

## Phylogenetic analysis to compare populations of acid tolerant bacteria isolated from the gastrointestinal tract of two different prawn species *Macrobrachium rosenbergii* and *Penaeus monodon*

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**Abstract.** Probiotic is every preparation of microbe cell components which are beneficial to the health and life of a host. The aim of this study was to evaluate the genetic differences of candidate probiotic bacteria in the digestive tract of *Macrobrachium rosenbergii* and *Penaeus monodon* using phylogenetic analysis of 16S rDNA sequences. The method used morphological, biochemical test and molecular identification using 16s rDNA. 16S rDNA sequences was compared using a BLAST search to the data base in Gen Bank. Eleven (11) candidate probiotic bacteria was isolated from *M. rosenbergii*. Isolate Iso A had 99% homology to *Bacillus* sp. BDU4. Ten (10) candidate probiotic bacteria was isolated from *P. monodon*. There were none digestive track bacteria reported in Gen Bank which had more than 96% homology to the isolates from *P. monodon*. The *P. monodon* isolates we conclude as original probiotic bacteria isolates from Riau province.

**Key Words:** Probiotic bacteria, *Macrobrachium rosenbergii*, *Penaeus monodon*, *Bacillus*.

**Introduction.** The use of drugs or antibiotics in fisheries production as a prevention of diseases is being reduced due to regulations prohibiting any residues of these chemicals found in the fish. The use of antibiotics or antimicrobials as a additives in animal feed lately decreased, it has been banned in some countries due to two main factors. First, the possibility of antibiotics contaminating residues will be toxic for the consumers. Secondly, antibiotics can increase the number of resistant microorganisms in the human or animal body (especially pathogenic bacteria such as *Salmonella* sp., *Escherichia coli* and *Clostridium perfringens*) (Feliatra et al 2011).

Probiotics are products composed by microbes or microscopic natural foods that are beneficial and gives effect to increase the balance of intestinal tract microbial communities in the host animal. Probiotics in aquaculture have a role in increasing the growth rate, and improving the immune system by changing the bacterial community of the intestines (Irianto & Austin 2003).

Probiotics can produce bacteriocins which work selectively against some pathogenic strains. They also produce lactic acid, acetic acid, hydrogen peroxide,

lactoperoxide, lipopolysaccharide, and several antimicrobials. In addition, important nutrients in the host immune system and metabolism, such as B vitamins (pantothenic acid), pyridoxine, niacin, folic acid, cobalamin, biotin and antioxidants such as K vitamins can be produced (Adam 2009).

According to Munoz-Quezada et al (2013), probiotics must be able to control the digestive tract and increase the health of its host through metabolic activity. In particular, probiotics must survive with the acid conditions and high levels of salt with 2.0 and 7.0 pH as a control.

Feliatra et al (2004) found nine species of bacteria that have the potential as probiotics in the gastrointestinal tract of *Ephinephelus fuscogutatus*. They are *Lactococcus* sp., *Carnobacterium* sp., *Staphylococcus* sp., *Bacillus* sp., *Eubacterium* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Micrococcus* sp. and *Bifidobacterium* sp. Those bacteria have a potential as probiotic because of having resistance at pH 2 as a main indicator of probiotic bacteria. According to Yulvizar (2013) there are five isolates which have a potential as probiotics from the stomach and intestines of *Rastrelliger* sp. All the isolates consist of three genera of the bacteria such as *Micrococcus*, *Staphylococcus*, *Bacillus* and one species of bacteria that is *Hafnia alvei*. Yulvizar et al (2015) found three isolates have the ability to inhibit and eliminate the pathogenic bacteria in aquaculture systems.

Modabberi et al (2014) examined the effect of different doses of probiotic Bactocell in the diet of rainbow (*Oncorhynchus mykiss*) trout on growth parameters and bacterial flora. These authors found that the probiotic Bactocell is promising candidate to affect the weight gain and capable of improving the efficacy of bacterial flora of rainbow trout and Sharibi et al (2015) found the use of Bactocell as dietary supplement notably increases growth and nutritional parameters in the benni fish fingerlings. The optimal level could be the 0.2 grams of probiotic Bactocell per kg of diet. In this direction, incorporating 0.2 grams of Bactocell per kg diet stands the chance to improve the growth and nutritional performance of benni fish fingerlings.

