UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND GROWTH OPTIMIZATION OF MARINE FUNGI, AND ANTIBACTERIAL AND BIOFILM REMOVAL ACTIVITIES OF ITS EXTRACTS

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FSTM 2016 15

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By

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

January 2016

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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Chairman : Assoc. Prof. Nor Ainy Mahyudin, PhD
Faculty : Food Science and Technology

Marine derived fungi known as source of bioactive compounds and many were found to produce compounds with antibacterial and biofilm removal activities. The objectives of this study are; 1) to characterize fungal isolates, 2) to study the effect of media, temperature and salinity on fungal growth, 3) to determine antibacterial activity of marine fungal extracts against food-borne bacteria; *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium* and 4) to examine biofilm removal activity of marine fungal extracts. Four fungal isolates; PR1T4, PP2L4, PR3T13 and PR5T4 isolated from Pulau Redang and Pulau Payar Marine Parks, Malaysia, were characterized using morphological and molecular identification. All isolates were subjected to study of the effect of media (PYGA, MEA and PDA), temperatures (25°C and 37°C) and salinities (0% and 15% of NaCl) on their growth. Antibacterial activity of all fungal extracts was tested against the selected bacteria using agar well diffusion assay. Test surface was done on stainless steel disc (SSD); adhesion, detachment and biofilm formation of tested bacteria. Screening of fungal extracts potential as biofilm removal agents was done against tested bacteria. The isolates were identified as *Penicillium citrinum* (PR1T4), *Sarocladium strictum* (PP2L4), *Aspergillus sydowii* (PR3T13) and *Aspergillus* species (PR5T4). All isolates responded differently on all growth media but showed general preference to Malt Extract Agar (MEA) with additional of 15% sodium chloride, while 25°C was a preferable growth temperature ($p>0.05$) by all isolates. All isolates (PR1T4, PP2L4, PR3T13 and PR5T4) cultured in Malt Extract Broth (MEB) displayed significantly ($p<0.05$) higher antibacterial activity against *S. aureus* (16.36±0.36mm, 27.57±0.81mm, 22.60±0.62mm and 15.82±0.49mm, respectively) and *L. monocytogenes* (21.45±0.21mm, 22.34±0.78mm, 21.30±0.50mm and 17.27±0.38mm, respectively) compared to cultures on other media. Ethyl acetate (EA) was the best extraction solvent as crude extracts gave significantly ($p<0.05$) highest antibacterial activity compared to other solvents. All bacteria shown similar pattern of adhesion on SSD where their log CFU/cm^2 readings were increased from 24 to 72 hours. The highest count of adhered cells (log CFU/cm^2) on SSD was recorded by *E. coli* (8.65±0.11) followed by *S. aureus* (8.14±0.04) and *L. monocytogenes* (8.01±0.07) while the lowest was displayed by *S. typhimurium* (7.91±0.08) after 72 hours of incubation. Cell detachment from SSD showed a linear decreased of bacterial counts

from 1st to 5th contacts. For example, adhered cell counts (log CFU/cm^2) of *E. coli* reduced from 6.05±0.07 (1st contact) to 0.00 (4th contact) after 24h of incubation. All bacteria except *S. typhimurium*, showed highest viable counts (log CFU/cm^2) (6.80-7.38) on Day 6 and the counts decreased to lowest (3.98-4.25) on the Day 15 while *S. typhimurium* reached its maximum counts (7.26±0.05) after 9 days of incubation and decreased to 5.10±0.09 on Day 15. In conclusion, all isolates demonstrated promising bioactivities with isolate PR1T4 (*P. citrinum*) as the most potential candidate to be explored as new antibacterial and biofilm removal agents against food-borne bacteria.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENCIRIAN DAN PENGOPTIMUMAN PERTUMBUHAN KULAT MARIN SERTA AKTIVITI ANTIBAKTERIA DAN PENYINGKIRAN BIOFILM OLEH EKSTRAKNYA

