MOLECULAR CLONING OF THE *CHLORAMPHENICOL ACETYLTRANSFERASECAT* GENE

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MOLECULAR CLONING OF THE *CHLORAMPHENICOL ACETYLTRANSFERASE CAT* GENE

By

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ABSTRACT

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Mitochondria are a membrane enclosed organelles found in eukaryotic cells. Since they contain protein synthesising machinery, mitochondria are able to synthesise its own proteins. Besides, the number of mitochondria in each eukaryotic cell varies depends on the types of organisms and tissue. Theoretically, the gene expression increases as a result of the mitochondria abundance in each cell. The delivery of exogenous DNA into the mitochondrion is a technical challenge. Fusion of the mitochondrial targeting sequence (MTS) to the foreign gene allows the introduction of gene into matrix of mitochondria. A successful transformation requires a selectable marker, which is an essential feature of the plasmid to identify the transformants. Chloramphenicol has been used to selectively inhibit prokaryote protein synthesis. Thus, *chloramphenicol acetyltransferase* (*CAT*) *gene* was chosen as the selectable marker for mitochondrial transformation. Overlap extension PCR is a fast and precise method to fuse two genes together compared to restriction-ligation method. Amplified *CAT* gene and MTS were fused together to create a chimeric using overlap extension PCR method. Ligated PCR products and *CAT* gene were separately cloned into

pENTR/D-TOPO using TOPO cloning reaction.The reaction mixtures were transformed into *E. coli*Mach1^{TM} T1^{R} strain via electroporation. The transformants were screened for the positive clone by colony PCR. However, none of the transformants contained the insert. This might be due to the primers combination used for colony PCR were not suitable based on the presence of intense primer-dimer bands in the PCR products.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

HO MEI SAN

APPROVAL SHEET

This project report entitled "**MOLECULAR CLONING OF THE*CHLORAMPHENICOL ACETYLTRANSFERASE CAT* GENE**" was prepared by HO MEI SAN and submitted as partial fulfilment of the requirements for the Degree of Bachelor of Science (Hons) in Biotechnology at UniversitiTunku Abdul Rahman.

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PERMISSION SHEET

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I hereby give permission to my supervisors to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

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LIST OF ABBREVIATINOS

Ade	Adenine
ATP	Adenosine triphosphate
bp	Base pair
CAT	Chloramphenicol acetyltransferase
Co-A	Coenzyme A
ccd	Control of cell death
DNA	Deoxyribonucleic acid
g	g-force/ earth's gravitational acceleration
gyrA	gyrase A
MPP	Matrix Presequence Protease
MTS	Mitochondrial Targeting Sequence
PCI	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase Chain Reaction
psi	Pound per square inch
PreP	Presequence Protease
RNase	Ribonuclease
rpm	Revolution per minute
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Mitochondria

Mitochondria originated from α-proteobacterium, an endosymbiont prokaryotic ancestor which have evolved and become organelles after invading primitive eukaryotic host cells (Daniell& Chase, 2004). It is the power house of eukaryotic cells which produce adenine triphosphate (ATP) through oxidative phosphorylation (Bevan&Lang, 2004). Mitochondria are phospholipid bilayers organelles that haveits own DNA content. The presence of large number of porins on the surface of outer layer of mitochondriapermits the entry of moleculeswhich are smaller than 5 kDa (Garcia-Saez, Fuertes, Suckale & Salgado, 2010). However, the inner mitochondria membrane is impermeable to charged or polar molecules. Therefore, special membrane transporters are required to transport molecules and ions into the matrix (Karp, 2009). Since mitochondria have their own genetic system, these organellesare able to encode proteins or mitochondrial components from the mitochondrial genome which are involved in respiration, oxidative phosphorylation, translation and transcription, and protein import (Bevan & Lang, 2004). Generally, mitochondria are present in hundreds per cell and have multiple copies of the DNA in each mitochondrion (Krebs, Goldstein & Kilpatrick, 2011).

1.2 Mitochondrial Transformation

Evaluation of the effects of DNA modifications and analyse the changes of phenotype can be done through genetic transformation (Remacle & Matagne, 1998). Successful mitochondrial transformation has been achieved in the green alga *Chlamydomonasreinhardtii* and yeast *Saccharomyces cerevisiae* by using biolistic delivery system (Daniell & Chase, 2004). The ability of these species to grow on media requires active mitochondrial function, which is used as the selection of the mitochondrial transfromants in this species (Young & Koob, 2008). However, mitochondrial transformation on plant remains far from behind (Daniell & Chase, 2004). Difficulty of delivering foreign DNA into mitochondria and the limited availability of selectable markers on identification of mitochondrial transformants cause a technical challenge in mitochondrial transformation (Young & Koob, 2008). Without using the biolistic device to introduce the foreign gene into the mitochondrial, mitochondrial targeting sequence (MTS) can be used to direct the exogenous DNA into mitochondrial. Besides, potential selectable markers for mitochondrial transformation such as drug resistant gene can be developed (Young & Koob, 2008).

1.3 Mitochondrial Targeting Sequence (MTS)

Mitochondrial targeting sequence (MTS) or matrix targeting sequence isa fundamental amino sequence whichpresent in any organism's genomic DNA for transferring the pre-proteins across the outer and inner membrane into the matrix of mitochondria (Omura, 1998). These sequences are mainly located at the NH_2

terminus of the protein. However, MTS also can also be found in the COOH terminus of the protein (Neve & Ingelman, 2000). It is an N-terminal sequence which directs the fusion protein or the recombinant protein into the matrix of mitochondria (Omura, 1998). MTS is a highly positive charged and usually forms an amphiphilic secondary structure (Neve & Ingelman, 2000). The sequence of the MTS is in Figure 2.5.

According to Neveand Ingelman (2000), the MTS will first interacts with part of the translocase of the outer membrance (TOM) complex of the mitochondria that is the Tom20. Then, the highly negative charged amino acid residues from Tom20's surface will attract and bind to the positively charged MTS. This binding allows the protein to cross the lipid bilayer through the general translocation pore formed by the TOM complex. Proteins are then transferred to the translocase of the inner membrane (TIM) complex and to the matrix. In the matrix, the NH_2 terminal signal sequence of the protein will be removed by mitochondrial processing peptidase (MPP) (Regev, Yogev & Pines, 2008).

1.4 Transformation Selectable Marker

Development of selectable markers for transformation is the major factor to achieve a successful genetic manipulation. Selectable marker gene is a prerequisite features in the plasmid to precisely select transformed cells that have taken up the plasmid and enhance the transformation process (Reece, 2004). The type of selectable marker used depends on the type of cells work with. This is because some markers only act against prokaryotes as compared to others that have a broad spectrum of action.

1.5 The Use of ChloramphenicolAcetyltransferase as a Selectable Marker

Chloramphenicol is a highly stable antimicrobial which can pass through biological membranes to reach intracellular (Schwarz& White, 2005). It inhibits the prokaryotic translation by preventing the peptide bond formation. Since the protein translation in mitochondria is prokaryotic, thus protein synthesis is sensitive to chloramphenicol (Li, Ruf & Bock, 2010). Chloramphenicol acetyltransferase (CAT) is an enzyme encoded by chloramphenicol resistance gene (Reece, 2004). According to Li et al.(2010),CAT serves as a good choice of selectable marker in plant mitochondria transformation.

1.6 Objective of this study

It is hypothesised that chloramphenicolacetyltransferase can be expressed in yeast mitochondria and used as a selectable marker in yeast mitochondria. Mitochondriaarespecial and fundamental eukaryotic cellular organelles that manipulate several essential homeostatic function of the cell (Dakubo, 2010). Mitochondrion is an elaborate protein-synthesising machinery as it contains mitochondrial DNA in the matrix(Rastogi, 2005).The number of mitochondria per cell varies, which depends on the energy demand of the cell and types of organism. For instance, in yeast, there are 1 to 50 organelles of mitochondria per cell, whereas for *Chlamydomonas*, a unicellular alga only contains a few mitochondria per cell (Gupta, 2007 & Day, 2004). If the targeting DNA is in the mitochondria, the expression of the gene willbe increased substantiallyas compared to recombinant DNA in the cytoplasm. This will in turn lead to an increase in the yield of desired product.Therefore, mitochondrial targeting sequence (MTS) has to be cloned and incorporated into the plasmid to direct the *CA T* nto the matrix of mitochondria.

The ultimate aim of the project is clone the *CAT* gene with and without the MTS fusion, i.e. MTS-*CAT*. By cloning these two constructs, the effectiveness of cytoplasmically-localized and mitochondrial-localized CAT can be compared. This will help to determine if CAT is a suitable selectable marker for mitochondrial transformation. This is important because unlike in nuclear transformation, there are numerous copies of mitochondrial genomics in numerous mitochondria. Therefore, the mitochondrial transformation selectable marker, not only need to confer resistance to the selectable antibiotics, it also need to confer selective advantage to the replication and maintenance of the mitochondria genome that harbour this selectable marker, and thus the transgene.

CHAPTER 2

LITERATURE REVIEW

2.1 Cloning System: *E. coli* Strain

Escherichia coli is a Gram negative rod shaped bacterium commonly found in mammalian intestine (Manning, 2010). It is a facultative anaerobe which is able to survive in aerobic and anaerobic condition, with the optimum temperature at 37^{o}C. Besides, *E. coli* grows easily on chemically defined media and has a rapid growth rate (Sussman, 1997).

In media rich nutrients, *E. coli* replicates every 20-30 minutes in the exponential phase. Therefore, overnight incubation of single cell on agar plate is able to yield a single visible colony. The role of *E. coli* has been strengthened becomes the host for proliferation, controlling and characterization of recombinant DNA due to the simplicity of transformation ability and genetic manipulation of *E. coli*. (Casali,2003). This host strains include *E. coli* DB3.1, *E. coli* Mach 1 T1 Phage Resistant ($T1^{R}$), and so on.

2.1.1 *E. coli* DB3.1 Strain

The *E. coli* DB3.1 strain is a genetically modified *E. coli* strain and possesses the *gyr*A462 allele that enables the strain resistant to the toxicity of control of cell death

B (ccdB) protein. Moreover, this strain is streptomycin resistance which allowsto act as a selection. Plasmid carrying *ccdB* gene can transform and replicate in DB3.1 *E. coli* strain without killing the cells (Invitrogen Corporation, 2001). This *ccd* (*control of cell death*) is a system that consists of 2 proteins component, control of cell death A (ccdA) and ccdB encoded by *ccdA*gene and *ccdB* gene respectively.

Protein of ccdB is a naturally occurring inhibitor of DNA gyrase (Menzel&Gellert, 1994). SOS pathway and heat-shock responses were activated with the present of these proteins, causes cell-filamentation, degradation of chromosomes and cell death (Thomas, 2005). Biological responses of DNA damaging agent is due to induction of SOS (Bernard & Couturier, 1992).

However, ccdA is an antitoxin protein that inhibitsccdB protein from binding with DNA gyrase complex. In the absence of ccdA antidote, ccdB toxin inhibits DNA gyrase activity by binding to GyrA (Moat, Foster& Spector, 2002). According to Bernard and Couturier (1992), the SOS activation was suppressed with the presence of *gyr*A462. With this mutation, ccdB protein is unable to binds with gyrase A (GyrA) to inhibit the DNA gyrase activity. Therefore, replicating and transforming plasmid containing ccdB gene to the host with *gyr*A462 mutation or else the host will be killed by ccdB toxic protein.

2.1.2 *E. coli* Mach 1Phage Resistant ($T1^R$) Strain

Mach 1 Phage Resistant ($T1^R$) *E. coli* strain which is genetically modified from non-K12, wild type W strain and the doubling time of this strain is faster compared to other cloning strains. Moreover, this strain confers *endA1* mutation that enhances the quality and quantity of the plasmid and *recA* that reduces the non-specific recombination in cloned DNA (Invitrogen Corporation, 2006).

2.2 Cloning Vector Systems

Plasmids are extrachromosomal DNA molecules which generally are in circular, double-stranded and supercoiled form. It can be found in both bacteria and some yeasts. Besides, as compared to the host cell chromosome, plasmids are relatively smaller (Nicholl, 2002). They are different in size, from a few kilobases to several hundred kilobase pairs. (Dale &Schantz, 2007). According to Casali (2003), most of the plasmids remain in covalently closed circular, supercoiled form. But, open circular form does exist if one of the strand of the DNA double helix was nicked. Thus, the open circular form will tend to migrate slower than the supercoiled form in an agarose gel (Casali, 2003).

Plasmid confers phenotypes vary in their host cells, such as antibiotic resistance, antibiotic production, heavy-metal resistance, bacteriocin production, sugar fermentation, degradation of aromatic compounds, induction of plant tumours, haemolysin production, hydrogen sulphide production, sugar fermentation, host-controlled restriction and modification, and enterotoxin production (Old & Primrose,

1980). Generally, vectors designing for genetic engineering use these traits to act as selection of cells containing the plasmid (Nicholl, 2002).

