UNIVERSITI PUTRA MALAYSIA

CLONING THE PVUII ISOSCHIZOMER RESTRICTION MODIFICATION SYSTEM FROM JMH 1 (4) SM CLONE INTO ESCHERICHIA COLI

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CLONING THE *Pvu*II ISOSCHIZOMER RESTRICTION MODIFICATION SYSTEM FROM JMH 1(4) SM CLONE INTO *Escherichia coli*

By

LEE SWEE LING

A Project Report Submitted in Partial Fulfillment of the Requirement for the
Degree of Bachelor of Bioindustry Science in the
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DEDICATED TO

MY BELOVED FATHER
MY BELOVED MOTHER
MY FAMILY MEMBERS
& MY FIANCE
FOR THE SUPPORT AND CARE

ABSTRACT

Cloning the *Pvu*II isoschizomer restriction modification system from JMH 1(4) SM into *Escherichia coli* was studied. The presence of the isoschizomer for *Pvu*II in bacteria strain JMH 1(4) SM was verified. Selected commercially available restriction enzymes were used to construct restriction pattern of bacteria strain JMH 1(4) SM. DNA fragments of 2-5 kb DNA were obtained by digesting genomic DNA of bacteria strain JMH 1(4) SM by using selected restriction enzymes *Eco*RI, *Hin*dIII and *Fau*NDI. The DNA fragments were ligated into the linear plasmid pET23a (+) and transformed into TOP10F' *Escherichia coli*. The recombinant plasmids were extracted and digested with lysate of bacteria strain of JMH 1(4) SM to verify the presence of methylase gene. However, the presence of methylase activity was not confirmed by further transformations.

ABSTRAK

Kajian tentang mengklonkan isoschizomer *Pvu*II bagi sistem restriksi modifikasi daripada strain bakteria JMH 1(4) SM telah dijalankan. Kehadiran *isoschizomer Pvu*II dalam strain bakteria JMH 1(4) SM telah dikenalpastikan. Pelbagai jenis enzim restriksi komersial yang dipilih untuk memotong JMH 1(4) SM untuk mendapatkan pola restriksi. Fragmen DNA bersaiz 2-5 kb telah didapati dengan pemotongan ke atas genomik DNA bagi bakteria strain JMH 1(4) SM dengan menggunakan enzim restriksi yang dipilih iaitu *Eco*RI, *Hin*dIII and *Fau*NDI. Fragmen DNA telah digabungkan dengan plasmid pET23a (+) linear dan transfomasi ke dalam TOP10F' *Escherichia coli*. Rekombinan plasmid telah diekstrak dan dipotong dengan menggunakan lysate bakteria JMH 1(4) SM untuk mengenalpasti kehadiran gen methylase. Walau bagaimanapun, kehadiran aktiviti methylase tidak dapat dikenalpasti dengan transformasi selanjutnya.

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APPROVAL SHEET

I certify that this research project report entitled "Cloning of *Pvu*II Isoschizomers Restriction-modification System from JMH 1(4) SM Clone into *Escherichia coli*" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Bioindustry Science in the Faculty of Agriculture and Food Sciences, University Putra Malaysia Bintulu Campus.

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LIST OF ABBREVIATIONS

bp	Base-pair
BSA	Bovine Serum Albumin
CIAP	Calf Intestine Alkaline Phosphatase
EDTA	Ethylenediaminetetraacetic acid, Tetrasodium Salt
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent Assay
kb	Kilo-base-pair
LB	Luria Bertani
SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA

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CHAPTER 1

INTRODUCTION

1.1 Background

Biotechnology is the use of technology to control biological process to meet the societal needs. One can control the biological process when a cell copies its DNA every time it divides. Biotechnology is so varied, such as transgenic biotechnology, the use of biotechnology to establish criminals, of crime victims in forensic biotechnology, pharmaceuticals, genetic engineering and gene cloning. There are two important biotechnology tools to help in biological process, which are the restriction enzymes and the cloning vector such as plasmids.

Genetic engineering is getting more popular with the development of recombinant DNA technology. There are 3 major scientific developments that occurred roughly at the same time which can be considered as the cornerstones of the technology. The first step was the ability to introduce DNA into *Escherichia coli* which was known as transformation and subsequently selection of the transformed bacteria. The second was the ability to purify plasmid DNA in high yield. And the third was the discovery and purification of restriction enzyme. By using just three simple procedures, restriction enzyme can be produced.

In this study, the possibility to clone the *Pvu*II restriction endonuclease isoschizomer from bacteria strain JMH 1(4) SM into *Escherichia coli* was studied. The JMH 1(4) SM is a mesophilic bacteria grow at RT and was isolated from soil in Jementah, Johor. It was shown to contain *Pvu*II isoschizomer restriction

endonuclease. *Pvu*II restriction enzyme recognized 5'-CAG$^{\downarrow}$CTG-3' and cleave the DNA after the G to yield blunt ends, where as for isoschizomer from JMH 1(4) SM has not be characterized.

1.2 Objectives

The objectives of this study are listed as below:

1. Verification of *Pvu*II isoschizomer activity in JMH 1(4) SM.
2. Study of the restriction pattern of the genomic DNA from bacteria strain JMH 1(4) SM by using selected restriction enzymes.
3. Cloning of *Pvu*II isoschizomer restriction-modification system into plasmid pET23a (+).

CHAPTER 2

LITERATURE REVIEW

2.1 History

The first discovery of restriction enzymes was done by Werner Arber, Swiss microbiologist in 1968 (Robert and Cheng, 2002). In early 1970, Dan Nathans also discovered the restriction modification system by studying the genetics of bacteriophage or host interactions and the phenomenon of host resistance to phage infection (Nathans and Smith, 1975). *E.coli* greatly reduced the ability of bacteriophages to form plagues while the term "restriction" comes from the observation (Bertani and Weigle, 1953; Luria and Human, 1952). The protection of methylation involved transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) (Kuhnlein and Arber, 1972).

Hamilton O. Smith had discovered type II restriction enzymes, enzymes which cleave within a short symmetrical sequence (Smith and Wilcox, 1970). Daniel Nathans, an American molecular biologist who continued Smith's work, had demonstrated that type II enzymes could be useful in genetic studies and helped advance the DNA recombinant technique in the 1970s (Danna and Nathans, 1971). In 1978, the Nobel Prize in Physiology or Medicine was awarded to Werner Arber, Hamilton Smith and Daniel Nathans for the discovery of restriction enzymes and their application to problems of molecular genetics (Robert and Cheng, 2002).

2.2 Standard Nomenclature for Restriction Enzymes

Dr. Smith and Nathans suggested the now standard nomenclature for restriction enzymes which is according to the bacteria which they are derived. For example, *Bam*HI was the first restriction enzyme found in *Bacillus amyyloliquefaciens* strain H. The first letter '*B*' of *Bam*HI designates the genus '*Bacillus*' while the lower case letter '*am*' designate the species '*amyyloliquefaciens*'. H is representing the strain H (Smith and Nathan, 1973). For other example, *Eco*RI was isolated from *Escherichia coli* (strain RY13), *Hind*II and *Hind*III from *Haemophilus influenzae*, and *Xho*I from *Xanthomonas holcicola.*

2.3 Restriction-Modification Systems (RMS)

Restriction-modification systems (RMS) had been found in a wide variety of bacteria and the type II restriction endonucleases are an important tool in the field of molecular biology (Harumi *et al.*, 1996). Every bacterium has its own RMS. RMS consists of two components which are restriction endonuclease and modification-methylase. Restriction endonucleases break DNA by hydrolyzing the phosphodiester backbone on both strands, while modification methylase blocking the cleavage of restriction activity by adding a methyl group within the recognition sequences. RMS is the mechanism in bacteria to protect the cells from infection of foreign DNA molecules such as virus (Kwoh *et al.*, 1988). RMS possesses some of the properties that promote the survival of bacteria where this behavior can be described as having selfish behavior (Dawkins, 1989; Kobayashi, 2001).

There are five types of RMS which are Type I, Type II, Type IIS, Type III, and Type IV. These are divided based on enzyme subunit composition, co-factor

requirements, DNA specificity characteristics and reaction products (Wilson and Murray, 1991). Type II RMS is the simplest sequence-specific DNA enzymes used in creating recombinant DNA molecules (Pei Chung Hsieh *et al.*, 2000). Among the 232 different specificities, nearly half of the restriction modification system have been cloned and expressed (Robert and Macelis, 1998; Wilson, 1991). They usually consist of two independent polypeptides, a restriction endonuclease which cleaves DNA and a corresponding DNA methyltransferase (methylase), which protects endogenous DNA from endonuclease digestion by methylating the endonuclease recognition sequence (Nwankwo *et al.*, 1994). Type II restriction endonucleases recognize and cleave precisely within specific nucleotide sequences or very close to their recognition sequences (Roberts, 1990).

2.3.1 Restriction Endonuclease

Restriction endonucleases are enzymes that recognize and bind the recognized sequence of the foreign DNA before it cleaves within the recognition sequence at internal phosphodiester bond. Different restriction endonucleases have different affinity for different recognition sequences and will usually recognize 4-8 nucleotides of the recognition sequences and palindromic (Roberts and Macelis, 1996; Szybalski *et al.*, 1991).

2.3.2 Modification Metlylase

Methylase modification enables the bacteria to protect their own DNA by distinguishing it from foreign infecting DNA (Kelly and Smith, 1970). It chemically modifies the sequences by adding a methyl group ($-CH_3$) to the DNA sequences.

This methylation process will prevent the restriction endonuclease from cleaving their own modified DNA and cleave the unmodified DNA (Kobayashi, 2001).

2.4 Application

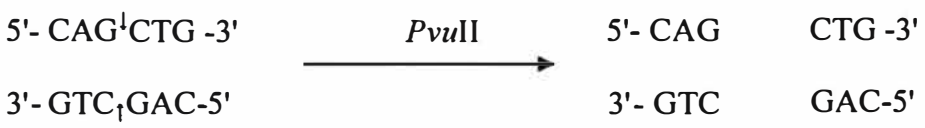
Restriction and modification system are very useful in genetic engineering and molecular biology. According to information from New England Biolabs, over 10,000 bacteria and archebacteria have been screened for restriction enzymes and more than 200 different sequence recognition specificities have been found (New England Biolabs Catalogue, 2001).

Restriction endonucleases are primary tools for molecular cloning. *Escherichia coli* had been manipulated to produce protein products of the cloned gene which was known as genetic engineering. Cloned genes can be used to transform a bacterium to produce useful phenotype such as in the development of bioremediation approaches that enable bacteria to clean up toxic chemicals resulting from environmental accidents (USEPA, 2000). Moreover, mutated genes had been introduced in transgenic animals to generate gene "knockouts" to mimic human disease in bioremedical research (Miesfeld, 1999). In addition, gene therapy using cloned genes to treat disease and improve human health is becoming important although in its infancy (Templeton and Lasic, 2000).

2.5 Isoschizomer for *Pvu*II Restriction Endonuclease

The *Pvu*II restriction endonuclease was discovered in *Proteus vulgaris*, is a rod-shaped Gram negative bacterium (a chemoheterotroph) and falcultative

anaerobes that inhabits in the intestinal tracts of animals and it can be pathogenic. *Pvu*II restriction endonuclease recognizes the DNA sequence as listed below:



The vertical arrows show the cleavage within the sequence after the G to yield blunt ends. *Pvu*II cuts neither CAGm4CTG nor CAGm5CTG (Manda and Robert, 2000). The *Pvu*II DNA methyltransferase (M *Pvu*II) generates N^4-methylcytosine at the internal C (Blumenthal, 1985; Butkus *et al.,* 1987), the *Pvu*II restriction endonuclease cleaves between the central two bases (Gingeras and Roberts, 1981) and the genes *Pvu*IIM and *Pvu*IIR are divergently oriented with respect to one another.

The isoschizomer is a group of restriction modifications that recognize the same recognition sequence but are isolated from different microbial sources. Restriction endonuclease isoschizomer may or may not cleave in the exact sequence as the compared restriction endonuclease. The modification methylase isoschizomer also may not chemically modify the same nucleotide sequences. There are two types of isoschizomer restriction endonuclease. Homoisochizomer restriction endonucleases recognize similar nucleotide sequences and cleave the sequence at the same site while neoscizomer cleaves at different sites (Hamablet, 1989).

CHAPTER 3

METHODOLOGY

3.1 Research Materials

3.1.1 *Pvu*II Isoschizomer Bacterial Strains: JMH 1(4) SM

The bacteria strain used is JMH 1(4) SM was provided by Dr. Chee Hui Yee from Vivantis Technologies. The JMH 1(4) SM was shown contain isoschizomer for *Pvu*II. This is a mesophilic bacteria which grows at room temperature and was isolated from soil samples in Jementah, Johor.

3.1.2 Competent *Escherichia coli*: Top 10F'

Escherichia coli used was TOP10F' with genotype F'[lacI^q Tn10 (tetR)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 λ-. The cells were grown at 37 °C in LB media with tetracycline to retain plasmid which contain F´ episome with tetracycline resistance gene and allows isolation of ssDNA from vector that have an f1 origin of replication. In addition, the F' carries the lacI^q repressor to inhibit transcription from lac promoter. This strain was also provided by Dr. Chee Hui Yee.

3.1.3 Plasmids

Plasmid used was pET23a (+) which was provided by Dr. Chee Hui Yee. The pET-23a-d (+) vectors carry an N-terminal T7•Tag® sequence and an optional C-terminal His•Tag® sequence. The pET-21a-d (+) contain the "plain" T7 promoter instead of the T7lac promoter and with the absence of the lacI gene. Unique sites are shown on the circle map (Figure 1). Since the sequence is numbered by the pBR322

convention, the T7 expression region is reversed on the circular map. Beside that, the cloning or expression region of the coding strand transcribed by T7 RNA polymerase is as shown. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand.

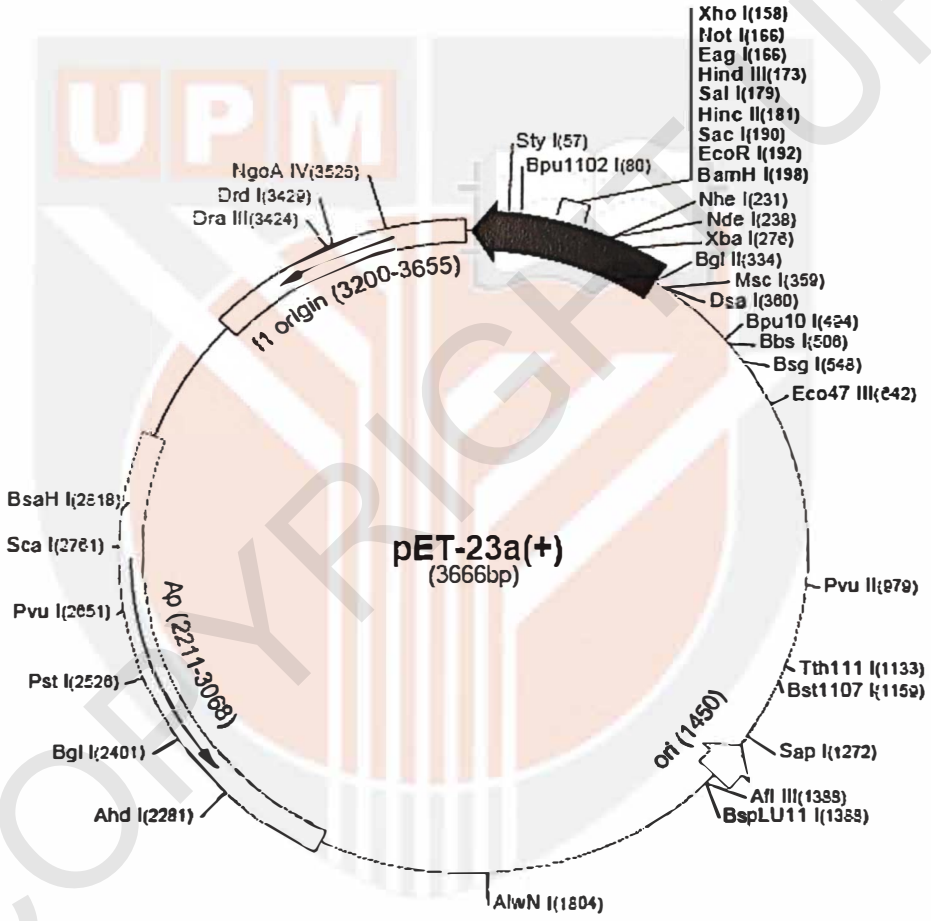


Figure 1: pET-23a (+) (adapted from Novagen)

3.1.4 Restriction Enzymes

Various Restriction Enzymes used were from Vivantis Technologies (Malaysia) and Fermentas Inc. (USA). Selective restriction enzyme was used to

perform at multiple cloning sites on plasmid pET23a (+) which include *Bgl*I, *Cci*NI, *Eco*RI, *Fau*NDI, *Hin*dIII, *Pst*I, *Sal*I and *Xba*I. They have single cutting site on plasmid pET23a (+). The other restriction enzymes that have been used in restriction pattern analysis include *Ava*II, *Pvu*I, *Bme*18I, *Bsn*I, *Bsp*19I, *Bss*MBI, *Eco*RV, *Kpn*I, *Sfi*274I and *Sma*I. They perform more than one cutting site at the multiple cloning sites on plasmid pET 23a (+). All the restriction digestions were done at suitable conditions suggested by the manufacturer.

