



**DEVELOPMENT OF LACTO-FERMENTED FUNGAL GROWTH
INHIBITOR COATING TO EXTEND THE SHELF LIFE AND ENHANCE THE
QUALITY OF MANGO (*Mangifera indica* L.)**

By

FERNANDO HEWAGE RANJITH PIYASIRI

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

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DEDICATION

This thesis is dedicated to

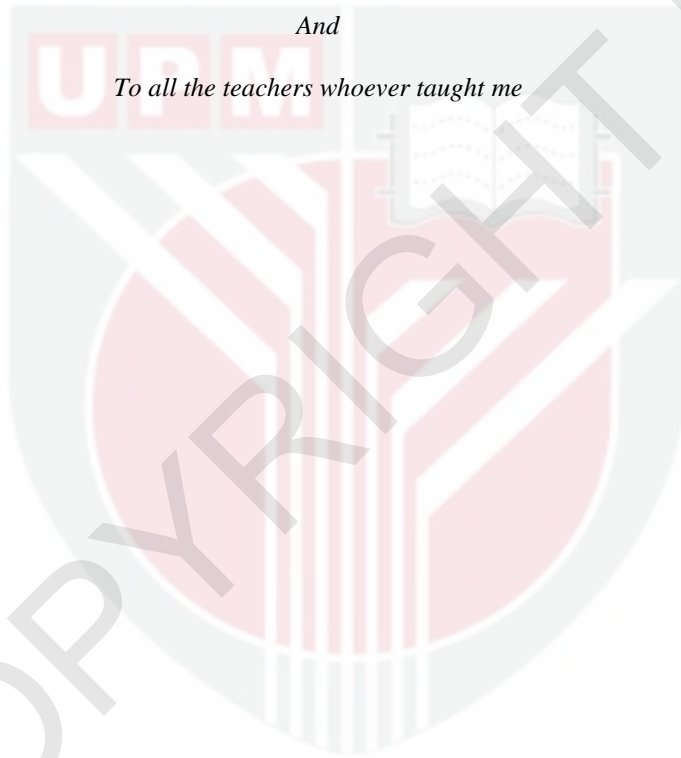
All I love

Specially,

To My mother, father, and beloved kids and wife

And

To all the teachers whoever taught me



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

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February 2022

Chairman : Associate Professor Anis Shobirin Meor Hussin, PhD
Faculty : Food Science and Technology

Mango is a tropical dessert fruit that is consumed directly without further processing. Fungal diseases limit mangoes' postharvest life, which requires applying preservation techniques to reduce postharvest losses. Consumers have higher health risks from the chemicals used in postharvest preservation. The main objective of this research was to produce a natural antifungal coating formulation for controlling postharvest spoilage fungi in mango. The fungal growth inhibitors produced by five selected lactic acid bacteria (LAB) strains cultivated in agricultural by-products were screened against five spoilage fungi in mango. The Lacto-fermentation conditions, namely, substrate ratio (10-30%), fermentation time (42- 96 h), and temperature (31- 37°C) of palm kernel cake (PKC) fermented with *Lactobacillus plantarum* ATCC8014 (PKCL1) and *Lactobacillus fermentum* ATCC9338 (PKCL2), were optimized to enhance the antifungal ability against *Colletotrichum gloeosporioides* DSM62136 and *Botryodiplodia theobromae* DSM62078 under response surface methodology (RSM) procedure. The antifungal compounds in PKCL1 and PKCL2 were identified using 1H-Nuclear magnetic resonance (NMR) spectroscopy-based metabolomics analysis. The antifungal action mechanisms were determined by inter cellular compounds' leakage, ergosterol synthesis, and fungal cell morphology. PKCL1 and PKCL2 were incorporated with polysaccharide-based polymers to develop the formulations of edible coatings with higher stability. Besides, the influence of coating formulations on mango fruit quality and postharvest life were evaluated. The LAB strains *L. plantarum* ATCC8014, and *L. fermentum* ATCC9338 were grown in PKC, and pineapple peel (PP) exhibited significantly strong *in vitro* antifungal activity against *C. gloeosporioides* DSM62136 and *B. theobromae* DSM62078. *In vivo* results showed that mango treated with PKCL1 and PKCL2 had the lowest disease incidence, disease severity, and total conidia concentration. The optimal fermentation conditions were; substrate ratio (24.75% and 30% (w/v)), fermentation time (96 h and 85.5 h) and temperature (37°C) for PKCL1 and PKCL2 respectively. The higher lactic acid concentrations, moderate acetic acid concentrations, and bioactive peptides were obtained in PKCL1 and PKCL2. Significant differences were observed in

