CHAPTER 5

PREPARATION OF INSERT DNA, LIGATION, PACKAGING AND TITERING THE LIBRARY

5.1 INTRODUCTION

Genomic library is a population of independent DNA insert containing all the necessary sequence information which represents the total genetics of the organism allowing DNA propagation in the host cell (Abcouwer, 2001). One of the most important aspects in DNA cloning is the insert size. There are various commercially available vectors with different capacity ranging from a few kb to 100kb to cater for different purpose as one insert size does not meet all needs. For example conventional plasmids hold up to 10kb of DNA, λ bacteriophage hold up to 20kb whereas cosmids can hold up to 45kb (Shashikant *et al.*, 1998). The somatic cell hybrid technology allowed the cloning of whole chromosomes without reference to regional location or linkage order. Cloning of subchromosomal fragments enables the assignment of genes to regions as small as 1Mb and provides information on gene order.

For instance, *E. coli* with a genome size of 4.6x 10^6 base pair is fragmented into 17kb fragment lengths where 820 clones is needed to find the particular gene of interest compared to a 35kb if fragment length that which will produce 410 clones with a 95% probability. This is calculated based on the formula below:

$$N = \frac{\ln (1 - \mathrm{P})}{\ln \left[1 - \left(\frac{a}{b}\right)\right]}$$

Where,

N= the number of clones needed;

P= the probability of that any gene will be present

a= the average size of the DNA fragments inserted into the vector

b= the total size of the genome

Bacteriophage usually contains multiple cloning sites (Figure 5.1) and is used as a vector due to its high infectivity and clone style propagation. Most viral genome is not essential for lytic growth which enables the substitution with foreign gene segments. Among some of the advantages of bacteriophage includes: (1) high cloning efficiency; (2) relatively large insert-size and; (3) suitability for screening using nucleic acid probes. The commercially available bacteriophage vectors supplied as pre-digested and modified arms have simplified the construction of genomic libraries (Wang *et al.*, 2003).

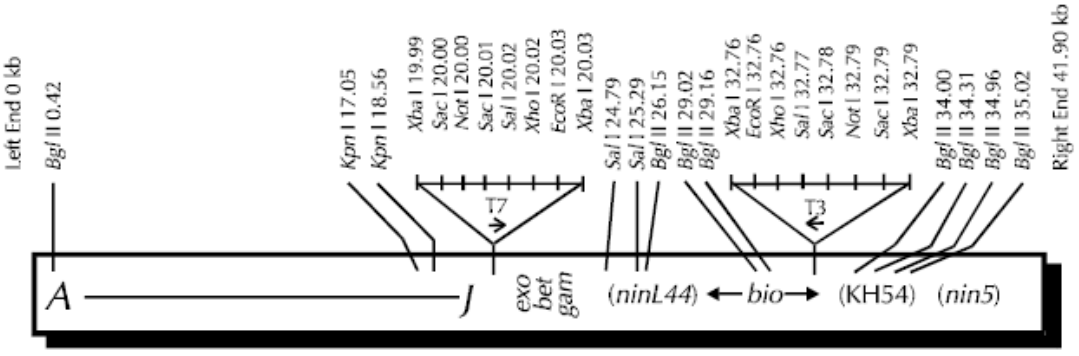

Fig. 5.1: Lambda FIX II vector with multiple cloning sites.

For this study Lambda FIX II/*Xho* I Partial Fill-In (Stratagene) was chosen as the vector which had been digested with *XhoI* enzyme and partially filled in with dCTP and dTTP, leaving 3'-CT-5' overhangs. It can accommodate 9-23kb fragments. Gigapack III XL Packaging Extract, an *in vitro* packaging extract specially designed for use in constructing

genomic library was used to form an infectious viral particle. Rahman *et al.*, (2007) had used a similar vector in constructing the genomic library of rice.

Once the phage infects the bacteria by injecting its DNA, the linear double stranded DNA will be circularised at the cos site and incorporated into the genome of bacteria or take over the metabolism of the bacteria producing many progeny and killing the bacteria by lysing the cell membrane. Due to its evolutionary history of infecting bacteria, the success of introducing recombinant DNA into the host is much higher compared to the transformation of the plasmid into the bacteria (Chauthaiwale *et al.*, 1992 and Mulhardt and Beese M.D, 2007).

The aims of this chapter are to:

i. Prepare the insert DNA

ii. Ligate the insert DNA into Lambda Fix II vector

iii. Form an infective viral particle using the Gigapack III XL Packaging Extract

iv. Titer the library

5.2 MATERIALS AND METHODS

5.2.1. Preparation of Reagents

10mM dGTP

10µl of 100mM dGTP (Finnzymes) was added into 90µl sterile distilled water. The solution was stored at -20°C.

10mM dATP

10µl of 100mM dATP (Finnzymes) was added into 90µl sterile distilled water. The solution was stored at -20°C.

TE buffer (10mM Tris-Cl, 1mM EDTA)

This buffer was prepared according to Section 4.2.

