PRODUCTION AND PARTIAL PURIFICATION OF LIPASE PRODUCED BY *Burkholderia cenocepacia* ST8 USING USED ENGINE OIL AS SUBSTRATE

By

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Thesis Submitted to the School of Graduate Studies,
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Chairman : Arbakariya Bin Ariff, PhD
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Pollution of environment from the impacts of improper disposal of used engine oil (UEO) that lead to the endangering of biota had become a global environmental issue nowadays. UEO contains aliphatic and aromatic hydrocarbon mixtures. The bioconversion of UEO into useful and cost-effective product (i.e., lipase) via microbial fermentation, can reduce the risk of releasing UEO into the environment seems to be very attractive strategy.

The aim of this study was to investigate the production, optimization, and partial purification of extracellular lipase by an indigenous *Burkholderia cenocepacia* ST8 in submerged fermentation using shake flask. The effect of lipid and the components of UEO employed for enhancement of lipase production were investigated. Statistical-based approaches were employed to optimize the fermentation medium based on UEO for the improvement of lipase production by *B. cenocepacia*. The crude lipase from *B. cenocepacia* was also partially purified by solvent/salt-based aqueous two-phase systems (ATPS) using UEO-based fermentation broth as feedstock.

Preliminary results showed that lipase production by *B. cenocepacia* using UEO was induced in the presence of Tween 80 in the culture. The role of UEO fractions (hydrocarbon content) in the presence of Tween 80 in relation to the enhancement of lipase production was also studied. On individual hydrocarbon system, aliphatic hydrocarbon was found to be more favorable as compared to aromatic hydrocarbon in the enhancement of lipase production. The use of the mixtures of aliphatic (C15 or C16) and aromatic hydrocarbon gave substantial increment in growth of *B. cenocepacia* and lipase production as compared to basal medium (BM). Lipase production could be improved by the addition of C40 or benzene, in BM containing

UEO. Response surface methodology (RSM) predicted the optimized media constituents to be 2.28% Tween 80 (v/v), 2.26% UEO (v/v), 0.79% nutrient broth (w/v) and 1.33% gum arabic (w/v), with actual observed lipase activity of 216 U/mL. The RSM-optimized system as well as ANN-GA optimized system revealed approximately 1.6-fold increment in lipase production as compared to non-optimized medium. Nutrient broth and Tween 80 were found to be the most important variables that greatly influenced the lipase activity. Partial purification of lipase was obtained with a purification-fold of 4.8 and R_T of 141% in a 1-propanol/sodium citrate ATPS system.

Enhancement of lipase production is associated with the types of hydrocarbons, individual and in combination as well as the presence of surfactant. The combined effects of aliphatic and aromatic hydrocarbons (UEO content) in the presence of Tween 80 can enhance lipase production. RSM and ANN-GA approaches are effective tools for predicting and optimizing fermentation medium for lipase production. Solvent/salt-based ATPS considered as economically feasible and potential downstream method to purify lipase with high enzyme recovery from UEO-based fermentation broth as feedstock.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENULENAN SEPARA LIPASE DARIPADA *Burkholderia cenocepacia* ST8 MENGGUNAKAN MINYAK ENJIN TERPAKAI SEBAGAI SUBSTRAT

Oleh

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Pencemaran alam sekitar akibat daripada pembuangan minyak enjin terpakai (UEO) yang tidak terancang boleh menyebabkan ancaman terhadap biota. Isu pencemaran alam sekitar di peringkat global juga ditintik-beratkan pada masa kini. UEO mengandungi campuran hidrokarbon alifatik dan aromatik. Dengan penukaran UEO kepada produk yang berguna dan kos efektif (iaitu, lipase) melalui penapaian mikrob, dapat mengurangkan risiko UEO dibebaskan ke persekitaran, merupakan strategi yang boleh dilaksanakan.

Tujuan kajian ini adalah untuk menyiasat penghasilan, pengoptimuman, dan penulenan separa lipase ekstraselular daripada *Burkholderia cenocepacia* ST8 dalam fermentasi terendam menggunakan kelalang kon. Kesan lipid dan komponen UEO yang digunakan untuk meningkatkan penghasilan lipase telah diselidik. Pendekatan statistik digunakan untuk menentukan komposisi media berasaskan UEO yang memberikan optimal produksi lipase oleh *B. cenocepacia*. Enzim mentah dari *B. cenocepacia* telah ditulenkan secara separa melalui sistem dua fasa berair (ATPS), pelarut organik hidrofilik / garam dengan menggunakan media fermentasi berasaskan UEO.

