MOLECULAR CLONING OF *CCOAOMT* GENOMIC SEQUENCE FROM *ACACIA MANGIUM* SUPERBULK TREES VIA PCR

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This thesis is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours in Resource Biotechnology

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2010

ACKNOWLEDGEMENTS

With completion of this thesis, there are many who deserve a word of appreciation. First, I would like to express my sincere appreciation and deepest gratitude to my supervisor, Dr. Ho Wei Seng, Faculty of Resource Science and Technology, UNIMAS, whom had provided me the opportunity to perform and complete my final year project research in Forest Genomics and informatics Laboratory. Thanks a million for his countless advice, patience and guidance, definitely deserves more recognition than a word of merit. Also, I would like to thank to my co-supervisor, Dr. Pang Shek Ling, researcher from the Sarawak Forestry Corporation, for her advice and guidance. Their patience on the support side was vital in succession of this thesis.

Extended assistance from the postgraduates in Forest Genomics and informatics Laboratory is much treasured too. My truthful gratitude goes to especially, Miss Tchin Boon Ling for her guidance, advices, and patience. She had dispensed wisdom on assortment of basic molecular techniques which helped me a lot in performing the research. She spent many hours of meticulous care in helping me to complete the research besides giving me the very important knowledge on how to use the software and analyze my result data.

Next, I would like to thank all my friends who had been with me with their constant support throughout the progression of the thesis since day one. Last but not least, I would like to express my gratitude to my lovely family members who always supporting me, accommodating and understanding in every way.

Thank you to all who had made this thesis a success.

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Abstract

The lignin contents in wood are important determinant of the pulping quality of wood. Hence, the study was carried out to clone and sequence the *caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT)* gene from *Acacia mangium* superbulk trees which is responsible for lignin biosynthesis. Partial genomic DNA of *CCoAOMT* was isolated and amplified by using specific primers, CCoAOMT-PT1 and CCoAOMT-PT2. The product sizes for both of the primer were 1.6 kb approximately. After examined on a 1.5% agarose gel, DNA was purified using gel extraction kit and then ligated into pGEM® -T Easy Vector. The ligation was transformed into competent cells, *Escherichia coli* JM 109. White colonies were observed and colony PCR was performed in using M13 forward and reverse sequence primers. The positive clone with recombinant plasmid was then isolated and purified. Purified plasmid was sent for sequencing and then the data was analyzed by using Chromas Lite and NCBI BLASTn search application. The identity of the consensus sequence was found 100% similar to *Acacia aurifuliformis x Acacia mangium CCoAOMT* gene with the E-value of 1e-63. Motif GXXXGYS which was proposed to be a SAM binding domain was found in the partial amino acid sequence of *CCoAOMT*.

Key Words: *Acacia mangium* superbulk, *caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT)*

Abstrak

Kandungan lignin dalam kayu merupakan komponen yang penting untuk menentukan kualiti kayu. Kajian telah dilakukan untuk mengklon dan menjujukkan gen caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT) daripada pokok Acacia mangium superbulk yang berfungsi untuk mensintesiskan lignin. CCoAOMT DNA serpihan telah diasing dan diamplifikasikan dengan menggunakan primer-primer yang telah disediakan, CCoAOMT-PT1 dan CCoAOMT-PT2. Saiz produk untuk kedua-dua primer adalah sepanjang kira-kiranya 1.6 kb. DNA telah dipurifikasikan dengan menggunakan kit pengekstrak jel dan seterusnya diikatkan ke dalam pGEM® -T Easy Vector. Ikatan DNA dengan pGEM® -T Easy Vector telah ditransformasikan ke dalam sel berkebolehan tinggi, E. coli JM 109. Koloni putih telah ditemui dan digunakan untuk memjalankan colony PCR di mana primer M13 telah digunakan. Gerombolan positif yang mengandungi plasmid recombinan telah diasingkan dan jujukan DNA diperolehi telah dianalisiskan dengan menggunakan Chromas Lite dan NCBI BLASTn. Identiti jujukan DNA telah menunjukkan 100% persamaan dan E-value sebanyak 1e-63 dengan gen Acacia aurifuliformis x Acacia mangium CCoAOMT. Motif GXXXGYS yang dicadangkan ialah SAM penyambung domain telah ditemui daripada jujukan separa asid amino.