Phenol-chloroform

This solution was prepared according to Section 4.2.2

10 X STE buffer

5.84g of NaCl (Sigma) and 2.92g of EDTA (Sigma) was dissolved in 50ml of sterile distilled water. 20ml of Tris-HCL (1M, pH7.5) was added to the solution and volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

1 X STE buffer

0.1ml of 10X STE buffer was added to 0.9ml of sterile distilled water. The solution was stored at room temperature.

70% (v/v) Ethanol

This solution was prepared according to Section 4.2.2

Tris-Cl (1M, pH7.5)

30.28g of Tris-base (Sigma) was dissolved in 200ml sterile distilled water, pH was adjusted to pH 7.5 using concentrated hydrochloric acid and volume made up to 250ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Gelatin (2% w/v)

2g of Bovine gelatine (Merck) was dissolved in 10ml of sterile distilled water. The solution was stored at room temperature.

SM buffer

2.9g of Sodium Chloride (Sigma) and 1g of Magnesium Sulfate Heptahydrate (Merck) were dissolved in 300ml of sterile distilled water. 25ml of Tris-Cl (1M, pH 7.5) and 2.5ml of Gelatin (2% w/v) were added into the solution and made up to 500ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

NZY broth

10g of NZ Amine (Merck), 5g of Yeast Extract (Merck), 5g of Sodium Chloride (Sigma) and 2g of Magnesium sulphate heptahydrate (Merck) were added into a beaker containing 100ml sterile distilled water, pH was adjusted to 7.5 with Sodium hydroxide pellets (Merck) and volume made up to 1000ml with sterile distilled water. The solution was autoclaved.

NZY agar plate

15g of bactoagar powder (BD) was added to 1000ml of NZY broth and autoclaved. Solution was cooled to 60°C and poured into plates. The plates were then placed in a plastic and sealed. The sealed plastic containing the plates were stored at 4°C.

NZY supplemented with 0.2% maltose

250ml of NZY broth was autoclaved and allowed to cool down to 40°C before adding 2.5ml of sterile 20% maltose. The solution was the stored at room temperature.

20% Maltose

20g of Maltose (Merck) was dissolved in 100ml of sterile distilled water and filter sterilized using 0.2µm pore size (Sartorius). The solution was the stored at room temperature.

10mM $MgSO_4$

0.2465g of Magnesium sulphate heptahydrate (Merck) was dissolved in 100ml of sterile distilled water. The solution was the stored at room temperature.

NZY top agarose

1.75g of agarose powder (Promega) was added into 250ml of NZY broth and autoclaved.

5.2.2. Preparation of the Insert DNA

5.2.2.1. Pilot Scale Partial Digestion

Each tube (Table 5.1) containing 2.5µg of DNA was mixed with 10X *Bam*HI buffer, 10mg/ml BSA, 10mg/ml RNase A, sterile distilled water and different ratio of enzyme (Table 5.2); 0.05U/µg, 0.10U/µg, 0.15U/µg, 0.20U/µg, 0.25U/µg and 0.30U/µg. The contents were gently mixed by pipetting before dispensed into each tube. The tubes were incubated in a 37°C water bath for 90 minutes. 5µl of loading dye (EURx) was added into the tubes to stop the reaction at the end of incubation period.

Table 5.1: DNA master mix cocktail. The following table shows the quantity of component added into each tube.

Tube	Per reaction	For 6 reactions
2.5µg DNA (µl)	2.4	14.4
10X *Bam*HI buffer (µl)	0.50	3.0
10mg/ml BSA (µl)	0.15	0.9
10mg/ml RNase A (µl)	0.15	0.9
Sterile distilled water (µl)	Made up to 5µl	Made up to 30µl

Table 5.2: Serial dilution of *Bam*HI to genomic DNA of *H. undatus.* The following table shows the volume of restriction enzyme required to add into each tube to produce different dilution ratio.

Tube	1	2	3	4	5
DNA(µg)	2.5	2.5	2.5	2.5	2.5
Dilution (U/µg DNA)	0.05	0.10	0.15	0.20	0.25
Unit of restriction enzyme	0.25	0.50	0.75	1.00	1.25
Volume of 0.1U/µl restriction enzyme (µl)	2.5	5	7.5	10.0	12.5

5.2.2.2 Agarose Gel Electrophoresis

The best dilution of unit of enzyme per microgram of DNA that yielded highest intensity of fragments between 9-23 kb was identified through agarose gel electrophoresis. Samples from Section 5.2.2.1 were loaded to agarose gel. The agarose gel was prepared as described in Section 4.2.5.

5.2.2.3. Full Scale Partial Digestion

In this study, 0.25U/µg yielded the highest intensity of fragments between 9-23 kb (Figure 5.2). Hence the ratio was used for full scale partial digestion. Ten tubes (Table 5.3) containing 5µg of DNA was mixed with 10X *Bam*HI buffer, 10mg/ml BSA, 10mg/ml RNase A and 0.25U of *Bam*HI enzyme (Table 5.4). The contents mentioned above were gently mixed by pipetting before dispensed into each tube. The tubes were incubated at 37°C for 90 minutes and at 65°C for 20 minutes in water bath to denature the *Bam*HI enzyme. The content from the 10 tubes were pooled to make 1 tube after the *Bam*HI enzyme was denatured. The pooled 50µg genomic DNA insert was precipitated by mixing with 1ml of 100% (v/v) ethanol and centrifuged for 10 minutes at 14,000rpm. The supernatant was decanted and the DNA pellet was washed with 1ml of 70% (v/v) ethanol. The mixture was further centrifuged for 1 minute at 14,000rpm. The supernatant was then decanted and the pellet was air-dried and resuspended in 100µl of TE buffer.

