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# Isolation and Identification of Bacteria Micro Flora of White Shrimp, *Litopenaeus vannamei*, with Antagonistic Properties Against *Vibrio* Species

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## ABSTRACT

The emergence of antibiotic-resistant bacterial pathogen has led aquaculture attentions to the use of probiotics as an alternative to antibiotics. This study was conducted to isolate bacterial micro flora from digestive tract of healthy juvenile white shrimp, *Litopenaeus vannamei* based on antagonistic activity against shrimp pathogen, *Vibrio parahaemolyticus*. In this study, potential probiotic strains were isolated using replica plating method to screen the bacteria with antagonism properties. Six isolates were identified as *Pseudomonas* sp. using conventional biochemical tests and Biolog GN microplates. In addition three *Vibrio* species including *V. parahaemolyticus, Vibrio alginolyticus* and *Vibrio cholerae* were isolated from moribund shrimp and used as pathogen in this study. The pathogenicity of three *Vibrio* species was tested on shrimp *L. vannamei* by injecting 0.1 mL of $10^7$ CFU $mL^{-1}$ of each pathogen into the third abdominal segment. Among three *Vibrio* species only *V. parahaemolyticus* found to be highly virulent to shrimp with 43% cumulated mortality after 10 days. Subsequently, all *Pseudomonas* sp. isolates were tested for antibacterial activity against three *Vibrio* species using cross streak assay. Strong antibacterial activity was recorded for *Pseudomonas* sp. isolates number 5, 7, 15 and 30 against three pathogens. In addition a reasonable antibacterial activity was observed for isolates number 9 and 12. On the basis of great antibacterial activity of *Pseudomonas* sp. isolates these species may be considered for future challenge experiments in shrimp as a very promising alternative to the use of antibiotics.

**Key words:** *Litopenaeus vannamei*, vibriosis, antibacterial activity, *Pseudomonas* sp.

## INTRODUCTION

Shrimp farming is one of the significant aquaculture activities in many tropical countries with a rapid increasing of productions. However, this industrial development has been accompanied with some environmental impacts, which negatively have influenced shrimps and their aquatic environment. The rate of diseases in aquaculture industry has increased due to the intensification of aquaculture activities (Shariff *et al*., 2001). Pathogenic bacteria have also been involved in this crisis. *Vibrio* species are among the most important bacterial pathogens of cultured shrimp, responsible for up to 100% stricken. Species such as *Vibrio harveyi, Vibrio anguillarum, V. parahaemolyticus* and *Vibrio vulnificus* have been frequently associated with mortalities both in hatcheries and grow out ponds (Baticados *et al*., 1990; Mohney *et al*., 1994).

Traditionally, application of chemical compounds and antimicrobial drugs has been considered to control the infectious problems in penaeid hatcheries. For instance chlorine is widely used in hatcheries and ponds; however, it is able to stimulate the development of multiple antibiotic resistance genes in bacteria (Balcazar *et al*., 2006). In addition, Moriarty (1997) already stated that the use of antibiotics in aquaculture for disease control has been accompanied by potential negative consequences including drug resistance arising in microorganisms through adaptation or by genetic exchange. Therefore the use of probiotic bacteria as an alternative to antibiotics and antimicrobial drugs has been suggested to control and manage the disease problems. Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2001). A wide range of probiotics have been examined for use in aquaculture such as lactic acid bacteria (Balcazar *et al*., 2007; Vaazquez *et al*., 2005), *Pseudomonas* sp. (Alavandi *et al*., 2004; Chythanya *et al*., 2002; Vijayan *et al*., 2006), *Shewanella algae* (Zadeh *et al*., 2010; Shakibazadeh *et al*., 2008) *Bacillus* spp. (Lalloo *et al*., 2007; Zokaeifar *et al*., 2012a; Far *et al*., 2009) and commercial probiotics (Balasundaram *et al*., 2012).

A common way to select probiotics is to perform identification of candidate bacteria and *in vitro* antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products (Zokaeifar *et al*., 2012b). Given this the present study aimed to isolate beneficial antagonistic bacteria as probiotics from digestive tract of health juvenile white shrimp, *Litopenaeus vannamei*. In addition candidate antagonism bacteria were identified using conventional biochemical tests and also Biolog Microlog software.

