fishing-khaolak.com/fish_species/freshwater_species/striped_snakehead.html

Flint, E. B. and Suslick, K. S. (1991). "The temperature of cavitation," Science. 253, 1397-1399.

Food and Agriculture Organization of the United Nations. Retrieved Oct 22, 2019 from http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/

Formo, M. W. (1945). "Ester reactions of fatty materials," Journal of the American Oil Chemists' Society. 31, 548-559.

Frankel, E. N. (1981). "Volatile lipid oxidation products," Progress in Lipid Research. 22,1-33.

Freedman, B. and Pryde, E. H. (1982). "Fatty esters from vegetable oils for used as a diesel fuel," In Proceeding of Internatonal Conference on Plant and Vegetable Oil as Fuels. (pp. 117-122). ASAE, St. Joseph : MI.

Fukuda, H., Kondo, A. and Noda, H. (2001). "Biodiesel fuel production by transesterification of oils," Journal of Bioscience and Bioengineering. 92, 405-416.

Gargouri, Y., Julien, R., Sugihara, A., Verger, R. and Srada, L. (1984). "Studies on the inhibition of pancreatic and microbial lipases by proteins," Biochimica et Biophysica Acta (BBA). 795, 326-331.

Gargouri, Y., Piéroni, G., Riviére, C., Srada, L. and Verger, R. (1986). "Inhibition of lipases by proteins: A binding study using dicaprin momolayers," Biochemistry. 25, 1733-1738.

Garner, C. W. and Smith, L. C. (1970). "A convenient purification of porcine pancreatic lipase free of proteolytic activity," Archives of Biochemistry and Biophysics. 140, 503-507.

Gebicka, L. and Gekicki, J. L. (1997). "The effect of ultrasound on heme enzymes in aqueous solution," Journal of Enzyme Inhibition and Medical Chemistry. 12, 133-141.

Genest, P. A. and Borst, P. (2007). "Analysis of telomere length variation in *Leishmania* over time," Molecular and Biochemical Parasitology. 151, 213-215.

Geoffry, K. and Achur, R. N. (2018). "Screening and production of lipase from fungal organism," Biocatalysis and Agricultural Biotechnology. 14, 241-253.

Georgogianni, K. G., Katsoulidis, A. K., Pomonis, P. J., Manos, G. and Kontominas, M. G. (2009). "Transesterification of rapeseed oil for the production of biodiesel using homogeneous and heterogeneous catalysis," Fuel Processing Technology. 90, 1016-1022.

Gharat, N. and Rathod, V. K. (2013). "Enzyme catalyzed transesterification of waste cooking oil with dimethyl carbonate," Journal of Molecular Catalysis B: Enzymatic. 88, 36-40.

Gharat, N. and Rathod, V. K. (2013). "Ultrasound assisted enzyme catalysed transesterification of waste cooking oil with dimethyl carbonate," Ultrasonics Sonochemistry. 20, 900-905.

Gjellesvik, D. R., Lombardo, D. and Walther, B. T. (1992). "Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): Purification and properties," Biochimica et Biophysica Acta (BBA). 1124, 123-134.

Gjellesvik, D. R., Lorens, J. B. and Male, R. (1994). "Pancreatic carboxylester lipase from Atlantic salmon (*Salmon salar*) cDNA sequence and computer-assisted modelling of tertiary structure," European Journal of Biochemistry. 226, 603-612.

Gjellesvik, D. R., Raae, A. J. and Walther, B. T. (1989). "Partial purification and characterization of triglyceride lipase from cod (*Gadus morhua*)," Aquacuture. 79, 177-184.

Gog, A., Roman, M., Tosa, M., Paizs, G. and Irimie, F. D. (2012). "Biodiesel production using enzymatic transesterification-current state and perspectives," Renewable Energy. 39, 10-16.

Gogate, P. R. and Kabadi, A. M. (2009). "A review of applications of cavitation in biochemical engineering/biotechnology," Biochemical Engineering Journal. 44, 60-72.

Goja, A. M., Yang, H., Cui, M. and Li, C. (2013). "Aqueous two-phase extraction advances for bioseparation," Bioprocessing & Biotechniques. 4, 1000140.

Gorman, L. A. and Dordiek, J. S. (1992). "Organic solvents strip water off enzymes," Biotechnology and Bioengineering. 39, 392-397.

Granados, M. L., Proves, M. D. Z., Alonso, D. M., Mariscal, R., Galisteo, F. C., Tost, R. M., Santamaría, J. and Fierro, J. L. G. (2007). "Biodiesel from sunflower oil by using activated calcium," Applied Catalysis B: Environmental. 73, 317-326.

Grochulski, P., Li, Y., Schrag, J. D., Bouthillier, F., Smith, P., Harrison, D., Rubin B. and Cygler, M. (1993). "Insights into interfacial activation from an open structure of *Candida rugose* lipase," Journal of Biological Chemitry. 268, 12843-12847.

Guidoni, A. A., Bendouka, F., De Caro, J. D. and Rovery, M. (1981). "Characterization of the serine reacting with diethyl *p*-nitrophenyl phosphate in porcine pancreatic lipase," Biochimica et Biophysica Acta (BBA). 660, 148-150.

Guldhe, A., Singh, B., Mutanda, T., Permaul, K. and Bux, F. (2015). "Advances in synthesis of biodiesel via enzyme catalysis: novel and sustainable approaches," Renewable and Sustainable Energy Reviews. 41, 1447-1464.

Guldhe, A., Singh, P., Kumari, S., Rawat, I., Permaul, K. and Bux, F. (2016). "Biodiesel synthesis from microalgae using immobilized *Aspergillus niger* whole cell lipase biocatalyst," Renewable Energy. 85, 1002-1010.

Gulomova, K., Ziomek, E., Schrag, J. D., Davranov, K. and Cygler, M. (1996). "Purification and characterization of a *Penicillium* sp. lipase which discriminates against diglycerides," Lipids. 31, 379-384.

Gupta, R., Gupta, N. and Rathi, P. (2004). "Bacterial lipases: an overview of production, purificationand biochemical properties," Applied Microbiology and Biotechnology. 64, 763-781.

Hanh, H. D., Dong, N. T., Okitsu, K., Nishimura, R. and Maeda, Y. (2009). "Biodiesel production through transesterification of triolein with various alcohols in an ultrasonic field," Renewable Rnergy. 34, 766-768.

Hanh, H. D., Dong, N. T., Okitsu, K., Nishimura, R. and Maeda, Y. (2009). "Biodiesel production by esterification of oleic acid with short-chain alcohols under ultrasonic irradiation condition," Renewable Energy. 34, 780-783.

Hasan, F., Shah, A. A., Javed, S. and Hameed, A. (2010). "Enzymes used in detergents: Lipase," African Journal of Biotechnology. 9, 4836-4844.

Hayyan, A., Alam, M. Z., Mirghani, M. E. S., Kabbashi, N. A., Hakimi, N. I. N. M., Siran, Y. M. and Tahiruddin, S. (2011). "Reduction of high content of free fatty acid in sludge palm oil via acid catalyst for biodiesel production," Fuel Processing Technology. 92, 920-924.

Herbert, R. A. (1992). "The perspective on the biotechnological potential of extremophiles," Trends in Biotechnology. 10, 395-402.

Heu, M. S., Kim, H. R. and Pyeun, J. H. (1995). "Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*)," Comparative Biochemistry and Physiology Part-B: Biochemistry and Molecular Biology. 112, 557-567.

Hide, W. A., Chan, L. and Li, W. H. (1992). "Structure and evolution of the lipase superfamily," Journal of Lipid Research. 33, 167-178.

Hingu, S. M., Gogate, P. R. and Rathod V. K. (2010). "Synthesis of biodiesel from waste cooking oil using sonochemical reactor," Ultrasonics Sonochemistry. 17, 827-832.

Hoekman, S. K., Broch, A., Robbins, C., Ceniceros, E. and Natarajan, M. (2012). "Review of biodiesel composition, properties, and specifications," Renewable and Sustainable Energy Review. 16, 143-169.

Huddleston, J., Veide, A., Köhler, K., Flanagan, J., Enfors, O.-S. and Lyddiatt, A. (1991). "The molecular basis of partitioning in aqueous two-phase systems," Trends in Biotechnology. 9, 381-388.

