Evaluation of a Novel Halophilic Lipase Secretion by *Marinobacter litoralis* SW-45 for Butyl Esters Synthesis from Palm-based Oils

by

Haliru Musa
(1441111384)

A thesis submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy

School of Bioprocess Engineering
UNIVERSITI MALAYSIA PERLIS

2018

ACKNOWLEDGMENT

All praise and adoration is due to Almighty Allah, the most gracious, the most merciful, whose infinite and boundless mercy have seen me through my PhD programme. I would like to start my acknowledgement by appreciating my supervisor, Dr. Farizul Hafiz Kasim for his unflinching moral and financial support, constructive criticism and his academic expertise which aided the successful completion of my PhD research programme. I am equally indebted to my former co-supervisor, late Assoc. Prof. Dachyar Arbain (May his gentle soul rest in Jannat Firdausi) for his professional guidance, and whose immense knowledge and wealth of experience moulded me into the young researcher I am today. Even though he was only around for a short period of time, his down-to-heart nature and fervent professional advice is duly appreciated. I can't but also acknowledge the input of my current co-supervisor, Dr. Ahmad Anas Bin Nagoor Gunny, who incidentally was Assoc. Prof. Dachyar's former PhD student, and who eventually took over the reign from my late co-supervisor. Your academic impact and succour was highly appreciated.

At this juncture, I would also like to appreciate the constant effort and mentorship of Assoc. Prof. Subash Bose Chandra, whose huge experience in paper publication ensured that I got all my papers published in reputable and high impact journals. I am indeed grateful for your constant support. My indebtedness also goes to all the lab technicians that ensured I had the needed research chemicals, consumables, and technical support during my laboratory work. I also will not fail to appreciate the support of my colleagues in the post graduate room. May Almighty Allah bless all of you in abundance.

Finally, my sincere and unending appreciation goes to my dad, Alhaji Pharm. Musa Dangana Sa'adu, my loving mum, Hajiya Gogo Safiya Musa, and my step-mum, Hajiya Fatima Musa for their unflinching love and support. To all my siblings, who I can't mention all by name, I acknowledge your loving support. I dedicate this piece to all of you. It is arduous to enlist the names of all those that positively influenced and inspired me in the pursuit of my doctorate degree. But Allah, the all-knowing will reward you in manifolds.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CCD	Central Composite Design
CDS	Coding sequence
CPKO	Crude Palm Kernel Oil
CPO	Crude palm Oil
CTAB	Cetyl trimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FAEs	Fatty Acid Esters
FCCCD	Face-Centered Central Composite Design
FFA	Free Fatty Acids
FID	Flame Ionization Detector
GC	Gas Chromatography
IS	Internal Standard
MLL	*Marinobacter litoralis* lipase
NCBI	National Centre for Biotechnology Information
OD	Optical Density
OFAT	One-Factor-At-A-Time
PB	Plackett-Burman
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethyl sulfonyl fluoride
P-NPL	Para nitrophenyl laurate
P-NPP	Para nitrophenyl palmitate
REA	Relative Enzyme Activity
RNA	Ribonucleic acid
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate
SMF	Submerged Fermentation
SSF	Solid State Fermentation
TG	Triacylglycerol
TLL	*Thermomyces lanuginosus* lipase
WGS	Whole Genome sequence

Penilaian Rembesan Lipase Halofilik Baru oleh Marinobacter litoralis SW-45 untuk Sintesis Butil Ester daripada Minyak Berasaskan Sawit