protein, DNA, and sugar concentrations in fungi cells when treated with PKCL1 or PKCL2. Furthermore, significant reductions of ergosterol and remarkable morphological changes were observed in treated fungi. Out of 10 polysaccharide-based polymers tested, chitosan (CH) was the best polymer to carry and sustainable release of antifungal compounds. CH coating containing PKCL1 and PKCL2 exhibited the prominent inhibition zones against *C. gloeosporioides* DSM62136 and *B. theobromae* DSM62078, and they controlled the growth of both fungi on mango. Both coating formulations did not negatively affect the mango quality. The quality and nutritional parameters were not significantly different among coating formulations and CH only coating but significantly different from the uncoated fruits. The results of all experiments showed that the antifungal compounds produced by Lacto-fermentation of PKC with *L. plantarum* ATCC8014 and *L. fermentum* ATCC9338 exhibit a great potential to control *C. gloeosporioides* DSM62136 and *B. theobromae* DSM62078. Moreover, these antifungal compounds can be employed as natural coatings incorporating CH polymer to prevent anthracnose and stem-end rot in mango, enhancing shelf life and quality.

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

**PEMBANGUNAN LAPISAN PERENCAT PERTUMBUHAN KULAT SECARA
PENAPAIAN LAKTO UNTUK MENINGKATKAN JANGKA HAYAT DAN
KUALITI MANGGA (*Mangifera indica* L.)**

Oleh

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Mangga adalah pencuci mulut tropika yang dimakan secara langsung tanpa pemrosesan selanjutnya. Penyakit kulat mengehadikan jangka hayat mangga selepas penuaian dimana memerlukan penggunaan teknik pemeliharaan untuk mengurangkan kerugian selepas penuaian. Terdapat risiko yang lebih tinggi terhadap kesihatan pengguna oleh bahan kimia yang digunakan dalam pemeliharaan selepas penuaian. Objektif utama penyelidikan ini adalah untuk menghasilkan formulasi salutan antikulat semulajadi untuk mengawal kulat selepas mangga dituai. Sebatian antikulat yang dihasilkan oleh lima bakteria asid laktik (LAB) yang dikultur daripada sisa produk pertanian telah disaring terhadap lima kulat terhadap mangga. Syarat-syarat penapaian Lacto, iaitu nisbah substrat (10- 30%), masa penapaian (48- 96h), dan suhu (31- 37°C) kek kernel sawit (PKC) ditapai dengan *Lactobacillus plantarum* ATCC8014 (PKCL1) dan *Lactobacillus fermentum* ATCC9338 (PKCL2) dioptimumkan untuk meningkatkan aktiviti antikulat terhadap *Colletotrichum gloeosporioides* DSM62136 dan *Botryodiplodia theobromae* DSM62078 menggunakan metodologi Kaedah Respon Permukaan (RSM). Kompoun antikulat PKCL1 dan PKCL2 telah dikenal pasti menggunakan analisis proteomik magnetik nuklear (NMR) yang berasaskan spektroskopi bagi menentukan mekanisme tindakan antikulat melalui kebocoran sebatian antara sel, sintesis ergosterol, dan morfologi sel kulat. PKCL1 dan PKCL2 digabungkan dengan polimer berasaskan polimer untuk membangunkan formulasi salutan boleh dimakan dengan kestabilan yang lebih tinggi. Selain itu, kesan rumusan salutan terhadap kualiti mangga dan jangka hayatnya telah dinilai. *L. plantarum* ATCC8014 dan *L. fermentum* ATCC9338 telah ditumbuh pada PKC dan kulit nanas (PP) menunjukkan aktiviti antikulat yang tinggi terhadap *C. gloeosporioides* DSM62136 dan *B. theobromae* DSM62078. Keputusan *in vivo* menunjukkan bahawa mangga yang dirawat dengan PKCL1 dan PKCL2 menunjukkan kadar terendah bagi penyakit, keterukan penyakit, dan kepekatan conidia. Syarat-syarat penapaian yang optimum adalah; nisbah substrat (24.75% dan 30% (w/v)), masa penapaian (96 h dan 85.5 h) dan suhu (37°C) masing-masing untuk PKCL1 dan PKCL2. Kepekatan asid laktik yang lebih tinggi, kepekatan sederhana asid asetik, dan