Kata kunci: Acacia mangium superbulk, caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT)

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LIST OF ABBREVIATIONS

C3H	Coumarate-3-hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamoyl alcohol dehydrogenase
CCR	Cinnamoyl CoA reductase
COMT	Caffeic acid/5-hyroxyferulic acid O-methyl transferase
CCoAOMT	Caffeoyl-coenzyme A-O-methyltransferase
cDNA	Complementary DNA
F5H	Ferulate 5-hydroxylation
ORF	Open Reading Frame
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
4CL	Hydroxycinnamate: CoA ligase
SAM	S-adenosyl-l-methionine

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CHAPTER I

INTRODUCTION

Acacia mangium is an adaptable and fast growing tropical tree which was first introduced to Malaysia in 1960s for the purpose of producing general utility timber (Sahri *et al.*, 2002). The species has become a major plantation tree species in Southeast Asia since the last two decades. The species is economic importance as the wood can be used for production of furniture, veneers, plywood, pulp and paper making. Besides, *A. mangium* is being widely used for reforestation and soil rehabilitation in degraded soil due to its ability to associate with soil rhizobia and fix nitrogen from atmosphere. *Acacia mangium* superbulk is a genetic improved clone of *Acacia mangium* with more excellent traits (Tchin, 2009).

Lignin is the second most abundant plant natural product found in mostly in cell walls (Zhong *et al.*, 1998). It is made up by three important basic units, called monolignols including ρ-coumaryl, coniferyl, and sinapyl (Kopriva & Rennenberg, 2000). From agro-economical point of view, lignin is considered as negative factor which can affect paper manufacture and limits the digestibility of forage crops (Chen *et al.*, 2001). The lignin residues are known to lower the paper quality by causing discoloration and low brightness level of the pulp (Chiang *et al.*, 1988). It is problematic to degrade or remove the lignin from cellulose because the removal of lignin during pulping process must not cause damage of the polysaccharide components of wood (Chen *et al.*, 2001). In addition, kraft pulping is mostly carried out through chemical delignification process which is environmentally unfriendly because large amount of chemicals are required such as sodium hydroxide (NaOH), sodium sulfide (Na_2S), chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), hypochlorite (NaOCl), oxygen (O_2), or ozone (O_3) (Chen *et al.*, 2001). Moreover, the lignin extraction process will partly degrade cellulose and results in lower quality of pulp and paper strength (Chen *et al.*, 2001).

Many genes have been known to be involved in the lignin biosynthesis pathway. The genes including cinnamyl alcohol dehydrogenase (CAD), caffeoyl-coenyzme A-O-methyltransferase (CCoAOMT), cinnamate 4-hydroxylase (C4H), cinnamoyl-CoA reductase (CCR), phenylalanine ammonia-lyase (PAL) and others. In this study, main focus was the caffeoyl-coenyzme A-O-methyltransferase (CCoAOMT) gene from *Acacia mangium* superbulk trees. The gene plays important role in methoxylation of lignin precursors and previous studies had carried out and proved that the down-regulation of the *CCoAOMT* gene was able to reduce the lignin content and composition.

The method used in this study for the isolation and amplification of the gene of interest was to clone the PCR amplified gene by inserting it into another DNA molecule, pGEM® – T Easy Vector that can be replicated in living cells. Competent cell *Escherichia coli* JM109 was used as the host cell which then replicated and produced a clone. The vector with the foreign desired piece of DNA also replicated, and thus amplified the desired DNA into a large number which then could be purified for sequencing analysis.

Therefore, in this study, the DNA of *Acacia mangium* superbulk was isolated to amplify and clone the *CCoAOMT* gene. The purified plasmid DNA was sent for sequencing and analyzed by using software. The objective of this study is to clone and sequence the *CCoAOMT* gene which is responsible for lignin biosynthesis.