Based on these findings, this study was conducted to find out the genetic diversity of probiotics in the digestive tract of *Macrobrachium rosenbergii* and *Penaeus monodon*. Isolated probiotic candidate bacteria were characterized by phenotype characterization (morphological observation, physiological and biochemical test) and genotype characterization based on phylogenetic analysis of 16S rDNA sequences and nucleotide sequence analysis with the Basic Local Alignment program Search Tool (BLAST) against sequences published in GenBank.

**Material and Method.** This study was conducted on October 2013 at the Microbiology and Biotechnology Laboratory of Fisheries and Marine Sciences Faculty, University of Riau, Pekanbaru to isolate the candidate probiotic bacteria. Furthermore, molecular examination, purification gel electrophoresis and sequencing DNA were done at the Institute of Biotechnology Serpong, Tangerang, Banten.

The samples of the study were *Macrobrachium rosenbergii* (15-30 cm in length), obtained from fish farmers derived from Teratak Buluh, Pekanbaru, Riau and *Penaeus monodon* (7-9 cm in length) samples derived from shrimp farms in Bengkalis, Riau. They were included in the ice box with 5°C then they were brought to Marine Microbiology laboratory. Nutrient Agar (NA) and physiological solution pH 2 were used as the media to isolate and purify the bacteria. Test Gram staining test was done with crystal violet solution, distilled water, iodine solution, 95% ethyl alcohol and safranin solution. The solution of 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used in the catalase test. MR-VP media and methyl red indicator were used in the methyl red test. Oxidative/fermentative (O/F) medium was used in O/F test. SIM medium (Sulfide-Indol-Motility) was used in the Indole, H<sub>2</sub>S and motility test. Simmon's citrate media was used in the citrate agar test. Triple Sugar Iron Agar (TSIA) media was used in the use of sugar test. In additions, NA medium was used to test the growth of bacteria at different temperatures.

For DNA isolation cultures of probiotic candidate bacteria were grown in 4 mL of liquid Luria-Bertani medium (LB) at 37°C for 24 hours. The cultures were then moved to 1.5 mL microtubes and centrifuged at a speed of 13,000 rpm (revolutions per minute) at

4°C for 2 minutes. The supernatant was discarded, and the pellet dried. Dissolved in 40 µL of Tris-EDTA (TE) buffer solution with 8 pH and 20 µL of lysozyme added to the solution. The solution was inverted 3-5 times, and incubated for 10 minutes at 37°C. After incubator 50 µL of 10% Sodium dodecyl sulfate (SDS) was added and mixture was gently rotated (20 rpm) for 5-10 minutes on a rotamix rotator. Added 550 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the mixture and the mixture further rotated gently (20 rpm) for 10 minutes. The mixture was then centrifuged at 7.000 rpm for 10 minutes. The upper aqueous phase was transferred to a new microtube, and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the tube. The mixture was gently rotated (20 rpm) on a rotamix rotator for 10 minutes. The mixture was then centrifuged at 7.000 rpm for 10 minutes. The upper aqueous phase was transferred to a new microtube and 3 M sodium acetate was added (40% volume) to the aqueous phase. Two times volume of 99% cold alcohol was then added to this solution to precipitate the DNA, and the solution chilled at -20°C for 30 minutes. The precipitated DNA was centrifuged at 13.000 rpm, 4°C for 10 minutes and the supernatant discarded. The DNA pellet was washed in 1 mL 70% cold ethanol centrifuged for another 10 minutes at 13.000, 4°C. The supernatant discarded, and resulting DNA pellet was then air dried. The pellet was dissolved in 40 µL TE-buffer (pH 8) and 2.5 µL of RNase solution were added and homogenized using vortex mixer and incubated at 37°C for 1 hour. Finally, the DNA of probiotic candidate bacteria was ready to be used, or stored -20°C.