## MATERIALS AND METHODS

**Location of study and rearing condition:** This study was conducted in the Hatchery complex, Department of Aquaculture, Faculty of Agriculture, University Putra Malaysia. Healthy shrimp larvae, *L. vannamei*, were provided from local hatchery and reared in aquaria measuring 0.85×0.38×0.24 $m^3$ for one month to eliminate the weaker ones. The larvae were fed with *Artemia* and commercial pellet feed, from Vietnam containing 38% crude protein at daily rate of 8% b.wt., three times daily. During rearing period, pH, salinity and temperature was monitored daily using a pH meter (YSI, USA) and a hand refractometer (Atago, 8808).

***Artemia* preparation:** *Artemia* cysts (supreme Plus, Golden Mark Brand) were hatched in conical fiberglass tanks with continues strong aeration under the optimal hatching condition (2 g $L^{-1}$; 20-30 ppt; 25-30°C) a day before feeding. *Artemia* cysts hatched after 18-24 h of incubation. Newly hatched *Artemia* nauplii were harvested by covering the top portion of the tank with a black material to let nauplii settle at the bottom. Accumulated *Artemia* nauplii were then siphoned out from the bottom of hatching tanks thoroughly. *Artemia* nauplii were then filtered with plankton net (80-120 μm) and washed thoroughly before being fed to the larvae.

**Isolation of bacterial strain as pathogen:** *Vibrio parahaemolyticus, V. alginolyticus* and *V. cholerae* which were isolated from digestive tract of moribund shrimp were used for antagonism test. Briefly digestive tract of moribund shrimp were dissected aseptically and homogenized in sterile distilled water. Samples were then serially 10-fold diluted in Normal Saline Solution (NSS) and subsequently 0.1 mL of each dilution was plated in Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar, Difco). After 24 h incubation in 30°C single colonies were picked and preserved in 15% glycerol at -80°C for further investigation and identification.

**Shrimp challenge test with isolated pathogen bacteria:** Shrimp, *L. vannamei* was challenged with isolated *Vibrio* to confirm the bacterial pathogenicity using injection method. Healthy juveniles weighing approximately 4 g were acclimatized for one week in 20 ppt seawater. Shrimps were then distributed into 70 L glass aquaria of 15 animals each with a constant aeration. Three groups of shrimp in triplicates were separately injected with 0.1 mL of $10^7$ CFU $mL^{-1}$ of *V. parahaemolyticus, V. alginolyticus and V. cholera*. The third group was shrimp injected with 0.1 mL of sterile NSS as control. All shrimps were injected at the third abdominal segment using 1 mL sterile insulin syringe (29 G). Cumulated mortality was monitored daily for up to 15 days.

**Isolation and screening for bacteria with antibacterial activity:** Shrimps were washed gently in sterile distilled water to remove loosely adhering particles. Samples were dissected aseptically to remove digestive tract. All samples were homogenized in sterile distilled water and diluted up to $10^{-16}$. Aliquot of 0.1 mL from each dilution were spread plated in two replicate. To ensure maximum recovery of cultivable bacteria, samples were cultured in marine blood agar, Tryptic Soya Agar, TSA (Difco, US). After incubation single colonies were replica plated onto plates containing 10 mL Muller Hinton Agar (MHA) supplemented with 1% NaCl and *V. parahaemolyticus* which was previously grown overnight in LB broth (Difco, USA). Plates were then incubated for 24 h at 30°C. Clear zones around replica-plated bacteria were interpreted as antagonistic activity against the pathogen and they were picked from the original plates. Antagonistic strains were stored in glycerol at -80°C for further investigation.

