UNIVERSITI MALAYSIA KELANTAN

Heterologous Expression of Lipase in *Escherichia coli*

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "Heterologous Expression of Lipase in *Escherichia coli*" by Nur Farhana binti Jamaludin, matric number, F15A0139 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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_____

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Heterologous Expression of Lipase in *Escherichia coli*

ABSTRACT

Lipase enzyme are being used in industrial purpose since long time ago especially in food processing, dairy making, oleochemical, detergent, pharmaceutical and so on due to its properties in hydrolyzing fats and oils as well as synthesizing organic compounds. However, the production of the enzyme is quite expensive and requires more research to produce an economical lipase enzyme. Therefore, in order to increase the efficiency of lipase enzyme, most researchers started introducing genetic manipulation to the lipase genes. Thus, this study aims in cloning two different lipase genes in *Escherichia coli*. The two lipase genes consist of *Bacillus subtilis* (BsubL) and *Candida antarctica* (CalB) were homologously recombined in *Saccharomyces cerevisiae* and heterologously expressed in *E. coli* host. The lipase expression was observed through disc diffusion assay. However, there were no expressions on the agar even after induced with 0.4 mM IPTG. For further confirmation, the transformants carrying CalB and BsubL lipase genes were sent for DNA sequencing and resulted with 97.50% identity for pET2U-CalB and 98.54% identity for pET2U-BsubL, respectively. Hence, it proves that both lipase genes were correctly inserted in the expression vector, pET2U. Therefore, further study on expression level of the inserted gene should be carried out in the future.

Keywords: lipase, *Escherichia coli*, homologous recombinant, cloning, heterologous expression

Pengekspresan Lipase Secara Heterologos dalam *Escherichia coli*

ABSTRAK

Enzim lipase digunakan dalam tujuan perindustrian sejak lama dahulu terutama dalam pemprosesan makanan, pembuatan tenusu, oleokimia, detergen, farmaseutikal dan sebagainya disebabkan sifatnya dalam menghidrolisis lemak dan minyak serta mensintesis sebatian organik. Walau bagaimanapun, pengeluaran enzim agak mahal dan memerlukan lebih banyak penyelidikan untuk menghasilkan enzim lipase yang berekonomi. Oleh itu, untuk meningkatkan kecekapan enzim lipase, kebanyakan penyelidik mula memperkenalkan manipulasi genetik kepada gen lipase. Oleh itu, kajian ini bertujuan mengklon dua gen lipase yang berbeza dalam *Escherichia coli*. Kedua-dua gen lipase terdiri daripada *Bacillus subtilis* (BsubL*)* dan *Candida antarctica* (CalB) akan dimasukkan ke dalam hos vektor secara rekombinan homologi dalam *Saccharomyces cerevisiae* dan seterusnya diekspres di dalam hos *E. coli*. Pengekspresan enzim lipase telah diperhatikan dengan kaedah resapan agar. Walau bagaimanapun, tiada ekspresi enzim lipase pada agar walaupun selepas diaruhkan dengan 0.4 mM IPTG. Untuk pengesahan selanjutnya, rekombinan DNA yang membawa gen lipase CalB dan BsubL telah dihantar untuk penjujukan DNA dan masing-masing menghasilkan identiti 97.50% untuk pET2U-CalB dan 98.54% identiti untuk pET2U-BsubL. Oleh itu, ia membuktikan bahawa kedua-dua gen lipase telah dimasukkan dengan betul ke dalam hos vektor, pET2U. Justeru, kajian lanjut perlu dilakukan bagi mengesan tahap ekspresi gen tersebut dalam hos heterologos.

Kata kunci: lipase, *Escherichia coli*, rekombinan homolog, pengklonan, ekspresi heterologos

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

CalB	*Candida antarctica* lipase B
BsubL	*Bacillus subtilis* lipase
LiOAc	Lithium oxide acetate
rpm	Revolutions per minute
°C	Degree celcius
pH	Potential of hydrogen
mM	Millimolar
mL	Milliliter
w/w	Weight/weight
%	Percentage
µg	microgram
g	Gram
µL	Microliter
L	Litre
µM	Micro molar
V	Voltage
mA	Milli ampere
sec	Second
µFd	Microfarad
Ω	Ohm

CHAPTER 1

INTRODUCTION

1.1 Research Background

In recent years, enzymes and microorganisms are widely used in industrial purpose. The benefits of enzyme have been explored by previous scientist in order to produce a better functional enzyme. Each enzyme have special characteristic which turn out to give benefits to the human. One of the well-known significant enzymes is lipase (triacylglycerol acylhydrolases, EC 3.1.1.3). Lipase is a universal enzyme that can be derived from mammalian species or microbial species. They are ubiquitously produced by plants (Villeneuve, 2003), animals (Kurtovic, Marshall, Zhao & Simpson, 2009) and microorganisms (Mehta, Bodh & Gupta, 2017). Apart from that, lipase has unique properties as it is able to react under aqueous and non-aqueous conditions. This is due to their potential to use relatively a wide spectrum of substrates, high extreme temperature stability, organic solvent, pH and as well as do not require cofactor (Andualema and Gessesse, 2012). They are known to catalyze the synthesis of ester linkages in long-chain triacylglycerol with concomitant release of the constituent acid and alcohol moieties (Takeuchi, Geraldine & Mala, 2008). The activity of lipase results in the hydrolysis of

triacylglycerol to glycerol and free fatty acids at the liquid water interface (Hermansyah, Wijanarko, Dianursanti, Gozan, Wulan & Arbianti, 2010).

Lipases are known with its diversity in term of applications in the industry. With respect to their biochemical nature, lipases have a variety of applications for example food, detergents, cosmetic, organic synthesis, perfumery, pharmaceuticals and medical diagnostics (Gandhi, 1997). Based on 'Lipase' (2017), food industry has set the highest demand with value of less than US$ 100 million by the end of 2017. In addition, lipase enzyme also has been commercialized since three decades ago by a company names NOVO Nordisk. Along with time, more company started to produce lipase enzyme in order to compete with the market demand. Hansen Holdings A/S, Koninklijke DSM N.V. and so on are the examples of company that sold enzymes (PRNewswire, 2018). Hence, more research also have been made in order to produce the best lipase with high cost-efficiency and less time consuming (Gao, Su, Lin, Jiang, Ma & Wei, 2009). Up until the future, more novel lipases will be found, hence causing a strong competition among them.

Nowadays, many studies have proved the efficiency of every type of lipase producer. However, microorganisms lipase producer is preferable to be chose as main lipase source. This is due to its characteristic that makes the production more convenience as well as ease of availability. Furthermore, in this era of technology, genetic manipulation is the fastest way to produced commercial enzyme. Apart from high efficiency in the production, genetic manipulation also helps to alter the gene sequence of some microbial lipase producer to produce high quality of lipase enzymes. This study also was conducted in order to evaluate the activity of lipase enzyme with different source by heterologous expression in *Escherichia coli*.

1.2 Problem Statement

Nowadays, lipase received a great demand in industrial biocatalyst such as detergent, cheese production, fats and oils, pharmaceutical and so on. It is expected to be increased in the next few years as the world's population is increasing. Therefore, enzymes are the most chosen as they are favorable towards environment compared to conventional. However, there is problem arise regarding the utilization of enzyme in the industry which is expensive. Therefore, it is necessary to find economical routes for enzyme production as well as high productivity with less amount of enzyme used.

1.3 Hypothesis

a. Lipase enzymes from *Candida antarctica* and *Bacillus subtilis* can be heterologously expressed in *E. coli.*

b. Lipase activity can be observed from the clones.

1.4 Objectives

This study was conducted in order,

a. To cloned two different lipase genes from *C. antarctica* and *B. subtilis* in *E. coli*.

b. To evaluate the heterologous expression of lipase in *E. coli*

1.5 Scope of Study

This study is focusing on the heterologous expression of lipase gene from different sources into *E. coli*. The lipase genes of *C. antarctica* and *B. subtilis* consist of lipase gene which are *C. antarctica* Lipase B (CalB) and *B. subtilis* lipase (BsubL) were amplified and cloned into expression vector pET2U by homologous recombination in *Saccharomyces cerevisiae* and further transformed and expressed into *E. coli*. Lastly, the clones were analyzed for lipase activity.

1.6 Significance of Study

a. Understanding towards gene amplification and heterologous expression of the lipase gene in *E. coli*.

b. The successful lipase expression method might be useful for future research to increase the production of enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipases

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are enzymes that hydrolyze fats and oils by releasing free fatty acids, diglycerides, monoglycerides, and glycerol. Lipases have a very similar fold although they have low primary sequence identity. Apart from lipases, other enzymes also have similar structural features, such as protease, esterase, dehalogenase, peroxidase and epoxide hydrolases, which are composed of $\alpha\beta$ hydrolase family (Anobom et al., 2014). The $\alpha\beta$ hydrolases active site consists of one catalytic acidic residue (aspartic or glutamic acid), and one histidine residue. In lipases, the nucleophile has been characterized as a serine residue (Anobom et al, 2014; Bordes et al, 2010; K.-E. Jaeger, Dijkstra & Reetz, 1999).

In addition, according to Guo et al, (2015), lipases generally adopt the α/β hydrolase fold, while their catalytic site (Ser-His-Asp/Glu) is conserved and usually occluded from the solvent by an α-helix region. This region is usually called the 'lid' and regulates the catalytic activity by structural changes. Bordes et al, (2010) stated that, in order to design an enzyme for specific applications, understanding towards the structural determinants involved in their selectivity is vital. According to Anobom et al, (2014), this

domain can adopt two distinct conformations which are opened and closed states. In opened states, the active site becomes reachable revealing a wide hydrophobic surface that makes the enzyme functional while in closed states, the active site is not reachable towards solvent and, as a consequence, enzyme's surface is predominantly hydrophilic rendering the lipase inactive (Bordes et al, 2010; Ranaldi et al, 2010).

2.1.1 Source of Lipase

Lipases are universal in almost entire living organisms and can be produced by plants, mammalian and microorganisms. According to Villeneuve Pierre (2003), as compared to microbial enzyme, plant enzymes are less likely to be used in the production of restructured fats. For example, wheat germ lipase from rice is commercially available, but, it is mainly active in a short chain triacylglycerol such as triacetin and tributyrin (Villeneuve, 2003). Other than that, as stated by Kurtovic, Marshall, Zhao and Simpson (2009), the ruminant's pancreas and serous glands mostly the young kid, calf, and lamb, as well as the pig's pancreas have been significant sources of lipases for flavor development in cheeses and other dairy products. However, nowadays, microbial enzyme is the most chosen enzyme in the industry as it is easily accessible and can be produce in bulk in low cost (Mehta et al, 2017). In addition, they have many commercial applications due to convenience in their production, facilitates in genetic manipulation and enormous diversity in term of characteristic and specificities (Kurtovic et al, 2009).

The main microorganisms used consist of fungi, bacteria and yeasts. Usually, soil contaminated with spillage from the products of oil and dairy involving fungal species have the possibility to secrete lipases in order to undergoes fats and oils degradation (Villeneuve,

2003). Apart from that, bacterial lipase also can be isolated from petroleum contaminated soil (Carvalho et al, 2008). According to Sharma, Chisti, Chand & Banerjee, (2001) microorganisms that produce lipase have been found in variety habitats, such as, dairy plants, industrial wastes and soil contaminated with oil and oil seeds among others.

