



Faculty of Resource Science and Technology

**ISOLATION AND CHARACTERIZATION OF PARTIAL GENOMIC SEQUENCE  
OF CYSTEINE SYNTHASE (*CSase*) GENE FROM PETAI BELALANG (*Leucaena  
leucocephala*)**

**Athirah Binti Abdul Rahman**

**35538**

**Bachelor of Science with Honours  
(Resource Biotechnology)  
2015**

## **ACKNOWLEDGEMENT**

This project consumed huge amount of work, research and dedication. Still, implementation would not have been possible if did not have a support of many individuals and organizations. Therefore, I would like to extend my sincere gratitude to all of them.

First of all, thank to my supervisor, Dr. Ho Wei Seng for giving me opportunity to accomplish my final year project research, encouragement, valuable advice, financial and logistical support and for providing necessary guidance concerning projects implementation.

I am also grateful to Ms. Natalie anak Gali and Mr. Lai Nan Keong for devoted their time, provision of expertise, and technical support in the implementation. Without their superior knowledge and experience, this project would like in quality of outcomes, and thus their support has been essential.

Nevertheless, I would like to express my gratitude toward my families, lab members and colleagues for their kind co-operation and encouragement which help me in completion of this project.

## **DECLARATION**

I hereby declare that this Final Year Project report 2015 entitled “Isolation and characterization of partial genomic sequence of cysteine synthase (*CSase*) gene from petai belalang (*Leucaena leucocephala*)” is based on my original work except for the quotation and citations which have been fully acknowledge also, declare that it has not been or concurrently submitted for any other degree at UNIMAS or other institution of higher learning.

---

**Athirah Binti Abdul Rahman**  
**Department of Molecular Biology**  
**Faculty of Resource Science and Technology**  
**Universiti Malaysia Sarawak**

## TABLE OF CONTENTS

<b>ACKNOWLEDGMENTS</b>	I
<b>DECLARATION</b>	II
<b>TABLE OF CONTENTS</b>	III
<b>LIST OF ABBREVIATIONS</b>	V
<b>LIST OF TABLES</b>	VI
<b>LIST OF FIGURES</b>	VII
<b>ABSTRACT/ ABSTRAK</b>	IX
<b>1.0 INTRODUCTION</b>	1
<b>2.0 LITERATURE REVIEW</b>	
2.1 Selection of species studies	3
2.1.1 Family Fabaceae (Leguminosae)	3
2.1.2 <i>Leucaena leucocephala</i>	4
2.2 Cysteine	7
2.3 Cysteine synthase	8
<b>3.0 MATERIALS AND METHODS</b>	
3.1 Plant material	10
3.2 DNA extraction and purification from <i>Leucaena leucocephala</i>	10
3.2.1 Chemicals and reagents	10
3.2.2 DNA extraction protocol	10
3.2.3 DNA purification protocol	11
3.3 Agarose gel electrophoresis (AGE)	12

3.4 DNA quantification	12
3.5 Primer design	12
3.6 PCR optimization	13
3.7 PCR product purification	14
3.8 Agarose gel electrophoresis quantification of PCR product (DNA)	14
3.9 DNA sequencing and data analysis	15
<b>4.0 RESULTS AND DISCUSSION</b>	
4.1 Reagents preparation	16
4.2 DNA extraction of <i>Leucaena leucocephala</i>	16
4.3 DNA purification of <i>Leucaena leucocephala</i>	19
4.4 Agarose gel electrophoresis	20
4.5 Primer design	21
4.6 PCR optimization	22
4.7 PCR product purification	24
4.8 DNA sequencing and data analysis	25
<b>5.0 CONCLUSIONS AND RECOMMENDATIONS</b>	28
<b>REFERENCES</b>	29
<b>APPENDICES</b>	
Appendix A	32
Appendix B	39
Appendix C	43

## LIST OF ABBREVIATIONS

A	Ampere
bp	Base pair
<i>CSase</i>	Cysteine synthase gene
cDNA	Complementary DNA
CTAB	Cetyl trimethyl ammonium bromide
CIA	Chloroform-isoamyl alcohol
ddH <sub>2</sub> O	Double-distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
mRNA	Messenger RNA
Ng	Nanogram
Rpm	Revolution per minute
T <sub>m</sub>	Melting temperature
Tris-HCL	Tris-hydrochloride
UV	Ultra violet
V	Volt
μl	Microliter