Keputusan awal menunjukkan bahawa penghasilan lipase dari *B. cenocepacia* menggunakan UEO dapat dipertingkatkan dengan kehadiran Tween 80 dalam medium kultur. Pecahan UEO (kandungan hidrokarbon) disertai dengan Tween 80 boleh dikaitan dengan peningkatan penghasilan lipase. Pada sistem hidrokarbon individu, hidrokarbon alifatik didapati lebih baik dibandingkan dengan hidrokarbon aromatik dalam peningkatan penghasilan lipase. Penggunaan campuran hidrokarbon

alifatik (C15 atau C16) dan aromatik memberikan peningkatan yang tinggi untuk produksi lipase dari *B. cenocepacia* berbanding dengan medium basal (BM). Penghasilan lipase boleh dipertingkatkan dengan menambah C40 atau benzena dalam BM yang mengandungi UEO. Metodologi permukaan respons (RSM) memilih komposisi media, iaitu 2.28% Tween 80 (v / v), 2.26% UEO (v / v), 0.79% kaldu nutrien (w / v) dan 1.33% gum arabic (w / v)), dengan penghasilan lipase yang maksimum adalah sebanyak 216 U / mL. Sistem yang dioptimumkan melalui RSM dan sistem yang dioptimumkan melalui ANN-GA menunjukkan peningkatan produksi lipase sebanyak 1.6 kali ganda berbanding dengan medium yang tidak dioptimumkan. Kaldu nutrien dan Tween 80 adalah pemboleh ubah terpenting dalam mempengaruhi produksi lipase. Penulenan separa enzim lipase dari *B. cenocepacia* memberi lipatan penulenan sebanyak 4.8 kali dan hasil pemulihan (R_T) sebanyak 141% melalui sistem 1-propanol / natrium sitrat, ATPS.

Peningkatan aktiviti lipase adalah dikaitkan dengan jenis hidrokarbon, individu dan kombinasi serta kehadiran surfaktan. Kesan gabungan hidrokarbon alifatik dan aromatik (kandungan UEO) dengan kehadiran Tween 80 boleh meningkatkan penghasilan lipase. Kaedah RSM dan ANN-GA adalah berkesan untuk meramalkan dan mengoptimumkan medium penapaian bagi penghasilan lipase. ATPS dianggap boleh dilaksanakan dari segi ekonomi dan kaedah pemprosesan hiliran ini adalah berpotensi untuk menulenkan lipase dengan pemulihan enzim yang tinggi dari media fermentasi berasaskan UEO.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATPS	aqueous two-phase systems
BSA	bovine serum albumin
BRANN	Bayesian regularization
× *g*	force of gravity
h	hour
LMANN	Levenberg-Marquardt
MW	molecular weight
NaOH	sodium hydroxide
NaCl	sodium chloride
HCl	hydrochloride acid
PAHs	polyaromatic hydrocarbons
PEG	polyethylene glycol
p-NP	*p*-nitrophenol
sp.	species
TBA	tributyrin agar
TPH	total petroleum hydrocarbon
UEO	used engine oil
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Environmental pollution occurred due to the difficulty of reuse or disposal of used engine oil (UEO) (Ibrahim, 2016). The UEO investigated in this study (also known as used motor/lubricating/crankcase/spent oil) is obtained from the crankcase of internal combustion automotive engines in local automobile repair workshops. In general, the major UEO components consisted of aliphatic and aromatic hydrocarbons (Irwin et al., 1997). UEO also contains greater amount of heavy polycyclic aromatic hydrocarbons (PAHs) and metals than fresh oil (Vazquez-Duhalt, 1989). These constituents of UEO would lead to chronic (long term) health hazards including carcinogenicity and mutagenicity; and also require several years/decades for recovery in some ecosystems results in chronic problems to biota (Vazquez-Duhalt, 1989; Hewstone, 1994; Irwin et al., 1997; Bidoia, 2010; Al-Hawash et al., 2019). Large amounts of UEO is generated from automobile workshops, however, little of this amount is recycled, and disposed by dumpling or incineration, lead to adversity to the environment (Koma et al., 2001). Thus, there is an essential need for appropriate governing or recycling to avoid the hazardous threat of UEO towards the environment (Kalyani & Pandey, 2014).