CHAPTER II

LITERATURE REVIEW

2.1 *Acacia mangium* superbulk

Acacia mangium, species of family Leguminoseae, is originated from northern Australia, eastern Indonesia and Papua New Guinea. It was first introduced to Malaysia in 1960s for the purpose of producing general utility timber (Sahri *et al.*, 2002). The species has been planted extensively throughout Southeast Asia and Malaysia because of its fast growth, good form and utilization potential of the wood. Besides, it can easily grow on nitrogen-deficient soils due to the ability to associate with soil rhizobia and fix nitrogen from atmosphere. In 1976, Sabah Forestry Development Authority (SAFODA) was established to reforest nearly 200,000 hectares of degraded lands that resulted from bad shifting cultivation activity and the species mostly planted was *A. mangium* (Varmola, 2002). As shown in the Table 2.1 below was the scientific classification of *Acacia mangium.*

(a) (b)

Figure 2.1: (a) *Acacia mangium* branch and pods (Starr, 2003). (b) The plantation of 6-year-old *Acacia mangium* at Benakat, South Sumatra, Indonesia (Inoue, 2001).

Table 2.1: Scientific classification of *Acacia mangium*

Scientific Classification	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Leguminoseae
Subfamily	Mimosoideae
Tribe	Acacieae
Genus	Acacia
Species	*A. mangium*

However, *A. mangium* is no longer famous as main forest plantation species in Peninsular Malaysia due to some detectable diseases like heart rot, root rot, and phyllode rust (Lee, 2004). Therefore, *A. mangium* was studied and genetically improved to form a new species known as *Acacia mangium* superbulk (Tchin, 2009).

2.2 Lignin Biosynthesis Pathway

Lignin is a complex phenolic polymer which plays important role in physiological functions in plants. It composed of a mixture of ρ-hydroxyphenyl, guaiacyl (G) and syringyl (S) units which derived from monolignols ρ-coumaryl, coniferyl and sinapyl alcohol. The composition of lignin produced in a plant is determined by the amount present of these three units of monolignols. Higher proportions of sinapyl to guaiacyl units produce wood with lower lignin content (Kues, 2007). The lignin content is known to have an impact on the paper and pulp industry because the lignin residues in the wood fibers cause discoloration and reduces paper brightness (Chiang *et al.*, 1988).

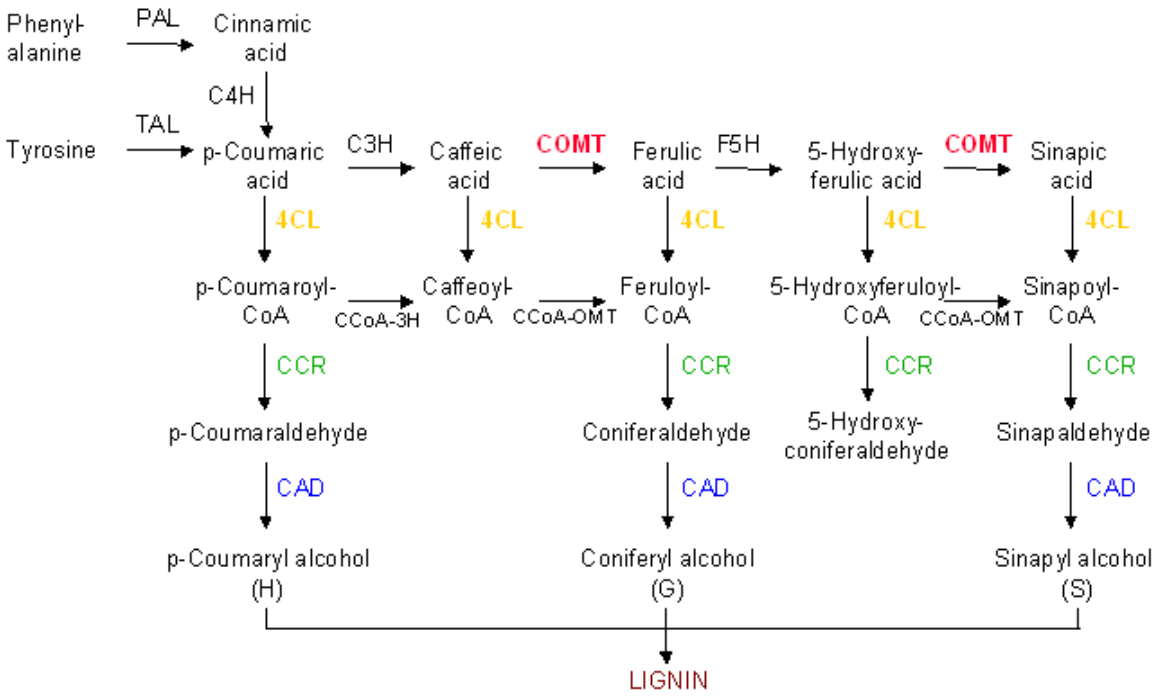

Figure 2.2: The Lignin Biosynthesis Pathway (Spangenberg *et al.*, 2001)