## LIST OF TABLES

Table 3.1	PCR reaction mixture	13
Table 3.2	PCR profile	13
Table 4.1	Primer design for cysteine synthase of <i>L. leucocephala</i>	22
Table 4.2	BLAST search result of amplified 372 bp PCR product	26

## LIST OF FIGURES

Figure 2.1	<i>Leucaena leucocephala</i> tree with a white popinac flower	5
Figure 1.2	Young seed and pod that can be eaten raw	6
Figure 2.3	Outline of cysteine biosynthesis in plant cell	8
Figure 2.4	Mimosine and cysteine biosynthetic pathway	9
Figure 4.1	(a) <i>Leucaena leucocephala</i> sample  (b) <i>Leucaena leucocephala</i> leaves after running with tap water, wipe with tissue to dry	16
Figure 4.2	Three layer of aqueous phases after addition of CIA	17
Figure 4.3	DNA pellet formed after centrifugation	18
Figure 4.4	Concentrated and smearing band formed	19
Figure 4.5	Aqueous layer formations after addition of CIA	20
Figure 4.6	Concentrated DNA band formed after purification	21
Figure 4.7	DNA concentration optimization	23
Figure 4.8	Lane 3: PCR product of 500 ng/ $\mu$ l DNA concentration in 100 $\mu$ l of total reaction	24
Figure 4.9	Lane 1: Purified PCR product, ~1,300 bp	25
Figure 4.10	Nucleotide sequence of 372 bp <i>CSase</i>	26

Figure 4.11 Comparison of cysteine synthase gene from full length genomic DNA of *Glycine soja* (EF535995.1), mRNA sequences from *Glycine max* (LOC547631) and *L. leucocephala* (KF754356.1). Coloured cylinders represent exon region, while black cylinder represent intron region.

27

# ISOLATION AND CHARACTERIZATION OF PARTIAL GENOMIC SEQUENCE OF CYSTEINE SYNTHASE (*CSase*) GENE FROM PETAI BELALANG (*Leucaena leucocephala*)

**Athirah Binti Abdul Rahman**

Resource Biotechnology Programme  
Faculty of Resource Science and Technology  
University Malaysia Sarawak

## ABSTRACT

Cysteine synthase (*CSase*) is an important enzyme for cysteine biosynthesis and occurred at three different organelle of plant cell such as mitochondria, cytosol and chloroplast. Petai belalang or scientifically known as *Leucaena leucocephala* is a leguminous plant belonging to Fabaceae family. It provides worldwide function such as feed-stock for ruminant, cropping system, charcoal and small furniture. The aim of this study was to isolate and *in silico* characterize the partial genomic sequence encode for cysteine synthase from *L. leucocephala*. Total DNA from the young leaf of petai belalang was isolated and purified by using the modified CTAB method. According to the conserved region of *CSase* gene from multiple alignment of mRNA sequence from five plant species; *Glycine max*, *Solanum tuberosum*, *Citrullus lanatus*, *Arabidopsis thaliana* and *L. leucocephala* and using *L. leucocephala* mRNA sequence of cysteine synthase gene as a template, the forward and reverse primers were designed. Then, the DNA fragment was amplified by using the standard polymerase chain reaction (PCR). The amplicon which was ~1,300 bp was sequenced and the sequence was edited to 372 bp. The genomic DNA showed 99 % homology with Cy-OAS-TL of *L. leucocephala* analyzed by BLASTn. The intron-exon regions was determined by multiple alignment and the *CSase* amplicon only consist of coding region. Throughout this study, the cysteine synthase sequence can be used as a foundation to understand the molecular aspect of petai belalang tree.

Key words: *Leucaena leucocephala*, Cysteine synthase, polymerase chain reaction (PCR), BLASTn.