Hyun, J., Kothari, H., Herm, E., Mertenson, J., Treadwell, C. R. and Vahouny, G. V. (1969). "Purification and properties of pancreatic juice cholesterol esterase," Journal of Biological Chemistry. 244, 1937-1945.

Iijima, N., Tanaka, S. and Ota, Y. (1998). "Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*," Fish Physiology and Biochemistry. 18, 59-69.

Islam, M. A., Parveen, F., Hossain, K., Khatun, S., Karim, Md. R., Kim, G. S., Absar, N. and Haque, Md. S. (2009). "Purification and biochemical characterization of lipase from the dorsal part of *Cirrhinus reba*," Thai Journal of Agricultural Science. 42, 71-80.

Izquierdo, M. S., Socorro, J., Arantzamendi, L. and Hernadèz-Cruz, C. M. (2000). "Recent advances in lipid nutrition in fish larvae," Fish Physiology and Biochemistry. 22, 97-107.

Jaeger, K. E., Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M. and Misset, O. (1994). "Baterial lipases," FEMS Microbiology Reviews. 15, 29-63.

Jegannathan, K. R., Yun-Lee, L., Chan, E. S. and Ravindra, P. (2010). "Production of biodiesel from palm oil using liquid core lipase encapsulated in κ-carrageenan," Fuel. 89, 2272-2277.

Jena, P. C., Raheman, H., Prasanna, K. G. and Machavaram, R. (2010). "Biodiesel production from mixture of mahua and simarouba oils with high free fatty acids," Biomass and Bioenergy. 34, 1108-1116.

Jin, W., Broedl, U. C., Monajemi, H., Glick, J. M. and Rader, D. J. (2002). "Lipase H, a new member of the triglyceride lipase family synthesized by the intestine," Genomics. 80, 268-273.

Johansson, G. (1998). Affinity partitioning of proteins using aqueous two-phase system, in: J.C. Janson, L. Ryden (Eds.), Protein purification: Principles, High-resolution methods, and applications. London: Wiley.

Johansson, H. O., Karlstrom, G. and Tjerneld, F. (1997). "Temperature-induced phase partitioning of peptides in water solutions of ethylene oxide and propylene oxide random copolymers," Biochimica et Biophysica Acta (BBA). 1335, 315-325.

Johansson, H.-O., Karlström, G., Tjerneld, F. and Haynes, C. A. (1998). "Driving forces for phase separation and partitioning in aqueous two-phase systems," Journal of Chromatography B: Biomedical Sciences and Applications. 711, 3-17.

Johansson, H.-O., Karlström, K., Tjerneld, F. and Haynes, C. A. (1998). "Driving forces for phase separation and partitioning in aqueoustwo-phase systems," Journal of Chromatography B. 711, 3-17.

Joseph, B., Shrivastava, N. and Ramteke, P. W. (2012). "Extracellular cold-active lipase of *Microbacterium luteolum* isolated from Gangotri glacier, western Himalaya: Isolation, partial purification and characterization," Journal of Genetic Engineering and Biotechnology. 10, 137-144.

Julita, A., Zhu, K., Patkar, S. A., Vind, J., Svensden, A. and Kinnunen, P. K. J. (2000). "Detergent-induces conformational changes of *Humicola lanuginosa* lipase studied by fluorescence spectroscopy," Journal of Biophysics. 78, 1634-1642.

Kadkhodaee, R. and Povey, M. J. W. (2008). "Ultrasonic inactivation of *Bacillus* [alpha]-amylase. I. Effect of gas content and emitting face of probe," Ultrasonics Sonochemistry. 15, 133-142.

Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E. and Fukuda, H. (1999). "Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent," Journal of Bioscience and Bioengineering. 88, 627-631.

Karimi, M. (2012). "Immobilization of lipase onto mesoporous magnetic nanoparticles for enzymatic synthesis of biodiesel," Biocatalysis and Agricultural Biotechnology. 8, 182-188.

Karra-Châabouni, M., Bouaziz, I., Boufi, S., do Rego, A. M. B. and Gargouri, Y. (2008). "Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: Activity and stability studies," Colloids and Surfaces B: Biointerface. 66, 167-177.

Keera, S. T., El Sabagh, S. M. and Taman, A. R. (2018). "Castor oil biodiesel production and optimization," Egyptian Journal Petroleum. 27, 979-984.

Keim, G. I. (1945). "Process for treatment of fatty glycerides," US Patent 2. 383-601.

Kepka, C., Collet, E., Persson, J., Ståhl, Å., Lagerstedt, T., Terneld F. and Veide, A. (2003). "Pilot-scale extraction of an intracellular recombinant cutinase from *E. coli* cell homogenate using a thermoseparating aqueous two-phase system," Journal of Biotechnology. 103, 165-181.

Ketnawa, S., Benjakul, S., Martínez-Alvarez, O. and Rawdkuen, S. (2014) "Thermoseparating aqueous two-phase system for the separation of alkaline proteases from fish viscera," Separation Science and Technology. 49, 2158-2168.

Kim, H. J., Kang, B. S., Kim, M. J., Park, Y. M., Kim, D. K., Lee, J. S. and Lee, K. Y. (2004). "Transesterification of vegetable oil to biodiesel using heterogeneous base catalyst," Catalysis Today. 93, 315-320.

Kim, M. H., Kim, H. K., Lee, J. K., Park, S. Y. and Oh, T. K. (2000). "Thermostable lipase of *Bacillus Stearothermophilus*: High-level production, purification, and calcium-dependent thermostability," Bioscience, Biotechnology, and Biochemistry. 64, 280-286.

Kimtun, P.,Yunu, T., Paichid, N., Klomklao, S., Prasertsan, P. and Sangkharak, K. (2017). "Study of chemical constituents from palm fruit (*Elaeis guineensis*) after harvesting and the application of palm lipase for biodiesel production," Thaksin University Journal. 20, 1-9.

Klibanov, A. M. (1997). "Why are enzyme less active in organic solvents than in water?," Trends in Biotechnology. 15, 97-101.

Klomklao, S. and Benjakul, S. (2018). "Two trypsin isoforms from albacore tuna (*Thunnus alalunga*) liver: Purification and physicochemical and biochemical characterization," International Journal of Biological Marcromolecules. 107, 1864-1870.

Klomklao, S., Benjakul, S. and Kishimura, H. (2010). "Proteinases in hybrid catfish viscera: Characterization and effect of extraction media," Journal of Food Biochemistry. 34, 711-729.

Klomklao, S., Benjakul, S. and Visessanguan, W. (2004). "Comparative studies on proteolytic activity of splenic extract from three tuna species commonly used in Thailand," Journal of Food Biochemistry. 28, 355-327.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B. K. (2006). "Purification and characterization of trypsin from the spleen of oftongol tuna (*Thunnus tonggol*)," Journal of Agricultural and Food Chemistry. 54, 5617-5622.

Klomklao, S., Kishimura, H., Yabe, M. and Benjakul, S. (2007). "Purification and characterization of two pepsin from stomach of Pectoral rattail (*Coryphaenoides Pectoralis*)," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 147, 682-689.

Knothe, G. Gerpen, J. V. and Krahl, J. (2005). The Biodiesel Handbook. Illinosis : American Oil Chemists society Press.

Komaromy, M. and Schotz, M. (1987). "Cloning of rat hepatic lipase cDNA: Edidence for a lipase gene family," Proceedings of the National Academy of Sciences of the United States of America. 84, 1526-1530.

Kotogán, A., Zambrano, C., Kecskeméti, A., Varga, M., Szekeres, A., Papp, T., Vágvölgyi, C. and Takó, M. (2018). "An organic solvent-tolerant lipase with both hydrolytic and synthetic activities from the oleaginous fungus *Mortierella echinosphaera*," International Journal of Molecular Sciences. 19, 1129.

Kuby, S. A. (1991). Effect of pH and temperature. Boca Raton. Boston : CRC Press, Inc.

Kuepethkaew, S., Sangkharak, K., Benjakul, S. and Klomklao, S. (2017). "A laundry detergent-stable lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas: Effect of extraction media and biochemical characterization," International Journal of Food Properties. 20, 769-781.