2.1.2 Lipase-Catalyzed Reactions

Lipases are well-known with its catalytic activity in variety reactions and they have wide substrate specificity. The reactions catalyzed by lipases can be divided into hydrolysis and synthesis as follows:

a. Hydrolysis of fats and oils

Triglycerides lipases catalyze the hydrolysis of water-immiscible triglycerides and interface between water-lipid. In the presence of excess water, complete hydrolysis reaction can be observed (Hermansyah et al, 2010). Generally, the hydrolysis of fats and oils is an equilibrium reaction. Hence, it is possible to modify the direction of reaction by improving the reaction condition. In addition, the forward and reverse reactions are controlled by water content. This is due to lipase able to catalyze ester synthesis reaction in non- aqueous conditions. (K -E Jaeger et al, 1994).

b. Synthesis of organic compounds

Esterification is a process in which lipase catalyzes the reaction of alcohols with acids to produce esters and water. While, transesterification is a process involving fats and oils in which can be specified depends on the type of acyl acceptor (K. -E Jaeger et al., 1994). Triglycerides are hydrolyzed into methyl-ester and glycerol in the presence of a catalyst. Esterification and transesterification reactions are favorable to react if there is absence or lack of water.

2.2 Industrial Applications of Lipase

Microbial lipase have gained special attention in the industry due to their variety of ability such as can remained under extreme temperature, pH and organic solvents (Mehta et al, 2017). Currently, microbial enzymes have been steadily grown due to diversified applications in many areas such as pharmaceuticals, cosmetics, biocatalysts and agrochemical industry. In addition, as stated by Gupta, Kumari, Syal & Singh (2015) the current market value of lipases is $100 million and the market is growing rapidly with 8.2% CAGR 593 (compound annual growth rate) due to their emerging uses in 594 industries. Hence, it shows that microbial enzyme is significant in this era.

Firstly, lipases have variety functions in the food processing and dairy industry. For example, low calorific value of lipids and milk fat, modifications of fats and oils, lipid's structural, and synthesis of fatty acid ester (Gupta et al., 2015). Mostly, commercial lipase produce are used in flavor enhancement in dairy products and food processing. For

example, milk products, baked food and beer (Sindhu, Binod, Sabeela, Amith, Anil et al, 2018). Other than that, the ester produce from short chain fatty acid has been widely utilized as flavouring agent in the food industry (Aravindan, 2007). For example, 'tempeh', a popular traditional fermented food that utilized lipase from *Rhizopus oligosporous* (Shurtleff & Aoyagi, 2001).

In term of application of lipase in the dairy industry, lipase is significant in cheese processing. Lipase is used extensively in this industry due to its capability to hydrolyze the milk fat. Today's applications include flavor development of cheese, the quickening of cheese ripening as well as manufacturing of cheese-like products. The free fatty acids that are generated by the action of lipase on milk fat endow many dairy products, particularly soft cheese with their specific flavor characteristic. Thus, the addition of lipase that primarily released short-chain (mainly C4 and C6) fatty acids leads to the development of a sharp and tangy flavor (Vulfson, Woolley & Petersen, 2011).

At present, lipases have not been played a significant role in household detergent. This is due to the lack of enzymes that are sufficiently stable and active under alkaline conditions (Vulfson, Woolley & Petersen, 2011). However, the accelerating trend towards the lower laundering temperature led to a much higher demand for such preparations. In additions, with the aid of recent intensive screening activity and followed by the research among the scientist on the genetic manipulation of lipase gene have resulted in the introduction of several suitable preparations for the lipase to be remained in the alkaline condition. For example, Novo Nordisk's 'Lipolase' (*Humicola* lipase expressed in *Aspergillus oryzae*). Undoubtedly, we can see lipases from novel sources as well as genetically engineered enzymes with improved characteristics as shown in Table 2.1.

Table 2.1 Microbial lipases used in household detergents

Origin of lipase	Product name	Year of introduction	Company (location)
Fungal			
Humicola lanuginosa	Lipolase	1988	NOVO-Nordisk (Denmark)
Bacterial			
Pseudomonas mendocina	Lumafast	1992	Genencor (USA)
Pseudomonas alcaligenes	Lipomax	1995	Gist-Brocades (The Netherlands)
Pseudomonas glumae	n.a	n.a	Unilever (The Netherlands)
Pseudomonas species	n.a	n.a	Solvay (Belgium)
Bacillus pumilus	n.a	n.a	Solvay (Belgium)

n.a no annotations

Source: K.E- Jaegar et al (1994)

2.3 Cloning of Bacterial and Yeast Lipase Producer Gene

2.3.1 Bacterial and Yeast Producer Lipase Gene

Many studies showed that bacteria and yeast harbor many genes that responsible in the production of lipase. *B. subtilis* is the example of bacterial lipase producer, while, *C. antarctica* is the example of yeast lipase producer. *B. subtilis* is gram-positive bacteria. It is ubiquitously found in nature especially soil in which makes it easier to be access. Bacteria have become a very significant stereoselective biocatalyst used in organic chemistry due to its properties that have high region-selectivity and enantioselectivity. Therefore, high-level production of these biocatalyst needs more research and knowledge on gene expression folding and secretion (K.-E. Jaeger et al., 1999).

Besides, *Pseudomyza antarctica* or its new name, *Candida antarctica* is also one of the most widely used in industry due to its excellent properties of high stereoselectivity and stability (Ujiie, Nakano, & Iwasaki, 2016). In most research, CalB is highly expressed in *Pichia pastoris, Saccharomyces cerevisiae, Aspergillus oryzae* and *Yorrowia lipolytica* in which they act as recombinant protein expression host. However, the expression resulted in the formation of insoluble inclusion bodies (Yang, Liu, Dai & Li, 2013). Hence, according to the research made by Ujiie et al., (2016), they have come out with a genuine primary sequence in which CalB can be expressed in *E. coli* without the addition of amino acid residue at the N- and/or C-termini in which the recombinant protein is not genuine with respect to the primary sequence.

2.3.2 Synthetic DNA Fragment

In recent years, there has been accelerated number of research in term of gene synthesis. In order to make the enzymatic error correction and genome assembly methods works efficiently, these new technologies have put synthetic biology into a higher level. With the utilization of synthetic biology by expanding the capacity to construct, manipulate and analyze DNA provide the power to design, manipulate or even producing artificial living systems (Ma, Tang, & Tian, 2012). According to article from Gewin (2013), genome-editing techniques will remain the go-to choice for most applications that require a small number of genetic alterations, whereas genome design will be useful for specialized applications, such as recoding an entire genome to incorporate new amino acids.

2.3.3 Homologous Recombination in *S. cerevisiae*

Homologous recombination is significant mechanism by which transferred DNA is incorporated into the prokaryotes or eukaryotes by transformation (Gietz & Woods, 2001; Kung, Retchless, Kwan, & Almeida, 2013). According to Kung et al., (2013) there are several factors that can affect the gene transfer; firstly, the lack of uptake DNA sequence, short DNA sequenced and preferentially bound by competent cells. Therefore, in donor DNA, those can prevent the gene to bind efficiently and transport the DNA across the membrane. In order to efficiently transform the intact cells, several methods have been done in order to developed and improved. For example, the combinations of alkali cations (Li^+) and polyethylene glycol (PEG) can increase the chance of plasmid DNA uptake by intact yeast cells and heat shock were essential for the transformation (Gietz & Schiestl, 2007; Gietz & Woods, 2001; Gietz, Schiestl, Willems, & Woods, 1995; Gietz & Woods, 2002). Li+ utilization in the study can resulted in faster and easier transformation which causes most researchers to applying this method in their studies (Gietz & Woods, 2001).

2.3.4 *Escherichia coli* as A Host for Heterologous Protein Expression

Generally, there are various types of eukaryotic microorganisms that can be used as recombinant protein expression host such as *P. pastoris, S. cerevisiae, A. oryzae* and *Y. lipolytica* (Høegh, Patkar, Halkier, & Hanse, 1995; Cereghino & Cregg, 2000; Madzak, 2015). As mention by Chen, (2012), the *E. coli* expression system still dominating bacterial expression system in which most of them still remain to be used in the laboratory investigations as well as initial development in commercial activities. *E. coli* is the most option for heterologous host due to its ease of genetic manipulation, availability of large number of mutant host strains and cloning vector system as well as its ability to grow at high density of inexpensive substrates (Baneyx, 1999; Ujiie et al., 2016). However, there are some problems regarding the heterologous expression system in which the ligation step in cloning needs to undergoes many trials in order to success. Therefore, in order to improve the technique, cloning strategy by the research from (Dickschat, Pahirulzaman, Rabe & Klapschinski, 2014) will be implied in this research due to their successfulness in cloning bacterial terpene cyclases in *E. coli* by applying homologous recombination in yeast in their studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

The chemicals and reagents that were used in this study are sterile distilled water (SDW), tryptone, sodium chloride, yeast extract, yeast nitrogen based, agar, ampicillin, potassium chloride, magnesium sulphate, glucose, adenine sulphate, ammonium sulphate, supplement mixture minus uracil, proteinase K, 5X One Taq standard reaction buffer, 10 mM dNTPs, one Taq DNA polymerase, template DNA, 5 μM forward primer, 5 μM reverse primer, restriction enzymes; *Eco*RI, *Xho*l and *Eco*RV, 10X fast digest buffer, 0.1 M lithium acetate, 1.0 M lithium acetate, polyethylene glycol (PEG) 3500, salmon sperm carrier DNA, isopropyl-ß-D-1-thiogalactopyranoside (IPTG), trypticase soy broth, olive oil and rhodamine B.

3.1.2 Microbial strain

Table 3.1: Microbial strains used in study.

Strain	Genotype	Source
S. cerevisiae YPH499	MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1	SIRIM Berhad
E. coli TOP10	F- mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 ara 139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG	SIRIM Berhad
E. coli BL21	F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS) pLysS[T7p20 orip15A](CmR)	SIRIM Berhad

3.1.3 Equipment and Apparatus

Equipment and apparatus that were used for this study are pipette, agar plate, inoculating loop, centrifudge tube, electroporation cuvette, cork borer, beaker, measuring cylinder, incubator shaker (Lab Companion), Class II Biological Safety Cabinet (LabGard), incubator (Memmert), thermal cycler machine (Kyratec), MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology), Zymoprep II Yeast Plasmid Miniprep Kit (Zymoresearch) and Gene Pulser Electroporator (Bio-Rad).

3.2 Methods

3.2.1 Strains and Culture Condition

In this experiment, *Saccharomyces cerevisiae* strain YPH499 were used as the assembly host for homologous recombinant, while, *Escherichia coli* strain BL21 and TOP10 were used as the expression host for heterologous expression of lipase.

3.2.1.1 *S. cerevisiae* Strain YPH499

S. cerevisiae strain YPH499 were grown in liquid YPAD medium in which consist of 1% of yeast extract, 2% of tryptone, 2% glucose and 0.04% of adenine sulphate or on YPAD agar plates which consist of 1.5% of agar and the following YPAD medium at 30°C. Next, the *S. cerevisiae* transformants were selected on SM-ura medium (0.17% of yeast nitrogen based, 0.5% of ammonium sulphate, 2% of glucose, 0.077% of nutritional sup minus uracil and 2% agar) or on YPAD agar plates (YPAD medium plus 1.5% agar).