peptida bioaktif di perolehi di PKCL1 dan PKCL2. Perbezaan yang signifikan diperhatikan dalam protein, DNA, dan kepekatan gula dalam sel kulat apabila dirawat dengan PKCL1 atau PKCL2. Tambahan pula, pengurangan ketara ergosterol dan perubahan morfologi yang luar biasa diperhatikan dalam kulat yang dirawat. Daripada 10 polimer berasaskan polimer yang diuji, chitosan (CH) adalah polimer terbaik untuk membawa dan pelepasan berterusan sebatian antikulat. Lapisan CH yang mengandungi PKCL1 dan PKCL2 mempamerkan zon perencatan yang menonjol terhadap *C. gloeosporioides* DSM62136 dan *B. theobromae* DSM62078, dan mereka mengawal pertumbuhan kedua-dua kulat pada mangga. Kedua-dua formulasi salutan tidak memberi kesan negatif kepada kualiti mangga. Parameter kualiti dan pemakanan tidak jauh berbeza di kalangan formulasi salutan dan CH hanya salutan tetapi jauh berbeza daripada buah-buahan yang tidak dikenali. Hasil daripada semua eksperimen menunjukkan bahawa sebatian antikulat yang dihasilkan oleh Lacto-fermentation PKC dengan *L. plantarum* ATCC8014 dan *L. fermentum* ATCC9338 mempamerkan potensi yang lebih tinggi untuk dikawal *C. gloeosporioides* DSM62136 dan *B. theobromae* . DSM62078 Selain itu, sebatian antikulat ini boleh digunakan sebagai salutan antikulat semulajadi yang diperbadankan dengan polimer CH untuk mengawal antraknosa dan reput batang dalam mangga dengan meningkatkan hayat dan kualiti mangga.

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LIST OF ABBREVIATIONS

AA	Ascorbic Acid
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
°C	Degree Celsius
CAT	Catalase
CEP	Cell- Envelop Proteinases
CFS	Cell Free Supernatant
CFU	Colony Forming Units
CH	Chitosan
CMC	Carboxymethyl cellulose
CN	Carrageenan
CO ₂	Carbon Dioxide
DI	Disease Incidence
DNA	Deoxyribonucleic acid
D ₂ O	Deuterium Oxide
DPPH	1, 1-Diphenyl-2-picrylhydrazyl
DSI	Disease Severity Index
DSMZ	German Collection of Microorganisms and Cell Cultures
DSS	Sodium trimethylsilylpropanesulfonate
EDTA	Ethylenediaminetetraacetic acid
FCR	Folin-Ciocalteu Regent
FDA	Food and Drug Administration
FFS	Film Forming Solution