**Polymerase Chain Reaction (PCR).** The PCR reaction was done by using 8 µL DNA of each probiotic candidate bacteria as template, and adding 23 µL of H<sub>2</sub>O, 4 µL of 10x Taq polymerase, 0.5 µL of 12mM dNTPmix, 2 µL for each of universal primers consisting of forward primer 24F (5'AGAGTTTGATCCTGGCT-3') and reverse primer 1541R (5'AAGGAGGTGATCCAGCCGCA-3') and 0.5 µL Taq polymerase. PCR conditions were: hot start for 2 minutes at 94°C, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 40 seconds at 50°C, and strand extension for 1 minute at 72°C.

**Purification of DNA PCR fragments.** After PCR, the DNA obtained was separated on a 1% agarose gel by electrophoresis. PCR DNA bands were purified from the agarose gels using the Qiagen High Pure PCR Purification Kit (Japan), following instructions by the manufacturer.

**Sequencing, BLAST and phylogenetic analysis.** Purified PCR products were sequenced with ABI 3010 Genetic Analyzer XL Applied Biosystems. Sequencing was done at the Institute for Biotechnology Serpong, Tangerang, Banten. BLAST analysis was done using the tools and data of GenBank accessed at <http://www.ncbi.nlm.nih.gov/blast>. DNA sequences were aligned using CLUSTALX 2.1. (Larkin et al 2007). Phylogenetic trees were generated using the tools provided by CLUSTAL X 2.1., and trees were visualized using TreeView X version 0.5.0.

**Results.** From the digestive tract of *M. rosenbergii* and *P. monodon* 21 probiotic candidate bacteria could be isolated, consisting of 11 isolates derived from *M. rosenbergii* and 10 isolates from *P. monodon*. From the 21 probiotic candidates bacteria isolates, 12 isolates were gram positive, 6 isolates were gram negative and 3 isolates were gram positive leads to negative (Table 1).

Based on the observation of morphological and biochemical potential bacterial strains found in the digestive tract of *M. rosenbergii* and *P. monodon*, each strain showed different characteristics from each other. Colonies morphology was dominated by round and irregular shapes. The dominant colors of the colony were creamy with various kinds from creamy beige thick, yellowish and dull beige. The shape of the cells were dominated by round and followed by rods. For gram staining of *M. rosenbergii*, equal numbers were found between gram positive and negative bacteria, where as the strains of *P. monodon* were dominated by gram positive bacteria (Table 1).

Table 1

The results of morphological observations and biochemical potential of bacterial strains found in the digestive tract of *Macrobrachium rosenbergii* and *Penaeus monodon*

Sample															
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>Macrobrachium rosenbergii</i>															
Iso A	Irregular and spread	FC	rod	Carbol-fuchsin stain	+	+	-	+	O	±	+	-	+	-	-
Iso B	round	CC	round	Gram stain	-	+	-	+	O	+	+	-	+	+	+
Iso C	round	FC	round	Gram stain	-	-	-	+	O	±	-	+	+	+	-
Iso D	round	FC	round	Gram stain	-	-	-	+	O	±	-	+	+	-	-
Iso E	round	FC	round	Methylene blue stain	+	-	-	+	O	+	+	+	+	-	-
Iso F	Irregular	FC	rod	Pappenheim's stain	+	-	-	+	O	±	-	-	+	+	-
Iso G	Irregular	CC	rod	Gram stain	+	-	-	+	O	+	-	-	+	-	+
Iso H	round	YC	rod	Hiss capsule stain	-	-	-	+	O	±	-	-	+	-	-
Iso I	Irregular	CC	rod	Gram stain	+	-	-	+	O	±	-	-	+	-	-
Iso J	Fibrous	CC	rod	Carbol-fuchsin stain	-	-	-	+	O	+	-	-	+	+	-
Iso K	round	YC	rod	Gentian violet stain	+	-	-	+	O	±	+	-	+	+	-
<i>Penaeus monodon</i>															
HQS1	round	FC	rod	Gram stain	+	+	-	+	F	+	+	-	-	-	-
HQS2	round	CC	rod	Pappenheim's stain	-	+	-	+	O-	+	-	-	-	-	+
HQS3	Irregular	FC	rod	Vn	Vn	+	-	+	F	+	-	-	-	-	+
HQS4	round	FC	rod	Vn	Vn	+	-	+	F	+	-	-	-	-	+
HQS5	round	FC	rod	Gram stain	+	+	-	+	F	+	-	-	-	-	-
HQS6	round	FC	rod	Gram stain	+	-	-	+	F	+	-	+	-	-	-
HQS7	Irregular	CC	rod	Gram stain	+	+	-	+	F	+	-	-	-	-	-
HQS8	Irregular	YC	rod	Gram stain	+	-	-	+	F	+	-	+	-	-	-
HQS9	Irregular	CC	rod	Vn	Vn	+	-	+	F	+	-	+	-	-	-
HQS10	Irregular	CC	rod	Gram stain	+	+	-	+	F	Green	+	+	-	-	+