Kuepethkaew, S., Sangkharak, K., Benjakul, S. and Klomklao, S. (2017). "Use of TPP and ATPS for partitioning and recovery of lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas," Journal of Food Science and Technology. 54, 3880-3891.

Kuepethkaew, S., Sangkharak, K., Benjakul, S., and Klomklao, S. (2017). "Optimized synthesis of biodiesel using lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas," Renewable Energy. 104, 139-147.

Kurtovic, I., Marshall, S. N., Zhao, X. and Simpson, B. K. (2009). "Lipase from mammals and fishes," Aquacult Reviews in Fisheries Science & Aquaculture. 17, 18-40.

Kurtovic, I., Marshall, S. N., Zhao, X. and Simpson, B. K. (2009). "Lipase from mammals and fishes," Reviews in Fisheries Science. 17, 18-40.

Kyger, E. M., Wiegand, R. C. and Lange, L. G. (1989). "Cloning of bovine pancreatic cholesterol esterase/lysophospholipase," Biochemical and Biophysical Research Communications. 164, 1302-1309.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (1987). "Rules for optimization of biocatalysis in organic solvents," Biotechnology and Bioengineering. 30, 81-87.

Laemmli, U. K. (1970). "Cleavage of structure protein during the assembly of the head of bacteriophage T4," Nature. 277, 680-685.

Lawonyawut, K. (1995). Hybridization and genetic manipulation in *Carias* catfish. Doctoral dissertation Doctor of Phisophy. Scotland : University of Stirling.

Lee, S., Posarac, D. and Ellis, N. (2011). "Process simulation and economic analysis of biodiesel production processes using fresh and waste vegetable oil and supercritical methanol," Chemical Engineering Research and Design. 89, 2826-2642.

Legay, M., Gondrexon, N., Person, S. L., Boldo, P. and Bontemps, A. (2011). "Enhancement of heat transfer by ultrasound: Review and recent advances," International Journal of Chemical Engineering. 1-17.

Leger, C., Bauchart, D. and Flanzy, J. (1977). "Some properties of pancreatic lipase in *Salmo gairdnerii* Rich.: K_m Effect of bile salts and Ca^{2+}, gel filtrations," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 57, 359-363.

Li, Q. and Yan, Y. (2010). "Production of biodiesel catalyzed by immobilized *Pseudomonas cepacia* lipase from *Sapium sebiferum* oil in micro-aqueous phase," Applied Energy. 87, 3148-3154.

Liang, X. F., Ogata, H. Y. and Oku, H. (2002). "Effect of dietary fatty acids on lipoprotein lipase gene expression in the liver and visceral adipose tissue of fed and starved red sea bream *Pagrus major*," Comparative Biochemistry and Physiology-Part A: Molecular and Integrative Physiology. 132, 913-919.

Lie, Ø. and Lambersten, G. (1985). "Digestive lipolytic enzymes in cod (*Gadus morhua*): Fatty acid specificity," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 80, 447-450.

Lima, V. M. G., Krieger, N., Mitchell, D. A. and Fontana, J. D. (2004). "Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents," Biochemical Engineering Journal. 18, 65-71.

Lin, L., Cunshan, Z., Vittayapadung, S., Xiangqian, S. and Mingdng, S. (2011). "Oppporunities and challenges for biodiesel fuel," Applied Energy. 88, 1020-1031

Liu, J., Deng, L., Wang, M., Nie, K., Liu, L., Tan, T. and Wang, F. (2014). "Lipase catalyzed synthesis of medium-chain biodiesel from *Cinnamonum camphora* seed oil," Chinese Journal of Chemical Engineering. 22, 1215-1219.

Liu, K. (1994). "Preparation of fatty acid methyl ester for gas chromatographic analysis of lipids in biological material," Journal of the American Oil Chemists' Society. 71, 1179-1187.

Lluyemi, F. B., Hanafi, M. M., Radziah, O. and Kamarudin, M. S. (2010). "Nutritional evaluation of fermented palm kernel cake using red tilapia," African Journal of Biotechnology. 9, 502-507.

López-López, S., Nolasco, H. and Vega-Villasante, F. (2003). "Characterization of digestive gland esterase-lipase activity of juvenile redclaw crayfish *Cherax quadricarintus*,"Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 135, 337-347.

Lopresto, C. G., Naccarato, S., Albo, L., De Paola, M., Chakraborty, S., Curcio, S. and Calabrò, V. (2015). "Enzymatic transesterification of waste vegetable oil to produce biodiesel" Ecotoxicology Environmental Safety. 121, 229-235.

Lowe, M. E. (1999). Lipase and phospholipase protocols. Totowa : Humana Press Inc.

Ma, F. and Hanna, A. (2001). "Biodiesel production: a review," Bioresource Technology. 70, 1-15.

Mahamuni, N. N. and Adewuyi, Y. G. (2010). "Application of Taguchi method to investigate the effects of process parameters on the transesterification of soybean oil using high frequency ultrasound," Energy & Fuels. 24, 2120-2126.

Mankura, M., Kayama, M. and Saito, S. (1984). "Wax ester hydrolysis by lipolytic enzymes in pyloric caeca of various fishes," Bulletin of the Japanese Society for the Science of Fish. 50, 2127-2131.

Margesin, R. and Schinner, F. (1994). "Properties of cod-adapted microorganisms and their potential role in biotechnology," Journal of Biotechnology. 33, 1-14.

Martinez, C., Nicholas, A., van Tibeurgh, H., Egloff, M. P., Cudrey, C., Verger, R. and Cambillau, C. (1994). "Cutinase, a lipolytic enzyme with a preformed oxyanion hole," Biochemistry. 33, 83-89.

Mason, T. J. and Lorimer, J. P. (1988). Sonochemistry. New York: Eillis Horwood Limited.

Mathimani, T., Uma, L. and Prabaharan, D. (2015). "Homogenous acid catalyzed transesterification of marine microalga *Chlorella* sp. BDUG 91771 lipid – an efficient biodiesel yield and its characterization," Renewable Energy. 81, 523-533.

Mehrasbi, M. R., Mohammadi, J. and Peyda, M. (2017). "Covalent immobilization of *Candida antarctica* lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil," Renewable Energy. 101, 593-602.

Meneghetri, S. M. P., Meneghetti, M. R., Wolf, C. R., Lima, G. E., de Lira Silva, L., Serra, T. M., Cauduro, F. and de Oliveira, L. (2006). "Biodiesel from castor oil: A comparison of ethanolysis versus methanolysis," Energy & Fuels. 20, 2262-2265.

Michelsen, K. G., Harmon, J. S. and Sheridan, M. A. (1994). "Adipose tissue lipolysis in rainbow trout, *Oncorhynchus mykiss*, is modulated by phosphorylation of triacylglycerol lipase," Comparative Biochemistry and Physiology-Part B: Comparative Biochemistry. 107, 509-513.

Mittelbach, M. and Tritthart, P. (1988). "Diesel fuels derived from vegetable oils. III. Emission tests using methyl esters of used frying oil," Journal of the American Oil Chemists' Society. 65, 1185-1187.

Mittelbach, M., Pokits, B. and Silberholz, A. (1992). Production and fuel properties of fatty acid methyl esters from used frying oil. St .Joseph : ASAE.

Montoya-López, A., Moreno-Arias, J., Tarazona-Morales, A., Olivera-Angel, M. and Betancur, J. (2019) "Body shape variation between farms of tilapia (*Oreochromis* sp.) in Colombian Andes using landmark-based geometric morphometrics," Latin American Journal of Aquatic Research. 47, 194-200.

Mukherjee, J. and Gupta, M. N. (2016). "Lipase coated clusters of iron oxide nanoparticles for biodiesel synthesis in a solvent free medium," Bioresource Technology. 209, 166-171.

Mukherjee, K. D. and Hill, M. J. (1990). "Triacylglycerol lipase from rape (*Brassica napus* L.) suitable for biotechnological purposes," Applied Biochemistry and Biotechnology. 26,1-10.

Mukundan, M. K., Gopkumar, K. and Nair, M. R. (1985). "Purification of lipase from hepatopancreas of oil sardine (*Sardinella longiceps* Linnaceus) and its characteristic and properties," Journal of the Science of Food and Agriculture. 36, 191-203.