3.1.5 Antibiotic Selection

Selected antibiotics from AMRESCO in appropriate concentrations were used when needed such as Ampicilin (50 mg/mL), Chloramphenicol (34 mg/mL), Kanamycin sulphate (10 mg/mL), Streptomycin (10 mg/mL) and Tetracycline Hydrochloride (5 mg/mL). The concentrations of antibiotic used were according standard protocol (Sambrook and Russel, 2001).

3.1.6 Kits

3.1.6.1 GF-1 Bacterial DNA Extraction Kits

The GF-1 Bacterial Extraction Kits perform rapid and efficient extraction purification of high molecule weight genomic DNA from either Gram-negative or Gram-positive bacteria. This kit contains GF-1 columns, 2 mL collection tubes, resuspension buffer 1 (Buffer R1), resuspension buffer 2 (Buffer R2), bacterial genomic binding buffer (Buffer BG), wash buffer (concentration needed to diluted with absolute ethanol as suggested by manufacturer) and elution buffer (10 mM Tris-HCl, pH8.5) from Vivantis Technologies.

3.1.6.2 GF-1 Plasmid DNA Extraction Kits

The GF-1 Plasmid DNA Extraction Kits perform rapid and efficient extraction of high copy and low copy plasmid DNA from bacteria without the need for precipitation or organic extractions. This kit contains GF-1 columns, 2 mL collection tubes, solution 1 (S1) where S1 needs to store at 2°C to 8°C after the addition of RNase A, solution 2 (S2), neutralising buffer (Buffer NB), wash buffer (diluted with absolute ethanol as suggested by manufacturer), RNase A (DNase Free) and elution buffer (100 mM Tris-HCl, pH 8.5) from Vivantis Technologies.

3.1.6.3 GF-1 DNA Gel Recovery Kits

The GF-1 DNA Gel Recovery Kits perform rapid purification of DNA bands ranging from 100bp to 10kb from all grades of agarose gel in TAE (Tris-acetate/EDTA) or TBE (Tris-borate/EDTA). This kit contains GF-1 columns, 2 mL collection tubes, gel DNA binding buffer (Buffer GB), wash buffer (diluted with absolute ethanol as suggested by manufacturer) and elution buffer (10 mM Tris-HCl, pH 8.5) from Vivantis Technologies.

3.2 Research Methods

3.2.1 Maintenance and Stab Culture

All the provided bacteria strains were grown weekly by culturing onto a selective medium with added selective antibiotic at appropriate concentration if necessary, whereas the stab cultures were prepared to maintain the master culture. Bacteria strain JMH 1(4) SM was subcultured weekly in LB/4 medium (2.5 g/L tryptone soya broth from OXIOD, 1.25 g/L yeast extract from OXIOD, 2.5 g/L NaCl from R&M Chemical, and 15 g/L Agar from DIFCO). TOP10F' *Escherichia coli* was cultured in LB medium (10 g/L typtone from OXIOD, 5 g/L yeast extract from OXIOD, 10 g/L NaCl from R&M Chemical, and 15 g/L Agar from DIFCO) with tetracycline. TOP10F' with pET23a (+) was cultured in LB medium with Ampicilin (50mg/mL, stock concentration).

Stab cultures were prepared according to QIAGEN guidelines. Bacteria strain can be stored in stab culture for up to 1 year. Briefly, LB/4 agar was prepared and autoclaved. Then, LB/4 agar was cooled to below 50°C. A total of 1 mL agar was poured into a 2mL screw-cap vial under sterile condition and left to solidify. Then, a single colony from freshly streaked plate was picked and stabbed it deep down into the agar several times using an inoculation loop. The vial with the cap slightly loosened was incubated at 37°C for 8 to 12 hours. After that, the vials were sealed with parafilm and stored at 4°C.

Bacteria were sub-cultured by streaking on LB or LB/4 agar plate. Antibiotic as added if necessary. A single colony was picked and streaked on agar plate using

inoculation loop. The plate was placed up-side down and incubated at 37°C for 8-12 hours. After that, the plates were sealed with parafilm and stored at 4°C.

3.2.2 Strain Verification for Isoschizomer *Pvu*II

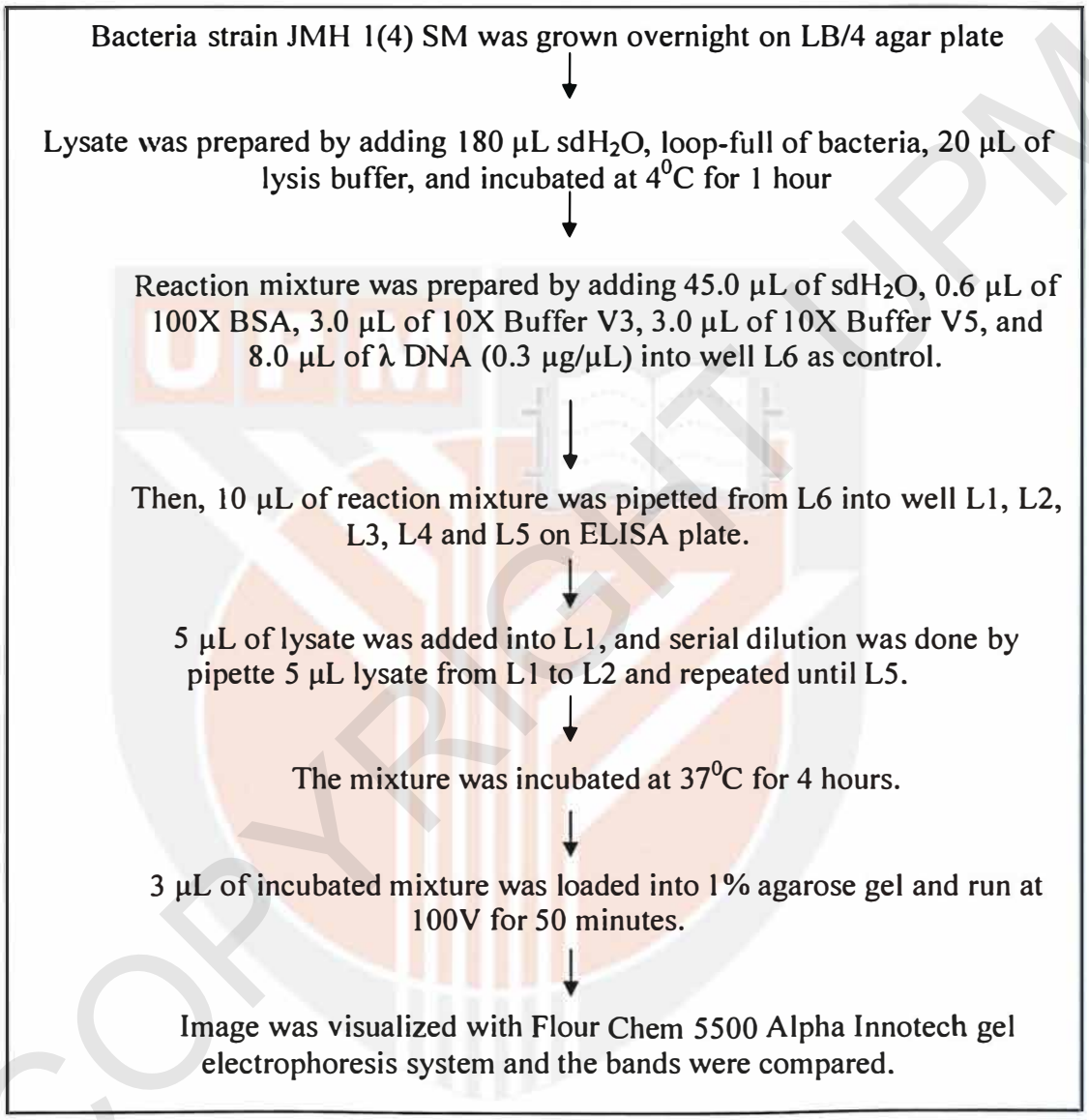


Figure 2: Flow Chart for Strain Verification

The strain verification protocol was suggested by Dr. Chee Hui Yee. Bacteria were cultured overnight on LB/4 agar plate (2.5 g/l typtone, 1.25 g/l yeast extract, 2.5 g/l NaCl and 15 g/l Agar) at room temperature. Briefly, 180 µL distilled water

was pipetted into a 1.5 mL micro-centrifuge tube. Then a loop full of colonies was collected from overnight cultured bacteria agar plate and put into the microcentrifuge tube containing the water, and the inoculation loop was stirred until all the bacteria had been transferred into the water and the solution became cloudy. Then, 20 µL of 10x lysis buffer [200 mM Tris-Cl (pH 7.5 at 30°C), 0.1 % Triton-X (v/v) from Fisher Scientific; 20 mg/mL lysozyme from Vivantis] was added. The lysate mixture was incubated at 4°C for 1 hour.

Then, reaction buffer was prepared by adding 45.0 µL sterile distilled water, 0.6 µL BSA 10x, 3.0 µL Buffer V3 10x [50 mM Tris-Cl (pH7.5 at 30°C), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT], 3.0 µL Buffer V5 10x [30 mM Tris-acetate (pH7.9 at 30°C), 10 mM Mg-acetate, 60 mM K-acetate, 1 mM DTT] from Vivantis Technologies and 8.0 µL lambda DNA 0.3 µg/µL [10 mM Tris-HCl (pH8.0) and 1 mM EDTA] from Fermentas Inc. into microcentrifuge tube. Then, 10 µL of reaction buffer was pipetted into well L1, L2, L3, L4, L5 and L6 as control in the ELISA plate. After 1 hour incubation time, 5 µL of the lysate was transferred into ELISA plate containing 0.17 µg (10 µL of reaction buffer) of the lambda DNA prepared in reaction buffer. The lysate and the reaction mixture was mixed well and 4x serial dilutions where undiluted lysate was transferred 5 µL from well L1 to L2 through L5 which contained 0.17 µg of lambda DNA (10 µL), and the L6 served as control. The lysate-reaction mixture was incubated at 37°C in Stuart Orbital Incubator S150 for 4 hours.

After 4 hours, 1 kb DNA Ladder from Fermentas Inc. was loaded into the first well on gel and followed by 2 µL of 6x gel loading dye from Fermentas Inc.

was added into each well in ELISA plate and 3 µL of DNA was loaded from each well into 1.0% agarose gel (1st Base) in 0.5 x TBE buffer for gel electrophoresis system from Thermo Electron Corporation. The 0.5 x TBE buffer was diluted from 5 x TBE Buffer (54 g/L of Tris base from AMRESCO, 27.5 g/L of Boric Acid from R&M Chemical, 20mL of 0.5 M EDTA from R&M Chemicals). The gel was run at 120 V for 60 minutes or until the tracking dye (Bromophenol blue) reached 3/4 of the gel length. Then, the gel was stained with Ethidium Bromide (20 µL/L) from AMRESCO after dilution in dH_2O. The stained gel was then visualized by using Alpha Innotech Fluor Chem 5500 and digestion pattern of the test DNA compared by referring to Fermentas Inc. and Vivantis Technologies catalogues for the digestion pattern of the *Pvu*II.

3.2.3 DNA Extraction

Suspension
Cells was resuspended in 100 µL buffer R1

↓

Lysozyme Treatment
20 µL of lysozyme was added and mixed gently. The mixture was incubated at 37^{0}C for 20 minutes.

↓

Centrifuge at 10,000 x g for 3 minute
Supernatant was discarded.

↓

Protein Denaturation
Cells were resuspended in 180 µL buffer R2 and 20 µL Proteinase K was added and incubated at 60^{0}C for 20 minutes.

↓

Removal of RNA
20 µL of RNase A was added. The mixture was incubated at 37^{0}C for 5 minutes.

↓

Homogenization
440 µL of buffer BG was added and mixed thoroughly. The mixture was incubated at 60^{0}C for 10 minutes.

↓

Precipitation
The mixture was cooled and 200 µL of absolute ethanol was added and mixed immediately.

↓

Loading to column
The sample was transferred to column

↓

Centrifuge at 10,000 x g for 1 minute
Flow through was discarded.

↓

Column washing
A total volume of 750 µL wash buffer was added and left at room temperature for 2 minutes.

↓

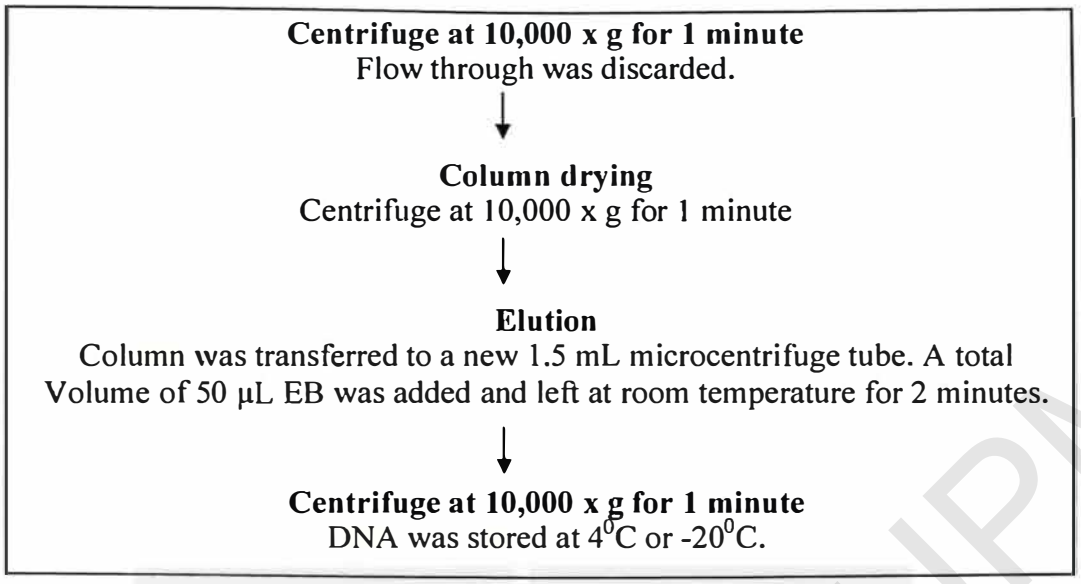


Figure 3: Flow Chart of DNA Extraction

DNA bacteria were extracted by using GF-1 bacterial extraction kits from Vivantis Technologies. Briefly, bacteria from overnight culture in LB/4 broth (1.5 mL) was extracted and pipetted into 1.5 mL microcentrifuge tubes. Then, the tubes were centrifuged using Eppendoft centrifuge 5415D at 6,000 x g for 5 minutes. The supernatant was decanted.

Then, the pellet was completely resuspended in 100 µL Buffer R1 by pipetting up and down. After that, 20 µL Lysozyme [50 mg/mL of storage buffer (1 mM $CaCl_2$, 20 mM Tris base, pH 8.0)] was added into the cell suspension and mixed gently. Then, the mixture was incubated at 37°C for 20 minutes. The digested pellet was centrifuged at 10,000 x g for 3 minutes. The supernatant was decanted completely. Cell pellet were resuspended in 180 µL buffer R2 followed by adding 20 µL Proteinase-K [20 mg/mL of 10 mM Tris-Cl (pH 8.0)] and was incubated at 60°C for 20 minutes in a shaking waterbath or with occasional manual mixing every

5 minutes. Then, a total of 20 µL RNase-A (DNase-Free, 20 mg/mL in dH_2O) was added to clear lysate and was incubated at 37°C for 5 minutes.

Then, 2 volumes (~440µL) buffer BG were added and mixed thoroughly by inverting the tubes several times until a homogeneous solution was obtained. The solution was incubated for a further 10 minutes at 60°C. After cooling down at room temperature for approximately 5 minutes, a total of 200 µL absolute ethanol (R&M Chemical) was added and mixed immediately and thoroughly. Then, the sample was transferred into a column assembled in a clean 2 mL collection tube and was centrifuged at 10,000 x g for 1 minute. The flow through was discarded. After that, 750 µL wash buffer was added to wash the column and was centrifuged at 10,000 x g for 1 minute. The flow through was discarded and was centrifuged at 10,000 x g for 1 minute to removal residual ethanol. Then, the column was transferred to a new 1.5 mL microcentrifuge tube.

Finally, 100 µL pre-heated EB (10 mM Tris-HCl, pH 8.5) was added and left at room temperature for 30 minutes. The eluted DNA was centrifuged at 10,000 x g for 1 minute. Finally, the extracted DNA was stored at 4°C or -20°C.

