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Characterization of potential probionts from blue swimming crab *Portunus pelagicus* and its antagonistic activity against *Vibrio harveyi*

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Abstract

The aim of this study was to isolate local bacteria as potential probiont in controlling the growth of pathogenic *Vibrio harveyi*. Eleven potential bacteria were successfully isolated from the haemolymph of four healthy *Portunus pelagicus*. All of the isolates were identified as *Bacillus amyloliquefaciens* by series of biochemical test using triple sugar ion test, oxidase and catalase test, followed by Internal Transcribe Spacer (ITS) gene sequence analysis. This isolate was able to inhibit the growth of *V. harveyi* in *in-vitro* screening assay by using well diffusion assay with the strong antagonistic activity from 7 to 16 mm. The potential strains at 10^8 CFU/ml showed highest inhibition response towards *V. harveyi* after co-cultured for 48 hr. The isolates produced four major extracellular enzymes which were amylase, protease, gelatinase and lipase and able to form biofilm after 24 hr of cultured. Thus, *B. amyloliquefaciens* showed important characteristics as probiotic which worth to be carried out for *in vivo* challenge assay.

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Introduction

The blue swimming crab, *Portunus pelagicus* (Linnaeus 1758) also known as flower crab which is one of the species that being cultured in Malaysia. These crabs form a basis of important aquaculture commercial and recreational fisheries (Talpur *et al.,* 2011). However, disease outbreaks and environmental factors have been blamed for some of the variation in crab production. Many blue crab mortalities are attributed to systemic bacterial infections, especially when the animals are subjected to crowded and confined conditions (Gretchen and Carl, 1992). Microbial infections have been a major concern of crab culture especially at larval and juvenile stages. The zoeal stages of blue crabs are often exposed to pathogenic bacteria such as *Vibrio* sp. and *Pseudomonas* sp. (Jithendran *et al.,* 2009). The genus *Vibrio* such as *V. harveyi*, *V. parahemolyticus*, *V. cholerae* and *V. vulnificus* were commonly found in blue crab (Wang, 2010). According to Laferty *et al.,* (2015), certain infectious diseases are common in the marine culture and it could affect the quality and survival of the some species. For examples, wild infected disease species would transferred the disease to farm species and back to wild environment. This circle of infectious are continues from recent areas to another and also to other species.

Several options have been made to overcome this bacterial disease and one of the ways is antibiotics (Heppell and Davis, 2000). An antibiotic has been identified as best, effective and cheapest methods that are reliable to be used in large amount. However, the antibiotics residues remained in culture tissues after harvesting process (Shakila *et al.,* 2006). A wide range, intensive, and misuse of antibiotic in infected culture led to raise a resistance phenomenon among the target pathogen (Catry *et al.,* 2003; Kümmerer, 2004). The failure of treatments can cause mortality to the culture (Molina-Aja *et al.,* 2002).

As an alternative, probiotics has been used which may reduce the frequency of microbial proliferation or diminish the sternness of disease eruptions. Numerous researches have reported encouraging results in the application of probiotics in aquaculture (Vine *et al.,* 2006; Wang *et al.,* 2010).

Consequently, the use of probiotics in the aquaculture is increasing with the demand for more environmentally-friendly aquaculture practices (Gatesoupe, 1999). The use of probiotics is prevalent in the aquaculture industry as means of improving water quality by balancing bacterial load and replacing the use of antibiotics/biocides, production of supplemental digestive enzymes, lower incidence of diseases, improved survival, and improved immune response (Verschuere *et al.,* 2000). However, the effectiveness of the probiotic also depends on reaction and responds of the host and other surrounding microbes.

There are two ways that probiotics can be directly deliver into cultured organism. It can be blended with feed and administrated orally to enhance beneficial microbial flora of the cultured gut. Secondly, probiotics could proliferate in water medium and exclude the pathogenic bacteria by consuming all available nutrients. Other than that, probiotic therapy and dietary intake need to be align and in precise concentration for them to work perfectly and not only for single health benefit (Reid, 2016). In aquaculture field, probiotic is commonly isolated from fish and shrimp. However, several criteria should be considered including the test based on *in vitro* and *in vivo* which could combined together for better understanding of characteristics and properties of the every potential probiotic (Blajman *et al.,* 2015).