Murray, H. M., Gallant, J. W., Perez-Casanova, J. C., Johnson, S. C. and Douglas, S. E. (2003). "Ontogeny of lipase expression in winter flounder," Journal of Fish Biology. 62, 816-833.

Naganagouda, K. and Mulimani, V. H. (2008). "Aqueous two-phase extraction. (ATPE: An attractive and economically viable technology for downstream processing of *Aspergillus oryzae*α-galactosidase)," Process Biochemistry. 43, 1293-1299.

Naganthran, A., Masomian, M., Rahman, R. N. Z. R. A., Ali, M. S. M. and Nooh, H. M. (2017). "Improving the efficiency of new automatic dishwashing detergent formulation by addition of thermostable lipase, protease and amylase," Molecules. 22, 1577.

Nakpong, P. and Woothikanokkhan, S. (2010). "Roselle (*Hibiscus sabdariffa* L.) oil as an alternative feedstock for biodiesel production in Thailand," Fuel. 89, 1806-1811.

Narkhede, N., Brahmkhatri, V. and Patel, A. (2014). "Efficient synthesis of biodiesel from waste cooking oil using solid acid catalyst comprising 12-tungstosilicic acid and SBA-15," Fuel. 135, 253-261.

Navvabi, A., Razzaghi, M., Fernandes, P., Karami, L. and Homaei, A. (2018). "Novel lipase discovery specifically from marine organisms for industrial production and practical applications," Process Biochemistry. 70, 61-70.

Ng, H. S., Tan, C. P., Mokhtar, M. N., Ibrahim, S., Ariff, A., Ooi, C. W. and Ling, T. C. (2012). "Recovery of *Bacillus cereus* cyclodextrin glycosyltransferase and recycling of phase components in an aqueous two-phase system using thermo-separating polymer," Separation and Purification Technology. 89, 9-15.

Nini, L., Sarda, L., Comeau, L. C., Boitard, E., Dubès, J. P. and Chanhinian, H. (2001). "Lipase-catalysed hydrolysis of short-chain substrates in solution and in emulsion: A kinetic study," Biochimica et Biophysica Acta (BBA). 1534, 34-44.

Noureddini, H. and Zhu, D. (1997). "Kinetics of transesterification of soy bean oil," Journal of the American Oil Chemists' Society. 74, 1457-1463.

Noureddini, H., Gao, X. and Philkana, R. S. (2005). "Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil," Bioresource Technology. 96, 769-777.

Ogino, H., Nakagawa, S., Shinya, K., Muto, T., Fujimura, N., Yasuda, M. and Ishikawa, H. (2000). "Purification and characterization of organic solvent-stable lipase from organic solvent-telerant *Pseudomonas aeruginosa* LST-03,: Journal of Bioscience and Bioengineering . 89, 451-457.

Ollis, D. L., Cheah, E., Cyler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G. and Goldman, A. (1992). "The α/β hydrolase fold," Protein Engineering. 5, 197-211.

Olsen, R. E., Henderson, R. J. and Pederson, T. (1991). "The influence of dietary lipid classes on the fatty acid composition of small cod *Gadus morhua* L. juveniles reared in an enclosure in northern Norway," Journal of Experimental Marine Biology and Ecology. 148, 59-76.

Onukwuli, D. O., Emembolu, L. N., Ude, C. N., Aliozo, S. O. and Menkiti, M. C. (2017). "Optimization of biodiesel production from refined cotton seed oil and its characterization," Egyptian Journal of Petroleum. 26, 103-110.

Osório, N. M., Ferreira-Dias, S., Gusmão, J. H. and Fonseca, N. M. R. (2001). "Response surface modeling of the production of omega-3 polyunsaturated fatty acids-enriched fats by a commercial immobilized lipase," Journal of Molecular Catalysis B: Enzymatic. 11, 677-686.

Ozbek, B. and Ulgen, K. O. (2000). "The stability of enzymes after sonication," Process Biochemistry. 35, 1037-1043.

Panaitov, I. and Verger, R. (2000). Enzymatic reaction at interfaces: Interfacial and temporal organization of enzymatic lipolysis. New York : Marcel Dekker.

Park, E. Y., Sato, M. and Kojima, S. (2008). "Lipase-catalyzed biodiesel production from waste activated bleaching earth as raw material in a pilot plant," Bioresource Technology. 99, 3130-3135.

Patchimpet, J., Sangkharak, K. and Klomklao, S. (2019a). "Lipolytic activity of viscera extract from three freshwater fish species in Phatthalung, Thailand: Comparative studies and potential use as dishwashing detergent additive," Biocatalysis and Agricultural Biotechnology. 19, 101143.

Patchimpet, J., Sangkharak, K. and Klomklao, S. (2019b). "Thermoseparating aqueous two-phase system for lipase recovery and partitioning from Nile tilapia viscera: Biochemical properties and effect of ultrasound," Separation and Purification Technology. In submitted.

Patchimpet, J., Sangkharak, K. and Klomklao, S. (2019c). "Optimization of process variables for the production of biodiesel by transesterification of used cooking oil using lipase from Nile tilapia viscera," Renewable Energy. In submitted.

Patton, J. S., Navenzel, J. C. and Benson, A. A. (1975). "Specificity of digestive lipases in hydrolysis of wax esters and triglycerides studies in anchovy and other selected species," Lipids. 10, 575-583.

Patton, J. S., Waener, T. G. and Benson, A. A. (1977). "Partial characterization of bile salt-dependent triacylglycerols lipase from the leopard shark pancreas," Biochimica et Biophysica Acta (BBA). 486, 322-330.

Peng, R., Lin, J. and Wei, D. (2010). "Purification and characterization of an organic solvent-tolerant lipase from *Pseudomonas aeruginosa* CS-2," Applied Biochemistry and Biotechnology. 162, 733-743.

Pereira, M., Wu, Y.-T., Venâncio, A. and Teixeira, J. (2003). "Aqueous two-phase extraction using thermoseparating polymer: A new system for the separation of endo-polygalacturonase," Biochemical Engineering Journal. 15, 131-138.

Perez-Casanova, J. C., Murray, H. M., Gallant, J. W., Ross, N. W., Doudlas, S. E. and Johnson, S. C. (2004). "Bile salt-activated lipase expression during larval development in the haddock (*Melanogrammus aeglefinus*)," Aquacuture. 235, 601-617.

Persson, J., Johansson, H.-O. and Tjerneld, F. (1999). "Purification of protein and recycling of polymers in a new aqueous two-phase system using two thermoseparating polymers," Journal of Chromatography A. 864, 31-48

Pfennig, A., Schwerin, A. and Gaube, J. (1998). "Consistent view of electrolytes in aqueous two-phase systems," Journal of Chromatography B. 711, 45-52.

Picker, M. D. and Griffiths C. L. (2011). Alien and invasive Animals- A south African Perspective. Cape Town : Randomhouse/Struik.

Pogorevc, M., Stecher, H. and Faber, K. (2002). "A caveat for the use of log *P* values for the assessment of the biocompatibility of organic solvents," Biotechnology Letters. 24, 857-860.

Ponjarat, J., Singchat, W., Monkheang, P., Suntronpong, A., Tawichasri, P., Sillapaprayoon, S., Ogawa, S., Muangmai, N., Baicharoen, S., Peyachoknagul, S., Parhar, I. and Na-Nakorn, U. (2019). "Evidence of dramatic sterility in F_1 male hybrid catfish [male *Clarias gariepinus* (Burchell, 1822) x female *C. macrocephalus* (Günther, 1864)] resulting from the failure of homologous chromosome pairing in meiosis I," Aquaculture. 505, 84-91.

Poonsin, T., Sripokar, P., Benjakul, S., Simpson, B. K., Visessanguan, W. and Klomklao, S. (2016). "Major trypsin like-serine proteinases from albacore tuna (*Thunnus alalunga*) spleen: Biochemical characterization and the effect of extraction media," Journal of Food Biochemistry. 41, 1-9.

Poosumas, J. (2014). Ultrasound assisted reactor for biodiesel production from purified palm oil on calcium oxide heterogeneous catalyst. Master's thesis Master of Engineering. Chulalongkorn University.

