UNIVERSITI PUTRA MALAYSIA

SYSTEMATIC ANALYSIS OF A MONOTERPENE SYNTHASE FROM BANGUN-BANGUN (*Plectranthus amboinicus lour. spreng)*

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FBSB 2021 14

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

June 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Plectranthus amboinicus (Lour.) Spreng, is an aromatic medicinal herb noted for its therapeutic and nutritional properties attributed by the presence of terpenoids. To date, molecular research on terpenoids biosynthesis from this herb is still limited and the terpene synthases responsible for terpenoids production in *P. amboinicus* have yet to be described. Terpenoids have high demands in their applications as flavours and fragrances in food and cosmetics, and also as therapeutic agents in pharmaceutical products. Unfortunately, nature rarely produces these compounds in abundance. Hence, isolating the desired gene from the *P. amboinicus* for production of the terpenoid compounds in a suitable heterologous system poses an alternative way to overcome such limitations in the natural sources. The aim of this study was to isolate and characterise the *P. amboinicus* terpene synthase. Among the approaches involved are profiling of volatiles that contributed to the distinctive aroma of *P. amboinicus* conducted using GC-MS, accompanied by characterisation of the recombinant terpene synthase and terpenoids production in an *Escherichia coli* system, gene expression analysis *in planta* using quantitative real-time PCR and *in silico* structure prediction using homology modelling and protein docking. Volatiles profiling of *P. amboinicus* leaves revealed the presence of 46 % monoterpenes and 53 % sesquiterpenes comprising of α-bergamotene, carvacrol, caryophyllene, ρ-cymene, γ-terpinene and terpene alcohols. A putative monoterpene synthase coding sequences (designated as *PamTps1*) with an open reading frame of 1797 bp encoding a predicted protein of 598 amino acids with more than 60 % identity to known terpene synthases of Lamiaceae was isolated. A soluble recombinant *Pam*Tps1 with estimated size of ~79 kDa was successfully expressed *via* pET-32b(+) expression vector in *Escherichia coli* Rosetta™ 2(DE3) system at 28 °C with 0.5 mM IPTG induction. Enzyme characterisation revealed that the *Pam*Tps1 catalysed the conversion of geranyl pyrophosphate (GPP, C_{10}) and farnesyl pyrophosphate (FPP, C_{15}) into linalool and nerolidol, respectively. Biochemical properties of *Pam*Tps1 exhibited the

highest activity at an optimal pH and temperature of 6.5 and 30 °C, respectively, in the presence of 20 mM magnesium as a cofactor. The Michaelis-Menten constant (K_m) and catalytic efficiency (k_{cat}/K_m) were 16.72 ± 1.32 µM and 9.57 x 10^{-3} μM^{-1} s^{-1}, respectively showed that the *Pam*Tps1 had a higher binding affinity and specificity for GPP instead of FPP as expected for a monoterpene synthase. The recombinant *E. coli* harbouring *PamTps1* produced 13.6 ± 0.2 µg/ml and 10.6 ± 0.1 µg/ml linalool and nerolidol after 72 h and 24 h incubation, respectively. These outcomes validated the multi-substrate use of this enzyme in producing linalool and nerolidol, both in the *in vivo* and *in vitro* systems. Transcript expression analysis revealed that *Pam*Tps1 was up-regulated in leaves (42-fold of expression level) instead of stems which were associated with linalool emission following a diurnal rhythm regulated by circadian clock of the plant. Homology modelling of *Pam*Tps1 was predicted using BPPS (1N24) with 67 % sequence identity as a template. *Pam*Tps1 active site pocket analysis revealed nine aromatic residues (W268, Y272, Y299, F371, Y378, Y379, F447, Y517, and Y523) that defined the hydrophobic walls of the active site cavity and shaped the active site for proper substrate binding and folding. While residues from the RRx_8W motif, RxR motif, H-α1, and J-K loops formed the active site lid, protecting the highly reactive carbocationic intermediates from solvents. The dual-substrates used by *Pam*Tps1 were hypothesised due to the architecture and residues lining the catalytic site that can accommodate larger substrate (FPP) as demonstrated by protein modelling and docking analysis. The *Pam*Tps1 herein was identified as a linalool/nerolidol synthase, and this is the first study describing isolation and characterisation of such substrate promiscuity terpene synthase from the *P. amboinicus*. This study provides an initial insight into terpenoids biosynthesis in this herb that can be exploited for production of these natural products using metabolic engineering in both microbial and plant systems.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