As so far, well study have been done on interaction between host and bacteria, however specific study should be considered based on geographical area and also local need. Thus, this study was undertaken to isolate the potential local bacteria from adult's blue crab to inhibit the growth of pathogenic *V. harveyi* and subsequently be used as potential probiont for aquaculture.

Materials and methods

Adult living blue crab, *Portunus pelagicus* (two male and two female) with weight ranging 100- 200g were collected from Centre of Marine Science (COMAS) UPM, Port Dickson, Negeri Sembilan.

The blue crabs were kept alive with water and oxygen supply during transportation to the Fish Health Laboratory, Faculty of Agriculture, UPM, Serdang in polypropylene box.

Isolation of potential bacteria

The potential bacteria were isolated from seven different parts of the crabs. Crabs were washed with sterile saline water to remove any contaminant. Gut, muscle, and gill were aseptically removed using a sterile sharp knife and homogenized by using sterilized mortar and pastel.

The haemolymph was collected using 23-gauge needle by penetrating the intersegment membrane between the posterior of the carapace and the abdomen after the site disinfected by alcohol. The samples were serially diluted with PBS. A 100µl of sample from each dilution was plated on Tryptic Soy Agar (TSA, Difco™ Merck, Germany) with addition of 1.5% of NaCl and spread evenly by using sterile glass hockey stick. All the plates were incubated at 30°C for 24h. Total of 119 pure colonies were picked by choosing different morphology and colors. Then, all the isolates were streaked onto Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Difco™ Merck, Germany) to discard vibrios.

Detection of antagonistic activity

The antagonistic activity of the potential probionts against *V. harveyi* was determined using spot lawn, disc diffusion and well diffusion assay with minor modifications.

Spot lawn assay

The potential isolates and pathogenic *V. harveyi* were cultured individually in 10 ml of TSB for overnight at 30°C. Then the isolates were washed three times using sterilized saline and centrifuged (Eppendorf, 5804R) at 5000 rpm for 10 minutes. Potential isolates were adjusted to 10^6 CFU/ml while pathogenic *V. harveyi* was adjusted to 10^5 CFU/ml. A 100µl of *V. harveyi* was swabbed evenly onto the surface of TSA agar plate and allowed to dry for 15 minutes. A 3µL of the potential candidate was spotted on top of the agar surface and incubated at 30°C for overnight. The clear zone was observed and measured.

Disc diffusion assay

Potential isolates and *V. harveyi* were prepared using the same method as spot lawn assay. Then, 100µl overnight culture of *V. harveyi* was transferred onto the surface of TSA agar plate and swabbed evenly.

The plates were allowed to dry for 15 minutes and the disc was dipped into overnight culture of potential isolates and placed onto the TSA plate using sterile forceps. A commercial antibiotics disc, Streptomycin (S10) was used as positive control while sterile distilled water as a negative control. The plates were incubated at 30°C for overnight. After incubation, the size of the inhibition zones was measured.

Well diffusion assay

A 100 µl overnight culture of *V. harveyi* was adjusted to 10^5 CFU/ml and transferred onto the surface of TSA agar, spread evenly and allowed to dry for 15 minutes. Then, wells were made on the agar by punched (5mm) into the agar and 20µl overnight culture of potential isolates (10^6 CFU/ml) was filled up into the well. All the plates were incubated at 30°C for overnight and diameter of inhibitory zone was measured. A commercial antibiotic, Streptomycin was used as positive control and sterile water was used as negative control.

Co-culture assay

Potential isolates at concentration of 10^6 and 10^8 CFU/ml were co-cultured with *V. harveyi* (10^5 CFU/ml) individually and incubated at 30°C for overnight with shaking (140rpm). Samples were taken at different incubation time (0, 6, 12, 24, 48, and 96hr). A 10µl of culture from each inoculum from different time interval was spread on TCBS and incubated at 30°C for overnight. The numbers of colonies grown in the plates were observed and recorded.

