UNIVERSITI PUTRA MALAYSIA

ENGINEERING Lactococcus lactis AS CELL FACTORY FOR THE PRODUCTION OF PLANT LIMONENE

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By

NURUL `AISHAH BINTI SHAILI

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

October 2019

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DEDICATION

This work is humbly dedicated to my late mother, Pn Jamiah Kordi, who passed away on 22nd Nov 2019. You are my source of inspiration, you taught me to persevere and prepared to face the challenges with faith and humility. You have been with me every single step of this tough journey. It hurts to think that you are not here anymore but I always feel your presence to motivate me to strive for my goals in life. Thank you for your unconditional love, sacrifices, prayers, and advices. You will always be in my heart and prayers.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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October 2019

Chairman : Professor Raha binti Abdul Rahim, PhD
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Limonene and perillyl alcohol (POH) are plant monoterpenes which give a significant contribution to the aroma of most essential oils due to its pleasant fragrance. Both compounds are synthesized via two pathways known as the mevalonate or 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway by metabolizing geranyl pyrophosphate (GPP) substrate. These compounds have been studied to exhibit an anti-cancer effect against several types of cancer including colorectal cancer. However, isoprenoid metabolic engineering work has been mostly studied in *Escherichia coli.* To date there is no report on limonene and POH production in *Lactococcus lactis*. In this study, *L. lactis* was developed as a heterologous host for the production of limonene using the recombinant *L. lactis* as an oral delivery for anti-cancer compounds against colorectal cancer in the future. Limonene synthase (LS) and cytochrome P450 alkane hydroxylase (*ahpGHI*) genes were cloned and expressed in *L. lactis* NZ9000 host. Western blot analysis using mouse IgG His-Tag monoclonal antibody revealed a successful LS expression by *L. lactis* with the size of ~56 kDa. The expression of *ahpGHI* gene by *L. lactis* was not detected by Western blot despite induction time up to 48 h. According to RT-PCR analysis, the *ahpGHI* gene was transcribed only after 24 h post induction, although protein expression of ahpGHI gene remained unresolved. GC-MS analysis result showed that limonene production was optimum after 24 h post induction. However, production of limonene was still low (~4.0 ppm) and did not reach the LD_{50} value required to inhibit the proliferation of cancer cells. Therefore metabolic engineering was attempted to increase the production of limonene by introducing the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and *mvk* genes in the bacterial host. There are two types of HMGR that was

used which are *mvaA* gene from *L. lactis* and *HMG1* from baker's yeast. The constructs containing *mvaA* and *mvk* genes from *L. lactis* was described as pNZ:LSMM plasmid, whereas the one from baker's yeast written as pNZ:LS:*HMG1* plasmid. The recombinant *L. lactis* carrying pNZ:LSMM plasmid successfully enhanced the limonene production over three folds (4.9-15.1 ppm) after 24 h of induction. Trypan blue exclusion test was conducted to investigate the viability of SW480 colorectal cancer cell after treatment with recombinant *L. lactis* producing limonene. From the result it showed that the cell viability was decreased in dose-dependent manner with the highest inhibition effect observed at 29.2% after 4 h of treatment at MOI 1500:1. The outcomes of this study shed light on the potential of food grade *L. lactis* for plant proteins and bioactive compounds production which prospectively leads to a delivery system for anti-cancer compounds.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

KEJURUTERAAN *Lactococcus lactis* SEBAGAI KILANG SEL UNTUK PENGHASILAN LIMONENE TUMBUHAN

Oleh

NURUL 'AISHAH BINTI SHAILI

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Pengerusi : Profesor Raha binti Abdul Rahim, PhD
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Limonene dan perillil alcohol (POH) adalah monoterpene tumbuhan yang menyumbang secara signifikan kepada aroma minyak pati kerana harumannya yang menyenangkan. Kedua-dua sebatian ini dihasilkan melalui dua laluan iaitu laluan mevalonate atau 2-C-metil-D-eritritol-4-fosfat (MEP) dengan memetabolisasi geranyl pirofosfate (GPP). Sebatian-sebatian ini juga telah dilaporkan mempunyai sifat anti-kanser terhadap beberapa jenis kanser termasuk kanser kolorektal. Walau bagaimanapun, kerja-kerja kejuruteraan metabolik isoprenoid banyak dikaji dalam *Escherichia coli.* Sehingga kini tidak ada laporan yang diterbitkan mengenai penghasilan limonene dan POH dalam *Lactococcus lactis*. Dalam kajian ini, *L. lactis* telah dibangunkan sebagai hos heterologus untuk penghasilan limonene dengan menggunakan *L. lactis* rekombinan sebagai penghantaran secara oral untuk sebatian anti-kanser terhadap kanser kolorektal. Gen limonene synthase (LS) dan cytochrome P450 alkane hydroxylase (*ahpGHI*) telah diklon dan diekspreskan ke dalam hos *L. lactis* NZ9000. Analisis Western blot menggunakan IgG His-Tag antibodi monoklonal tikus berjaya mengekspreskan LS gen dengan saiz protein ~56 kDa. Ekspresi gen *ahpGHI* oleh *L. lactis* tidak dapat dikesan oleh Western blot walaupun dengan masa induksi sehingga 48 jam. Menurut analisis RT-PCR, gen *ahpGHI* hanya ditranskripsikan selepas 24 jam induksi, walaupun ekspresi protein masih tidak dapat diselesaikan. Hasil analisa GC-MS menunjukkan penghasilan limonene adalah optimum selepas 24 jam induksi. Walau bagaimanapun, penghasilan limonene masih rendah (~4.0 ppm) dan tidak mencapai nilai LD_{50} yang diperlukan untuk menghalang percambahan sel kanser. Oleh itu, kejuruteraan metabolik dilakukan untuk meningkatkan penghasilan limonene dengan memasukkan 3-hydroxy-3-methylglutaryl