3.2.1.2 *E. coli* Strain BL21 and TOP10

E. coli strain BL21 and TOP10 were grown in Luria-Bertani (LB) medium which consist of 0.5% of yeast extract, 1% tryptone and 0.5% sodium chloride. As for agar plate, 1.5% of agar was added to the medium. Then, 100 µg/mL of ampicillin (Amp) were added to the culture medium as a selection antibiotic.

3.2.2 Cloning Protocol

3.2.2.1 Lipase Gene Fragment

Two lipase genes were identified through literature searching. The two lipase genes are from; *B. subtilis* (Shi et al, 2010) and *C. antarctica* (Ujiie et al, 2016). The sequences were obtained from National Center for Biotechnology Information (NCBI). Next, synthetic DNA fragment were designed by Apical Scientific Sdn. Bhd. based on the lipase gene DNA sequences as follows:

a. *Bacillus subtilis* Strain FS1403 (639 bp)

```
  1 ATGAAATTTG TAAAAAGAAG GATCATTGCA CTTGTAACAA TTTTGATGCT GTCTGTTACA
 61 TCGCTGTTTG CGTTGCAGCC GTCAGTAAAA GCCGCTGAAC ACAATCCAGT CGTTATGGTT
121 CACGGCATTG GAGGGGCATC ATTCAATTTT GCGGGAATTA AGAGCTATCT CGTATCTCAG
181 GGCTGGTCGC GGGACAAGCT GTATGCAGTT GATTTTTGGG ACAAGACAGG CACAAATTAT
241 AACAATGGAC CGGTATTATC ACGATTTGTG CAAAAGGTTT TAGATGAAAC GGGTGCGAAA
301 AAAGTGGATA TTGTCGCTCA CAGTATGGGG GGCGCGAACA CACTTTACTA CATAAAAAAT
361 CTGGACGGCG GAAATAAAGT TGCAAACGTC GTGACGCTTG GCGGCGCGAA CCGTTTGACG
421 ACAGGCAAGG CGCTTCCGGG AACAGATCCA AATCAAAAGA TTTTATACAC ATCCATTTAC
481 AGCAGTGCCG ATATGATTGT CATGAATTAC TTATCAAGAT TAGATGGTGC TAGAAATGTT
541 CAAATCCATG GCGTTGGACA CATCGGCCTT CTGTACAGCA GCCAAGTCAA CAGCCTGATT
601 AAAGAAGGGC TGAACGGCGG GGGCCAGAAT ACGAATTAA
```

b. *Candida antarctica* Lipase B (1020 bp)

```
  1 ATGAAATACC TGCTGCCGAC CGCTGCTGCT GGTCTGCTGC TCCTCGCTGC CCAGCCGGCG
 61 ATGGCCCTGC CGAGCGGTAG CGATCCGGCG TTTAGCCAGC CGAAAAGCGT TCTGGATGCA
121 GGCCTCACGT GTCAGGGTGC GAGCCCGAGC AGCGTTAGCA AACCGATTCT GCTGGTTCCG
181 GGTACGGGTA CGACCGGTCC GCAGAGCTTT GATAGCAATT GGATTCCGCT GAGCACGCAA
241 CTCGGCTATA CCCCGTGTTG GATTAGCCCG CCGCCGTTTA TGCTGAATGA TACCCAGGTG
301 AATACCGAAT ATATGGTGAA TGCGATTACC GCGCTGTATG CGGGTAGCGG TAATAATAAA
361 CTGCCGGTGC TGACCTGGAG CCAGGGTGGT CTGGTGGCGC AGTGGGGCCT GACCTTTTTT
421 CCGAGCATTC GTAGCAAAGT GGATCGTCTG ATGGCGTTTG CGCCGGATTA TAAAGGCACC
481 GTGCTGGCGG GTCCGCTGGA TGCGCTGGCG GTGAGCGCGC CGAGCGTGTG GCAGCAGACC
541 ACCGGTAGCG CGCTGACCAC CGCGCTGCGT AATGCGGGTG GTCTGACCCA GATTGTGCCG
601 ACCACCAATC TGTATAGCGC GACCGATGAA ATTGTGCAGC CGCAGGTGAG CAATAGCCCG
661 CTGGATTCGA GCTATCTGTT TAATGGTAAA AACGTTCAAG CACAGGCGGT GTGTGGTCCG
721 CTGTTTGTGA TTGATCATGC GGGTAGCCTG ACCAGCCAGT TTAGCTATGT GGTGGGTCGT
781 TCGGCACTGC GTAGCACCAC CGGCCAGGCG CGTAGCGCGG ATTATGGCAT TACCGATTGT
841 AATCCGCTGC CGGCGAATGA TCTGACCCCG GAACAGAAAG TTGCCGCTGC TGCCCTGCTG
901 GCTCCAGCGG CGGCAGCCAT TGTGGCGGGT CCGAAACAGA ATTGTGAACC GGATCTGATG
961 CCGTATGCGC GTCCGTTTGC GGTGGGTAAA CGTACCTGTA GCGGTATTGT GACCCCGTAA
```

3.2.2.2 Amplification of Lipase Gene

Polymerase Chain Reaction (PCR) for lipase gene was carried out in a 25 µL reaction (Table 3.2). The lipase genes were amplified from synthetic DNA fragment by PCR using primers (Table 3.3) that produced 30-base 5' overlaps with the flanking vector sequence. Primer pairs were designed based on the DNA sequences from NCBI. PCR were performed with a Kyratec thermal cycle machine with the program in Table 3.4.

Table 3.2: PCR reaction mixture.

Component	**25 µL reaction**
Sterile distilled water	Top up to 25 µL
5X One *Taq* standard reaction buffer	5.00 µL
10 mM dNTPs	0.50 µL
One *Taq* DNA polymerase	0.15 µL
Template DNA	2.50 µL
5 µM forward primer	2.50 µL
5 µM reverse primer	2.50 µL

Table 3.3: The lipase primers

Primer name	DNA Sequence (5' -----> 3')	Gene
CalB-Fwd	CCCGCGAAATTAATACGACTCACTATAGGGATGAAATACCTGCTGCCGAC	CalB
CalB-Rev	CCCGTTTAGAGGCCCCAAGGGGTTATGCTATTACGGGGTCACAATACCGC	
Bsub-Fwd	CCCGCGAAATTAATACGACTCACTATAGGGATGAAATTTGTAAAAAGAAG	BsubL
Bsub-Rev	GGCCCCAAGGGGTTATGCTATTAATTCGTATTCTGGCCCCCGCCGTTCAG	

Table 3.4: PCR program setup

Process	Temperature	Time length	Cycle
Pre-denaturation	94 °C	20 seconds	1x
Denaturation	94 °C	20 seconds	20x
Annealing	65 °C	15 seconds	
Synthesis of new DNA	72 °C	*	
Synthesis of new DNA	72 °C	5 minutes	1x

* The extension time, 72 °C was set according to source of DNA used; 15 sec/1kb of plasmid or 30sec/1kb of genomic DNA

Lastly, the PCR products were visualized on 1% agarose gel in 1x TAE buffer at 80V, 400 mA for 30 minutes.

3.2.2.3 Restriction Enzyme Digestion

Restriction enzyme digestions was carried out using Thermo Scientific FastDigest Restriction Enzymes with 2 µL (analytical) or 15 µL (preparative) plasmid miniprep DNA (Table 3.6). The enzymes that were used are *Eco*RI and *Xho*l. The digestion took 1 hour at 37 °C. The electrophoresis was analyzed on 1% agarose gel in 1X TE buffer at 80V, 400 mA, for 30 minutes. For preparative digestion, the digested plasmid was purified using MEGAquick-spin^{TM} Total Fragment DNA Purification Kit according to the manufacturer's protocol.

Table 3.5: Restriction enzyme digestion mixture.

Reagents	**Analytical digestion**	**Preparative digestion**
DNA plasmid	2.0 µL	15.0 µL
10X Fast digest buffer	1.0 µL	2.0 µL
Restriction enzymes	0.2 µL	2.0 µL
Sterile distilled water	6.8 µL	1.0 µL
Total volume	10.0 µL	20.0 µL

3.2.2.4 Cloning of Lipase Gene into pET2U Vector

The lipase genes, *C. antarctica* Lipase B (CalB) and *B. subtilis* lipase (BsubL) were cloned into vector pET2U (Figure 3.1) by homologous recombination in *S. cerevisiae*.

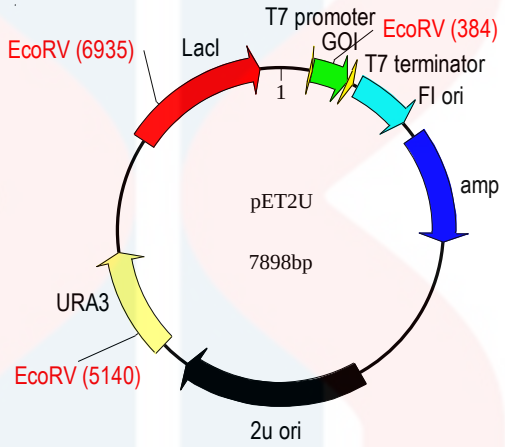

Figure 3.1: Plasmid map of pET2U vector

Genetically engineered plasmid that designed purposely for the cloning, storing DNA or genome modification basically consists of an origin of replication, selection marker and multiple cloning sites. The pET2U vector which is replicable in both *S. cerevisiae* and *E. coli* were used in this study. It consists of strong T7 promoter and terminator, genes of ampicillin and URA3 for selection in *E. coli* (amp) and *S. cerevisiae* respectively. The gene of interest (GOI) was inserted downstream the strong T7 promoter. With the pET2U vector, gene cloning can be carried out by homologous recombination in *S. cerevisiae* followed by heterologous expression in *E. coli*.

3.2.2.5 Homologous Recombination in *S. cerevisiae*

Homologous recombination in *S. cerevisiae* was carried out using the LiOAc/SS carrier DNA protocol from Gietz & Schiestl (2007) with some modifications. A single colony of *S. cerevisiae* strain YPH499 was inoculated in YPAD liquid medium (10 mL) at 30 °C and 120 rpm overnight as a starter culture. The starter culture was used to inoculate YPAD liquid culture (40 mL) and incubated for 4 – 5 hours at 30 °C, 120 rpm. Next, the culture was centrifuged at 4, 625 rpm for 5 minutes. The pelleted cells were washed with 25 mL sterile distilled water (SDW), and centrifuged for another 5 minutes. Then, the mixture was resuspended in 1 mL of 0.1 M aqueous LiOAc. The suspension was transferred into a 1.5 mL microcentrifuge tube and centrifuged for 15 seconds at 13,200 rpm. The pelleted cells were resuspended in 400 µL of 0.1 M LiOAc. 50 µL of the resulting suspension was used for each transformation process. Again, the cell was centrifuged at top speed for 1 minute in order to get pellet and the following solutions were added afterwards; PEG 3500 (50% v/v, 240 µL), 1.0 M LiOAc (36 µL), salmon sperm carrier DNA (50 µL, 2 mg mL^{-1}), 34 µL of transforming DNA (~1 µg of linearized pET2U vector and equimolar amount of PCR product). The mixtures were vortexed vigorously to re-suspend the cells. Then, it was incubated at 30 °C for 30 minutes, followed by 42 °C for 30 minutes. The cells were centrifuged at 13, 200 rpm for 15 seconds and were gently resuspended in 600 µL SDW. Lastly, 300 µL of the suspensions were spread on SM-ura medium and incubated at 30 °C for 2 days.