Table 5.3: DNA master mix cocktail for full scale digestion. The following table shows the quantity of component added into each tube.

Tube	Per reaction	For 10 reactions
5µg DNA (µl)	4.8	48.0
10X *Bam*HI buffer (µl)	1.0	10.0
10mg/ml BSA (µl)	0.1	1.0
10mg/ml RNase A (µl)	0.1	1.0
Sterile distilled water (µl)	Made up to 10µl	Made up to 100µl

Table 5.4: Dilution of *Bam*HI to genomic DNA of *H. undatus* prepared for 1 tube. 12.5 µl of 0.1U/µl restriction enzyme were added to all 10 tubes.

Contents	Tube
DNA(µg)	5
Dilution (U/µg DNA)	0.25
Unit of restriction enzyme	1.25
Volume of 0.1U/µl restriction enzyme (µl)	12.5

5.2.2.4. Partial End Fill With dGTP, dATP and Klenow Polymerase

Samples (Section 5.2.2.3) was partially filled-in according to Stratagene fill-in kit instruction; 30µl of 10X fill-in buffer, 5µl 10mM dATP, 5µl 10mM dGTP, 15U of Klenow polymerase and volume made up to 300µl with sterile distilled water. The mixture was added into a tube and incubated in room temperature at 22°C for 15 minutes.

150µl of 1X STE buffer, 50µl of 50X STE buffer and 500µl of phenol-chloroform were then added into the tube, vortexed and spun for 2 minutes at 14,000 rpm. The upper aqueous phase was transferred into a new tube. The addition of 500µl of phenol-chloroform were then added into the tube, vortexed and spun for 2 minutes at 14,000 rpm.

This step was repeated if there is an interface. 500µl of chloroform was added into the tube, vortexed and spun for 2 minutes at 14,000rpm. The upper aqueous phase was transferred into a new tube and 1ml of 100% (v/v) ethanol was added and incubated at -20°C for 30 minutes. The tube was then centrifuged for 10 minutes at 4°C at 14,000rpm. The supernatant was decanted and the DNA pellet was mixed with 1ml of 70% (v/v) ethanol. The mixture was further centrifuged for 1 minute at 14,000rpm. The supernatant was decanted and the pellet was air-dried and resuspended in 25µl of TE buffer.

5.2.3. DNA Quantification

The purity and the concentration of the DNA sample was determined as described in Section 4.2.4.

5.2.4 Ligation of the Insert DNA to Lambda Phage DNA

Ligation mixture containing 1.0µg of the Lambda Fix II *Xho I* predigested DNA (Stratagene), 1µg insert, 0.5µl of 10X ligase buffer (NEB), and 1µl of T4 DNA Ligase (NEB) was prepared and volume made up to 5µl with sterilized distilled water. The ligation mixture was incubated overnight at 4°C and ready for use in packaging.

5.2.5. Packaging

Two and a half microliter of the ligated DNA (5.2.4) was added to the packaging extract by gentle pipetting. The tube was incubated at room temperature (22ºC) for 2 hours after which 500µl of SM buffer was added followed by 20µl chloroform. The tube was spun briefly and the supernatant, containing the phage and ready for tittering, was stored at 4°C.

5.2.6. Titering of Library

5.2.6.1. Growth and Preparation of Host Strain

A three microliter of *E. coli* strain XL-1 Blue MRA (P2) (Stratagene) was streaked on NZY agar plate. The plate was inverted and incubated overnight at 37°C. Single colony of E. coli strain XL-1 Blue MRA (P2) (Figure 5.2) was isolated into 5ml of NZY supplemented with maltose (0.2% w/v) and grown overnight at 30°C with shaking at 225rpm by using Shellab orbital shaking incubator (S14). Upon incubation, the cells were spun down at 1000rpm for 10 minutes. The medium was decanted and the cell pellet was resuspended in 2.5ml of 10mM $MgSO_4$ and diluted till OD_{600}=0.5.

5.2.6.2. Preparation of the λ Bacteriophage

Two hundreds microliter of XL-1 Blue MRA (P2) at OD_{600}=0.5 was added to individual sterile culture tube in a test tube rack. Ten microliter of λ bacteriophage was added into each sterile culture tube containing the XL-1 Blue MRA (P2) cells. The test tube rack was placed in a 37°C water bath for 20 minutes. Three mils of NZY top agarose was poured into each tube containing the λ phage and E. coli strain XL-1 Blue MRA (P2) after the incubation period. The tube was removed from the rack and quickly flicked before pouring onto the NZY plates. The top agarose was allowed to solidify before inverting and overnight incubation at 37°C.