Priyanka, B. S., Rastogi, N. K., Raghavarao, K. S. M. S. and Thakur, M. S. (2012). "Downstream processing of luciferase from fireflies (*Photinus pyralis*) using aqueous two-phase extraction," Process Biochemistry. 47, 1358-1363.

Pryde, E. H. (1984). "Vegetable oils as fuel alternatives-symposium overview," Journal of the American Oil Chemists' Society. 61, 1609-1610.

Quinlan, P. and Moore, S. (1993). "Modification of triglycerides by lipases: Process technology and its application to production of nutritionally improved fats," Inform. 4, 580-585.

Rahimi, M., Aghel, B., Alitabar, M., Sepahvand, A. and Ghasempour, H. R. (2014). "Optimization of biodiesel production from soybean oil in a microreactor," Energy Conversion and Management. 79, 599-605.

Rahman, I. A., Rahman, R. N. Z. R. A., Salleh, A. B. and Basri, M. (2013). "Formulation and evaluation of an autonatic dishwashing detergent containing T1 Lipase," Journal of Surfactants and Detergents. 16, 427-434.

Rahman, R. N. Z. A., Baharum, S. N., Salleh, A. B. and Basri, M. (2006). "S5 Lipase: an organic solvent tolerant enzyme," Journal of Microbiology. 44, 583–590.

Raita, M., Arnthong, J., Champreda, V. and Laosiripojana, N. (2015). "Modification of magnetic nanoparticle lipase designs for biodiesel production from palm oil," Fuel Processing Technology. 134, 189-197.

Rakkan, T., Suwanno, S., Paichid, N., Yunu, T., Klomklao, S. and Sangkharak, K. (2017). "Optimized synthesis method for transesterification of residual oil from palm oil mill effluent and lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas to environmentally friendly biodiesel," Fuel. 209, 309-314.

Ramadhas, A. S., Jayaraj, S. and Muraleedharan, C. (2005). "Characterization and effect of using rubber seed oil as fuel in the compression ignition engines," Renewable Energy. 30, 795-803.

Ramakrishman, V., Goveas, L. C., Suralikerimath, N., Jampani, C., Halami, P. M. and Narayan, B. (2016). "Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization," Biocatalysis and Agricultural Biotechnology. 6, 19-27.

Ranganathan, S. V., Narasimhan, S. L. and Muthukumar, K. (2008). "An overview of enzymatic production of biodiesel," Bioresource Technology. 99, 3975-3981.

Rasco, B. A. and Hultin, H. O. (1988). "A comparison of dogfish and porcine pancreatic lipases," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 89, 671-677.

Rathelot, J., Julien, R., Canioni, P., Coeroli, C. and Sarda, L. (1975). "Studies on the effect of bile salt and colipase in enzymatic lipolysis. Improved method for the determination of pancreastic lipase and colipase," Biochimie. 57, 1117-1122.

Reyero, I., Arazamendi, G., Zabala, S. and Gandía, L. M. (2015). "Kinetics of the NaOH-catalyzed transesterification of sunflower oil with ethanol to produce biodiesel," Fuel Processing Technology. 129, 147–155.

Rivera-Pérez, C., del Toro, M. A. N. and García-Carreño, F. (2011). "Purification and characterization of an intracellular lipase from pleopods of whiteleg shrimp (*Litopenaeus vannamei*)," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 158, 99-105.

Robinson, H. W. and Hodgen, C. G. (1940). "The biuret reaction in the determination of serum protein. I. A study of the condition necessary for the production of the stable color which bears a quantitative relationship to protein concentration,"Journal of Biological Chemistry.135, 707-725.

Rokhina, E. V., Lens, P. and Virkutyte, J. (2009). "Low-frequency ultrasound in biotechnology: State of the art," Trends in Biotechnology. 27, 298-306.

Rosa, P. A. J., Azevedo, A. M. A., Sommerfeld, S., Mutter A. and Aires-Barros, M. R. (2009). "Application of aqueous two-phase systems to antibody purification: A multi stage approach," Journal of Biotechnology. 139, 306-313.

Rovery, M., Boudouard, M. and Bianchetta, J. (1987). "An improved large scale procedure for the purification of porcine pancreatic lipase," Biochimica et Biophysica Acta (BBA). 525, 373-379.

Rudd, E. A., Mizuno, N. K. and Brockman, H. L. (1987). "Isolation of two forms of carboxylester lipase (cholesterol esterase) from porcine," Biochimica et Biophysica Acta (BBA). 918, 106-114.

Sae-leaw, T. and Benjakul, S. (2018). "Lipase from liver of seabass (*Lates calcarifer*): Characteristics and the use for defatting of fish skin," Food chemistry. 240, 9-15.

Sahar, Sadaf, S., Iqbal, J., Ullah, I., Bhatti, H. N., Nouren, S., ur-Rehman, H., Nisar, J. and Iqbal, M. (2018). "Biodiesel production from waste cooking oil: An efficient technique to convert waste into biodiesel," Sustainable Cites and Society. 41, 220-226.

Sanjid, A., Masjuki, H., Kalam, M., Rahman, S., Abedin, M. and Palash, S. (2013). "Impact of palm, mustard, waste cooking oil and *Calophyllum inophyllum* biofuels on performance and emission of CI engine" Renewable and Sustainable Energy Reviews. 27, 664-682.

Santos, F. F. P., Malveira, J. Q., Cruz, M. G. A. and Fernandes, F. A. N. (2010). "Production of biodiesel by ultrasound assisted esterification of *Oreochromis niloticus* oil," Fuel. 89, 275-279.

Saxena, R. K., Davidson, W. S., Sheoran, A. and Giri, B. (2003). "Purification and characterization of an alkaline thermostable lipase from *Aspergillus caneus*," Process Biochemistry. 39, 3239-3247.

Sbihi, H. M., Nehdi, I. A., Blidi, L. E., Rashid, U. and Al-Resayes, S. I. (2015). "Lipase/enzyme catalyzed biodiesel production from *Prunus mahaleb*: A comparative study with base catalyzed biodiesel production," Industrial Crops and Products. 76, 1049-1054.

Schluck, A., Maurrer, G. and Kula, M.-R. (1995). "Influence of Electrostatic Interactions on Partitioning in Aqueous Polyethylene Glycol/Dextran," Biphasic Systems: Part I. Biotechnology Bioeng. 46, 443-451.

Schrag, J. D. and Cygler, M. (1997). "Lipases and alpha/beta hydrolase fold," Methods in Enzymology. 284, 85-107.

Schwab, A. W., Dykstra, G. J., Selke, E., Sorenson, S. C. and Pryde, E. H. (1988). "Diesel fuel from thermal decomposition of soybean oil," Journal of the American Oil Chemists' Society. 65, 1781-1786.

Senphan, T and Benjakul, S. (2014). "Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp," Separation and Purification Technology. 129, 57-63.

Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A. Yusof, Y. A., Chen, S. K. and Ling, T. C. (2011). "Direct recovery of lipase derived from *Burkholderia cepacia* in recycling aqueous two-phase flotation," Separation and Purification Technology. 80, 577-584.

Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K. and Ling, T. C. (2012). "Primary recovery of lipase derived from *Burkholderia cenocepacia* strain ST8 and recycling of phase components in an aqueous two-phase system," Biochemical Engineering. 60, 74-80.

Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K. and Ling, T. C. (2012). "Extractive fermentation for improved production and recovery of lipase derived from *Burkholderia cepacia* using a thermoseparating polymer in aqueous two-phase systems," Bioresource Technology. 116, 226-233.

Sim, J. H., Kamaruddin, A. H. and Bhatia, S. (2010). "Biodiesel (FAME) productivity, catalytic efficiency and thermal stability of lipozyme TL IM for crude palm oil transesterification with methanol," Journal of the American Oil Chemists' Society. 27, 1027-1034.

Simpson, B. K. and Haard, N. F. (1984). "Trypsin from the Greenland cod (*Gadus ogac*). Isolation and comparative properties," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 79, 613-622.

Singh, B., Guldhe, A., Rawat, I. and Bux, F. (2014). "Towards a sustainable approach for development of biodiesel from plant and microalgae," Renewable & Sustainable Energy Reviews. 29, 216-245.