Lipases (EC 3.1.1.3), serine hydrolyses that catalyze both synthesis and hydrolysis of esters from glycerol and free fatty acids at lipid-water interface. The global industrial enzymes market was predictable at a global value comprised between USD $5000 million and USD $5500 million in 2016 (Guerrand, 2017). The lipases ranked third among current commercialized enzymes after carbohydrases and proteases (Celligoi, 2017; Guerrand, 2017). Lipases are one of the important industrial biocatalysts. This is promoted by lipase's unique characteristics such as stability towards high temperature, pH and organic solvents, high substrate specificity, ability to react in aqueous and non-aqueous media, and also demonstrated regio-, chemo- and enantio-selective in reactions (Saxena et al., 1999; Sarmah et al., 2018). Thus, lipases have gained increased interest in various fields of industrial applications such as pharmaceuticals, biodiesel, food, flavor industry, detergent, agrochemicals, bioremediation, cosmetic, fine chemicals, derivatives of esters and amino acid, biosensor, and organic synthesis industry (Saxena et al., 1999; Houde et al., 2004; Hasan et al., 2006; Sarmah et al., 2018). Lipases are produced by plants, animals, and microorganisms. Microbial lipases are largely produced via fermentation by numerous bacteria, fungi and yeast such as *Pseudomonas*, *Bacillus*, *Burkholderia*, *Aspergillus*, *Candida*, and *Rhizopus* (Ghosh et al., 1996; Gupta et al., 2004). The production of bacterial lipases is mainly extracellular and produced by submerged fermentation (Gupta et al., 2004). *Burkholderia* lipases have earned much attention due to its unique characteristics such as thermal stability; solvent tolerant stability; enantio-selectivity and substrate specificity, making them suitable for industrial application (Dandavate et al., 2009; Wang et al., 2009a). *Burkholderia* strains are also able to degrade various hydrocarbons and its associated substituted (e.g., n-alkanes, toluene, phenanthrene, pyrene, naphthalene, pristane) under aerobic conditions (Yuste et al., 2000; Andreolli et al., 2011; Andreolli et al., 2013; Chen et al., 2013).

At present, only one report is available in the literature on lipase production using microbial degradation of UEO. This study revealed that the compounds like lipase, complex alcohol, and organic acids were produced by an isolated hydrocarbon-degrading bacterium GS-3 using UEO as carbon source; and optimization of lipase production was investigated, which the best lipase activity (0.097-0.221 U/mL) was achieved by nitrogen source of urea, addition of Tween 80, removal of $FeCl_3$, and under conditions of pH 7 and 30°C (Mahmood et al., 2017). A few other reports aimed at the removal of hydrocarbons by bioremediation of the polluted sites, in which, crude enzymes, including lipases (together with other enzymes, such as alkane hydroxylase, and esterase) were secreted during the microbial biodegradation of UEO (Balaji et al., 2014; Kadri et al., 2018a; Kadri et al., 2018b). For instance, Kadri et al. (2018b) reported the production of crude enzymes (lipases as well as alkane hydroxylase, esterase, which involved in bioremediation) by *Alcanivorax borkumensis* using petroleum hydrocarbons (i.e., hexane, hexadecane and motor oil). *A. borkumensis* showed excellent growth on motor oil with highest lipase production of 71 U/mL and motor oil degradation of 75% after 72 h. To the best of our knowledge, the possibility of using used engine oil (UEO) for the production of lipase by microorganism such as *Burkholderia* spp. in submerged fermentation has not been reported in the literature. UEO consists many types of alkane and their preferences to supports growth of *Burkholderia* spp. and lipase production have not yet identified. The use of UEO as fermentation substrate for production of valuable bioproduct, such as lipase, may not only reduce the cost of raw materials but also an approach to solve problem with the disposal of UEO to the environment. If UEO is disposed of inappropriately and released into the environment, they can harm humans, plants, animals, fish and shellfish.

According to the Freedonia group report, the demand of Malaysia for automotive engine oil is expected to grow at a compound annual rate of 2.9% between 2016 and 2021 to 202,000 metric tons. And the increasing demand growth is due to a spike in motor vehicle ownership, increased annual distanced traveled of various vehicles, and follow equipment manufacturing oil drain interval by consumers (Kellenberger, 2018). Malaysia's Department of Environment had licensed waste oil recovery facilities for properly managing used oil but incineration remains to be the most popular disposal method (Hassan et al., 2014; Kellenberger, 2018). And, UEO pollution problems remain unsolved due to lack of enforcement of the law (Hassan et al., 2014). Aja et al. (2016) reported that 133,261 metric tons of oil and hydrocarbons wastes (including UEO, waste hydraulic oil, waste mineral oil, oil tanker sludge, etc.) were generated in 2011. Thus, UEO which is rich in organic compounds can be used as a source of carbon and energy in microbial lipase production. *Burkholderia* spp. is known to produce lipase (Gupta et al., 2004), and well-studied for the degradation of aliphatic and aromatic petroleum hydrocarbons (UEO components) (Mohanty & Mukherji, 2008a; Mohanty & Mukherji, 2012; Revathy et al., 2015). It is hypothesized that *B. cenocepacia* ST8 may support good growth and lipase production simultaneously with microbial degradation of UEO as carbon source. Exploring and applying the UEO as low cost raw material for the effective production of lipase using *B. cenocepacia*, and partial purification of UEO-based fermentation crude feedstock is needed due to its environmentally-friendly and economically.

