PRELIMINARY ANALYSIS OF LINALOOL EXTRACTED FROM *Aspergillus*- FERMENTED PINEAPPLE WASTES TOWARDS *Staphylococcus aureus*

LEE CHEE YAN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTH AND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2016

NON-PLAGIARISM DECLARATION

By this letter I declare that I have written this thesis completely by myself, and that I have used no other sources or resources than the ones mentioned.

I have indicated all quotes and citations that were literally taken from publications, or that were in close accordance with the meaning of those publications, as such. All sources and other resources used are stated in the references.

Moreover I have not handed in a thesis similar in contents elsewhere.

In case of proof that the thesis has not been constructed in accordance with this declaration, the Faculty of Health and Life Sciences has the right to consider the research thesis as a deliberate act that has been aimed at making correct judgment of the candidate's expertise, insights and skills impossible.

I acknowledge that the assessor of this item may, for the purpose of assessing this item,

- reproduce this assessment item and provide a copy to another member of the University; and/or,
- communicate a copy of this assessment item to a plagiarism checking service (which may then retain a copy of the assessment item on its database for the purpose of future plagiarism checking).

In case of plagiarism the examiner has the right to fail me and take action as prescribed by the rules regarding Academic Misconduct practiced by INTI International University.

LEE CHEE YAN	
Name	Signature
I13003103	16/12/2016
I.D.Number	Date

DECLARATION

I hereby declare that the work in this proposal is my own except for quotations and summaries which have been duly acknowledged, and completed under the supervision of Ms. Emily Quek Ming Poh.

LEE CHEE YAN	MS. EMILY QUEK MING POH
I13003103	(SUPERVISOR)

ACKNOWLEDGEMENT

Firstly, I would like to show my appreciation to my supervisor Miss Emily Quek Ming Poh who showed her patience and support throughout my final year project. Guidance from her was much appreciated when I faced doubts while conducting my study and dissertation writing. Specially gratitude to lecturers, laboratory assistances and friends from INTI International University Faculty of Health and Life Sciences who helped me when I need especially Dr Geeta Selvarajah, Miss Quah Hui Hsien, Mr Ng Peng Wah and Miss Abegal Then. Without help and moral support from them, my final year project might not be completed successfully. Lastly, encouragement from both my parents are much appreciated especially when I feel discouraged while carrying out the project.

ABSTRACT

Linalool is a monoterpene used widely in fragrance and household products industries. Linalool also has anti-microbial activity against *Staphylococcus aureus*. This study aimed to determine the optimal fermentation duration for both blended core and crown of Josapine using *Aspergillus brasiliensis*, to quantify the linalool content in the *Aspergillus*-fermented blended core and crown of Josapine, and to screen the anti-microbial potential of linalool extracted from both fermented blended core and crown of Josapine towards *S. aureus*. Production of linalool in this study was obtained through the fermentation of Josapine core and crown for three fermentation periods namely, Day-4, Day-8 and Day-12. After fermentation, the fermentation broths of the blended core and crown were extracted for linalool using dichloromethane. The crude extracts of the fermented core and crown were separated using thin layer chromatography (TLC) and mobile phase methanol-ethylacetate. The presence of linalool in the isolated extracts were detected and visualized with phosphomolybdic acid (PMA) dye. Then, the absorbance of TLC-isolated linalool was measured at 300 nm using UV-spectrophotometer. The *S. aureus* culture was used to screen anti-microbial activity of TLC-isolated linalool. Fermentation using the blended core and crown showed significant differences in yielding linalool on Day-4 and Day-8 based on independent samples *t*-test. The concentration of linalool extracted from non-fermented blended crown and core were 21.548 mg/mL and 14.964 mg/mL respectively. Linalool concentration extracted from Day-4 fermented core and crown were 24.560 mg/mL and 15.773 mg/mL respectively. On Day-8, 1.481 mg/mL and 11.663 mg/mL linalool were extracted from respective fermented core and crown. The anti-microbial activity screening using TLC-isolated linalool from both fermented blended crown and core showed no inhibition of the growth for *S. aureus*. As a conclusion, Day-12 of fermentation was not chosen for linalool extraction as the percentage of viability lower was than Day-0. The linalool concentration extracted from the fermented blended core on Day-4 was found highest compared to non-fermented blended core while continuous decrement found in crown. Hence, crown was found not desirable to produce linalool through fermentation compared to core. Anti-microbial activity of linalool was proven using linalool standard (6 mg/mL) but not TLC-isolated linalool as the reactivity of linalool reduced due to the bound PMA.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{300}	Absorbance at 300 nm
ANOVA	One-way analysis of variance
A.brasiliensis	*Aspergiluus brasiliensis*
A. niger	*Aspergillus niger*
ATCC	American Type Culture Collection
CFU/mL	Colony forming unit/ millilitre
cm	centimetre
$C_{10}H_{18}O$	Chemical formula of linalool
Degree Celsius	°C
FDA	Food and Drug Administartion
g	gram
GRAS	Generally Recognized as Safe
hrs	hours
L	litre
LB	Luria-Bertani
MARDI	Malaysian Agriculture Research and Development Institute
mg/mL	milligram/millilitre
µL	microlitre
mL	millilitre
mm	millimetre
mins	minutes
MPIB	Malaysian Pineapple Industry Board
nm	nanometer
PBS	Phosphate buffered saline