Identification of potential candidates

Observation of colony morphology

The potential isolates were cultured on the TSA at 30°C for 24h. Gram staining was carried out and the morphology of forming colonies on the plate was observed.

Acid fast staining

A single colony of the overnight culture was picked and smeared onto the glass slide contained a drop of sterile water. Then, the glass slide was passed through the heat to fix the bacterial cells. Next, carbol fuchsin dye reagent was applied on the glass slide covered with paper towel and heated for five minutes. Then, the slide was rinsed with water for 30 seconds and decolorized by placing a drop of acid alcohol and allowed to sit for 10 seconds. After that, the slides were washed directly with tap water for five seconds and counterstained with methylene blue for two minutes. Finally, glass slides were rinsed with water for 30 seconds and blotted dry.

Spore staining

Prepared glass slide fix with isolate was prepared similar to method mentioned in 2.4.2. Then, malachite green was applied on top of the paper towel of the slides and steam for five minutes. Next, the slide was rinsed with water for 30 seconds and counter stained with safranin for one minute. Lastly, the glass slide was rinsed with water and blotted dry.

Biochemical test

The biochemical tests conducted were triple sugar iron test (TSI), oxidase test, and catalase test. The entire tests were followed according to Cappuccino and Sherman, (2011).

Triple sugar iron test (TSI)

Pure cultured of potential isolates were stabbed deep into the bottom of the tube containing TSI media. The tubes were closed tightly and incubated at 37°C for 24hr. The color changes of the medium were observed and recorded.

Oxidase test

A small amount of potential bacteria was obtained by using a sterile swab and placed on sterile filtered paper. A drop of reagent was placed on top of the culture. Changes and reaction within 10-30 seconds was observed and recorded.

Catalase test

A dropped of H_2O_2 (3%) was put on clean slide. One loop full of bacterial growth was emulsified with the H_2O_2 drops. Then the bubble or foaming were observed and recorded.

Sequencing analysis of the internal transcribed spacergene (ITS)

The total genomic DNA of potential probionts was extracted by using the Genaeid™ Genomic DNA Mini Kit. ITS gene was amplified by using polymerase chain reaction method (PCR) from the purified DNA. The primers that have been used are (5'-CGGTGAATACGTTCCCGGGYCTTG-3') for forward and (5'-TTTCRCCTTTCCCTCACGGTA-3') (Diyana-Nadirah and Ina-Salwany, 2016) for reverse primers. The PCR amplification was performed by using Master cycler Gradient PCR system (Eppendorf, Hamburg, Germany) by initial denaturation at 94°C for three minute, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing temperature of the primers at 50°C for one minute, and extension at 72°C for 90 seconds. Negative control with no DNA template was included in every set of reactions. PCR products were analyzed using 1% agarose gel electrophoresis. A 100bp DNA ladder (Fermentas, USA) was used as DNA standard in this study. The purified DNAs were sequenced by First Base Laboratory Sdn. Bhd. Malaysia.

The determined sequences were analyzed using Basic Local Alignment Search Tool (BLAST) program (www.ncbi.nlm.gov/blast/). Then the gene sequence were compare to other known sequence from GenBank database using BLASTn (Nucleotide Basic Local Alignment Search Tool) searches of National Centre for Biotechnology Institute. Bacteria that showed 99 to 100% GenBank similarities on the ITS sequences are members of the same species, while 97 to 99% GenBank similarities are for members of the same genus (Drancourt *et al.*, 2000).

Potential probiont characteristics

Extracellular Enzymes Production

The tests conducted were starch hydrolysis, casein hydrolysis, gelatin hydrolysis and lipases hydrolysis. The entire tests were followed according to Cappuccino and Sherman, (2011).

Starch hydrolysis

The potential isolates were streaked on top of the starch agar and incubated overnight at 30°C. After overnight, iodine solution was applied on top of the starch agar and allowed to sit for 10min. The presence of clear halos surrounding colonies was observed and recorded.