## ABSTRAK

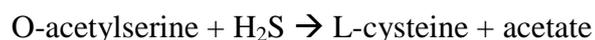
Cysteine synthase (*CSase*) merupakan enzim penting untuk cysteine biosintesis dan berlaku pada tiga organel berbeza pada sel tumbuhan seperti mitokondria, sitosol dan kloroplas. Petai belalang atau nama saintifiknya *Leucaena leucocephala* adalah tumbuhan kekacang daripada keluarga Fabaceae. Tumbuhan ini menyediakan pelbagai fungsi secara meluas seperti makanan bagi haiwan ternakan, sistem tanaman, arang dan perabot kecil. Tujuan kajian ini adalah untuk mengasingkan dan *in silico* mencirikan separa genomik sequence untuk cysteine synthase dari *L. leucocephala*. Jumlah DNA yang telah diekstrak dan dibersihkan daripada daun muda petai belalang dengan menggunakan kaedah CTAB diubahsuai. Berdasarkan conserved region daripada penjajaran secara berganda mRNA sequence antara lima spesies tumbuhan; *Glycine max*, *Solanum tuberosum*, *Citrullus lanatus*, *Arabidopsis thaliana* dan *L. leucocephala* dan urutan mRNA cysteine synthase gene *L. leucocephala* digunakan sebagai template untuk mereka bentuk forward dan reverse primer. Kemudian, DNA diampifikasikan menggunakan kaedah tindakbalas berantai polimerase (PCR) standard. Amplicon yang bersaiz ~1,300 bp telah dianalisis dan urutan DNA telah diedit kepada 372 bp. Genomik DNA menunjukkan 99 % homologi dengan Cy-OAS-TL *L. leucocephala* melalui BLASTn. Bagi menentukan inton dan exon, penjajaran secara berganda telah dijalankan dan *CSase* amplicon hanya terdiri daripada coding region sahaja. Melalui kajian ini, sequence cysteine synthase boleh digunakan sebagai asas untuk memahami aspek molekul pokok petai belalang.

Kata kunci: *Leucaena leucocephala*, Cysteine synthase, tindakbalas berantai (PCR), BLASTn.

## 1.0 INTRODUCTION

*Leucaena leucocephala* or commonly known as petai belalang is widely distributed in tropical country such as Malaysia and it is belong to Fabaceae or leguminous family (Little, 1970). It has been proved that this leguminous tree have worldwide function such as cropping system through contour strips at steep slope that helps for erosion control, fuel production, fodder for ruminants, the young leaves and seeds can also be used for human consumption but in small amount as the young leave contain toxic, mimosine, that may lead to diseases such as goiter (Shelton & Brewbaker, 2014).

Through sulphur assimilation process by the plant, inorganic sulphate ion in the soil is reduced and produced cysteine (Singh, 1998). Cysteine synthesis occurred at three different place of plant; mitochondria, cytosol and chloroplast (Singh, 1998; Gotor et al., 1997). Besides that, cysteine acts as sulphur donor for amino acid methionine and integrates with protein and antioxidant, glutathione (GSH). Hence, it is precursor of biomolecules, for examples, vitamins, cofactor and defense molecules such as glucosinolates and phytoalexins (Álvarez et al., 2012). Biosynthesis of cysteine required a lots of enzymes and substrates, such as ATP Sulfurylase, APS Reductase, Sulphite reductase, APS kinase and O-acetylserine(thiol)-lyase (Singh, 1998). Cysteine synthase also known as O-acetylserine(thiol)-lyase is the key enzyme for cysteine synthesis. Reaction between O-acetylserine and hydrogen sulphide subrates (Bettati et al., 2000) will form L-cysteine and acetic acid (Yamaguchi et al., 1998).



Nowadays, studies have been done on cysteine synthase in physiological and molecular aspect in a few plant species such as spinach (Saito et al., 1994), *Arabidopsis thaliana* (Register et al., 1995) and *Capsicum Annuum* (Romersg et al., 1992). Up to now, there is no information on isolation and partial genomic sequence of cysteine synthase from petai belalang.

Therefore, the objective of this study were to isolate the DNA encode for cysteine synthase from *L. leucocephala* and to *in silico* characterize the partial genomic sequence of cysteine synthase from *L. leucocephala*.

## 2.0 LITERATURE REVIEW

### 2.1 Selection of species studied

#### 2.1.1 Family Fabaceae (Leguminosae)

Fabaceae is one of the families for flowering plant (angiosperm) which is commonly known as legume family, pea family or bean family. Among angiosperm families, Fabaceae is the third largest family after Orchidaceae and Asteraceae that composed of more than 650 genera and approximately 20,000 species of trees, shrubs, vines and herbs (Doyle, 1994).

Furthermore, Fabaceae family contributes in ecological and economic terms as legumes can increase soil nitrogen and act as sources of livestock such as peas and beans (Wojciechowshi et al., 2006; Lavin, 2001). Fabaceae is widely distributed around the world especially in seasonally dry, tropical country and high dessert to lowland rainforest (Lavin, 2001). Fabaceae can be divided into three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae (Doyle, 1994). Categorization of the families are based on flower; size, symmetry, sepal and pollen besides the presence of pleulogram and root nodules (Wojciechowshi et al., 2006).