***In vitro* antagonism assay by cross streak method:** For the study of inhibition by cross-streak method, an 18 h cultured of candidates bacteria in LB (Difco, US) were streaked as 2 cm thick band, across the diameter of the Muller-Hinton agar plate, MH (Difco, US). After incubation for 24 h at 30°C, the growth bacteria were scraped with a sterile slide. The remaining bacteria were killed by exposure to 5 mL chloroform ($CHCl_3$) poured on the glass lid and left for 15 min by keeping the medium inverted over the lid. The plates were then air dried for about 10 min to remove any residual chloroform. Then three different pathogens (*V. parahaemolyticus, V. alginolyticus* and *V. cholerae*) were used to evaluate the antagonist ability of the candidate bacteria and the same bacteria was streaked as positive control and the LB broth medium as negative control, perpendicular to candidates band using sterile swab dipped in an 18 h old culture of pathogen bacteria regulated by spectrophotometer to McFarland concentration (625 nm, OD: 0.08-0.1). Final concentration by this regulation was around $10^6$ CFU $mL^{-1}$. The resulted plates were incubated for 24 h at 30°C. The linear zone of inhibition was recorded in each case (Chythanya *et al*., 2002).

**Identification of bacterial strains:** Conventional biochemical methods followed by Biolog GN microplates (Biolog, Hayward, CA, USA) (Olsson *et al*., 2004) were applied for all identifications. After the Gram-staining and morphological characteristics, strains were subjected to phenotypic tests for conventional identification, including catalase, oxidase, motility, urease, citrate, indole, Voges-Proskauer, $H_2S$ production, glucose, inositol, manitol and reduction ability of nitrate to nitrite.

## RESULTS

**Water quality parameters:** The temperature was maintained at 26-28°C and the range of water salinity of the water was 25-28 g $L^{-1}$. Total ammonium (0-0.1 mg $L^{-1}$), nitrite (0-0.05 mg $L^{-1}$) and pH (7.0-7.6) were stable and within acceptable ranges (Boyd and Tucker, 1998).

**Identification of bacterial isolates:** All bacterial isolates including pathogens and candidate antagonistic isolates were identified using conventional biochemical test followed by Biolog GN microplates and Microlog Software. Identification of pathogenic bacteria using biochemical tests showed the assessment of three species of Vibrionaceae including *V. parahaemolyticus, V. alginolyticus* and *V. cholera* (Table 1). On the other hand, candidate antagonistic strains were identified as *Pseudomonas* sp. using biochemical tests. Bacterial identification using biochemical tests were confirmed with identification using Biolog Microlog Software where the same identifications were resulted.

**Isolation and assessment of pathogenic bacterial strains:** Based on the morphological observations in TCBS agar, three different species including *V. parahaemolyticus, V. alginolyticus* and *V. cholera* were successfully isolated and identified. Subsequently they were subjected for their virulent effects and pathogenicity on white shrimp *L. vannamei* using the injection method. Cumulated mortality of shrimp infected with *V. parahaemolyticus* was recorded 43% after 10 days of injection, however no mortality was observed for those shrimp injected with *V. alginolyticus* and *V. cholera*. These results clearly revealed the pathogenicity effect of *V. parahaemolyticus* for white shrimp *L. vannamei*.

**Isolation and assessment of antagonistic bacterial strains:** A total of 43 bacteria were isolated from digestive tract of shrimp, *L. vannamei*. They were tested for their possibility of antibacterial activity against *V. parahaemolyticus* using replica-plating method. Only six bacterial isolates produced clearing zones of different sizes. To confirm the antagonistic activity of isolates, cross streak assay was carried out. Out of six antagonistic isolates, four isolates (isolates: 5, 7, 15 and 30) displayed strong inhibitory activity against *V. parahaemolyticus, V. alginolyticus* and *V. cholera* (Fig. 2). Results of antagonistic test of four *Pseudomonas* sp. isolated bacteria showed great antagonistic activity against three pathogens (Fig. 2) while the other two *Pseudomonas* sp. (isolates: 9 and 12) did not exhibit strong antagonistic ability against three pathogens

Table 1: Biochemical characteristics of pathogens and candidate antagonistic bacteria

| | | | | *Pseudomonas* isolate | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Test | *V. parahaemolyticus* | *V. alginolyticus* | *V. cholerae* | 5 | 7 | 9 | 12 | 15 | 30 |
| Gram | - | - | - | - | - | - | - | - | - |
| Oxidase | + | + | + | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | + | + | + |
| Motility | + | + | + | + | + | + | + | + | + |
| Fermentative | + | + | + | + | + | + | + | + | + |
| Urease | - | - | - | - | - | - | - | - | - |
| Citrate | + | + | + | + | + | + | + | + | + |
| Indole | + | + | + | - | - | - | - | - | - |
| VP | - | - | - | - | - | - | - | - | - |
| $H_2S$ | - | - | - | - | - | - | - | - | - |
| Glucose | + | + | + | - | - | - | - | - | - |
| Inositol | + | - | - | + | + | + | + | + | + |
| Manitol | + | + | + | - | - | - | - | - | - |
| Nitrate reduction | + | + | + | + | + | + | + | + | + |

