SCREENING AND CHARACTERISATION OF POTENTIAL LIPASE PRODUCING STRAINS FROM ARCTIC SAMPLES

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2018

SCREENING AND CHARACTERISATION OF POTENTIAL LIPASE PRODUCING STRAINS FROM ARCTIC SAMPLES

by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

January 2018

ACKNOWLEDGEMENT

In the name of Allah, Most Gracious Most Merciful. All praises to Him for allowing me to get here. Acknowledgement will always be my favourite part to write. I owe the biggest thank you to my parents and siblings, for when financial was critical and moral was down—they helped me through. Rafidah Rasol, Nazuha Roslan and my favourite aunty Nidyaletchmy—you guys know how much our friendship means to me and we saw ourselves at both our highest and lowest point. Rasyidah Asilah, my Rasyid—I thank you for always being there. Thank you for being my temporary ATM machine and super-efficient driver whenever we took a long drive out. Thank you to lab 406 members especially Alyaa and Siang Yu for everything. Not to forget, staff of School of Biological Sciences and the members of lab 405, lab 414 (especially Abang Faisal) and lab 409 for all the help they gave and the laughter we shared. My new friends from VCRU (Khadija, Fatinah, Atikah and the rest) and Kak Nas, thank you for the memories. I am saving the best for last. To my wonderful supervisor, Dr Rashidah Abdul Rahim, a huge thank you to you for all this time. Your beauty goes beyond your pastel shades wardrobe and your warm hugs. I know I am not easy to deal with, but you showed so much patience and thank you for sharing your knowledge and wisdom. I know at this point there is already too many thank you's but I do not think it will be enough to convey how grateful I am to have these people, from friends to teachers, the Indian aunty that made the most delicious fried chicken and curry, the aunty who helped to clear our lab and was always so warm, Kak Sabariah from the school's store, to every random people that directly or indirectly was there through this, from the bottom of my heart, thank you. This was not easy, but it was definitely worth it. Alhamdulillah.

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ABSTRAK

Mikroorganisma psikrotoleran wujud secara meluas di persekitaran semulajadi sejuk dari laut dalam ke kawasan kutub dan memiliki mekanisme sinergistik yang membenarkan mereka beradaptasi untuk hidup dalam sejuk. Memiliki enzim aktif sejuk yang telah berkembang ialah salah satu strategi asas dalam pengadaptasian. Antara pelbagai enzim sejuk ialah enzim lipase, sejenis enzim yang berkebolehan menghidrolisis trigliserol membebaskan asid lemak. Lipase mikrobial telah mendapat perhatian yang ketara untuk kegunaan yang meluas dalam industri. Walau bagaimanapun, penghasilan enzim secara pukal terus daripada mikrob asal tidak boleh dilaksanakan kerana kos penyelenggaran yang mahal lantas tidak mampu untuk memenuhi permintaan tinggi dari industri. Pembangunan bagi penghasilan lipase secara cekap masih diperlukan untuk biokatalisis lipase sampai ke potensi penuh. Kajian ini dijalankan untuk menyaring dan mengenalpasti strain psikrotoleran penghasil lipase yang sesuai untuk pengekspresan di dalam sistem pengekspresan perumah heterolog sebelum pencirian protein rekombinan. Berbekalkan idea itu dalam minda, DNA enam mikroorganisma dilabel sebagai ARB 1A, ARB 1B, ARB 1C, ARB 10, ARC 8A dan ARC 8B telah diekstrak melalui kaedah CTAB yang diubahsuai dan digunakan untuk analisis penjujukan 16S rDNA. Pewarnaan Gram mendedahkan lima daripada enam *isolates* sebagai Gram negative. Mereka dikenalpasti sebagai bakteria tergolong dalam empat genera iaitu *Pseudomonas* sp., *Arthrobacter* sp., *Janthinobacterium* sp., dan *Acinetobacter* sp., dan telah disaring untuk aktiviti lipase. Aktiviti lipase dikesan

menggunakan asai sensitif dan khusus menggunakan minyak zaitun sebagai substrat. ARB 1B (*Arthrobacter* sp.), ARB 10 (*Pseudomonas* sp.) dan ARC 8B (*Janthinobacterium* sp.) telah dikesan positif lipase. ARB 10, memiliki 97% persamaan dengan *Pseudomonas* sp., telah dipilih untuk pengklonan. Gen lipase diamplifikasi melalui kaedah tindak balas rantai polimerase menggunakan set primer spesifik direka untuk menyasarkan gen diingini. Gen teramplifikasi telah diklonkan ke dalam vektor pGEM®-T Easy menggunakan *E. coli* JM109 sebagai sel perumah dan didapati memiliki 92% persamaan dengan gen lipase daripada *Pseudomonas fluorescens* melalui penjujukan. Gen lipase itu, ARB_*lip10*, telah disub-klon ke dalam sistem pengekspresan pCold^{TM} I dengan *E. coli* BL21(DE3) sebagai sel perumah dan dianalisis melalui SDS-PAGE. Lipase rekombinan yang diekpres dipulihkan di dalam pecahan tidak larut dengan anggaran berat molekukar 55.34 kDa. Jujukan pentapeptida terpelihara, GHSLG, telah ditemui dan triad pemangkin dibentuk oleh Ser^{207}, Asp^{255} dan His^{333} telah dikenalpasti. Pengekspresan dengan plasmid caperon, pTf16 telah separa berjaya memperoleh lipase rekombinan dalam pecahan larut. Asai lipase melalui kaedah kolorimetrik mengesan aktiviti tertinggi 1867.80 U/mL dalam pecahan tidak larut oleh sistem pengekspresan pCold. Aktiviti tertinggi direkodkan oleh pengekspresan protein dengan plasmid caperon ialah 748.46 U/mL. Memandangkan kerja penulinan tidak dioptimumkan dalam kajian ini, ia dicadangkan untuk kajian masa depan. Kajian ini telah membentangkan beberapa fahaman asas untuk ARB_lip10 daripada *Pseudomonas* sp. dimanipulasi dan diasah bagi memenuhi kehendak beberapa industri. Kajian bioinformatik boleh dilaksanakan untuk menyiasat ciri struktur lipase teragregat dalam badan inklusi dan memahami mekanisme aksi bagaimana dapatan tertinggi direkod dari pecahan tidak larut pula, kontra daripada kepercayaan meluas.