coenzyme A reductase (HMGR) dan *mvk* gen ke dalam hos bakteria. Terdapat dua jenis HMGR yang digunakan iaitu gen *mvaA* dari *L. lactis* dan *HMG1* dari yis pembuat roti. Konstruk yang mengandungi gen *mvaA* dan *mvk* dari *L. lactis* digambarkan sebagai pNZ:LSMM, manakala yang dari yis pembuat roti ditulis sebagai pNZ:LS:*HMG1*. *L. lactis* rekombinan yang mengandungi pNZ:LSMM berjaya meningkatkan pengeluaran limonene melebihi tiga lipatan (4.9-15.1 ppm) selepas 24 jam induksi. Ujian trypan blue exclusion telah dilakukan untuk mengkaji daya tahan sel kanser kolorektal SW480 selepas dirawat dengan *L. lactis* rekombinan yang menghasilkan limonene. Keputusan menunjukkan penurunan daya tahan sel dalam cara yang bergantung kepada dos dengan kesan perencatan yang paling tinggi diperhatikan pada 29.2% selepas 4 jam rawatan di MOI 1500: 1. Hasil kajian ini menunjukkan potensi *L. lactis* gred makanan untuk menghasilkan protein tumbuhan dan sebatian bioaktif yang secara prospektif membawa kepada sistem penghantaran untuk sebatian anti-kanser.

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

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LIST OF ABBREVIATIONS

~	Approximately
µF	Capacitance
µl	Microlitre
µM	Micromolar
atoB	Acetoacetyl-CoA synthase
bp	Base pair
BSA	Bovine Serum Albumin
BVOE	Blood orange volatile oil emulsion
$CaCl_2$	Calcium chloride
CYP450	Cytochrome P450
DAB	3,3′-diaminobenzidine
dH_2O	Distilled water
DMAPP	Dimethylallyl pyrophosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleotide acid
dNTPs	Deoxyribonucleotide triphosphates
DXP	Deoxyxylulose-5-phosphate
FaNES	Linalool/nerolidol synthase
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
GC-MS	Gas chromatography-mass spectrometry
GGPP	Geranyl geranyl pyrophosphate
GM17	M17 supplemented with 0.5% glucose
GPP	Geranyl pyrophosphate
GPPS	Geranyl pyrophosphate synthase
GRAS	Generally recognized as safe
h	Hour
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase

HMGS	3-hydroxy-3-methylglutaryl coenzyme A synthase
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
idi	Isopentenyl diphosphate isomerase
IE	Ionization voltage
IFN-γ	Interferon gamma
IL-	Interleukin
IPP	Isopentyl pyrophosphate
kb	Kilo base pairs
kDa	Kilodalton
kV	Kilovolt
L	Litre
LAB	Lactic acid bacteria
LcS	*L. casei* Shirota
LPS	Lipopolysaccharides
LS	Limonene synthase
M	Molar
MCS	multiple cloning site
MEP	2-C-methyl-D-erythritol-4-phospahte/ Non-mevalonate
mg	Milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
min	Minute
ml	Mililitre
mm	Milimetre
mM	Milimolar
MMP	Matrix metalloprotease
Mn^{2+}	Manganese
MVA	Mevalonate
mvk	Mevalonate kinase
NCBI	National Center for Biotechnology Information

ng	Nanogram
NICE	Nisin induced expression system
nM	Nanomolar
^{o}C	Degree celsius
OD	Optical density
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline-tween 20
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PI3K	Phosphoinositide 3-kinase
PMD	Phosphomevalonate decarboxylase
PMK	Phosphomevalonate kinase
POH	Perillyl alcohol
ppm	Part per million
PVDF	Polyvinyllidene difluoride
RE	Restriction endonuclease
RNA	Ribonucleotide acid
ROS	Reactive oxygen species
RT	Retention time
s	Seconds
SAAT	Alcohol acyltransferase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGM17	M17 supplemented with 0.5% glucose and 0.5M sucrose
SPME	Solid-phase microextraction
TAE	Tris-acetate, EDTA
TGS	Tris-glycine-SDS
TNF-α	Tumor necrosis factor alpha
TTFC	Tetanus toxin fragment C
V	Volt
v/v	Volume per volume