Phenylalanine is an essential amino acid that acts as starting material in lignin biosynthesis pathway. In the presence of phenylalanine ammonia-lyase (PAL), phenylalanine will undergo deamination to produce cinnamic acid. Then, the cinnamic acid will be hydrolyzed to ρ-Coumaric acid, which catalyzed by cinnamate-4-hydroxylase (C4H) enzyme. The coumaric acid formed will then join to coenzyme A by hydroxycinnamate: CoA ligase (4CL) and reduced by cinnamoyl CoA reductase (CCR) and cinnamoyl alcohol dehydrogenase (CAD) to ρ-coumaryl alcohol. Through polymerization process, ρ-coumaryl alcohol will be converted to hydroxyphenyl lignin.

However, ρ-coumaric acid is also a compound for the synthesis of other monolignans. Through hydroxylation and methylation process by coumarate-3-hydroxylase (C3H) and caffeic acid/5-hyroxyferulic acid O-methyl transferase (COMT) respectively, ferulic acid will be synthesized. Ferulic acid can also be ligated to coenzyme

A by 4CL and then reduced by CCR and CAD to form coniferyl alcohol which then polymerized to guaiacyl lignin. On the other hand, ferulic acid can be hydroxylated by ferulate 5-hydroxylation (F5H) and methylated by COMT to sinapic acid. Alternatively, the series of hydroxylation and methylation process can be performed on the CoA derivates of the phenylpropanoids by ρ-coumaroyl CoA 3-hydroxylase (CCoA3H) and caffeoyl CoA O-methyltransferase (CCoAOMT). Due to the low affinity of 4CL for sinapic acid, sinapyl alcohol seems to be formed through this alternative pathway.

2.3 *Caffeoyl-coenzyme A-O-methyltransferase* (*CCoAOMT*) gene

In general phenylpropanoid metabolism, caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT) plays important role in methoxylation of lignin precursors. CCoAOMT methylate hydroxyl groups at the aromatic ring which determine the extractability of lignin (Kues, 2007). According to Kopriva and Rennenberg (2000), the downregulation of the enzyme has become a successful approach to reduce lignin content in transgenic tobacco. A total of 33% to 47% of total lignin content was reduced with slightly higher S/G ratio (Zhong *et al.,* 1998). In addition, the depression of *CCoAOMT* gene had shown no visible abnormal growth of the plant (Zhong *et al.*, 1998). However, the vessel walls were severely deformed but still seem to transport water solutes in sufficient amounts for normal plant growth development (Zhong *et al.*, 1998). Besides tobacco, the cDNAs of this gene have been isolated from a number of species, such as *Zinna* (Ye *et al*., 1994), *Stellaria longipes* (Zhang *et al.*, 1995), and *vitis vinifera* (Busam *et al*., 1997).

2.4 Molecular cloning

According to Mullis (1990), the first cloning experiment was unequivocally demonstrated by Hamilton Smith and co-workers that restriction endonucleases cleave a specific DNA sequence. Daniel Nathans was then used restriction endonucleases to map the simian virus 40 (SV40) genome and to locate the origin of replication. The breakthrough drew attention to the great potential of restriction enzyme for DNA work. Hybrid of phage λ and the SV 40 mamamalian DNA virus genome was one of the first engineered recombinant DNA molecules. The first eukaryotic gene was cloned in 1974 where the amplified ribosomal RNA (rRNA) genes from the South African clawed frog *Xenopus laevis* were cut by restriction enzymes and ligated to a bacterial plasmid.