SCREENING AND CHARACTERISATION OF POTENTIAL LIPASE PRODUCING STRAINS FROM ARCTIC SAMPLES

ABSTRACT

Psychrotolerant microorganisms are widespread in natural cold environments ranging from deep sea to polar region and have a synergistic mechanism that allows them to adapt living in the cold. Possessing evolved cold active enzymes is one of the basic strategies of adaptation. Among the various cold enzymes is cold active microbial lipase, an enzyme that is able to hydrolyse triglycerols releasing fatty acids. Microbial lipases have gained notable attention for their widespread uses in industries. However, bulk production of enzymes direct from original microbe source is not feasible due to high maintenance cost thus will not be able to cater to high industrial demands. Developments for efficient lipase productions are still necessary for lipase biocatalysis to rise to its full potential. This study was conducted to screen and identify suitable psychrotolerant lipase producing strain to be expressed in a heterologous host expression system before characterising the recombinant protein. With that in mind, six microorganisms labelled as ARB 1A, ARB 1B, ARB 1C, ARB 10, ARC 8A and ARC 8B's DNAs were extracted via modified CTAB method and applied to 16S rDNA sequence analysis. Gram staining revealed five out of six isolates were Gram negative. They were identified as bacteria belonging to four genera *Pseudomonas* sp., *Arthrobacter* sp., *Acinetobacter* sp., and *Janthinobacterium* sp., and were screened for lipase activity. Lipase activity was detected using sensitive and specific plate assay using olive oil as substrate. ARB 1B, ARB 10 and ARC 8A were tested as lipase positive. ARB 10, having 97% similarity to *Pseudomonas* sp. was selected for cloning. Lipase

gene was amplified by PCR method using specific set of primers that was designed to target the gene of interest. The amplified gene was cloned into pGEM®-T Easy vector using *E. coli* JM109 as the host cells and was found to have 92% similarity to the lipase gene of *Pseudomonas fluorescens* by sequencing. The lipase gene, *lip10* was sub-cloned into pCold^{TM} I expression system with *E. coli* BL21(DE3) as the host cells and was analysed by SDS-PAGE. The expressed recombinant lipase was recovered in insoluble fraction with an estimated molecular weight of 55.34 kDa. The conserved pentapeptide sequences, GHSLG was found and the catalytic triad was identified; formed by Ser^{207}, Asp^{255} and His^{313}. Chaperone plasmid expression with pTf16 managed to partially recover the recombinant protein into the soluble fraction. Lipase assay via colorimetric method detected the highest activity to be 1867.80 U/mL in the insoluble fraction of pCold^{TM} I expression system. The highest activity recorded for chaperone co-expression was 748.46 U/mL from recombinant lipase recovered in the soluble fraction. Since purification work was not optimized in this study, it is recommended to do so for future studies. This study has laid some basic understandings for ARB_lip10 from *Pseudomonas* sp. to be further manipulated and polished to meet the requirements of several industries. Bioinformatics studies can be conducted to investigate further the structural features of lipase aggregated into inclusion bodies and to understand the mechanism of action on how the highest yield was recorded from the insoluble fractions instead, contrary to widespread beliefs.

CHAPTER 1

INTRODUCTION

Living in a permanently cold habitat would have been a constant struggle to anyone or anything. Microorganisms in general have shown wonders through their abilities to tackle extreme living issues and are termed extremophiles.

Obligate psychrophiles are defined by Morita (1975) as organisms that have optimal temperature for growth at 15°C or lower, with a maximum of 20°C and the minimal at 0°C or below. Facultative psychrophiles or also known as psychrotolerant organisms are defined as organisms having the ability to grow at temperature close to 0°C, able to grow well above 20°C with optimal temperature being 20°C or higher (Gounot, 1986). Cold adapted enzymes produced by psychrophiles are receiving notable attention specifically for their unique characteristics. One of the enzymes currently on the rise is lipase. Lipase which is scientifically known as triacylglycerol hydrolases (E.C.3.1.1.3) has the ability to catalyse the hydrolysis of long-chain acylglycerols in aqueous emulsions (Leonov, 2010), releasing glycerol and fatty acids.

Lipases gain notable attention worldwide due to its interesting characters and versatility. Multiple approaches towards its utilization with broad range of substrate and reaction specificity, and working well in the absence of a cofactor make lipases largest industrial use remain the hydrolysis of fats and oils (Leonov, 2010).

Enzymes global market value back in late 1960s was reported to be a few millions dollars, with 60% of them being produced in Europe (Sharma *et al.,* 2001). In 2015, industrial enzymes market was worth USD 8.18 billion (“Enzymes Market by

Type", 2016) and the numbers are positively expected to shoot up to USD 17.50 billion in 2024 ("Enzymes Market Size", 2016). One of the limiting factors in this market lies in the shortage of lipases that possess specific processing traits required for industries. The drawback in obtaining cold-active microbial lipases is that the organisms responsible for producing them are hard to maintain and sometimes even failed to be cultured under laboratory conditions. Large scale fermentation of psychrophilic wild strains requires bulky cost to sustain at low temperature (Ali *et al.,* 2013).

Recombinant DNA technology fills in the gap and assists in the massive production of desired enzymes via heterologous expression system. The ease in cultivation technology of mesophilic host will allow the production of enzymes cost efficiently. With enzymes market projected to grow even bigger in the coming years, enzymes with interesting characters that can meet with industrial demands are on the rise and research are worth to be conducted in hope many more novel genes could be isolated, cloned, expressed and studied. In this study, a psychrotolerant lipase gene was isolated from Arctic bacteria and identified before being cloned and expressed into a suitable expression system using *E. coli* BL21(DE3) as the host cells.