Casein hydrolysis

Potential isolates were streaked onto skim-milk agar and incubated at 30°C for 24hr. After incubation, plates were flooded with 1% acid hydrochloric (HCl) and the diameter of the clear zones was measured.

Gelatin hydrolysis

The potential isolates were cultivated on the TSA at 30°C for overnight. Then, colonies were touched using a sterile inoculating needle and stabbed directly down the center of the tube to 10mm from the bottom. The negative control tube with uninoculated isolate also included. The tubes were incubated at 30°C for 24hr. Then the tubes were examined and transferred to a refrigerator for 24hr. After that, chilled tubes were gently inverted to test for solidity.

Lipases hydrolysis

All the potential isolates were inoculated into 10ml of TSB for overnight at 30°C. Then the cultured were washed three times using sterilized saline and centrifuged (Eppendorf, 5804R) at 5000 rpm for 10min. Then 3µl of the potential candidate was spotted on top of the trybutyrin agar (HiMedia, India) surface and incubated at 30°C for 24h and diameter of inhibitory zone was measured.

Hemolysin activity

This method was based on Vesper and Vesper (2004) with modification. The potential probionts were cultivated on the TSA at 30°C for overnight. One colony was harvested from the fresh grown plate culture and streaked on plates containing blood-based agar supplemented with 5% (w/v) sheep sterile blood and 3% (w/v) NaCl. Plates were incubated at 37°C for 24 hr and clear zone were observed.

Biofilm formation

Biofilm formation was assessed by crystal violet (CV) staining based on Belas *et al.,* 2009 with modification.

Potential isolates were grown for 24hr at 30°C in TSB with shaking. Then 2ml of culture were transferred into 2 ml of fresh TSB in glass culture tubes and allowed to grow at 30°C without shaking for 24h, 48h, 72h and 96h. After incubation, the liquid culture was discarded and rinsed with sterile seawater to remove loosely attached cells. The biofilm attached to the test tube wall were stained with 1ml of CV solution for 30min at room temperature. Unbound dye was removed with a subsequent wash using sterile seawater. A 1:1 solution of dimethyl sulfoxide (DMSO) and ethanol (95% [vol/vol]) was used to elude the dye, and measured by spectroscopy at 550nm using a Bio-Rad Model 680 reader.

Results

Isolation of potential probionts

A total of 119 isolates were successfully isolated from different part of the crab as in Table 1. All isolates undergone first screening on TCBS plate agar to eliminate *Vibrio* species. Nineteen isolates were not grown on TCBS and were tested for their antagonistic activity for selecting potential probiotic strain.

Table 1. Total of isolates isolated from different part of crab.

Organs	Total of isolates
Hepatopancrease	24
Gill	23
Muscle	6
Ovary	12
Testis	10
Carapace	17
Haemolymph	27
Total	119

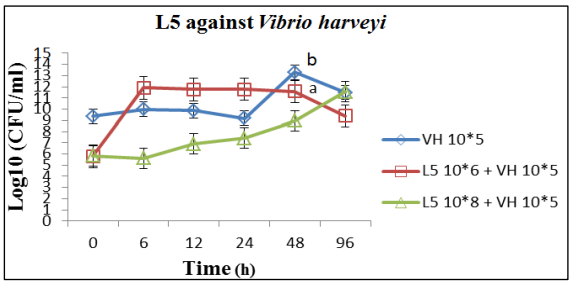
Antagonistic activity against V. harveyi

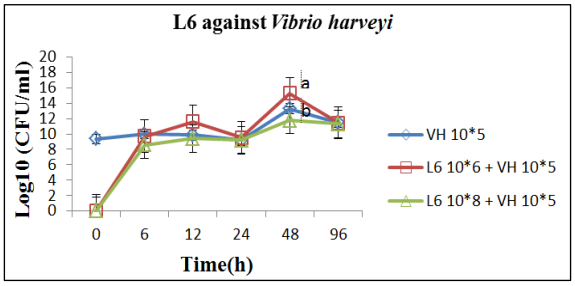
Eleven isolates had positive antagonistic activity against *V. harveyi* with clear zones ranging from 7 to 16 mm in series of *in vitro* plate assays (Table 2). In the co-culture assay, all eleven potential isolates with different concentrations were proven to have different potential in inhibiting the growth of *V. harveyi* (Fig. 1). Among all, isolate L13 showed the highest reduction. Meanwhile, three isolates (L1, L5 and L6) at 10^6 CFU/ml reduced the number of vibrios after 48 hr of incubation.