ANALISIS SISTEMATIK MONOTERPENA SINTASE DARI POKOK BANGUN-BANGUN (*Plectranthus amboinicus* Lour. Spreng)

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Plectranthus amboinicus (Lour.) Spreng, dari keluarga Lamiaceae, merupakan herba aromatik yang mempunyai ciri-ciri terapeutik dan nilai pemakanan yang tinggi disebabkan kehadiran bahan terpenoid semulajadi. Sehingga kini, penyelidikan molekul mengenai biosintesis terpenoid dari genus ini masih terhad dan terpena sintase yang bertanggungjawab untuk penghasilan terpenoid oleh *P. amboinicus* masih belum dihuraikan. Terpenoid mempunyai permintaan pasaran yang tinggi sebagai perisa dan pewangi dalam makanan dan kosmetik, dan juga sebagai agen terapeutik dalam produk farmaseutikal. Malangnya, alam semula jadi jarang menghasilkan bahan ini dengan banyak. Oleh itu, pemencilan gen dari *P. amboinicus* bagi tujuan penghasilan terpenoid dalam sistem heterologus yang sesuai merupakan kaedah alternatif bagi mengatasi keterbatasan tersebut dalam sumber semula jadi. Tujuan kajian ini dijalankan adalah untuk memencilkan dan pencirian terpena sintase dari *P. amboinicus*.
Di antara pendekatan yang digunakan adalah memprofil volatil yang menyumbang kepada aroma pokok *P. amboinicus* menggunakan kaedah GC-MS, disertai dengan pencirian fungsi terpena sintase melalui ekspresi protein rekombinan dalam sistem *Escherichia coli*, analisis ekspresi gen di dalam tumbuhan menggunakan kaedah kuantitatif tindak balas berantai polimerase masa nyata dan meramal model protein dengan menggunakan pemodelan homologi dan dok protein secara dalam silico. Profil volatil daun *P. amboinicus* menunjukkan kehadiran 46 % monoterpena dan 53 % sesquiterpena yang terdiri daripada α-bergamotena, karvakrol, kariofilena, ρ-simena, γ-terpinena, humulena dan alkohol terpena. Gen monoterpena sintase (ditetapkan sebagai *PamTps1*) yang mempunyai jujukan nukleotida 1797 bp dan mengekodkan protein sebanyak 598 asid amino dengan identiti persamaan lebih dari 60 % terhadap terpena sintase dari Lamiaceae berjaya dipencilkan. *Pam*Tps1 rekombinan dengan anggaran saiz ~79 kDa berjaya diekspreskan dengan menggunakan sistem ekspresi pET-32b(+) pada suhu 28 °C dengan induksi IPTG pada kepekatan 0.5 mM. Pencirian enzim menunjukkan bahawa *Pam*Tps1