1 - isolate; 2 - colonies forms; 3 - colonies color (FC - faded cream, CC - concentrated cream, YC - yellowish cream); 4 - cell shape; 5 - type of coupling; 6 - Gram; 7 - catalase; 8 - motilitas; 9 - different temperatures (28-37°C); 10 - O/F; 11 - Indole; 12 - Metil Red; 13 - Citrat; 14 - Glucose; 15 - Sucrose; 16 - H<sub>2</sub>S.

PCR products using primers for 16S rDNA formed DNA bands on agarose gel electrophoresis with molecular weights between 1500 to 2000 *base pairs*/bp (Figures 1 and 2). The size was suitable with the expectation of the 16S rDNA genes of bacteria (Sabdono & Rajasa 2006). The results of BLAST of potential probiotic bacteria derived from *M. rosenbergii* indicated that only isolates A had a degree of homology of 99% with the bacterial strain *Bacillus* sp. BDU4, while 10 others bacterial isolates only had a level of homology between 91-96% with the *Bacillus* genus. Likewise, the result of BLAST candidates from *P. monodon* was also leading to the *Bacillus* genus. For digestive isolates from *P. monodon*, there were none which had homologous isolates approaching 97%, so it was concluded that all isolates were putative new isolates not reported yet in Gen Bank. All isolates from *P. monodon* showed homology to *Bacillus* sp. with the closest homology of 96% to *Bacillus cereus* NC7401 reported by Takeno et al (2012).