2.5 Case study

Day *et al.* (2008) performed a study regarding the down-regulation of Caffeoyl coenzyme A O-methyltransferase (CCoAOMT) in *Linum usitatissimum* flax plants. Antisense strategy was used in the study and the chemical analyses revealed that the lignin quantity was reduced. Also, the syringyl/guaiacyl (S/G) lignin monomer ratio was modified which associated with altered xylem organization, reduced cell-wall thickness and the appearance of an irregular xylem phenotype. In addition, UV microspectroscopy also revealed that CCoAOMT down-regulation induced alterations in xylem cell-wall structure and the lignin fractions as well as the xylem cell size and identity.

In addition, Wei *et al.* (2008), had carried out a study on the pulping performance of transgenic poplar with depressed Caffeoyl-CoA O-methyltransferase (CCoAOMT). The pulping performance of 3 year old field-grown transgenic poplar (*Populus tremula x Populus alba*) was evaluated. The lignin content in transgenic poplar with anti-sense

CCoAOMT was found decreased about 13%. On the other hand, S/G ratio was shown slightly increased. Through chemical analysis, the transgenic poplar had significantly less benezene-ethanol extractive than the control wood. However, there was no significant differences were found in contents of ash, cold water extractive, hot water extractive, 1% NaOH extractive, holocellulose, pentosans and cellulose. Besides, the down-regulation of *CCoAOMT* expression was proved to improve the fiber quality via fiber assay. Also, kraft pulping showed that lower lignin in transgenic poplar led to notable better pulp quality and increased pulp yield.

According to Zhong *et al.* (1998), antisense approach was carried out to further understand the methylation steps in lignin biosynthesis to confirm that CCoAOMT is essential for lignin biosynthesis. In the study, transgenic tobacco plants with a substantial reduction in CCoAOMT and the plants with a simultaneous reduction in both CCoAOMT and caffeic acid *O*-methyltransferase (CAOMT) were generated. Alteration was made for the *O*-methyltransferase (*OMT*) gene expression and the consequence of this alteration was investigated. Lignin analysis showed that the reduction in CCoAOMT alone resulted in dramatic decrease in lignin content and also led to a dramatic alteration in lignin composition. Both guaiacyl lignin and syringyl lignin were reduced but guaiacyl lignin was preferentially reduced, giving the result of an increase in the S/G (syringyl/guaiacyl) ratio. The reduction in both CCoAOMT and CAOMT was investigated and resulted in a further decrease in total lignin content.

CHAPTER III

MATERIALS AND METHODS

3.1 Collection of Plant Materials

The leaf samples from *Acacia mangium* superbulk trees were collected from the UNIMAS's arboretum. Two samples were used in the study namely SB9 and SB2.

3.2 DNA Isolation

Firstly, two percent (v/v) of mercaptoethanol was added into 50ml Falcon tube which containing 5ml of CTAB buffer which had already contained polyvinylpyrrolidone. The CTAB extraction buffer was preheated at 65^{o}C for 30 minutes. Next, 1 g of fresh leaves was cut into small pieces and weighted. The tissues were frozen rapidly in liquid nitrogen and ground into a fine powder with a pestle and mortar as the liquid nitrogen boils off. Then, the powder was transferred as fast as possible into a Falcon tube containing preheated CTAB extraction buffer and mixed gently by inverting the tube ten times. The sample was incubated for 30 minutes at 65^{o}C.

After incubation, the tube was removed from the water bath and allowed to cool to ambient. Then, 600 µl of the mixture was transferred into 1.5 ml of microcentrifuge tubes and following that, equal volume of CIA (24:1) was added into each microcentrifuge tube. The contents were mixed gently to a single phase. Then, contents were centrifuged at 13,000 rpm for 10 minutes to obtain supernatant. After that, the aqueous phase (the top phase) was transferred to a new microcentrifuge tube and the CIA extraction step was repeated. Then, 2/3 volume of cold isopropanol was added into the tube and stored at -20^{o}C for 30 minutes.

After that, the tubes were centrifuged at 13,000 rpm for 15 minutes to obtain the pellet DNA. Then, the supernatants were poured off and the pellets were washed with 1 ml of 70% ethanol. The tubes were mixed gently and stored at -20^{o}C for 30 minutes. After frozen, the tubes were centrifuged at 13,000 rpm for 2 minutes. The supernatants were discarded and the pellets were air-dried at room temperature by inverting the tube. Then, the dried pellets were resuspended in the 50 µl of ultrapure water and stored at -20^{o}C.