+: Positive reaction, -: Negative reaction

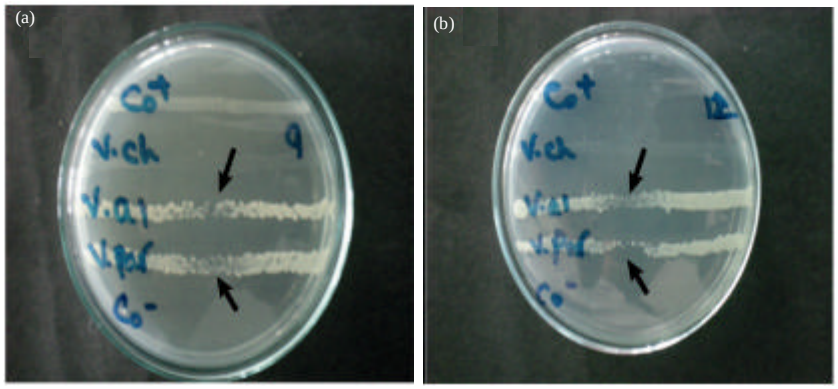


Fig. 1(a-b): Low inhibitory effect of two *Pseudomonas* sp. isolates No., (a) 9 and (b) 12 against three pathogens *V. parahaemolyticus* (V. par), *V. alginolyticus* (V. al) and *V. cholerae* (V. ch) in cross-streak method, $Co^+$: Positive control, $Co^-$: Negative control, Arrows show the linear zone of inhibition

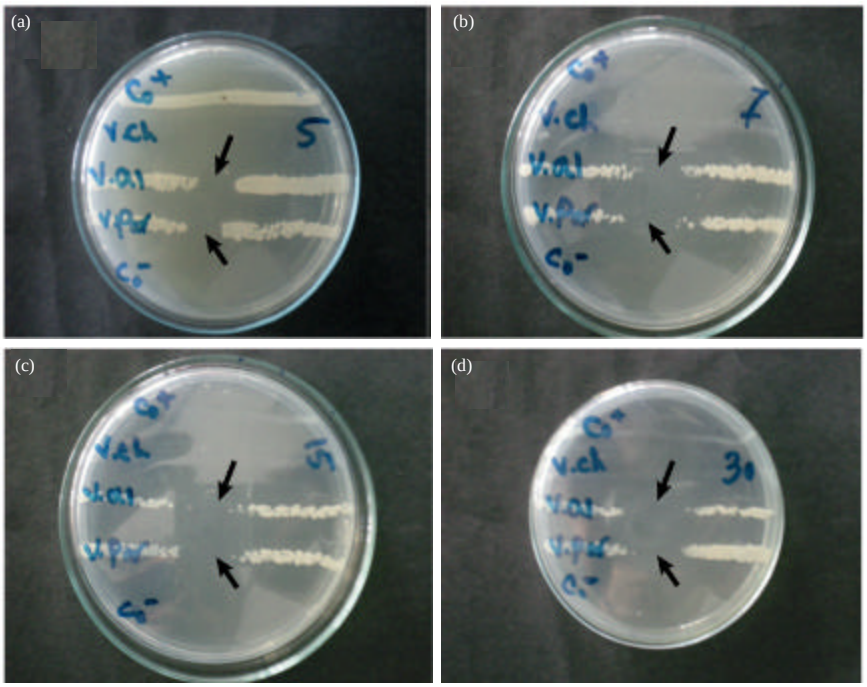


Fig. 2(a-d): Inhibition zone of four isolated *Pseudomonas* sp. isolates No., (a) 5, (b) 7, (c) 15 and (d) 30 against *V. parahaemolyticus* (V. par), *V. alginolyticus* (V. al) and *V. cholerae* (V. ch) in cross-streak method, $Co^+$: Positive control, $Co^-$: Negative control, Arrows show the linear zone of inhibition