This study was conducted to meet the list of objectives as stated below;

1. Screening and identification of psychrotolerant lipase producing strains from Arctic soil and sediment samples.

2. Cloning, expression and characterisation of a psychrotolerant lipase from Arctic bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Biodiversity of Arctic

The northern region on the Earth consisting of ocean and land is permanently cold and is home to a vast array of biodiversity. The Arctic is famously known to be the home of the iconic Polar bears has an average winter temperature as low as -40°C.

The concept of biodiversity encompasses aspects of biology ranging from large-scale ecosystems down to the molecular level, when variability in genetics is used to characterize diversity within and among populations of species. The genetic component of biodiversity is often not distributed evenly across the geographic range of species and the major cause for such structure in Arctic is may be due to evolutionary history and processes related to geography, variable climate and strong ties seasonally available resources.

Arctic is host to many globally significant populations which include more than half of the world's shorebird species, 80% of the global goose populations, several million reindeer and caribou, and many unique mammals such as the infamous polar bear (Hohn & Jaakkola, 2010).

The organisms surviving within the sea ice are consequently small and mostly dominated by bacteria, unicellular plants and animals. A certain type of algae named diatoms, are considered as the most important primary producers inside the ice with more than 200 species available. In the past 100 years, the average Arctic temperatures have increased at almost twice the average global rate. Arctic warming, with its many and increasing impacts on flora, fauna and habitats, has triggered the panic button in

identifying and filling the knowledge gaps on various aspects of Arctic biodiversity and monitoring (Hohn & Jaakkola, 2010).

2.2 Psychrophiles

In 1902, Schmidt-Nielsen first proposed the term psychrophiles, referring to organisms he managed to grow at 0°C. Not long after, his works revealed that these organisms can also survive higher temperature and the term received objection from Muller in 1903 (Morita, 1975). Ingraham and Stoke (1959) tried to precisely re-define the term in which they stated psychrophiles are microorganisms able to grow rapidly enough at 0°C to become macroscopically visible, further sub-dividing them into strict (or obligate) and facultative psychrophiles.

Microorganism is knowingly characterized by its cardinal temperatures which are the maximum, optimum and minimum growth temperature (Gounot, 1986). Psychrophiles are generally termed as organism having growth temperature close to the freezing point of water, with an optimum temperature at 15°C. Obligate psychrophiles are distinctly characterized by its inability to tolerate temperature above 20°C, whilst facultative psychrophiles or psychrotolerant being the opposite, having manage to have fast growth rates above 20°C (Cavicchioli *et al.*, 2002).

Psychrotrophic microorganism are widespread in natural environments whilst psychrophilic microorganism are restricted to permanently cold habitats (Gounot, 1986). Irrespective to how the definitions have changed over decades, 'psychro' microorganisms equal to cold-adapted and they exhibit characteristics polar opposite to those of other thermal classes. Unlike its mesophilic counterparts, psychrophiles have

specific activity a magnitude higher (D'Amico *et al.*, 2003) and have catalytic efficiency over a temperature range of roughly 0-30°C with high thermosensitivity (Feller and Gerday, 1997).

How do psychrophiles combat extreme challenges to continue surviving is of major interest. The ability to grow at temperature close to the freezing point of water indicates that psychrophiles have successfully overcome two main challenges; low temperature because the rate of biochemical reactions will be exponentially affected and viscosity of aqueous environment which increases by a factor higher than two between 37°C and 0°C (D'Amico *et al.*, 2006). Fundamental cellular processes of metabolism, replication, transcription and translation are crucially adapted to withstand the cold, similarly for all other components of the cell from membranes and transport systems to intacellular solutes (Cavicchioli *et al.*, 2002).

2.3 Cold adaptation of psychrophiles

Adaptational properties of psychrophiles could be attributed to some major factors. Psychrophiles carry within them enzymes that work wonders even in extreme cold temperature. Cold enzymes tend to have a lower activation energy which leads to high catalytic efficiency. As temperature drops in psychrophiles, cold-shock proteins enable specific continued activities. This is well attributed to an enhanced flexibility of the protein structure (Deming, 2002).

For a psychrophile to survive, it has to be flexible. Psychrophiles have managed to overcome the barriers in surviving the cold by making fatty acid changes. Psychrophiles are known to possess higher proportions of polyunsaturated fatty acids compared to its mesophilic counterparts (Reddy *et al.*, 2009). At cold temperatures, the

polyunsaturated fatty acids increase which in turn reduce the melting point of membrane and greatly enhance its fluidity in cold-adapted *Rhodotorula glutinis* (He *et al.*, 2015). Eight psychrotrophic yeasts were able to grow in temperature ranging from 4 to 28°C. When the temperature dropped to 4°C, a decrease in monounsaturated fatty acids and an increase in polyunsaturated fatty acids simultaneously occurred, accounting for 20% of the total biomass (Řezanka *et al.,* 2016).

Some psychrophiles possess antifreeze proteins (AFP) or also recognised as ice-binding protein (Maayer *et al.,* 2014). Ice-binding proteins possess the ability to modify the crystal structure of an ice thus inhibiting its growth. Prior to freezing, AFPs lower the freezing point of water, a mechanism called thermal hysteresis, where the melting point of water remained unaltered. In frozen state, these proteins inhibit snow ice re-crystallization (Margesin and Miteva, 2011). Antifreeze proteins are also unique feature of fish from Antarctic and Arctic (Tutino *et al.,* 2009). The production of exopolysaccharide (EPS) is also a promising mechanism to avoid freezing of cells. High level of EPS was observed in psychrophiles under extreme cold conditions, where it decreases the freezing point of water and temperature for ice-nucleation (Maayer *et al.,* 2014).