3.2.2.6 Yeast Plasmid MiniPrep

The yeast plasmid was extracted from the transformation plate in Section 3.2.2.5 using Zymoprep Yeast Plasmid Miniprep ll according to the manufacturer's protocol.

3.2.2.7 *E. coli* Transformation by Electroporation

1 µl of yeast DNA was added to a 40 µl of *E. coli* BL21 and *E. coli* TOP10 electrocompetent cells and the mixture was transferred to a precooled electroporation cuvette. After pulsing at 2.5 kV, 25 µFd capacitance and 400 Ω resistance using a Bio-Rad Gene-Pulser, 200 µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was immediately added to the cells and mixed. Then, it was incubated at 37 °C for 1 h. The cells were spread on LB plate supplemented with ampicillin and incubated at 37 °C overnight.

3.2.2.8 Screening of *E. coli* Transformants

The *E. coli* transformants were screened by colony PCR and restriction enzyme digestion in order to make sure the gene is correctly inserted into the expression vector, pET2U. Colony PCR was carried out by following procedures in Section 3.2.2.2 with cell lysate as the PCR template. The cells were toothpicked from well-spaced *E. coli* colonies on transformation plates and were resuspended in 25 µL of 50 µg/mL Proteinase K in TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA) and heated at 55 °C for 15 minutes and

80 °C for 15 minutes. Next, the suspension was centrifuged at 140,000 rpm for 3 minutes and 2.5 µL of the supernatant was used as template for analytical PCR.

3.2.2.9 Expression of Lipase

A single colony of the recombinant strain *E. coli* BL21 and *E. coli* TOP10 harboring pET2U-CalB and pET2U-BsubL were inoculated into 20 mL fresh LB broth supplemented with 100 µg/mL ampicillin. Then, 0.4 mM of Isopropyl-ß-D-1-thiogalactopyranoside (IPTG) was added to the broth cultures and incubated at 37 °C, 120 rpm for 4 hours. The cultures were immediately used for lipase assay.

3.3 Lipase Assay

Lipase assay was conducted using agar-diffusion assay. Lipase agar medium containing 8 g/L of trypticase soy broth, 4 g/L of yeast extract, 3 g/L of sodium chloride, 20 g/L of agar, 30 ml/L of olive oil and 2 mg/L of rhodamine B was freshly prepared for determination of lipase activity. A well was made using a cork borer at the center of the agar plates and 20 µL of the 4 hours induced cultures were pipetted into the well. Lastly, an orangish fluorescent halos around the well was observed after incubated at 37 °C overnight.

3.4 Sequencing and Analysis of Lipase

The recombinant plasmids were sent to First Base Laboratories Sdn. Bhd. for sequencing using primers specifically for CalB and BsubL genes (Table 3.4). The DNA sequence was analyzed using BLAST from National Center of Biotechnology (NCBI) while the lipase DNA sequence pET2U-CalB and pET2U-BsubL were aligned using ClustalW (Multiple Sequence Alignment).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 DNA Purification for PCR Product of *Candida antarctica* Lipase B

The gene from *C. antarctica* lipase B (CalB) and *B. subtilis* lipase (BsubL) were successfully amplified during the PCR process. As seen on Figure 4.1, PCR for *B. subtilis* lipase gene shows the expected size with single band observed on the gel.

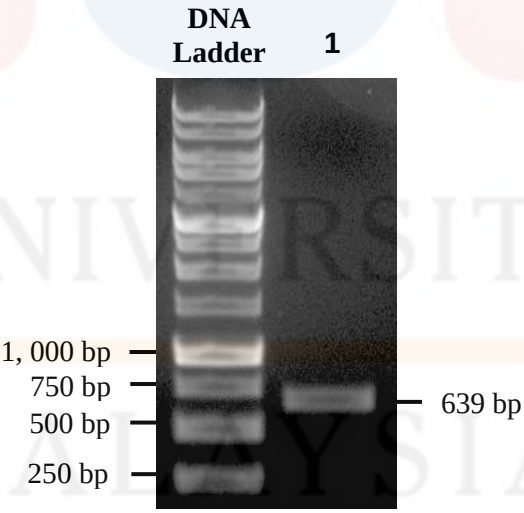

Figure 4.1: PCR product of BsubL gene. Lane 1 shows the expected size for BsubL at 639 bp

However, the PCR on CalB gene lead to many non-specific DNA products of varying sizes (Figure 4.2). The purity and concentration of the DNA product is crucial for cloning purposes. The formation of multiple bands usually is due to the unspecific amplification or annealing temperature as well as unsuitable primer concentration. The primer concentration should be reduced that will make the annealing become more specific thus eliminate incorrect amplifications (National Diagnostics, 2011).

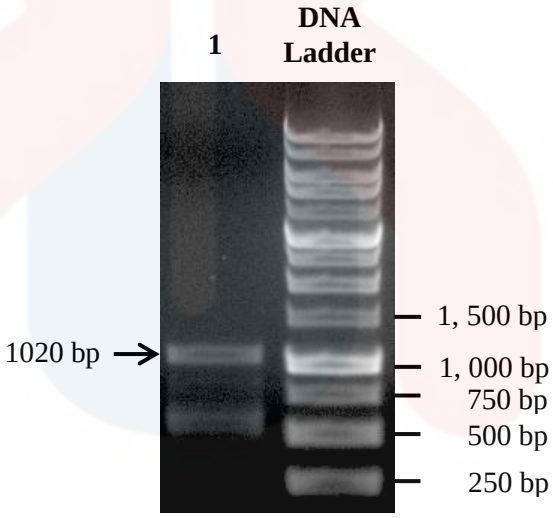

Figure 4.2: PCR product of CalB gene. Lane 1 shows two bands appeared for CalB (expected band at 1020 bp and non-specific bands at 500 bp – 750 bp)

Gel purification was conducted in order to get the correct DNA product for CalB. The resulted purified DNA of CalB can be seen on Figure 4.3.

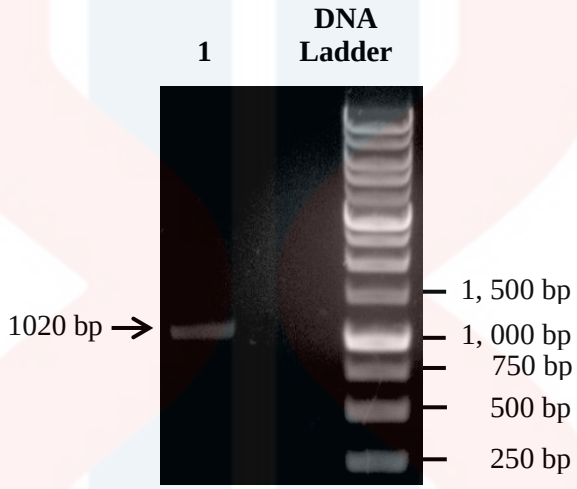

Figure 4.3: PCR product of purified CalB gene was visualized on 1% agarose gel. Lane 1 shows the correct size with single band at 1020 bp.

4.2 Digestion of pET2U Vector for Homologous Recombination in *S. cerevisiae*

Restriction enzyme digestion allows a plasmid vector to be cut at specific area with the aid of enzymes in which it will allow the gene of interest to be inserted into the vector. In this study, two enzymes were used; *Eco*RI and *Xho*I in order to make sure the plasmid vector, pET2U is successfully digested. The linearized pET2U vector was visualized on agarose gel in order to confirm the complete digestion (Figure 4.4). A linearized plasmid will generally give a single band at difference molecular weight compared to the undigested plasmid. As seen on Figure 4.4, the digested plasmid did give a linear band while, the undigested plasmid have smearing band. This is because, an intact vector with or without insert is circular in shape and under stress, so its lowest conformation of energy is knotted and twisted, but, once the insert is cut out, there is only one conformation (linear form) as tension was destroyed (Vikash, n.d.)

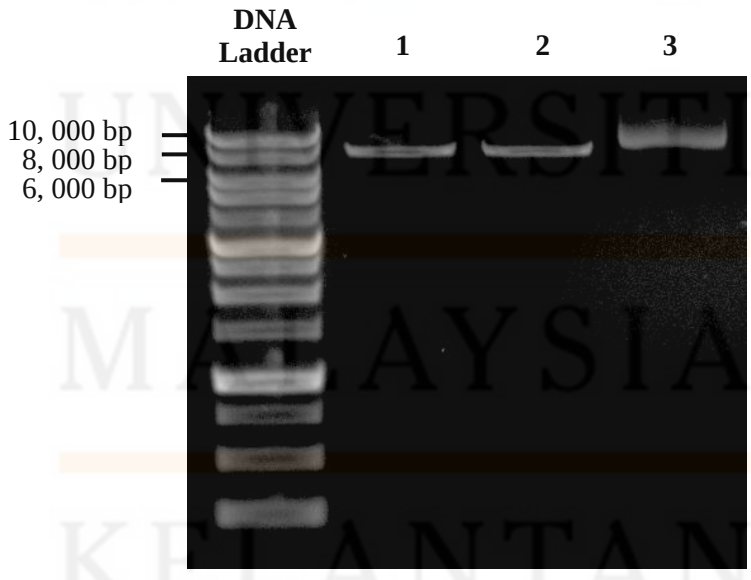

Figure 4.4: The digested pET2U vector (lane 1 and lane 2) and undigested pET2U (control, lane 3) were visualized on 1% agarose gel.

4.3 Heterologous Expression of Lipase in *E. coli* by Homologous Recombination in Yeast

Cloning by homologous recombination in *S. cerevisiae* is an efficient and cost-effective way apart from other recombinant DNA technology methods (Joska, Mashruwala, Boyd & Belden, 2014). This study utilized the previously constructed yeast-*E.coli* shuttle vector, pET2U (Figure 3.1) (Pahirulzaman, K. A. K., unpublished data). The pET2U vector comprise of 2μ origin of replication and URA 3 gene for replication and selection in *S. cerevisiae*, respectively.

Homologous recombination occurs via double-stranded-break-repair mechanism (Orr-Weaver & Szostak, 1985). The repair system allows insertion of DNA sequences which can efficiently join a linearized vector and DNA fragment(s), provided with 20 – 30 bp overlap homologous sequence between them (Hua, Qiu, Chan, Zhu & Luo, 1997). The gene of interest, CalB and BsubL was inserted downstream the strong T7 promoter by homologous recombination in *S. cerevisiae* and transformed into *E. coli*.

E. coli is a common prokaryotic expression host and the most chosen heterologous protein producer by most researchers (Gomes, Byregowda, Veeregowda & Balamurugan, 2016). The expression of this system is proved to be the quickest, cheapest and easiest. For instance, it takes only 20 – 30 minutes to grow (Snustad & Simmons, 2010). In this study, the recovered plasmid from yeast DNA was transformed into the *E. coli* by electroporation.