(Fig. 1). The linear zones of inhibition for isolate number 5 (Fig. 2a) were 5, 10 and 14 mm for *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*, respectively. The linear zones of inhibition activity for isolate number 7 (Fig. 2b) were 15, 14 and 12 mm for *V. parahaemolyticus*,

*V. alginolyticus* and *V. cholera,* respectively. The linear zones of inhibition for isolate number 15 (Fig. 2c) were 15, 16 and 13 mm for *V. parahaemolyticus*, *V. alginolyticus* and *V. cholera,* respectively. The linear zones of inhibition for isolate number 30 (Fig. 2d) were 16, 17 mm for *V. parahaemolyticus*, *V. alginolyticus,* respectively, without any zone for *V. cholera.*

## DISCUSSION

In selecting a potential probiotic strain for beneficial health effects on the host, many criteria must be examined. Considering the infectious problems in shrimp aquaculture, antagonism properties against pathogen could be a key factor of candidate strains. Although, isolation and screening of potential probiotics is usually a long process and time-consuming, the replica-plating method could be used successfully for the isolation of bacterial strains with antibacterial properties (Hjelm *et al*., 2004). In the present study candidate probiotic bacteria were isolated during a course of one week, using replica plating method, based on the antibacterial activity against shrimp pathogens.

Before the experiment was conducted we isolated and identified the pathogen bacteria from moribund shrimp and subsequently their pathogenicity to white shrimp, *L. vannamei* was examined. Among the isolated pathogens only *V. parahaemolyticus* showed highly virulent to shrimp, however, all pathogen isolates were subjected for cross streak antagonism assay.

Six out of 43 isolated strains exhibited inhibitory activity in replica plating method; however, using the cross streak method to confirm the antagonism properties, four isolates showed strong inhibitory activity against *V. parahaemolyticus*, *V. alginolyticus* and *V. cholera.* Although the other two isolates did not show strong antagonistic activity (Fig. 1) against pathogens, but the reasonable inhibitory activity of these two isolates might suggest these bacteria as candidate probiotics. All candidate isolates were identified as *Pseudomonas* sp. using two methods of conventional biochemical tests and Biolog GN microplates using Microlog Software.

Isolation and antagonistic activity of *Pseudomonas* sp. against pathogens has been reported by literatures. For instance, Alavandi *et al.* (2004) isolated *Pseudomonas* sp. PM11 as candidate probiotics from the gut of farm-reared sub adult shrimp and tested for their effect on the immunity indicators of black tiger shrimp. This study reported promising *in vitro* results for shrimp culture. In another study, Irianto and Austin (2002) reported the inhibitory roles of *Pseudomonas* I-2 against *V. harveyi and V. fluvialis* in shrimp culture. Additionally, Gram *et al.* (1999) reported that a *Pseudomonas fluorescens* AH2 reduced the mortality of rainbow trout (*Oncorhynchus mykiss*) infected with a pathogenic *V. anguillarum* strain.

*In vitro* promising achievements were resulted from the present study where strong anti-vibrio activities of four *Pseudomonas* sp. isolates were recorded. Vijayan *et al.* (2006) reported the production of siderophores by *Pseudomonas* PS-102, responsible for inhibition of pathogenic vibrios and *Aeromonas* spp. Therefore it can be concluded that the inhibition of pathogens in the present study might be due the siderophore production of *Pseudomonas* sp. isolated from digestive tract of white shrimp, *L. vannamei.* Gatesoupe (1997) already stated that harmless bacteria with the ability to produce siderophores have been used as probiotics to compete and overgrow pathogenic microbes.

## CONCLUSION

We report on isolation and identification of six candidate probiotics, *Pseudomonas* sp. which showed probiotic properties in terms of inhibitory activity against pathogenic *Vibrio* species. This could be a promising result for shrimp aquaculture that has been affected by vibriosis during the recent years.

Further experiments, including the application of candidate probiotics and shrimp challenged with pathogens, could provide valuable information of probiotic potential abilities of these candidates for shrimp aquaculture facilities.

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