3.2.4 Restriction Enzyme Digestion Pattern

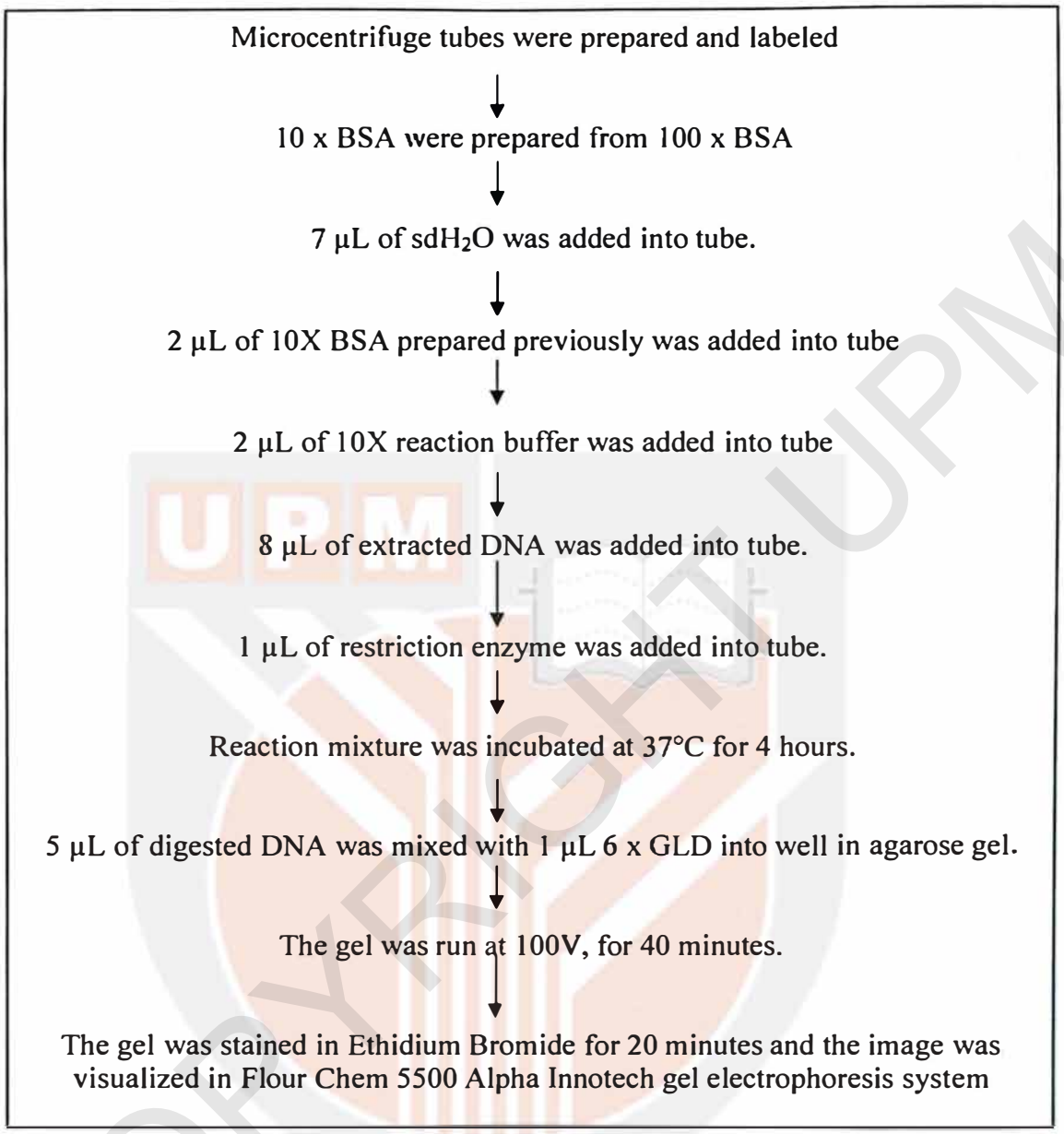


Figure 4: Flow Chart of Restriction Enzyme Digestion Pattern

Various restriction enzymes was used to obtained restriction enzyme digestion pattern. The restriction enzyme digestion was performed on microcentrifuge tubes with appropriate reaction buffer. Restriction enzymes used are *Asu*NHI, *Bam*HI, *Bgl*I, *Bme*18I, *Bmt*I, *Bse*X3I, *Bsn*I, *Bsp*19I, *Bss*MI, *Bst*MBI, *Eco*RI, *Eco*RV, *Cci*NI, *Fau*NDI, *Hin*dIII, *Kpn*I, *Psp*124I, *Psp*124BI, *Pst*I, *Rsa*I, *Sal*I, *Sfi*274I, *Sma*I, and *Xba*I. The reaction mixture was preformed as specifically suggested by the manufacturer (Appedix A.4).

Then the reaction mixture was incubated at 37°C for 4 hours. After that, 1 µL 6x loading dye and 5 µL of test DNA were mixed and loaded from each well of microcentrifuge tubes into 0.8% agarose gel in 0.5 x TBE for gel electrophoresis (Thermo Electron Corporation). The gel was run at 100 V for 50 minutes. The gel was subjected to stain in ethdium bromide for 20 minutes. The image was visualized by using Fluor Chem 5500 from Alpha Innotech. The digestion pattern was observed and the suitable restriction enzyme which can cut the tested DNA to obtain a DNA fragment around 2-5 kb was determined.

3.2.5 DNA Recovery Gel from Agarose Gel

Gel Electrophoresis

DNA sample on agarose gel electroporesis was run. The desired band was cut and the nett weight of agarose gel was determined.

↓

Solubilization

5 volume buffer BG: 1 volume gel was added. The gel was incubated at 50^0C until the gel was melted completely.

↓

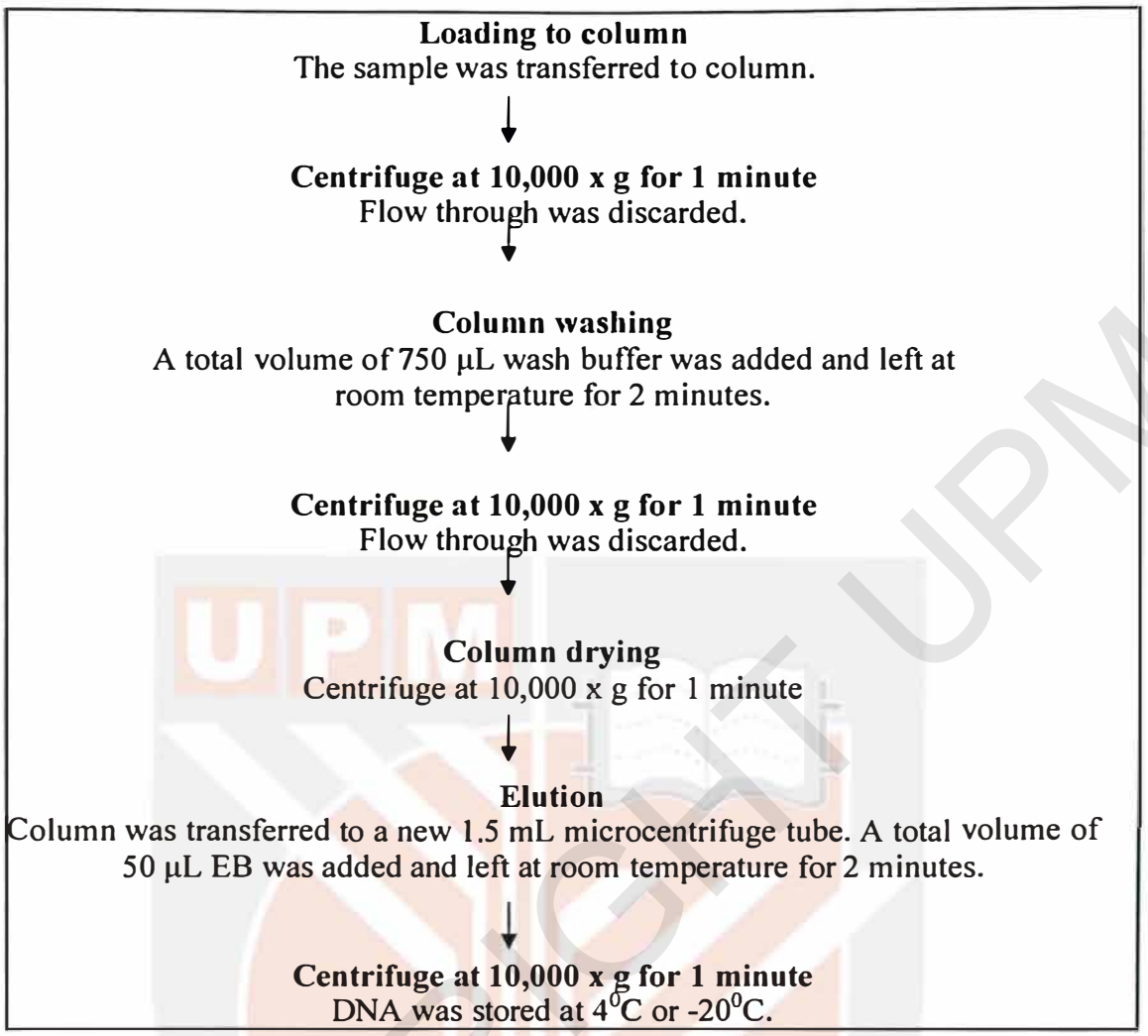


Figure 5: Flow Chart of DNA Recovery Gel from Agarose Gel

DNA fragments around 2-5 kb was recovered by using GF-1 gel DNA recovery kits from Vivantis Technologies. The DNA sample with suitable restriction enzyme digestion was run on 0.8% agarose gel electrophoresis for 50 minutes to separate DNA fragments and ethidium bromide staining was performed for DNA visualization.

The region between 2-5 kb was determined by using the fluorescence ruler on the gel tray after staining. Agarose gel containing the desired DNA region was cut into smaller slices. Then, the gel slices was placed into microcentrifuge tubes

and the weight of each gel slice was recorded. Five volumes of buffer GB was added to 1 volume of gel slice (every gel slice of mass 0.1 g will have a volume of 100 µL). The gel slice in the microcentrifuge with Buffer GB (Gel DNA Binding Buffer) was melted at 50°C using heating and cooling block from Grant-Bio for not more than 5 minutes and until the gel melted completely with mixing occasional to ensure complete solubilization. The samples would turn to yellow (pH 7.0 or below). The samples were then was transferred into a column assembled in a clean 2 mL collection tube and centrifuged at 10,000 x g by using Eppendoft centrifuge 5415D for 1 minute. The flow through was discarded. The remaining solution was transferred into the column and the steps above were repeated until all the solution was transferred.

Then, 750 µL wash buffer was added into the column and left for 2 minutes to thoroughly remove any residue agarose. The collection tube was centrifuged at 10,000 x g for 1 minute and flow through was discarded. The collection tube was then centrifuged again at 10,000 x g for 1 minute to remove residual ethanol. Lastly, the column was placed into a clean 1.5 mL microcentrifuge tube and 50 µL Elution Buffer (10 mM Tris-HCl, pH 8.5) was applied directly onto column membrane and left to stand for 2 minutes. The microcentrifuge with column was centrifuged at 10,000 x g for 1 minute to elute DNA and the DNA was stored at 4°C.

3.2.6 Manual Plasmid Extraction

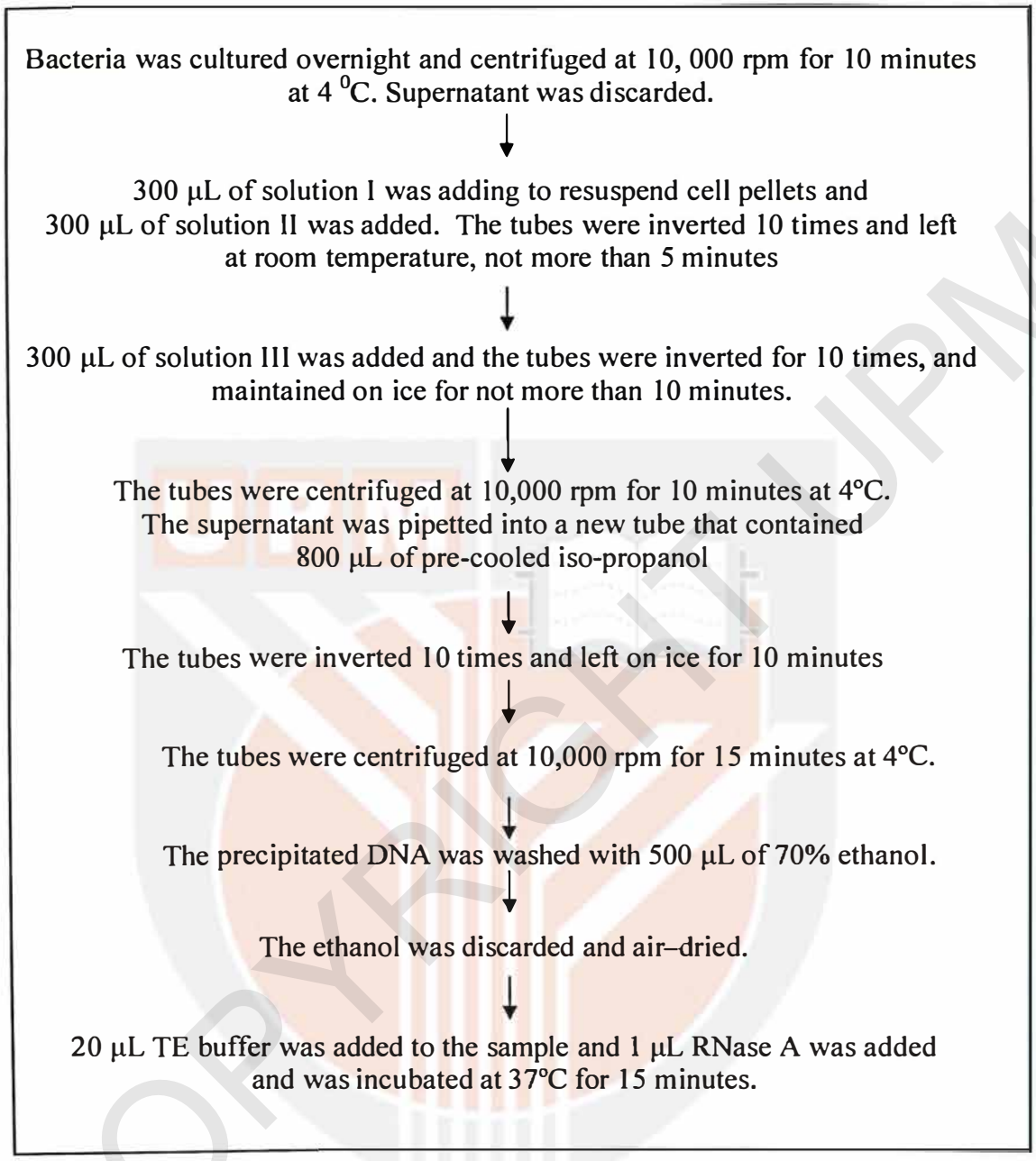


Figure 6: Flow Chart of Manual Plasmid Extraction

Plasmid pET23a (+) in TOP10F' *Escherichia coli* from overnight culture (1.5 mL) was extracted by using the modified alkaline lysis miniprep procedure (Ioannou *et al.*, 1996), with the solution I, II and III in the ratio 1:1:1. The solution I [50 mM glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA (pH 8.0) and the pH was adjusted to pH 8.0] Solution I was prepared in batches, autoclaved and store at 4°C. Solution II: 0.2 N NaOH (freshly diluted from 10 N stock), 1% (w/v) SDS (diluted from 20% stock). Solution II was prepared fresh and used at room temperature. Solution III (3M potassium acetate, pH 5.5 was adjusted using glacial acetic acid and stored at 4°C).

In brief, 1.5 mL of culture cell was pipetted into microcentrifuge tubes, and centrifuged in Thermo electron corporation centrifuge machine at 10,000 rpm, 4°C for 10 minutes. Cell pellets were re-suspended in 300 µL solution I. Then, the supernatant was discharged and 300 µL of solution II was added into microcentrifuge tubes. The tubes were inverted 10 times to mix well and left at room temperature for not more than 5 minutes. Then, 300 µL solution III was added into tubes and the tubes were inverted for 10 times and maintained on ice for not more than 10 minutes. The tubes were centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was pipetted into new tubes which containing 800 µL pre-cooled isopropanol. The tubes were inverted 10 times and left on ice for 10 minutes to allow the DNA to be precipitated.

Samples were then centrifuged at 10,000 x g, 4°C for 15 minutes. The precipitated DNA was washed with 500 µL of 70% ethanol. The ethanol was discarded and air–dried. Finally, 20 µL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH

8.0) was added to the sample and 1 µL RNase was added and incubated at 37°C for 15 minutes. For small plasmids, sample DNA was stored at -20°C.