Mainly plasmids confer one or more origin of replication in order to propagate independently of the bacterial chromosome of the cell (Brown, 2006). Moreover, there were 2 cloning methods which are currently used for gene insertion into the plasmid. Restriction enzyme cloning method has been used since 1972 until now. This method requires a specific restriction enzyme to cut the specific sequence (restriction sites) in the vector and outside of the gene of interest. However, this method is not a very efficient tool as it requires several factors, i.e. restriction enzymes, restriction sites on both vector and gene to be cloned,and ligaseto make a successful cloning (Caldwell, Williams & Caldwell, 2006).

In 2000, recombination cloning was invented and patented by Invitrogen Corporation. It is also commonly known as Gateway Cloning (Caldwell et al., 2006). Gateway technology enhances the efficiency of DNA fragment into vector system by using the site-specific recombination ability of bacteriaphage lambda (Invitrogen Corporation, 2006). The gene of interest is able to incorporate into any Gateway vector if there is the presence of Gateway recognition sequence with the target gene (Caldwell et al., 2006).

2.2.1 Plasmid pUGW2

pUGW2 is a gateway-compatible vector conferring ampicillin and chloramphenicol resistance, and *ccdB*gene. This plasmid will only replicate in host that had *gyr*A462 allele such as DB3.1 *E. coli* strain. Figure 2.1 below shows the structure of pUGW2.

2.2.2 pENTR/D-TOPO Cloning System

The cloning strategy of using pENTR/D-TOPO enable the direct cloning of a blunt-end PCR product into the plasmid vector. This method is achieved without any enzymes like ligase and restriction enzyme. The size of pENTR/D-TOPO is 2580bp (Invitrogen Corporation, 2006).The pENTR.D-TOPO vector contains the origin of pUC for high copy-number and kanamycin resistance gene as a selection of plasmid in *E. coli*. Besides, this plasmid also has *attL1* and *attL2* sites for sites-specific recombination of the entry clone with a Gateway destination vector (Figure 2.2) (Invitrogen Corporation, 2006).

2.3 Antibiotic Resistance Marker Genes

Antibiotic resistance genes are used as a selection to identify transformed cells that contain recombinant DNA. Cells that have these genes are able to survive and growing in particular antibiotic (Harwood &Wipat, 2001). There are 3 types of antibiotic resistance and each requires different solution (Drlica & Perlin, 2011). The first type of antibiotic resistance is acquired resistance. According to Drlic and Perlin (2011), mutant cells arise spontaneously i.e. about one out of a million cells per generation, or resistance gene transferred from other microbes.

Disseminated resistance is the second type of resistance. This resistance is already present in the pathogen before the treatment startsand is highly visible. Normally, the action taken to stop the spreading of pathogen is halting transmission. The third type of antibiotic resistance is antibiotics which have no effect on pathogen species, intrinsically resistant. The only way to cure this pathogen is to develop vaccines or practice infection control (Drlica & Perlin, 2011).

2.3.1 The *Chloramphenicol Acetyltransferase* (*CAT*) Gene

Chloramphenicol was naturally isolated from a *Streptomyces* sp. from the soil sample collected in a mulched field near Caracas, Venezuela (Schwarz & White, 2005). It is water and lipid soluble, which has a broad spectrum of action, bacteriostatic towards gram-negative rods and bactericidal against pneomococci (Harvey, Champe& Fisher, 2007). Chloramphenicol is a highly specific and potent inhibitor of protein synthesis by binding to 70S ribosome. It also forms the peptide bond between amino acids that inhibits the peptidyltransferase reaction (Schwarz & White, 2005). Generally, chloramphenicol acetyltransferase inactivates chloramphenicol and leads to chloramphenicol resistance (Byarugaba, 2010). Figure 2.3 shows the sequence of *CAT* gene.

With the presence of antibiotic inactivating enzyme i.e. CAT, bacterial is resistant to chloramphenicol as the enzyme catalyses the acetyl-CoA-dependent acetylation of chloramphenicol at the 3-hydroxyl group (Schwarz & White., 2005). Acetyl group is

transferred to the C3 position of the chloramphenicol by CAT followed by shifting of position from C3 to C1. Now, the second acetylation step can proceed due to the availability of C3 position (Figure 2.4). Both mono- or di-acetylated chloramphenicol molecules do not have antimicrobial activity as they do not bind to bacterial ribosomes andis not an inhibitor of peptidyltransferase. Moreover, CAT enzymes

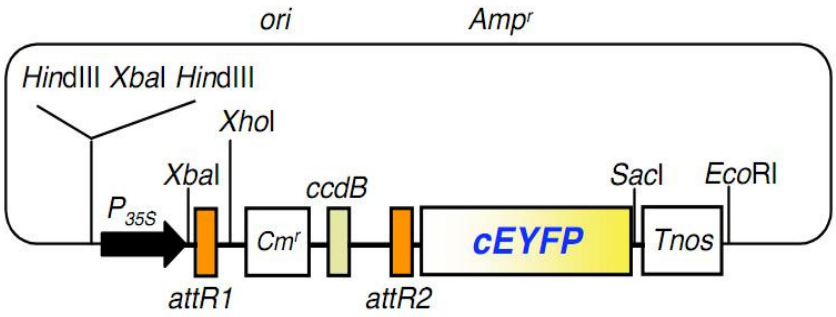

Figure 2.1: The structure of pUGW2.

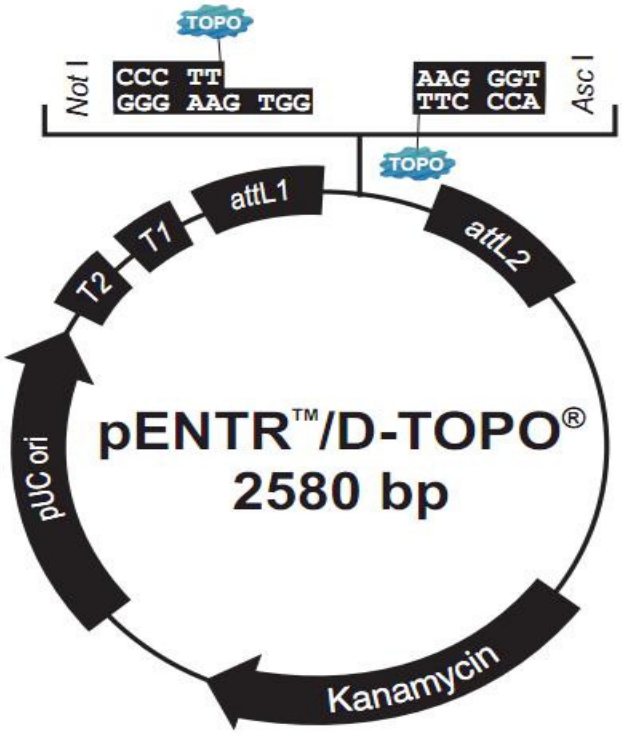

Figure 2.2: The features of pENTR/D-TOPO from Invitrogen Corporation.

ATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCA
TCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAA
CCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAA
ATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGA
ATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATA
TGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACG
TTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACAC
ATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCT
AAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGT
TTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCC
GTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCC
GCTGGCGATTCAGGTTCATCATGCCGTCTGTGATGGCTTCCATGTCGGCAG
AATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGT
AA

Figure 2.3: The sequence *CAT* gene.

Chloramphenicol + Acetyl-CoA → 3-Acetoxy chloramphenicol + CoA

3-Acetoxy chloramphenicol ↔ 1-Acetoxy chloramphenicol

1-Acetoxy chloramphenicol + Acetyl-CoA →1, 3-Acetoxy chloramphenicol + CoA

Figure 2.4: The chemical reaction of CAT towards chloramphenicol.

unable inactivate a chloramphenicol derivative, florfenicol (Roberts & Schwarz, 2009). Chloramphenicol resistance is mediated by mono- and di-acetylation by CAT enzymes. Substitution of C3 position with a fluorine residue causes the structure of the acceptor site for acetyl groups altered. This modification causes CAT enzymes to inactivate florfenicol resistant. Therefore, chloramphenicol resistance is caused by the CAT activity which is susceptible to florfenicol (Schwarz & White., 2005).

2.4 Mitochondrial Targeting Sequence (MTS) of Mitochondrial Tryptophanyl Synthetase (MS)

Mitochondrial tryptophanyl-tRNAsynthetase is named MS, WARS2 or mitochondrial TRPRS. It is an enzyme that exists in mitochondria, which catalyses the animoacylation of tRNA with tryptophan. MS contains a signal peptide for mitochondrial import which is an N-terminal mitochondrial targeting signal (National Center for Biotechnology Information [NCBI], 2009). A surveyrecorded that mitochondrion consists of over 1000 different proteins and only 1-2% of the proteins are synthesised within the organelle itself (Rapaport&Nargang, 2004). Nuclear genes coded for most of the mitochondrial membrane and synthesised on free ribosomes. After being synthesised, the post-translational proteins are only directed into the mitochondrion (Bolsover, Hyams, Shephard, White &Wiedemann, 2004). Therefore, mitochondrial biogenesis requires the importation of preproteins into the organelle and by doing so, it is also important for eukaryotic cell viability. Presequence or mitochondrial targeting sequence (MTS) is an N-terminal extension which is synthesised to the precursor proteins that are destined for the matrix

(Rapaport&Nargang, 2004). The length of MTS is commonly 20-60 amino acids (Lee, Neupert, & Stuart, 2000). Cytosolic proteins can be directed into the mitochondria by fusion of MTS to the non-mitochondrial proteins such as dihydrofolatereductase (DHFR).

Presequence are carried by the proteins destined for the mitochondria matrix. MTS structurally forms an amphipathic helix and receptor protein in the outer membrane recognises this sequence. The translocons in the outer and inner mitochondrial membrane interact with the hydrophobic and positively charged faces of amphipathic helix (Curan& Koehler, 2004). The protein unfolded and move across the outer and inner mitochondrial membrane. Matrix processing peptidase (MPP) is used to cleave MTS as it enters the matrix of mitochondria (Lee et al., 2000). According to research, during mitochondrial localization, 18 amino acids were cleaved off (Jørgensen, Søgaard, Rossing, Martensen & Justesen, 2000). Besides, presequence protease (PreP) is used to degrade the free targeting sequence (Glaser & Whelan, 2010). The protein refolded once the protein is translocated (Bolsover et al., 2004). Figure 2.5 shows the nucleotide sequence of MTS and Figure 2.6 is the amino acid sequence of MTS and the cleavage site upon enter into mitochondria matrix.

ATGTCGAATAAGCAGGCGGTTCTGAAGTTAATCAGTAAAAGGTGGATAAG

CACAGTGCAACGTGCCGATTTTAAGCTGAATTCCGAAGCGCTTCATAGTA

ATGCT

Figure 2.5: The nucleotide sequence of MTS.

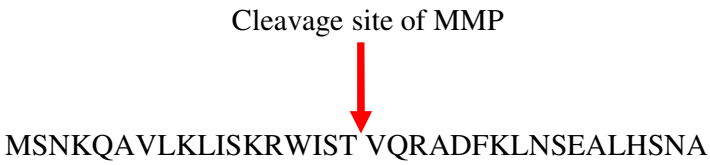

Figure 2.6: The protein sequence of MTS and the cleavage site.

2.5 Overlap Extension PCR

Cleaving of DNA fragment with restriction enzymes and ligate the fragments to the cloning vector with DNA ligase to obtain recombinant DNA are the common approach used in genetic engineering. A new strategy has been developed for fusion of DNA fragments by using polymerase chain reaction (PCR) (Yolov&Shabarova, 1990). This method is known as ligation PCR, fusion PCR or overlap extension PCR. Overlap extension PCR allows fast and precise fusion of desired fragments (Bryksin et al., 2004). Two desired DNA fragments are amplified separately from respective template or target gene with one of the primers of each gene contains homologous sequence. Thus, two DNA fragments amplified have a short homologous region. The two PCR products are mixed and undergo PCR process, which are denaturation, annealing and extension. The annealing process allows the fusions of the complementary sequence of the two DNA fragments followed by extension to synthesise the full length of ligated fragments by DNA polymerase (Reetz& Jaeger, 2000). Amplification of the fused DNA fragment is performed using standard PCR (Charlier et al., 2003). Figure 2.7 illustrates how overlap extension PCR work.

CHAPTER 3

MATERIALS AND METHODS

3.1 The Summary of Procedure for Molecular Cloning of a *Chloramphenicol Acetyltransferase* Gene

Mediums, reagents and solutions required in this experiment were prepared prior to begin this research. After the preparation, *E. coli* Mach1 T1 and DB3.1 strains were recovered for preparation of competent cells. Plasmid containing *CAT* gene was transformed into *E. coli* DB3.1 strain for recovery and increased the copy number of plasmid which acted as the template for amplification of *CAT* gene. Meanwhile, amplification of MTS was performed by using genomic DNA that was extracted from recovered yeast L40 strain. *CAT* gene and MTS were fused together via overlap extension PCR technique. *CAT* gene with and without MTS were separately transformed into pENTR/D-TOPO and cloned into *E. coli* Mach1 using electroporation method.