FW	Fresh Weight
g	Gram
GAE	Galic Acid Equivalent
GRAS	Generally Recognize As Safe
HPLC	High Performance Liquid Chromatography
Hz	Hertz
CIE	Commission International Elcairage
IN	Inulin
kDa	Kilo Daltons
Km	Menten Constant Value
KSP	Potassium persulphate
LAB	Lactic Acid Bacteria
LC	Liquid Chromatography
LDPE	Low-density Polyethylene
LSD	Least Significant Difference
M	Molar
Mb	Megabase
MCC	Microcrystalline Cellulose
MD	Maltodextrin
MEB	Malt Extract Broth
MFC	Minimal Fungicidal Concentration
MIC	Minimal Inhibition Concentration
mM	Millimolar
MRSB	De Man Rogosa and & Sharpe Broth

MS	Mass Spectroscopy
MT	Metric Tonnes
NBT	Nitro-blue tetrazolium
NMR	Nuclear Magnetic Resonance
O ₂	Oxygen
OP	Orange Peel
OPA	O-phthalaldehyde
PB	Phosphate Buffer
PDA	Potato Dextrose Agar
PE	Pectin
PKC	Palm Kernel Cake
POD	Peroxidase
PP	Pineapple Peel
PVC	Polyvinyl Chloride
PVPP	Polyvinylpyrrolidone
RB	Rice Bran
RCBD	Randomize Complete Block Design
RH	Relative Humidity
RNA	Ribosenucleic Acid
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
SA	Sodium Alginate
SAS	Statistical Analysis System
SEM	Scanning Electron Microscope

SOD	Superoxidase Dismutase
SP	Starch Potato
SS	Starch Soluble
TA	Titrateable Acidity
TE	Trolox Equivalent
TPC	Total Phenolic Content
TSS	Total Soluble Solids
UK	United Kingdom
US	United State
UV	Ultra Violet
V _m	Maximum Respiration Rate
WHO	World Health Organization
WI	Whiteness Index
WR	Watermelon Rind
WVP	Water Vapour Permeability
X _i	Independent Variables
Y _i	Dependant Responses
μg	Micro Gram
ΔE	Total Colour Difference
%	Percentage
μL	Microliter
μmoles	Micromoles

CHAPTER 1

INTRODUCTION

Mango (*Mangifera indica* L.) is prevalent due to its excellent taste and excellent nutrient profile, including vitamins, minerals, and phytochemicals. It is an important tropical fruit belonging to the family of Anacardiaceae. Mango has a significant commercial value in the global export market, in recent years, it has taken a high economic value with more than 1.69 billion US \$ annual export value (Muhammad Siddiq, Jeffrey K. Brecht, 2017; G. Shu, Shi, Chen, Ji, & Meng, 2017). During the last decades, mango production and international trade have increased rapidly, though the postharvest losses are also grown in all steps of the mango supply chain (Singh, Singh, Sane, & Nath, 2013). Therefore, there is a requirement for systematic storage and apply postharvest preservation techniques to reduce these losses in the mango industry.

Fungal diseases are one of the major problems that involve elevated postharvest losses of mango. Fungal diseases make significant economic losses and possible health problems for consumers by producing secondary metabolites like mycotoxins. Major postharvest fungal diseases of mango have been reported as anthracnose (*Colletotrichum gloeosporioides*), stem end rot (*Botryodiplodia theobromae*, *Lasiodiplodia theobromae*, *Dothiorella dominicana*, or *Phomopsis mangifera*), and black mold disease (*Aspergillus sp.*). Several fungal infestations like *C. gloeosporioides* begin at the flowering stage and endophytically colonize and live with protection of epidermis tissues (de Oliveira Costa et al., 2010; Prusky, Alkan, Mengiste, & Fluhr, 2013). Consequently, quiescent infestations are challenging to identify and prevent at the first stages of mango development. Therefore, these postharvest diseases seriously affect the postharvest stage during mango ripening, reducing the postharvest life and limiting long-distance commercial transportation and export. For a long time, many technologies such as chemical, physical, and biological approaches have been practised to prevent the fungal diseases of mango. Many of these technologies support to extend the shelf life to approximately 2- 4 weeks. However, chemical applications have been popular during the past three decades because of simple practising ability, high effectiveness, and comparatively low cost (Mahajan, Caleb, Singh, Watkins, & Geyer, 2014). However, the social rejection and community protests have limited application chemicals, which prompted the exploration for alternative methods to prevent fungal diseases (Onaran & Yanar, 2016; Palou, 2018; Wisniewski, Droby, Norelli, Liu, & Schena, 2016).