According to Mitra (2010), most of Caealpiniaeeae subfamily is shrub and climbing tree, some are herbs. Sepals and petals of the flower are five in number and it may be free or united imbricate aestivation. *Saraca indica* and *Tamarindus indica* are the examples of Caelpiniaceae subfamily. Mimosaceae subfamily also consists of shrubs, herbs and trees. The flower is actinomorphic that is radial symmetry. Examples of Mimosaceae are *Mimosa pudica* and *Acacia arabica*. Next, Papillionaceae is consisting of perennial shrubs and herbs, some of it are climbers. The calyx contains five sepals that are

united at the base and hairy. Besides that, five separated petal, butterfly in shape are located at the corolla. *Arachis hypogea* and *Pisum sativum* are examples of this subfamily.

Morphologically, the Fabaceae is characterized by compound leaf, usually pinnate compound, palmate compound and trifoliolate. Besides its leaves, the fruits for Fabaceae is commonly a pod or legume which when it dries it will split open and discharge its seed for germination. The structure of the fruit is depending on how the pods are dispersed such as water-dispersed, outer layer consist many air cell for water buoyancy, wind-dispersed, that have wings that vary in size and shape, and through animals fur. The pod is small in size and might consist of one or dozens of seeds (Lavin, 2001).

### **2.1.2 *Leucaena leucocephala***

*Leucaena leucocephala* or locally known as petai belalang or white popinac (Figure 2.1) is widely distributed in tropical country, such as Malaysia (Little, 1970). According to Shelton and Brewbaker (2014), this plant is originated from Central America and Yucatan Peninsula of Mexico. It is then introduced into Australia during late 19<sup>th</sup> century. This plant is thorn-less and long-lived shrub as it is drought tolerance; *L. leucocephala* have deep root that extend 5 m to absorb underground water.



**Figure 2.1** *Leucaena leucocephala* tree with a white popinac flower  
Adapted from *Leucaena leucocephala*, by D. Valke, 2007, Retrieved from  
<http://www.flickr.com/photos/91314344@N00/348704834>

Besides that, *L. leucocephala* is also known as ‘miracle tree’ because of its worldwide function. The leaves have bipinnate structure that is highly nutritious for ruminants. In addition, this plant can be used in cropping system through contour strips at steep slope that helps for erosion control. Medium light hardwood is produced in massive amount can contribute in production of low moisture and high heating value of fuel. Moreover, it can be used as charcoal that released a small amount of ash and smoke. Parquet flooring and small furniture are some of the products from this plant. Climbing tree such as pepper used *L. leucocephala*’s pole as a frame to climb. This plant is also popular as the young leaves and seeds can be used for human consumption (Figure 2.2). Immature seeds from young green pod can be eaten raw or cooked in small consumption due to mimosine content that is toxicity (Shelton & Brewbaker, 2014).



**Figure 2.2** Young seed and pod that can be eaten raw.  
Adapted from Jumbie Bean, White lead Tree by G. Deane, (n.d.), Retrieved from  
<http://www.eattheweeds.com/leucaena-leucocephala-food-and-fodder-2/>

Mimosine is synthesis by lysine (Shann & Alan, 1986) and high amount in *L. leucocephala* foliage and pods, although this plant is highly nutritious for ruminant but only small proportion in animal's diet is recommended due to its allelopathic effects and it deters cell division (Vestena et al., 2001). For rumen, mimosine will be breakdown into DHP (3 hydroxyl-4-(1H)-pyridone) with assist of microbe in its stomach. DHP need to undergo biochemical reaction until it is non-toxic. Accumulation of DHP molecule can lead to disease such as goiter that eventually will cause hair loss, excess synthesis of saliva, loss of appetite, listlessness and loss of weight (Shelton & Brewbaker, 2014).

Biomass is highly demand and the biggest energy supplier in most develops country. Through anaerobic fermentation of *L. leucocephala*, methane-rich gas is produced and electric power system can be generated economically (Bassam, 2010). Besides that, *L. leucocephala* has biofuel potential due to the kernel consists 15-20% of fatty acid. The seed oil can be used as a biofuel for diesel engines (Meena et al., 2013). Recently, East

Coast Economic Region (ECER) will construct world's first bio-methionine and thiochemicals plant at Kertih BioPolymer Park, Terengganu. This project involves Korea's CJ CheilJedang (CJ)'s bio-methionine plant and France's Arkema's thiochemicals platform. L-methionine will be produced through fermentation process from *Acacia mangium* and *Leucaena leucephala* which is used in manufacturing of animal feed (ECER Receives World's First Integrated RM2 Billion Bio-Methionine & Thiochemicals Plant, 2012).