The Lambda FIX II system operates based on spi (sensitive to P2 inhibition) selection. Lambda phages containing active *red* and *gam* genes on the stuffer fragment are unable to grow on host strains that contain P2 phage lysogens such as XL1-Blue MRA (P2). When the stuffer fragment is replaced by an insert, the recombinant Lambda FIX II vector

becomes Red–/Gam–, and the phage is able to grow on the P2 lysogenic strain. Hence, in the Lambda FIX II system, only recombinant phages are allowed to grow. The titer was determined by using the following formula (Rahman *et al.*, 2007):

$$\frac{\text{Number of plaques (pfu)} \times \text{Dilution factor} \times 1000\ (\mu l/ml)}{\text{Volume of the } \lambda \text{ bacteriophage added to the cells } (\mu l)}$$

5.3 RESULTS

5.3.1 Pilot Scale Partial Digestion

In the pilot scale partial digestion, total DNA of 2.5µg was digested with different ratio of enzyme; 0.05U/µg, 0.10U/µg, 0.15U/µg, 0.20U/µg, 0.25U/µg and 0.30U/µg to obtained the DNA fragments of 9-23Kb. This step is critical as the length is the insert accommodation range of the Lambda FIX II vector. The ratio of 0.30U/µg of *Bam*HI enzyme that yielded majority of fragments in the 9-23Kb range as compared to other enzyme ratio (Figure 5.2). Therefore the ratio of 0.30U/µg of *Bam*HI enzyme was chosen to proceed in full scale partial digestion.

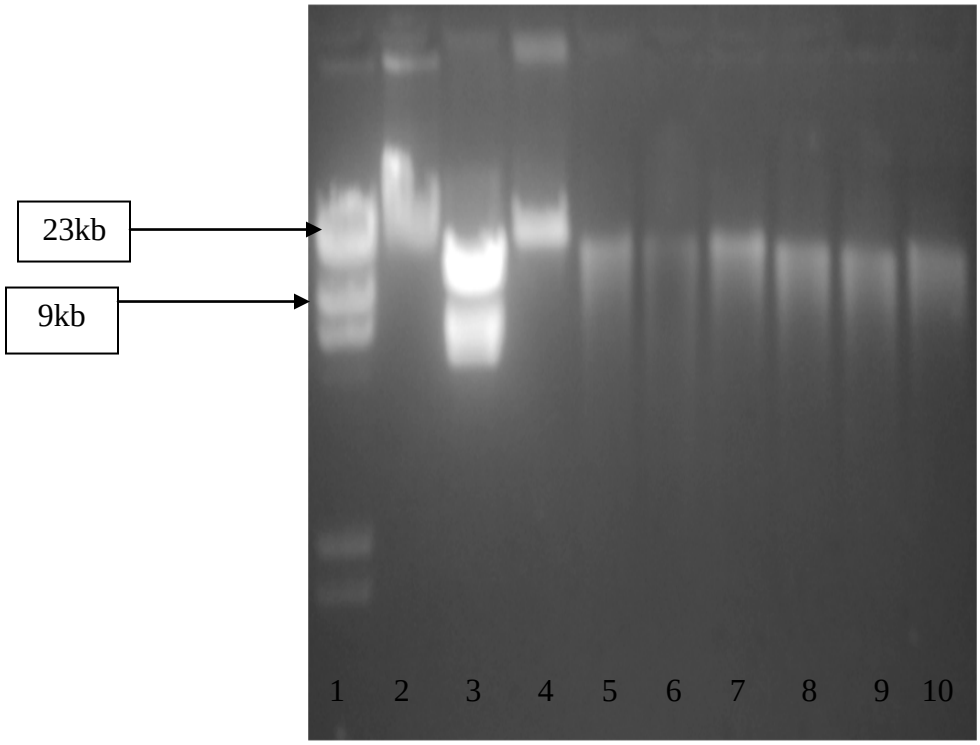

Fig. 5.2: Restriction enzyme digestion of *H. undatus* genomic DNA using different units of BamH1. Lane 1: *HindIII* digest ladder; Lane 2: lambda DNA; Lane 3: lambda DNA digested with *BamHI*; Lane 4: genomic DNA; Lane 5-10: Genomic DNA of *H.undatus* digested with *BamHI* with 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30U/µg respectively. The ratio of 0.30U/µg of *Bam*HI enzyme that yielded majority of fragments in the 9-23Kb range as compared to other enzyme ratio.

5.3.2 DNA Quantification of Purified DNA Insert

After the partial fill-in step, DNA was quantified using UV-spectrophotometer producing a reading at OD 260 and OD 280 of 0.014 and 0.008 respectively. Therefore the purity of the partially filled-in insert DNA was 1.75 (A_{260}/A_{280}) while the concentration of the insert prior ligation with the vector was 0.7µg/µl.

5.3.3 Growth and Preparation of Host Strain

For the growth and preparation of the host strain, a single colony was successfully obtained as shown in Figure 5.3.