ABSTRAK

Ester asid lemak pada masa sekarang dihasilkan dengan esterifikasi melalui teknologi kimia, melibatkan penggunaan asid mineral yang toksik sebagai pemangkin, dan memerlukan penggunaan tenaga yang tinggi. Teknologi enzim memberi alternatif proses yang lebih mesra alam melibatkan penggunaan mikrobial lipase sebagai pemangkin, dan mempunyai kelebihan daripada proses kimia yang konvensional dari segi kondisi proses yang sederhana, proses yang ringkas dan kualiti produk. Kecenderungan terkini yang menerima pakai pendekatan yang lebih hijau terhadap penghasilan bahan oleokimia bernilai tinggi telah mendorong keperluan untuk meneroka dan menambah baik secara konsisten ekstremozim dengan ciri-ciri novel sebagai biopemangkin yang stabil untuk digunakan dalam proses bioteknologi. Oleh itu, dalam kajian ini, sejumlah 56 strain bakteria halofilik dan 9 strain kulat halofilik diasingkan dari pelbagai persekitaran masin, dan disaring untuk perembesan lipase pada agar tributirin. Strain bakteria halofilik SW-45 yang merembeskan lipase tertinggi (0.603 U/mL) bagi SMF dalam keadaan teraduk dipilih untuk kajian pengoptimuman selanjutnya. Strain yang merembeskan lipase tertinggi ini disumberluarkan bagi tujuan pengenalpastian molekul, dan dinamakan *Marinobacter litoralis* SW-45. Gabungan kaedah bukan statistik satu-faktor-pada-satu-masa (OFAT) serta pengoptimuman statistik Plackett-Burman (PB) dan Reka Bentuk Komposit Berpusat Muka (FCCCD) digunakan untuk menambah baik penghasilan lipase halofilik oleh strain tersebut. Penghasilan tahap optimum lipase halofilik diperoleh dengan menggunakan 3.0 g/L maltosa, 1% (v/v) minyak zaitun, 30 °C suhu pertumbuhan dan 4% isi padu inokulum (v/v), dan pengoptimuman oleh FCCCD menunjukkan penambahbaikan sebanyak 1.7 kali ganda bagi penghasilan lipase halofilik daripada 0.603 U/mL kepada 1.0307 U/mL. Kajian selanjutnya menunjukkan pencirian biokimia, serta kajian keserasian dan kestabilan bagi lipase ekstrasel yang dioptimumkan dalam pelarut organik, dan ia menunjukkan enzim bersifat termostabil dan mempunyai keserasian yang tinggi dengan pelarut. Seterusnya, lipase *Marinobacter litoralis* SW-45 (MLL) telah berjaya digunakan untuk sintesis butil ester daripada minyak sawit mentah (CPO) dan minyak isirung sawit mentah (CPKO) masing-masing dalam sistem heptana dan sistem bebas pelarut menggunakan proses hidropengesteran. Proses hidropengesteran telah dijalankan melibatkan hidrolisis enzim permulaan [*Thermomyces lanuginosus* lipase (TLL)] CPO dan CPKO kepada asid lemak bebas (FFA) diikuti oleh pengesteran bermangkin-mikrob bagi FFA tulen dengan butanol (penerima asil) masing-masing dalam sistem berpelarut dan sistem bebas pelarut untuk mensistesis butil ester. Di bawah keadaan pengesteran optimum 40 °C dan 45 °C; 150 rpm dan 230 rpm; 50% (v/v) kepekatan biopemangkin; 1:1 dan 5:1 butanol: FFA, 9% dan 15% (w/v) NaCl; dan 60 min dan 15 min masa tindak balas bagi sistem pengesteran FFA perolehan-CPO dan -CPKO, penukaran ester maksimum yang diperoleh ialah masing-masing 62.2% dan 69.1%. Analisis kromatografi gas mengesahkan butil ester yang terbentuk adalah butil heksanoat, butil laurat dan butil stearat. Kesimpulannya, keputusan yang diperoleh daripada kajian ini menunjukkan lipase halofilik mempunyai potensi yang cerah untuk

digunakan bagi biosintesis butil ester yang mempunyai kepentingan dalam industri daripada stok suapan CPO dan CPKO, dan ia boleh menjadi alternatif menarik bagi industri oleokimia.