Sivaramakrishnan, R. and Incharoensakdi, A. (2017). "Direct transesterification of Botryococcus sp. catalyzed by immobilized lipase: Ultrasound treatment can reduce reaction time with high yield of methyl ester," Fuel. 191, 363-370.

Smichi, N., Fendri, A., Chaâbouni, R., Rebah, F. B., Gargouri, Y. and Miled, N. (2010). "Purification and biochemical characterization of an acid-stable lipase from the pyloric caeca of Sardine (*Sardinella aurita*)," Applied Biochemistry and Biotechnology. 162, 1483-1496.

Smichi, N., Gargouri, Y., Miled, N. and Fendri, A. (2013). "A grey mullet enzyme displaying both lipase and phospholipase activities: Purification and characterization," International Journal of Biological Macromolecules. 58, 87-94.

Sonare, N. R. and Rathod, V. K. (2010). "Transesterification of used sunflower oil using immobilized enzyme," Journal of Molecular Catalysis B: Enzymatic. 66, 142-147.

Sonntag, N. O. V. (1979). Structure and composition of fats and oils. New York : John Wiley and Sons.

Soria, A. C. and Villamie, M. (2010). "Effect of ultrasonic on the technological properties and bioactivity of food: A review," Trends in Food Science and Technology. 21, 323-331.

Sprules, F. J. and Price, D. (1950). "Production of fatty esters," US Patent 2. 366-494.

Sridharan, R. and Mathai, I. M. (1974). "Transesterification reactions," Journal of Scientific and Industrial Research (JSIR). 33, 178-187.

Sriket, C., Benjakul, S., Visessanguan, W., Hara, K., Yoshida, A. and Liang, X. (2012). "Low molecular weight trypsin from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*): Characteristics and biochemical properties," Food Chemistry. 134, 351-358.

Steel, R. G. D. and Torrie, J. H. (1980). Principles and Procedures of Statistics. New York: McGraw-Hill.

Su, F., Li, G. L., Fan, Y. L. and Yan, Y. J. (2015). "Enhancing biodiesel production via a synergic effect between immobilized *Rhizopus oryzae* lipase and Novozym 435," Fuel. 137, 298-304.

Subhedar, P. B. and Gogate, P. R. (2014). "Enhancing the activity of cellulose enzyme using ultrasonic irradiations," Journal of Molecular Catalysis B: Enzymatic. 101, 108-114.

Subhedar, P. B., Botelho, C., Ribeiro, A., Castro, R., Pereira, M. A., Gogate P. R. and Cavaco-Paulo, A. (2015). "Ultrasound intensification suppresses the need of methanol excess during the biodiesel production with Lipozyme TL-IM," Ultrasonic Sonochemistry. 27, 530-535.

Sutkar, V. S. and Gogate, P. R. (2009). "Design aspects of sonochemical reactors: Techniques for understanding cavitational activity distribution and effect of operating parameters," Chemical Engineering Journal. 155, 26-36.

Sztajer, H., Lünsdorf, H., Erdmann, H., Menge, U. and Schmid, R. (1992). "Purification and properties of lipase from *Penicillium simplicissium*," Biochimica et Biophysica Acta (BBA). 1124, 253-261.

Takahashi, K., Hatano, M. and Sakura, Y. (1996). "Lipase from neon flying squid hepatopancreas: Purification and properties," Food Chemistry. 57, 515-521.

Takeda, Y., Aono, R. and Doukyu, N. (2006). "Purification, characterization, and molecular cloning of organic-solvent-tolerant cholesterol esterase from cyclohexane-tolerant *Burkholderia cepacia* stain ST-200," Extremophiles. 10, 269-277.

Tan, T., Lu, J., Nie, K., Deng, L. and Wang, F. (2010). "Biodiesel production with immobilized lipase: a review," Biotechnology Advances. 28, 628-634.

Thongprajukaew, K., Kovitvadhi, S., Kovitvadhi, U. and Preprame, P. (2017). "Effects of feeding frequency on growth performance and digestive enzyme activity of sex-reversed Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)," Agricultural and Natural Resoueces. 51, 292-298.

Tocher, D. R. and Sargent, J. R. (1984). "Studies in triacylglycerol, wax ester and sterol ester hydrolases in intestinal caeca of rainbow trout (*Salmon garidnerii*) fed diets rich in triacylglycerols and wax esters," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 77, 561-571.

Tomke, P. D. and Rathod, V. K. (2015). "Ultrasound assisted lipase catalyzed synthesis of cinnamyl acetate via transesterification reaction in a solvent free medium," Ultrasonics Sonochemistry. 27, 241-246.

Unajak, S., Meesawat, P., Paemanee, A., Areechon, N., Engkagul, A., Kovitvadhi, U., Kovititvadhi, S., Rungruangsak-Torrissen, K. and Choowongkomon, K. (2012). "Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.)," Food Chemistry. 134, 1533-1541.

Ungcharoenwiwat, P. and H-Kittikun, A. (2015). "Purification and characterization of lipase from *Burkholderia* sp. EQ3 isolated from wastewater from a canned

fish factory and its application for the synthesis of wax esters," Journal of Molecular Catalysis B: Enzymatic. 115, 96-104.

van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N., Verger, R. and Cambillau, C. (1993). "Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography," Nature. 362, 814-820.

Veljković, V. B., Avramović, J. M. and Stamenković, O. S. (2012). "Biodiesel production by ultrasound-assisted transesterification: state of the art and the perspectives," Renewable and Sustainable Energy Reviews. 16, 1193-1209.

Verger, R. (1980). "Enzyme kinetics of lipolysis," Methods in Enzymology. 64, 340-392.

Verger, R. (1984). Pancreatic lipases. New York : Elsevier.

Verger, R. (1997). "Interfacial activation of lipases: Facts and artifacts," Trends in Biotechnology. 15, 32-38.

Verger, R. and de Haas, G. H. (1976). "Interfacial enzyme kinetics of lipolysis," Annual Review of Biophysics and Bioengineering. 5, 77-117.

Verger, R., Mieras, M. C. E. and de Haas, G. H. (1973). "Action of phospholipase A at interfacial," Journal of Biological Chemistry. 248, 4023-4034.

Verma, N., Thakur, S. and Bhatt A. K. (2012) "Microbial lipases: Industrial applications and properties (a review)," International Research Journal of Biological Sciences. 1, 88-92.

Waghmare, G. V. and Rathod, V. K. (2016). "Ultrasound assisted enzyme catalyzed hydrolysis of waste cooking oil under solvent free condition," Ultrasonics Sonochemistry. 32, 60-67.

Wang, C. S. (1980). "Purification of human milk bile salt-activated lipase by cholic acid-coupled Sepharose 4B affinity chromatography," Analytical Biochemistry. 105, 398-402.

Wang, C. S. and Harstuck, J. A. (1993). "Bile salt-activated lipase. A multiple function lipolytic enzyme," Biochimica et Biophysica Acta (BBA). 1166, 1-19.

Wang, C. S. and Harstuck, J. A. (1993). "Bile salt-activated lipase. A multiple function lipolytic enzyme," Biochimica et Biophysica Acta (BBA). 1166,1-19.

Wang, C. S. and Lee, M. (1985). "Kinetic properties of human milk bile salt-activated lipase: Studies using long chain triacylglycerol as substrate," Journal of Lipid Research. 26, 824-830.

Wang, J. X., Huang, Q. D., Huang, F. H., Wang, J. W. and Huang, Q. J. (2007). "Lipase-catalyzed production of biodiesel from high acid value waste oil using ultrasonic assistant," Chinese Journal of Biotechnology. 23, 1121-1128.

Wang, X., Yu, X. and Xu, Y. (2009). "Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from *Burkholderia cepacia* ATCC 25416," Enzyme and Microbial Technology. 45, 94-102.

Watanabe, T. (1982). "Lipid nutrition in fish," Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology. 73, 3-15.

Whitaker, J. R. (1994). Effect of pH on rates of enzyme-catalyzed reactions. New York : Marcel Dekker Inc.

Winkler, U. K., d'Arcy, A. and Hunziker, W. (1990). "Structure of human pancreatic lipase," Nature. 343, 771-774.

Wion, K. L., Kirchgessner, T. G., Lusis, A. J., Schotz, M. C. and Lawan, R. M. (1987). "Human lipoprotein lipase complementary DNA sequence," Science. 235, 1638-1641.