3.3 DNA Purification

The isolated DNAs were purified by using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacture's protocol. Firstly, the DNA samples were topped up to a volume of 600µl with double-distilled water. Next, 3 µl of 4 mg/ml RNase solution was added into each of the microcentrifuge tube containing DNA and the mixtures were incubated at 37^{o}C for 15 minutes. Then, the tubes containing mixture were allowed to cool to room temperature for 5 minutes. After that, 200 µl of protein precipitation solution were added to each tube and then mixed gently. The tubes were centrifuged for 3 minutes at 13,000 rpm. Then, the supernatants were transferred into new microcentrifuge tubes containing 600 µl of room temperature isopropanol, mixed gently and stored at room temperature for 30 minutes. After that, the mixtures were centrifuged for 10 minutes at 13,000 rpm. The supernatants were discarded and 600 µl of 70% ethanol were added into each tube and mixed gently. Then, the mixtures were centrifuged again for 1 minute. After that, the supernatants were discarded and the pellets were air-dried at room temperature. Lastly, 40 µl of ultrapure water was added into each of the tubes and resuspended by flinking. The quality of DNA was analyzed on 0.8% agarose gel.

3.4 DNA Quantification

The purified DNAs were quantified by using Lambda 25 UV/VIS Spectrophotometer (Perkin Elmer, USA). Three microlitres of purified DNA was transferred into quartz curette and then diluted with 2997 µl of double distilled water with the dilution factor of 1000x. Then, the absorbance readings were measured at wavelengths of 230nm, 260 and 280.

3.5 DNA Amplification (PCR)

Amplification was carried out by using specific primer set designed from cDNA sequence of CCoAOMT (EU275979.1) (Appendix B) from *Acacia* hybrid. The details of both of the primer sets were described as the table shown below.

Table 3.5 (a): Primer set 1 (CCoAOMT-PT1)

Primer	CCoAOMT-PT1
Forward Sequence	5'GAACCCATGAAGGAGTTGAGA 3'
Reverse sequence	5'AGCCGTTCCATAAGGTGTTGT 3'
Forward T_a	57.6^oC
Reverse T_a	59.3^oC
Expected product size	415 bp

Table 3.5 (b): Primer set 2 (CCoAOMT-PT2)

Primer	CCoAOMT-PT2
Forward Sequence	5' ACACTGGCTACTCCTTGCTCG 3'
Reverse sequence	5' TCCGCTACAACTCATTACTTT 3'
Forward T_a	60.4^oC
Reverse T_a	58.3^oC
Expected product size	508 bp

Later, the PCR product was examined on 1.5 % agarose gel and 100bp DNA ladder (Promega, USA). The amplification of *CCoAOMT* was performed in Mastercycler Gradient Thermal Cycler (Eppendorf; Germany) and the profile was set as Table 3.5 (a) and (b) shown below.

Table 3.5 (c): PCR reaction mixture

Reagent	**Stock concentration**	**Final concentration**	**Final volume**
ddH_2O	-	-	10.25 µl
10x PCR buffer	10 x	1 x	2.5 µl
dNTP	2 mM	0.2 mM	2.5 µl
$MgCl_2$	50 mM	1.5 mM	0.75 µl
Forward primer	5 pmol	2 pmol	2 µl
Reverse primer	5 pmol	2 pmol	2 µl
DNA	10ng/µl	30ng/µl	3 µl
Taq DNA polymerase	0.5 U/µl	1U/µl	2 µl
Total			**25 µl**

Table 3.5 (d): PCR reaction cycles, temperatures and durations

Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	95^{o}C	2 min	
Denaturation	94^{o}C	45 s	
Annealing	63^{o}C	45 s	35
Extension	72^{o}C	1 min	
Final extension	72^{o}C	10 min	

3.6 Purification of DNA from Agarose Gel

The PCR product was transferred into pre-weight microcentrifuge tube. Then, the tube contained gel fragment was weighed again to obtain the amount of gel. After that, 3 volumes of Buffer QG were added to 1 volume of gel and incubated for 10 min at 50^{o}C or until the gel was totally dissolved. Later, 1 gel volume of isopropanol was added and

mixed by flicking. A QIAquick spin column was placed in a 2 ml collection tube. The DNA mixture was transferred into the column and centrifuged for 1 min. The flow through was discarded and the column was placed back to the collection tube. Then, 750 µl of Buffer PE was added into the column and centrifuged for 1 min. The flow through was discarded and centrifuged again for another 5 min. After that, the column was placed into a clean 1.5 ml microcentrifuge tube. Then, 50 µl of distilled water was added to the centre of the membrane and centrifuged for 1 min. Later, the DNA was examined on a 1.5% agarose gel.