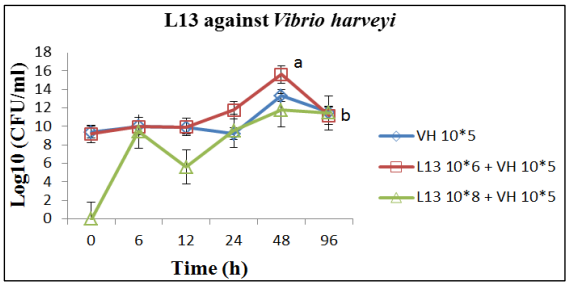
The other seven isolates (L8, L9, L10, L11, L12, L13 and L14) at concentration 10^8 CFU/ml showed significant decreased of vibrios numbers after 48hr of incubation. However, no reduction of vibrios was demonstrated by isolate L15 at both concentrations.

Table 2. Diameter of inhibition zone (± colony/disc/well size) by potential isolates against *Vibrio harveyi* in series of plates assay.

Potential probionts	Spot lawn assay	Disc diffusion	Well diffusion
L1	12±5	16±6	11±5
L5	11±5	13±6	10±5
L6	11±5	9±6	10±5
L8	9±5	8±6	10±5
L9	11±5	7±6	11±5
L10	10±5	12±6	10±5
L11	12±5	6±6	11±5
L12	13±5	9±6	11±5
L13	6±5	12±6	12±5
L14	13±5	13±6	13±5
L15	9±5	16±6	13±5
Streptomycin (S10)	15±5	20±6	19±5
sdH2O	0	0	0







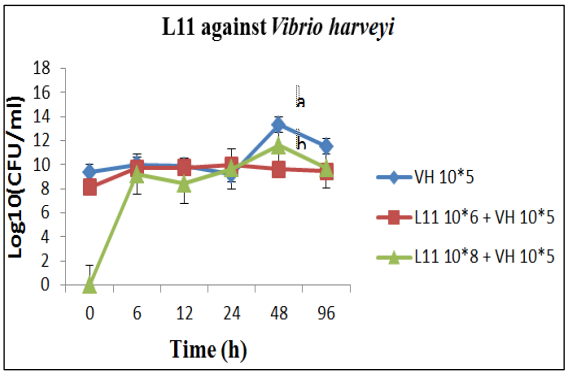


Fig. 1. Growth pattern of four different potential isolates at concentration 10^6 and 10^8 CFU/ml after co-cultured with *Vibrio harveyi* against time.
*VH: *Vibrio harveyi* ; L1, L5, L6 and L13: Potential probiotic.

Identification of potential probionts

Identification of the isolates based on morphology, staining and biochemical characteristics are shown in Table 3. Results demonstrated that all 11 isolates were Gram positive with rod shape.

Meanwhile, in plate the morphology was circular with milky white in color. All isolates showed positive results in oxidase, catalase and TSI assay. Furthermore, all the isolates contained spores and non-acid fast strains. In addition, the isolates were found to secrete amylase, gelatinase, lipase and negative in hemolysin production.

The Internal Transcribe Spacer (ITS) gene sequencing for all eleven isolates were determined by Polymeraese Chain Reaction (PCR). The amplification of ITS gene presented as a band with expected size of 1000bp nucleotides.

The identity percentage range from ITS BLAST analysis was choosing from range of 91-99%. The ITS BLAST results showed the similarities to the GeneBank with the samples were from *Bacillus amyloliquefacien* strain (Accession no. CP011686.1).

Biofilm formation of the probionts showed all isolates had the adhesive properties at 24hr and decreased after extension incubation time after 24 hr (Table 4).

Table 3. Biochemical characteristics of the potential isolates.