Evaluation of a Novel Halophilic Lipase Secretion by Marinobacter litoralis SW-45 for Butyl Esters Synthesis from Palm-based Oils

ABSTRACT

Fatty acid esters are currently synthesized by esterification via chemical technologies involving the use of toxic mineral acids as catalysts, and requiring high energy consumption. The alternative enzyme technology which is a greener process involves the use of microbial lipase as catalysts, and holds enormous advantages of moderate reaction conditions, process simplification, and product quality over the conventional chemical process. The current trend of adopting a greener approach towards the synthesis of high-value oleochemicals has prompted the need to consistently explore and improve extremozymes with novel qualities as a stable biocatalyst to function in biotechnological processes. Hence, in this study, a total of 56 halophilic bacteria and 9 halophilic fungal strains were isolated from various saline environments and screened for lipase secretion on tributyrin solid agar. The halophilic bacterial strain SW-45 which secreted the highest lipase (0.603 U/mL) in SMF under agitation condition was chosen for further optimization studies. This highest lipase-secreting strain was outsourced for molecular identification and was designated as *Marinobacter litoralis* SW-45. A combinatorial non-statistical one-factor-at-a-time (OFAT) method and statistical optimization of Plackett-Burman (PB) and face-centered central composite design (FCCCD) approaches were adopted to improve halophilic lipase production by the strain. The optimum level halophilic lipase production was obtained with 3.0 g/L maltose, 1% (v/v) olive oil, 30 °C growth temperature, and 4% inoculum volume (v/v), and the optimization by FCCCD revealed 1.7-fold improvement in the halophilic lipase production from 0.603 U/mL to 1.0307 U/mL. The study further demonstrated biochemical characterization, as well as compatibility and stability studies of the optimized extracellular lipase in organic solvents, which revealed that the enzyme is thermostable and highly solvent compatible. Subsequently, *Marinobacter litoralis* SW-45 lipase (MLL) was successfully utilized for butyl esters synthesis from crude palm oil (CPO) and crude palm kernel oil (CPKO) in heptane and solvent-free systems, respectively using hydroesterification process. The hydroesterification process performed involved initial enzymatic [*Thermomyces lanuginosus* lipase (TLL)] hydrolysis of CPO and CPKO to free fatty acids (FFAs), followed by microbial-catalytic (MLL) esterification of the purified FFAs with butanol (acyl acceptor) in solvent and solvent-free systems respectively to synthesize butyl esters. Under optimal esterification conditions of 40 °C and 45 °C, 150 rpm and 230 rpm, 50% (v/v) biocatalyst concentration, 1:1 and 5:1 butanol: FFA, 9% and 15% (w/v) NaCl, and 60 min and 15 min reaction time for CPO- and CPKO-derived FFA esterification system, the maximum ester conversion of 62.2% and 69.1% was attained, respectively. Gas chromatography analysis confirmed that the butyl esters formed are butyl hexanoate, butyl laurate, and butyl stearate. In conclusion, the results obtained in the current study show that the halophilic lipase has promising potential to be used for biosynthesis of butyl esters of industrial importance from CPO and CPKO feedstock, which could be an attractive alternative for the oleochemical industry.

CHAPTER 1

INTRODUCTION

1.1 Background Information

Fatty acid alkyl esters are derivatives of free fatty acids (FFA) which are obtained either by reaction of FFA with short- or long-chain alcohols (through esterification process) or by transesterification of triglycerides with alcohols (M. Rani & Prakash, 2010). Fatty acid esters such as butyl esters are important compounds used as emulsifiers, emollients (butyl oleate, myristyl myristate, oleyl erucate, cetyl ricinoleate), detergents (butyl stearate), flavour and fragrance (butyl caprylate, butyl valerate) and thickeners in food, pharmaceuticals, detergents, biodiesel and cosmetic industry (Abdelmoez & Mustafa, 2014; Kiss, 2014).