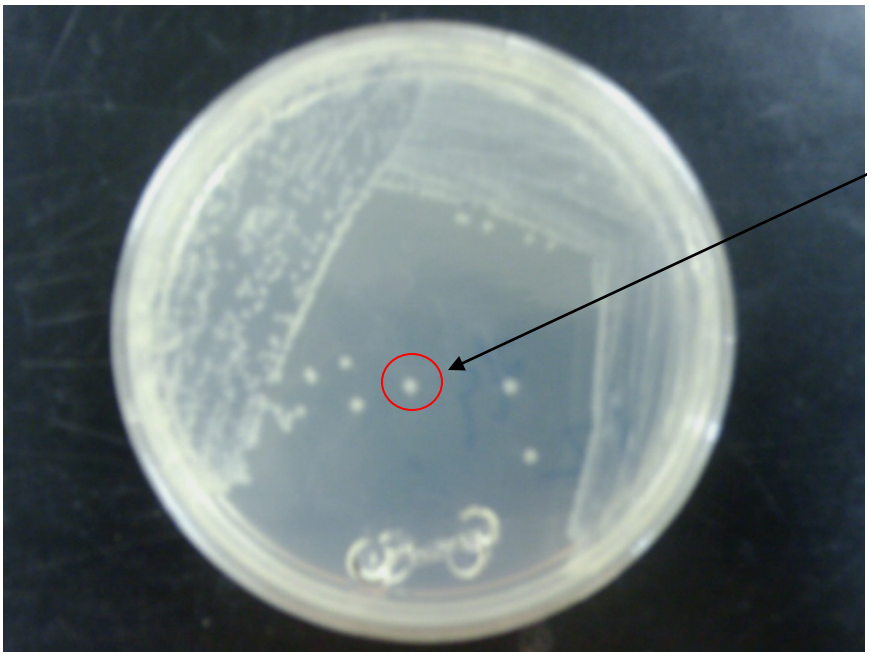

Fig. 5.3: Image of the growth of the host strain XL1-Blue (circled) on NZY plate after overnight incubation at 37°C in order to obtain the single colony. The single colony was then used for the host strain.

5.3.4 Titering of Library

There were approximately 400 plaques (Figure 5.4) formed in the petri dish using 10µl bacteriophage from packaging. The resulting titer for *H. undatus* genomic library was 4 x10^4 pfu/ml using the formula below:

$$\frac{\text{Number of plaques (pfu)} \times \text{Dilution factor} \times 1000\ (\mu l/ml)}{\text{Volume of the } \lambda \text{ bacteriophage added to the cells } (\mu l)}$$

$$= \frac{400 \text{ pfu} \times 1 \times 1000\ \mu l/ml}{10\ \mu l}$$

$$= 4 \text{ x} 10^4 \text{ pfu/ml}$$

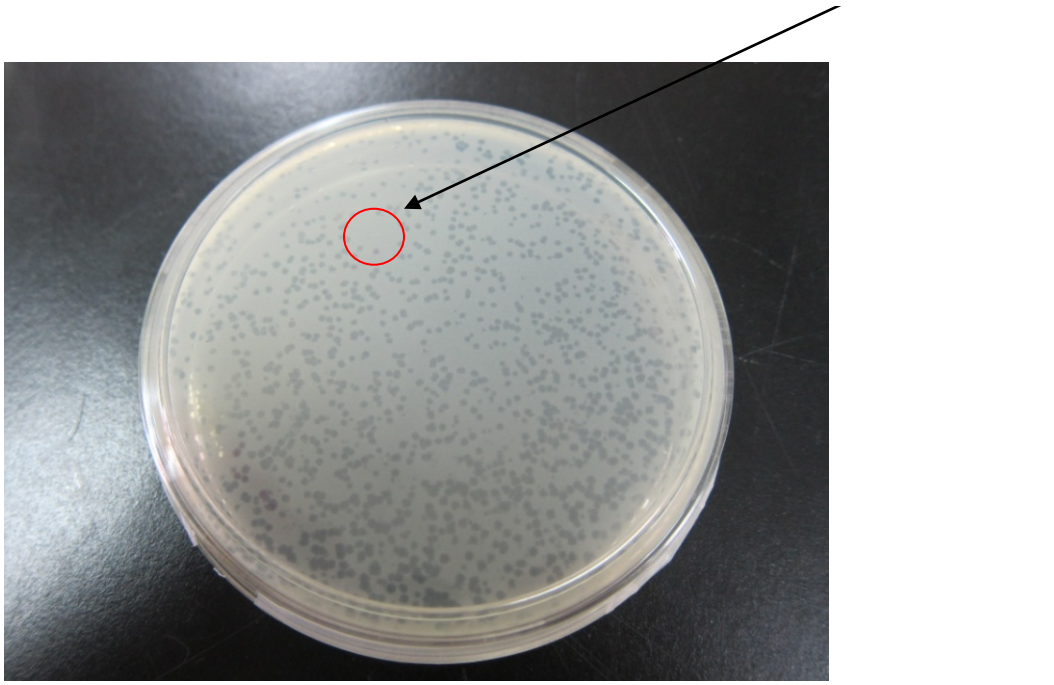

Fig. 5.4: Image of the plaque formation on the NZY plate using 10µl bacteriophage after overnight incubation at 37°C. The red circle showed few of the many plaques that formed in this petri dish.

5.4 DISCUSSION

The prerequisite for restriction digest is that the DNA has to be of high purity and intact. This is crucial as broken fragments of DNA will not produce the desired result and low quality of DNA such as the presence of polysaccharide will hinder restriction digestion. Based on Fig 5.2 (Lane 3), the DNA obtained in this partial digestion was intact.