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pH	Potential of hydrogen
PMA	Phosphomolybdic acid
R_f	Rate of flow
rpm	Revolutions per minute
S. aureus	*Staphylococcus aureus*
TLC	Thin Layer Chromatography
v/v	Volume/ volume
w/v	Weight/volume
%	Percent

CHAPTER 1

INTRODUCTION

Fungus is widely used in producing various fermented food and beverages as well as in producing useful chemicals in bulk (Forti, Mauro, Cramarossa, Filippucci, Turchetti & Buzzini, 2015). *Aspergillus brasiliensis* is the fungal strain commonly used in the production of organic acids such as citric acid (Liaud et al., 2015). This fungal strain is classified as Generally Recognized as Safe (GRAS) organism (Perrone et al., 2007). The most important reason of choosing this fungus in this study is due to the public acceptance to fungus in which this microorganism is commonly used in food production unlike other 'fermentative' microorganisms such as bacteria (U.S. Food and Drug Administration [FDA], 2015). Moreover, there are no proofs on the substance produced by this fungus showing any adverse side effect and endanger public health after consumption.

According to Malaysian Pineapple Industry Board (MPIB) (2016), there are three main varieties of pineapple planted in Malaysia namely, Mauritius, Sarawak and Gandol. Due to the popularity, a hybrid pineapple Josapine was chosen as the material for this study due to its all-year-round availability. Approximately 452,000 tonne/ metric pineapples were produced in 2015 and these pineapples have generated wastes such as the crown, core and peel (MPIB, 2016). These pineapple wastes have many different uses such as production of linalool, phenolic anti-oxidants and ethanol (Barretto, Moreira, Santos, Narain & Santos, 2013; Upadhyay, Lama & Tawata, 2010). In this study, both core and crown of pineapple wastes were used as the fermentation substrates of *A. brasiliensis* in hope to generate linalool. Apart from its availability, ripe Josapine produces sweet aroma and contains high level of sugars which allowed fungal cells such as *A. brasiliensis* to ferment the wastes of Josapine in order to produce linalool (Phoophuangpairoj & Srikun, 2014). According to Wei et al. (2011), higher contents of aroma were proven to be present in the non-fermented pineapple core and pulp such as β-caryophyllene, copaene and valencene. Hence, there is potential research value in determining the quantity of linalool in the fermented pineapple wastes. The linalool quantity was believed to be higher in the fermented wastes compared to the non-fermented wastes as pineapple wastes were able to

undergo bioconversion to produce valuable materials such as linalool through fermentation (Dhanasekaran, Lawanya, Saha, Thajuddin & Panneerselvam, 2011). Another reason of using pineapple wastes to conduct liquid phase fermentation is to diversify the use of pineapple wastes rather than throwing them away after the pulps were consumed.

Linalool is monoterpene which can be found in most of the aromatic plants such as ginger grass and lavender (Seol, Kang, Lee & Seol, 2016; Aprotosoaie, Hancianu, Costache & Miron, 2014). Linalool is widely used in production of fragrance, cosmetic products as well as household products due to its pleasant floral scent (Aprotosoaie et al., 2014). Besides that, anti-microbial and insect-repellant properties had been found in linalool (Beier, Byrd, Kubena, Hume, McReynolds, Anderson & Nisbet, 2014). Linalool was able to disrupt formation of *Staphylococcus aureus* by suppressing the biofilm-forming genes (Federman, Ma & Biswas, 2016a; Federman, Joo, Almario, Salaheen, & Biswas, 2016b). Thus, *S. aureus* was chosen as the study subject to test the anti-microbial activity of linalool because this microbe was normally found on mammal's skin and hair and would cause infection when having wounds (Mandal, 2012). By conducting fermentation using pineapple wastes, linalool maybe extracted in a higher concentration and able to contribute more to the related industries mentioned above.