Wright, H. J., Segur, J. B., Clark, H. V., Coburn, S. K., Langdon, E. E. and Dupuis, R. N. (1994). "A report on ester interchange," Oil Soap. 21, 145-148.

Wu, X. Y., Jääskeläinen, S. and Linko, Y. Y. (1996). "An investigation of crude lipases for hydrolysis, esterification, and transesterification," Enzyme and Microbial Technology. 19, 226-231.

Xu, X. (2000). "Production of specific structure triacylglycerols by lipase-catalyzed reaction. A review," European Journal of Lipid Science and Technology. 102, 287-303.

Yachmenev, V., Condon, B., Klasson, T. and Lambert, A. (2009). "Acceleration of the enzymatic hydrolysis of corn stover and sugar cane bagasse celluloses by low intensity uniform ultrasound," Journal of Biobased Materials and Bioenergy. 3, 25-31.

Yadav, G. D. and Lathi, P. S. (2006). "Intensification of enzymatic synthesis of propylene glycol monolaurate from 1,2-propanediol and lauric acid under microwave irradiation: kinetics of forward and reverse reactions," Enzyme and Microbial Technology. 38, 814-820.

Yadav, R. P., Saxena, R. K., Gupta, R. and Davidson, W. S. (1998). "Purification and characterization of a regiospecific lipase from *Aspergillus terreus*," Biotechnology and Applied Biochemistry. 28,243-249.

Yan, Y., Bornscheuer, U. T. and Schmid, R. D. (2002). "Efficient water removal in lipase-catalyzed esterification using a low-boiling point azeotrope," Biotechnology and Bioengineering. 78, 31-34.

Yang, W., He ,Y., Xu, L., Zhang, H. and Yan, Y. (2016). "A new extracellular thermos-solventstablelipase from *Burkholderia ubonensis* SL-4: indentificaiton, characterization and application for biodiesel production," Journal of Molecular Catalysis B: Enzymatic. 126, 76-89.

You, O., Yin, X., Zhou, Y. and Zhang, Y. (2013). "Biodiesel production from jatropha oil catalyzed by immobilized *Burkholderia cepacia* lipase on modified attapulgite," Bioresource Technology. 148, 202-207.

Yu, D. Tian, L., Wu, H., Wang, S., Wang, Y., Ma, D. and Fang, X. (2010). "Ultrasonic irradiation with vibration for biodiesel production from soybean oil by Novozym 435," Process Biochemistry. 45, 519-525.

Zarai, Z., Ali, M. B., Fendri, A., Louati, H., Mejdoub, H. and Gargouri, Y. (2012). "Purification and biochemical properties of *Hexaplex trunculus* digestive lipase," Process Biochemistry. 47, 2434-2439.

Zarai, Z., Bacha, A. B., Horchani, H., Bezzine, S., Zouari, Z., Gargouri, Y. and Mejdoub, H. (2010). "A novel hepatopancreatic phospholipase A_2 from *Hexaplex trunculus* with digestive and toxic activities," Archives of Biochemistry and Biophysics. 494, 121-129.

Zhang, L., Sun, S., Xin, Z., Sheng, B. and Liu, Q. (2010). "Synthesis and component confirmation of biodiesel from palm oil and dimethyl carbonate catalyzed by immobilized-lipase in solvent-free system," Fuel. 89, 3960-3965.

Zhang, Q., Zheng, Z., Liu, C., Liu, C. and Tan, T. (2016). "Biodiesel production using lipase immobilized on epoxychloropropane-modified Fe_3O_4 sub-microspheres.," Colloids and Surfaces B: Biointerfaces. 140, 446-451.

Zhang, Y., Dube, M. A., McLean, D. D. and Kates, M. (2003). "Biodiesel production from waste cooking oil: Economic assessment and sensitivity analysis," Bioresource Technology. 90, 229–240.

Zhao, L., Budge, S. M., Ghaly, A. E., Brooks, M. S. and Dave, D. (2012). "Partition of pepsinogen from the stomach of red perch (*Sebastes marinus*) by Aqueous Two Phase Systems: Effects of the Salt Type and Concentration," Journal of Food Processing & Technology. 3, 1000180.

Zhao, L., Budge, S. M., Ghaly, A. E., Brooks, M. S. and Dave, D. (2013). "Partition of pepsinogen from the stomach of red perch (*Sebastes marinus*) by aqueous two phase systems: Effects of PEG molecular weight and concentration," Enzyme Engineering. 2, 1000108.

Zhao, L., Buge, S. M., Ghaly, A. E., Brooks, M. S. and Dave, D. (2011). "Extraction, purification and characterization of fish pepsin: A critical review," Journal of Food Processing & Technology. 2, 1-4.

Ziejewski, M. Z., Kaufman, K. R. and Pratt, G. L. (1983). Vegetable oil as diesel fuel. USA : USDA.

Ziejewski, M., Kaufman, K. R., Schwab, A. W. and Pryde, E. H. (1984). "Diesel engine evaluation of a nonionic sunflower oil-aqueous microemulsion," Journal of the American Oil Chemists' Society. 61, 1620-1626.

APPENDIX

APPENDIX A

Preparation for Polyacrylamide Gel Electrophoresis

1) Stock Reagent

- 30% Acrylamide - 0.8% Bisacrylamide Solution

  Acrylamide 29.20 g

  N,N'-Methylene – Bis Acrylamide 0.80 g

  Adjust volume to 100 ml with distilled water

- 1.5 M Tris-HCl pH 8.8

  Tris (Hydroxymethyl) - Aminomethane 18.17 g

  Adjust pH to 8.8 with 1 N HCl and final volume to 100 ml with distilled water

- 0.5 M Tris-HCl pH 6.8

  Tris (Hydroxymethyl) - Aminomethane 6.05 g

  Adjust pH to 6.8 with 1 N HCl and final volume to 100 ml with distilled water

- Tris – Glycine Electrode Buffer

  Tris (Hydroxymethyl) – Aminomethane 3.00 g

  Glycine 14.40 g

  SDS 1.0 g

  Dissolve in distilled water to 1,000 ml to give final pH to be 8.3

2) SDS-PAGE

4% Stacking Gel

30% Acrylamide – 0.8% Bis	665	µl
0.5 M Tris-HCl Buffer, pH 6.8	1,250	µl
Distilled Water	3,000	µl
10% SDS	50	µl
10% Ammonium Persulfate	25	µl
TAMED	3	µl
Total Volume	4,993	µl

12% Running Gel

30% Acrylamide – 0.8% Bis	4,000	µl
1.5 M Tris-HCl Buffer, pH 8.8	2,500	µl
Distilled Water	3,345	µl
10% SDS	100	µl
10% Ammonium Persulfate	50	µl
TAMED	5	µl
Total Volume	10,000	µl

APPENDIX B

Standard Curve

1. Standard Curve of BSA for Calculation of Protein Concentration by Bradford Method

Standard curve of BSA was prepared by diluting the stock of BSA solution to final concentrations of 0-100 µg/ml. The absorbance of the obtained solutions was measured at 595 nm. The absorbent value was plotted against the BSA concentration.