Right from the time immemorial, esters were synthesised from oils and fats through micro-emulsification, thermal cracking (pyrolysis), blending and transesterification. However, transesterification or esterification (as the case may be) is now the preferred method for industrial production of fatty esters (Borges & Díaz, 2012). Transesterification involves the reaction between triacylglycerols (TAG) in oil and fat with an alcohol leading to formation of fatty alkyl esters and glycerol as a by-product (Chouhan & Sarma, 2011). While esterification involves the reaction of FFAs from oil and fat with alcohol to form fatty esters and water as by-product (Kiss, 2014). Whichever case, both esterification and transesterification require the presence of a catalyst to accelerate the rate of reaction and to increase the reaction yield. Generally, the catalysts used for fatty esters production can be grouped into three namely; acid catalyst, alkaline catalyst and enzyme. Acid and alkaline catalyst is further sub-divided into homogenous and heterogeneous catalysts (Gorji & Ghanei, 2014). A homogeneous catalyst is a catalyst that functions in a reaction system in which both the reactants and the catalyst are in the same liquid phase, while heterogeneous catalyst functions (mostly as solid) by interacting with the reactants in a different (either gas or liquid) phase (Dicks & Hent, 2014).

Homogeneous acid-catalyzed esterification involving sulphuric acid was the first approach used for fatty ethyl ester (biodiesel) production from crude palm oil feedstock using ethanol as acyl acceptor (Gebremariam & Marchetti, 2017). This approach entails the reaction of a TAG from oil with alcohol as an acyl acceptor and using acid (usually sulphuric acid or hydrochloric acid) as catalyst to catalyse the formation of esters and by-product, glycerol. Acid-catalysed esterification/transesterification approach is most suitable for waste oils because of its insensitiveness to high FFA content, as well as the ability to catalyse both transesterification and esterification reaction at the same time. Despite these advantages, its major limitations include; susceptibility to low water quantity, corrosiveness, product recovery setbacks (Saifuddin, Samiuddin, & Kumaran, 2015), and that the reaction proceeds at a much slower rate (Talha & Sulaiman, 2016). On the other hand, alkali- or base-catalysed reaction (transesterification) proceeds at a faster rate than the acid-catalysed reaction (Gebremariam & Marchetti, 2017), high ester conversion rate, low or no water formation (Talha & Sulaiman, 2016) and the base catalyst are not as corrosive as the acid catalyst counterpart. Hence, homogeneous base catalyst is commonly and preferably adopted for industrial production of esters (Talha & Sulaiman, 2016). The base catalysts commonly used for transesterification include; alkali, alkaline hydroxides (NaOH and KOH), potassium and sodium carbonates and alkaline metal alkoxides (sodium and potassium methoxide and ethoxide) (Gebremariam & Marchetti, 2017; Jamil et al., 2018). Alkali-catalysed transesterification is faced with certain drawbacks such as undesirable saponification reaction of oil due to high FFA content, thus leading to reduction in ester yield as well as difficulty in product separation and recovery, and high energy requirement (Gebremariam & Marchetti, 2017; Parawira, 2010). In general, the use of homogeneous catalysts require product washing, thus, usually produce large amount of wastewater which needs treatment to alienate environmental impact, product purification and recovery of catalyst is also considered problematic (Saifuddin et al., 2015). All these constraints makes homogeneous catalysis unfavourable and in turn contribute to high cost of ester production (Jamil et al., 2018).