Bio-preservation is an alternative technology for controlling the growth of harmful microorganisms and is safe for consumers and the environment (Lamont, Wilkins, Bywater-Ekegård, & Smith, 2017; Muhialdin, Saari, & Hussin, 2020; Roselló et al., 2013). For a longer time, LAB was used as a bio preservative agent for the natural preservation of food. The use of the antagonism activity of LAB to control the spoilage fungi in fresh produce was becoming an exciting research field with the social demand for chemical-free foods (Jeddi et al., 2014; Li et al., 2015; Trias, Bañeras, Montesinos, & Badosa, 2008). The use of LAB and/or their metabolites as bio preservatives showed significant results against a broad range of fungi, which improve the postharvest life,

quality and sensory properties of different fruits (Barrios-Roblero et al., 2019; Anna Marín, Plotto, Atarés, & Chiralt, 2019). The antifungal activity of LABs has been well documented with the production of different antifungal metabolites such as organic acids, bioactive peptides, fatty acids, and hydrogen peroxide (Magnusson & Schnürer, 2001; Muhialdin, Hassan, Bakar, & Saari, 2016). As examples, many of the earlier studies revealed the antifungal capability of different LAB strains on various fresh produces (Marín et al., 2019; El-Mabroc et al., 2012; Barrios-Roblero et al., 2019; Trias, Bañeras, Badosa, & Montesinos, 2008; Sathe, Nawani, Dhakephalkar, & Kapadnis, 2007)

The production of antifungal compounds through the Lacto-fermentation depends on significant factors such as substrate, bacteria strains, temperature, pH, and fermentation time (Janakiram Naveena, Altaf, Bhadrappa, & Reddy, 2004; Sridevi, Padmaja, Sahitya, Vardhan, & Rao, 2015). The nutrition profile of substrates is highly affected by antifungal production (Ricci et al., 2019), and Pessione & Cirrincione (2016) reported that a higher amount of bio-active peptides are produced in protein-rich substrates. The vital issue of LAB fermentation is the higher substrate cost due to the high nutrition requirement of LAB (Wang, Yan, Wang, Zhang, & Qi, 2012). Agricultural by-products are sustainable solutions for the substrate cost of Lacto-fermentation because of the high potential to use as a substrate with large amounts of carbohydrates, proteins, minerals, vitamins, and higher moisture contained in them (Rodriguez Couto, 2008; Shalini & Gupta, 2010).

The use of Lacto-fermented antifungal compounds directly in food systems has been reported few barriers and limitations. Some of the critical characteristics of antifungal compounds like amphipathicity, polarity, hydrophobicity, aromatic residues, and position of charged enforce the hydrophobic nature, low stability, and higher binding ability with unstable molecules (Lee et al., 2014; Min & Krochta, 2005; Sagaram et al., 2011; Treviño-Garza et al., 2015). Antifungal compounds with edible coatings are promising technologies that improve both molecules' antifungal activity, stability, and other essential properties. Natural biopolymers such as proteins, polysaccharides, lipids, resins, and mixtures can produce active coatings. Such coatings can be combined with antifungal compounds (Karaca, Pérez-Gago, Taberner, & Palou, 2014; Tavassoli-Kafrani, Shekarchizadeh, & Masoudpour-Behabadi, 2016). Several previous studies reported that the use of different antifungal compounds incorporation with varying materials of coating; chitosan and plant gel aloe-vera (Palou, Ali, Fallik, & Romanazzi, 2016), pectin (Treviño-Garza et al., 2015), alginate (Narsaiah et al., 2014), sericin (Chimvaree et al., 2019), whey protein (Min & Krochta, 2005) and tapioca starch (Sanjurjo, Flores, Gerschenson, & Jagus, 2006).

