UNIVERSITI PUTRA MALAYSIA

EFFECT OF DETERGENT, HERBS AND SPICES ON THE GROWTH OF Vibrio parahaemolyticus BIOFILM IN SEAFOOD

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By

ELEXSON NILLIAN

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

September 2014

Dedicated to God for the love, wisdom and strength

Dedicated to my family and my relatives for their unconditional love and endless support

Dedicated to my friends for the wonderful friendship, love and joy

Dedicated to everyone whom have invested their lives in my life

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

EFFECTOF DETERGENT, HERBS AND SPICES ON THE GROWTH OF *Vibrio parahaemolyticus* BIOFILM IN SEAFOOD

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September 2014

Chairman: **Professor Son Radu, PhD**
Faculty: **Food Science and Technology**

The aim of this study was to investigate the effects of detergent, herbs, and spices on antimicrobial activity and specificity to mitigate the growth of *V. parahaemolyticus* biofilm. A total of 394 samples were collected from wet markets (n=201) and hypermarkets (n=193), consisting of cockles (*Anadara granosa*), clams (*Mya arenaria*), shrimps (*Penaeous spp.*) and squids (*Loligo opalescens)*. The prevalence of 9.14% (36/394) of positive *toxR* genes, 63.8% of positive isolates contained virulent genes of *V. parahaemolyticus tdh+*, 22.2% of *V. parahaemolyticus trh+*, and 5.56% (2/36) of positive isolates in both *V. parahaemolyticus tdh+* and *trh+* had been detected. The thirty six (n=36) isolates were further tested with antibiotic susceptibility test and they werefound to be resistant to at least seven out of eighteen antibiotics. *V. parahaemolyticus* isolates (97.2%) showed Multiple Antibiotics Resistance index > 0.2, indicating that these isolates might have originated from high risk source. Next, RAPD-PCR was carried out to investigate the relationship between genetic diversity and clonal among the strains. As dendogram was generated among the four groups, group Type B was the largest group that possessed 92.86% (13/14) of isolates from bivalve seafood, such as cockles and clams. The samples were derived from wet markets and they are closely related. RAPD-PCR fingerprinting and antibiotic resistance profiling indicated multiple antibiotics resistant *V. parahaemolyticus* were wide-spread among the groups. The isolates were examined in the assessment of biofilm producer at different temperatures. *V. parahaemolyticus* could form biofilm and presence of biofilm was detected at 37 ºC (optimum temperature), followed by room temperature (25 ºC), and 4°C (chiller temperature). The representatives of clonal from each group, which wereresistant antibiotics of*V. parahaemolyticus*, were examined for effect of detergents. Out of the three detergents; Detergent 1 (Linear Alkylbenzene sulphonate based), Detergent 2 (Quaternary Ammonium based), and Detergent 3 (Sodium Hydroxide based) with minimum concentration of 100 mg/ml (100,000 µg / ml), Detergent 1 was found to be the most effective. As for Detergent 1, the Minimum Inhibition Concentrations (MICs) ranged from 97.656 - 1562.5 µg/ml, while the Minimal Bactericidal

Concentrations (MBCs) were at 781.25 - 3125 µg/ml. Meanwhile, inhibition time assay showed that the bactericidal activity of detergent was fast-acting against antibiotics resistant *V. parahaemolyticus* at 8× MIC within 1 hr and the reduction in CFU/ml was 3 log units (99.9%) with P value < 0.05 was considered statistically significant. The growth of antibiotics resistant *V. parahaemolyticus* biofilm was inhibited at 1562.5 – 6250 µg/ml and was eradicated at 3125 – 12500 mg/ml. Then, the study was furthered by using natural antimicrobial herbs and spices in inhibiting and in preventing the growth of *V. parahaemolyticus* biofilm. Out of the twenty herbs and spices, four of them, whichwere cloves, star anise, lemon leaves, and curry leaves, showedsome activities towards the antibiotics resistant isolates. At a minimum of 10 mg/ml (1000 µg/ml), clove was the most effective as it showed MICs at 19.531-78.125 µg/ml and MBC at 78.125-625 µg/ml. The killing time activity with cloves was fast-acting against antibiotic resistant *V. parahaemolyticus* at 8× MIC in 0.5hr time, while 1 hr, 4 hr, and 8 hr with star anise, lemon leaves, and curry leaves extracts. The reduction in CFU/ml was 3 log units (99.9%). Besides, cloves demonstrated concentration that ranged from 78.125 – 156.25 µg/ml, and 312.5 – 625 µg/ml to inhibit and to totally eradicate the growth of antibiotic resistant *V. parahaemolyticus* biofilm. Thus, these findings can be used to mitigate the resistance of biofilm growth in any food supply chain related to seafood in future.