VEGF	Vascular endothelial growth factor
VMPSTS	Vanda mimi palmer sesquiterpene synthase
w/v	Weight per volume
YPD	Yeast extract-peptone-dextrose
µg	Microgram
Ω	Resistance

CHAPTER 1

INTRODUCTION

Isoprenoids are the largest class of natural compounds that possess attractive characteristics such as flavour, fragrance and medically active compounds. Two metabolic pathways known as mevalonate (MVA) and 2-C-methyl-D-erythritol-4-phospahte (MEP) are utilized by living organism for synthesizing isoprenoids through the universal precursors' isopentyl diphosphate (IPP) and dimethylallyl pyrophospahte (DMAPP). These compounds are mainly present in plants and the extraction from plant material remains as the main production mode. However, this natural extraction is no longer efficient in order to meet the expanding demand of many isoprenoids due to high cost and slow growth rate. The best strategy to overcome this challenge is through engineering of microbial host for heterologous isoprenoids production. Microbes are ideal hosts for isoprenoids production, because of their fast growth rate; require minimal resources, and naturally synthesizing the building blocks of IPP and DMAPP. Therefore, the production of plant isoprenoids in microbial cell factory is of great advantage as many genetic tools are also available for microbial engineering. Previous studies also had reported the usage of microbial hosts for the production of isoprenoids as they dominate most of the enzymes involved in the metabolic pathways for isoprenoids production (Jongedijk *et al.,* 2016; Niu *et al.,* 2017; Ward *et al.,* 2018)

Lactococcus lactis is a Gram positive lactic acid bacterium that has been used extensively in the fermentation of food products. Over the last two decades, this bacterium has gained interest due to its ability as a heterologous host for bioactive compounds production such as enzymes, peptides and vaccine antigens. *L. lactis* is also recognized as one of the bacterium that has been categorized as GRAS (Generally Regarded as Safe) due to its non-pathogenic and non-invasive characteristics. *L. lactis* is also known as non-colonizing bacteria which make it suitable as delivery vehicle to carry therapeutic agents into the gastrointestinal tract. Apart from being food-grade, this Gram-positive bacterium utilizes the mevalonate pathway for isoprenoid production unlike other prokaryotes that uses the MEP pathway. Presently, most isoprenoid metabolic engineering works have been done in *Escherichia coli* which is no longer a suitable host for further treatment in human as this strain produces endotoxin. Furthermore, *L. lactis* also possess an efficient expression system named as Nisin Induced Expression System (NICE), where the expression of desired gene located downstream of the nisin promoter is induced by the addition of nisin.

In this study, limonene and perillyl alcohol from the monoterpene group (C_{10} isoprenoid), with reported anti-proliferative properties were targeted for production in *L. Lactis* as our candidate compounds against colorectal cancer cells. Limonene is synthesized by limonene synthase by metabolizing the geranyl pyrophosphate (GPP) substrate which can be found naturally in all organisms including *L. lactis*. Meanwhile, perillyl alcohol (POH) is the oxidation product of limonene by cytochrome P450 hydroxylase. Metabolic engineering was performed to improve the production level of limonene by introducing 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). HMGR is knows as the rate-limiting enzyme for the mevalonate pathway and is one of the most finely regulated enzymes which convert HMG-Co to mevalonate. The third key enzyme of the mevalonate pathway is mevalonate kinase that converts mevalonate into mevalonate-5-phosphate. The high amount of *mvaA* associates with the high yield of mevalonate by HMGR which is then used as substrate for *mvk* to increased the desired amount of monoterpene (Song *et al.,* 2014).

Following the gene expression study, the cytotoxicity of recombinant *L. lactis* producing limonene against the cell viability SW480 colorectal cancer cell lines were tested by trypan blue exclusion assay. To the best of our knowledge, to date there is no oral delivery treatment available using live recombinant *L. lactis* producing limonene targeting colorectal cancer.

Thus, the general objective of this study is to produce biosynthetic limonene using *L. lactis* and the specific objectives are:

a) to express LS gene and improve the production level of limonene by metabolic engineering *L. lactis* NZ9000,
b) to validate the production of limonene by GC-MS analysis, and
c) to test the toxicity effect of recombinant *L. lactis* producing limonene against SW480 colorectal cancer cell line

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PUBLICATION

Poster presentations:

Nurul 'Aishah Shaili, Adelene Song Ai Lian, Siti Sarah Othman, Lionel In Lian Aun, and Raha Abdul Rahim. Engineering *Lactococcus lactis* as cell factory for the production of plant anti-cancer compounds, limonene and perillyl alcohol. International Congress of Malaysian Society for Microbiology, 7-9 December 2015, Penang, Malaysia.

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