Isolate	Morphology	Gram stain	Acid fast stain	Spore stain	Catalase	Oxidase	TSI	Amylase	Lipase	Protease	Gelatinase	Hemolysin
L1	Rod	+	-	+	+	+	+	+	+	-	+	-
L5	Rod	+	-	+	+	+	+	+	+	-	+	-
L6	Rod	+	-	+	+	+	+	+	+	-	+	-
L8	Rod	+	-	+	+	+	+	+	+	-	+	-
L9	Rod	+	-	+	+	+	+	+	+	-	+	-
L10	Rod	+	-	+	+	+	+	+	+	-	+	-
L11	Rod	+	-	+	+	+	+	+	+	-	+	-
L12	Rod	+	-	+	+	+	+	+	+	-	+	-
L13	Rod	+	-	+	+	+	+	+	+	-	+	-
L14	Rod	+	-	+	+	+	+	+	+	-	+	-
L15	Rod	+	-	+	+	+	+	+	+	-	+	-

Table 4. Biofilm-forming ability of isolates at different time growth against *Vibrio harveyi*.

Isolates	Hours				
	0	24	48	72	96
Vibrio harveyi	0.20 ± 0.00^{a}	1.79 ± 0.05^{e}	1.73 ± 0.12^{e}	0.94 ± 0.06^{d}	0.88 ± 0.04^{f}
L1	0.20 ± 0.00^{a}	1.80 ± 0.06^{e}	1.69 ± 0.05^{e}	0.86 ± 0.08^{bc}	0.81 ± 0.04^{ef}
L5	0.20 ± 0.00^{a}	1.54 ± 0.07^{cd}	0.84 ± 0.18^{c}	1.40 ± 0.40^{e}	0.51 ± 0.04^{bc}
L6	0.20 ± 0.00^{a}	2.49 ± 0.11^{f}	0.85 ± 0.03^{c}	0.55 ± 0.10^{abc}	0.61 ± 0.08^{bcde}
L8	0.20 ± 0.00^{a}	1.54 ± 0.03^{cd}	1.42 ± 0.11^{d}	0.52 ± 0.02^{ab}	0.49 ± 0.01^{ab}
L9	0.20 ± 0.00^{a}	1.68 ± 0.10^{de}	0.21 ± 0.01^{a}	0.32 ± 0.07^{a}	0.31 ± 0.06^{a}
L10	0.20 ± 0.00^{a}	0.85 ± 0.04^{a}	0.55 ± 0.03^{b}	0.33 ± 0.01^{a}	0.49 ± 0.14^{ab}
L11	0.20 ± 0.00^{a}	1.15 ± 0.04^{b}	1.07 ± 0.03^{c}	0.84 ± 0.03^{bc}	0.91 ± 0.06^{f}
L12	0.20 ± 0.00^{a}	1.36 ± 0.11^{c}	0.45 ± 0.03^{b}	0.59 ± 0.01^{abc}	0.72 ± 0.11^{cdef}
L13	0.20 ± 0.00^{a}	1.49 ± 0.01^{cd}	0.94 ± 0.09^{c}	0.50 ± 0.00^{ab}	0.81 ± 0.00^{def}
L14	0.20 ± 0.00^{a}	0.78 ± 0.01^{a}	0.53 ± 0.03^{b}	0.56 ± 0.04^{abc}	0.44 ± 0.05^{ab}
L15	0.20 ± 0.00^{a}	1.15 ± 0.04^{b}	0.52 ± 0.07^{b}	0.60 ± 0.01^{abc}	0.59 ± 0.04^{bcd}

*Different superscript indicates significant different ($p<0.05$).

Discussion

Probiotic is defined as a "live microbial feed supplement which beneficially affects host animal by improving its intestinal microbial balance" (Fuller, 1989). Meanwhile Salminen *et al.,* 1999 stated "probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host". In this study, the selection of probiotic was done by isolating potential bacteria from healthy adult *P. pelagicus* and evaluated their inhibitory ability against pathogenic *V. harveyi* as target pathogen. A previous study found that *V. harveyi* is one of the marine pathogen which can infected vertebrates and shellfish which could lead to weakness and death of the species (Pai, 2006). Softshell, lethargy, necrotic and discolored of skin appeared as early symptom of the disease. According to Ashokkumar and Mayavu (2014), the infected culture also resulting in reduced growth rate, poor feed consumption, loss of body weight in due course.