## 2.2 Cysteine

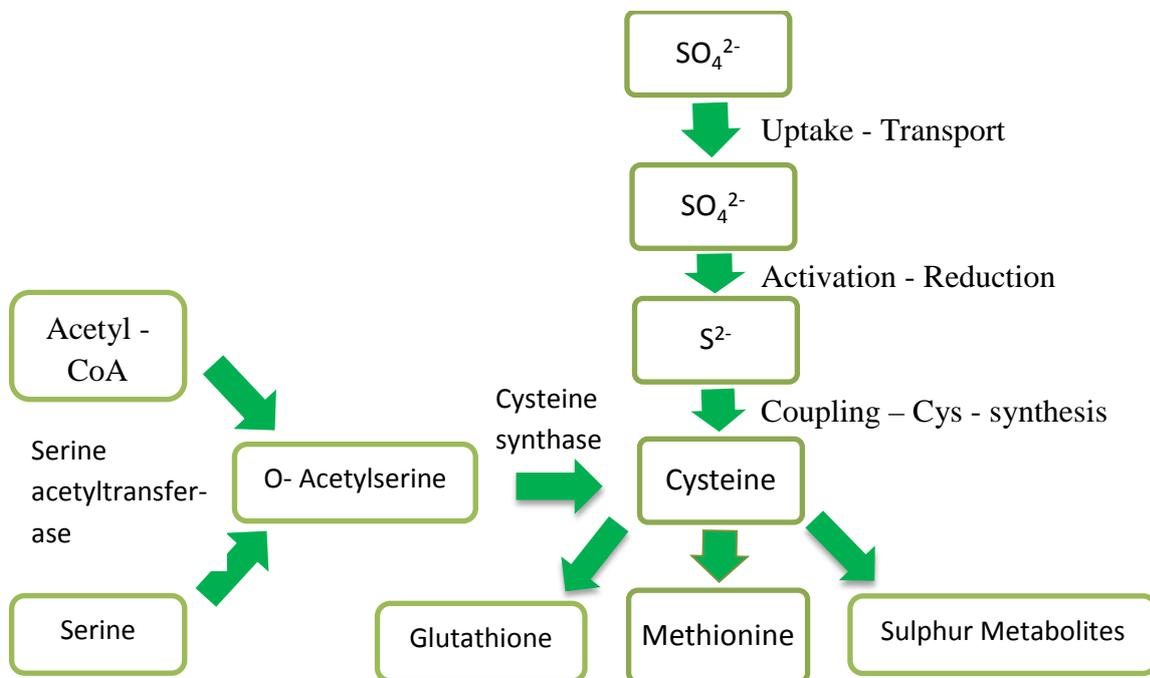
Cysteine is an amino acid that consist sulphur at the R terminus, other than methionine. Cysteine residues are crucial for stabilization of protein structure especially tertiary and quaternary protein conformation via disulfide bridges (Wirtz & Droux, 2005). According to Álvarez et al. (2012), amino acid methionine received sulphur ion from cysteine and integrates with protein and antioxidant, glutathione (GSH). In plant physiology, sulphur played an important role for growth. Synthesis of sulfur-containing amino-acids through reduction of sulfate to sulphide can be done by assimilation of inorganic sulfate by plants, bacteria and fungi (Wirtz & Droux, 2005). Cysteine biosynthesis affects sulphur cycle in the environment by incorporate inorganic sulphur from the soil and air into cysteine. There are two biochemical pathways for cysteine synthesis (Figure 2.3); transport, activation and reduction of sulfate to sulphide and supplying amino acid serine and coupling with hydrogen sulphide to form cysteine (Singh, 1998).

Sulphate ion ( $\text{SO}_4^{2-}$ ) is abundant in soil and it is the most oxidative agent. Hence, it is the most stable form of sulphur atom. According to Scott (2013), sulphur uptake by the roots is in the form sulphate ion and converts to cysteine when it is transported to the leaves. This is due to leaves contain high amount of ATP and reduced ferredoxin during photosynthesis process. Hence, sulphate ion will reduced to form sulphide ion ( $\text{S}^{2-}$ ) after

undergo a few processes at the chloroplast. Throughout this process, a lot of proteins, specifically enzymes play major role for cysteine synthesis such as ATP sulfurylase, APS reductase, sulphite reductase, APS kinase and O-acetylserine(thiol)-lyase (Singh, 1998).