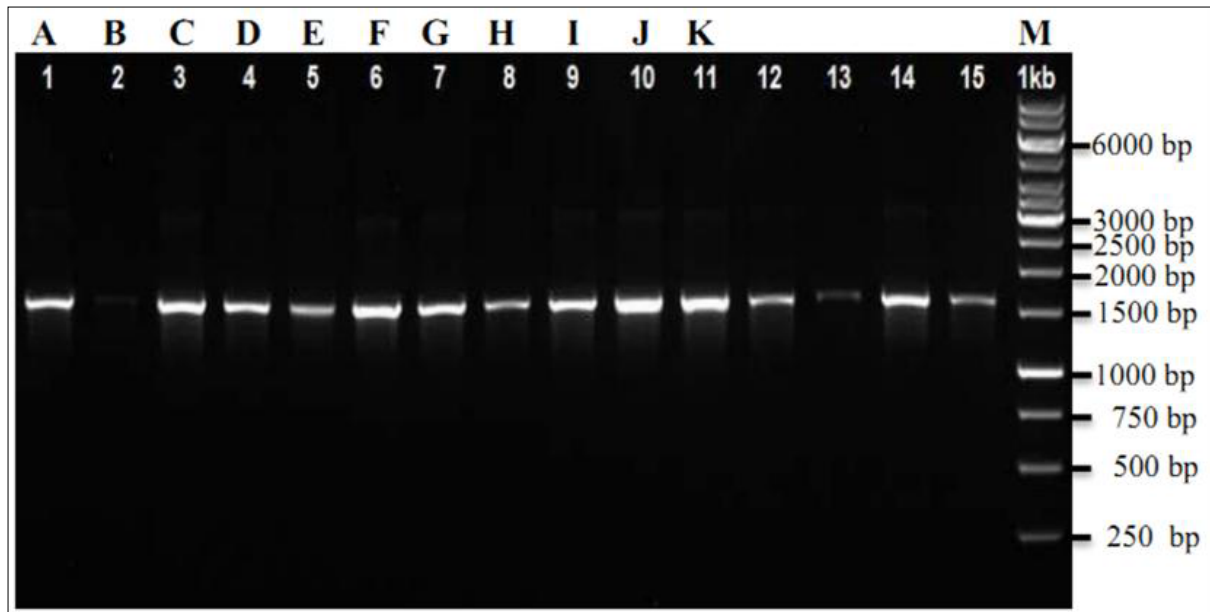


Figure 1. The results of gel agarose electrophoresis of 16S rRNA PCR products of bacteria isolated from the digestive tract of *Macrobrachium rosenbergii* (isoA - Iso K).

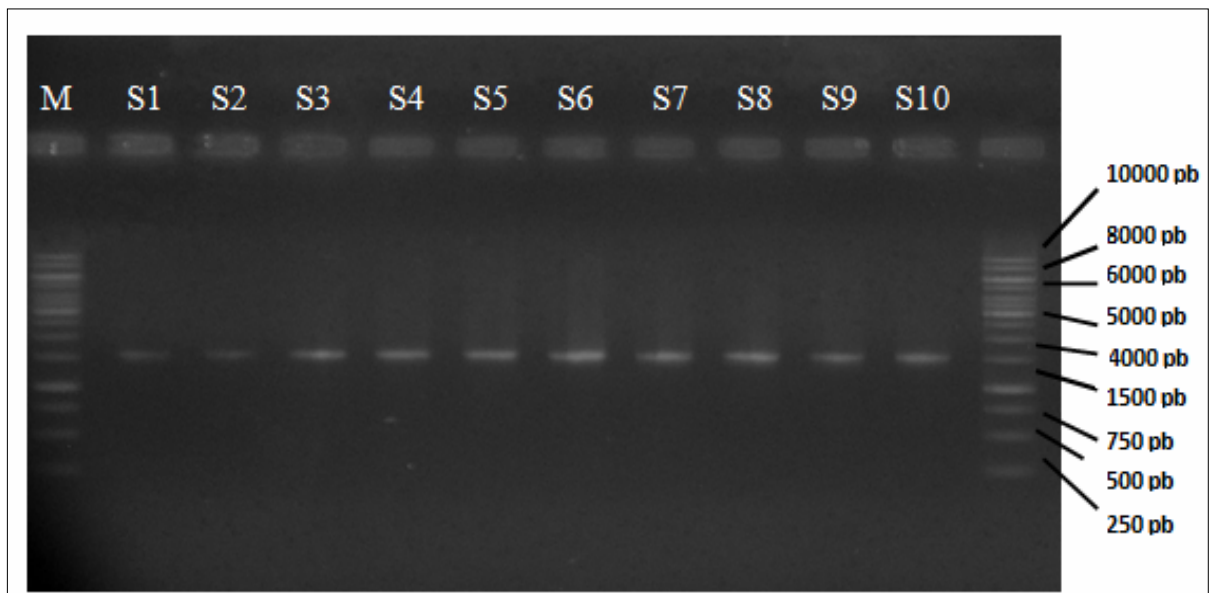


Figure 2. The results of gel agarose electrophoresis of 16S rRNA PCR products of bacteria isolated from the digestive tract of *Penaeus monodon* (HQS1- HQ S10).

**Discussion.** Based on molecular characteristics between species or strains of the same species, phylogenetic tree was useful to show the genetic relationship of each species. All analyzed samples make a group. Isolates Iso consisted of Iso I and Iso F have a level of genetic relationship with the bacteria *B. cereus* strains (Figure 3), where as isolates HQ S7 has genetic relationship with *Bacillus subtilis* strain SRS-35GU056813.1. According to Blackwood & Buyer (2004) there are at least two kinds of subspecies of *B. subtilis*, *B. subtilis* and subspecies *subtilis* and *B. subtilis* subspecies *spizizenii*. There have previously done some researches related to the benefits of these bacteria which could associate to the inside and outside of the body of another living creature (Silva et al 2013).