Enzymes flexibility is contributed by synergistic changes in structural features. Modeling of 3-dimensional genome protein homology of *Colwellia psychrerythraea* suggests changes to proteome composition which may be directly involved in enhancement of enzyme adaptation at lowered temperature (Gianese *et al.,* 2001). The crystal structures of cold enzymes are similar with mesophilic and thermophilic homologues. They do not possess atypical conformations but rather increase their flexibility by undergoing structural modifications instead (Violot *et al.,* 2015). The

activity-stability-flexibility relationship is widely approved as the hypothesis of their adaptation to cold, raising understanding that the enzyme activity is attributed to its increased flexibility in counteracting the cold (Johns and Samero, 2004). Reduction in arginine/lysine residues is often less uniformly distributed in psychrophilic than in mesophilic enzymes. Intramolecular salt bridges are stabilized by a member of charged surface residues. This in return will promote an increase in solvent interaction (Maiangwa *et al*., 2015). Additional surface loops, substitution of proline residues by glycine in surface loops, decreased in inter-domain and subunit interactions and lesser aromatic interactions all together play a role in giving flexibility to the active site and adjoining regions (Cavicchioli *et al.,* 2002). Thermolability is directly related to flexibility thus explains the high thermolability of cold enzymes.

In their natural environments, psychrophiles are engaged in frequent freeze-thaw events and occasionally experience fluctuations in temperature. The evolution of a series of crucial adaptation mechanisms that involve reproduction, metabolic activity and protection strategies to name a few permits the growth of these microorganism at a temperature where others would most likely experience fatal freezing effect (Margesin & Miteva, 2011).

2.4 Lipases

Formally known as triacylglycerol hydrolases (E.C.3.1.1.3), lipase is an enzyme that catalyses the hydrolysis of fats and oils (Ji *et al.*, 2015). Ubiquitous in nature; plants and animals produce lipases as well. However, lipases from microorganism are the most biotechnologically employed due to the ease of cultivation technology which allows

rapid, mass productions. Genetic manipulation is somehow simpler in microorganisms compared to plants and animals.

Cold active lipases are particularly attractive with huge potentials to be manipulated in industrial applications. First, they possess the ability to catalyse numerous reactions. Second, they have high catalytic activity which makes them work efficiently at low temperature. They can be produced in high yield in microorganism via recombinant technology approach. Quite a number of structures have been solved which would allow rational design of enzymes in biomolecular engineering and function with co-factors. These features are especially useful in detergent formulations, food processing and fine chemistry catalysis (Jaeger & Eggert, 2002).

Cold-active lipases have high thermosensitivity. Whilst this is not a much desired trait to other temperature classes, cold lipases from psychrophiles are of major interest because the enzyme reactions can be inactivated by a slight increase in temperature offering economic benefits by eliminating the requirement of costly heating steps (Cavicchioli *et al.*, 2002).

Advancement in lipase based technology contributes to the fast-paced usage of lipases in various technical industries. Exploitation of lipase to enhance flavours, textures and shelf-life in food industries, the removal of lipid stains in detergent industries, the removal of different hydrophobic and lipid fractions in paper industries and environmental friendly synthesis of polyester make lipases even better biocatalyst with greater potentials.

2.5 Application of lipases

Cold enzymes are applied in several industries, ranging from food to environmental applications. A number of psychrophile proteins are already available in the market place. One example is Puratos, a Belgian origin company which sells cold adapted protein for bread baking (Zimmer, 2013). Table 2.1 shows some of the commercially available cold-active enzymes in several industries (Sarmiento *et al.,* 2015) and Table 2.2 shows some of the commercially available lipases manufactured by leading global enzyme suppliers (Guerrand, 2017).

The applications of cold lipases also offer economic benefits in reducing the production costs by saving energy through mild heat-inactivating step (Ali *et al*., 2013; De Santi *et al*., 2014). Due to cold enzymes high thermosensitivity, mild head treatment also helps in preserving the quality of product and conserving the nutritional values (Tutino *et al*., 2009; Ali *et al*., 2013) instead of using chemical extractions that will in turn have adverse effects on reaction yields and end product (Maiangwa *et al*., 2015). Possessing high specific activity also reduced the amount of enzymes required to initiate the reaction (Gerday *et al.,* 2000).

In brewing and wine industries for example, cold enzymes are highly desired than enzymes of mesophilic counterparts (Gerday *et al.,* 2000) to avoid undesirable side reactions that exist in higher temperature (Babu *et al.,* 2007). Cold lipases are beneficial in developing flavours due to their unique features (Tutino *et al.,* 2009). At lowered temperature, there is a significant reduced in risk of contamination and flavours could be retained. They can also be applied as flavor modifying in production of fermented food and cheese manufacturing (Groudieva *et al*., 2004).

Table 2.1: Examples of commercially available cold active enzymes adapted from Sarmiento *et al.*, (2015).