### 2.3 Cysteine synthase

In this research, we will focus more on cysteine synthase that is crucial for cysteine synthesis in plant. Cysteine biosynthesis undergoes two-step reaction (Figure 2.3); serine acetyltransferase is acquired in the first reaction that assists in production of O-acetylserine from acetyl-CoA which acts as acetyl donor and serine through acetylation process. O-acetylserine(thiol)-lyase (OAS-TL) also known as cysteine synthase is an enzyme that is categorized in transferase family. Hence, it functions as a terminal step for cysteine biosynthesis specifically L-cysteine (Yamaguchi et al., 1998) that catalyzes O-acetylserine and hydrogen sulphide through  $\beta$ -replacement reaction (Bettati et al., 2000). This reaction causes acetic acid to release. Synthesis of cysteine occurred at three major parts of the plant such as mitochondria, chloroplast and cytosol (Singh, 1998; Gotor et al., 1997).

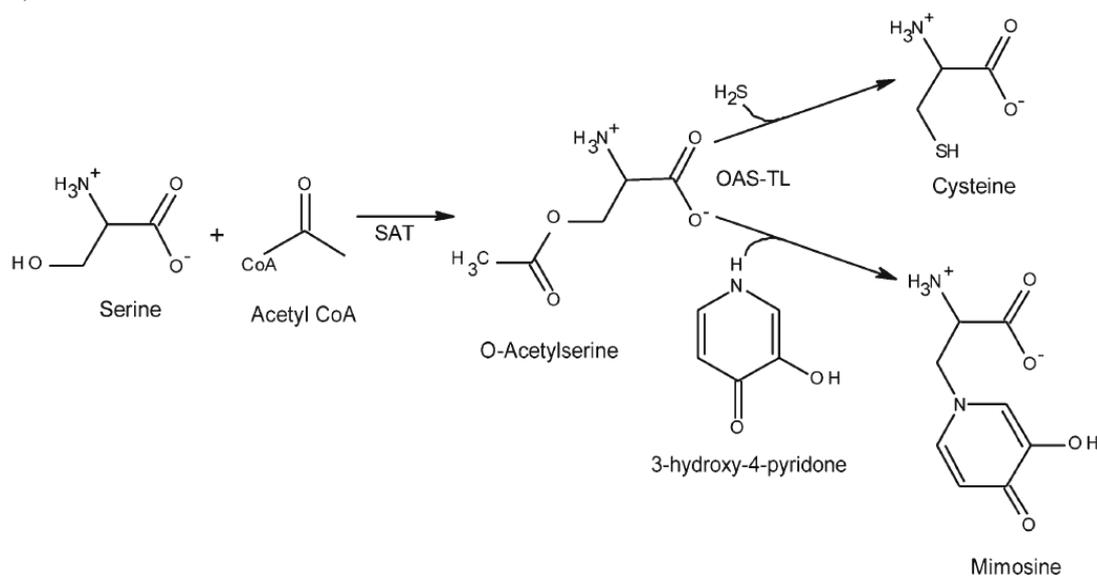


**Figure 2.3** Outline of cysteine biosynthesis in plant cell.

Adapted from *Plant Amino Acids: Biochemistry and Biotechnology* (p. 268), by B. K. Singh, 1998, CRC Press.

Pyridoxal 5'-phosphate (PLP) is a cofactor that binds with O-acetylserine forming amino acrylate (OAL-TL) as intermediate (Singh, 1998). Rolland et al. (1996) reported that, OAL-TL consist of two catalytically non-equivalent active site due to subunit-subunit interaction. Besides that, PLP-dependent enzyme can be classified into three groups,  $\alpha$ ,  $\beta$  and  $\gamma$  families according to their chemical characteristic and basis of sequence and structural features in five distinct fold types. Cysteine synthase has been classified in  $\beta$ -family and fold type II respectively (Bettati et al., 2000).

In addition,  $\beta$ -substitute alanines a nonprotein amino acid class is naturally produce by many different plant species form several of plant secondary metabolites including L-mimosine in *Leucaena leucocephala* and *Mimosa* species. Hence, understanding of the mimosine biosynthetic pathway is important to develop a mimosine-free and protein rich *L. leucocephala*. OAS-TL involves in both mimosine and cysteine biosynthesis in *L. leucocephala* and the two steps of cysteine biosynthesis is identical to mimosine biosynthesis except, a heterocyclic ring (3-hydroxy-4-pyridone(3H4P)) will be replaced with sulfide (Yafuso et al., 2014).