3.2 Bacteria Strains, Plasmids, and Yeast

Bacterial strains were used for transformation purposes and recovery of plasmid. Besides, the plasmid and yeast are used as template for PCR. The materials used were provided by Dr H.L Wong. Table 3.1 is to show bacterial strains, yeast strain and expression vectors used in this study.

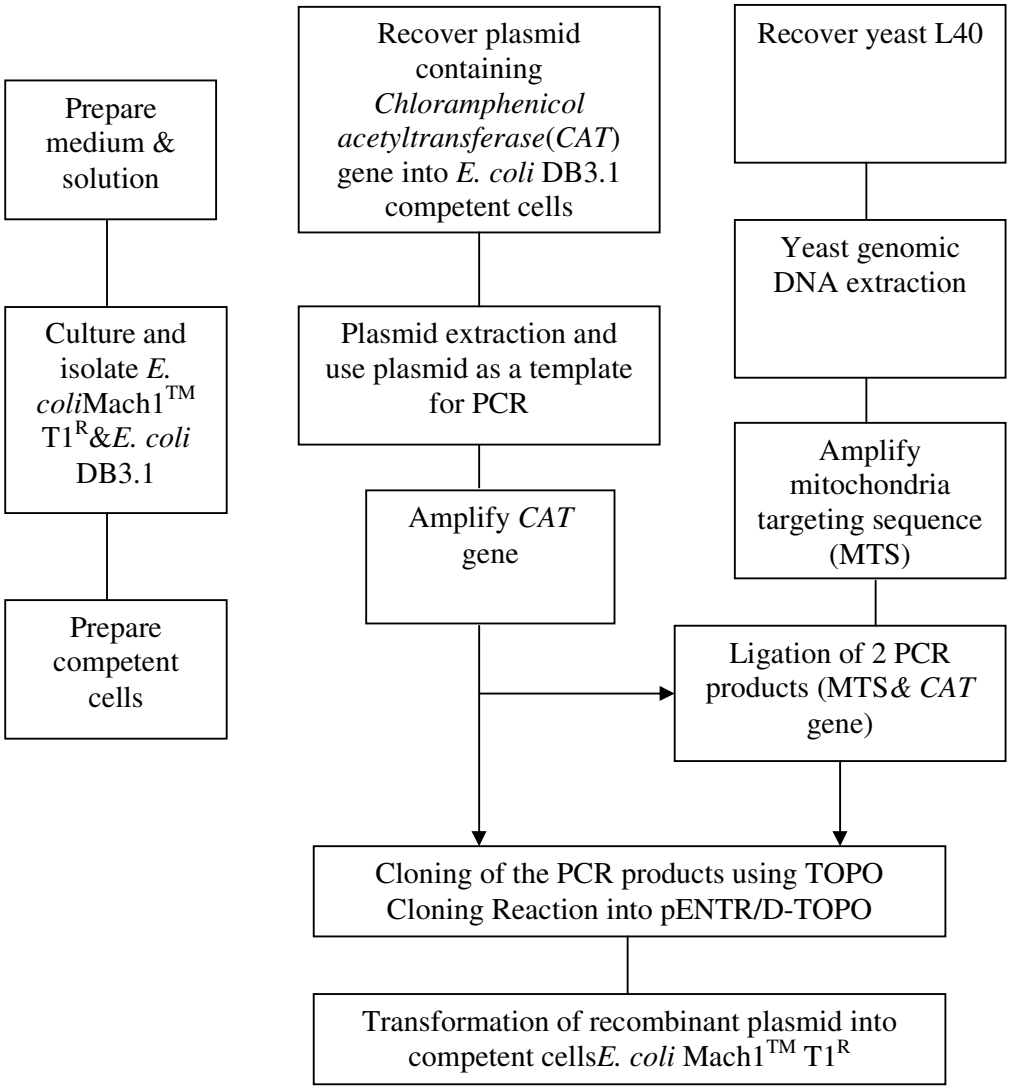

Figure 3.1: The flow chart of molecular cloning of a *chloramphenicol acetyltransferase* gene.

3.3 General Materials and Reagents, and Equipments

Chemical reagents and solvents used in this study were supplied by the Department of Science, UniversitiTunku Abdul Rahman. The manufacturers of the chemicals are listed in Table 3.2. However, the equipments and machines used in this study were provided by Dr H.L. Wong, and Department of Science, UniversitiTunku Abdul Rahman. The equipments furnished are listed in Table 3.3.

3.4 Media

3.4.1 2x YT Medium

NaCl ---------- 5 g

Tryptone ---------- 16 g

Yeast extract ---------- 10 g

The ingredients were dissolved with distilled water to a final volume of 1L and the pH was adjusted to 7.0. For solid medium, 15g of agar powder was added followed by autoclaving

3.4.2 SOC Medium

This medium was used to recover transformed competent cells after electroporation and allowed the antibiotic resistance gene in the plasmid to express.

Table 3.1: Bacterial Strains, Yeast Strain, and Plasmids.

	Features
E. coli DB3.1	Contains *gyr*A462 allele and streptomycin resistance gene
E. coli Mach1^{TM} T1^{R}	Fast growing strain
Yeast L40	Contains MTS
pUGW2	Contain *chloramphenicol acetyltransferase* resistance gene

Table 3.2: List of Chemicals and the Manufacturers of the Chemicals Used in This Study.

Chemical reagents and solvents	Manufacturer
100 bp DNA marker	NacalaiTesque
1 kb DNA marker	Fermentas, NAG Research Laboratory
95% Ethanol	Copens Scientific (M) Sdn. Bhd.
Absolute Ethanol	HmbG® Chemical
Acetic Acid	SYSTERM
Adenine Hemisulfate Salt (Ade), minimum 99%	SIGMA
Agar Powder	R & M Chemical
Agarose Powder	Vivantis
Boric Acid	QRëC^{TM}
Calcium Chloride Dehydrate	QRëC^{TM}
D(+) Glucose	Bendosen
DNA Polymerase (Prime STAR & Ex Taq)	Takara

Table 3.2:List of Chemicals and the Manufacturers of The Chemicals Used in This Study (Continue).

Chemical reagents and solvents	Manufacturer
DNA Polymerase Buffer (Prime STAR & Ex Taq)	Takara
dNTPs (2mM)	TOYOBO
(Ethylenediaminetetraacetic acid) EDTA	QRëC™
Ethidium Bromide	Bio Basic Inc
Glycerol	QRëC™
Hydrochloric Acid	MERCK
Isopropanol	MERK
10x Loading Dye	Fermentas
Magnesium Chloride, MgC_2 (Colony PCR)	Fermentas
Magnesium Chloride, MgC_2	QRëC™
Na_2-EDTA	SYSTERM
Phenol/Chloroform/Isoamylalcohol (PCI)	CALBIOCHEM
Potassium Acetate	MERCK
Potato Dextrose Agar	Pronadisa
Potato Dextrose Broth	Pronadisa
Sodium Acetate	SYSTERM
Sodium Chloride	SYSTERM
Sodium Hydroxide	R & M Chemical
Tris	Vivantis
Tryptone	Pronadisa
Yeast Extract	Scharlau Microbiology

Table 3.3: List of Equipments and Machines Used in This Study.

Equipments	Manufacturer
-20^{o}C Refrigerator	Toshiba
-80^{o}C Refrigerator	ESCO
ABJ Analytical Balance	KERN
Autoclave Steriliser	HIRAYAMA
Electrophoresis System	Takara Bio Inc
Electroporation Cuvette	Eppendorf
Electroporator 2510	Eppendorf
Evaporator	EYELA Centrifugal Evaporator
Hotplate Stirrer	Stuart
Imagin System	UVP
Incubator	Memmert
Laminar Flow (Horizontal)	ESCO
Microcentrifuge	Thermo Electron Corporation
Micropipette	Gilson
Microwave	Sharp
Nanophotometer	Implen
PCR Machine	Biometra T-Personal Thermocycler
Shaking Incubator	N-Biotek
Table Top Refrigerated Centrifuge	Dynamica
UV Transilluminator	UVP
UV/Vis Spectroscopy	Bio-Rad
Water Bath	Memmert

Sodium chloride ---------- 0.5 g

Tryptone ---------- 20 g

Yeast extract ---------- 5 g

Distilled water was added to final volume 950 mL

250mM Potassium chloride ---------- 10 mL

The pH of the medium was adjusted to 7.0 with 5N sodium hydroxide followed by topping up to 1L using deionised water. After being autoclaved, 5mL of 2M sterilised magnesium chloride and 20mL of 1M sterilised glucose were added into medium before used.

3.4.3 Potato Dextrose (PD) Medium

Potato Dextrose Agar was used for cultivation and enumeration of yeasts whereas Potato Dextrose Broth was used to cultivate yeasts. Potato dextrose agar (PDA) was prepared by mixing 7.8 g of PDA powder with 200 mL distilled water in the Schott glass bottle. Autoclaved medium was added with 3mL of 0.2% sterilised adenine hemisulfate and poured into agar plates. The same procedure was used to make potato dextrose broth (PDB) by replacing 7.8 g of PDA powder with 5.3 g of PDB powder.

3.5 Stock Solutions and Buffers

3.5.1 Antibiotic Stock Solutions

The antibiotics used in this study are listed in Table 3.4 whereas antibiotic stock solutions prepared are as shown in Table 3.5. All the antibiotic stock solutions were filter-sterilised by using 0.2 µm sterile syringe filter and stored at 4^{o}C.

3.5.2 5X Trisborate EDTA (TBE) Buffer

Tris-base ---------- 53.0 g

Boric acid ---------- 27.5 g

0.5M EDTA ---------- 20 mL

Distilled water added to final volume 1 L.

The buffer was autoclaved and diluted to 0.5X for agarose gel and as electrophoresis buffer.

3.5.3 TE Buffer (pH 8.0)

This buffer was used to resuspend pellet after extraction and purification of DNA.

1 M Tris-HCl (pH 8.0) ------- 1 mL

0.5 M EDTA (pH 8.0) -------- 200 µL

Distilled water was added to final volume 100 mL

TE buffer was sterilised by autoclaving.

Table 3.4: The Antibiotics Used.

Antibiotic	Salt	Form	Source
Ampicillin (Ac)	Ampicillin sodium	Powder	Wako Pure Chemical Industries Ltd.
Chloramphenicol (Cm)	-	Powder	NacalaiTesque Inc.
Kanamycin (Km)	Kanamycin sulfate	Powder	Wako Pure Chemical Industries Ltd.
Streptomycin (Sm)	Streptomycin sulfate	Powder	Wako Pure Chemical Industries Ltd.

Table 3.5: Stock Solution of Antibiotics.

Antibiotic	Stock solution (mg/mL)	Solvent used	Volume of stock (mL) per litre of medium	Final concentration (µg/mL)
Ampicillin (Ac)	50	Distilled water	1	50
Chloramphenicol (Cm)	30	70% ethanol	2	15
Kanamycin (Km)	50	Distilled water	1	25

3.6 Sterilization and De-contamination of Biological Materials

Sterilization of heat-stable mediums, reagents, glassware, polypropylene tubes, micropipette tips, and toothpicks were achieved by using autoclave machine at 121^{o}C, 15 psi for 15 minutes and 20 minutes for liquid and solid items respectively. Onthe other hand, heat-sensitive solutions such as antibiotics and adenine were filter-sterilised through 0.2 µm sterile syringe filter

The waste products and contaminated materials were autoclaved at 121^{o}C, 15 psi for 30 minutes before disposal. Besides, the used glassware was autoclaved for de-contamination.

3.7 Recovery of Bacterial and Yeast Strains

Recovery of cells was to revive the cells and obtain single isolated colony before inoculation into the broth medium for further experiments. Glycerol stock of*E. coli* DB3.1 strain, *E. coli* Mach1^{TM} T1^{R}strain and yeast L40 from were re-streaked on streptomycin agar plate, empty plate and PDA respectively and incubated overnight at 37^{o}C. Bacterial and yeast that were able to grow on respective plates were used to prepare competent cells. Competent cells were used for cloning the plasmid. It was a must to harvest the cells in the log phase in order to achieve high transformation efficiency of competent cells. During the whole procedure of preparing the competent cell, the cells had to be kept in ice.

3.7.1 Chemically Competent Cells via Calcium Chloride ($CaCl_2$) Treatment

Single colony of bacterial cells was inoculated into 2 mL 2xYT broth and incubated overnight with agitation at 250rpm, 37°C. The OD of overnight culture was measured using UV/V spectrophotometer at 600nm. The culture was diluted with 2xYT to make the OD of the cell approximately 0.1. The culture was then continued to agitate at 250 rpm and incubated at 37°C until the OD of the cell reached around 0.7. The cells were spun down at 9000 rpm, 4°C for 10 minutes and supernatant was removed. The pellet was washed with 0.1 M sterile $CaCl_2$, and was spun down again followed by discarding supernatant. This step was repeated twice before the pellet was resuspended with 400 µL of competent cells mixture (20 mM $CaCl_2$, 80 mM $MgCl_2$, and 10% glycerol). 40 µL of competent cells were aliquoted into microcentrifuge tube, froze in liquid nitrogen for 15 seconds and stored at -80°C refrigerator for future use.