3.2.7 Plasmid Extraction by Using Kits

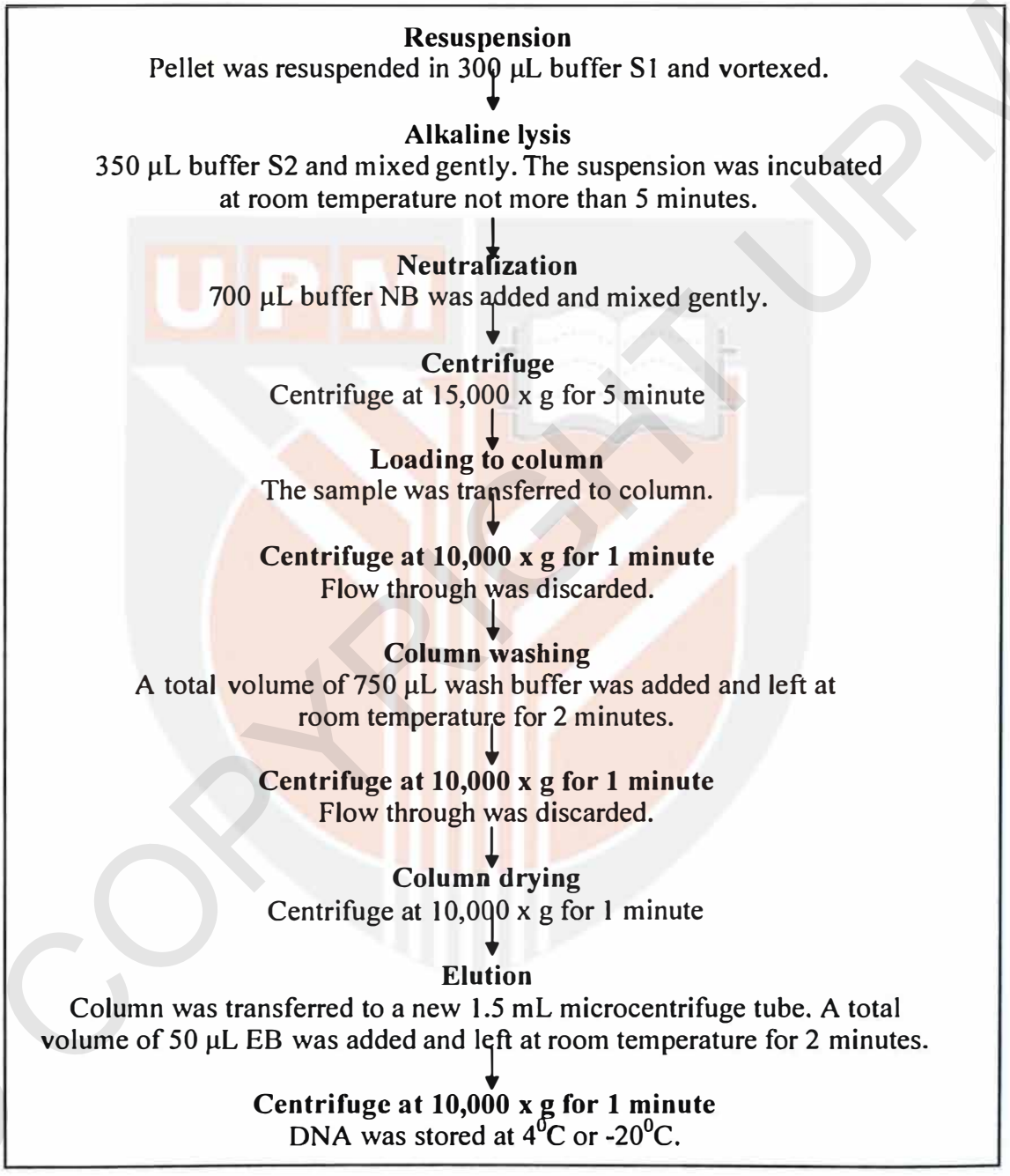


Figure 7: Flow Chart of Plasmid Extraction by Using Kits

Plasmid Extraction was performed using GF-1 plasmid extraction kits from Vivantis Technologies. *Escherichia coli* TOP10F'containing plasmid pET23a (+) was incubated overnight in LB medium with appropriate antibiotic at 37°C with agitation at 250 rpm. Then, a 1.5 mL plasmid-containing bacterial cell was pipetted into micro-centrifuge tubes, and centrifuged in Eppendoft centrifuge 5415D at 6,000 x g for 5 minutes. The supernatant was decanted completely. Then, 350 µL S1 (Solution 1 was provided in the kits and RNase A was added previously before use) was added into the pellet and the cells was resuspended completely by vortexing or pippetting. The suspension was then transferred to a clean 1.5 mL microcentrifuge tube. A 350 µL Solution 2 was added and cells was mixed gently by inverting tube several times (4-6 times) to obtain a clear lysate. Then, the suspension was incubated on ice for not more than 5 minutes.

After that, 700 µL of Buffer NB was added and mixed gently by inverting the tube several times (4-6 times) to neutralize the lysate until a white precipitate forms. Then, the white precipitate was centrifuged at 15,000 x g for 5 minutes. A total of 700 µL of the supernatant was transferred into a column assembled in a clean 2 mL collection tube. The supernatant was centrifuged at 10,000 x g for 1 minute. The flow through was discarded. The remaining solution was transferred into the column and above steps repeated until all solution was transferred.

A total of 700 µL wash buffer was added into the column and centrifuged at 10,000 x g for 1 minute. The flow through was discarded. Then, the column was centrifuged at 10,000 x g for 1 minute to remove the residual ethanol. Finally, the column was placed into a clean 1.5 mL microcentrifuge tube. A total of 100 µL of

elution buffer (10 mM Tris-Cl, pH 8.5) was added directly onto the column membrane and left to stand for 1 minute. Then, the column was centrifuged at 10,000 x g for 1 minute to elute DNA and stored at 4°C or -20°C.

3.2.8 Preparation of Cloning Vector pET-23a (+)

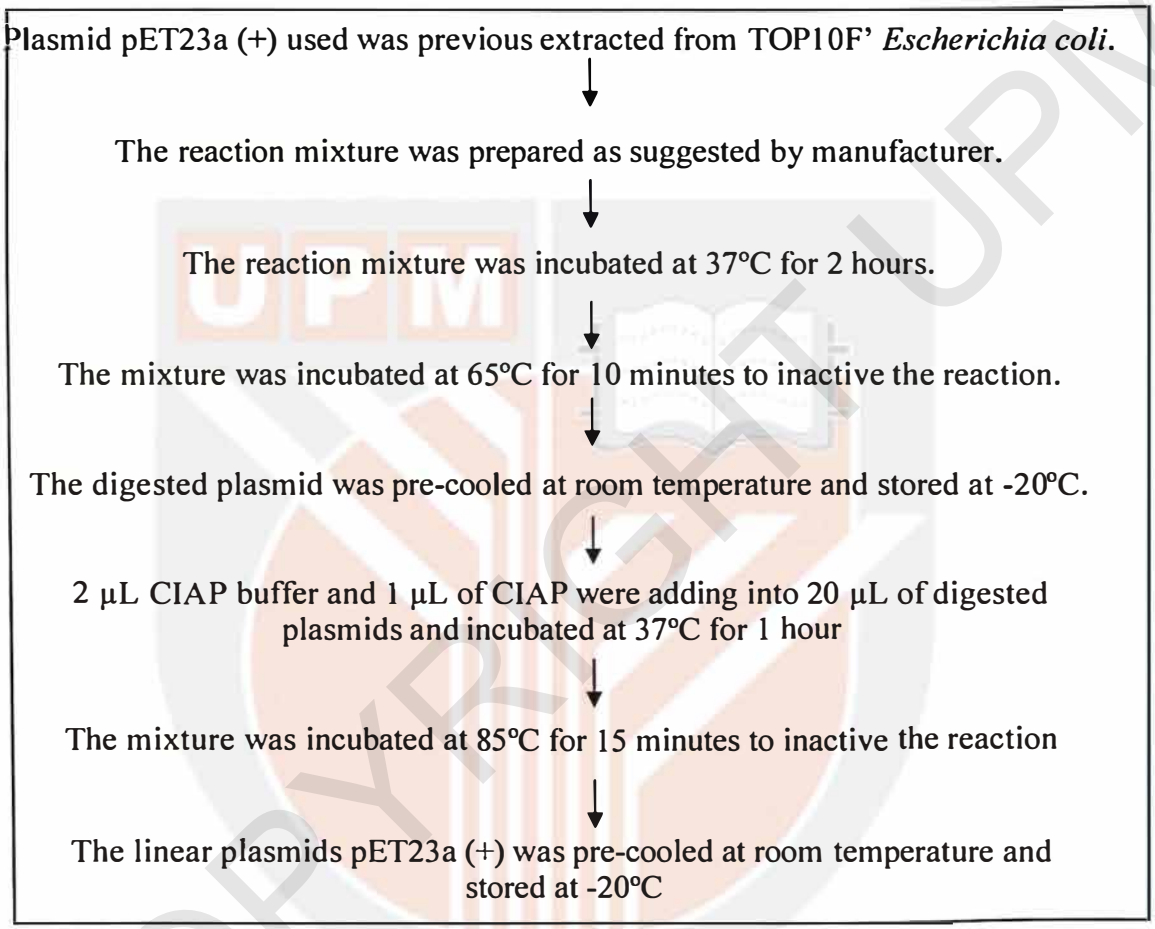


Figure 8: Flow Chart of Preparation of Cloning Vector pET-23a (+)

Plasmid pET23a (+) used was previous by extracted from TOP10F' *Escherichia coli*. The reaction mixture was prepared as suggested by the manufacturer. The reaction mixture was incubated at 37°C for 2 hours. After the incubation, the mixture was incubated at 65°C for 10 minutes to inactive the reaction. The digested plasmid pET23a (+) was pre-cooled at room temperature and stored at -20°C. The linear plasmids were then incubated at 37°C for 1 hour by adding 20 µL

of digested plasmids, 2 µL Calf Intestine Alkaline Phosphatase (CIAP) buffer and 1 µL of CIAP from Vivantis Technologies. After the incubation, the mixture was incubated at 85°C for 15 minutes to inactive the reaction. The linear plasmids pET23a (+) were pre-cooled at room temperature and stored at -20°C prior to subsequent usage.

3.2.9 Ligation of Fragment DNA into pET23a (+)

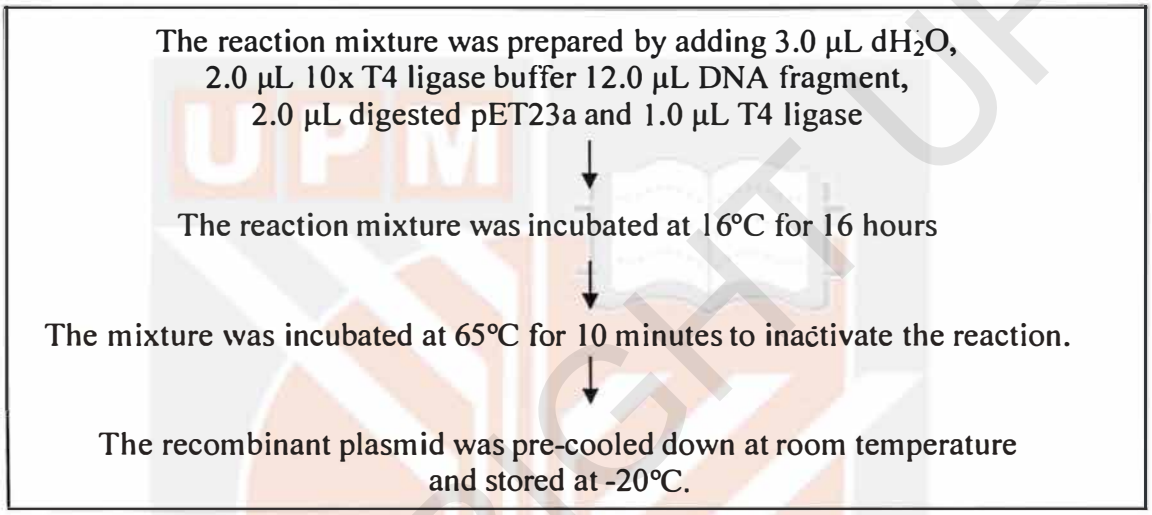


Figure 9: Flow Chart of Ligation of Fragment DNA into pET23a (+)

Ligation used was a modified protocol (Crowe *et al.*, 1991). The reaction mixture was prepared by adding 3.0 µL dH_2O, 2.0 µL 10x T4 ligase buffer [50 mM Tris-HCl (pH 7.8 at 25°C) 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA] from Vivantis Technologies, 12.0 µL DNA fragment, 2.0 µL *Eco*RI digested pET23a and 1.0 µL T4 ligase from Vivantis Technologies. The reaction mixture was incubated at 16°C for 16 hours on a heating and cooling block from Grant-Bio. For ligation of the sticky end the incubation temperature was 16°C. Meanwhile, for the blunt end the incubation temperature recommended was 4°C. After the incubation, the mixture was incubated at 65°C for 10 minutes to inactivate the

reaction. The recombinant plasmid was pre-cooled down at room temperature and stored at -20°C.

3.2.10 Preparation and Induction of Calcium Chloride Transformation

5 mL bacterial culture was diluted in 100 mL LB broth and incubated at 37°C with agitation at 250 rpm.

↓

When the OD_{600} value reached to 0.4-0.5, 40 mL cell culture was aliquot into pre-cooled 50 mL centrifuge tubes each.

↓

The tube was then centrifuged at 4000 rpm for 10 minutes at 4°C

↓

The supernatant was discarded and pellet was maintained on ice.

↓

The pellet was re-suspended with 20 mL of pre-cooled 100 mM $CaCl_2$ and the suspension was kept on ice for 5 minutes.

↓

The suspension was then centrifuged at 4,000 rpm for 10 minutes at 4°C

↓

The supernatant was discarded. The pellet was re-suspended very gently in 4 mL of 100 mM $CaCl_2$

↓

The suspension was then centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatant was discarded.

↓

140 µL of DMSO was added into centrifuge tubes and swirled to mix well.

↓

The suspension was maintained on ice for 20 minutes.

↓

Aliquots were frozen in pre-cooled isopropanol and stored at -80°C

↓

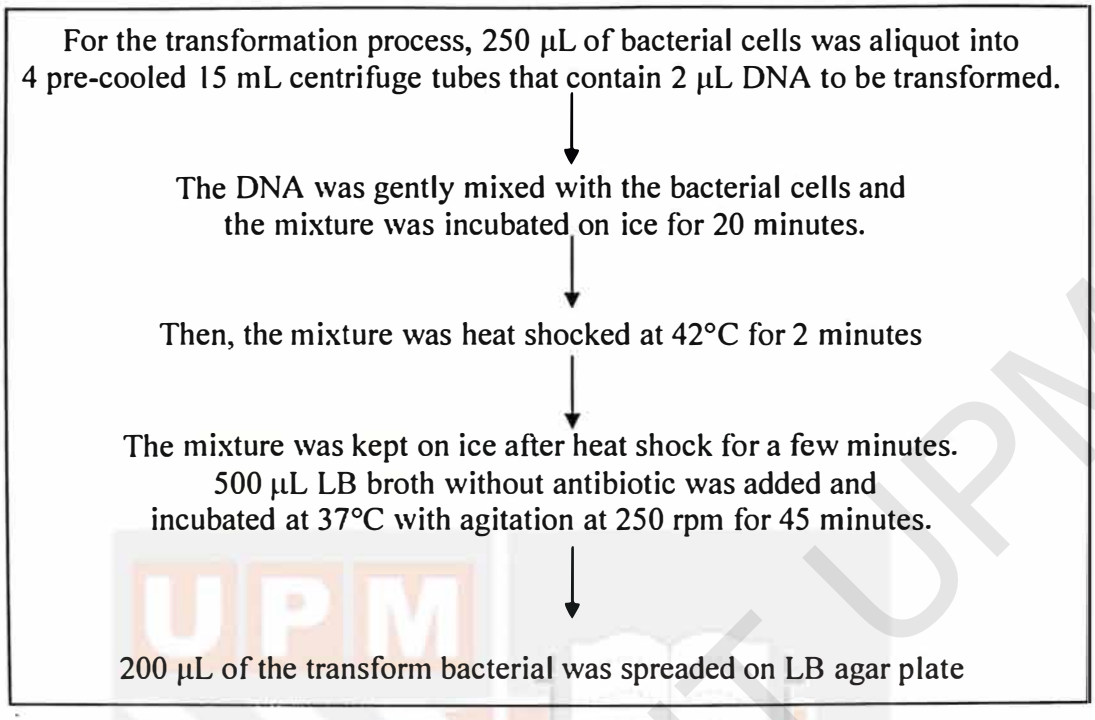


Figure 10: Flow Chart of Preparation and Induction of Calcium Chloride Transformation

$CaCl_2$ transformation of *Escherichia coli* TOP10F' with recombinant was according to standard protocols (Sambrook and Russell, 2001). In brief, the selected overnight bacterial culture was diluted 1:20 in fresh LB broth (5 mL overnight cells diluted in 100 mL LB broth) in 1 L flask and incubated at 37°C with agitation at 250 rpm. Bacterial cell growth was monitored by taking OD_{600} readings using Varian, Cary 50 Conc. UV-Visible Spectrophotometer approximately after 1 hour of incubation time.

When the OD_{600} value reached to 0.4-0.5, 40 mL cell culture aliquot was transfereed into each of two sterile pre-cooled 50 mL centrifuge tubes. The cell cultures were centrifuged at 4000 rpm for 10 minutes at 4°C in a Hettich Mikro 22-R benchtop centrifuge. The supernatant was discarded while the pellets in the tubes were maintained on ice. Then, the bacterial pellet was gently re-suspended with 20

mL of pre-cooled 100 mM $CaCl_2$ (1/2 the culture volume) by using a pre-cooled pipette and the suspension was kept on ice for 5 minutes. The bacterial suspension was then centrifuged at 4,000 rpm for 10 minutes at 4°C in Hettich Mikro 22-R benchtop centrifuge. The supernatant was discarded. The bacterial pellet was re-suspended very gently in 4 mL of 100 mM $CaCl_2$ (equivalent to 1/10 of the original volume). The bacterial suspension was then centrifuged at 4,000 rpm for 10 minutes at 4°C in Hettich Mikro 22-R benchtop centrifuge. The supernatant was discarded. A total of 140 µL of DMSO from AMRESCO was added into centrifuge tubes and tubes were swirled to mix well. The suspension was maintained on ice for 20 minutes. Aliquots were frozen in isopropanol which had been pre-cooled at -80°C and stored at -80°C.