Oleh

NOOR IFATUL HANIM BINTI MAT DAUD

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Pengerusi : Prof. Madya Nor Ainy Mahyudin, PhD
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Kulat pencilan marin terkenal sebagai sumber sebatian aktif dan dikenalpasti banyak menghasilkan sebatian dengan aktiviti antibakteria dan agen penyingkiran biofilm. Objektif kajian ini adalah untuk; 1) pencirian pencilan kulat, 2) mengkaji kesan media, suhu dan kemasinan terhadap pertumbuhan kulat, 3) menentukan aktiviti antibakteria oleh ekstrak kulat pencilan marin terhadap bakteria bawaan makanan iaitu *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* dan *Salmonella typhimurium*, serta 4) mengkaji aktiviti penyingkiran biofilm oleh ekstrak kulat pencilan marin. Empat pencilan kulat; PR1T4, PP2L4, PR3T13 dan PR5T4 telah dipencilkan dari Taman Laut Pulau Redang dan Pulau Payar, Malaysia, seterusnya dicirikan menggunakan pengenalpastian morfologi dan molekular. Kesan media (PYGA, MEA dan PDA), suhu (25°C dan 37°C) dan kemasinan (0% dan 15% of NaCl) di kaji terhadap pertumbuhan semua pencilan. Aktiviti antibakteria dilakukan terhadap bakteria terpilih menggunakan cerakin sebaran telaga agar. Ujian permukaan termasuklah pelekatan, penyingkiran dan pembentukan biofilm oleh bakteria terpilih telah dilakukan pada cakera keluli tahan karat (SSD). Penyaringan potensi ekstrak kulat marin sebagai agen penyingkiran biofilm telah dilakukan terhadap bakteria terpilih. Pencilan kulat PR1T4, PP2L4, PR3T13 dan PR5T4 telah dikenalpasti masing-masing sebagai *Penicillium citrinum* (PR1T4), *Sacroladium strictum* (PP2L4), *Aspergillus sydowii* (PR3T13) dan *Aspergillus* species (PR5T4). Semua pencilan bertindak balas secara berbeza terhadap semua media terpilih tetapi secara umumnya memilih agar Ektrak Malt (MEA) dengan penambahan 15% sodium klorida manakala semua pencilan memilih suhu 25^{o}C ($p>0.05$) seagai suhu tumbesaran. Pencilan (PR1T4, PP2L4, PR3T13 dan PR5T4) dikultur dalam larutan Malt Ekstrak (MEB) menunjukkan aktiviti antibakteria tertinggi yang signifikan ($p<0.05$) terhadap *S. aureus* (16.36±0.36mm, 27.57±0.81mm, 22.60±0.62mm dan 15.82±0.49mm, mengikut turutan) dan *L. monocyogenes* (21.45±0.21mm, 22.34±0.78mm, 21.30±0.50mm dan 17.27±0.38mm, mengikut turutan) berbanding media yang lain. Etil asetat (EA) merupakan larutan pengekstrakan terbaik yang menghasilkan ekstrak dengan aktiviti perencatan bakteria tertinggi yang signifikan ($p<0.05$) berbanding larutan pengekstrakan yang lain. Semua bakteria menunjukkan corak pelekatan yang sama pada SSD dimana bacaan log CFU/cm^2 bertambah apabila tempoh inkubasi bertambah dari 24 jam ke 72 jam. Bacaan lekatan sel (log CFU/cm^2) tertinggi di rekodkan selepas

72 jam inkubasi oleh *E. coli* (8.65±0.11) diikuti oleh *S. aureus* (8.14±0.04) dan *L. monocytogenes* (8.01±0.07) manakala *S. typhimurium* menunjukkan bacaan terendah (7.91±0.08). Penanggalan sel dari SSD menunjukkan kiraan bakteria berkurang apabila bilangan sentuhan meningkat (1-5sentuhan). Contohnya, bilangan lekatan sel (log CFU/cm^2) *E. coli* berkurang dari 6.05±0.07 (sentuhan pertama) kepada 0.00 (sentuhan keempat) selepas 24 jam inkubasi. Pembentukan biofilm menunjukkan semua bakteria kecuali *S. typhimurium* menunjukkan kiraan bakteria tertinggi (6.80-7.38 log CFU/cm^2) pada hari ke 6 dan kiraan tersebut menurun ke tahap terendah (3.98-4.25 log CFU/cm^2) pada hari ke 15 manakala *S. typhimurium* mencapai kiraan maksimum (7.26±0.05 log CFU/cm^2) pada hari ke 9 dan menurun ke 5.10±0.09 log CFU/cm^2 pada hari ke 15. Kesimpulannya, semua pencilan menunjukkan bioaktiviti yang menggalakkan dengan pencilan PR1T4 (*P. citrinum*) sebagai calon berpotensi untuk di diketengahkan sebagai agen baru antibakteria dan penyingkiran biofilm terhadap bakteria bawaan makanan.