3.7.2 Electrocompetent Cells

Isolated single colony of bacterial cells was inoculated in 2 mL of 2xYT broth for overnight incubation with agitation at 250 rpm, 37°C. Electrocompetent cells were prepared by diluting the overnight incubated cultureto ~0.1 OD at 600 nm with 2xYT. The culture was shaken at 250rpm, 37°C until the OD reached about 0.7 at 600 nm. The cells were spun down at 9000 rpm, 4°C for 10 minutes and supernatant was removed.

The pellet was washed with equal volume of 10% glycerol followed by spinning down the pellet and then the supernatant discarded. This step was repeated with half and 1/25 volumes of 10% glycerol. After the washing procedure, the pellet was resuspended with 400 µL of 10% glycerol and 40 µL was aliquoted into microcentrifuge tubes, froze in liquid nitrogen for 15 seconds before being stored in refrigerator at -80^{o}C.

3.8 Recovery of Plasmid

Plasmid recovery was done by using heat shock method. 0.5 µL of vector was added into 40 µL of competent cells and gently mixed. The tube was incubated on ice for 30 minutes followed by being immersed onto 42^{o}C water bath for 90 seconds. The tube was incubated on ice again for 20 minutes and 250 µL of SOC medium was added prior to incubating at the shaker for 1 hour, 250 rpm, at 37^{o}C. A hockey stick was used to spread 100 µL of transformation mixture on agar plate containing appropriate antibiotics and incubated at 37^{o}C overnight.

3.9 Solution Used in DNA Isolation

3.9.1 Solution I

0.5M EDTA (pH8.0) -------- 2 mL

1M Tris-HCl (pH8.0) -------- 2.5 mL

Glucose ---------- 0.9 g

Distilled water was added to the final volume of 100 mL and autoclaved. Solution I was used to increase the osmotic pressure outside the cells, maintain a constant pH and protect the DNA from DNAse.

3.9.2 Solution II

1N NaOH ---------- 3 mL

15% SDS ---------- 1 mL

Distilled water was added to the final volume of 15 mL and this solution need not to be autoclaved. The purpose of using this solution was to cause cells to be ruptured, destroyed the lipid membrane, solubilised cellular proteins and also denatured the DNA into single strands.

3.9.3 Solution III

5M Potassium acetate ---------- 60.0 mL

Acetic acid ---------- 11.5 mL

Distilled water was added to final volume 100 mL and autoclaved. Solution II neutralised the pH, allowing the renature of DNA strands, and precipitation of cellular debris. During the renaturation, the chromosomal DNA was trapped in the precipitate. Thus, only plasmid DNA remained in the solution.

3.9.4 DNA Extraction Solution

25mM EDTA ---------- 250 µL

250mMNaCl ---------- 1250 µL

200mMTris HCL (pH7.5) ---------- 1000 µL

0.5% SDS ---------- 125 µL

Distilled water was added to final volume 5 mL and filtered sterilised.

3.10 DNA Extraction

3.10.1 Plasmid

Plasmid was extracted from transformed *E. coli* which had been incubated overnight in 2 mL of 2x YT broth containing suitable antibiotics. Next, 1.5 mL of the culture was transferred into microcentrifuge tube and the cells were pellet down via centrigufation at 5,000 rpm, 4^{o}C, for 10 minutes. Supernatant obtained was discarded and the pellet was resuspended with 100 µL of ice-cold Solution I, mixed by vortexing. Then, 200 µL of Solution II was added, gently mixed and incubated on ice for 2 minutes before adding 150 µL Solution III and being left on ice for 5 minutes.

After the incubation, the solution was spun down at 15,000 rpm, 4^{o}C for 10 minutes. Supernatant was transferred into new microcentrifuge tube and equal volume of phenol/chloroform/isoamyl alcohol (PCI) was added. The mixture was mixed by vortexing and spun down at 15,000 rpm, at room temperature for 5 minutes. The aqueous layer was transferred into a new tube and 2 volumes of 100% ethanol were added. This tube was incubated on ice for 2 minutes followed by centrifugation. After recovering the pellet, 300 µL of 70% ethanol was used to rinse the pellet and spin for 3 minutes at 15,000 rpm, 4^{o}C. The supernatant was discarded and pellet was dried prior to resuspending with appropriate volume of TE buffer.

3.10.2 Genomic DNA

This method used for yeast genomic DNA extraction was modified from Bust n' Grab protocol by Harju, Fedosyuk and Peterson (2004). The yeast genome was extracted from overnight culture of cells in PD broth. The cells were pellet down by centrifugation at 14,000 rpm for 5 minutes. The pellet was resuspended with 400 µL of DNA extraction solution. The tube was incubated on ice for 2 minutes followed by 5 minutes incubation on 95^{o}C water bath. This procedure was repeated before vortexing.

After that, 400 µL of PCI was added and vortexed before being centrifuged for 3 minutes at room temperature, 14,000 rpm. The upper aqueous phase was transferred to a new tube and the PCI process was repeated. 800 µL chilled 100% ethanol added to the solution and mixed by inversion. The tube was incubated at room temperature for 5 minutes and

centrifuged for 5 minutes at room temperature with14,000 rpm. The recovered pellet was washed with 0.5 mL 70% ethanol and spun down to remove supernatant. The pellet was air-dried at room temperature and resuspended with appropriate volume of TE buffer.

3.11 RNase Treatment

RNase treatment was used to remove RNA residues in the solution after DNA isolation. RNase A was added to extract DNA and incubated at 37^{o}C for at least 30 minutes. In order to recover nucleic acid, equal volume of PCI was added and mixed by vortexing. After centrifugation for 2 minutes, at room temperature, 15,000 rpm, the upper aqueous phase was transferred into new tubes.

After that, 1/10 volume of sodium acetate and 2.5 volume of 100% ethanol was added into the tube. The mixture was mixed by vortexing and being left on the bench for 30 minutes followed by 30 minutes of centrifugation at 4^{o}C, 15,000rpm. 10 volumes of 70% ethanol were added after the recovery of the pellet. The solution was spun down at 15,000rpm, 4^{o}C for 2 minutes and supernatant was removed. The pellet was dried using a vacuum evaporator and resuspended with a suitable amount of TE buffer.

3.12 Polymerase Chain Reaction (PCR)

Each PCR reaction for amplification was 20 µL. The ingredients for each reaction were 0.2 µL of DNA template, 5X Prime STAR buffer, 0.2 mMdNTPs, 0.4 µM of forward and

reverse primers, 0.2 µL Prime STAR and sterile deionised water. Amplification of the *CAT* gene and MTS of the MS gene (MTS-MS) were performed by using a same reaction mix except for the forward and reverse primers, amplification parameter and DNA template.

3.12.1 Oligonucleotides Primers Used for Gene Amplification

Forward and reverse primers were used to amplify the gene. F-CAT-EH and R-CAT-ES primers were used in the PCR of *chloramphenicol acetyltransferase*(*CAT*) gene whereas F-MS-MTS and R-MS-MTS-CAT were used to amplify the MTS. Fusion of MTS and *CAT* gene was carried out using a PCR machine without the needs of any primers. However, forward primer of MTS and reverse primer of *CAT* gene were needed to amplify the ligated product.

***Chloramphenicol Acetyltransferase (CAT)* Gene**

The condition used to amplify this gene was:

96°C : 15 sec (Initial denaturation)

96°C : 15 sec (Denaturation)
50°C : 30 sec (Annealing) } 5 cycles
70°C : 1 min (Extension)

96°C : 15 sec (Denaturation)

55°C : 30 sec (Annealing) 25 cycles

70°C : 1 min (Extension)

70°C : 1 min (Final extension)

10°C : ∞

3.12.2 Tryptophanyl-tRNA-synthetase of Mitochondrial Targeting Sequence (MS-MTS)

The gene was amplified using the following parameter:

96°C : 15 sec (Initial denaturation)

96°C : 15 sec (Denaturation)
57°C : 10 sec (Annealing) } 5 cycles
70°C : 30 sec (Extension)

96°C : 15 sec (Denaturation)
60°C : 10 sec (Annealing) } 25 cycles
70°C : 30 sec (Extension)

70°C : 1 min (Final extension)

10°C : ∞

3.12.3 Overlap Extension PCR of MTS with *CAT* gene

Overlap extension PCR was divided into two phases. In the first phase, MTS and *CAT* gene were ligated as at the 3' end of MTS and 5' end of *CAT* gene containing complementary sequence which allows them to anneal. The fusion of two genes was performed with the aid of forward primer of MTS i.e. F-MS-MTS and reverse primer of *CAT* gene i.e. R-CAT-ES. The second phase of PCR was conducted by amplified the PCR product offirst phase overlap extension PCRto increase the quantity. For the first PCR, the reaction mix contains genes to be ligated (MTS and *CAT* gene), 5X Prime STAR buffer, 2 mM dNTPs, and sterile water. No primers were needed in first PCR and the parameter used was as following:

96°C : 1 min (Initial denaturation)

96°C : 15 sec (Denaturation)
65°C : 30 sec (Annealing) } 10 cycles
70°C : 1 min (Extension)

70°C : 3 min (Final extension)

10°C : ∞

After the first PCR, the PCR product acted as template and the reaction mix was prepared as mentioned in Section 3.12. F-MS-MTS and R-CAT-ES primers were used for the second PCR and the condition was:

96°C : 1 min (Initial denaturation)

96°C : 15 sec (Denaturation)

66°C : 30 sec (Annealing) } 25 cycles

70°C : 50 sec (Extension)

70°C : 3 min (Final extension)

10°C : ∞

3.13 TOPO Cloning Reaction

TOPO cloning reaction was to insert the gene of interest into the vector. According to Invitrogen, the molar ratio of PCR product:TOPO vector used in the cloning reaction for *CAT* gene with and without MTSrespectively was 2:1. Since the cloning was done using electroporation method, therefore, the salt solution used was diluted 1:4 with distilled water. The reaction material and volume used are recorded in Table 3.7. The prepared TOPO cloning reaction was mixed gently and incubated at room temperature (22-23°C) for 15 minutes. After the incubation, the reaction was placed on ice and ready to be transformed.

3.14 Transformation of Recombinant DNA

Electroporator cuvette was pre-chilled before being used whereas for the SOC medium, it was pre-warmed in 37^{o}C. The microcentrifuge tube containing 40 µL of thawed Mach1^{TM}T1^{R} and 2 µL cloning reaction was gently mixed and incubated for 6 minutes. The cells were transferred to the chilled cuvette and electroporated using the Eppendorfelectroporator.Immediately after the electroporation, 250 µL of pre-warmed SOC medium was added into the cuvette and the mixture was transferred into a new microcentrifuge tube. The mixture wasagitated and incubated at 37^{o}C for one hour to allow the expression of the antibiotic gene. 100 µL of each culture, was used to spread on pre-warmed selective plateand incubated overnight at 37^{o}C.

Table 3.7: Reagents Used of TOPO Cloning Reactions.

Reagents	**Volume (µL)**
PCR Product	0.5 to 4
Diluted Salt Solution (1:4)	1
Sterile Water	Add to a final volume of 5
pENTR/D-TOPO	1
Final Volume	6

Source: Invitrogen Corporation.

3.15 Colony Screening

All single isolated colonies developed from overnight incubation of transformed cells were selected for colony PCR. Selected colonies were picked by using sterile toothpicks and then inoculated on master plate. The remaining cells on the toothpick were placed into the respective labelled PCR tubes. The tubes were incubated in 95^{o}C water bath for 10 minutes.

At the same time, a PCR premix was prepared. Each reaction containing 10X Ex Taq buffer, 2 mM NTPs, forward and reverse primers, Ex Taq, and sterile water. The final volume of each reaction was 10 µL. The forward primer and reverse primer used for the screening were F-M13 and R-CAT-ES respectively. The screening parameter is as follows:

94^{o}C : 3 min (Initial denaturation)

94^{o}C : 30 sec (Denaturation)

55^{o}C : 1 min (Annealing) } 35 cycles

72^{o}C : 1 min (Extension)

72^{o}C : 5 min (Final extension)

CHAPTER 4

RESULTS

4.1 Recovery of *E. coli* Strains and Yeast

4.1.1 *E. coli* strains

Two *E. coli* strains used in this projectwere*E. coli* DB3.1 and *E. coli* Mach 1 $T1^R$. They were used for cloning purposes. *E. coli* DB3.1 and *E. coli* Mach 1 $T1^R$ strain were successfully recovered from glycerol stock in which recovery of *E. coli* DB3.1 was achieved by streaking the cells on streptomycin plate while recovery of *E. coli* Mach 1 $T1^R$ strain were done by streaking the cells on 2x YT plate without antibiotics. Single colonies from a successful recovered *E. coli* strains were inoculated in 2xYT broth for the preparation of electrocompetent cells. The growth of *E. coli* DB3.1 and *E. coli* Mach 1 $T1^R$ strains on their respective plate are shown in Figure 4.1 and Figure 4.2.