For the transformation process, 250 µL aliquots of the re-suspended bacterial cells were transferred into four pre-cooled 15 mL centrifuge tubes containing 2 µL DNA to be transformed. The DNA was gently mixed with the bacterial cells and the mixture was incubated on ice for 20 minutes. Then, the mixture was heat shocked at 42°C for 2 minutes by putting the tubes in a 42°C water bath. The mixture was kept on ice after heat shock for a few minutes. The transformation mixture was added with 500 µL LB broth without antibiotic and incubated at 37°C with agitation at 250 rpm for 45 minutes. Finally, 200 µL of the transformed bacterial was spread on LB agar plates with Ampicillin (50 mg/mL).

3.2.11 Preparation and Induction of Electro-competent Cells

5 mL of bacteria culture was diluted in 250 mL LB broth and incubated at 37°C with agitated at 250 rpm.

↓

When the OD_{600} reading reached to 0.4-0.5, the cell culture was divided equally into two 250 mL centrifuge tubes and kept at -20°C for 20 minutes

↓

The cells were centrifuged at 4,000 rpm for 25 minutes at -5°C

↓

The supernatant was discarded and the pellet was resuspended in one volume of ice-cold 10% glycerol (w/v)

↓

The supernatant was discarded and the pellet were resuspended in 10mL ice-cold 10% glycerol (w/v)

↓

The suspension was transferred into a single 50 mL centrifuge tube, and then topped up to 40 mL using ice cold 10% glycerol (w/v).

↓

This suspension was centrifuged at 4,000 x g for 15 minutes at -5°C

↓

The supernatant was discarded and 40 mL ice-cold 10% glycerol (w/v) was added and centrifuge at 4,000 x g for 15 minutes at -5°C.

↓

The supernatant was discarded and the cell pellet was resuspended in 400 µL of ice-cold 10% glycerol (w/v).

↓

The suspension was maintained on ice for 20 minutes.

↓

Aliquots were frozen in isopropanol which had been pre-cooled at -80°C and stored at -80 °C.

↓

For the electroporation process, all the materials were maintained on ice.

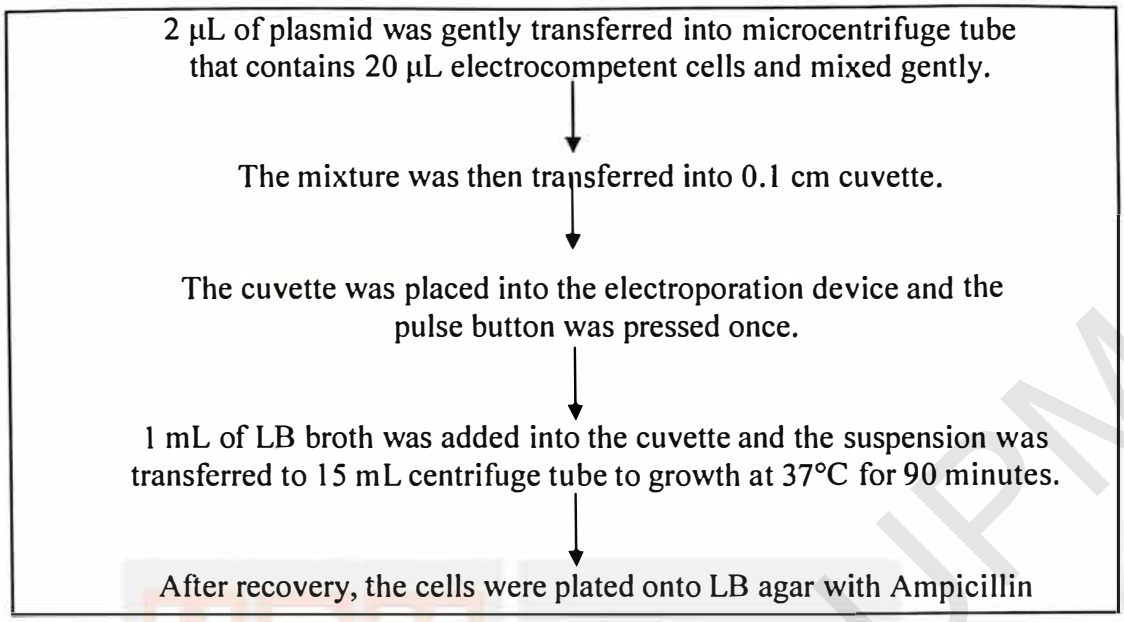


Figure 11: Flow Chart of Preparation and Induction of Electro-competent Cells

A total of 5 mL of overnight *Escherichia coli* TOP10F' culture was incubated 250 mL LB medium containing appropriate antibiotic in a 2 L volumetric flask at 37°C, and agitated at 250 rpm. Bacterial cell growth was monitored by taking OD_{600} readings by using Varian, Cary 50 Conc. UV-Visible Spectrophotometer approximately after 2 hours of incubation.

When the OD_{600} reading reached to 0.4-0.5, the cell culture was divided equally into two 250 mL centrifuge tubes and kept at -20°C for 20 minutes to slow down the cell growth. The cells were swirled at frequent intervals to prevent ice formation. Then, the cells were centrifuged at 4,000 rpm for 25 minutes at -5°C, using acceleration value and deceleration value of 6. The supernatant was discarded and the pellet was resuspended in one volume of ice-cold 10% glycerol (w/v) (equal to the original culture volume) and pelleted as before. The supernatant was discarded and the pellets from both 250 mL centrifuge tubes were then resuspended in 10 mL ice-cold 10% glycerol (w/v) and transferred into a single 50 mL centrifuge tube, and

then topped up to 40 mL using ice cold 10% glycerol (w/v). This suspension was centrifuged at 4,000 x g for 15 minutes at -5°C in Hettich, Mikro-22R. The supernatant was discarded and the pellet resuspended in 40 mL ice-cold 10% glycerol (w/v) and centrifuged again at 4,000 x g for 15 minutes at -5°C in the same centrifuge. Then, the supernatant was discarded and the cell pellet was resuspended in 400 µL of ice-cold 10% glycerol (w/v). The suspension was maintained on ice for 20 minutes. Aliquots were frozen in isopropanol which had been pre-cooled at -80°C and stored at -80 °C.

For the electroporation process, the electroporation device, Gene Pulser Xcell from Bio-rad was warmed up for 30 minutes before use. The current was set at 1.8 kV for 0.1 cm cuvette and 2.5 kV for 0.2 cm cuvette for transformation. All the materials including the cuvette, electrocompetent cells and recombinant plasmid were maintained on ice. For the electroporation process, 2 µL of plasmid was gently transferred into microcentrifuge tubes that containing 20 µL electrocompetent cells and mixed gently. The mixture was then transferred into 0.1 cm cuvette. The cuvette was placed into the electroporation device and the pulse button was pressed once. After that, 1 mL of LB broth was immediately added into the cuvette and the suspension was transferred to 15 mL centrifuge tube to growth at 37°C for 90 minutes. After recovery, the cells were plated onto LB agar with Ampicillin (50 mg/mL, stock concentration).

3.2.12 Pre-screening for the Presence of Methylase

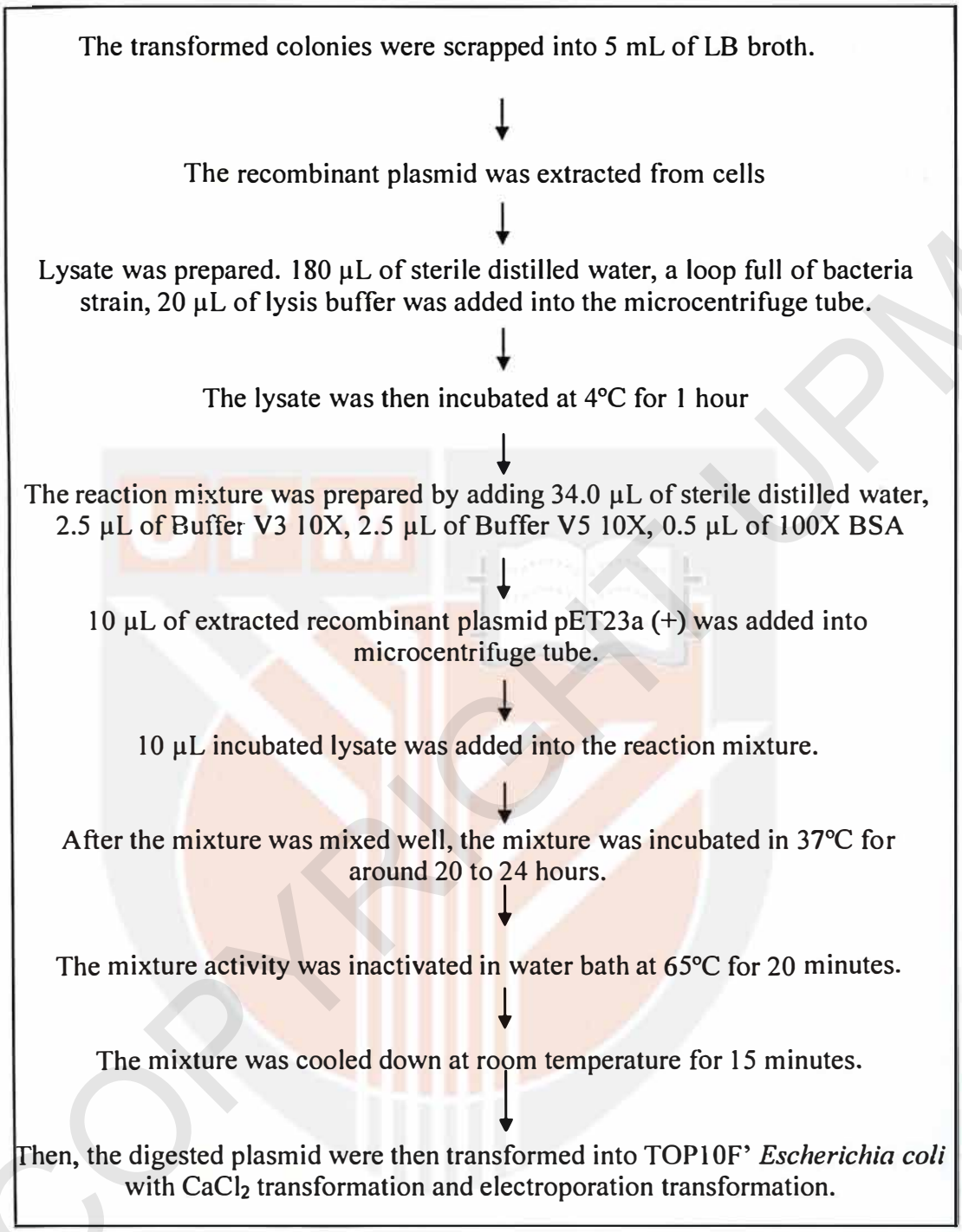


Figure 12: Flow Chart of Pre-screening for the Presence of Methylase

The transformed of recombinant plasmid pET23a (+) containing 2-5 kb of fragments DNA in TOP10F' *Escherichia coli* was screening for the presence of methylase activity. The transformed colonies were scrapped into a 15 mL

centrifugetube containing 5 mL of LB broth. The plasmid extraction was done according to the standard protocol of modified alkaline lysis miniprep procedure (Ioannou *et al.*, 1996) and GF-1 plasmid extraction kits from Vivantis Technologies. After obtained the extracted recombinant plasmid, lysate of bacteria strain JMH 1(4) SM was prepared. A full loop of bacteria strain that was cultured overnight was transferred into 180 µL of sterile distilled water in a microcentrifuge tube. The bacteria cells were resuspended by pipetting up and down. Then, 20 µL of lysis buffer (200 mM Tris-Cl, pH 7.5 at 30°C, 0.1% (v/v) Triton-X, 20 mg/mL lysozyme) was added into the microcentrifuge tube. The lysate was then incubated at 4°C for 1 hour.

Then, the reaction mixture was prepared by adding 34.0 µL of sterile distilled water, 2.5 µL of 10X Buffer V3 [50 mM Tris-Cl (pH 7.5 at 30°C), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT] from Vivantis Technologies, 2.5 µL of 10X Buffer V5 [30 mM Tris-acetate (pH 7.9 at 30°C), 10 mM Mg-acetate, 60 mM K-acetate, 1 mM DTT] from Vivantis Technologies, 0.5 µL of 100X BSA from Vivantis Technologies and followed by adding 10 µL of extracted recombinant plasmid pET23a (+) with 2–5 kb of DNA fragment into microcentrifuge tube. Then 10 µL incubated lysate was added into the reaction mixture. After the mixture was mixed well, it was incubated iat 37°C for around 20 to 24 hours.

Then, the mixture was inactivated in a water bath at 65°C for 20 minutes. The mixture was cooled down at room temperature for 15 minutes. Then, the digested plasmid were then transformed into TOP10F' *Escherichia coli* with $CaCl_2$ transformation and electroporation transformation.

CHAPTER 4

RESULTS

4.1 Strain verification

The verification of bacteria strain JMH 1(4) SM was done by digesting the lambda DNA in the restriction enzyme (RE) containing lysate from the cell JMH 1(4) SM. The restriction pattern was analyzed using agarose gel electrophoresis (Figure 13 and 14).

From the figures, it can be observed that after 4x serial dilutions, the restriction bands become more prominent from L1 to L5. There was 1 band allocated at more than 10,000 bp, 1 band around 10,000 bp, 4 bands around 4,000 to 5,200 bp, 1 band around 2,500 bp, 1 band around 1,700 bp, 2 bands around 1000 bp and 4 bands around 500 bp. There was only 1 band at the control region.

The restriction pattern of λ DNA digestion using RE obtained from bacteria strain JMH 1(4) SM was similar to the restriction pattern obtained from λ DNA digested by *Pvu*II which referring to the (catalogue of Fermentas Inc., Canada).

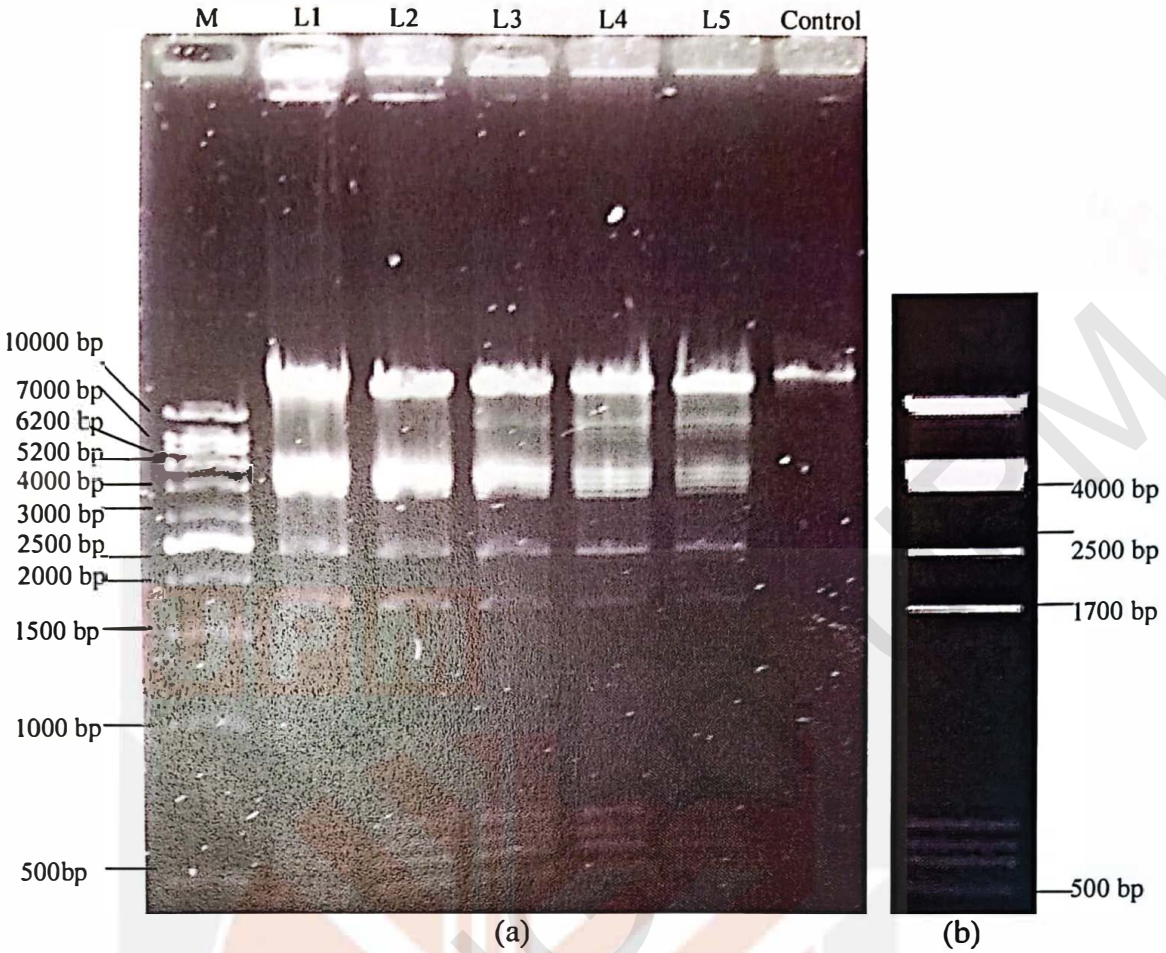


Figure 13: Strain Verification of JMH 1(4) SM 1.