For example, in Malaysia, crab culture especially during *P. pelagicus* larvae culture production, low or zero survival and high mortality rate occurred due to bacterial infections particularly cause by *V. harveyi* (Talpur *et al.,* 2011). Dan *et al.,* (2015) reported that necrosis usually can be found in larvae, post-larvae or adults crab with high mass mortality during early larvae stages in seed production rearing. This disease was also known as 'black spot', 'brown spot', burnt spot', 'shell disease', or chitinolytic bacterial disease (Jithendran *et al.,* 2009). It was caused by the invasion of chitinolytic bacteria, which break down the chitin of the exoskeleton and eventually lead to erosion and melanisation (dark brown or black pigmentation) at the site of infection.

As a consequences, none of the farmers in Malaysia are willing to culture the blue crab rather than wild catches to avoid loss of their investment. This scenario will lead to overharvesting to meet consumer need without any awareness to culture them.

For decades, farmers and aquaculturist has always using antibiotic as the solution to the bacterial infections. They are used as traditional strategy for fish diseases management (as prophylactic and to treat the infection) and also for the improvement of growth and efficiency of feed conversion (Pandiyan *et al.,* 2013). However, a prolong and frequent used of these antibiotics can lead to emergence and spread of antibiotic resistance bacteria (Nomoto, 2005) and pass to human which could rises a health problem if not handling properly (Van den Bogaard and Stobberingh, 2000). Alternative approaches for controlling microbial disease are needed and probiotic is considered since it is more environmentally friendly methods.

In these study isolation process of 119 bacteria strains were isolated from muscle, carapace, heamolymph, hepatopancrease, gills, ovary, and testis based on the different morpholoy forming on the agar. However only 11 isolates were identify as potential probiont since they were non-vibrios and showed positive interaction in *in vitro* test. *In vitro* test is a one of the preferred of antagonism test to select probiotics bacteria. Antagonistic activity of bacteria is a prevalent phenomenon which one bacterium species suppresses the development or inhibits the growth of other microorganisms (Semenov *et al.,* 2007). In this study, all potential probionts were identified as *Bacillus amyloliquefaciens* which were isolated from heamolymph of blue crab. A previous study by other researcher were successfully isolated three *Bacillus* strains; *Lactobacillus. plantarum, Lactobacillus salivarius* and *Lactobacillus rhamnosus* from the gut of *P. pelagicus* as a potential probiotic (Talpur *et al.,* 2012). Occasionally, *Bacillus* spp. are important microbe which not only act as probiotic but also produce biological control agent (Donio *et al.,* 2014). They are widely found in natural environment and proven to be safe for consumption.

Numereous studies have been done on *B. amyloliquefaciens* but a few are from aquaculture especially in blue crab. Other researcher also found *B. amyloliquefaciens* from different host such nile tilapia which suggest that *B. amyloliquefaciens* could help in increasing the size of the intestinal villi, increased number of goblet cells and enhancing the homeostatic state of the fish (Silva *et al.,* 2015). Other than that, there was also a study on the *B. amyloliquefaciens* isolated from activated sludge of a polluted river. They found that these bacteria are efficient as remediation in clearing nitrate in aquaculture water (Xie *et al.,* 2013). Study done by (Drablos *et al.,* 1999; Morikawa *et al.,* 1992; Perez *et al.,* 1993) found that most of the *Bacillus* spp. produce polypeptides (bacitracin, gramicidin S, polymyxin, and tyrothricin) that are active against a broad range of Gram positive and Gram negative bacteria.

In this study, the candidates exerted zone of inhibition in spot lawn assay, disc diffusion assay, and well diffusion assay against pathogenic *V. harveyi.* Based on study done (Hu *et al.,* 2010), the inhibitory effect of *Bacillus* is mainly due to the production of antimicrobial proteins and antibiotics as well as chemical compounds synthesized by secondary metabolism pathways competition for essential nutrients and adhesion sites.