4.1.2 Yeast L40 Strain

Yeast L40 from glycerol stock was restreaked onto the PDA plate with 0.003% Ade. Many white single colonies of yeast L40 were observed. A single colony of yeast L40 was picked and inoculated in PD broth for genomic extraction. Yeast L40 grown on a PDA plate is shown in Figure 4.3.

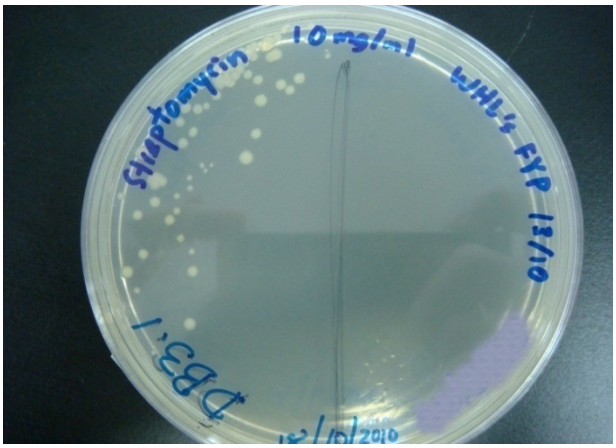

Figure 4.1: Growth of *E. coli* DB3.1 on 10 µg/ml streptomycin 2x YT plate.

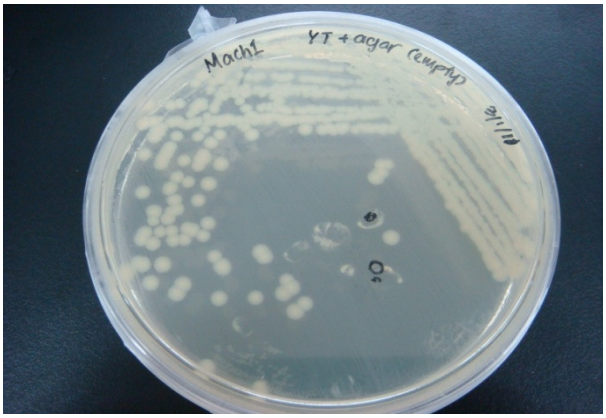

Figure 4.2: Growth of *E. coli* Mach 1 $T1^R$ strain on 2x YT plate.

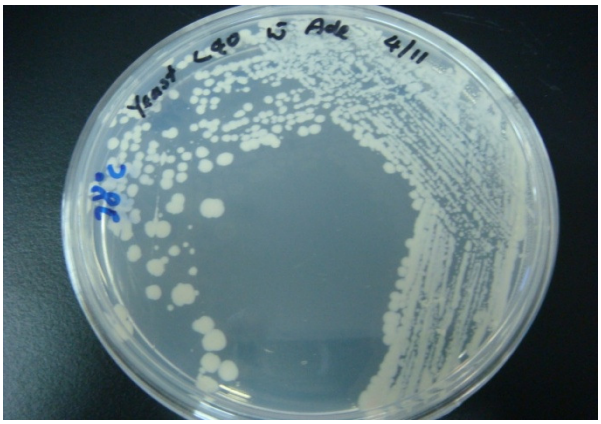

Figure 4.3: Growth of Yeast L40 on PDA plate containing 0.003% Ade.

4.2 DNA Extraction

4.2.1 pUGW2

pUGW2 which confers *chloramphenicol acetyltransferase (CAT)* gene was transformed into *E. coli* DB3.1 strain and plated on agar plate containing 50 µg/ml ampicillin and 15 µg/ml chloramphenicol antibiotics. After overnight incubation, single colony was isolated before being inoculated into 2xYT broth with ampicillin and chloramphenicol antibiotics. The plasmid was extracted from the overnight broth culture and two DNA bands were observed from the gel image (Figure 4.4). From the gel image, the approximate size of the plasmid was 6 kb. The concentration and the purity of the pUGW2 extracted are recorded in Table 4.1.

4.2.2 Yeast L40 Genomic DNA

Genomic extraction from yeast L40 was achieved. The observed DNA band was expected to be larger than 15 kb. This was in accordance with the agarose gel image

shown in Figure 4.5 in which the position of the observed DNA band was higher than the 1kb DNA ladder.

4.3 Polymerase Chain Reaction (PCR)

4.3.1 *Chloramphenicol Acetyltransferase (CAT)* Gene

The extracted vector, pUGW2 served as a template in amplifying*CAT* gene. Different concentrations of template were used to optimise the condition of PCR using Biometra-

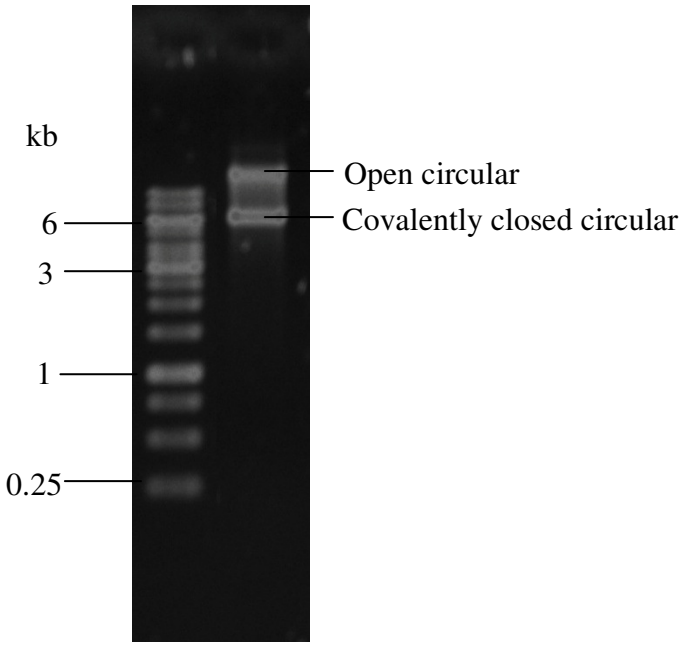

Figure 4.4: Extracted pUGW2 from *E. coli* DB3.1 strain.

Lane 1: 1 kb DNA ladder

Lane 2: Isolated pUGW2

kb

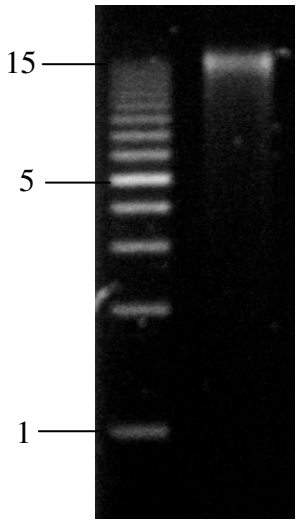

Figure 4.5: Extracted yeast L40 genomic DNA.

Lane 1: D-1000 bp DNA marker

Lane 2: Yeast L40 genomic DNA

T Personal Thermocycler machine. The agarose gel image (Figure 4.6) showed that at all concentration of template show a distinct band was observed with no non-specific bands. The concentration of the undiluted template was recorded in Table 4.1. According to the 1kb DNA ladder, the size of the PCR product was between 500 to 750 base pairs. The PCR product was purified for cloning purposes (Figure 4.7).

4.3.2 Matrix Targeting Sequence (MTS)

PCR of MTS was done by using yeast L40 genome as template. The parameter for amplification of MTS was optimised. As a result of the optimisation, undiluted template (10^0) and 10^1 of diluted template wereused for PCR. The concentration of the undiluted template was recorded in Table 4.1. A single band for both different concentration of yeast genome was observed on the gel image (Figure 4.8). The DNA ladder used was 100

bp. From the indication of ladder size, the length of the band was between 100-200 bp. Furthermore, the MTS PCR product was purified and the gel image shows in Figure 4.9. 1 kb DNA ladder was used in this gel image.

4.3.3 Ligation PCR of MTS with *CAT* gene

Ligation of MTS to *CAT* gene was completed using PCR. The process of ligation PCR is illustrated in Figure 4.10. Firstly, the annealing temperature used for second PCR was 55^{o}C. Since there are non-specific bands (Figure 4.11), optimization of annealing temperature wasdone.Afteroptimisation,66^{o}C of annealingtemperature was the

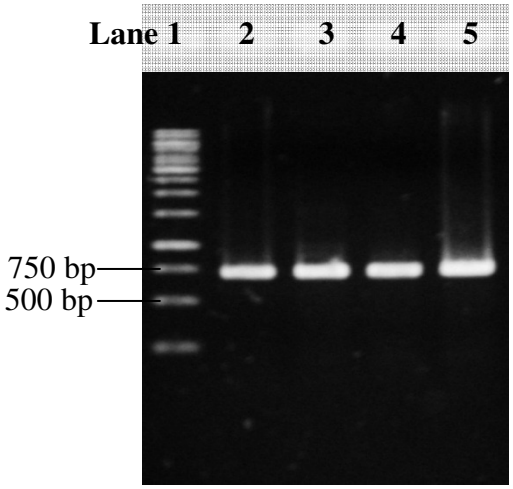

Figure 4.6: Optimization of PCR condition for *CAT* gene using pUGW2 as template.

Lane 1: 1 kb DNA ladder

Lane 2: PCR product from 1: 1000 dilution of template.

Lane 3: PCR product from 1:100 dilution of template.

Lane 4: PCR product from 1:10 dilution of template.

Lane 5: PCR product from undiluted template.

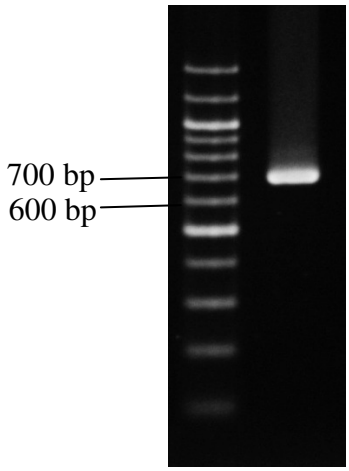

Figure 4.7: Purified *CAT* gene after PCR.

Lane 1: 100 bp DNA ladder

Lane 2: Purified *CAT* gene.

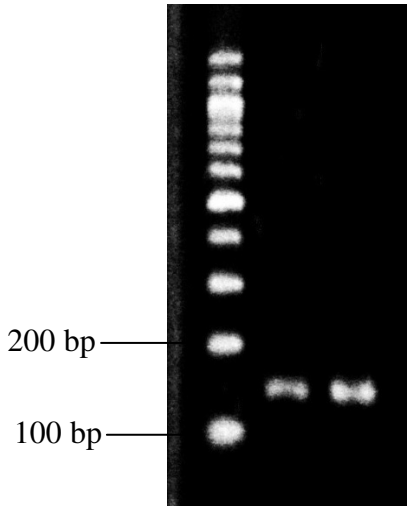

Figure 4.8: PCR of MTS DNA fragment from different concentration of yeast L40 genomic DNA.

Lane 1: 100 bp DNA ladder

Lane 2: PCR MTS from undiluted template

Lane 3: PCR MTS from 1:10 dilution of template

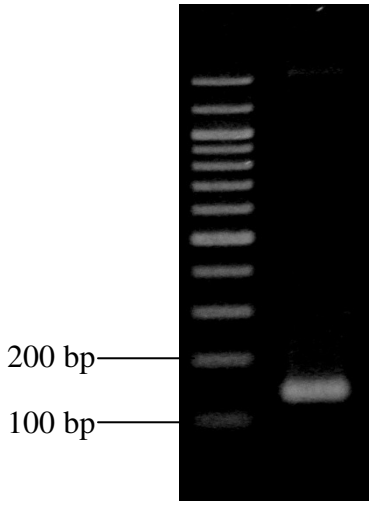

Figure 4.9: Purified MTS DNA fragment.

Lane 1: 100bp DNA ladder

Lane 2: Purified MTS PCR product

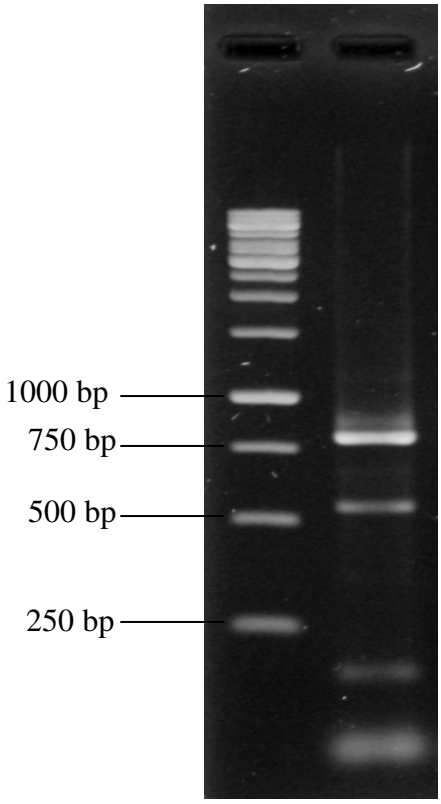

Figure 4.11: PCR product of *CAT* gene with MTS using the annealing temperature 55^{o}C.

Lane 1: 1 kb DNA ladder

Lane 2: Fused*CAT* gene with MTS

suitable condition for second PCR to produce single band without any non-specific bands (Figure 4.12). As compared to the 100 bp DNA ladder, the band size was between 700 to 800 bp. The concentration and the purity of the ligated PCR products are recorded in Table 4.1.