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim, Alhamdulillah, Allahuakbar. All praises to Allah, the Almighty. I managed to complete this study under Allah's blessings. Everything happens with Allah's permission.

First of all, I would like to say a bouquet of thanks to my mother, Wan Esah Wan Ahmad for her endless supports, advices and encouragements. My infinity love and thanks goes to my late father, Mat Daud Jusoh for always being in my heart. To my brothers and sisters; Luqman, Zulkarnain, Hafizi, Haslina, Zainatul Adian and Syafikah, big hug for all of you for supporting me especially in financing my study and being good listeners. I would like to say thanks to my supervisor, Assoc. Prof. Dr. Nor Ainy Mahyudin for her guidance. It was a nice experience working under her supervision. Then, I would like to extend my appreciative thanks to member of supervisory committee; Prof. Dr. Fatimah Abu Bakar, Prof. Dr. Nazamid Saari and Dr. Wan Norhana Nordin for their advices, guidance and sharing of knowledge.

To my laboratory mates in Food Safety and Quality Laboratory especially Selvi, A'isyah, Lai, Jaini, Farah, Zhet, Hafiz, Mardhiyah and Najwa, thanks for your support, helps and encouragement, may Allah bless all of us and it was a great chapter of my life being around all of you. My outmost thanks go to all staff in the Faculty of Food Science and Technology for their kind services and helps. My special thanks go to Mr. Azman, Mrs. Aziatum, Mrs. Noordiana and Mrs Norliza for their guidance and teachings. I would like to thank Universiti Putra Malaysia for financial support through Graduate Research Fellowship (GRF) throughout my study. I also would like to acknowledge Ministry of Higher Education for their financial support through MyBrain15 scheme.

Last but not least, I want to thanks everyone who has participated either directly or indirectly in this project. I appreciate all your supports and advices and may Allah repay all your deeds and kindness. Everything happens with His consent, Alhamdulillah.

I certify that a Thesis Examination Committee has met on 15 January 2016 to conduct the final examination of Noor Ifatul Hanim binti Mat Daud on her thesis entitled "Characterization and Growth Optimization of Marine Fungi, and Antibacterial and Biofilm Removal Activities of its Extracts" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BHB	Brain Heart Broth
CFU/cm^2	Colony Form Unit per Centimeter Square
CFU/ml	Colony Form Unit per Mililiter
$CHCL_3$	Chloroform
CCL_4	Carbon Tetrachloride
cm	Centimetre
d	Day
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EtoAc	Ethyl acetate
EFSA	European Food Safety Authority
ECDC	European Centre for Disease Prevention and Control
FCS	Food Contact Surface
g	Gram
h	Hour
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
min	Minute
mg/mL	Milligram per mililiter
mm	Milimeter
$mmol^{-1}$	micromolar per liter
ml	Mililiter
NaCl	Sodium chloride
RTE	Ready-to-eat
SSD	Stainless Steel Disc
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization
µg	Microgram
µl	Microliter
µg/mL	Microgram per mililiter

CHAPTER 1

INTRODUCTION

Microbiology is a study of microorganisms' life. Large numbers of these microscopic creatures have been exploited, manipulated and engineered in order to produce functional microorganisms or their by-products. These achievements have drawn worldwide attentions from industry players in many sectors. Terrestrial environment was previously chosen by researchers as source of microorganisms for metabolites production. Flora, fauna and soil have been screened for presence of known and new species of microorganisms. After decades of study and research on terrestrial environment, the finding of new compounds became lower in number and the compound formulation and structures appeared repetitive (Davidson, 1995). Microorganisms evolve and undergo various mutation processes to be adaptive to changing environments. The adaptation process will lead to occurrence of microbial resistance against present antimicrobial agents and drugs. Microbial resistance occurs in many fields including health, food and also agriculture (Hall-Stoodley *et al*., 2004). In health sector, this resistance caused available compounds become less effective on emerging diseases and infections. Agriculture sector faces production loss as many pathogens including bacteria, fungi and virus cannot be killed or controlled by available antimicrobial compounds and at the same time, there are emergences of new diseases. In food industry, microbial resistances to temperature, acidity and other preservation methods have made food become easily spoiled and deteriorated by foodborne pathogens especially bacteria and fungi thus leads to elevating number of food diseases and illnesses (Hall-Stoodley *et al*., 2004; Frank, 2001; Shi and Zhu, 2009).