**Figure 2.4** Mimosine and cysteine biosynthetic pathway.

Adapted from "An O-Acetylserine (thiol) Lyase from *Leucaena leucocephala* is a cysteine synthase but not a mimosine synthase" by J. T. Yafuso et al., 2014, *Applied Biochemistry and Biotechnology* 173. Copyright 2014 by Springer Science+Business Media New York

## **3.0 MATERIALS AND METHODS**

### **3.1 Plant material**

Fresh young leaves tissues of *Leucaena leucocephala* were collected and stored at -80 °C in liquid nitrogen for DNA extraction.

### **3.2 DNA extraction and purification from *Leucaena leucocephala***

#### **3.2.1 Chemicals and reagents preparation**

The reagents that were used for DNA extraction and purification were liquid nitrogen, 2X CTAB buffer, 2%  $\beta$ -mercaptoethanol, chloroform-isoamyl alcohol, isopropanol, wash buffer, phenol-chloroform-isoamyl alcohol, 70% ethanol, ammonium acetate, 1M Tris-HCL, 0.5 EDTA and TAE buffer.

#### **3.2.2 Total genomic DNA isolation of *Leucaena leucocephala***

Young leaves of petai belalang tree were collected and cleaned the leaves surface with tap water to remove dust and foreign substance. Then, the bipinnate petai belalang leaves with approximately 1 g and pre-chilled pestle and mortar was used to grind the leaves with liquid nitrogen until powdery formed.

Next, the fine powder was transferred to 15/50 ml Falcon tube containing 6 ml of CTAB buffer and 120  $\mu$ l  $\beta$ -mercaptoethanol that was preheat in 60 °C water bath and incubated for 2 hours in 60 °C water bath. The tube was inverted in 10 minute interval. The slurry was allowed to cool at room temperature and equal volume of chloroform-isoamyl alcohol was added followed by mixing gently for 15 minutes. Then, the tube was centrifuged for 15 minutes at 4,000 rpm at room temperature forming a three layer aqueous phase. The aqueous phase was transferred into a clean 15/50 ml Falcon tube and equal volume of CIA was added and the mixing and centrifuged step was repeated. Again, the

aqueous layer was transferred into a new 15/50 ml Falcon tube and 2/3 volume of cold isopropanol was added, and gently mixed and was kept at -20 °C for overnight.

By centrifugation at 1,500 rpm for 15 minutes in room temperature, the nucleic acid was collected the next day. The supernatant was discarded and 1 ml of wash buffer was added for 1 hour. Then, the pellet was transferred into a clean 1.5 µl microcentrifuge tube together with the wash buffer. Centrifuged again for 10 minutes at 13,000 rpm and the supernatant was removed and allowed the pellet to air dry. After that, the pellet was dissolved with double distilled water and 3 µl of the DNA sample was run in a 0.8% agarose gel together with *Lambda-HindIII* and 100 bp.

### **3.2.3 DNA purification**

The microcentrifuge tube containing dissolve pellet was topped up to 600 µl of double distilled water and 3 µl of RNase A was added to each DNA sample and incubated for an hour at 37 °C in water bath. An equal volume of phenol-chloroform-isoamyl alcohol was added into the DNA sample, and the tube was inverted gently for 15 minutes followed by centrifugation at 13,000 rpm for 15 minutes. The aqueous phase was transferred into a clean microcentrifuge tube and equal volume of CIA was added, the tube was inverted gently for 15 minutes and centrifuged at 13,000 rpm for 15 minutes. The transfer of aqueous phase into a new microcentrifuge tube step was repeated and again the aqueous phase was transferred into a new 1.5 µl microcentrifuge tube. Two volumes of cold ethanol and ammonium acetate were added to a final concentration of 2.5 M. The tube was inverted gently for a few times and allowed to precipitate at -20 °C for overnight.

The next day, the DNA was recovered by centrifuged at 13,000 rpm for 15 minutes and the supernatant was poured off. The pellet was washed with cold 70% ethanol for at least 1 hour at -20°C. The tube was centrifuged again at 13,000 rpm for 15 minutes, the

supernatant was discarded and the pellet was allowed to air dried. Then, the pellet was re-suspended with double distilled water and store at -20 °C.

### **3.3 Agarose gel electrophoresis (AGE)**

The total DNA isolated from the petai belalang leaf was determined by running at 0.8% agarose gel with 2.5 µl of 100 bp (Solis BioDyne, Estonia), and mixing of 1 µl of loading dye into each 3 µl of DNA sample and 3 µl of *Lambda-HindIII* as a marker. After that, the gel was stained with ethidium bromide (EtBr) and de-stained in distilled water. Under UV transilluminator, the presence of DNA band was detected.