Hagstrom et al (2000) stated that the isolates which have 16S rDNA sequence similarity greater than 97% may represent the same species. For those who have similarities between 93-97% sequence could represent the identity at the level of genus but they are probably different at the species level. Possibly other isolates with homology

under 97% are putative probiotic bacteria that have not been reported yet in Gen Bank. Additional analysis, using other marker genes still needs to be done, to verify this. Isolates bacterial: Iso D, Iso C, Iso A, Iso G, Iso K and Iso E, formed separate clusters. Based on the genetic relationship, the closest bacteria was *B. cereus* strains JDA1GU056812.1, but closer to the HQ S9, HQ S3, HQ S6, HQ S1, HQ S8 which clustered together with *Bacillus cereus* JDA-1GU056812.1 (Figure 3).

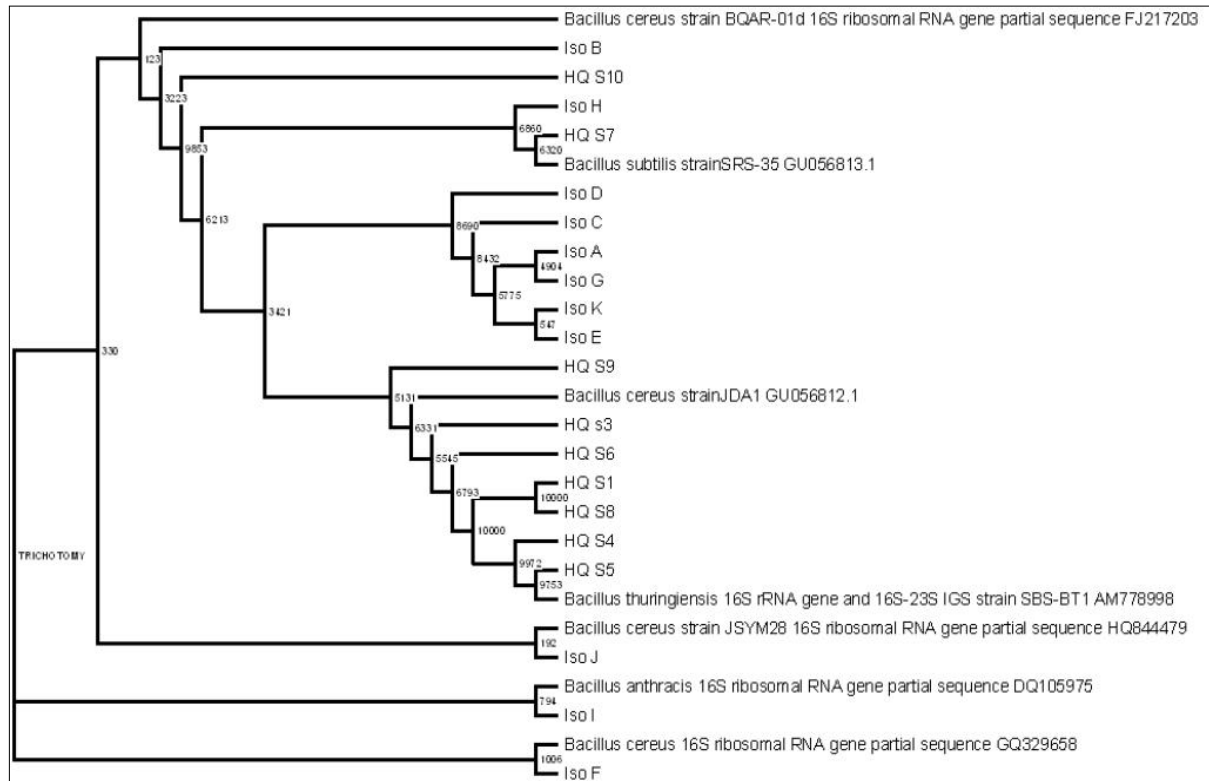


Figure 3. Phylogenetic tree (cladogram) potential isolate probiotic bacteria from the digestive of *Macrobrachium rosenbergii* (Iso A - Iso K) and *Penaeus monodon* (HQ S1-S10).