Market	Enzyme	Commercially available	Uses
Molecular Biology	Alkaline phosphatases	Antarctic phosphatase (New England Biolabs Inc.)	Dephosphorylation of 5′ end of a linearized fragment of DNA
	Uracil-DNA N-glycosylases (UNGs)	Uracil-DNA *N*-glycosylase (UNG) (ArcticZymes), Antarctic Thermolabile UDG (New England Biolabs Inc.)	Release of free uracil from uracil-containing DNA
	Nucleases	Cryonase (Takara-Clontech)	Digestion of all types of DNA and RNA
Detergent	Lipases	Lipoclean®, Lipex®, Lipolase® Ultra, Kannase, Liquanase®, Polarzyme®, (Novozymes)	Breaking down of lipid stains
	Proteases	Purafect® Prima, Properase®, Excellase (Genencor)	Breaking down of protein stains
	Amylases	Stainzyme® Plus (Novozymes), Preferenz™ S100 (DuPont), Purafect® OxAm (Genencor)	Breakdown starch-based stains
	Cellulases	Rocksoft™ Antarctic, Antarctic LTC (Dyadic), UTA-88 and UTA-90 (Hunan Youtell Biochemical), Retrocell Recop and Retrocell ZircoN (EpyGen Biotech), Celluzyme®, Celluclean® (Novozymes)	Wash of cotton fabrics
	Mannanases	Mannaway® (Novozymes), Effectenz™ (DuPont)	Degradation of mannan or gum
	Pectate lyases	XPect® (Novozymes)	Pectin-stain removal activity
Textile	Amylases	Optisize® COOL and Optisize NEXT (Genencor/DuPont)	Desizing of woven fabrics
	Cellulases	Primafast® GOLD HSL IndiAge® NeutraFlex, PrimaGreen® EcoLight 1 and PrimaGreen® EcoFade LT100 (Genencor/DuPont)	Bio-finishing combined with dyeing of cellulosic fabrics
Food and beverages	Pectinases	Novoshape® (Novozymes), Pectinase 62L (Biocatalysts), Lallzyme® (Lallemand)	Fermentation of beer and wine, breadmaking, and fruit juice processing
Other	Catalase	Catalase (CAT), (Swissaustral)	Textile, research, and cosmetic applications

Table 2.2: Commercially available lipases by leading global enzymes suppliers adapted from Guerrand (2017).

Enzyme producer	Product	Application
Novozymes	Lipase 435 Lipolase	Multipurpose lipases Detergents
DSM	Gumzyme Maxapal 42	Oil degumming Egg processing
AB Enzymes	Rohalase PL, Rohalase F, Veron Hyperbake	Baking
Amano	Lipase DF	Baking

One of the common uses of cold lipases in domestic application is in the detergent industry. Lipases are added into the detergent formulation and mainly used in household and industrial laundry to remove oil stains from fabrics at the temperature of tap water (Babu *et al.,* 2007; Tutino *et al.,* 2009). Roughly, around 1000 tons of lipase are incorporated in detergents for the hydrolysis of fats yearly (Bell *et al*., 2002). Cold enzymes can potentially reduce the environmental load of detergent products. Washing at lowered temperature reduces energy consumption which concurrently reduces the content of other less desirable chemicals (Hasan *et al.,* 2006). Cold washing also reduces the wear and tear of fabrics besides protecting the colour of textiles (Babu *et al.,* 2007; Ali *et al.,* 2013).

The use of microorganism to tackle environmental contamination issues appears to be a more feasible option to physicochemical methods. Bacterial lipases are actively involved in degrading organic pollutants in domestic sewage and anaerobic digesters (Gerday *et al.,* 2000; Hasan *et al.,* 2006). Introduction of psychrotropic microbial degraders of hydrocarbons, lipids and oils into the environment could curb environmental contamination in temperate regions (Babu *et al.,* 2007).

Lipases have promising roles in the organic synthesis of high-end short chain esters applied in pharmaceuticals for preparation of bulk drug substances (Maiangwa *et al*., 2015). With no requirement of cofactors and a wide range of substrate specificity, lipases own significant potentials in several industries including pharmaceutical (Bae *et al.,* 2014). Lipase from *Yarrowia lipolytica* has potential to act as biocatalyst in food, environment and pharmaceutical industries (Brígida *et al.,* 2014). Bell *et al.,*(2002) reported the usage of *Serratia marcescens* lipases on a large scale in producing drugs such as Diltiazem.

2.6 Cloning and lipase gene expression in heterologous host system

A variety in designs of expression vectors with distinct affinity tag sequences cater the fusion to almost any target protein in cloning and expression in a microbial host (Maiangwa *et al.,* 2015). *E. coli* expression system is undoubtedly the most popular one to date. Even since twelve years ago, according to the data from protein data bank (PDB), 80% at approximation of the proteins used to solve 3D structures were prepared in *E. coli* expression system while T7 based pET expression system is the most used in preparation of recombinant protein (Sørensen & Mortensen, 2005). Table 2.3 shows some of cold adapted recombinant lipases that have been previously studied (Maiangwa *et al.,* 2015).

The expression of recombinant proteins in cells in which the occurrence is not natural defined heterologous protein production (Fakruddin *et al.,* 2013). Recombinant LipA (r-LipA) from *Sorangium cellulosum* was expressed in *E. coli* BL21 (DE3) and its soluble form was purified using Ni-NTA affinity chromatography. Analysis of the purified protein using LC-ESI-MS/MS revealed peptides that matched with LipA's deduced amino acid sequences, successfully proving the purified protein was the r-LipA expressed heterogeneously (Cheng *et al.,* 2011). Xuezheng *et al.,* (2010) cloned and expressed two cold-active lipases from *Psychrobacter* sp. G isolated from the Antarctic via heterologous system. Clear haloes were formed on an LB tributyrin plate, indicating functional expression of lipolytic enzymes by the two lipases identified as *Lip-1452* and *Lip-948.*

Table 2.3: Some of cold adapted recombinant lipases that have been previously studied adapted from Maiangwa *et al.*, (2015).