M : 1 kb DNA ladder
L1 : λ DNA digestion by cell lysate of JMH 1(4) SM
L2 : 3 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L3 : 9 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L4 : 27 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L5 : 81 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
C : Control

Agarose Gel Electrophoresis images of (a) Serial dilution of λ DNA digestion by cell lysate of JMH 1(4) SM. This agarose gel was run at 1.0% agarose in 0.5 x TBE buffer, 120 V for 50 minutes. (b) λ DNA digested by *Pvu*II in Fermentas Inc. catalogue. This gel was run at 1.0% agarose.

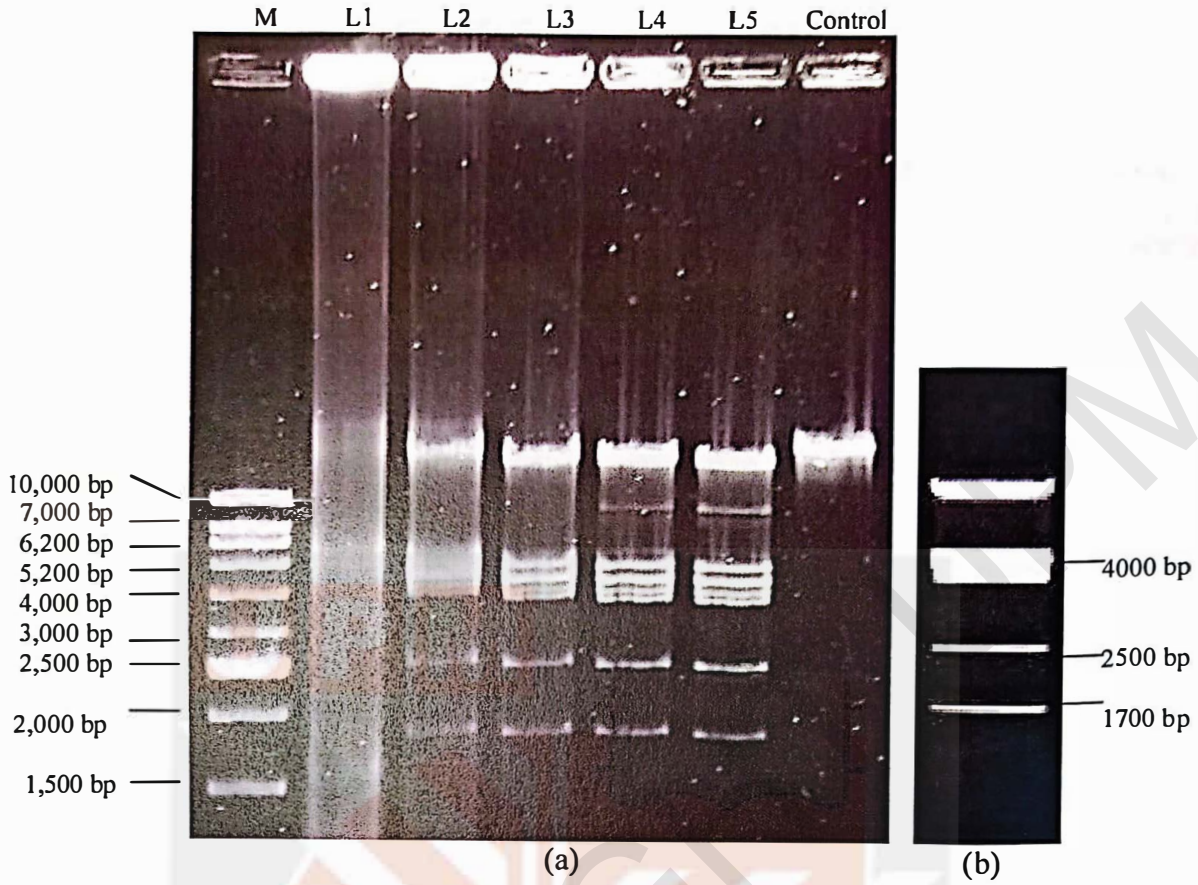


Figure 14: Strain verification of JMH 1(4) SM 2

M : 1 kb DNA ladder
L1 : λ DNA digestion by cell lysate of JMH 1(4) SM
L2 : 3 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L3 : 9 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L4 : 27 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
L5 : 81 x of dilution of λ DNA digestion by cell lysate of JMH 1(4) SM
C : Control

Agarose Gel Electrophoresis images of (a) Serial dilution of λ DNA digestion by cell lysate of JMH 1(4) SM. This agarose gel was run at 1.0% agarose in 0.5 x TBE buffer, 120 V for 60 minutes. (b) λ DNA digested by *Pvu*II in Fermentas Inc. catalogue. This gel was run at 1.0% agarose.

4.2 Restriction Pattern Analysis

After strain verification, the restriction pattern of genomic DNA from strain JMH 1(4) SM was obtained using selected restriction enzymes which were complementary to the multiple cloning sites (MCS) of plasmid pET23a (+) in figure 15. Other selected RE were used to digest with bacteria strain to obtained restriction pattern for further analysis (Figure 15, 16, 17 and 18). The restriction enzyme mixture was incubated for 4 hours before subjected to gel electrophoresis.

As observed from Figure 4, *Eco*RI enabled the digestion to produce significant DNA fragments around 2-5 kb. The *Hin*dIII and *Fau*NDI were able to produce DNA fragments around 2-5 kb due to the restriction pattern observed (Figure 15). In this study, *Eco*RI, *Hin*dIII and *Fau*NDI were chosen based upon the prominent DNA fragments around 2-5 kb produced after digestion.

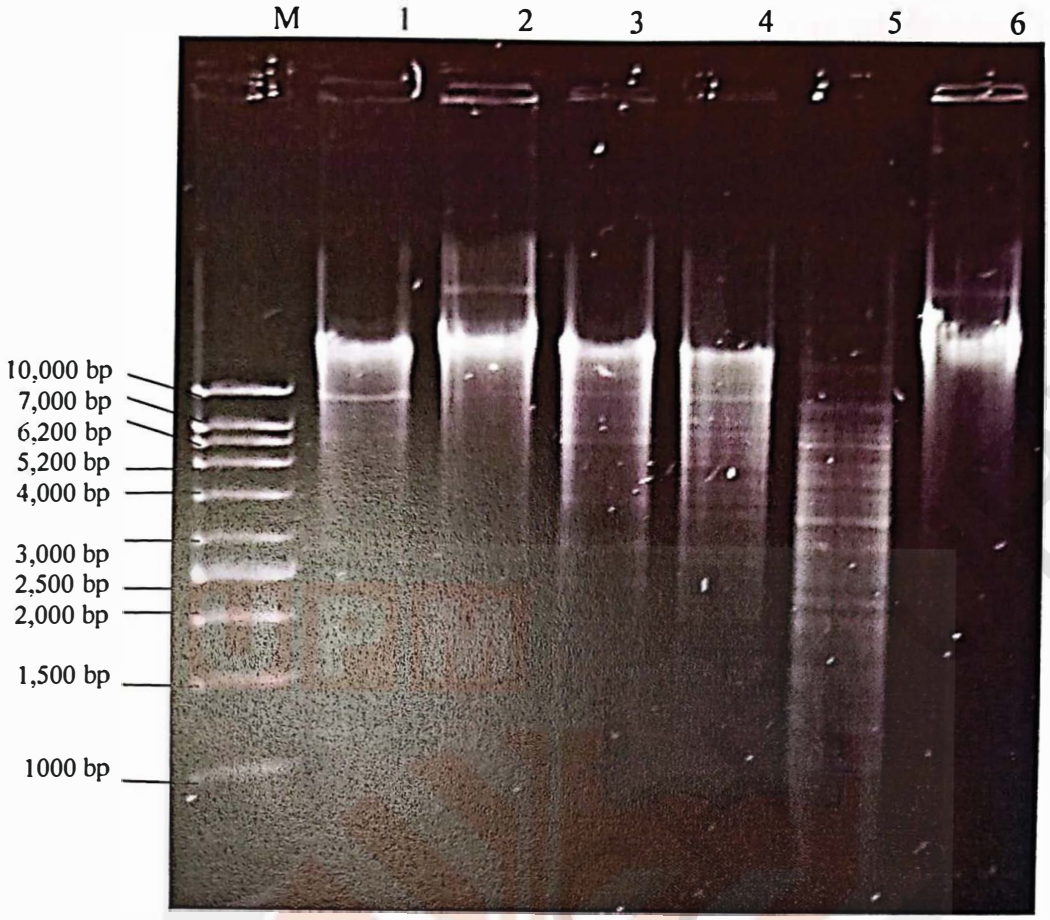


Figure 15: Restriction pattern analysis of genomic DNA JMH 1(4) SM using *Bgl*I, *Cci*NI, *Eco*RI, *Fau*NDI, *Hind*III, *Pst*I.

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Bgl*I
2 : Genomic DNA of JMH 1(4) SM digested with *Cci*NI
3 : Genomic DNA of JMH 1(4) SM digested with *Eco*RI
4 : Genomic DNA of JMH 1(4) SM digested with *Fau*NDI
5 : Genomic DNA of JMH 1(4) SM digested with *Hind*III
6 : Genomic DNA of JMH 1(4) SM digested with *Pst*I

Agarose Gel Electrophoresis of bacteria strain JMH 1(4) SM digested with selected restriction enzymes. This gel was run at 0.8% agarose in 0.5 x TBE buffer, 100 V for 50 minutes.

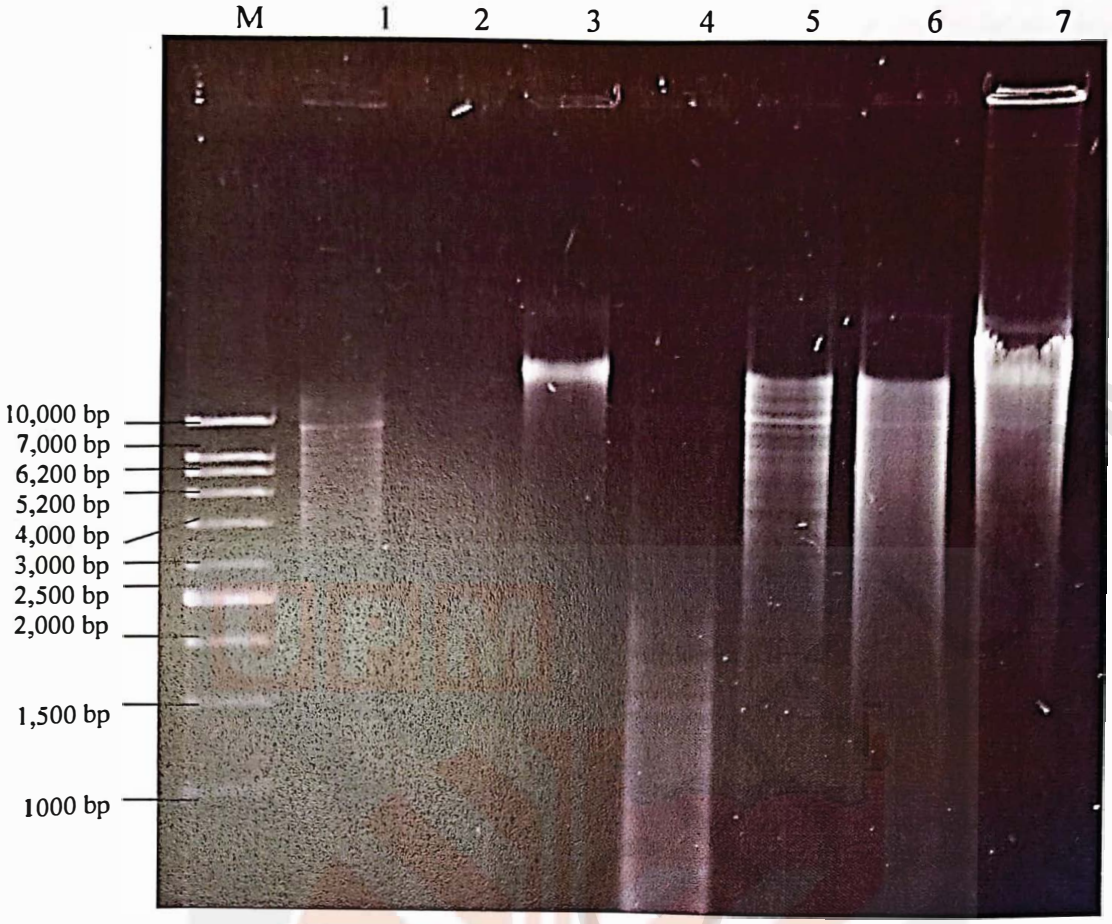


Figure 16: Restriction pattern analysis of genomic DNA JMH 1(4) SM using *Bme*18I, *Bsn*I, *Bsp*19I, *Bss*MI, *Eco*RV, *Kpn*I, *Sal*I.

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Bme*18I
2 : Genomic DNA of JMH 1(4) SM digested with *Bsn*I
3 : Genomic DNA of JMH 1(4) SM digested with *Bsp*19I
4 : Genomic DNA of JMH 1(4) SM digested with *Bss*MI
5 : Genomic DNA of JMH 1(4) SM digested with *Eco*RV
6 : Genomic DNA of JMH 1(4) SM digested with *Kpn*I
7 : Genomic DNA of JMH 1(4) SM digested with *Sal*I

Agarose Gel Electrophoresis of bacteria strain JMH 1(4) SM digested with selected restriction enzymes. This gel was run at 0.8% agarose in 0.5 x TBE buffer, 100 V for 50 minutes.

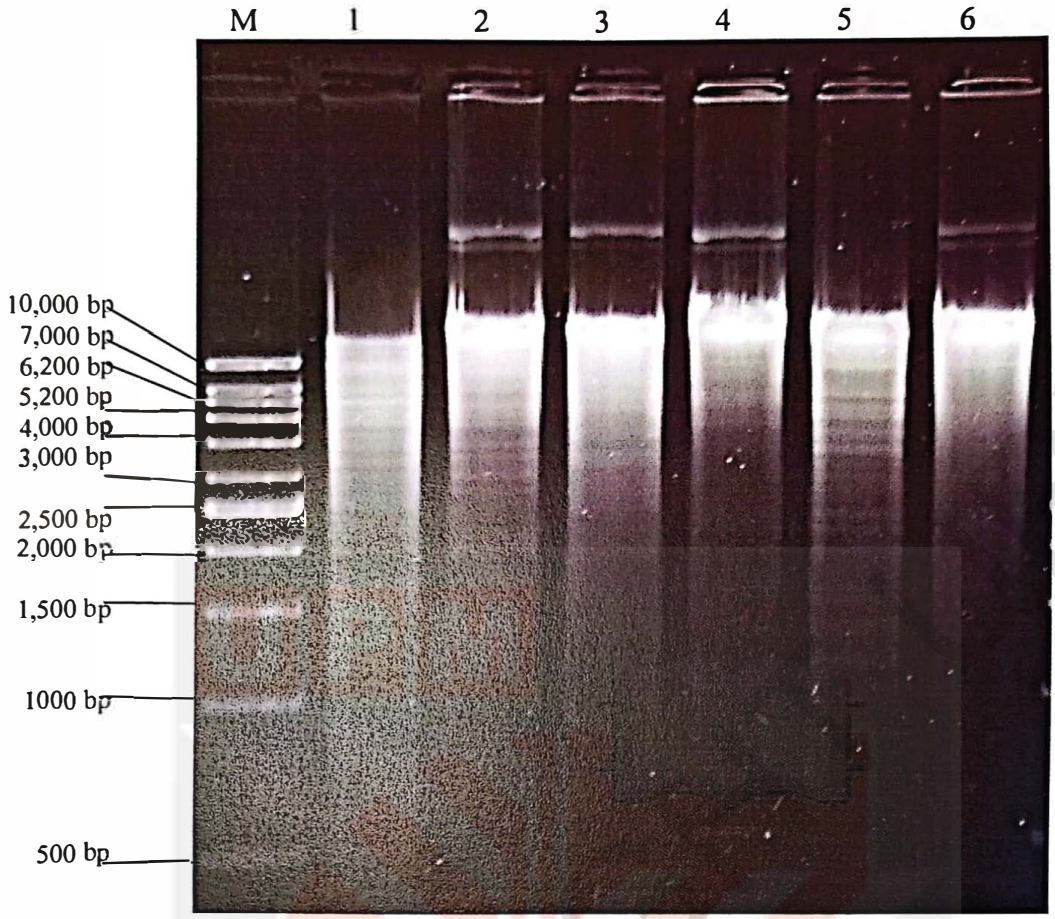


Figure 17: Restriction pattern analysis of genomic DNA JMH 1(4) SM using *Ava*II, *Pvu*I, *Sfr274*I, *Sma*I, *Sal*I, *Xba*I.

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Ava*II
2 : Genomic DNA of JMH 1(4) SM digested with *Pvu*I
3 : Genomic DNA of JMH 1(4) SM digested with *Sfr274*I
4 : Genomic DNA of JMH 1(4) SM digested with *Sma*I
5 : Genomic DNA of JMH 1(4) SM digested with *Sal*I
6 : Genomic DNA of JMH 1(4) SM digested with *Xba*I

Agarose Gel Electrophoresis of bacteria strain JMH 1(4) SM digested with selected restriction enzymes. This gel was run at 0.8% agarose in 0.5 x TBE buffer, 100 V for 50 minutes.