### **3.4 Agarose gel electrophoresis quantification of DNA**

By comparing the DNA band with the *Lambda-HindIII* marker (ThermoScientific, Lithuania), the concentration of DNA was determined. 1.05 µl of purified DNA sample was transferred into 1.5 µl microcentrifuge tube and 298.95 µl of ddH<sub>2</sub>O was added for dilution and stored at -20 °C.

### **3.5 Primer design**

Data mining of nucleotide sequence of cysteine synthase (*CSase*) gene from closely related species were obtained from NCBI GenBank database. Four types of plant species mRNA sequences such as *Glycine max*, *Solanum tuberosum* , *Citrullus lanatus* and *Arabidopsis thaliana* including *Leucaena leucocephala* were selected for multiple sequence alignment by using CLC sequence viewer 7.5 (CLC Bio, Denmark).

Based on the conserved regions of cysteine synthase genes from the multiple alignment result, the forward and reverse primers in 20 bp length were designed by Primer3web version 4.0.0 (<http://primer3.ut.ee/>). The parameters for the primer design such as GC

content, melting temperature, and primer length were adjusted before sent to Integrated DNA Technologies, Singapore, for primer synthesis.

### 3.6 PCR optimization

Gradient PCR was conducted using MasterCycler Gradient Thermal Cycler (Eppendorf, Germany) ranging from 41.4 °C to 63.4 °C. Along that, DNA and *Taq* DNA polymerase optimization was undergone to detect the cysteine gene. A total of 25 µl volume reaction was prepared in PCR tube and the PCR parameters and profile are shown as follows:

**Table 3.1** PCR reaction mixture

Reagent	Stock concentration	Final concentration	Final volume
ddH <sub>2</sub> O	-	-	
PCR buffer	10 x	1 x	2.5 µl
dNTP	2.0 mM	0.2 mM	2.0 µl
Forward primer	2.5 pmol/µl	5 pmol	2.0 µl
Reverse primer	2.5 pmol/µl	5 pmol	2.0 µl
Template DNA	100 ng/µl	500 ng/µl	5.0 µl
<i>Taq</i> DNA polymerase	0.5 U/µl	2.0 U/µl	4.0 µl
<b>Total</b>			25.0 µl

**Table 3.2** PCR profile

Stage	Temperature	Duration	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	94 °C	30 s	} 30
Annealing	52.4 °C	30 s	
Extension	72 °C	1 min	
Final extension	72 °C	10 min	1

Next, the amplified PCR products were examined by 1.5 % agarose gel together with 100 bp DNA ladder.

### **3.7 PCR product purification**

By using GeneJET Gel Extraction Kit (ThermoScientific, Lithuania), the PCR product was purified after undergo gel electrophoresis. An empty 1.5 µl microcentrifuge tube was weighed and the DNA band was excised from the gel under UV light and placed the gel sliced in the 1.5 µl microcentrifuge tube.

The gel sliced was weighed and 770 µl amount of membrane binding solution per 0.77 g of the gel sliced was added. Then, the gel was completely dissolved by incubated at 60 °C in water bath and let it cooled down at room temperature. 600 µl of the solution was transferred into the Minicolumn tube and centrifuged for 1 minute at 13,000 rpm, the step was repeated until all the solution was completely transferred. The supernatant was discarded and 100 µl of membrane binding buffer was added and left for 1 minute. Then, the Minicolumn tube was centrifuged again for 1 minute at 13,000 rpm. And the supernatant was removed. 700 µl of wash buffer was added and left for 1 minute followed by centrifugation at 13,000 rpm for 1 minute. The supernatant was discarded and the empty Minicolumn tube was centrifuged again to remove the wash buffer completely.

After that, the supernatant was discarded and the Minicolumn was reinserted into a collection tube. An amount of 30 µl of double distilled water was added and centrifuged again for 1 minute at 13,000 rpm. Next, the Minicolumn tube was removed and the collected DNA was stored at -20 °C.

### **3.8 Agarose gel electrophoresis quantification of PCR product (DNA)**

The purified DNA was determined by running a 1.5 % of agarose gel. A total of 1 µl of purified DNA was mixed with 1 µl of loading dye, 2.5 µl of 100 bp and 1 µl of *Lambda-HindIII* was used as a marker. The gel was run at 40 V and 50 A for 90 minutes. Then the gel was stained in ethidium bromide for a few seconds and de-stained in distilled water for