memangkinkan penukaran geranil pirofosfat (GPP, C_{10}) dan farnesil pirofostat (FPP, C_{15}) kepada linalool dan nerolidol. Pencirian biokimia *Pam*Tps1 menunjukkan aktiviti tertinggi pada pH 6.5 dan suhu optimum 30°C, dengan kehadiran 20 mM magnesium sebagai kofaktor. Pemalar Michaelis-Menten (K_m) dan kecekapan pemangkin (k_{cat}/K_m) adalah sebanyak 16.72 ± 1.32 µM dan 9.57 x 10-3 µM-1 s-1, masing-masing, menunjukkan bahawa *Pam*Tps1 mempunyai kadar pengikatan dan kecekapan pemangkin yang lebih tinggi terhadap GPP, seperti yang diramalkan untuk monoterpena sintase. *E. coli* rekombinan yang membawa gen *PamTps1* berupaya menghasilkan 13.6 ± 0.2 µg / ml dan 10.6 ± 0.1 µg / ml linalool dan neroliol setelah 72 jam dan 24 jam inkubasi. Keputusan ini mengesahkan kebolehanan enzim ini yang menggunakan dwi substrat dalam menghasilkan linalool dan nerolidol baik dalam sistem *in vivo* dan juga *in vitro*. Analisis pengekspresan gen menunjukkan bahawa *PamTps1* dihasilkan lebih tinggi pada daun berbanding batang (tingkatan pengkspresan 42 kali ganda) yang dikaitkan dengan pelepasan linalool mengikut irama diurnal yang diatur oleh rentak sirkadian tanaman ini. Pemodelan homologi *Pam*Tps1 diramalkan dengan menggunakan BPPS (1N24) yang mempunyai persamaan identiti sebanyak 67 % sebagai templat. Analisis poket tapak aktif *Pam*Tps1 telah mengenal pasti sembilan residu aromatik (W268, Y272, Y299, F371, Y378, Y379, F447, Y517 dan Y523) yang menggariskan dinding hidrofobik tapak aktif, dan membentuk laman aktif untuk pengikatan dan pelipatan substrat yang betul. Sementara residu dari motif RRx8W, RxR, gegelung H-α1 dan J-K membentuk penutup tapak aktif yang melindungi perantara yang sangat reaktif dari pelarut. Kebolehan *Pam*Tps1 untuk menggunakan pelbagai substrat dianggarkan kerana tapak aktifnya yang dapat menampung substrat yang lebih besar (FPP) berdasarkan seni bina protein dan residu yang melapisi tapak aktif, seperti yang ditunjukkan oleh model protein dan analisis dok. *Pam*Tps1 dikenalpasti sebagai enzim linalool/nerolidol sintase, dan ini merupakan kajian pertama yang menerangkan pemencilan dan pencirian terpena sintase dari *P. amboinicus*. Kajian ini memberikan maklumat awal mengenai biosintesis terpenoids dalam herba ini dan ianya mempunyai potensi besar untuk dimanfaatkan bagi penghasilan produk semula jadi menggunakan teknik kejuruteraan metabolik dalam sistem mikrob dan tumbuhan.

ACKNOWLEDGEMENTS

In the name of Allah, the most Gracious, the most Merciful. First and foremost, I would like to thank God for bringing me to this path and for giving me the strength and perseverance to finally completing this long journey towards pursuit of knowledge.

I would like to express my gratitude and appreciation to my supervisor, Assoc. Prof. Dr. Janna Ong Abdullah for her encouragement and guidance throughout my study. Thank you for giving me your full trust in carrying out this research under your supervision. To all my supervisory committee members, Prof. Dr. Raha Abdul Rahim, Assoc. Prof. Dr. Suriana Sabri, and Assoc. Prof. Dr. Lai Kok Song, thank you for your guidance and valuable advices.

A word of thank you cannot fully describe my gratitude to my parents and siblings who have been there and supported me throughout this journey. Thank you for all the encouragement and prayers that accompanied me along this journey. To all my friends at Malaysia Genome Institute (MGI) and Universiti Putra Malaysia (UPM), thank you for sharing this journey with me and lending me a helping hand and ear by listening to my rent when I faced with difficulty. It always works like a charm talking to friends to find a solution to my problems.

My appreciation to the Ministry of Higher Education (MOHE) for MyPhD scholarship and FRGS grant (FRGS/2/2014/STWN10/UPM/02/1), and Universiti Putra Malaysia for Putra Grant (GP-IPS/2015/9463800). Thank you.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABREVIATIONS

3'-UTR	3'-Untranslated region
5'-UTR	5'-Untranslated region
AACT	Acetoacetyl-CoA thiolase
APS	Ammonium persulfate
bLins	Bacterial linalool/nerolidol synthase
bp	Base pair
BPPS	Bornyl diphosphate synthase
CAI	Codon adaptation index
CASTp	Computed Atlas of Surface Topography of Proteins
cDNA	Complementary DNA
CMK	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DomPRED	Protein Domain Prediction server
DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
E	Amplification efficiency
EDTA	Ethylenediaminetetraacetic acid
FPP	Farnesyl diphosphate/pyrophosphate
FPPS	Farnesyl diphosphate synthase
GAP	Glyceraldehydes-3 phosphate
GC-MS	Gas Chromatography-Mass Spectrometry
GF	Gel filtration chromatography