Partial digestions of the genomic DNA were carried out with different amounts of enzyme for 90 minutes to optimize the conditions in order to obtain DNA fragments in the range of 9-23kb before proceeding to full scale digestion. Generating genomic DNA within 9-23 Kb was very crucial since it is the insert accommodation range of the Lambda FIX II vector. This strategy was similar to Van Buuren *et al.,* 1992. Full scale digestion was carried out using 50µg genomic DNA in 10 tubes instead of using single tube as the DNA pellet cannot be fully dissolved and the resulting solution will be too viscous for restriction enzyme to access the DNA. Furthermore, it increases the surface/volume ratio in order to improve access of the restriction enzyme to the high molecular weight DNA. The other way to obtain the desired fragment is by varying the time of digestion with the same amount of enzyme. In the pilot scale partial digestion, 0.1U/µl *Sau3A* incubated at 37°C for 10-60 minutes was used to digest the DNA (Rahman *et al.*, 2007). The resulting fragments length depends on the location of recognition site and the digestion time. If incubation is prolonged, full digestion will occur which will lead to fragment that is too long to be cloned into the vector or too short if the recognition site at the end of the DNA molecule.

In the partial digestion, *BamHI* will cleave at the recognition sequence GGATCC which generate overhang 5'–GATC-3' molecule which have to be further treated by partial fill-in

reaction according to the Klenow Fill-In Kit (Stratagene) which used Klenow polymerase with the fill-in buffer, dATP and dGTP, because the Lambda FIX II vector (Stratagene) had been digested with *XhoI* enzyme and partially filled in with dCTP and dTTP which leaving 3'-CT-5' overhangs. This will generate compatible end and prevents it from self ligate.

Production of cohesive end is most desirable in cloning processes as the resulting end can be readily ligated to the vector provided both fragments were compatible as opposed to shearing the DNA using mechanical force which often leads to production of blunt end molecules which will require further treatment to convert blunt ends of DNA inserts into sticky ends for efficient ligation. This is due to inefficient of ligation of blunt-ended DNA fragments with vector DNA. Many methods have been developed such as addition of linkers followed by digestion with restriction enzymes to create cohesive termini, the addition of adaptors and the addition of a homopolymer tail (Nagesha *et al.,* 1996). These additional steps often results in loss of genomic samples through purification.

Usually partial digestion will be followed by size selection step, separation of digested DNA through agarose gel electrophoresis. DNA fragments that fall within the desired range will be excised and purified before proceeding to ligation. Although there are various methods, the size-fractionated DNA fragments recovered by electroelution were the most preferred to the common method of melting gel slices followed by agarose treatment. By this method, the yielding DNAs were comparatively less degraded and more amenable to ligation. In addition, a further advantage of the electroelution method is the ability to use regular high melting agarose, which makes gel handling easier than the low melting agarose required for enzyme-based DNA extraction. TAE buffer systems have been employed in

the electrophoretic separation of partially digested DNA, as borate ions (TBE) may inhibit ligation reaction used in the construction of BAC libraries (He *et al.,* 2003).

Size selection was omitted in the this construction of genomic library because the Gigapack III XL Packaging Extract is an in vitro packaging extract which specially designed for use in constructing genomic library. It is designed in such a way that preferentially size selects for extra large insert, which eliminates the step to size select the genomic insert and avoid the losses of genomic samples through purification. However, there were some digested genomic sample loses during purification in the full scale digestion was inevitable. The insert DNA can be used straight for ligation process to generate a recombinant molecule and subsequently package to form infectious viral particles.

Before proceeding to ligation, the concentration of insert DNA have to be at 0.2 µg/µl or greater as suggested by the Stratagene manual. It favours concatemers and optimal packaging efficiencies are obtained when the lambda DNA is in that confirmation. Therefore, the concentrations have to be determined in advance before proceeding to ligation. As the suggested concentration of 0.2 µg/µl or greater by the Stratagene manual, 0.7 µg/µl of DNA insert was obtained with a purity of 1.75. The pure DNA has a purity of about 1.8. The slight difference in the purity may be due to impurities that remained after purification affecting the OD reading. The success of constructing genomic library depends on the efficient ligation which depends on the available compatible ends (Rahman *et al.,* 2007). The higher the amount of compatible ends with the vector reflects the more efficient the ligation. The higher the number of pfu/ml of the library means the higher the efficiency

the ligation process. Hence, successful ligation can only be found out after the transduction process.

The XL1-Blue MRA (P2) cells was cultured in the presence of maltose, this sugar will induce the maltose operon that contains gene (lam b) that codes for the bacteriophage lamda receptor, side of attachment for the lamda to inject recombinant DNA to the cell. The cells were cultured to stationary phase by calibrating the spectrophotometer by measuring the OD_{600} of a growing culture. Only recombinant phage can grow because the selection was based on spi selection. Each plaque (Fig 5.4) contained phage derived from a single infecting phage and the plaque size do not increase in size when the bacterial lawn was fully grown and reached stationary phase (Rahman *et al.,* 2007 and Sambrook, 1989).