Iso J bacteria had a cluster with *B. cereus* strains JSYM2816S, and Iso I had the same cluster with the *Bacillus anthracis*, while Iso F had the same cluster with the *B. cereus* (Figure 3). *Bacillus* sp. was proteolytic bacterium that could break down proteins into amino acids. Amino acid was used to multiply, so that it could increase the feed protein and decrease the crude fiber. Moreover, this bacterium was also able to decipher disaccharides and polysaccharides into simple sugars which had pectinolytic nature with a capability in producing a complex carbohydrate pectin (Anggraini et al 2012).

Bacteria S5 and S4 were closer to the cluster of *Bacillus thuringiensis* (Figure 3). This bacterium could produce two types of toxin: crystals toxin (crystal, Cry) and cytolytic toxin (cytolytic, Cyt). Cyt toxin could strengthen Cry toxin widely used to improve the effectiveness in controlling insects. Several studies have been able to isolate and purify the bacteriocin of *Bacillus* sp.: cerein produced by *B. cereus* (Oscariz & Pisabarro 2000), and toxin produced by *B. thuringiensis* (Paik et al 1997).

According to Doi & Mcgloughlin (1992), two main properties which distinguish *Bacillus* from other bacteria forming endospores are the ability of *Bacillus* to live aerobic (although some of them are facultative anaerobes) and the majority of its kind to produce catalase (positive catalase). Endospores produced by *Bacillus* had a high resistance to chemical and physical factors, such as extreme temperatures, alcohol, and so on. These kinds entirely contained dipicolinic acid (DPA) and they had a degree of unparallel dormancy on the other life forms. The spores brought the development cycle where vegetative cells could form spore then it could germinate into vegetative cells. While the S9 isolates candidate had genetic relationship with *B. cereus*.

*B. cereus* bacterium was one of the pathogen agents having a great potential to be used as biological control. This bacterium has a specific host which is not harmful for the natural enemies of pests and other non-target organisms, easily biodegradable by the environment and its pathogenicity can be increased by genetic engineering techniques (Khetan 2001). *B. cereus* could grow well on NA medium incubated in the laboratory which had an average temperature of 28.8°C. According to Gordon et al (1973) *B. cereus* can grow well at a maximum temperature of 35 to 45°C and a minimum temperature of 10 to 20°C. The *B. cereus*' surface colonies that grow on NA media looks shiny and reflective. Eight-day bacteria can make a foul odor (Binoto 2001). *B. cereus* had the ability to do lysis toward the Cyanobacteria (BGA) at a concentration of 10<sup>3</sup> cells mL<sup>-1</sup> in waterpool. It could generate Cyanobacteriocyte so that it was suitable to be used as a biological control toward blooming blue green algae.

The kinds of *Bacillus* (*B. cereus*, *B. clausii* and *B. pumilus*) are included in the five commercial probiotic products for colonization, immunostimulation, and antimicrobial activity (Duc et al 2004). Twenty-one (21) probiotic candidate bacteria of *Bacillus* types were expected to be used to improve the productivity of fish and shrimp farming either for growing or improving the carrying capacity of the pool or pond.

**Conclusions.** Isolation of 21 strains of bacteria have potential as probiotics. They were found in the digestive tract of *Macrobrachium rosenbergii* and *Penaeus monodon*. Only one isolate had 99% homology to *Bacillus* sp. It could be ascertained as the same species because the lowest limit of the same species was 97%, while there was not found homologous bacterial approaching 97% from the other 20 bacteria isolates so that it can be said that they were bacteria who's 16S rDNA sequences have not been reported yet in Gen Bank. Because 20 of these bacteria had a survival at pH 2 and were able to develop well as the main indicators as probiotic bacteria, it could be concluded that they had the potential as probiotics.

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