4.3.4 Concentration and Purity of Products

Table 4.1: The Concentration and Purity of PCR Products

Product	Concentration (ng/µL)	A260	A280	Purity
pUGW2	383	0.773	0.412	1.894
CAT gene	245	0.294	0.494	1.690
MTS fragment	135	0.180	0.288	1.667
CAT gene with MTS	200	0.297	0.426	1.476

4.4 Colony PCR

4.4.1 *Chloramphenicol Acetyltransferase*(*CAT*) Gene

Purified *CAT* gene was cloned into pENTR/D-TOPO and transformed into Mach1^{TM} T1^{R} via electroporation with the time constant 4.6 milliseconds at 1600V. After an overnight incubation on 50µg/ml kanamycin and 15µg/ml chloramphenicol antibiotics plate at 37^{o}C,

the grew colonies(Figure 4.13) were used for screening. The colonies that had been screened is in Figure 4.14.

4.4.2 Product of Overlap Extension PCR

CAT gene with MTS was purified after amplification. Recombinant plasmids, pENTR/D-TOPO with purified fused PCR product were transformed into Mach1^{TM} T1^{R}electrocompetent cells and the time constant for 1600V was 4.4 milliseconds. . The transformed cells were plated on agar plate containing 50µg/ml kanamycin and 15µg/ml chloramphenicol antibiotics. Isolated single colonies were picked to analyse positive transformant (Figure 4.15). The results of colonies screened are shown in Figure 4.16.

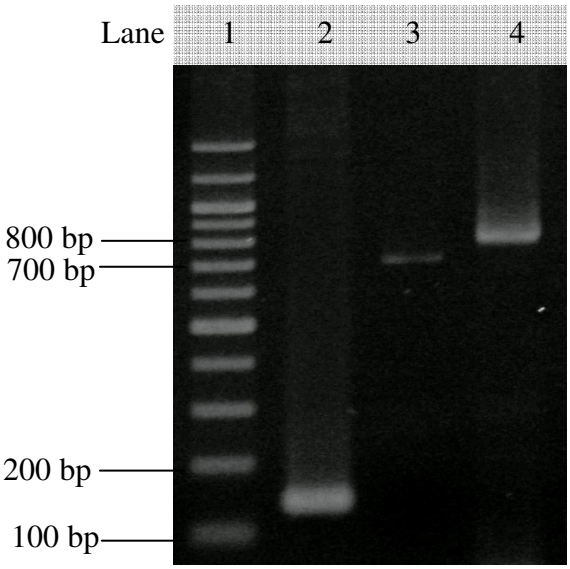

Figure 4.12: MTS,*CAT* gene and.*CAT* gene with MTS

Lane 1: 100bp DNA ladder

Lane 2: Purified MTS

Lane 3: Purified *CAT* gene

Lane 4: Fusion of MTS and *CAT* gene using PCR.

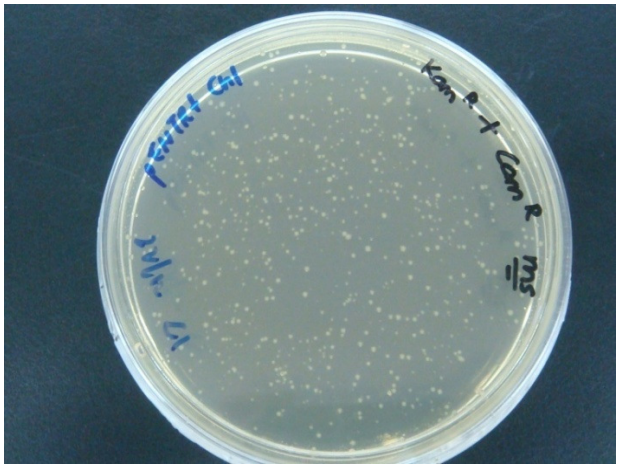

Figure 4.13: Transformed Mach1^{TM} T1^{R} with cloned pENTR/D-TOPO and *CAT* gene.

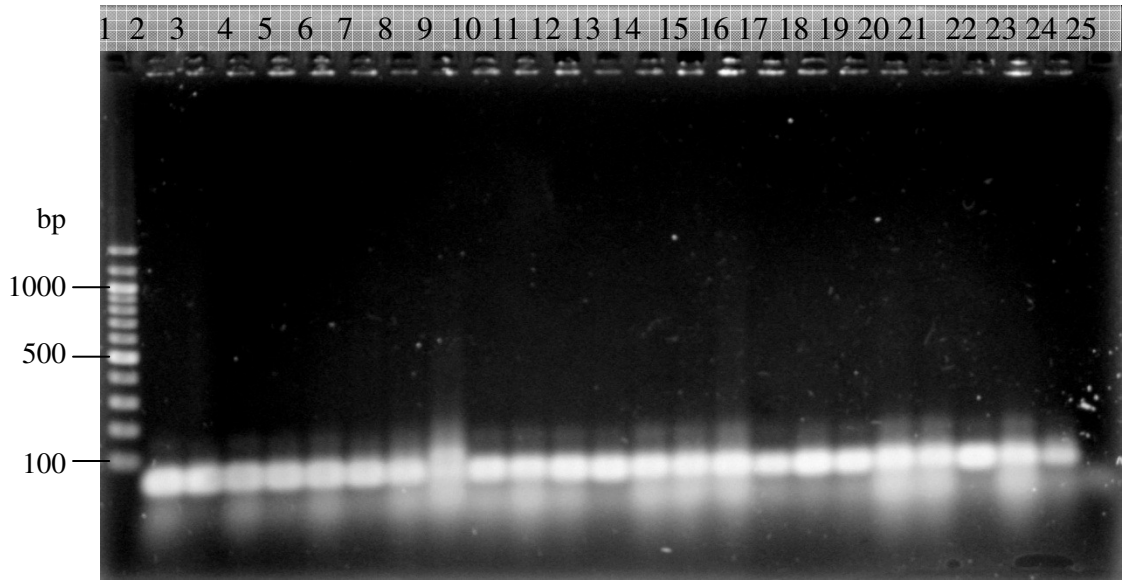

Figure 4.14: Screening of *CAT* gene from transformed Mach1^{TM} T1^{R}.

Lane 1: 100bp DNA ladder

Lane 2 to 24: Screening of transformed colony with *CAT* gene

Lane 25: Negative control (without bacteria cells)

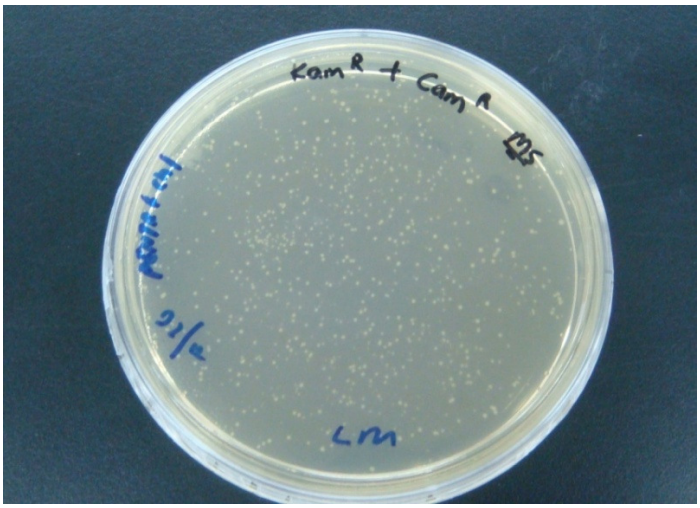

Figure 4.15: Transformed Mach1^{TM} T1^{R} with cloned pENTR/D-TOPO and *CAT* gene with MTS.

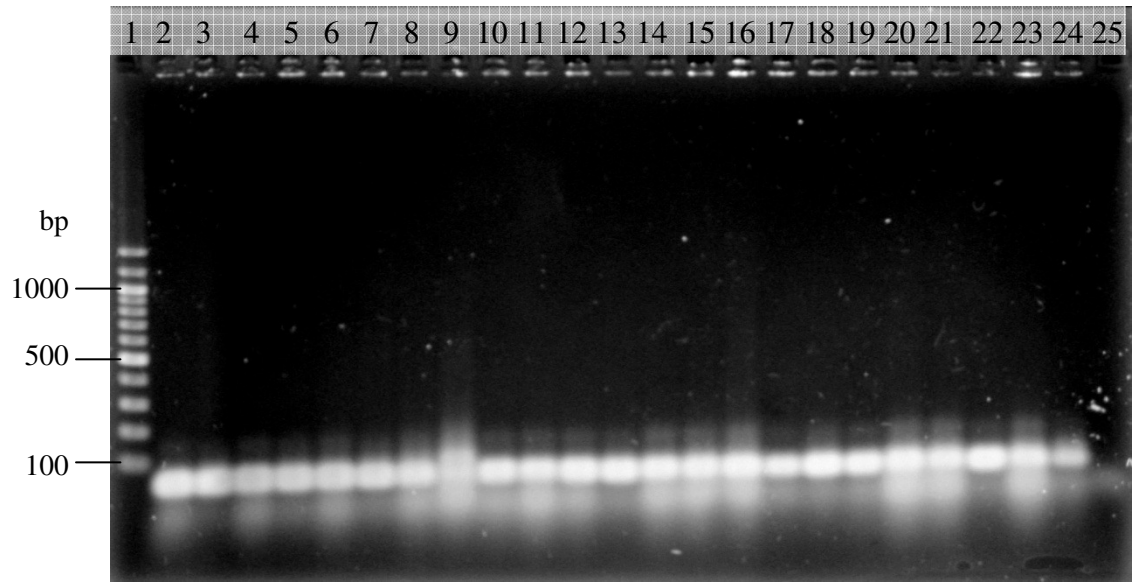

Figure 4.16: Screening of *CAT* gene with MTS from transformed Mach1^{TM} T1^{R}.

Lane 1: 100bp DNA ladder

Lane 2 to 24: Screening of transformed colony of*CAT* gene with MTS

Lane 25: Negative control (without bacteria cells)

CHAPTER 5

DISCUSSION

5.1 Recovery of Vector, pUGW2

pUGW2 was used as a template for *chloramphenicol acetyltransferase*(*CAT*)gene amplification as it conferred a *CAT* resistant gene. Plasmid was replicated to attain sufficient amount of template by retransforming the plasmid into the host cells, which is *E. coli* DB3.1 strain. Then the plasmid was extracted from transformed cell after a successful transformation and the presence of this plasmid was verified through agarose gel electrophoresis.

According to Micklos, Freyer&Crotty, (2003), few bands might be observed in the lane of agarose gel containing only undigested plasmid DNA. The migration of DNA molecule wasaffected by the conformation of DNA molecules, size of DNA molecule and strength of the current (Wink, 2006). There were three major conformation of plasmid DNA, i.e., supercoiled, relaxed, and linear. Supercoiled molecule is a compact structure of coiled DNA helix that wrapped around a histone-like structure. Thus, the compact structure of the DNA molecule allows to migrate faster in the agarose gel (Micklos et al., 2003).

On the other hand, relaxed form of plasmid DNA is the slowest in migration. This is due to introduction of a nick by topoisomerase I into one strand of DNA helix. Nicking causes the release the torsional strand that holds the molecule in a supercoiled. The third conformation of plasmid is linear DNA, produced from the cut of restriction enzyme at the restriction site of plasmid DNA. Linear DNA migrates at an intermediate rate between supercoiled and relaxed plasmid (Micklos et al., 2003).

The gel image of extracted pUGW2 (Figure 4.4) showed that two bands were observed.Based on Micklos et al., (2003) findings, the band further from the loading well was covalently closed plasmid whereas the band nearer to the loading well was relaxed plasmid. The third form of plasmid DNA conformation, i.e., the linear form was not seen in the gel image as the extracted plasmid was uncut. Moreover, the concentration and purity of the extracted pUGW2 were 383 ng/µL and 1.894, respectively. The extracted plasmid was considered pure as it was in the range of 1.8 - 2.0 of A260/A280 ratio (Grody, Nakamura, Kiechle& Strom, 2009).

5.2 PCR Product

5.2.1 *Chloramphenicol acetyltransferase*(*CAT*) Gene

Optimisation of the parameter for *CAT* gene amplification was done by using different concentration of template, pUGW2. From the gel image (Figure 4.6), *CAT* gene was successfully amplified at various concentrations of template as single band was observed

without any non-specific band. Therefore, undiluted template was used to amplify *CAT* gene.

The amplified *CAT* gene was confirmed by running in an agarose gel through electrophoresis. The expected size of *CAT* gene is 660 bp (Mingoiaet al., 2007). Since primers were included in the amplification of *CAT* gene, thus, the size of PCR product should be 690 bp instead of 660 bp (the size of *CAT* gene). According to the band observed in Figure 4.7, the product that had been amplified from pUGW2 was *CAT* gene as the band size was around 700bp as compared to the 100bp DNA ladder.