Overall, a total of 46 colonies that are successfully grow, which are 30 colonies from TOP10 *E. coli*-CalB, 11 colonies from TOP10 *E. coli*-Bsubs and 5 colonies of BL21-Bsubs and none from BL21-CalB (Figure 4.5).

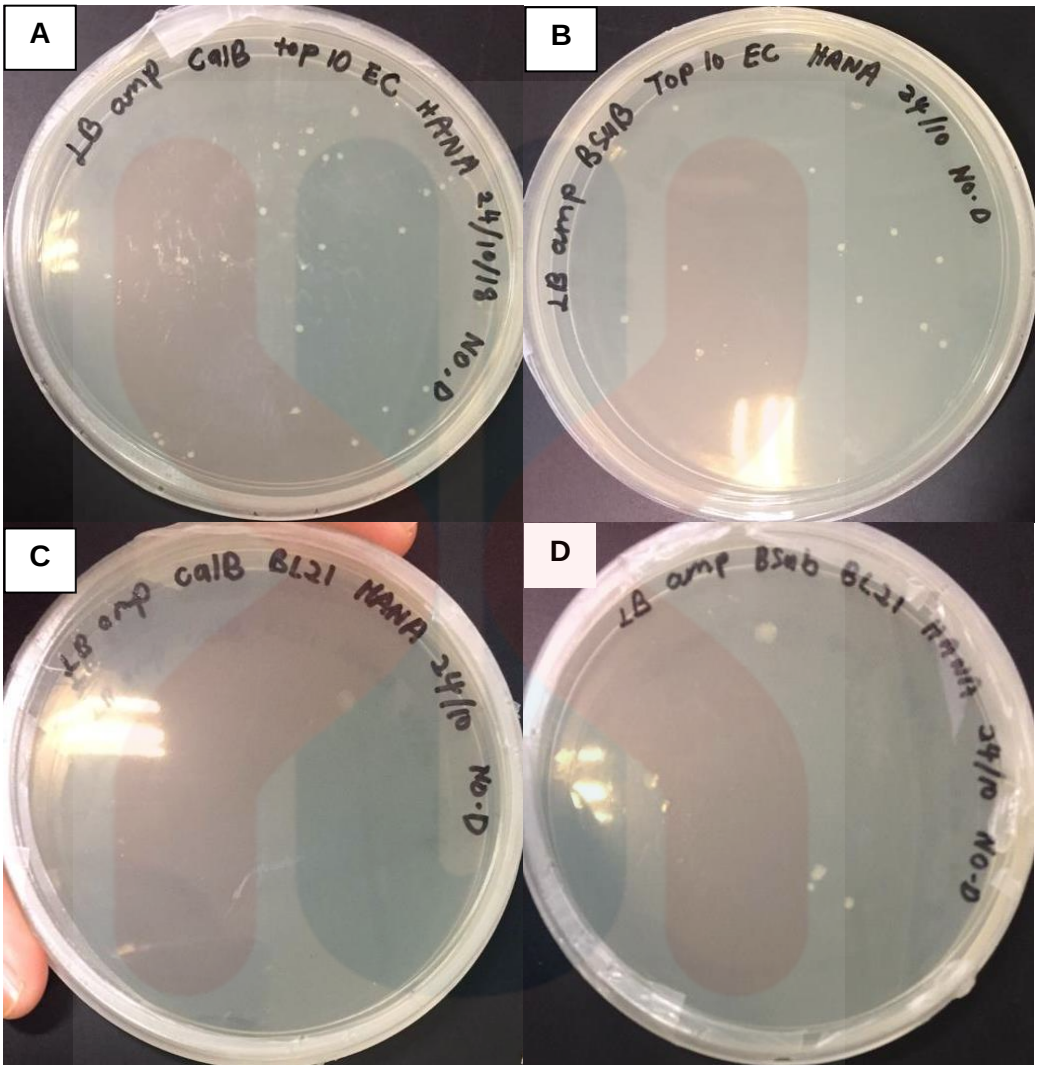

Figure 4.5: The *E. coli* transformation plates after overnight incubation (A) pET2U-CalB in TOP10 *E. coli* host (B) pET2U-BsubL in TOP10 *E. coli* host (C) pET2U-CalB in *E. coli* BL21 host (D) pET2U-BsubL in BL21 host.

Five colonies of recombinant *E. coli* TOP10 harboring pET2U-CalB were selected from transformation plate for analytical PCR using CalB primers (Table 3.3). This is to screen whether the transformants carrying the CalB gene (insert) or not. Only 1 colony gives expected DNA sizes at 1020 bp (Figure 4.6).

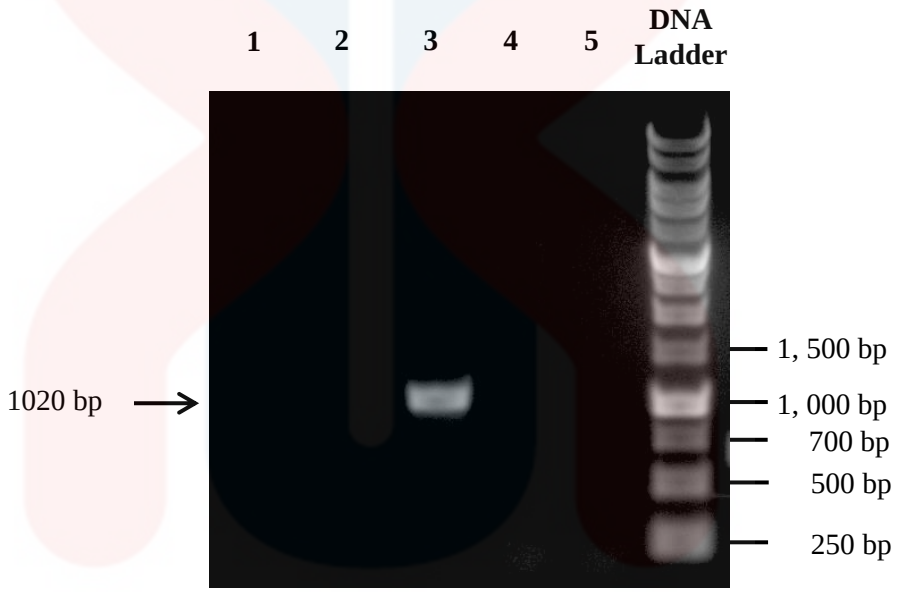

Figure 4.6: Analytical PCR on 5 colonies of pET2U-CalB transformants (*E. coli* TOP10 Host). Colony 3 (lane 3) shows the expected DNA product at 1020 bp.

Whereas, for pET2U-BsubL, four transformants from each *E.coli* TOP10 and *E. coli* BL21 hosts were screened by analytical PCR using BsubL's primer (Table 3.3). All colonies showed expected DNA sizes at 639 bp (Figure 4.7).

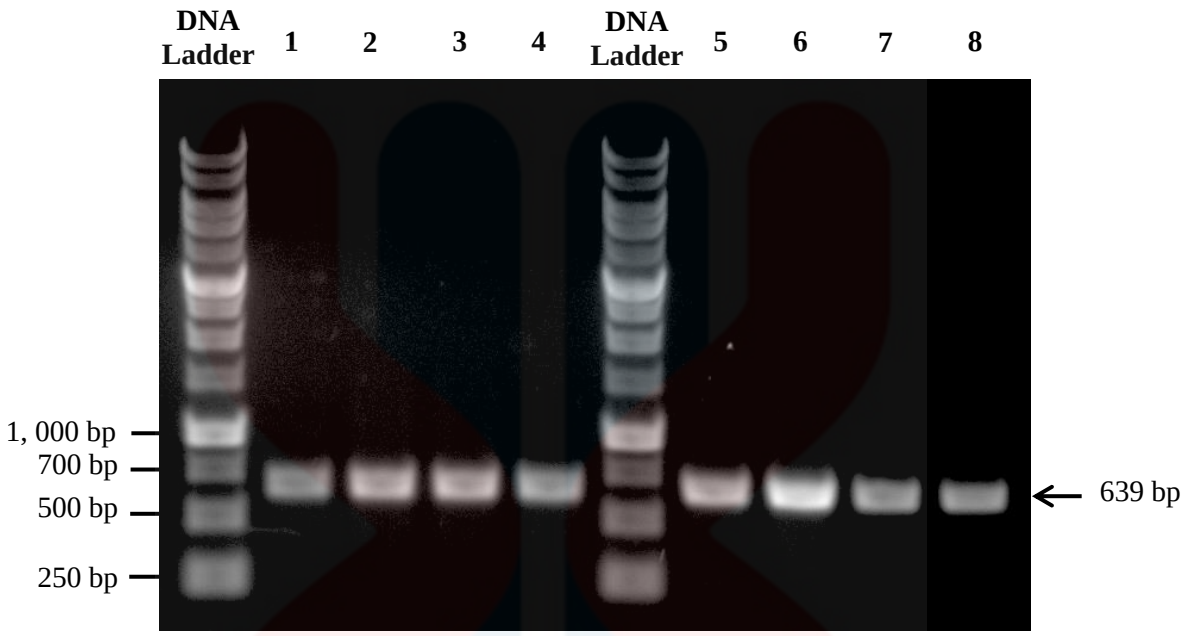

Figure 4.7: Analytical PCR on 4 colonies of pET2U-BsubL transformants; (lane 1 – 4, *E. coli* TOP10 host), (lane 5 – 8, *E. coli* BL21 host). All screened transformants show expected DNA sizes at 639 bp.

For further confirmation, the transformants were then digested with *Eco*RV. *Eco*RV cuts 3 times in pET2U vector without insert (Figure 3.1), whereas cut twice in pET2U-CalB. Analytical digestion with *Eco*RV yielded two fragments of 6898 bp and 1795 bp (Figure 4.8) confirming the insertion of CalB gene in the pET2U vector.

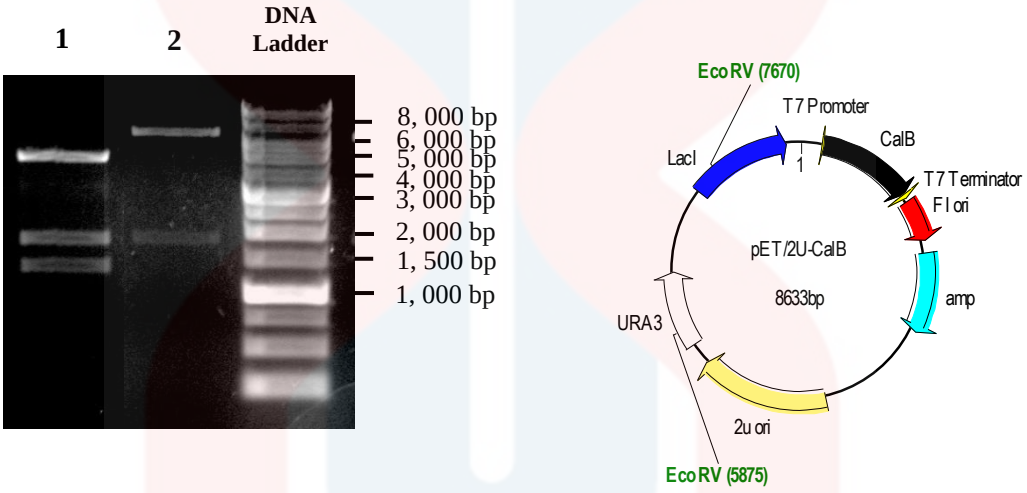

Figure 4.8: Map of plasmid pET2U-CalB (right). Restriction analysis of plasmid extracted from pET2U-CalB colony yielded 2 predicted fragments of 6898 bp and 1795 bp (lane 2), indicates that CalB gene is successfully inserted in the pET2U vector. Lane 1 is the digested pET2U vector without insert as control yielded 3 predicted fragments of 4756 bp, 1795 bp and 1347 bp.