Moreover, all of this eleven isolates were selected for co-culture assay in order to test their ability to inhibit the growth of *V. harveyi* in motile condition. In co-culture assay, the growth pattern of *V. harveyi* after pre-incubated with potential probionts at different concentration densities (10^6 and 10^8 CFU/ml) along with the inoculated periods interval. In this assay, each of the potential probiont showed ability in reduction of *V. harveyi.* The best concentration in inhibiting the growth of *V. harveyi* was at 10^8 CFU/ml.

The present study showed that the concentration of probiotics used need to be higher than *V. harveyi* in order for the probionts to inhibit the growth of pathogen significantly. These results were in line with study done (Vaseeharan and Ramasamy, 2003) suggested that the antagonist must be present at significantly higher levels than the pathogen and the degree of inhibition increased with the level of antagonist. A finding research by (Pham *et al.,* 2014), co-culture between candidate probiotic Strain NC257 had no significant effect on *V. harveyi* at concentration 1×10^5 CFU/mL and candidate NC297 showed moderate effect.

Other than that, various mechanisms have been proposed to explain the beneficial effects during selection of probiotics, including competition for adhesion sites, competition for nutrients, enzymatic contribution to digestion, and stimulation of the host immune response. Adhesion of probiotic bacteria to the host is considered important for health effects (Ouwehand *et al.,* 2000) and regarded a prerequisite for colonization (Alander *et al.,* 1999). In this study, adhesion of the potential probionts via biofilm formation assay were formed within 24hr and become lesser after 48hr of incubation. The bacteria will undergo adaptation to life and become resistant to antibiotic. Some of these bacteria will also increase in secondary metabolite production (O'Toole *et al.,* 2000). However, biofilm formation is a well-regulated developmental process and contribute to new complex community of organisms. A study done by (Emily, 2014), *Lactobacillus rhamnosus* and *Bacillus subtilis* isolates were able to produce biofilm formation at different time and different condition growth. Another research also found that *Bacillus* spp. were attached to the host via biofilm and competed with pathogen for adhesion site for colonization (Morikawa, 2006; Rengipipat *et al.,* 2000).

Moreover, in this present experiment, the potential isolates showed various productions of extracellular enzymes (gelatinases, lipases and amylases). The secretion of these types of extracellular enzymes was one of the main components for selection of the characteristic probiotic bacteria. Variety of microbes synthesizes and secretes different extracellular enzymes which allow the cells to communicate with their environment (Wetzel, 1991). In the absence of readily utilizable nutrients, bacteria which can secrete a variety of extracellular enzymes such as lipases, gelatinase and amylases to break down complex carbons sources and survive in its environment (Gadd 1988). In addition, a study by (Nimrat *et al.,* 2011) stated that *Bacillus* sp. with extracellular enzymes help in nutrient uptake and digestion in the host. Another study found that low extracellular enzymatic activities from potential probiotic isolates but positive in antagonistic activity against target pathogen (Askarian *et al.,* 2012).

The hemolysin test showed the potential probionts were non-hemolysin bacteria since there were no clear zones around the bacteria growth. Some of the *Bacillus* spp. produce hemolysins (Liu *et al.,* 2009), which could be a health risk to the host. Since the isolates were non-hemolysin bacteria, it considers as safe to use.

Conclusion

It is widely known that the indiscriminate use of antibiotics in aquaculture animal production contributes to the development of antibiotic-resistant strains (Khachatourians, 1998; Wegener *et al.,* 1999; Willis, 2000). An alternative way to replace antibiotics in aquaculture system is by using potential beneficial microorganisms such probiotics. The selection of probiotics in this study, was mainly focusing on the ability of the probiont in inhibiting the growth of *V. harveyi.* Potential probiont identified as *B. amyloliquefacien* demonstrated positive characteristics as potential probiont and worth to be carried out in *in vivo* assay to evaluate its potential in protecting host from *V. harveyi* infection.

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Conflict of interest

The author has no conflict of interest with respect to the content of this article.

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