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

KESAN DETERGEN, HERBA DAN REMPAH RATUS KE ATAS PERTUMBUHAN *Vibrio parahaemolyticus* BIOFILEM DALAM MAKANAN LAUT

Oleh

ELEXSON NILLIAN

September 2014

Pengerusi : Profesor Son Radu, PhD
Fakulti : Sains dan Teknologi Makanan

Penyelidikan ini bertujuan untuk mengenalpasti kesan detergen, herba dan rempah ratus dengan aktiviti mikrobial yang tinggi dan spesifikasi khusus terhadap pengurangan pertumbuhan bio filem *V. parahaemolyticus* . Sejumlah 394 sampel telah di kumpul daripada pasar borong (n=201) dan pasaraya (n=193) yang terdiri daripada kerang (*Anadara granosa*), kepah (*Mya arenaria*), udang (*Penaeous spp.*) dan sotong (*Loligo opalescens)*. Kelaziman sebanyak 9.14 % (36/394) positif gen t*oxR*, 63.8% kehadiran gen virulen *V. parahaemolyticus tdh+*, 22.2% *V. parahaemolyticus trh+*, dan 5.56% (2/36) pencilan *V. parahaemolyticus* mempunyai kedua-dua *tdh+* and *trh+* gen. Sejumlah tiga puluh enam (n= 36) pencilan tersebut telah di uji terhadap ujian keberkesanan antibiotik dan kepelbagaian rintangan kepada sekurangnya terhadap tujuh daripada lapan belas antibiotik yang di kenalpasti.Pencilan *V. parahaemolyticus* (97.2%) menunjukkan Kepelbagaian rintangan indek > 0.2, menunjukkan pencilan ini kemungkinan bersal daripada sumber yang berisiko tinggi. RAPD - PCR telah di lanjutkan dengan mengenal pasti kepelbagaian genetik dan pengkaitan klon di antara pencilan. Setelah dendogram di hasilkan, daripada empat kumpulan, kumpulan jenis B adalah kumpulan terbesar mengandungi 92.86% (13/14) pencilan terdiri daripada makanan laut seperti kerang dan kepah. Dimana diperolehi daripada pasar borong dan berkait rapat. Kaedah RAPD-PCR cap jari dan kepelbagain rintangan antibitotik profil menunjukkan pengandaan kepelbagaian rintangan antibiotik *V. parahaemolyticus* tersebar secara meluas di antara kumpulan. Pencilan diperiksa dengan mengakses penghasilan bio filem di dalam suhu yang berbeza *V. parahaemolyticus* boleh di bentuk dan terdapat pertumbuhan bio filem di 37 º C (suhu optimum), di ikuti oleh suhu bilik (25 ºC) and with 4° C (suhu pendingin). Wakil dari setiap klon daripada setiap kumpulan yang mempunyai pengandaan rintangan antibiotik *V. parahaemolitikus* telah di periksa ke atas kesan detergen. Daripada tiga pencuci, detergen 1, (berasaskan Linear Alkylbenzene sulphonate), detergen 2 (berasaskan Quaternary Ammonium) dan detergen 3 (berasaskan Sodium Hydroxide) mempunyai kepekatan minimum