To use agricultural by-products as substrates in LAB fermentation to produce natural fungal growth inhibitors, it should meet specific criteria including the higher capacity to produce antifungal compounds, consumer and environmentally friendly and low cost (Lamont et al., 2017; Shalini & Gupta, 2010; Wang et al., 2012). Besides, the Lacto-fermented fungal growth inhibitors should promise to incorporation with polysaccharide-based biopolymers to enhance the stability of antifungal compounds (Karaca et al., 2014; Palou et al., 2015; Tavassoli-Kafrani et al., 2016). Furthermore, literature shows only

minimal numbers of works about developing natural antifungal coating formulations for controlling the postharvest fungi in mango using Lacto-fermented agricultural by-products and their applications.

1.1 Problem Statements

To date, consumer demand is increasing for green-labeled (chemical-free) fruits and vegetables, including mango, due to higher health problems concomitant with chemicals (Prochloraz, Fludioxonil, Benomyl, and Topsin) used in the preservation (Onaran & Yanar, 2016). Some chemicals (Ex. Carbamate, organophosphates) have been prohibited worldwide including Malaysia, but still these chemicals use in the mango industry. Especially, mango is a dessert fruit that is consumed directly without further processing. There is a higher risk to expose the chemicals. Besides, the fungal diseases limit the mango's postharvest life, interrupting the mango export chain (Singh et al., 2013). Therefore, there is essential to develop a robust technology to reduce postharvest losses with minimum health hazards for consumers.

On the other hand, there were a higher number of studies for conducting the LAB and their antifungal compounds to preserve fruits. But, the higher production cost, low stability, and barriers for direct applications limit their practical uses (Sagaram et al., 2011; Treviño-Garza et al., 2015). Therefore, the production of natural fungal growth inhibitors and development of the edible coating formulations is recommended to overcome previously mentioned issues. Thus, improving antifungal activity and stability of natural coating formulations without negative impacts on the mango quality should be achieved in the application for the mangoes.

1.2 Hypothesis

Production of fungal growth inhibitors using Lacto-fermented agricultural by-products against postharvest spoilage fungi in mango will succeed as using a biopreservation agent. The response surface methodology will be enhanced the antifungal activity of fungal growth inhibitors via optimization of the fermentation conditions. The limitations for applying fungal growth inhibitors on mango will be reduced by encapsulation with polysaccharide biopolymers. Moreover, the encapsulated fungal growth inhibitors will be controlled the postharvest spoilage fungi in mango without negative impacts. The natural antifungal coating formulations will be successfully employed for the mango.

1.3 Objectives

1.3.1 General Objective

The general objective of this study was directed towards the development of fungal growth inhibitors, and formulation development of natural antifungal coating for control postharvest spoilage fungi in mango.

1.3.2 Specific Objectives

1. To screen the antifungal activity for Lacto-fermented fungal growth inhibitors produced by selected LAB strains cultured in agricultural by-products against postharvest spoilage fungi in mango.
2. To optimize the fermentation conditions to maximize the antifungal activity in fungal growth inhibitors.
3. To identify the antifungal compounds and determine the antifungal action mechanism.
4. To enhance stability and usability of fungal growth inhibitors with polysaccharide-based edible coatings.
5. To evaluate the effects of chitosan edible coatings combined with fungal growth inhibitors on mango quality and shelf life.

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