GGPP	Geranylgeranyl diphosphate
GGPPS	Geranylgeranyl diphosphate synthase
GMQE	Global Model Quality Estimation
GPP	Geranyl diphosphate/pyrophosphate
GPPS	Geranyl diphosphate synthase
HDR	4-hydroxy-3-methylbut-2-enyl-diphosphate reductase
HDS	4-hydroxy-3-methylbut-2-enyl-diphosphate synthase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
HS-SPME	Headspace-solid phase microextraction
IDI	Isopentenyl diphosphate isomerase
IMAC	Immobilised metal affinity chromatography
IPP	Isopentenyl diphosphate
IPTG	Isopropyl β- D-1-thiogalactopyranoside
kb	Kilo base pair
k_{cat}	Turnover number
k_{cat}/K_m	Catalytic efficiencies
kDa	Kilodalton
K_m	Michaelis-Menten constant
LB	Luria-Bertani
MCT	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
MDS	2C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase
MEGA	Molecular Evolution Genetic Analysis
MEP	Methylerythritol phosphate pathway
MES	2-(N-Morpholino) ethanesulfonic acid

MOPSO	3-Morpholino-2-hydroxypropanesulfonic acid
MVA	Mevalonate pathway
MVD	Mevalonate diphosphate decarboxylase
MVK	Mevalonate kinase
MW	Molecular weight
NRT	No-reverse transcription control
NTC	No-template control
ORF	Open reading frame
PamTps1	*Plectranthus amboinicus* terpene synthase 1 (gene)
*Pam*Tps1	*Plectranthus amboinicus* terpene synthase 1 (protein)
PCR	Polymerase chain reaction
PHYLIP	PHYLogeny Inference Package
PMK	Phosphomevalonate kinase
PPi	Pyrophosphate moiety
ProSA	Protein Structure Analysis server
PSIPRED	Protein Structure Prediction server
QMEAN	Qualitative Model Energy Analysis
qPCR	Quantitative real-time PCR
R^2	Correlation coefficient
RACE PCR	Rapid amplification of cDNA ends PCR
RMSD	Root mean square deviations
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
SAVES	Structural Analysis and Verification Server
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TCP	Total cell protein
TD PCR	Touchdown PCR
TPS	Terpene synthases
TQ	Thymoquinone
V_{max}	Maximum catalytic rate

CHAPTER 1

INTRODUCTION

1.1 Introduction

Terpenoids are the most abundant and structurally diverse natural products present in many plants with a wide array of applications in the food and pharmaceutical industries. Among the terpenoids, monoterpenes are associated with the conspicuous aromatic properties with a wide range of biological activities. The monoterpenes are known to possess antioxidant, antifungal, antiviral, anti-diabetic, anti-inflammatory and hepatoprotective activities (Ashrafizadeh et al., 2020; Wojtunik-Kulesza et al., 2019). Besides, the monoterpenes in essential oils also exhibited sedative effects as used in aromatherapy for a holistic healing treatment after having to cope with a busy and stressful lifestyle (Ali et al., 2015).

Plectranthus amboinicus or known as *Pokok Bangun-bangun* or *Ati-ati Hijau* in Malaysia, is one of the most sought out herbs owing to its numerous biological activities such as anti-inflammatory, anti-microbial, antineoplastic, larvicidal, analgesic and wound healing (Arumugam et al., 2016; Kumar et al., 2020). These therapeutic properties are said to be attributed by the phytochemical constituents mainly monoterpenes and sesquiterpenes that are present in this plant. Thus, this makes *P. amboinicus* an excellent source of terpenoids for scientific study.

While plants are the major sources of terpenoids, these valuable compounds are presented only at low amounts and are rarely sufficient for large-scale industrial application (Vickers et al., 2014). Furthermore, extraction from plants grown by traditional farming practices is risky as it is affected by geographical and seasonal variations, political intervention, natural catastrophes and crop diseases (Atanasov et al., 2015; Caputi and Aprea, 2011). The aforementioned factors subsequently affect quality and yield of the products, and do not guarantee a constant products supply. This might then lead to fluctuations in price of the raw materials and ultimately affects the cost of the products (Caputi and Aprea, 2012). Therefore, production of terpenoids through biotechnological processes is seen as a promising alternative towards sustainability by introducing the key enzyme and biosynthesis pathway into a heterologous host such as the microbial systems.