sebnayak 100 mg/ml (100000 µg/ml), detergen 1 di dapati paling efektif. Untuk detergen 1, Perencatan kepekatan minimum (MICs) diantara 97.656 - 1562.5 µg/ml manakala minimum kepekatan penbunuhan (MBCs) di antara 781.25 - 3125 µg/ml. Assay masa membunuh menunjukkan aktiviti pembunuhan pencuci adalah bertindak pantas melawan rintangan antibiotik *V. parahaemolyticus* di 8× MIC di dalam 1 jam dan pengurangan di CFU/ml adalah 3 log units (99.9%) dengan nilai $P < 0.05$ adalah di anggap tidak ada perbezaan signifikan statistik. Pertumbuhan bio filem rintangan antibiotik *V. parahaemolyticus* boleh di rencat di 1562.5 – 6250 µg/ml di basmi di 3125 – 12500 mg/ml. Kemudian, kajian telah di lanjutkan untuk memeriksa menggunakan herba dan rempah ratus untuk merencat dan menghalang pertumbuhan *V. parahaemolyticus* bio filem. Daripada dua puluh herba dan rempah ratus, empat termasuk cengkih, bunga lawang, daun limau dan daun kari menunjukkan aktiviti terhadap pencilan rintangan antibiotik. Di kepekatan minimum 10 mg/ml (1000 µg/ml), cengkih adalah paling efektif menunjukkan MICs di 19.531 - 78.125 µg/ml dan MBC 78.125 - 625 µg/ml. Aktiviti masa membunuh cengkih adalah pantas bertindak terhadap rintangan antibiotik *V. parahaemolyticus* di 8× MIC dalam 0.5 jam manakala 1 jam, 4 jam and 8 jam oleh ekstrak bunga lawang, daun limau dan daun kari, Pengurangan di CFU/ml adalah 3 log units (99.9%). Cengkih demontrasi kepekatan di antara 78.125 – 156.25 µg/ml dan 312.5 – 625 µg/ml untuk merencat dan membasmi sepenuhnya pertumbuhan bio filem *V. parahaemolyticus*. Maka, penemuan ini boleh digunakan untuk mengurangkan pertumbuhan kerintangan bio filem di dalam rantaian bekalan makanan berhubung dengan makanan laut di masa hadapan.

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I certify that a Thesis Examination Committee has met on 23 June 2014 to conduct the final examination of Elexson Nillian on his thesis entitled "Effect of Detergent, Herbs and Spices on the Growth of *Vibrio parahaemolyticus* Biofilm in Seafood" 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 Doctor of Philosophy.

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

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µl	-	Microliter
µm	-	Micrometer
µg	-	Microgram
ADI	-	Acceptable daily intake
ATCC	-	American Type Culture Collection
BAM	-	Bacteriological Analytical Manual
bp	-	base pair
CDC	-	Centre for Disease Control
D1		Detergent 1
D2	-	Detergent 2
D3	-	Detergent 3
DNA	-	Deoxynucleic acid
dNTP	-	deoxyribo nucleoside triphospate
EDTA	-	Ethylene diamine tetra-acetic acid
FAO	-	Food and Agricultural Organization
FDA	-	Food and Drug Administration
HIV	-	Human immunodeficiency virus
ICMSF	-	International Commission on Microbiological
MAR	-	Multiple Antibiotic Resistance
mg	-	milligram
$MgCl_2$	-	Magnesium Chloride
MIC	-	Minimum inhibitory concentration
MBC	-	Minimum inhibitory concentration
MBEC	-	Minimum bactericidal eradication concentration
mM	-	milli Molar
MOH	-	Ministry of Health
MPN	-	Most probable Number
MRO	-	Multiple resistant organism
NaCl	-	Sodium chloride
NCCLS	-	National Committee for Clinical Laboratory Standard
OD	-	Optical Density
PCR	-	Polymerase Chain Reaction
RAPD	-	Randomly Amplified Polymorphic DNA
SPB	-	Salt Polymyxin Broth
SMIC	-	Sessile Minimum inhibitory concentration
Taq	-	*Thermus aquaticus*
TBE	-	Tris-Boric acid-EDTA
TSA	-	Trypticase Soy Agar
TSB	-	Tryptic soy broth
U	-	Unit
U. S	-	United States
UV	-	Ultra violet
V	-	Volt
WHO	-	World Health Organization
WTO	-	World Trade Organization
x g	-	unit gravity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

In nature, most bacteria exist as plankton (free floating) cells or suspended cells (sessile cells), and some exist on surfaces. According to the National Institute of Health (NIH) in the United States, 80 % of microbial can form biofilm and this includes food borne pathogens, such as *Salmonella* spp., *Klebsiella* spp., *Pseudomonas* spp., *Campylobacter* spp., *Escherichia coli*, and *Listeria* spp. These bacteria are of special significance in ready-to-eat and minimally processed food products (Kim et al., 2006). Bacteria have the capability to attach themselves and to colonize surfaces in many natural and artificial surfaces, which are called biofilms.