CHAPTER 6

GENERAL DISCUSSION

The prerequisite for constructing a genomic library is to obtain good DNA. The isolation of high molecular weight DNA from plant is difficult due to several reasons; the plant cell wall is not easily removed by physical means without damaging the contents of the cell and the vacuole which contain degradative enzyme such as DNases and secondary metabolites such as phenolic, tannins and pigments which can damage DNA. Furthermore, *Hylocereus undatus* contains high amounts of polysaccharides which will bind to the DNA making it viscous and glutinous after the precipitation step during extraction. This poses a challenge in obtaining the genomic DNA.

To overcome these obstacles, careful selection of proper starting material and optimization of the protocol with the right DNA isolation buffer are required. Germinated seedlings with partially expanded leaves were used for the DNA extraction because fully expanded mature leaves are heavily loaded with polysaccharide and polyphenols which will interfere with DNA isolation. When the cells are disrupted, these contaminants will come into contact with the DNA resulting in poor quality DNA (Havania *et al.,* 2004). Besides, mature leaves are tougher making the grinding process more difficult (Couch and Fritz, 1990).

In this study, only six days were required to reach a germination rate of 100% at 25˚C under fluorescent light. The germination of Cactacean seeds is usually a fast event upon the availability of water. In arid conditions, the seed germination process can be shortened and the time for complete seed imbibitions is also short leading to a high germination rate.

Fluorescent light plays a role in promoting or regulating the germination process. This was evident in this study as it took a longer duration to reach 100% germination in the dark room. This fact was also supported by evidence which is more obvious when the seeds were kept in a dark room for a week and then exposed to fluorescent light. A sudden increase in germination rate can be observed in the second phase. Nevertheless, light is not a prerequisite factor for germination as the seed can still germinate but only at a much slower rate. Simao *et al.,* (2007) suggested that the phytochrome could control seed germination in *H. setaceus.* According to Pearson *et al.,* (2003) small seeds usually respond positively to the light influences, while large seeds usually respond positively to temperature fluctuations.

In this study, it was found that seeds obtained from fruits initially stored at cold temperature could also undergo normal germination process. However, the time taken to achieve complete germination is relatively longer as compared to those seeds obtained from fruits not stored at cold temperature. The seeds obtained from fruits initially stored at cold temperature required an extra 4 days to reach 100% germination. This may be due to the fact that the germination process is influenced by the enzyme activity regulated by temperature. Long storage period may lead to breakdown of the flavonoid which renders the seeds susceptible to fungus invasion and the surrounding mucilage may promote fungus growth. Hence part of the experiments was terminated early due to fungal contamination. In this study, the seed germination was limited to 30 days due to fungal contamination.

Seeds of *H. undatus* fruits not exposed to cold environment for one week have the best germination percentage when placed in continuous fluorescent light at room temperature.

This method is able to provide a readily available source of raw material for DNA extraction. There was no dormancy observed in this study as all seeds exposed to different environments achieved 100% seed germination. Although all three groups of seeds will ultimately reach the maximum endpoint of 100% seed germination, it is best to use seeds that are either acid washed or air dried to minimise fungal contamination.

The DNA extraction method was based on Lodhi *et al.*, 1994 with slight modifications. Sodium bis-sulfite, sodium sarkosyl and sorbitol were added into the extraction buffer. Sodium bis-sulphite function as an antioxidant while sodium sarkosyl function as detergent. Lodhi protocol was a macro-method which uses 5ml of extraction buffer for 0.5g of leaves with a ratio of 1ml for every 1g of leave. However, in this extraction only 0.7ml of isolation buffer was used for 0.1g which is sufficient to yield high quality DNA. Tissues extracted with CTAB in high salt condition suppressed the co-precipitation of polysaccharides and DNA (Murray and Thompson, 1980 and Lodhi *et al.*, 1994).

The quality of the genomic DNA is of utmost importance in genomic library construction. Isolation of genomic DNA using the modified DNA extraction protocol, gave a DNA yield of 975 µg/g of leaf material. The pellet was white in colour indicating that it was free from contaminants and was further confirmed by the spectrophotometer readings which gave a A_{260} nm/A_{280} nm ratio of 1.92. Furthermore the band observed from gel electrophoresis showed a sharp and intact band instead of fire type bands and the extracted DNA was approximately 50kb long as was sized against the lambda DNA. This result correlates with the spectrophotometer readings.

This yield is far better compared to the method by Tel-Zur *et al.,* (1999) which yielded 10 to 20µg DNA/g of fresh roots. Roots were chosen as the tissue source as they have lower viscosity of extracts as compared to other tissues such as stems (Tel-Zur *et al.,* 1999). However, as large quantities of the roots were required in this study, the low density plantation was unable to continuously provide the materials. In Diadema *et al.,* (2003), where the callus was used as starting material, an average DNA yield of 1.8mg/g was obtained. The leaf callus is a result of the absence of the differentiation phase in callus which in turn prevents the synthesis of secondary metabolites (Diadema *et al.,* 2003). However, generating callus requires time, space, the expertise in tissue culture and most importantly is costly.