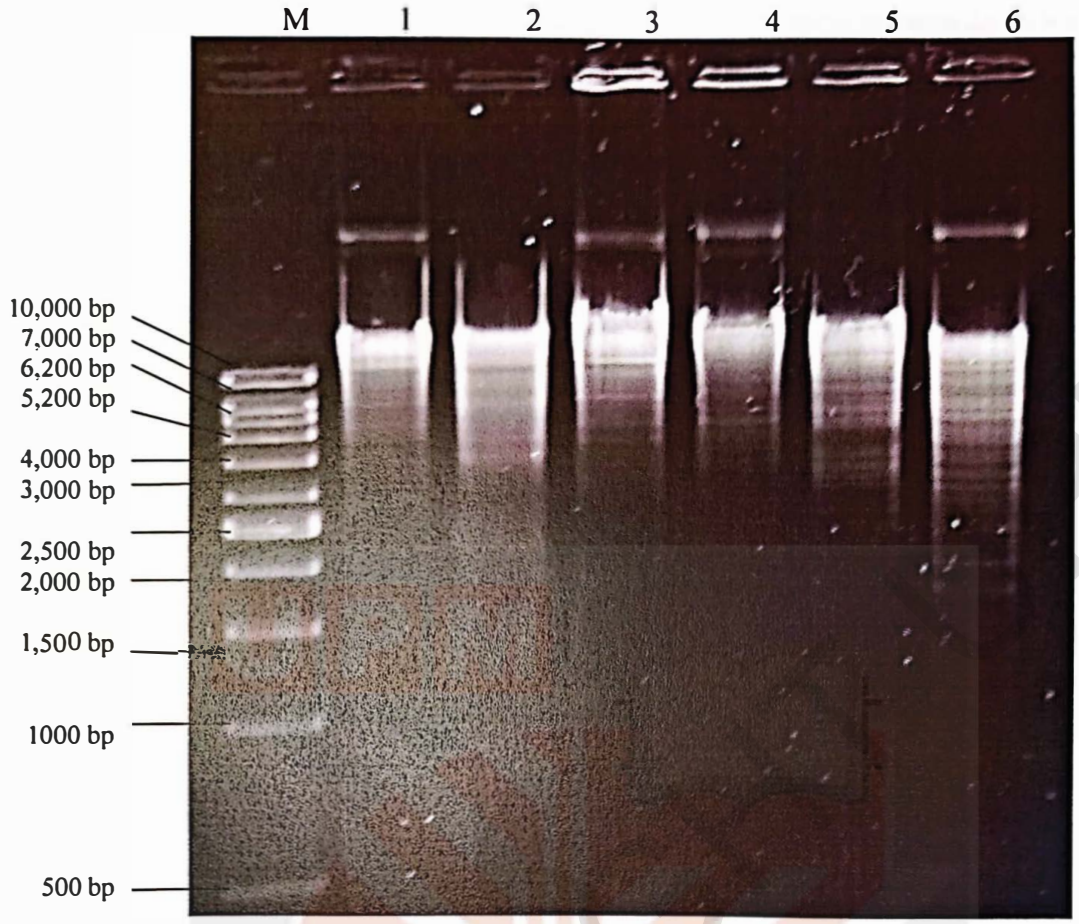


Figure 18: Restriction pattern analysis of genomic DNA JMH 1(4) SM using *Asu*NHI, Bg*l*II, *Bam*HI, BseX3I, *Psp*124I, *Bsp*19I.

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Asu*NHI
2 : Genomic DNA of JMH 1(4) SM digested with Bg*l*II
3 : Genomic DNA of JMH 1(4) SM digested with *Bam*HI
4 : Genomic DNA of JMH 1(4) SM digested with BseX3I
5 : Genomic DNA of JMH 1(4) SM digested with *Psp*124I
6 : Genomic DNA of JMH 1(4) SM digested with *Bsp*19I

Agarose Gel Electrophoresis of bacteria strain JMH 1(4) SM digested with selected restriction enzymes. This gel was run at 0.8% agarose in 0.5 x TBE buffer, 100 V for 50 minutes.

Table 1: Estimated Number and Size of Prominent Bands Obtained from Restriction Pattern Analysis

Restriction Enzymes	Number of prominent restriction bands	Size of DNA fragments (bp)
*Bgl*I	4	10000, 7000, 5200, 4700
*Cci*NI	1	10000
*Eco*RI	5	10000, 6200, 4000, 3000, 2500
*Fau*NDI	8	10000, 8000, 7000, 6200, 5000, 4000, 3500, 3000, 2500
*Hind*III	12	10000, 6200, 5200, 4000, 3200, 2700, 2300, 2000, 1700, 1500, 1300, 1000
*Pst*I	-	Smearing
*Bme*18I	9	10000, 8000, 7000, 6200, 5000, 4500, 4000, 2300, 2200
*Bsn*I	4	5200, 2300, 1300, 1200
*Bsp*19I	-	Smearing
*Bss*MI	4	2300, 2000, 1700, 1500
*Eco*RV	8	10000, 9000, 8000, 7200, 7000, 6200, 5200, 3500
*Kpn*I	1	10000
*Sal*I	2	10000, 5200
*Ava*II	10	8000, 7000, 6200, 4000, 1900, 1700, 1500, 1200, 1000, 500
*Pvu*I	7	8000, 4000, 3000, 2800, 2500, 2200, 1400
*Sfr*274I	4	6200, 3500, 2500, 1300
*Sma*I	4	6200, 5200, 2300, 700
*Sal*I	10	10000, 6200, 5200, 4500, 4000, 3500, 2500, 2300, 1500, 1300
*Sal*I	2	10000, 5200
*Asu*NHI	5	12000, 6200, 4500, 3500, 3000
*Bgl*II	4	10000, 7000, 6200, 4000
*Bam*HI	4	10000, 5200, 4500, 4000
*Bse*X3I	2	7000, 5200
*Psp*124I	5	6200, 5200, 5000, 4000, 3800

4.3 Gel DNA recovery

From the restriction pattern analysis, genomic DNA JMH 1(4) SM was digested with *Eco*RI, *Fau*NDI and *Hin*dIII restriction enzymes to obtain the 2–5 kb DNA fragment. The restriction pattern of JMH 1(4) SM digested with *Eco*RI is shown in Figure 19 while restriction patterns of JMH 1(4) SM digested with *Fau*NDI and *Hin*dIII are shown in Figure 20 and Figure 21, respectively. The portion of gel at the exact location that contained the 2–5 kb of DNA fragment was cut out and recovered using GF-1 DNA recovery kit from Vivantis Technologies. Figures 22, 23 and 24 show the 2–5 kb of DNA fragments of JMH 1(4) SM that were successfully recovered.

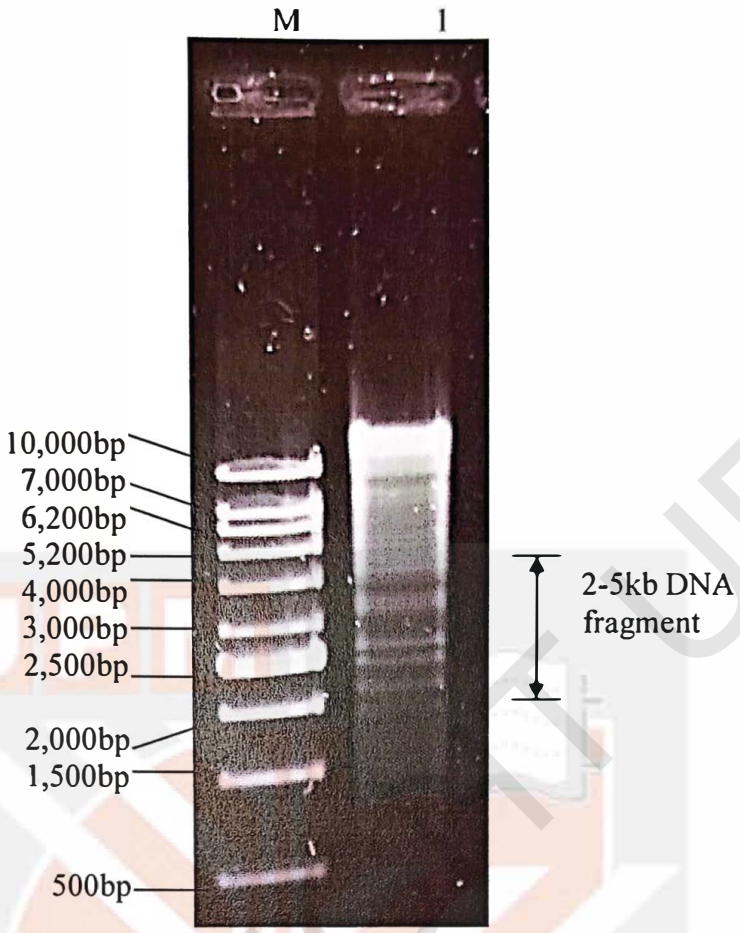


Figure 19: Agarose Gel Electrophoresis of genomic DNA of JMH 1(4) SM digested with *Eco*RI.

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Eco*RI

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes.

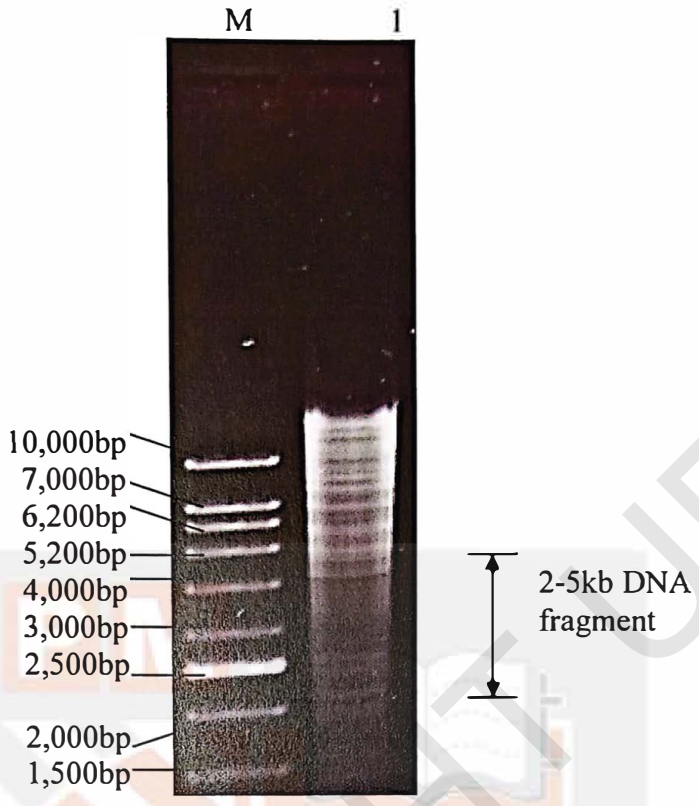


Figure 20: Agarose Gel Electrophoresis of genomic DNA of JMH 1(4) SM digested with *Fau*NDI

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Fau*NDI

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes.

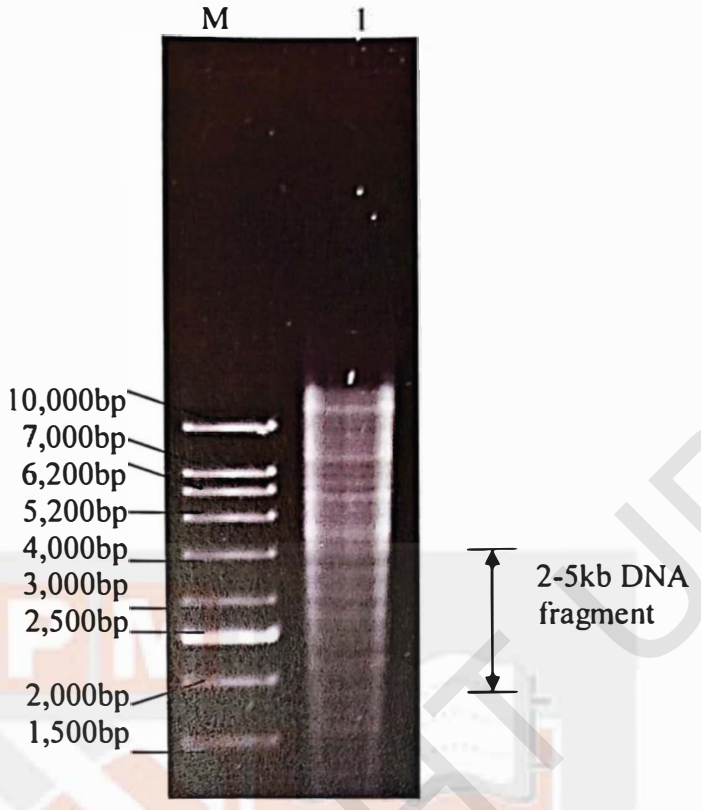


Figure 21: Agarose Gel Electrophoresis of genomic DNA of JMH 1(4) SM digested with *Hin*dIII

M : 1kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Hin*dIII

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes

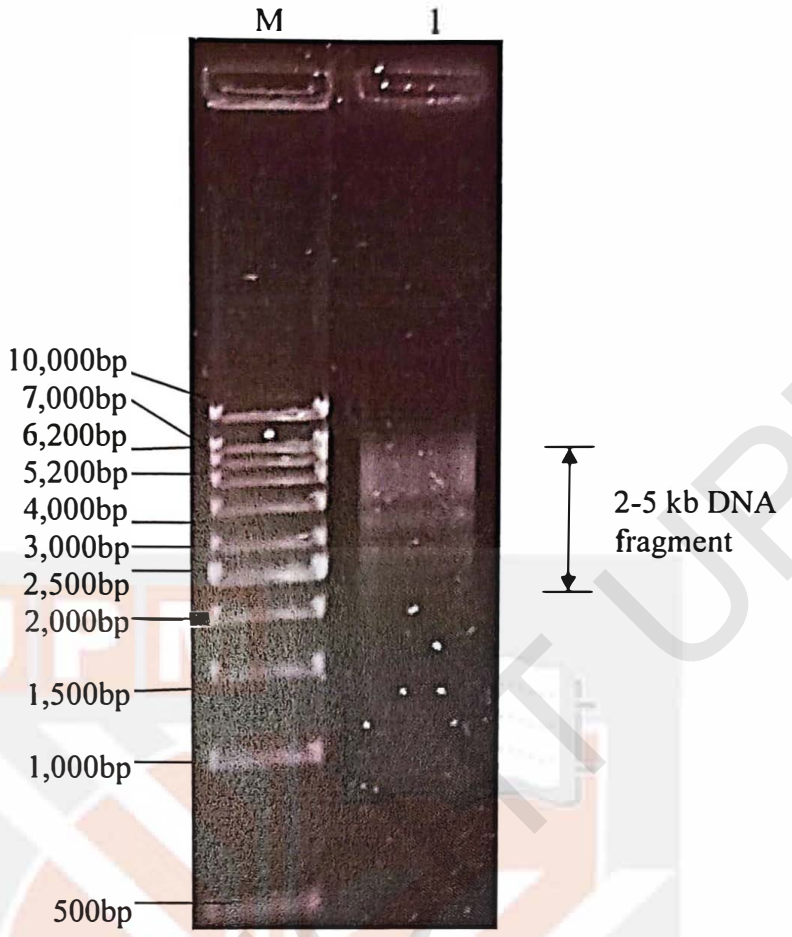


Figure 22: Agarose gel electrophoresis for 2–5 kb of recovered DNA fragment of JMH 1 (4) SM digested with *Eco*RI

M : 1 kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Eco*RI

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes.

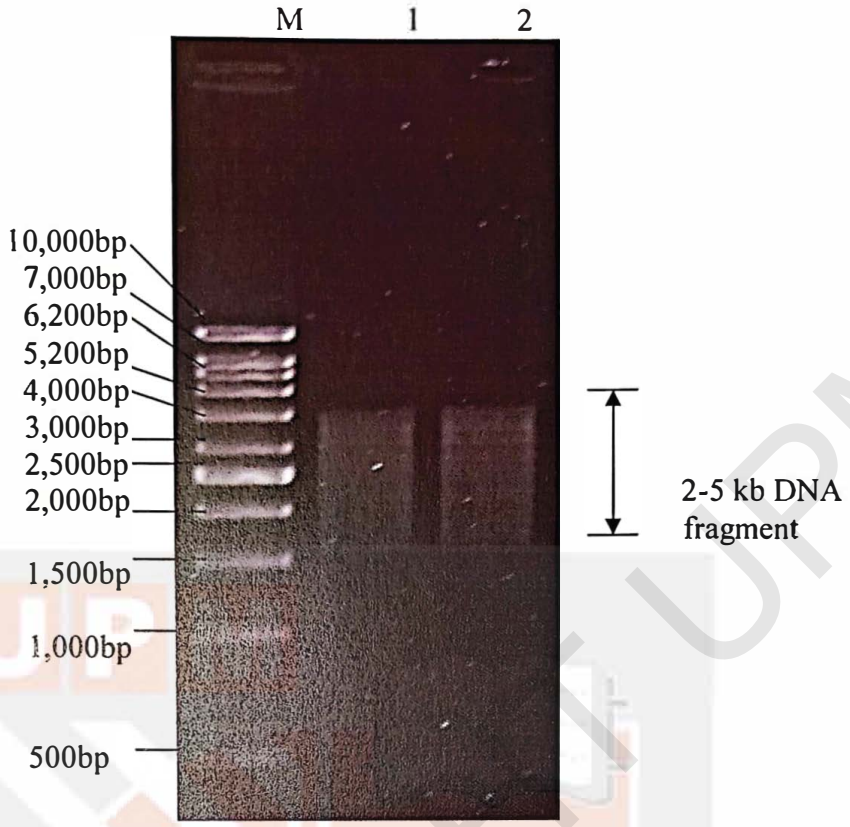


Figure 23: Agarose gel electrophoresis for 2–5 kb of recovered DNA fragment of JMH 1 (4) SM digested with *Fau*NDI

M : 1 kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Fau*NDI
2 : Genomic DNA of JMH 1(4) SM digested with *Fau*NDI

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes.