The same method was done to pET2U-BsubL. Analytical digestion with *Eco*RV yielded 2 fragments of 6457 bp and 1795 bp (Figure 4.9) confirming the insertion of BsubL gene in the pET2U vector.

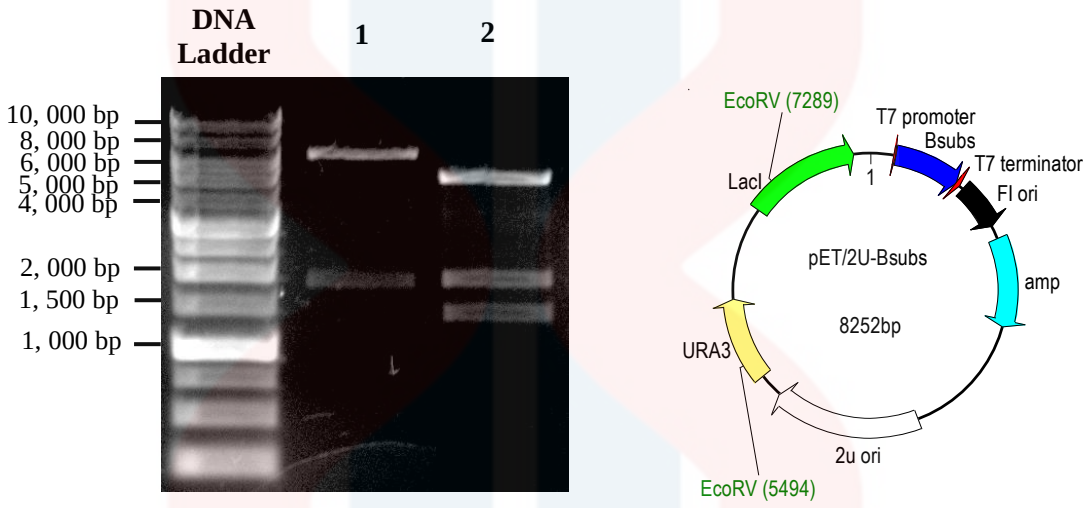

Figure 4.9: Map of plasmid pET2U-BsubL (right). Restriction analysis of plasmid extracted from pET2U-BsubL colony yielded 2 predicted fragments of 6457 bp and 1795 bp (lane 1), indicates that BsubL gene is successfully inserted in the pET2U vector. Lane 2 is the digested pET2U vector without insert as control yielded 3 predicted fragments of 4756 bp, 1795 bp and 1347 bp.

Both digestions towards the pET2U-CalB and pET2U-Bsubs proved that the lipase gene from *C. antarctica* and *B. subtilis* are successfully cloned into *E. coli* expression host. As the transformants are confirmed, lipase assay was conducted in order to observe the activity of the transformants. However, there is no halozones appeared on the agar. This indicates that the transformants did not have an expression. In order to induce the expressions, 0.4 mM of IPTG was added to the broth medium containing the transformants pET2U-CalB and pET2U-BsubL.

IPTG is a lactose metabolite that will triggers transcription of *lac operon*. Lactose entered *E. coli*, and converted to allolactose, an isomer of lactose. Then, allolactose binds to the lac repressor and releases the tetrameric repressorA from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon to form mRNA, followed by translation of mRNA. The gene coding for beta-galactosidase, a hydrolase enzyme is produced and catalyzes the hydrolysis of galactosides into monosaccharides (IPTG, 2013). Unfortunately, after induced with IPTG, no lipase expression was observed.

In this experiment, *Pseudomonas aeruginosa* has been used as a positive control since most of *P. aeruginosa* strains contain lipase (Mobarak-Qamsari, Kasra-Kermanshahi, & Moosavi-Nejad, 2011). As seen on Figure 4.10, orangish fluorenscene halozone only can be observed on the positive control plate, while, there were no halozone on other transformants *E. coli* plates. Hence, there are several factors that have been emphasized as the cause of no expression such as mutation and codon bias.

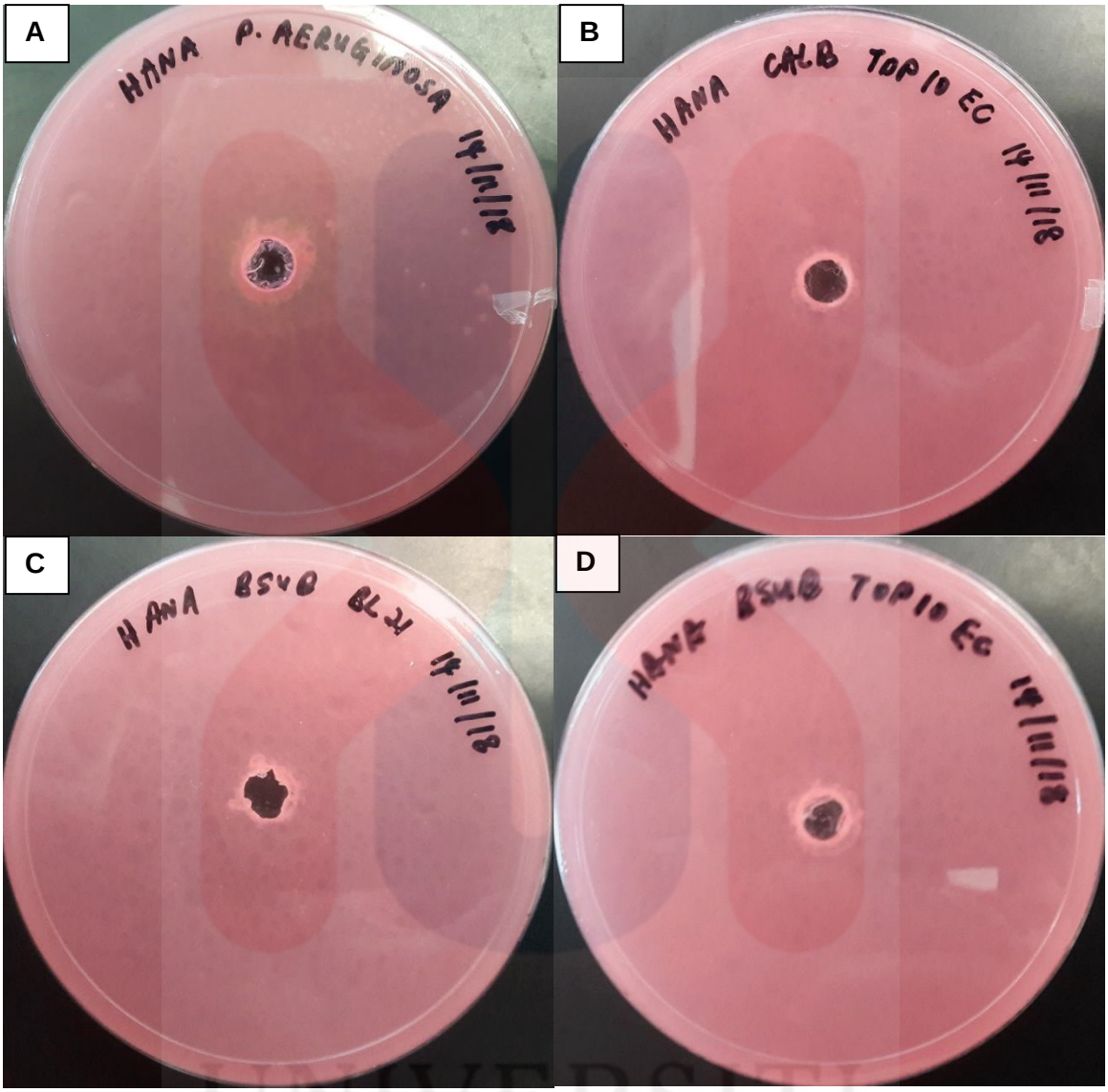

Figure 4.10: Disc-diffusion assay on *E. coli* transformants; (A) Positive control, (B) pET2U-CalB in *E. coli* TOP10 host (C) pET2U-BsubL in *E. coli* BL21 host, (D) pET2U-BsubL in TOP10 *E. coli* host.

Generally, protein expression vector that utilize the bacteriophage T7 promoter system has the capability to produce high level of protein. However, expression does not reliably achieve by this vector as there might be issue such as chromosomal mutation (Vethanayagam & Flower, 2005). The chromosomal mutation will diminish the level of functional level of T7 RNA polymerase, which results in declining expression in the plasmid. Apart from that, codon bias also may be the possibilities that causes of no expression. Codon bias arises when the frequency of occurrence of synonymous codons in the foreign coding DNA is significantly different from that of the host. At the moment of full synthesis of the recombinant protein, depletion of low-abundance tRNAs occurs. This deficiency may lead to amino acid misincorporation and/or truncation of the polypeptide, thus affecting the heterologous protein expression levels (Gustafsson, Govindarajan and Minshull, 2004). Thus, the transformants were sent for DNA sequencing for sequence confirmation.