Bacteria	Lipase		Cultivation temperature (°C)	MW/pH/temp optima (°C)
	Intracellular	Extracellular		
Acinetobacter baunmanii BD5	LipA		37	35 kDa/8.3/35
Acinetobacter sp.		No. 6	4	n.s./20
Aeromonas sp.		LPB 4	10	50 kDa/n.s./10
Pseudomonas sp. strain BII-I		lipP	37	33 kDa/8.0/45
Pseudomonas fluorescens KE38		KE38	25	43 kDa/8.0/20
Pseudomonas fragi IFO3456	rPFL		4-10	32.5 kDa/8.0/29
Pseudomonas fluorescens		Strain 38	4-10	155 kDa and 175 kDa/n.s
Pseudomonas sp. strain KB700A	KB-Lip		37	49 kDa/8.5/35
Pseudomonas sp. 7323	lipA		37	66 kDa/9.0/30
Psychrobacter sp. C18	LipX		20	35 kDa/8.0/30
Psychrobacter sp. Ant300	PsyEst		27	43 kDa/n.s./35
Psychrobacter pacificensis	Est10		22	24.6 kDa/7.5/25
Psychrobacter sp.	MBP		20	90 kDa/8.0/20
Psychrobacter sp. G	Lip-948		15	35 kDa/8.0/35
Psychrobacter sp. 7195	LipA1		30	5 kDa/9.0/30
Photobacterium sp. MA1-3	MA1-3		18	39 kDa/8.0/30
Photobacterium lipolyticum	M37		18	n.s./9.0/25
Pseudoalteromonas haloplanktis TAC125		Lip1	4 and 15	n.s./8.5/40
Stenotrophomonas maltophilia CGMCC 4254		SML	30	52 kDa/8.0/35
Streptomyces coelicolor A3(2)	EstC		16	35 kDa/8.5-9/35

As one of the most extensively used enzyme in industrial biocatalysis, a protein with 66% sequence identity to CalB of *Pseudozyma aphidis (*formally identified as *Candida antarctica)* designated *Ustilago maydis* lipase 2 (Uml2) was produced heterologously in *Pichia pastoris* (Buerth *et.al.,* 2014). Purification of extracellular Uml2 (Uml2-S125A) from supernatant of *P.pastoris* X-33 transformant cultures was conducted by IMAC using 1ml Ni-NTA agarose columns.

A complete sequence of lipase-coding gene fragment, *lip*E13 obtained by genome walking from Antartic seawater bacteria amplified by degenerate primers has closest identity to putative lipase of *Shewanella frigidimarina* NCIMB 400 (Parra *et.al.,* 2015). PCR product which encodes for mature lipase gene was cloned into pGEM®-T vector and subcloned into *E. coli* BL21(DE3)/ pET-22b(+) expression system. Purification of lipase from the periplasm was conducted according to the recommended manual for high-level expression and purification of 6x His-tagged proteins, using Ni-NTA agarose column.

In certain cases where the target protein becomes toxic to the host cells, mutant strains of BL21(DE3) called C41(DE3) and C43(DE3) are used to produce proteins. Over-production are seldom hard to acquire due to the toxicity of the target protein. These mutant host strains can grow to high saturation density, and production of proteins at an elevated level without side toxic effects can continue (Dumon-Seignovert *et al.,* 2004).

One of the major drawbacks in heterologous host expression system is the aggregation of expressed proteins into inclusion bodies. Inclusion bodies are usually unwanted and disregard as wasteproducts. De Pascale *et al.*,(2008) reported a problem of recombinant lipase aggregation as inclusion bodies when cloned into pET22b(+)

expression system. Expression via pMALc-2E vector was then conducted by cloning the lipase gene downstream the *malE* gene of *E. coli*. *malE* gene which encodes a protein for maltose-binding ensured a proper expression.

Denaturing agent such as 6M urea can be used to overcome inclusion bodies appearance in expression but in the case of lipases in subfamily I.1 and I.2, a chaperone protein knows as lipase specific foldase, *Lif* is usually required (Rashid *et al.*, 2001). Glogauer *et al.*,(2011) purified LipC12 using HiTrap Chelating HP affinity column which was concentrated by combining and dialyzing against Tris-HCl buffer. LipC12's protein sequence analysis revealed the absent of *Lif* despite belonging to lipase subfamily I.1. There also exist other strains from the same subfamily that lack the chaperone protein. Refolding by fractional dialysis can also enhance the purity and homogeneity of insoluble recombinant lipase (Maiangwa *et al.*, 2015).

2.7 Chaperone plasmid co-expression

The other alternative to recovering insoluble protein into the soluble fraction is by co-expressing the expression plasmid with a chaperone plasmid. By chaperone plasmid co-expression, the yield could be increased or decreased depending on the competency of chaperone plasmid with the expression plasmid. Defined as proteins that assist in the process of proper RNA folding to avert mis-folding or by fixing mis-folded species (Ermolenko & Makhatadze, 2002), this helper protein facilitates the folding of newly synthesized proteins and contributes to the system of quality check, altogether beneficial to refold aggregated, insoluble proteins (Shuo-Shuo *et al.*, 2011).

In the case of LIP-948 from *Psychrobacter* sp. G for example, the soluble protein formation showed varying degrees when co-expressed with five different kinds of chaperone plasmids pG-KJE8, pKJE7, pTf16, pG-Tf2 and pGro7 (Shuo-Shuo *et al.*, 2011). Several *Pseudomonas* lipases were also reported to require a chaperone protein for an orderly secretion and folding of the enzymes (Tutino *et al*., 2009).

Previous attempts to scale up expression to a commercially promising level of recombinant lipases from *Pseudomonas alcaligenes* were unachievable. The complicated process of folding and secretion of enzyme and the requirement of a specialised chaperone protein, LipB, limit the possibilities (Gerritse *et al.*, 1998).

Contrastly for Park *et al.*,(2009) the co-expression of an inactive lipase, BDLipA, from *Acinetobacter baumanni* BD5 with a lipase chaperone in refold procedures managed to obtain a highly active lipase. Another similar encounter was on the lipase gene Lip2Pc from *Psychrobacter cryohalolentis* $K5^T$ that formed inclusion body when expressed alone. After refolding with urea and β-mercaptoethanol and the co-expression of lipase specific foldase LifPc, the cold active lipase gene produced up to 6900 U/mg of specific activity (Novototskaya-Vlasova *et al.*, 2013).

2.8 Purification of recombinant protein

Receiving high attention worldwide, many recombinant lipases have been expressed, purified and characterized extensively. Methods to purify lipases mostly depended on nonspecific techniques such as precipitation, gel filtration, and ion exchange chromatography to name a few. Affinity chromatography is also widely performed to reduce individual purification steps required (Sharma *et al.*, 2001).