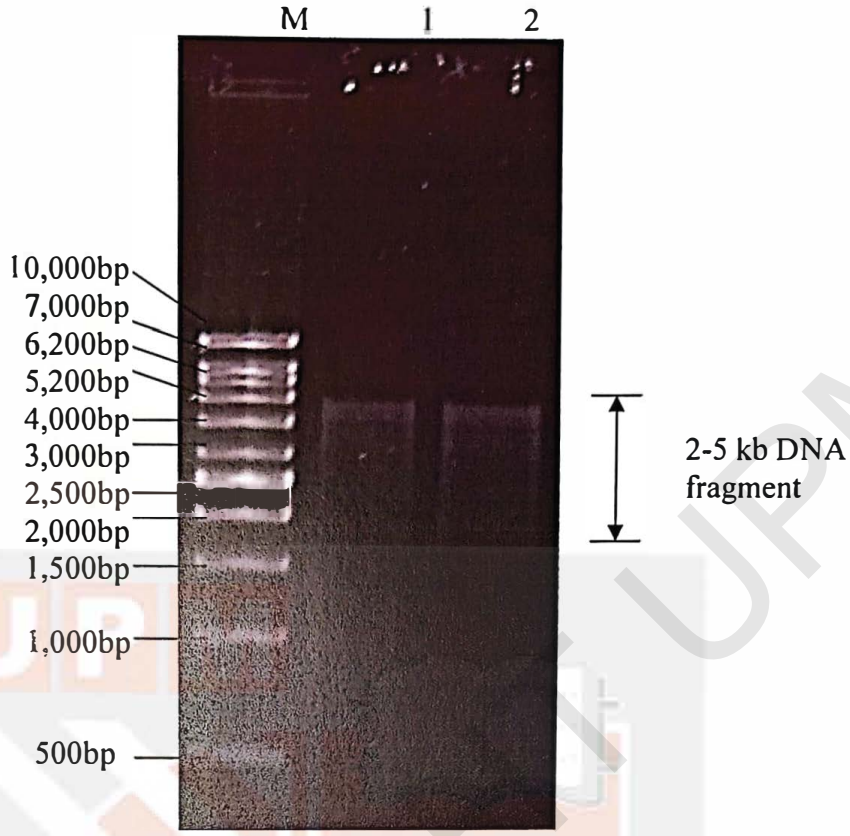


Figure 24: Agarose gel electrophoresis for 2–5 kb of recovered DNA fragment of JMH 1 (4) SM digested with *Hin*dIII

M : 1 kb DNA ladder
1 : Genomic DNA of JMH 1(4) SM digested with *Hin*dIII
2 : Genomic DNA of JMH 1(4) SM digested with *Hin*dIII

This gel was run at 0.8% agarose in 0.5 x TBE Buffer, 100 V for 50 minutes.

4.4 Ligation and Transformation

After the 2–5 kb of DNA fragments of JMH 1(4) SM were recovered, they were ligated into linearized plasmid pET23a (+) prior to digestion with *Eco*RI, *Bam*HI and *Fau*NDI restriction enzyme, respectively. The ligated plasmids were transformed into TOP10F' *Escherichia coli* using $CaCl_2$ transformation and electroporation transformation. The successful ligated plasmids will form colonies on the LB agar plates with Ampicilin (50 mg/mL, stock concentration) (Table 2). There is a single colony was successfully grown after the Calcium Choride Transformation.

Table 2: Number of colonies grown in LB agar after primary transformation

No.	Calcium Choride Transformation	Electoporation Transformation
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	1	-

4.5 Pre-screening for Methylase

The transformed recombinant plasmid was extracted and digested with cell lysate of the bacteria strain JMH 1(4) SM before re-transformed into TOP10F' *Escherichia coli*. The presence of methylase activity was verified by re-transforming the recombinant plasmid into TOP10F' *Escherichia coli* by $CaCl_2$ transformation and electroporation transformation. However, there were no colonies grown on LB agar after the secondary transformation process (Table 3).

Table 3: Number of colonies grown in LB agar after secondary transformation

No.	Calcium Choride Transformation
1	0
2	0
3	0

CHAPTER 5

DISCUSSION

5.1 Strain Verification

The provided bacteria strain JMH 1(4) SM was verified for the presence of *Pvu*II isoschizomer. The bacteria strain JMH 1(4) SM was lysed to obtain the lysate containing the *Pvu*II isoschizomer restriction endonuclease. By using lysate of bacteria strain JMH 1(4) SM to digest the lambda DNA (λ DNA), the restriction bands shown in the agarose gel were shown to be coherent with the Fermentas Inc. catalogue.

The λ DNA used for comparison revealed results comparable to the restriction pattern of λ DNA with *Pvu*II documented in the Fermentas Inc. catalogue. The gel images also indicated the high level of RE since complete restriction was obtained after 4x serial dilution. Agarose gel electrophoresis images of λ DNA digested by *Pvu*II isoschizomer in bacteria strain JMH 1(4) SM was run for extra 10 minutes, confirmed the separation of 4 bands around 4000 bp to 5000 bp.

Cleaner and better separated bands were obtained after serial dilution of the cell lysate. Smearing was found in L1 of Figure 13 and 14, was probably due to high concentration lysate where significant amounts of nucleases was present. Similar results were found with λ DNA digestion by using RMS from other bacteria strains. As the nucleases were diluted through serial dilution from L1 to L5, the cell lysate performed cleaner restriction bands. In L5, the bands were blurring, probably caused by decrease in the *Pvu*II isoschizomer RE in cell lysate from JMH 1(4) SM

following the dilution. There was a band at the control region in L6 of (Figure 13 and 14). This was due to the λ DNA without digestion which serve as control.

The restriction pattern obtained from λ DNA digested with cell lysate of bacteria strain JMH 1(4) SM which contain *Pvu*II isoschizomer RE performed a similar restriction pattern to the restriction pattern of λ DNA digested with *Pvu*II. Thus, the presence of *Pvu*II isoschizomer RE in bacteria strain JMH 1(4) SM was successfully verified.

5.2 Restriction Pattern Analysis

After the strain verification, the restriction pattern of genomic DNA of JMH 1(4) SM was analyzed using selected restriction enzymes which were chosen based on the Multiple Cloning Site (MCS) of the plasmid pET23a (+). The restriction enzymes that had been used were *Bgl*I, *Cci*NI, *Eco*RI, *Fau*NDI, *Hin*dIII, *Pst*I, *Sal*I and *Xba*I because they perform on MCS of pET23a (+). However, the RE did not present on MSC of pET23a (+) are *Bme*18I, *Bsn*I, *Eco*RV, *PvuI*, *Sma*I, *Bsp*19I, *Bss*MBI, *Kpn*I and *Sfr*274I.

Estimated number and size of restriction bands obtained from restriction pattern analysis were shown in Table 1. Suitable RE which can produce DNA fragments at 2-5 kb including *Asu*NHI, *Bam*HI, *Bgl*I, *Bme*18I, *Bsn*I, *Bsp*19I, *Bss*MBI, *Bxe*X3I, *Eco*RV, *Kpn*I, *Psp*124I, *Sfr*274I and *Sma*I. This information is valuable since it can be used as guideline in subsequent cloning steps. DNA fragment of 2-5 kb were of interest because most of the gene coding sequence of the bacteria is within this size. Some of the restriction patterns that were obtained did

not show clear bands probably due to several factors such as those RE is rare cutting enzymes such as *Cci*NI, improper incubation time and inadequate restriction enzymes used.

From the restriction pattern analysis of genomic DNA of JMH 1(4) SM, *Eco*RI, *Hin*dIII and *Fau*NDI were chosen for further cloning because they are present on MCS of the plasmid pET23a (+) and lots of prominent bands obtained around the 2-5 kb region in the restriction pattern analysis (Figure 15).

5.3 Ligation and Transformation

The desired DNA fragment was purified from gel electrophoresis and ligated into a cloning plasmid pET23a (+) prior to transformation into TOP10F' *E.coli*. Both plasmid pET23a (+) and the DNA fragment were then subjected to digestion with *EcoR*I, *Hin*dIII and *Fau*NDI respectively, to yield complementary sticky end. Before the ligation process, the linearized plasmid was treated with Calf Intestine Alkaline Phosphatase (CIAP). CIAP are used to catalyze the release of 5'- and 3'-phosphate groups from DNA, RNA and nucleotides, remove phosphate groups from proteins and prevent the ligation of the plasmid itself (Mossner *et al.*, 1980). After the transformation procedure, the efficiency of transformation was calculated on the basis of number of colonies formed after overnight incubation on plates onto LB agar with appropriate antibiotic. A single successful colony was transformed through the Calcium Chloride transformation of the *EcoR*I ligated plasmid (Table 2).

The low efficiency of ligation and Calcium Chloride transformation may due to several factors such as incomplete purified linearized pET23a (+), excess of

ligation mixture or larger volume of competent *E.coli* was used (Sawahel *et al.*, 1997). Thus, some of these considerations must be addressed to increase better performance of the transformation. The linearizing and CIAP treated pET23a (+) needed to be gel purified or at least phenol chloroform purified (Ausubel, 1995) to remove unwanted enzyme before being used in ligation. The ligation buffer must not freeze-thawed many times due to the presence of ATP, which is unstable and degraded by multiple freeze-thawed cycles. In addition, the buffer should completely melt and thawed before use. The ligation mixture should freshly prepared and used for transformation to prevent drastic drop in efficiency. Less volume of ligation mixture (2-5 µL) for transformation and smaller volume of competent cells (80 -100 µL) should be considered. The presence of inhibitor in ligation mix will affect the transformation. In order to obtain good transformation, the volume of ligated plasmid should be close to the competent cells. This is because; a larger volume of competent cells may result in loss of contact of the ligated plasmid to competent cells before heat-shock. Besides that, another way to increase the transformants is to divide the DNA fragments size into smaller size such as 2-3 and 3-5 kb, due to the difficulty of ligating bigger DNA fragments into plasmids.

5.4 Pre-screening for Methylase

The recombinant plasmid was extracted and digested with cell lysate of JMH 1(4) SM and transformed again into TOP10F' *Escherichia coli*. If the recombinant plasmid contains the gene coding for *Pvu*II isoschizomer methylase, it will not be digested by the *Pvu*II isoschizomer in the lysate of JMH 1(4) SM. Therefore, it will survive when transformed into TOP10F' *Escherichia coli*. Result in Table 3 showed that no colony was successful grown, probably due to the low efficiency of the

Calcium Chloride Transformation. In order to increase the performance of transformation, the RE from crude cells lysate of JMH 1(4) SM probably needed to be purified before digestion of the recombinant plasmid, because nucleases of the crude cells lysate will digest all the DNA including the recombinant plasmids during the digestion (Kharazmi, 2003).

CHAPTER 6

CONCLUSION

The bacteria strain JMH 1(4) SM had been successfully verified for the presence of the *Pvu*II isoschizomer restriction endonuclease. Various restriction patterns of genomic DNA of JMH 1(4) SM had been obtained by using selected commercially available restriction enzymes. Suitable RE which can produce 2-5 kb DNA fragments had been identified. Prominent DNA fragments around 2-5 kb were obtained by the digestion of genomic DNA JMH 1(4) SM using *Eco*RI, *Hin*dIII and *Fau*NDI. The DNA of 2-5 kb DNA fragments was successfully eluted and was ligated into the linearized plasmid pET23a (+) and was transformed into TOP10F' *Escherichia coli*. The isoschizomer *Pvu*II restriction modification system was not successfully cloned into *Escherichia coli* due to the methylase activity was not confirmed. However, the cloning of *Pvu*II isoschizomer RMS needed further research by using other suitable RE digested fragments from the restriction patterns. The restriction patterns had been obtained through this research will be very useful in future cloning efforts.

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APPENDICES

A.1 Table of Antibiotic Solutions.

Antibiotics	Stock solution		Working Concentration
	Concentration	Storage	Strigent Plasmid
Ampicilin	50 mg/mL in distilled H_2O	- 20°C	20 µL/mL
Chloramphenicol	34 mg/mL in ethanol	- 20°C	25 µL/mL
Kanamycin	10 mg/mL in distilled H_2O	- 20°C	10 µL/mL
Streptomycin	10 mg/mL in distilled H_2O	- 20°C	10 µL/mL
Tetracycline	5 mg/mL in ethanol	- 20°C	10 µL/mL

Note: Stock solutions of antibiotics were dissolved in steril H_2O by filtration through 0.22 µL filter.

A.2 Table of Unique Sites in pET23a (+)

Enzyme	Recognition	frequency	Position
*Bam*HI	G'GATC_C	1	199
*Bgl*II	A'GATC_T	1	335
*Bst*1107I	GTA'TAC	1	1160
*Ecl*136II	GAG'CTC	1	189
*Eco*47III	AGC'GCT	1	643
*Eco*52I	C'GGCC_G	1	167
*Eco*RI	G'AATT_C	1	193
*Hind*III	A'AGCT_T	1	174
*Mls*I	TGG'CCA	1	360
*Nde*I	CA'TA_TG	1	239
*Nhe*I	G'CTAG_C	1	232
*Not*I	GC'GGCC_GC	1	167
*Pdi*I	GCC'GGC	1	3528
*Pst*I	C_TGCA'G	1	2527
*Pvu*I	CG_AT'CG	1	2652
*Pvu*II	CAG'CTG	1	980
*Sac*I	G_AGCT'C	1	191
*Sal*I	G'TCGA_C	1	180
*Sca*I	AGT'ACT	1	2762
*Xba*I	T'CTAG_A	1	277
*Xho*I	C'TCGA_G	1	159

A.3 Table of Reaction Buffers.

Buffer	Contain
Buffer *Bgl*I	20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT
Buffer *Eco*RI	100 mM Tris-HCl (pH 7.5, at 25°C), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT
Buffer *Eco*RV	100 mM Tris-HCl (pH 8.5, at 30°C), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT
Buffer *Fau*NDI	33mM Tris-acetate (pH 7.9), 10mM Mg-acetate, 66 mM K-acetate, 1 mM DTT
Buffer V1	10mM Tris-HCl (pH 7.4, at 30°C), 10 mM $MgCl_2$, 1 mM DTT
Buffer V2	10mM Tris-HCl (pH 7.4, at 30°C), 10 mM $MgCl_2$, 1mM DTT
Buffer V3	50mM Tris-HCl (pH 7.4, at 30°C), 10 mM $MgCl_2$, 100 mM NaCl, 1mM DTT
Buffer V4	10mM Tris-HCl (pH 8.5, at 30°C), 100 mM KCl, 1mM DTT
Buffer V5	30mM Tris-acetate (pH 7.4, at 30°C), 10mM Mg-acetate, 60 mM K-acetate, 1 mM DTT
Buffer R	10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA
Buffer G	10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl, 0.1 mg/mL BSA

A.4 The Formulation of Reaction Mixture for Restriction Pattern Analysis

Restriction Enzyme	Reaction buffer (µL)	Sterile H_2O (µL)	DNA (µL)	10X BSA (µL)	Restriction Enzyme (µL)	Total (µL)
*Asu*NHI	2	7	8	2	1	20
*Bam*HI	2	7	8	2	1	20
*Bgl*I,	2	7	8	2	1	20
*Bgl*II	2	7	8	2	1	20
*Bse*X3I	2	7	8	2	1	20
*Bsp*124BI	2	7	8	2	1	20
*Cci*NI	2	7	8	2	1	20
*Eco*RI	4	5	8	2	1	20
*Fau*NDI	2	7	8	2	1	20
*Hin*dIII	2	7	8	2	1	20
*Pst*I	2	7	8	2	1	20
*Pvu*II	2	9	8	-	1	20
*Sal*I	2	7	8	2	1	20
*Sfr*274I	2	7	8	2	1	20
*Xba*I	2	7	8	2	1	20
*Bme*18I	2	7	8	2	1	20
*Bsn*I	2	7	8	2	1	20
*Bsp*19I	2	7	8	2	1	20
*Bss*MI	2	7	8	2	1	20
*Eco*RV	2	7	8	2	1	20
*Kpn*I	2	7	8	2	1	20
*Pvu*I	2	9	8	-	1	20
*Sma*I	2	7	8	2	1	20

PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Cloning the *Pvu*II isoschizomer restriction modification system from JMH 1(4) SM into *Escherichia coli*." by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, University Putra Malaysia Bintulu Campus and published in the form approved by the Faculty.

Lee Swee Ling

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