In furtherance, heterogeneous catalysis was later adopted as a potential alternative to alleviate and overcome the bottle-necks encountered in homogeneous-catalysed ester production. The use of heterogeneous (solid-based) catalyst for ester synthesis no doubt significantly reduced the cost of production because of the improvement over the drawbacks encountered in the homogeneous-catalysed esterification. For instance, utilization of heterogeneous acid-catalysed esterification for fatty acid ester production allowed continuous use of catalyst (Talha & Sulaiman, 2016), enabled simultaneous transesterification and esterification (Said, Ani, & Said, 2015), ease of product recovery procedure, and elimination of product (fatty acid esters) washing (Saifuddin et al., 2015). In addition to these advantages, heterogeneous base-catalytic transesterification reaction usually requires less operation unit number and ability of the reaction to proceed in moderate condition (Talha & Sulaiman, 2016). Nonetheless, heterogeneous base-catalytic transesterification still encounter sensitiveness to feedstock FFA and water content conundrum (Narasimharao, Lee, & Wilson, 2007), while heterogeneous acid-catalysis still requires high reaction temperature, complex catalyst synthesis process, and energy intensive process, thus leading to high production cost (Said et al., 2015).

Recently, there has been a shift in paradigm from traditional chemical process towards enzymatic approach for synthesis of esters due to the drawbacks (such as high energy requirements and environmental problems) posed by conventional chemical approach. Moreover, the shift is continuously attributed to several attractive advantages of enzymatic processes over the traditional chemical reactions which include biodegradability, derivatization from renewable resources, the capability to function under relatively mild temperature conditions, reduced secondary reactions, more efficient synthesis of single stereoisomers, high product (ester) purity, and low energy demand (Jemli, Ayadi-Zouari, Hlima, & Bejar, 2016). On the other hand, as mentioned earlier, chemically-catalysed reactions (esterification) are naturally non-selective, and operates at relatively high temperatures, thereby leading to undesired side reactions which causes product (esters) darkening and odour formation (Dhake, Thakare, & Bhanage, 2013; Hills, 2003). These unwanted by-products formed need to be removed via expensive and extensive post-purification processes in order to improve the quality of the esters (Hills, 2003). Although, enzymatic approach for ester synthesis has shown promising industrial potential to replace the traditional chemical method, the enzymatic approach is faced with certain limitations such as high cost of enzyme, long reaction time, and lower product yield due to lipase deactivation (Talha & Sulaiman, 2016). Lipase deactivation/inactivation and instability encountered in industrial processes is attributed to harsh reaction/reactor conditions of high temperature, deactivating impurities, physical forces, and high alcohol and solvent content (Saifuddin et al., 2015). Even though there are presently large quantity of commercial lipases available, large scale applications of lipase is hampered due to their relatively lower stability in harsh reaction conditions (Boehmwald, Muñoz, Flores, & Blamey, 2016).

Most biotechnological processes often function under harsh conditions such as extreme pH, elevated temperature and pressure, non-aqueous media, and oxidative conditions that normally inactivate mesophilic microbial enzymes (Jemli et al., 2016).

Therefore, enzymes from extremophiles have been proposed as suitable candidates to effectively function in the aforementioned harsh reaction conditions (Veit, 2004). An approach based on discovering novel enzymes with unknown or having unique structural features is highly important in developing new biotechnological products and facilitates the resolution of reactions that are not responsive to chemical synthesis with non-extremophilic enzymes (Ferrer, Golyshina, Beloqui, & Golyshin, 2007). Extreme environments are anticipated to produce a differential microbial diversity with unfamiliar cellular gene products harbouring captivating potentials and novel biocatalytic actions (Ferrer et al., 2007). Halophilic enzymes are considered as ideal alternative to catalyze reactions in harsh industrial conditions because of their polyextremophilic nature, that is, the capacity to be active and stable in more than one extreme conditions such as high salt concentrations, tolerance to wide range of pH, thermal denaturation, and organic solvent tolerance (Delgado-García, Valdivia-Urdiales, Aguilar-González, Contreras-Esquivel, & Rodríguez-Herrera, 2012; Munawar & Engel, 2013). Sinha & Khare (2014) investigated the influence of organic solvents on activity and structure of halophilic protease by *Bacillus* sp. EMB9. Their study revealed that NaCl and KCl imparted resistance to loss of activity by organic solvents, thus confirming the protective role of the salts on organic solvent tolerance and stability of the enzyme. Halophilic (salt-tolerant) lipases are reportedly advantageous especially with regards to their thermal stability and organic solvent tolerance, in addition to their halophilic traits (Salihu & Alam, 2015). In light of the aforementioned, the compatibility and stability of lipase in organic solvents as reaction media, as well as thermal stability are important features that are paramount for operational survival of biocatalyst in harsh hydrolytic and synthetic (esterification) reactions. These operational features or parameters are considered inherent properties of halophilic (salt-tolerant) lipases.