Due to time constraint, people will opt for dining out and ready to eat (RTE) foods especially those from fast food premises rather than homemade foods. They tend to give more attentions and demands on their food safety especially on the cleanliness and hygiene status of foods in order to maintain their health status. There is a clear link between food and health concern which has drawn considerable attentions of food researchers to find out on how certain compounds can be used to protect consumers against certain illness. The quality and safety of food have become main focus in food service and industry. Food safety programs and campaigns have been established to elevate the awareness among foods handlers and producers. Although prevention steps have been taken, foodborne illness still occurs as reported food poisoning cases are elevating by the incidence rate of 36.17 in 2009, 44.18 in 2010 and 56.25 in 2011 per 100,000 populations in Malaysia (MOH, 2009; 2010). The problem occurs due to lack of consistency in practicing hygiene during food production and preparation. Therefore, it is a big responsibility for food manufactures or handlers to make sure the safety of food products. Bacteria either from surrounding, raw materials or food handlers can be transmitted to food contact surface such as cutting board (Pérez-Rodríguez *et al*., 2008).

Improper wash of cutting board will leave food waste on its surface. Later, bacteria will utilize the food wastes as their nutrient source. Suitable growth conditions on cutting

board including temperature, pH, water activity and nutrient availability will promote the bacterial growth. They will reproduce and communicate with other bacteria via quorum sensing to build biofilm. Biofilm is a mixture of microorganisms embedded within a self-produced matrix of extracellular polymeric substance (EPS) which commonly adhere to each other and attached on surfaces (Sauer *et al*., 2007). The matrix of biofilm caused bacterial attachment on food contact surfaces became hard to be removed and cause losses in food industry (Brooks and Flint, 2008). Preparation of food using this contaminated cutting board will cause cross contamination, deteriorates the food and increases the number of food diseases and illnesses. For example, Vestby *et al.* (2009) stated that cross contamination occurs in domestic kitchen**.** Bacteria can adhere to contact surfaces and become persist over the time as they develop biofilm (Borucki *et al.*, 2003). The complexity of biofilm matrix has made biofilm become impenetrable by present and available antibacterial solutions. These problems trigger higher needs for novel bioactive compounds (Oliveira *et al*., 2006).

Marine environment known for its diverse organisms including microorganisms thus it become an alternative reservoir for biomining of new bioactive compounds. The large coverage of marine environment over the earth surface; approximately 70 % of total area gives a clear picture on how promising marine environment will offer to exploration of new microorganisms and compounds. Extreme conditions including salinity, high pressure, low light intensity, vast range of temperature and nutrients availability have made most marine creatures develop some unique characteristics for adaptation and protection towards their surroundings, thus make them unique and diverse. High diversity will lead to higher probability of isolation of new species and novel compounds (Liberra and Lindequist, 1995; Bhadury and Wright, 2004; Bugni and Ireland, 2004). In the past, limited literature on isolation and cultivation methods for marine microorganisms had put limitations in exploring marine environment and believed that marine microorganisms were uncultivable. However, a number of researchers in 1990s proved the fact was totally wrong (Davidson, 1995; Fenical, 1993; Kobayashi and Ishibashi, 1993; Okami, 1993). Invention of new technology has overcomes the obstacles of getting into the dark and deep side of oceans which was previously seemed impossible for exploration. A large number of new bioactive metabolites have been isolated and identified from marine microorganisms and their biological activities have been tested (Blunt, 2007; 2008; 2009; 2010).