In this project, isolation of full-length monoterpene synthase coding sequences was conducted followed by cloning and heterologous expression in an *Escherichia coli* system. The recombinant monoterpene synthase was

characterised to determine functionality and identity of the enzymatic product and its biochemical properties. Microbial production of terpenoids in an *E. coli* system was conducted to demonstrate the ability of the recombinant clone to produce terpenoids in a bench scale system. This study also investigated the expression pattern of the isolated transcript in different plant organs at different day/night conditions in correlation with the terpenoids formation. For better understanding of the structural basis of this plant terpene synthase, a three-dimensional homology model representing the *P. amboinicus* terpene synthase and docking pose of the substrate(s) in the active site was predicted to identify the key residues involved in the catalytic reaction.

1.2 Problem statement

Numerous researches on terpene synthases have focused on plants of economic importance such as lavender, mint, oregano and marjoram, thus leaving many enzymes that have yet to be explored. Until now, there has been no molecular research on terpenoids biosynthesis from *P. amboinicus*. The lack of knowledge of the corresponding terpene synthases responsible for terpenoid productions in *P. amboinicus* hampers further scientific investigation into the molecular mechanism that determines composition of the volatile terpenoids. In addition, the lack of 3-dimensional structure of *P. amboinicus* terpene synthase hindered understanding of the catalytic mechanism of this enzyme.

1.3 Objectives

The general objective of this study was to isolate and characterised the terpene synthase from a local herb, *Plectranthus amboinicus*. The specific objectives were:

1. To analyse the volatiles of *P. amboinicus* leaf and stem tissues under different day/night regimes
2. To characterise the *P. amboinicus* monoterpene synthase
3. To evaluate the correlation between expression of *P. amboinicus* monoterpene synthase and its enzymatic product(s) emission *in planta*
4. To identify functional residues involved in the protein-ligand interaction and catalysis

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BIODATA OF STUDENT

Nur Suhanawati binti Ashaari was born on 21st December 1982 in Ipoh, Perak, Malaysia. She acquired her primary education at SK Tepus, Perak and obtained her secondary education in SMK Raja Permaisuri Bainun, Ipoh and MRSM Balik Pulau, Pulau Pinang. She graduated from Universiti Teknologi Malaysia (UTM) with a Bachelor of Science in Industrial Biology, which she completed in 2004. Upon completion of her first degree, she also earned a Master's degree in Bioscience from UTM in 2010. After completion of her Master's degree, she joined Universiti Kebangsaan Malaysia (UKM) and later Malaysia Genome Institute (MGI) as a research scientist. Her interest in science and scientific experiments led her to pursue her Doctor of Philosophy in the area of molecular biology and genetic engineering while still maintaining her full-time job at the Malaysia Genome Institute.

LIST OF PUBLICATIONS

Peer-reviewed journal

Nur Suhanawati Ashaari, Mohd-Hairul Ab. Rahim, Suriana Sabri, Kok Song Lai, Adelene Ai-Lian Song, Raha Abdul Rahim, Wan Muhamad Asrul Nizam Wan Abdullah, Janna Ong Abdullah (2021). Kinetic studies and homology modeling of a dual-substrate linalool/nerolidol synthase from *Plectranthus amboinicus. Scientific Reports,* 11, 17094.

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Nur Suhanawati Ashaari, Mohd Hairul Ab. Rahim, Suriana Sabri, Lai Kok Song, Adelene Song Ai Lian, Raha Abdul Rahim, Janna Ong Abdullah. Molecular cloning and sequence characterization of a putative monoterpene synthase gene from Bangun-bangun plant (*Plectranthus amboinicus*), ASIA International Multidisciplinary Conference (AIMC 2017), 1-2 May 2017, UTM, Johor Baharu, Johor.

Patent filing

Janna Ong Abdullah, **Nur Suhanawati Ashaari**, Raha Abdul Rahim, Suriana Sabri, Lai Kok Song, Adelene Song Ai-Lian and Mohd Hairul Ab. Rahim. Recombinant Terpene Synthase. Patent Application PI 2019000052, filed 14 January 2019.