1.2 Problem Statement

Currently, esters of fatty acids are synthesised by esterification reaction between carboxylic acids and alcohols using the conventional chemical methods that employ various chemical catalysts such as strong mineral acids, alkaline, and metal-based catalysts to catalyse the reaction at considerably high temperature and pressure. The involvement of these harsh reaction conditions causes polymerization of oils and fats, as well as other by-products thereby resulting into darkening of esters and formation of undesirable odours. The presence of darkened unwholesome products arising from these side reactions are highly undesirable especially in cosmetic applications where high purity of emollient esters are desired. Expensive and extensive post-reaction purification procedures e.g. decolourization, bleaching, neutralization and steam treatment is thus usually required to remove these by-products and catalyst residues to ensure product acceptability. On the other hand, lipase-catalysed production of esters is considered a greener and attractive alternative to problematic chemical methods because lipases exhibit elevated degree of specificity and enantioselectivity for reactions e.g. esterification. Lipases catalyse reactions in neutral and mild conditions which conserves energy, thereby resulting into cleaner products with improved odour and colour. Enzymatic synthesis of esters is an environmentally friendly process and extensive post-reaction purification procedures are usually avoided because of the efficient nature of lipases. However, even though lipase-catalysed esterification have been embarked upon for a while now, the inability of lipase to efficiently catalyse esterification reaction due to their thermal and organic solvent instability, has been one of the major limitations.

1.3 Justification

Due to the limitations of thermal and solvent instability encountered in lipase-catalysed esters production, there is a need to search for robust lipases endowed with combination of novel qualities such as improved activity, organic solvent tolerance and thermal stability that can withstand the harsh operational conditions of moderately high temperature and high organic solvent concentrations that are employed in esterification reaction. Halophilic lipases which are group of extremozymes functioning optimally in high saline condition serve as excellent example of enzyme with the natural potential to act in such extremely harsh operational conditions.

1.4 Research Objectives

The research objectives that need to be accomplished are outlined below:

General objective

- To evaluate the efficiency of halophilic lipase in catalysing esterification reaction of hydrolysed crude oil of palm and kernel fruit for enhanced synthesis of butyl esters.

Specific objectives

1. To optimize halophilic lipase production by the selected microbial strain.
2. To characterize the optimized halophilic lipase produced by the selected microbial strain.
3. To assess the compatibility of the halophilic lipase with various organic solvents as reaction medium for esterification.

4. To evaluate the applicability of the halophilic lipase as biocatalyst for bioconversion of free fatty acids to corresponding fatty acid esters (Butyl esters).

1.5 Scope of Research

The research focus on the following:

1) This study uses Malaysian crude palm oil (CPO) and crude palm kernel oil (CPKO) as feedstock, which was initially hydrolysed to free fatty acids (FFAs) and subsequent esterification using crude halophilic lipase.
2) Isolation, screening and identification of halophilic lipase-secreting microorganisms from various saline environments.
3) This work only deal with crude halophilic lipase and the application of the crude lipase in characterization, compatibility and esterification studies.
4) Commercial lipase of *Thermomyces lanuginosus* was used as biocatalyst for hydrolysis of CPO and CPKO to corresponding FFAs, while crude halophilic lipase of *Marinobacter litoralis* SW-45 catalysed esterification of the FFAs to corresponding esters.
5) This research is limited to esterification of hydrolysed FFAs by crude halophilic lipase and analysis of the synthesised esters was performed using only Gas Chromatographic (GC) technique.