4.4 DNA Sequence Analysis on Transformants

The *E. coli* transformants harboring pET2U-CalB and pET2U-BsubL were sent to First Base Laboratories Sdn. Bhd. for sequencing using *C. antarctica* and *B. subtilis* forward primers, respectively. The DNA sequence was analyzed using BLAST from National Center of Biotechnology (NCBI). The multiple sequence alignment for pET2U-CalB and pET2U-BsubL are as in (Figure 4.11 and 4.12) respectively;

```
Plasmid         CTCCTCGCTGCCCAGCCGGCGATGGCCCTGCCGAGCGGTAGCGATCCGGCGTTTAGCCAGCCGAAAAGCG
CalB            CTCCTCGCTGCCCAGCCGGCGATGGCCCTGCCGAGCGGTAGCGATCCGGCGTTTAGCCAGCCGAAAAGCG
                ************************************************************************

Plasmid         TTCTGGATGCAGGCCTCACGTGTCAGGGTGCGAGCCCGAGCAGCGTTAGCAAACCGATTCTGCTGGTTCC
CalB            TTCTGGATGCAGGCCTCACGTGTCAGGGTGCGAGCCCGAGCAGCGTTAGCAAACCGATTCTGCTGGTTCC
                ************************************************************************

Plasmid         GGGTACGGGTACGACCGGTCCGCAGAGCTTTGATAGCAATTGGATTCCGCTGAGCACGCAACTCGGCTAT
CalB            GGGTATGGGTACGACCGGTCCGCAGAGCTTTGATAGCAATTGGATTCCGCTGAGCACGCAACTCGGCTAT
                ***** ********************************************************************

Plasmid         ACCCCGTGTTGGATTAGCCCGCCGCCGTTTATGCTGAATGATACCCAGGTGAATACCGAATATATGGTGA
CalB            ACCCCGTGTTGGATTAGCCCGCCGCCGTTTATGCTGAATGATACTCAGGTGAATACCGAATATATGGTGA
                ****************************************** ************************

Plasmid         ATGCGATTACCGCGCTGTATGCGGGTAGCGGTAATAATAAACTGCCGGTGCTGACCTGGAGCCAGGGTGG
CalB            ATGCGATTACCGCGCTGTATGCGGGTAGCGGTAATAATAAACTGCCGGTGCTGACCTGGAGCCAGGGTGG
                ************************************************************************

Plasmid         TCTGGTGGCGCAGTGGGGCCTGACCTTTTTTCCGAGCATTCGTAGCAAAGTGGATCGTCTGATGGCGTTT
CalB            TCTGGTGGCGCAGTGGGGCCTGACCTTTTTTCCGAGCATTCGTAGCAAAGTGGATCGTCTGATGGCGTTT
                ************************************************************************

Plasmid         GCGCCGGATTATAAAGGCACCGTGCTGGCGGGTCCGCTGGATGCGCTGGCGGTGAGCGCGCCGAGCGTGT
CalB            GCGCCGGATTATAAAGGCACCGTGCTGGCGGGTCCGCTGGATGCGCTGGCGGTGAGCGCGCCGAGCGTGT
                ************************************************************************

Plasmid         GGCAGCAGACCACCGGTAGCGCGCTGACCACCGCGCTGCGTAATGCGGGTGGTCTGACCCAGATTGTGCC
CalB            GGCAGCAGACCACCGGTAGCGCGCTGACCACCGCGCTGCGTAATGCGGGTGGTCTGACCCAGATTGTGCC
                ************************************************************************

Plasmid         GACCACCAATCTGTATAGCGCGACCGATGAAATTGTGCAGCCGCAGGTGAGCAATAGCCCGCTGGATTCG
CalB            GACCACCAATCTGTATAGCGCGACCGATGAAATTGTGCAGCCGCAGGTGAGCAATAGCCCGCTGGATTCG
                ************************************************************************

Plasmid         AGCTATCTGTTTAATGGTAAAAACGTTCAAGCACAGGCGGTGTGTGGTCCGCTGTTTGTGATTGATCATG
CalB            AGCTATCTGTATAATGGTAAAAACGTTCAAGCACAGGCGGTGTGTGGTCCGCTGTTTGTGATTGATCATG
                ********** ************************************************************

Plasmid         CGGGTAGCCTGACCAGCCAGTTTAGCTATGTGGTGGGTCGTTCGGCACTGCGTAGCACCACCGGCCAGGC
CalB            CGGGTAGCCTGACCAGCCAGTTTAGCTATGTGGCGGGCCGTTCGGCACTGCGTAGCACCACCGGCCAGGC
                ******************************** *** ******************************

Plasmid         GCGTAGCGCGGATTATGGCATTACCGATTGTAATCCGCTGCCGGCGAATGATCTGACCCCGGAACAGAAA
CalB            GCGTAGCGCGGATTATGGCATTACCGATTGTAATCCGCTGCCGGCGAATGATCTGACCCCGGAACAGAAA
                ************************************************************************

Plasmid         GTTGCCGCTGCTGCCCTGCTGGCTCCAGCGGCGGCAGCCATTGTGGCGGGTCCGAAACAGAATTGTGAAC
CalB            GTTGCCGCTGCTGCCCTGCTGGCTCCAGCGGCGGCAGCCATTGTGGCGGGTCCGAAACAGAATTGTGAAC
                ************************************************************************

Plasmid         CGGATCTGATGCCGTATGCGCGTCCGTTTGCGGTGGGTAAACGTACCTGTAGCGGTATTGTGACCCCGTA
CalB            CGGATCTGATGCCGTATGCGCGTCCGTTTGCGGTGGGTAAACGTACCTGTAGCGGTATTGTGACCCCGTA
                ************************************************************************

Plasmid         ATAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGG
CalB            ATAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGG
                ****************************************
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Figure 4.11: Nucleotide sequence alignment of the pET2U-CalB transformants (plasmid) with CalB gene (CalB). The region highlighted in yellow represents 97.5% identity.

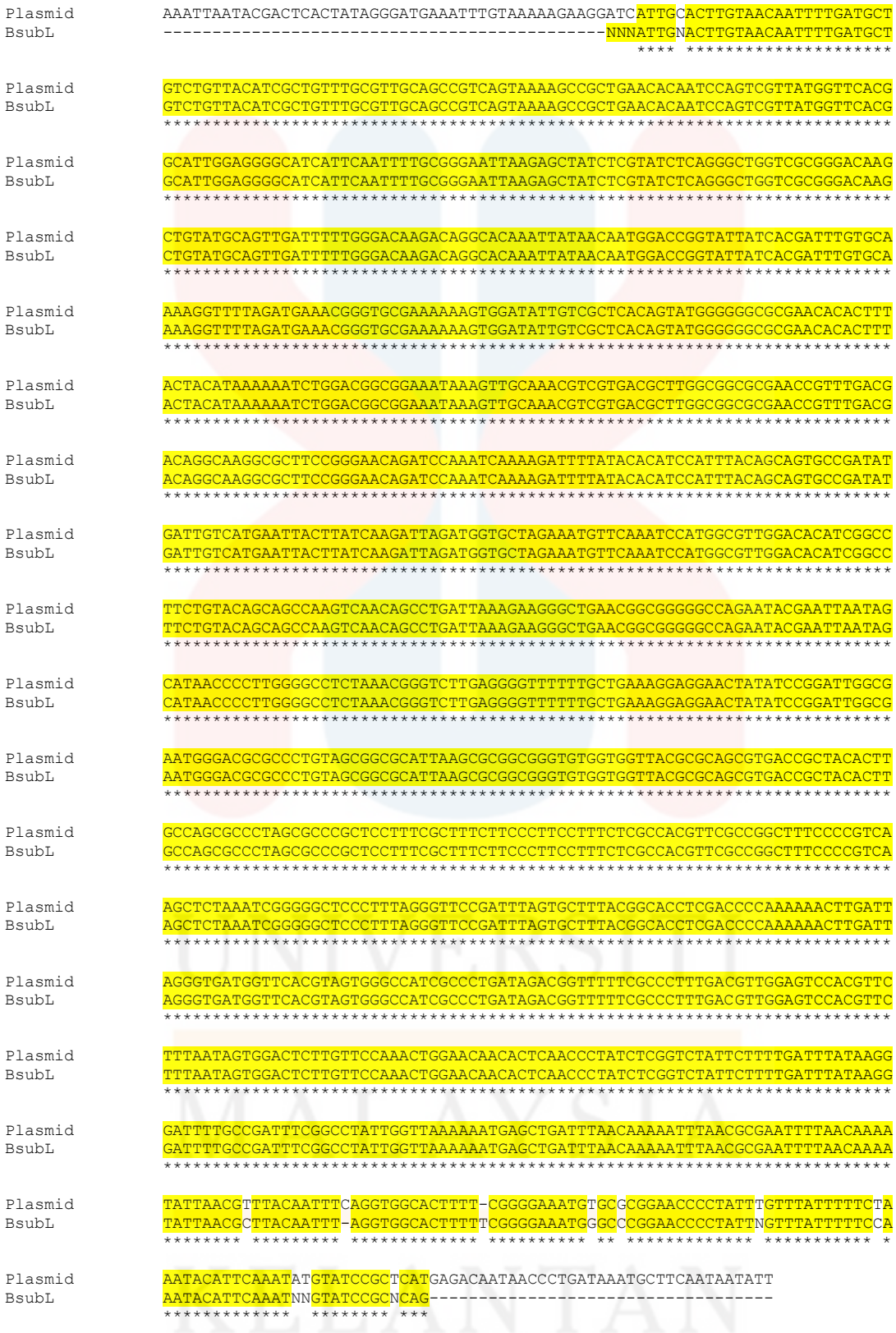

Figure 4.12: Nucleotide sequence alignment of the pET2U-BsubL transformants (plasmid) with BsubL gene (BsubL). The region highlighted in yellow represents 98.54% identity.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

With the advent of DNA recombinant technologies, cloning and expression of numerous genes in different system have been explored and improved. *E. coli* are the most attractive heterologous protein expression due to its cost efficiency, simple and rapid growth, while, yeast recombination is an improved technique to increase the DNA transformation efficiency. In this study, *Candida antarctica* Lipase B genes and *Bacillus subtilis* lipase gene were homologously recombined in *S. cerevisiae* and expressed in *E. coli* host. The homologous recombination of both genes into *E. coli* are successful as the analytical PCR and analytical digestion shows the expected sizes when visualized on 1% agarose gel. However, the heterologous expressions were not observed. In agar-diffusion assay, no activity or halozone from all the transformants *E. coli* were observed on the lipase agar even after induced with 0.4 mM of IPTG. This can be concluded that there is no expression among the transformants. For further confirmation, the recombinant *E. coli* harboring pET2U-CalB and pET2U-Bsubs were sent for sequencing. Result from the sequencing shows that CalB has the identity of 97.50% and Bsubs has the identity of 98.54%. Hence, there are no problems with the insertion of the lipase genes into the expression host, pET2U vector.

C. antarctica lipase B and *B. subtilis* lipase is a potential candidates for the biotechnological applications (Ujiie et al., 2015; Treichel et al., 2010). Therefore, further research on the expression of both lipase genes need to be done. As the cloning is successful, there might be problem with the expression host. For next research, trials can be done towards the temperature of incubation during induction of IPTG. Different range of temperatures comprise of 20 °C, 30°C and 37 °C can be done to each recombinant *E. coli* with the same concentration of IPTG. From this, we can identify the best temperature for lipase activity of both transformats (Ujiie et al., 2015). Apart from that, different concentration of IPTG also can be done in order to determine the best inducer concentration for lipase production. According to previous study, IPTG concentration used to induce gene expression range from 0.005 to 5.0 mM. However, strong induction can cause the formation of inclusion bodies as well as increase the death rate of host cells (Donovan, Robinson & Glick, 1996). Apart from that, longer induction time also may cause the nutrient content in the culture exhausted. For this reason, it is important to balance the induction capacity during the expression of the lipase gene. Finally, further study on the expression level of the inserted genes also should be carried out.

REFERENCES

Abdelmoez, W., & Mustafa, A. (2014). Oleochemical Industry Future through Biotechnology. *Journal of Oleo Science*, *63*(6), 545–554. https://doi.org/10.5650/jos.ess14022

Andualema, B., & Gessesse, A. (2012). Microbial lipases and their industrial applications. *Biotechnology*, *11*(3), 100-118.

Anobom, C. D., Pinheiro, A. S., De-Andrade, R. A., Aguieiras, E. C. G., Andrade, G. C., Moura, M. V, & Freire, D. M. (2014). From structure to catalysis: recent developments in the biotechnological applications of lipases. *BioMed Research International*, *2014*, 684506. https://doi.org/10.1155/2014/684506

Aravindan, R. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology*, *6*(April), 141–158. Retrieved from http://nopr.niscair.res.in/handle/123456789/3016

Baneyx, F. (1999). Recombinant protein expression in Escherichia coli. *Curr.Opin.Biotechnol.*, *10*(5), 411–421. https://doi.org/S0958-1669(99)00003-8 [pii]

Bordes, F., Barbe, S., Escalier, P., Mourey, L., André, I., Marty, A., & Tranier, S. (2010). Exploring the conformational states and rearrangements of Yarrowia lipolytica lipase. *Biophysical Journal*, *99*(7), 2225–2234. https://doi.org/10.1016/j.bpj.2010.07.040

Carvalho, N. B., De Souza, R. L., De Castro, H. F., Zanin, G. M., Lima, Á. S., & Soares, C. M. F. (2008). Sequential production of amylolytic and lipolytic enzymes by bacterium strain isolated from petroleum contaminated soil. *Applied Biochemistry and Biotechnology*, *150*(1), 25–32. https://doi.org/10.1007/s12010-008-8194-3

Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiology Reviews*, *24*(1), 45–66. https://doi.org/S0168-6445(99)00029-7 [pii]

Chancharoonpong, C., Hsieh, P. C., & Sheu, S. C. (2012). Production of enzyme and growth of Aspergillus oryzae S. on soybean koji. *International Journal of Bioscience, Biochemistry and Bioinformatics*, *2*(4), 228.

Chen, R. (2012). Bacterial expression systems for recombinant protein production: E. coli and beyond. *Biotechnology Advances*, *30*(5), 1102–1107. https://doi.org/10.1016/j.biotechadv.2011.09.013

Dickschat, J. S., Pahirulzaman, K. A. K., Rabe, P., & Klapschinski, T. A. (2014). An Improved technique for the rapid chemical characterisation of bacterial terpene cyclases. *ChemBioChem*, *15*(6), 810–814. https://doi.org/10.1002/cbic.201300763

Donovan RS, Robinson CW, Glick BR (1996). Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. *Journal of Industrial Microbiology and Biotechnology*. 16: 145-154

Gandhi, N. N. (1997). Applications of lipase. *Journal of the American Oil Chemists' Society*, *74*(6), 621–634. https://doi.org/10.1007/s11746-997-0194-x

Gao, B., Su, E., Lin, J., Jiang, Z., Ma, Y., & Wei, D. (2009). Development of recombinant Escherichia coli whole-cell biocatalyst expressing a novel alkaline lipase-coding gene from Proteus sp. for biodiesel production. *Journal of Biotechnology*, *139*(2), 169-175.

Gewin, V. (2013). News in focus. *Nature*, *495*(7438), 13–14. https://doi.org/10.1038/509015a

Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols*, *2*(1), 31–34. https://doi.org/10.1007/978-1-4939-0799-1_4

Gietz, R. D., Schiestl, R. H., Willems, A. R., & Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast*, *11*(4), 355–360. https://doi.org/10.1002/yea.320110408

Gietz, R. D., & Woods, R. A. (2001). Genetic transformation of yeast. *BioTechniques*, *30*(4), 816–831. https://doi.org/4120016

Gietz, R. D., & Woods, R. A. (2007). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in Enzymology*, *350*(2001), 87–96. https://doi.org/10.1016/S0076-6879(02)50957-5

Gomes, A. R., Byregowda, S. M., Veeregowda, B. M., & Balamurugan, V. (2016). An overview of heterologous expression host systems for the production of recombinant proteins. *Advance in Animal and Veterinary Sciences*, *4*(7), 346-356.

Guo, S., Xu, J., Pavlidis, I. V., Lan, D., Bornscheuer, U. T., Liu, J., & Wang, Y. (2015). Structure of product-bound SMG1 lipase: Active site gating implications. *FEBS Journal*, *282*(23), 4538–4547. https://doi.org/10.1111/febs.13513

Gupta, R., Kumari, A., Syal, P., & Singh, Y. (2015). Molecular and functional diversity of yeast and fungal lipases: Their role in biotechnology and cellular physiology. *Progress in Lipid Research*, *57*(January), 40–54. https://doi.org/10.1016/j.plipres.2014.12.001

Gustafsson, C., Govindarajan, S. and Minshull, J.(2004). Codon bias and heterologous protein expression. *Trends Biotechnology* 22, 346–353. doi:10.1016/j.tibtech.2004.04.006

Hermansyah, H., Wijanarko, A., Dianursanti, D., Gozan, M., Wulan, P. P., Arbianti, R., & Shibasaki-Kitakawa, N. (2010). Kinetic model for triglyceride hydrolysis using lipase. *Makara Journal of Technology*, *11*(1), 30-35.

Høegh, I., Patkar, S., Halkier, T., & Hansen, M. T. (1995). Two lipases from Candida antarctica: cloning and expression in Aspergillus oryzae. *Canadian Journal of Botany*, *73*(S1), 869-875.

Hua, S. B., Qiu, M., Chan, E., Zhu, L., & Luo, Y. (1997). Minimum Length of Sequence Homology Required forin VivoCloning by Homologous Recombination in Yeast. *Plasmid*, *38*(2), 91-96.

IPTG (January 8, 2013). IPTG: Triggers the transcription of lac operon. retreived on 28 November 2018 from http://agscientific.com/blog/2013/01/iptg-triggers-the-transcription-of-the-lac-operon/

Jaeger, K.-E., Dijkstra, B. W., & Reetz, M. T. (1999). Bacterial Biocatalysts: Molecular Biology, Three-Dimensional Structures, and Biotechnological Applications of Lipases. *Annual Review of Microbiology*, *53*(1), 315–351. https://doi.org/10.1146/annurev.micro.53.1.315

Jaeger, K. -E, Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M., & et, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, *15*(1), 29–63. https://doi.org/10.1111/j.1574-6976.1994.tb00121.x

Joska, T. M., Mashruwala, A., Boyd, J. M., & Belden, W. J. (2014). A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile. *Journal of Microbiological Methods*, *100*, 46-51.

Kiran GS, Shanmughapriya S, Jayalakshmi J, Selvin J, Gandhimathi R, Sivaramakrishnan S, Arunkumar M, Thangavelu T and Natarajaseenivasan K. (2008). Optimization of extracellular psychrophilic alkaline lipase produced by marine Pseudomonas sp. (MSI057). *Bioprocess and Biosystems Engineering*, *31*(5), 483–492. https://doi.org/10.1007/s00449-007-0186-0

Kung, S. H., Retchless, A. C., Kwan, J. Y., & Almeida, R. P. P. (2013). Effects of DNA size on transformation and recombination efficiencies in xylella fastidiosa. *Applied and Environmental Microbiology*, *79*(5), 1712–1717. https://doi.org/10.1128/AEM.03525-12

Kurtovic, I., Marshall, S. N., Zhao, X., & Simpson, B. K. (2009). Lipases from mammals and fishes. *Reviews in Fisheries Science*, *17*(1), 18–40. https://doi.org/10.1080/10641260802031322

Ma, S., Tang, N., & Tian, J. (2012). DNA synthesis, assembly and applications in synthetic biology. *Current Opinion in Chemical Biology*, *16*(3–4), 260–267. https://doi.org/10.1016/j.cbpa.2012.05.001

Madzak, C. (2015). Yarrowia lipolytica: recent achievements in heterologous protein expression and pathway engineering. *Applied Microbiology and Biotechnology*, *99*(11), 4559–4577. https://doi.org/10.1007/s00253-015-6624-z

Mehta, A., Bodh, U., & Gupta, R. (2017). Fungal lipases: A review. *Journal of Biotech Research*, *8*(1), 58–77.

Mobarak-Qamsari, E., Kasra-Kermanshahi, R., & Moosavi-Nejad, Z. (2011). Isolation and identification of a novel, lipase-producing bacterium, Pseudomnas aeruginosa KM110. *Iranian Journal of Microbiology*, *3*(2), 92.

Orr-Weaver, T. L., & Szostak, J. W. (1985). Fungal recombination. *Microbiological Reviews*, *49*(1), 33.

PRNewswire (2018). Global Industrial Enzymes Market 2016-2018 & 2025. Retrieved on 23 April 2018 from https://www.prnewswire.com/news-releases/global-industrial-enzymes-market-2016-2018--2025-300723583.html

Ranaldi, S., Belle, V., Woudstra, M., Bourgeas, R., Guigliarelli, B., Rocher, P., & Fournel, A. (2010). Amplitude of pancreatic lipase lid opening in solution and identification of spin label conformational subensembles by combining continuous wave and pulsed EPR spectroscopy and molecular dynamics. *Biochemistry*, 49(10), 2140–2149. https://doi.org/10.1021/bi901918f

Sindhu, R., Binod, P., Sabeela, B., Amith, A., Anil, K. M., Aravind, M., Sharrel, R., and Ashok, P., (2018) Applications of Microbial Enzymes in Food Industry. *Food Technology and Biotechnology*. Vol. 56 (1). doi: 10.17113/ftb.56.01.18.5491

Sharma, R., Chisti, Y., Chand, U., & Banerjee, U. C. (2001). Production, Purification, Characterization, and Applications of Lipases. *Biotechnology Advances*, *19*(8), 627–662. https://doi.org/10.1016/S0734-9750(01)00086-6

Shurtleff, W., & Aoyagi, A. (2001). History of Tempeh and Tempeh Products (1815-2011): Extensively Annotated Bibliography and Sourcebook. Soyinfo Center.

Snustad DP & Simmons MJ (2010). Principles of Genetics, Wiley 5th Edition, Wiley, New Jersey

Takeuchi, S., Geraldine, J., & Mala, S. (2008). Understanding Structural Features of Microbial Lipases—An Overview. *Analytical Chemistry Insights*, *3*, 9–19.

Treichel, H., de Oliveira, D., Mazutti, M., Di Luccio, M., Oliveira, J.V., 2010. A review on microbial lipases production. *Food Bioprocess Technology*. 3, 182–196.

Ujiie, A., Nakano, H., & Iwasaki, Y. (2016). Extracellular production of Pseudozyma (Candida) antarctica lipase B with genuine primary sequence in recombinant Escherichia coli. *Journal of Bioscience and Bioengineering*, 121(3), 303–309. https://doi.org/10.1016/j.jbiosc.2015.07.001

Vethanayagam, J. G., & Flower, A. M. (2005). Decreased gene expression from T7 promoters may be due to impaired production of active T7 RNA polymerase. *Microbial Cell Factories*, *4*(1), 3.

Vikash Kumar Dubey (n. d.) Lecture 27: Agarose Gel Electrophoresis for DNA analysis. *Proteomics & Genomics*. Retrieved on 8 December 2018 from https://nptel.ac.in/courses/102103017/module27/lec27_slide1.htm

Villeneuve, P. (2003). Plant lipases and their applications in oils and fats modification. *European Journal of Lipid Science and Technology*, *105*(6), 308–317. https://doi.org/10.1002/ejlt.200390061

Vulfson, E. N., Woolley, P. & Peterson, S. B. (2011). Chapter 13: Industrial applications of Lipase. United States of America. Cambridge University Press.

Yang, J.-K., Liu, L.-Y., Dai, J.-H., & Li, Q. (2013). de novo Design and Synthesis of Candida antarctica Lipase B Gene and α-Factor Leads to High-Level Expression in Pichia pastoris. *PLoS ONE*, *8*(1), e53939. https://doi.org/10.1371/journal.pone.0053939