According to Babu *et al.*, (2008), lipases from Antarctic bacteria usually faced failure in obtaining purified form due to the existence of lipopolysaccharides associated with the enzyme. Common prepurification processes for example ammonium sulphate precipitation, extraction using organic solvent or ultrafiltration, are oftenly practiced to concentrate the protein.

Two steps of purification procedure were conducted to purify a recombinant lipase from *P. pastoris* X33. Concentration of culture supernatant was firstly done by ultrafiltration followed by elution with imidazole in POROS MC 20µM column with Nickel (Liu *et al.,* 2012). Dey *et al.*, (2014) partially purified the crude enzyme of an extracellular lipase from *Pseudomonas* sp. ADT by ammonium sulphate precipitation and extensive dialysis. Partial purification of lipase from crude enzyme using different salt concentrations (20, 40, 50 and 60%) were used in the work of Shafqat (2015).

Lipases from *Bacillus pumilus* RK31, *Pseudomonas* sp. and *Thermosynthropha* are well purified in 60% saturation. Crude supernatant of cold active lipase from *P. lynferdii* NRRL Y-7723 obtained from hexane washing to remove oil clots residual was applied to DEAE anion-exchange chromatography column. The lipase was indeed stable in washing steps and its activity was enhanced by 30% (Bae *et al.*, 2014).

Aqueous Two-Phase Extraction (ATPE) is one of the suitable methods for bio molecules purification. Combining neutral pH with high water content and mild conditions, this method is however insufficient to purify proteins to homogeneity. Chromatography is often applied to acquire a homogenous preparation of lipase (Sikdar *et al.*,1991; Gupta *et al.,* 1999; Srinivas *et al.*,2002).

The latest alternative to ATPE is the purification of lipase using ionic liquids and salts which induce salting out. CALB enzyme yield of 95.9% with purification fold of

2.6 was attained with 1-methyl-3-octylimidazolium chloride [C(8)mim]Cl, reported as the highest purification achieved (Nagarajan, 2012). Maldonado *et.al.*,(2015) partially purified lipase from *Geotrichum candidum* by conducting two different purification approaches using 70% (v/v) ethanol and 80% (w/v) ammonium sulphate respectively, preceeded by lyophylization which plays a role in maintaining the enzymatic activity.

The concentration of lipase by ethanol was relatively faster and simpler compared to ammonium sulphate precipitation procedure. Several chromatographic procedures are commonly practised to obtain lipases of higher purity. It is also widely accepted that there exist no perfect methodology in purifying lipase and the possibilities are endless.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains

3.1.1 Psychrotolerant bacteria

Psychrotolerant bacteria used in this study were isolated from Arctic soil and sediment samples collected by the Polar Research Group (Polar@USM) Penang. Sampling took place in August 2011, on the northern coast of Hornsund, Wedel Jarlsberg Land, West Spitsbergen (°00'04"N, 15°33'37"E) (Rasol *et.al.*, 2014). The isolates are marked as ARB 1A, ARB 1B, ARB 1C, ARB 10, ARC 8A and ARC 8B.

3.1.2 Host cell, plasmid and antibiotic

Table 3.1 shows the list of host cells, plasmids and antibiotics used in this study.

Table 3.1: List of host cells, plasmids and antibiotics used in this study.

		Description	**Source**
Host cell	*E. coli* JM109	Cloning host of pGEM-T Easy vector (endA1, recA1, gyrA96, hsdR17 ($^{r}K^{-m}K^{-}$), 6(lac-proAB), relA1, supE44, thi, F'[lacI^{q}lacZ6M15proAB traD36])	Promega (USA)
	E. coli BL21(DE3)	Cloning host for pCold I^{TM} (F-ompThsdSB(rB-rnB-) gal dcm (DE3))	Lucigen (USA)
Plasmid	pGEM®-T Easy	Cloning vector	Promega (USA)
	pCold I^{TM}	Expression vector (cspA promoter driven expression)	Takara Bio. Inc. (USA)
Antibiotic	Ampicillin	Screening for clones for pGEM®-T Easy and pCold I^{TM}	Fisher Scientifics (USA)
	Chloramphenicol	Screening for clones for chaperone plasmid, pTf16	Fisher Scientifics (USA)

3.2 Medium

3.2.1 Tributyrin agar

Tributyrin agar was prepared by dissolving 28.0 g of NA (HiMedia, India) in 1 L of distilled water. 0.5% (v/v) of tributyrin was added and the solution was homogenized by using a homogenizer. Tributyrin agar was autoclaved at 121°C for 15 minutes and let cool before used.

3.2.2 Triolein agar

Triolein agar was prepared by dissolving 28.0 g of NA (HiMedia, India) in 1 L of distilled water. 1% (v/v) of triolein was added and the solution was homogenized by using a homogenizer. Triolein agar was autoclaved 121°C for 15 minutes and let cool before used.

3.2.3 Rhodamine B agar

Rhodamine B agar was prepared by dissolving 28.0 g of NA (HiMedia, India) and 1% (v/v) olive oil (Natural olive oil, extra virgin) was added and was homogenized using a homogenizer. After the agar has been autoclaved and cooled until warm to touch, 0.01% (w/v) of rhodamine B was added and mixed by rotating the bottle gently.

3.3 Temperature study

All six isolates, ARB 1A, ARB 1B, ARB 1C, ARB 10, ARC 8A and ARC 8B glycerol stocks were first revived in nutrient broth at 15°C, 180 rpm for five days. This temperature was chosen based on the optimum temperature for psychrophiles defined previously. A loophole of culture medium from each flask was then streaked onto nutrient agar and was incubated at 4°C. Psychrophiles generally should be able to grow

at temperature close to 0°C. Single colony for each isolate was then streaked onto nutrient agar plate in triplicates and stored at three different incubation temperatures; 4°C, 25±2°C and 37°C respectively.