Once the genomic DNA was successfully obtained, the next step was to digest the total DNA to a desired range of fragments which can be ligated to the selected cloning vector. The chosen vector was Lambda FIX II/*Xho* I Partial Fill-In (Stratagene) which had been digested with *XhoI* enzyme and partially filled in with dCTP and dTTP, leaving 3'-CT-5' overhangs which can accommodate 9-23kb fragment. Therefore the genomic DNA can be digested with any *Mbo I, Sau 3A, Bgl II* or *Bam HI* enzyme. The resulting fragments length depends on the location of the recognition site, the amount of restriction enzyme added and the digestion time.

Prolonged incubation which results in full digestion will lead to fragments that are too long to be cloned into the vector or too short when the recognition site is at the end of the DNA molecule. To overcome this problem, a pilot scale partial digestion can be used to obtain the desired fragments by varying enzyme concentrations or the time of digestion. A pilot

scale of partial digestion was set up using 2.5µg of DNA with different ratios of enzyme; 0.05U/µg, 0.1U/µg, 0.15U/µg, 0.2U/µg, 0.25U/µg and 0.30U/µg to obtain the DNA fragments of 9-23Kb. The ratio of 0.30U/µg of *Bam*HI enzyme yielded the majority of fragments in the 9-23Kb range as compared to other enzyme ratio. Therefore the ratio of 0.30U/µg of *Bam*HI enzyme was chosen to proceed in full scale partial digestion. Subsequently, full scale digestion was carried out using 50µg genomic DNA in 10 tubes rather than one tube alone as the DNA pellet cannot fully dissolve causing the solution to be viscous. Poor quality DNA such as presence of polysaccharides will hinder access of restriction digest.

The genomic DNA was digested with *BamHI* enzyme to produce the compatible end which can be used later in the ligation stage. The vector and the insert DNA must have compatible ends in order to construct a recombinant molecule. *BamHI* will cleave at the recognition sequence GGATCC which generates an overhang of GATC molecule. This molecule has to be modified to prevent them from self ligation. This was achieved by partial fill-in reaction using Klenow polymerase with the fill-in buffer, dATP and dGTP. Subsequently phenol extraction, ethanol precipitation was carried out and the precipitate was finally dissolved in an appropriate volume of TE buffer.

Partial digestion is usually followed by a size selection step which is done via separation of the digested DNA using agarose gel electrophoresis. DNA fragments that fall within the desired range will be excised out and purified before proceeding to ligation. Although various methods are available, the size-fractionated DNA fragments recovered by electroelution was preferred than the common method of melting gel slices followed by

agarose treatment. By this method, the yielded DNAs were comparatively less degraded and more amenable to ligation. In addition, a further advantage of the electroelution method is the ability to use regular high melting agarose, which makes gel handling easier than the low melting agarose required for enzyme-based DNA extraction. TAE buffer systems have been employed in the electrophoretic separation of partially digested DNA, as borate ions (TBE) may inhibit ligation reaction used in the construction of BAC libraries (He *et al.*, 2003).

Production of a cohesive end is most desired in the cloning process as the resulting end can be readily ligated to the vector provided both fragments are compatible. On the contrary shearing the DNA using mechanical force often leads to production of blunt end molecules which requires further treatment to convert blunt ends of DNA inserts into sticky ends for efficient ligation. This is due to inefficient of ligation of blunt-ended DNA fragments with vector DNA. There are many methods that have been developed such as addition of linkers followed by digestion with restriction enzymes to create cohesive termini, the addition of adaptors and the addition of a homopolymer tail (Nagesha *et al.,* 1996). These additional steps often results in losses of genomic samples through purification.

Size selection was omitted in the construction of genomic library in this study because the Gigapack III XL Packaging Extract, an in vitro packaging extract preferentially size selects for extra large inserts, thus eliminating the step to size select the genomic insert and avoiding the losses of genomic samples through purification. It was used to form an infectious viral particle to affect the host which was the XL1-Blue MRA (P2) bacteria. P2 was cultured in the presence of a maltose to a final stationary phase. The stationary phase

was confirmed by spectrometric measurements againts OD_{600} of the growing culture. The presence of maltose will induce the maltose operon that contains gene (lam b) that codes for the bacteriophage lamda receptor which is the attachment site for the lamda to inject recombinant DNA to the cell. Each plaque contained phage derived from a single infecting phage and the plaque did not increase in size when the bacterial lawn was fully grown and reached stationary phase (Rahman *et al.*, 2007 and Sambrook, 1989).

The insert DNA is then used for the ligation process to generate a recombinant molecule. Successful construction of genomic library depends on efficient ligation (Rahman *et al.*, 2007). Optimal packaging efficiencies are obtained when the lambda DNA is in concatemer confirmation. Before proceeding to ligation, the concentration of insert DNA has to be at 0.2 µg/ µl or greater as according to the Stratagene manual, this concentration favours DNA in concatemer confirmation. The higher the number of pfu/ml of the library means the higher the efficiency of the ligation process. The titer was determined using the formula: Number of plaques (pfu) × Dilution factor × 1000 (µl/ml) / Volume plated (µl). The titer was 40,000pfu/ml.

With the successful construction of the genomic library, genes of interest can be identified using probes by hybridization. For future studies, genes of interest can be used for direct transformation to investigate the effects of these genes on the plant traits.