Fungi are heterotrophic organisms that cannot produce their own foods as they lack of chlorophyll (de Hoog *et al*., 2000). They can grow well with or without presence of light. They require nutrients from their host. They can basically be grouped into five phyla; Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota (Alexopaulus *et al*., 2004). Fungi produce wide range of secondary metabolites with varying degree of bioactivity for examples the known ß-lactam antibiotic, Penicillin extracted from *Penicillium* sp has great ability in killing bacteria and Vancomycin produced by *Amycolatopsis orientalis* is active against a wide range of Gram-positive and Gram-negative bacteria, mycobacteria and fungi (Butler, 2004). Erythromycin produced by *Saccharopolyspora erythraea* has broad spectrum activity against gram-positive *cocci* and *bacilli* (Butler, 2004; Dewick, 2002). These findings have drawn attentions from researchers to explore the potentials of marine derived fungi and un-earth more new strains with variable functional compounds.

Marine fungi can be divided into two groups, facultative and obligate marine fungi Kohlmeyer and Kohlmeyer (1979). Facultative marine fungi originated from freshwater or terrestrial habitats which can tolerate the salinity of seawater while the obligate marine fungi are those originated from marine environment and need salinity for growth and secondary metabolites production (Kohlmeyer and Kohlmeyer, 1979). The term 'marine' is not suitable for those originated from terrestrial habitat, thus term marine derived fungi is widely used for this group of fungi. Although marine derived fungi cannot be considered as obligate marine fungi due to their terrestrial origins, most of them produce unknown and novel bioactive compound which cannot be isolated from terrestrial strains of the same taxa (Proksch *et al*., 2006). Bringmann *et al.* (2003) reported that *Penicillium chrysogenum* derived from *Ircinia fasciculate*, a marine sponge, produced new cytotoxic alkaloid identified as sorbicillactone A. This alkaloid cannot be isolated from terrestrial strains of *P. chrysogenum* or other *Penicillium* sp.. In 2002, Ebel *et al.* found aspergillitine and aspergione A – F from sponge-derived *Aspergillus niger*. These compounds cannot be isolated from terrestrial strains of *A. niger* (Lin *et al.*, 2001; Ebel *et al*., 2006).Marine-derived fungi became a new promising source of bio-active secondary metabolites (Bugni and Ireland, 2004). Marine derived fungi have proven to be an alternative source of antibacterial, antifungal, anticancer, anti-inflammatory, antiviral agents and antiplasmodial (Asolkar *et al*., 2002; Abdel-Lateff *et al*., 2003; Bugni and Ireland 2004; Gai *et al*., 2007; Kwong *et al*., 2006; Li *et al*., 2006; Samuel *et al*., 2011; Nanthaphong *et al*., 2014).

The difference between marine environment and laboratory condition has made fungal growth and yield becomes reduced (Proksch *et al.*, 2006). Optimization of fungal cultivation condition must be done in order to increase the yield of bioactive compounds. Fungal identification and characterization must take in first place as fungi vary widely in growth requirements. Some fungi need specific growth requirements (Li, 2007; Basu *et al*., 2015). Morphological identification is inadequate for fungal characterization (Geiser, 2004). Thus, molecular identifications such as comparative studies of the nucleotide sequences of ribosomal DNA (rDNA) genes have been used to identify the phylogenetic relationships of fungi. Internal transcribed spacer (ITS) region of ribosomal DNA is the most divergent and variable compared to othersequenced DNA regions and it can be amplified using universal primers (ITS 1 and ITS 4 primers) via Polymerase Chain Reaction (PCR) and the obtained sequenced can be aligned to present sequences in Basic Local Alignment Search Tool (BLAST) in order to find the matched sequences with identified species (Geiser, 2004; White *et al.*, 1990; Abd-Elsalam *et al*., 2003). Findings on marine derived fungi bioactivities suggested their potential to be explored as source of bioactive compounds. However, there is very limited report and literature on the isolation, characterization and bioactivity of marine derived fungi from Malaysia. For this study, four marine derived fungi have been isolated from invertebrates including sea fans, sea coral trees and tunicates and were collected from Pulau Payar and Pulau Redang Marine Parks.

The objectives of this study were:

1. To characterize marine fungal isolates using morphological and molecular identification.
2. To study the effect of media, temperature and salinity on fungal growth.

3. To determine antibacterial activity of isolated marine fungal extracts against foodborne bacteria; *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Salmonella* Typhi
4. To examine biofilm removal activity of marine fungal extracts.

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