Besides, lipase production was largely influenced by numerous factors like medium composition (carbon and nitrogen sources) and cultural conditions (pH, temperature, agitation speeds, size and age of inoculum) (Gupta et al., 2004; Hasan et al., 2009). Optimization of the fermentative yield of lipase is a required and vital step in efficient use of fermentation technology. To overcome the limitations of a single factor optimization process (e.g., laborious, time consuming), empirical methods such as statistical-based approach (response surface methodology) and artificial intelligence-based approach can be used efficiently to guarantees the optimal conditions determination (Desai et al., 2008).

The development of efficient and economical downstream processing techniques is vital for the success of production of industrial enzymes. As these processes are applied to biological products, these purification steps must be mild enough to preserve the enzyme activity as well as take consideration of other some factors (i.e., selectivity, recovery, compatibility, and throughput) for the separation of enzymes. Conventional downstream processing methods (i.e., ammonium sulfate precipitation, column chromatography, etc.) have the limitations such as commonly multi-steps, time-consuming, rendering low yield, requiring pre-treatment of sample, and are relatively expensive for large scale production (Aguirre-Ezkauriatza et al., 2010; Gu et al., 2012; Iqbal et al., 2016). ATPS is generally a simple, mild and efficient method for the separation and purification of numerous biomolecules such as proteins, enzymes, nucleic acids, virus, bionanoparticles, antibodies, and cell organelles (Asenjo & Andrews, 2011; Raja et al., 2012). ATPS consists of two liquid phases that are immiscible above a critical concentration. Purification of target enzymes are relied on the differential partitioning of the target enzymes to one phase and the contaminates to the other phase (Asenjo et al., 1994). A protein interacts with their surrounding molecules via hydrogen, ionic, and hydrophobic interactions, and other weak forces within a phase (Asenjo & Andrews, 2011). ATPSs have advantages such as scale-up potential, continuous operation, ease of process integration, low toxicity of phase forming constituents, and biocompatibility of the processes (Asenjo & Andrews, 2011; Ventura et al., 2011). ATPS is also economically viable (cost-effective) with high enzyme recovery in minimum operation unit steps, for direct comparison with the chromatographic process (Aguilar et al., 2006).

In this study, the production of lipase by *Burkholderia* strain fermentation was efficiently carried out using UEO, which containing a complex mixture of predominantly hydrocarbons. Herein, the main problem encountered is the residues of UEO components in fermentation broth might be inference with ammonium sulfate precipitation, dialysis even with column chromatography for the purification and recovery of lipase. And conventional liquid-liquid extraction method consists of water-organic solvent two-phase system was unsuitable for lipase purification due its protein denaturation and loss of enzyme activities (Mazzola et al., 2008), even it was applicable for the separation of UEO from UEO-containing fermentation broth. Solvent/salt-based ATPS was chosen as an alternative technique for the recovery of lipase from UEO-based fermentation crude feedstock. Solvent/salt-based-ATPS has been applied successfully to recover the organic compounds like 1-3-propanediol, and 2,3-butanediol from microbial sources with high recovery yields (> 91%) (Jiang et al., 2009; Li et al., 2010b; Li et al., 2011), thus it is a possible method used for separating the residual UEO, hydrocarbons components which are also known as organic compounds. Meanwhile, solvent/salt-based ATPS has also been applied for the recovery of solvent-tolerant enzymes such as lipase

and serine protease, with high recovery (> 96%) and purity (purification-fold > 11) (Ooi et al., 2009; Amid et al., 2012). *B. cenocepacia S*T8 lipase can tolerate to alcohol/solvent which make them applicable for enzyme recovery in solvent/salt ATPS. Hence, solvent/salt-based ATPS could provide mild and biocompatible extraction environment to recovery and purify of *B. cenocepacia* ST8 lipase simultaneously with the separation of UEO. Other advantages of solvent/salt-based ATPS include inexpensive phase-forming constituents, easy reutilization and constituents recovery, rapid phase-separation, high extraction efficiency, high polarity between the phases, low viscosity and low toxicity to environment (Ooi et al., 2009; Souza et al., 2015b; Rito-Palomares & Benavides, 2017). The major disadvantage of solvent/salt ATPS is that many proteins are incompatible with organic-solvent phase which lead to inactivate/denature of the enzymes, although it has been applied for the recovery of some enzymes (Rito-Palomares & Benavides, 2017).

The current research focused on the production and purification of extracellular lipase from an indigenous *B. cenocepacia* ST8 strain. The objectives of this study were:

(i) to investigate the effect of lipid and the components of UEO used for enhancement of the lipase production by *B. cenocepacia* ST8;

(ii) to optimize the medium composition (UEO-based fermentation medium as carbon source) using RSM and ANN-GA approaches for the improvement of the lipase activity yield of fermentation by *B. cenocepacia* ST8; and

(iii) to investigate the possibility of using aqueous two-phase system (ATPS) as potential downstream method for the partial purification of lipase from the optimized UEO-based crude feedstock.

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