Table 24 Absorbance at 595 nm of BSA Standard at Various Concentrations

BSA Concentration (µg/ml)	Absorbance at 595 nm
0	0.804
10	0.853
20	0.933
40	1.028
60	1.141
80	1.214
100	1.266

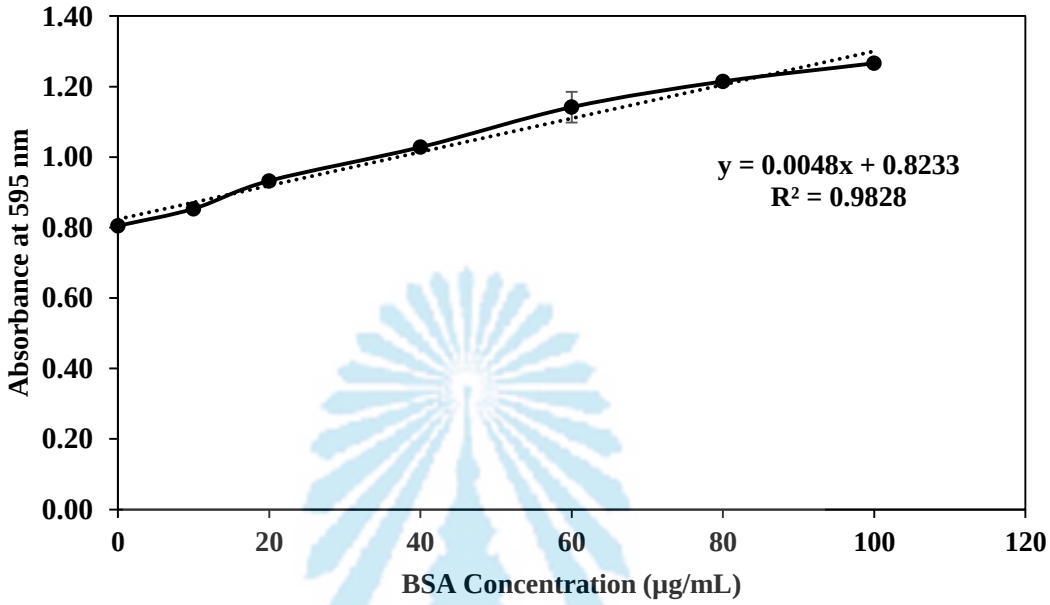


Figure 32. Standard Curve of BSA Concentration Against Absorbance at 595 nm.

2. Standard Curve of BSA for Calculation of Protein Concentration by Biuret Method

Standard curve of BSA was prepared by diluting the stock of BSA solution to final concentrations of 0-10 mg/ml. The absorbance of the obtained solutions was measured at 540 nm. The absorbent value was plotted against the BSA concentration.

Table 25 Absorbance at 540 nm of BSA Standard at Various Concentrations

BSA concentration (mg/ml)	Absorbance at 540 nm
0	0.066
2	0.173
4	0.281
6	0.393
8	0.484
10	0.581

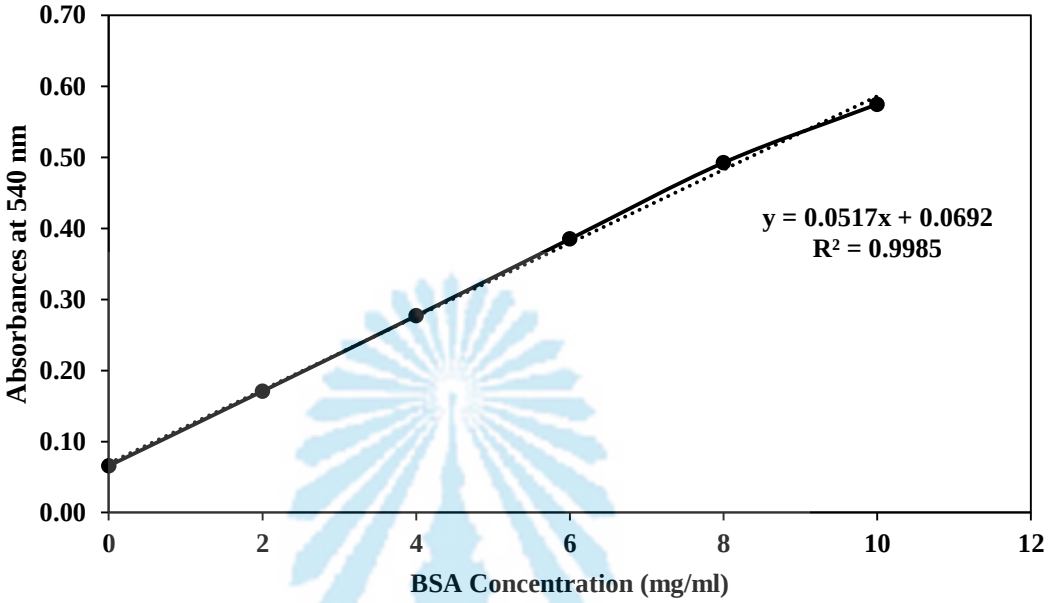


Figure 33. Standard Curve of BSA Concentration Against Absorbance at 540 nm.

CURRICULUM VITAE

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2. The Scholarship from the National Research Council of Thailand as of fiscal year 2019 (TSU 62 Ma. 01)

Awards

1. Recipient of 2nd place in the Poster Competition at International Conference on the 4th Industrial Revolution and its Impacts, March 28-30, 2019. Walailak University, Nakhonsithammarat, Thailand
2. Recipient of 2nd place in the Poster Competition at The 29th Thaksin University National Conference, May 9-10, 2019. Siam Oriental Hotel, Songkhla, Thailand.

List of Publication and Proceeding

Publications

1. **Patchimpet**, **J**., Sangkharak, K. and Klomklao, S. (2019). "Lipolytic activity of viscera extract from three freshwater fish species in Phatthalung, Thailand: Comparative studies and potential use as dishwashing detergent additive," Biocatalysis and Agricultural Biotechnology. 19, 101143.
2. **Patchimpet**, **J**., Sangkharak, K. and Klomklao, S. (2019). "Thermo separating aqueous two-phase system for lipase recovery and partitioning from Nile tilapia viscera: Biochemical properties and effect of ultrasound," Biochemical Engineering Journal. Submitted.
3. **Patchimpet**, **J**., Sangkharak, K. and Klomklao, S. (2019). "Optimization of process variables for the production of biodiesel by transesterification of used cooking oil using lipase from Nile tilapia viscera" Renewable Energy. Submitted.
4. **Patchimpet**, **J**., Sangkharak, K. and Klomklao, S. (2019). Ultrasonic enhancement of lipase-catalyzed transesterification for biodiesel production from used cooking oil," Fuel Process Technology. Submitted.

Proceedings

1. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2019). "Biochemical properties of partially purified lipase from Nile tilapia viscera," International Conference on the 4th Industrial Revolution and its Impacts, March 28-30, 2019. Walailak University, Nakhonsritammarat, Thailand.
2. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2019). "Enhancing the activity of partially purified lipase from Nile tilapia viscera using ultrasonic irradiation,". The 29th Thaksin University National Conference, May 9-10, 2019. Siam Oriental Hotel, Songkhla, Thailand.

3. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2019). "Process optimization of biodiesel production from used cooking oil using lipase from Nile tilapia viscera," The 21th Food Innovation ASIA Conference 2019, June 13-15, 2019. BITEC, Bangkok, Thailand.
4. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2019). "A Novel Lipase from Freshwater Fish Viscera for Use in Dishwashing Detergents," The 21th Food Innovation ASIA Conference 2019, June 13-15, 2019. BITEC, Bangkok, Thailand.
5. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2018). "Lipolytic activity of viscera extract from three fish species commonly used for processing in Thale Noi, Phatthalung province," The 20th Food Innovation ASIA Conference 2018, June14-16, 2018. BITEC, Bangkok, Thailand.
6. **Patchimpet, J.**, Sangkharak, K. and Klomklao, S. (2018). "Effect of extraction media on the recovery of lipase from viscera of Nile tilapia (*Oreochromis niloticus*)," The 20th Food Innovation ASIA Conference 2018, June 14-16, 2018. BITEC, Bangkok, Thailand.
7. **Patchimpet, J.**, Benabdulrasid, K., Jittreenop, R., Sarrathip S., Midumad, A., Chainapong, T. and Yooyen, T. (2017). "Identification of momogenea from some species of fishes from Thale Noi, Phatthalung Province," The 27th Thaksin University National Conference, May 3-6, 2017. BP Samila Beach Hotel, Songkhla, Thailand.
8. Sarrathip S., **Patchimpet, J.**, Benabdulrasid, K., Jittreenop, R., Midumad, A., Chainapong, T. and Yooyen, T. (2017). "Identification of acanthocephala from some species of fishes from Thale Noi, Phatthalung Province," The 27th Thaksin University National Conference, May 3-6, 2017. BP Samila Beach Hotel, Songkhla, Thailand.

9. Midumad, A., **Patchimpet, J.**, Benabdulrasid, K., Jittreenop, R., Sarrathip S., Chainapong, T. and Yooyen, T. (2017). "Identification of digenea from some species of fishes from Thale Noi, Phatthalung Province," The 27th Thaksin University National Conference, May 3-6, 2017. BP Samila Beach Hotel, Songkhla, Thailand.