5.2.2 Mitochondrial Targeting Sequence (MTS)

MTS DNA fragment was amplified by PCR from yeast L40 genomic DNA. Two concentration of yeast genomic DNA were used to act as template to optimise the condition for PCR which were undiluted and 1:10 dilution of yeast genomic DNA. From the results (Figure 4.8), single band was observed for both reactions. Hence, the amplification of MTS was performed by using undiluted template.

Figure 4.9 showed the size of the band was between 100 to 200 bp as compared to the 100 bp DNA ladder. According to Jørgensen et al. (2000), the expected size of MTS wasapproximately 35kDa, which was~105 nucleotide sequence. Therefore, the gene length of the PCR product of MTS including the primers was ~138 bp and the band

observed from the agarose gel (Figure 4.10) was between 100 – 200 bp. So, the product amplified was considered as MTS as the band in the gel was in the expected size range. Purification of PCR product was performed. The concentration and the purity of the PCR product were 135 ng/µL and 1.667, respectively. The purity of MTS PCR product was lower than expected 1.8, indicated the presence of protein residues in the PCR product. Even, so, the overlap extension PCR was not affected.

5.2.3 Overlap Extension PCR of Mitochondrial Targeting Sequence and *Chloramphenicol Acetyltransferase* Genes

Optimisation of the parameter for overlap extension PCR was done by varying the annealing temperature of the second PCR. The temperature tested was from 55^{o}C to 66^{o}C. The result showed that 66^{o}C of annealing temperature was able to produce a distinct band without any non-specific bands(Figure 4.12).

The total gene length of MTS and *CAT* gene was 788 bp including the forward and reverse primers. The forward and reverse primers used in amplification of ligated gene were F-MS-MTS and R-CAT-ES. From the gel image, the resulting band was roughly 800bp and the actual gene length of the ligated product including primers was 789bp. As a result, the 2 PCR products were successfully fused together and amplified.

Besides, the concentration and purification of the ligated genes were 200ng/µL and 1.476 respectively. The amplified PCR productwas not sufficiently pure. A plausible interpretation could be due to presence of primers and some dNTPs remained with the product.

5.3 Screening of Plasmid Containing Gene of Interest

5.3.1 Cloning of *Chloramphenicol Acetyltransferase (CAT)* Gene

Growth of many transformed colonies was observed on chloramphenicol(15 µg/mL) and kanamycin (50 µg/mL) antibiotics agar plate. The colonies were then used to analyse for positive clones (Figure 5.1). From the agarose gel image (Figure 4.14), no band was observed in the lane loaded with PCR product. The expected size of gene should be between 700 – 800 bp. The location of primers anneal to the plasmid is show at Figure 5.1. The causes of this problem might be due to contaminations and primers combination.

5.3.2 Cloning of Ligated MTS and *CAT* Gene

After the TOPO cloning reaction was carried out, the vector was transformed into Mach1^{TM} T1^{R} via electroporation. It was followed by an overnight incubation on the agar plate contained chloramphenicol (15µg/ml) and kanamycin (50µg/mL) antibiotics and growth of colonies were observed (Figure 4.15). Colony PCR was used to screen positive clones in transformed cells. However, the expected clones with the band size range of

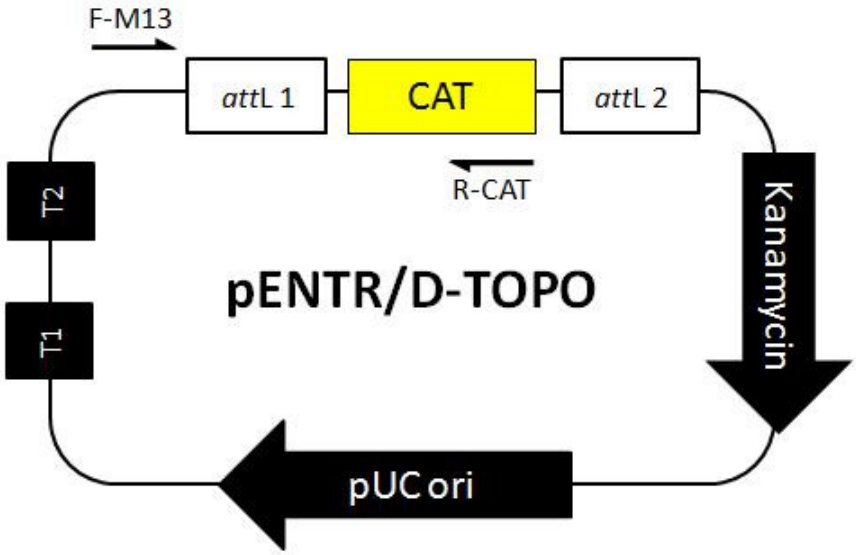

Figure 5.1: Structure of pENTR/D-TOPO withcloned*CAT* gene and the sites primers anneal for colony PCR.

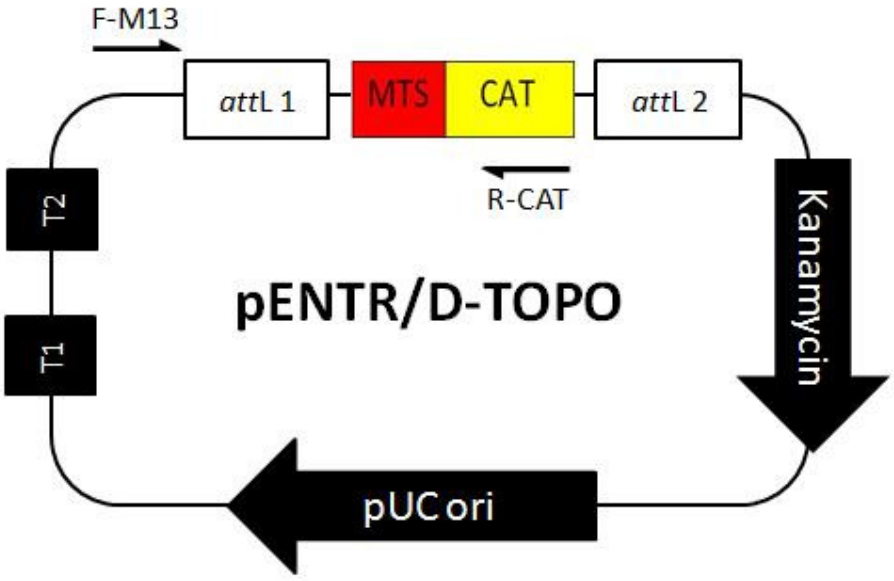

Figure 5.2: Structure of cloned *CAT* gene with MTS in the pENTR/D-TOPO and the sites of primers anneal for colony PCR.

800 – 900 bp were not successfullyscreened (Figure 4.16). Figure 5.2is thestructure of pENTR/D-TOPO with ligated gene cloned and the site of primers anneal. There were many solutions done to eliminate the possibilities that will affect the transformation.

5.3.3 Potential Problems and Solutions

Colony PCR is a method used to re-confirm positive inserts and the orientation of the insert (Capelli & Tschentscher, 2005). However, based on the transformed colonies obtained from colony PCR in Figure 4.13 and Figure 4.15, no positive clone (i.e. plasmid containing gene of interest) was screened from the plate even though the growth of many colonies were observed on each agar plate. A few possible causes might lead to this problem such as the purity of PCR product, contamination, the time constant of electroporation, etc.

Several procedures were performed to eliminate possible factors that arosefrom PCR products. PCR products could be contaminated during the preparation process or contaminants might be present in the solution used in the purification process (Invitrogen Corporation, 2006). Thus, all the solutions needed, reagents and primers used were re-prepared or replaced with new one. PCR of *CAT* gene, MTS and ligated MTS and *CAT* gene were performed by using the new solutions (Taylor& Noble, 1995). From Table 4.1, the purity of *CAT* gene, MTS and *CAT* gene with MTS were 1.690, 1.667 and 1.476, respectively. Since the A260/280 ratio is less than 1.7, this indicated some proteins remained in the PCR product after purification (Sanders& Miller, 2009). Thus,

purification process was carried out again to obtain purer PCR products. However, the problem remained unsolved.

The efficiency of the *E. coli*Mach1^{TM} T1^{R} competent cells used for transformation was 2.77x10^{9}. Growth of many colonies but without a positive clone was not due to the *E. coli*Mach1^{TM} T1^{R} resistance to chloramphenicol and kanamycin. After the competent cells preparation, the cells were tested on chloramphenicol and kanamycin antibiotics plate to ensure the competent cells were not contaminated and susceptible to chloramphenicol and kanamycin antibiotics. Commercially available *E. coli*Mach1^{TM} T1^{R} competent cells were used to substitute self-prepared competent cells to solve the encountered problem. Unfortunately, the same results were obtained by using commercial *E. coli* Mach1^{TM} T1^{R}competent cells, which was many colonies were observed on the plate but none of them contain the *CAT* gene with and without MTS. This condition suggested that the issue arosewas not caused by competent cells.

Moreover, the agar plates used for culturing were prepared in batch by batch. The agar plates prepared was incubated overnight at 37^{o}C before being used for plating to check for contamination. Besides, the antibiotics used were prepared in small amount. Contamination of antibiotics was ruled out as new antibiotic stocks were prepared for every batch of agar plate and sterilised via filter syringe.

According to Farrel (2010), primer-dimer formation will be favoured if the primers used are complementary to each other. Based on Table 3.6, complementary bases of F-M13

and R-CAT-ES were observed. Therefore, two primers tend to anneal to each other instead of annealing to the plasmid. This situation leads to amplification of primer-dimers rather than the gene of interest. As a result, intense primer-dimer bands were observed from the gel image of colony screening (Figure 4.14 & 4.16). Hence, another set of primers (F-CAT and R-M13) should be used to replace F-M13 and R-CAT (Figure5.3& Figure 5.4).

5.4 Future Work

Since molecular cloning of a *chloramphenicol acetyltransferase*(*CAT*) gene was unsuccessful and the actual possible source of failure was unable to be identified, therefore, it is suggested that all the solutions and reagents needed should be re-prepared. Besides, the *CAT* gene, mitochondrial targeting sequence (MTS) of tryptophanyl-tRNA-synthase (MS), and ligated MTS and *CAT* gene should be re-amplified to obtain a fresh PCR product for cloning reaction.

After being successfully screened of the *CAT* gene with and without MTS, the plasmids containing these genes will be extracted and sent for sequencing to confirm the cloned gene was the expected gene. Besides, the plasmid constructed containing *CAT* gene will be transformed again into yeast cytoplasm whilst the *CAT* gene with MTS will be transformed into mitochondria of yeast. The yeast transformants conferring *CAT* gene will be tested for the resistance level to the chloramphenicol antibiotic and the suitable antibiotic concentrations was determined for future usage. The level of expression of

CAT in the mitochondria will be compared to the expression of CAT in the cytoplasm. This comparison is to confirm whether expression of CAT in the mitochondria will be higher in the cytoplasm.

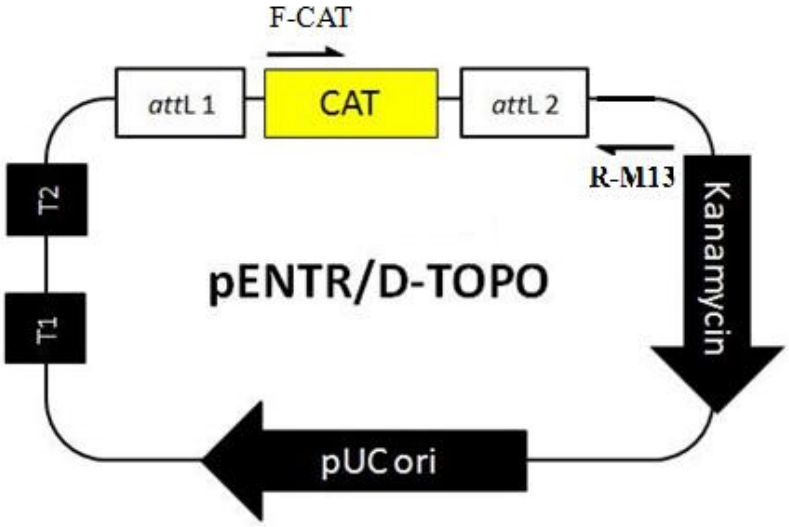

Figure 5.3: Location of another set of primer used, F-MS-MTS and R-M13 for vector containing *CAT* gene.