3.4 Gram staining

A drop of suspended culture was placed on a glass slide with an inoculation loop. The culture was air dried and was fixed over a gentle flame in a circular motion to avoid localised overheating. Applied heat helped the cell adhesion on the glass slide. Crystal violet stain was added covering the fixed culture and was let stand for 60 seconds. The stain was washed off by gently rinsing it with a stream of water. Iodine solution was then added over the smear and was let stand for 60 seconds. Iodine solution was washed off in a similar way to crystal violet. The smear was decolorized using 95% ethyl alcohol by slightly tilting the slide and dropping alcohol until the alcohol ran clear. The smear was immediately rinsed with water. Safranin was added as a counter stain and was let stand for 45 seconds. Safranin was washed off using a stream of water. The slide was the blot-dried using Kim wipes (KIMTECH Science, USA). Slides were viewed using a light microscope under oil immersion.

3.5 Genomic DNA extractions

The genomic DNA of all samples was extracted following modified CTAB method established by Doyle and Doyle (1991). Fifty mL of cell culture was centrifuged at 2,580 ×g (4,000 rpm) for 10 minutes. The supernatant was discarded. 4 mL of 2×CTAB was added into the tube and incubated at 55°C for 1 hour. The tube was inverted gently for every 20 minutes interval. 50 µL of lysozyme (50 mg/mL) was added and the culture was incubated at 37°C for 1 hour. 50 µL of 10% (v/v) SDS was added

and incubation at 37°C continued for 2 hours. 5 µL of RNase A (10 mg/mL) was added and culture was incubated at 37°C for 1 hour. 2 mL chloroform : isoamyl alcohol (24:1) was added and culture was incubated at 37°C, 180 rpm for 1 hour. The culture was then centrifuged at 2,580 ×g for 10 minutes and the supernatant was transferred into a new tube. 2 mL of choloform : isoamyl alcohol was added and culture was further shake and incubated at 37°C, 180 rpm overnight. The sample was centrifuged at 2,580 ×g for 10 minutes. The supernatant was transferred into 1.5 mL Eppendorf tubes. One volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added and mixed gently by inverting the tubes 10 times. The tubes were then centrifuged for 1 minute at 11,269×g. The upper layer was transferred into new tubes and the steps were repeated for 3 times. Similar steps were repeated but the phenol: chloroform: iso-amyl alcohol was replaced with chloroform: isoamyl alcohol (24:1). 2 volumes of 95% ethanol were added to precipitate the DNA. The samples were centrifuged for 20 minutes at 11,269 ×g. The supernatant was discarded and the pellets were left to air-dry. 30 µL of distilled water was added to re-suspend the pellets and extraction of DNA was now completed. Preparation of solutions are included in Appendix A.

3.6 Screening of lipolytic activity

Single colony from all six isolates was streaked onto screening agar plates of tributyrin, triolein and rhodamine B with olive oil as the substrate to observe for halo zone formation under UV light irradiation at 350nm wavelength via GelDoc (Thermofisher, USA). The plates were incubated at 25±2°C for three days. This temperature was chosen because this was the optimum temperature for growth (growth

was observed the fastest at this temperature) during temperature study. Those isolates that have managed to form clear zones were considered as positive lipase producers.

3.7 16S rDNA sequence analysis

DNA samples obtained via modified CTAB method (Section 3.5) was applied to Polymerase Chain Reaction (PCR) using universal primers of 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-CGG TTA CCT TGT TAC GAC TT-3' for 16S rRNA sequences. PCR products were purified and sent to First BASE Laboratory Sdn. Bhd. for sequencing. Sequences were then aligned via Mega6.0 and applied to Basic Local Alignment Search Tool (BLAST) to identify the species.

3.8 Lipase gene amplification by Polymerase Chain Reaction

3.8.1 Taq98^{TM} DNA Polymerase

Taq98^{TM} DNA Polymerase was used due to high DNA GC content (more than 60%) of the template DNA in this study. GC rich template is deemed challenging because GC bonds are stronger and requires higher melting temperature of the template (Frey *et al.,* 2008). Taq98^{TM} DNA Polymerase can withstand up to 98°C of denaturing temperature. The Taq98^{TM} used in this study was supplied by Lucigen Corporation (USA). Due to high GC content in organism' DNA, this Taq polymerase was used instead.

3.8.2 Reaction setup of Taq98TM DNA Polymerase

For a reaction setup with a final volume of 25 µL, 2.5 µL of 10 µM forward primer was mixed with 2.5 µL of 10 µM reverse primer, 1.0 µL of template DNA, 12.5

µL of Taq98™ Hot Start 2×Master Mix and sterile distilled water to a final volume of 25 µL.

3.8.3 Thermacycling condition of Taq98™ DNA Polymerase

The thermacycling condition for PCR run using Taq98™ DNA Polymerase consisted of six steps with a total cycle of 30. Initial denaturation was set at 98°C for 2 minutes, denaturation at 98°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, final extension at 72°C for 10 minutes and hold at 4°C indefinitely. Denaturation, annealing and extension were repeated for 29 times.

3.9 Primers design for *Pseudomonas* sp. lipase gene

Closely related species for *Pseudomonas* sp. were identified via BLAST. The lipase gene sequences of these closely related species were aligned and the conserved regions of these lipases were used to design the degenerate primers. Restriction sites for *Nde*I (CATATG) and *Bam*HI (GGATCC) were added to primers meant for expression. Six extra bases were added in front of the primers, before the restriction sites. The sequence of the forward primer for *Pseudomonas* lipase was lipF 5'-GGT GGT CAT ATG GGY GTY TTY GAC TA-3' and the sequence of the reverse primer was lipR 5'-GGT GGT GGA TCC GCY GAT GGA AAY YCC-3'. Underlined nucleotides represented the restriction sites.

3.10 PCR product purification

The PCR product was purified from excised gel by using HiYield™ Gel/PCR DNA Mini Kit supplied by Real Biotech Corporation (USA) following manufacturer's protocol.