Biofilm is known as a complex community that consists of microorganisms embodied in a matrix of extracellular polymeric material that they have generated. Thus, a mass or biofilm is established when bacteria adhere to the surface and produce extracellular polysaccharides (EPS). The EPS matrix consists of not only polysaccharides, but also proteins, which may be the major component in environmental and waste water biofilms and nucleic acids. This environment increases the resistance of bacteria to detergents and antibiotics as the dense extracellular matrix and the outer layer of cells protect the interior of the community (Merill, 2010).

Vibrio parahaemolyticus is a bacterium that is dangerous because it is one of the main causes of contamination in seafood, which can cause gastroenteritis worldwide (Lee, & Pogliano, 2007). *V. parahaemolyticus* is marine seafood borne pathogen, which causes gastroenteritis in humans (Miwatani, & Takeda, 1976). It has been recognized as a common cause of food borne illnesses in many Asian countries, including Japan, Taiwan, and China. According to Su and Liu (2007), it is the leading cause of human gastroenteritis in the United States and it is an unsafe seafood borne pathogen throughout the world.

Polymerase Chain Reaction (PCR) was adopted due to its specificity, easily automated, and its ability to amplify the amount of sample within few minutes if food poisoning outbreak occurs. Besides, in order to plan and to monitor targeted intervention strategies at preserving the therapeutic efficacy of antimicrobial agents, surveillance of antibiotic resistance is a key element to provide the latest information on the magnitude and the trends in resistance. On top of that, RAPD-PCR analysis was used to determine the relationship between antibiotic and isolates that were recovered from different habitats based on their RAPD-PCR fingerprints. Antibiotic resistance and RAPD proved to be

effective tools in characterizing and in differentiating the *V. parahaemolyticus* strains (Lesley et al., 2005; Tunung et al., 2007).

In addition, food processing environments provide a variety of conditions, which might favor the formation of biofilm with the presence of moisture, nutrients, and inocula of microorganism from raw materials. Bacterial colonization of food processing equipment and facilities is the main concern and is a potential source of contamination of foods that may lead to spoilage or transmission of food borne pathogens. Moreover, when biofilm detaches from the surfaces, individual microorganisms can easily spread.

In seafood food supply chain from pre harvest to marketable level, safe and effective detergent applied on the food contact surfaces and food facilities or equipment will ensure an acceptable decrease in microbial levels without the presence of toxic residuals or causing any damage for humans and useful forms of life. Natural antimicrobials nowadays are favorable concern to several issues pertaining to microorganisms. These include those that reduce the need of antibiotics and monitor microbial contamination in food. Due to the generally recognized as safe (GRAS) properties, herbs and spices have appeared to be natural antimicrobial, which could meet consumer demands for safe and healthier food consumption without any undesirable effect when applied in the food system.

In seafood industrial practice, it is necessary to develop a complete and a cost-effective cleaning program, which will inhibit the accumulation of both particulates and sessile bacterial cells present on the surfaces of food and processing line. Thus, in order to get rid of this problem, this study was carried out to investigate the effects of detergent, herbs, and spices on the growth of *V. parahaemolyticus* biofilm. Therefore, the objectives of this study were:

1. To determine the prevalence of *Vibrio parahaemolyticus,* regulator gene *toxR* gene, and virulence gene (*trh, tdh*) from seafood using polymerase chain reaction.

2. To profile *V. parahaemolyticus* based on antibiotics resistance and Random Amplified Polymorphism DNA–PCR fingerprinting.

3. To compare the formation of *V. parahaemolyticus* biofilms with varying temperatures: chiller temperature (4 ºC), room temperature (25 ºC), and optimum temperature (37 ºC).

4. To determine the effect of detergents against growth of antibiotic-resistant *V. parahaemolyticus* biofilm.

5. To determine the effects of herbs and spices against growth of antibiotic-resistant *V. parahaemolyticus* biofilm.

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