3.7 DNA Ligation

The purified PCR product was ligated into pGEM® -T Easy Vector System (Promega, USA) (Appendix C). Three to one ratio of the insert DNA to the vector was used. Quantity of PCR product required was calculated according to the formula below:

$$\text{Amount of PCR product required (ng)} = \frac{25\,\text{ng vector x kb of insert size}}{\text{size of vector, kb}} \text{ X } \frac{3}{1}$$

*****Size of pGEM-T Easy Vector is 3kb.

The ligation mixture was prepared in a microcentrifuge tube as shown in Table 3.7 and followed by incubation at 4^{o}C overnight.

Table 3.7: Ligation reaction mixture and volume

Reaction mixture	**Volume**
2X Rapid Ligation Buffer	2.5 µl
pGEM® -T Easy Vector (50ng/µl)	0.5 µl
Purified PCR product	1.5 µl
ddH_2O	-
T4 DNA ligases (3 weiss units/µl)	0.5 µl
Total	5.0 µl

3.8 Transformation

After ligation, the mixture was added into the microcentrifuge tube containing 25 µl of competent cells, *Escherichia coli* JM 109 and mixed gently by using pipette tip. Next, the cells were incubated on ice for 20 min and then heat shocked for 45s at 42^{o}C. Then, the cells were returned to the ice for 2 min. After that, 1ml of LB was added into the tube and the cells were incubated at 37^{o}C with shaking at 300 rpm for 3 hours and followed by centrifugation at 13,000 rpm for 10 min. After centrifugation, 800 µl of supernatant were discarded and the pellet was dissolved in the remaining broth. Then, 100 µl of the transformation culture was spread onto LB plates which contain 100 µg/ml ampicillin, 100µl of 0.1 mM IPTG and 20 µl of 50 mg/ml X-gal. Finally, the culture was incubated at 37^{o}C for 16 to 18 hours.

3.9 Blue/White Colony Screening

Pure white colonies were picked using sterile pipette tips and then inoculated into 1.5 ml microcentrifuge tube which contained 10 µl of distilled water. After that, colony PCR was performed by using Mastercycler Gradient Thermal Cycler (Eppendorf, Germany). The primers used were M13 forward and reverse and the reaction profile is shown in Table 3.9 (a) and (b) below.

Table 3.9 (a): Colony PCR reaction mixture

Reagent	Stock concentration	Final concentration	Final volume
ddH_2O	-	-	15.25 µl
10x PCR buffer	10 x	1 x	2.5 µl
dNTP	2 mM	0.2 mM	2.5 µl
$MgCl_2$	50 mM	1.5 mM	0.75 µl
M13 Forward primer	5 pmol	5 pmol	1 µl
M13 Reverse primer	5 pmol	5 pmol	1 µl
DNA	-	-	1 µl
Taq polymerase	0.5 U/µl	0.5U/µl	1 µl
Total			**25 µl**

Table 3.9 (b): Colony PCR reaction cycles, temperatures and durations

Stage	Temperature	Duration	Cycles
Initial denaturation	95^{o}C	5 min	
Denaturation	94^{o}C	30 s	
Annealing	55^{o}C	30 s	35
Extension	72^{o}C	30 s	
Final extension	72^{o}C	8 min	

The PCR products were then examined on 1.5% agarose gel. Positive clones were recultured in 50 ml Falcon tubes containing 5 ml LB Broth and 100 µg/ml ampicillin. Then, the culture was incubated at 37^{o}C with shaking for 16 to 18 hours.

3.10 Plasmid Isolation

The plasmid isolation was carried out using Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) according to the manufacture's protocol. Firstly, the overnight culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 5 min at room temperature. Next, the pellet was resuspended with 250 µl of Cell