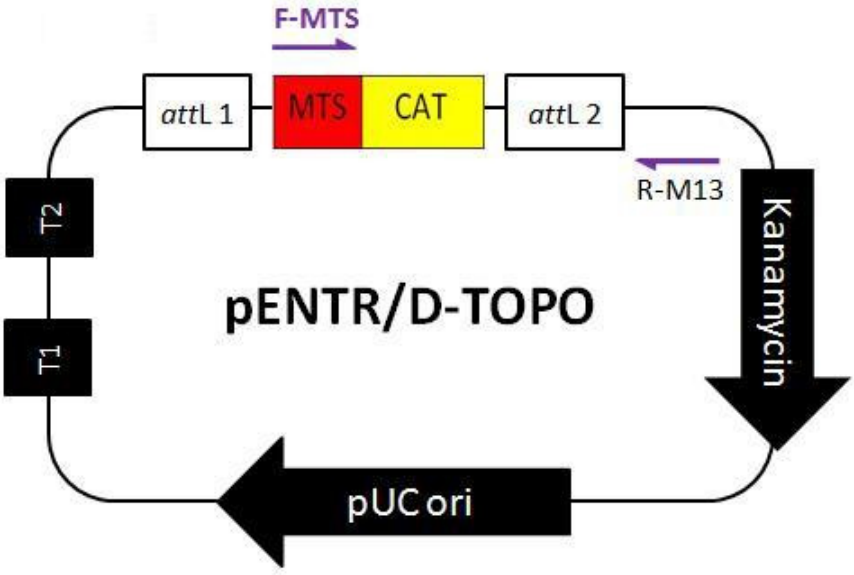

Figure 5.4: Location of another set of primer used, F-MS-MTS and R-M13 for vector containing *CAT* gene with MTS.

CHAPTER 6

CONCLUSIONS

The *CAT* gene and MTS were successfully amplified by PCR. Besides, fusion of MTS and *CAT* gene was also successfully performed by using overlap extension PCR.However, both *CAT* gene and MTS-*CAT* gene could not be screened from the transformed colonies. If the *CAT* gene successfully transformed into matrix of mitochondria with the aid of MTS, the host cell should be more resistant to chloramphenicol antibiotic. This is because of there is approximately 20 mitochondria per yeast cell which is able to carry out protein synthesis in the matrix (Birdsell& Wills, 2003, McKee, 2008). Hence, it is essential to measure the resistance of transformed yeast towards the different concentration of chloramphenicol to determine the most suitable concentration of chloramphenicol used for the selection in the future.

If CAT is successfully developed as a selectable marker for mitochondrial transformation and the mitochondrial targeting sequence (MTS) is used for directing protein into mitochondria matrix, it would ease the mitochondrial transformation procedure. Moreover, the high numbers of the mitochondria present in each eukaryotic cell would increase the transformation efficiency compared to the nuclear transformation. Besides, with the selectable marker for mitochondrial transformation, mitochondria mutant could be generated, allowing further study of mitochondrial genetics. Since mitochondria are maternally inherited, this technology also permits genetic modification

of plants without worrying the risk of spreading transgenes through the pollens of genetic modified plants.

REFERENCES

Bernard, P., & Couturier, M. (1992). Cell killing by the F plasmid ccdB protein involves poisoning of DNA-topoisomerase II complexes. *Journal of Molecular Biology. 226*(3), 735-745.

Bevan, R. B., & Lang, B. F. (2004). Mitochondrial genome evolution: The origin of mitochondria and of eukaryotes. In *Mitochondrial function and biogenesis* (p. 1). New York: Spinger.

Birdsell, J. A., & Wills, C. (2003).A review of contemporary models.In *Evolutionary biolog, Volume 33* (p. 73). New York: Springer.

Bolsover, S. R., Hyams, J. S., Shephard, E. A., White, H. A., &Wiedemann, C. G. (2004). *Cell biology: A short course*. Hoboken, NJ: John Wiley & Sons.

Brown, T.A. (2006). *Gene cloning and DNA analysis: An introduction.* (5thed.). Oxford: Blackwell Science.

Bryksin, A. V., Cabello, F. C., Ladisch, S., Nusinovich, Y. A., Shevchuk, N. A., & Sutherland, M. (2004). Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acid Research*, *32*(2), 1-20.

Byarugaba, D. K. (2010).Mechanisms of antimicrobial resistance.In *Antimicrobial resistance in developing countries* (p. 15). New York: Springer.

Caldwell, G.A., Williams, S.N., & Caldwell, K.A. (2006).*Integrated genomic: A discovery-based laboratory course.*West Sussex, England: John Wiley & Sons.

Capelli, C., &Tschentscher, F. (2005).Protocols for ancient DNA typing. In *Methods in Molecular biology: Forensic DNA typing protocols* (p. 265). New Jersey: Humana Press.

Casali, N., (2003). *Escherichia coli* host strains. In *E.coli plasmid vectors: Methods and applications*(p. 27). Totowa, NJ: Humana Press.

Charlier, N., Molenkamp, R., Leyssen, P., Vandamme, A. M., De Clercg, E.,Bredenbeek, et al. (2003). A rapid and convenient variant of fusion-PCR to construct chimeric flaviviruses.*Journal of Virological Method*, *108*(1), 67-74.

Curan, S. P., & Koehler, C. M. (2004). Mitochondrial biogenesis: Protein import into and across the inner membrane. In *Mitochondial function and biogenesis* (p. 37). Germany: Springer-Verlag Berlin Heidelberg.

Dakubo, G. D. (2010). Basis mitochondrial genetics, bioenergetics and biogenesis.In *Mitochondrial genetics and cancer* (p. 3). New York: Springer.

Dale, J.W., &Schantz, M.v. (2007).*From genes to genomes: Concepts and applications of DNA*.(2nd ed.). West Sussex, England: John Wiley & Sons.

Daniell, H., & Chase, C. (2004).Introduction to the molecular biology and biotechnology of plant organelles. In *Molecular biology and biotechnology of plant organelles*: *Chloroplast and mitochondria* (p. 1). Dordrecht, The Netherlands: Springer.

Day, D. A. (2004).Mitochondrial structure and function in plants. In *Plant mitochondria: From genome to function* (p. 1). Dordrecht, The Netherlands: Kluwer Academic.

Drlica, K., &Perlin, D.S. (2011).*Antibiotic resistance: Understanding and responding to an emerging crisis*. Bergen, NJ: Pearson Education.

Farrell, R. (2010). *RNA methodologies*. (4th ed.). London: Academic Press.

Garcia-Saez, A. J., Fuertes, G., Suckale, J., & Salgado, J. (2010).Permeabilization of the outer mitochondrial membrane by Bcl-2 proteins. In *Proteins membrane binding and pore formation: Advances in experimental medicine and biology, Volume 677* (p. 91). New York: Springer Science+business Media.

Glaser, E., & Whelan, J. (2010).Protein import into plant mitochondria.In *Plant mitochondria* (p. 261). New York: Springer.

Grody, W.W., Nakamura, R.M., Kiechle, F.L., & Strom, C. (2009).*Molecular diagnostics: Technique and applications for the clinical laboratory*. London: Academic Press.

Gupta, P. K. (2007). *Genetics classical to modern*. New Delhi: Rastogi.

Harju, S., Fedosyuk, H., & Peterson, K.R. (2004). Rapid isolation of yeast genomic DNA: Bust n' Grab. *BioMed Central Biotechnology: Methodology, 4*(8)

Harvey, R.A., Champe, P. C., & Fisher, B. D. (2007).*Lippincott's illustrated review: Microbiology*. Baltimore, MD: Lippincott Williams & Wilkins.

Harwood, C. R., &Wipat, A. (2001).Genetic engineering: Prokaryote. In *Basic biotechnology* (p. 72). Cambridge: Cambridge University Press.

Invitrogen Corporation. (2001). *Library efficiency DB3.1 competent cells.* California: Author.

Invitrogen Corporation. (2002). *Gateway pENTR vectors.* California: Author.

Invitrogen Corporation. (2006). *One Shot® Mach1™ –T1^R chemically competent E.coli.* California: Author.

Invitrogen Corporation. (2006). *pENTR™ directional TOPO® cloning kits.* California: Author.

Karp, G. (2009). *Cell and molecular biology: Concept and experiments*. (6thed). England: John Wiley and Sons.

Krebs, J. E., Goldstein, E. S., & Kilpatrick, S. T. (2011).*Lewin's Genes X*. Sudbury, MD: Jones and Battlett.

Jørgensen, R., Søgaard, T. M. M., Rossing, A. B., Martensen, P. M., &Justesen, J. (2000). Identification and characterization of human Mitochondrial Tryptophanyl synthetase.*Journal of Biological Chemistry*, *275*(22), 16820-16826

Lee, C. M., Neupert, W., & Stuart, R. A. (2000).Mitochondrial targeting signals. In *Protein, lipid and membrane traffic: Pathway and targeting* (p. 151). Amsterdam, The Netherlands: IOS Press.

Li, W., Ruf, S., & Bock, R., (2010).Chloramphenicol acetyltransferase as selectable marker for plastid transformation.*Plant Molecular Biology*, 1-9.

Manning, S.D. (2010). *Escherichia coliinfections.* (2nd ed.). New York: Chelsea House.

McKee, E. E. (2008). Assessing mitochondrial protein synthesis in drug toxicity screening.In *Drug-induced mitochondrial dysfunction* (p. 463). New Jersey: John Wiley & Sons.

Menzel, R., &Gellert, M. (Ed.). (1994). The biochemistry and biology of DNA gyrase. In *DNA topoisomerases: Biochemistry and molecular biology* (p. 39). San Diego: Academic Press.

Micklos, D. A., Freyer, G. A., &Crotty, D. A. (2003). *DNA science:A first course*. (2nd ed.). New York: Cold Spring Harbor Laboratory Press.

Mingoia, M., Vecchi, M., Cochetti, I., Tili, E., Vitali, L. A., Manzin, A., et al. (2007). Composite structure of *Streptococcus pneumoniae*containing the erythromycin efflux resistance gene *mef*(I) and the chloramphenicol resistance gene *catQ*. *American Society for Microbiology*, *51*(11), 3983-3987.

Moat, A.G., Foster, J.W., & Spector, M.P. (2002).*Microbiology physiology*(4th ed.). New York: Willey-Liss.

National Center for Biotechnology Information.(2009). *Tryptophanyl-tRNAsynthetase 2; WARS2*.Retrieved April 28, 2011 from http://www.ncbi.nlm.nih.gov/omim/604733.

Neve, E. P. A., &Ingelman, S. M. (2000).Identification and characterization of a mitochondrial targeting signal in rat cytochrome P450 2E1 (CYP2E1).*Journal of Biological Chemistry, 276*(14), 11317-11322.

Nicholl, D. S. T. (2002). *An introduction to genetic engineering.*(2nd ed.). Cambridge: Cambridge University Press.

Old, R.W., & Primrose, S.B., (1980).*Principles of gene manipulation: An introduction to genetic engineering.* Los Angelas: University of California Press.

Omura, T. (1998). Mitochondrial-targeting sequence, a multi-role sorting sequence recognised at all steps of protein import into mitochondria. *Journal of Biochemistry, 123*, 1010-1016.

Rapaport, D., &Nargang, F. E. (2004). Mitochondrial biogenesis: protein import into and across the outer membrane. In*Mitochondial function and biogenesis* (p. 37). New York: Springer-Verlag Berlin Heidelberg.

Rastogi, S. C. (2005). *Cell biology*.(2nd Ed.). New Delhi: New Age International.

Reece, R. J. (2004). *Analysis of genes and genomes*. West Sussex: John Wiley & Sons.

Reetz, M. T., & Jaeger, K. E. (2000).Superior biocatalysts by directed evolution. In *Biocatalysis: From discovery to application* (p. 31). New York: Springer-Verlag Berlin Heidelberg.

Regev, R. N., Yogev, O., & Pines, O. (2008). The mitochondrial targeting sequence tilts the balance between mitochondrial and cytosolic dual localization. *Journal of Cell Science, 121*, 2423-2431.

Remacle, C., &Matagne, R. F. (1998).Mitochondrial genetics.In *The molecular biology of chloroplasts and mitochondria in Chlamydomonas* (p. 661). Dordrecht, Netherlands: Kluwer Academic.

Roberts, M. C., & Schwarz, S. (2009). Tetracycline and chloramphenicol resistance mechanisms. In *Antimicrobial drug resistance: Mechanisms of drug resistance* (p. 188). New York: Human Press.

Sanders, E. R., & Miller, J. H. (2009).*I, microbiologist: A discovery-based undergraduate research course in microbial ecology and molecular evolution*. Washington: ASM Press.

Schwarz, S., & White, D. G. (2005).Phenicol resistance. In *Frontiers in antimicrobial resistance: A Tribute to Stuart B. Levy* (p.124). Washington: ASM Press.

Sussman, M. (Ed.). (1997). *Escherichia coli: Mechanisms of virulence.* Cambridge: Cambridge University Press.

Taylor, G. R., & Noble, J. S. (1995).Probe preparation by the polymerase chain reaction.In *Gene probes 1* (p172). New York: Oxford University Press.

Thomas, C.M. (Ed.). (2005). *The horizontal gene pool: Bacteria plasmids and gene spread*. Amsterdam: Harwood Academic.

Wink, M. (Ed.). (2006). *An introduction to molecular biotechnology: Molecular fundamentals, methods and application in modern biotechnology*. Weinheim: Wiley-VCH Verlag.

Yolov, A. A., &Shabarova, Z. A. (1990).Constructing DNA by polymerase recombination.*Nucleic Acid Research*, *18*(13), 3983-3986.

Young, G. Y., &Koob, M. D. (2008). Selection by drug resistance proteins located in the mitochondria of mammalian cells. In *National Institutes of Health*, *8*(5-6), 345-351.