The aims of this study are:

1. to determine the optimal fermentation duration for both blended core and crown of Josapine.
2. to quantify the linalool content in the *Aspergillus*-fermented broth of both blended core and crown of Josapine.
3. to screen the anti-microbial potential of linalool extracted from both *Aspergillus*-fermented blended core and crown of Josapine towards *S. aureus*.

CHAPTER 2

LITERATURE REVIEW

2.1 LINALOOL

Linalool, one of monoterpenes which is commonly extracted from plants and has chemical formula of $C_{10}H_{18}O$. It is also known as 3,7-dimethylocta-1,6-dien-3-ol (Marmulla, Cala, Markert, Schweder & Harder, 2016; Kern, Dkhil, Hendarsa, Ellis & Natsch, 2014). There are two stereoisomers of linalool present namely, (R)-(-)-linalool and (S)-(+)-linalool. Although these two stereoisomers are structurally different but their chemical reactions such as hydrogenation, esterification and dehydration are the same (Cotton, n.d.). Linalool can be produced via microbial fermentation and enzymatic biotransformation (Akacha & Gargouri, 2015). According to Kern et al. (2014), linalool can be detected through chromatography such as thin layer chromatography (TLC), gas chromatography and liquid chromatography.

It is a volatile organic compound that having pleasant sweet smell (Marques, Amorim, Silva-Junior, Sposito & Gloria, 2014). Linalool provides various types of smell depending on its structure. Lavender with some citrus sweet smell is from (S)-(+)-linalool while woody lavender smell found in (R)-(-)-linalool (Cotton, n.d.). Flowers and fruits normally produce different intensities of smell depending on its maturity stage. The more mature the flowers and fruits, the stronger the smell which indicated higher amount of linalool.

Linalool provides various benefits to the public such as anti-inflamatory, anti-oxidant and anti-microbial activities (Tabanelli, Montanari, Patrignani, Siroli, Lanciotti & Gardini, 2014). Lee (2013) has indicated the wide use of linalool in pharmaceutical, food and beverage industries and cosmetic industries. One of the significant applications of oxidized linalool in pharmaceutical industry is the patch test which is used to test the allergic reaction on dermatitis patients (Kern et al., 2014). Linalool and food additives were often added as one of the ingredients in perfumes, lotions, cosmetics and food and beverages industries.

2.2 *Aspergillus brasiliensis* FERMENTATION

A. brasiliensis formerly known as *A. niger* is black aspergillus which used in fermentation industries to produce citric acid (Varga, Kocsube, Toth, Frisvad, Perrone, Susca ... Samson, 2007). *A. brasiliensis* forms yellowish colour initially and turns black due to its growth (American Type Culture Collection [ATCC], 2016). It have some conidia on the surface and able to grow from temperature range of 20 to 25 degree Celsius (°C) under aerobic condition (ATCC, 2016). This fungus reproduces through asexual reproduction by forming spores which are the conidia as shown in Figure 2.1.

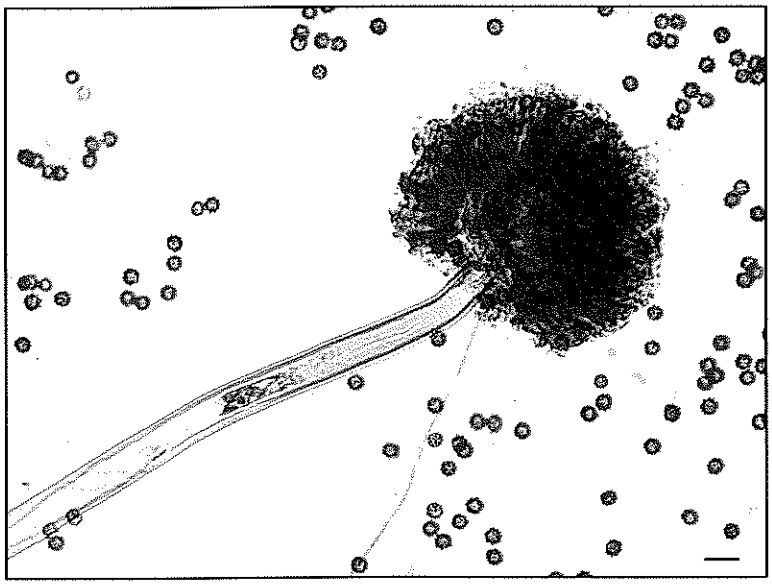

Figure 2.1 *Aspergillus brasiliensis*.
Source: https://www.inspq.qc.ca/en/moulds/fact-sheets/aspergillus-niger

A. brasiliensis is the largest contributor in citric acid production and at the same time it produces amylase, lipase, xylanase, protease and cellulase (Luis, 2013). Starchy and sugary substances are used during production of citric acid (Shahlaei & Pourhosein, 2013).