BIODIVERSITAS
Volume 20, Number 5, May 2019
Pages: 1339-1343

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d200524

Short Communication: Identification and characterization of nitrifying bacteria in mud crab (*Scylla serrata*) recirculation aquaculture system by 16S rRNA sequencing

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Manuscript received: 17 September 2018. Revision accepted: 25 April 2019.

Abstract. *Hastuti YP, Rusmana I, Nirmala K, Affandi R, Tridesianti S. 2019.* Identification and characterization of nitrifying bacteria in mud crab (*Scylla serrata*) recirculation aquaculture system by 16S rRNA sequencing. *Biodiversitas 20: 1339-1343.* Recirculation is one of the systems used for increasing aquaculture production, for example, is mud crab Scylla serrata culture. Aquaculture activities will produce organic nitrogen waste and can be affected to inorganic condition (nitrification bacteria). Nitrifying bacteria are capable of changing aerobically from NH_4^+ to NO_2^-(nitrite) and from $NO_2^-$to NO_3^-(nitrate). This current work aimed to identify and characterize nitrifying bacteria in mud crab (*S. serrata*) culture with a recirculated system. Nitrifying bacteria were isolated using serial dilution (10^{-1} until 10^{-5}) of open filter FB water sample previously cultured in a specific medium. Bacterial identification was performed using a procedure of Presto^{TM} Mini gDNA Kit (Geneid) for DNA extraction and Polymerase Chain Reaction (PCR) primer 63F and 1387R for 16S rRNA gene amplification. Molecular identification using 16S rRNA sequences, the nitrifying bacteria isolated from mud crab recirculation aquaculture system were 99% identified as a group *Pantoea calida*, *Pseudomonas stutzerii dan Halomonas* sp. Morphological characterization of the colony morphology showed that a mucoid appearance and appeared cream in color and large size. Furthermore, their cells were recognized as basil and Gram-negative.

Keywords: Bacteria, characterization, identification, nitrification, *Scylla serrata*

INTRODUCTION

Mud crab (*Scylla serrata*) is recognized as one of the exotic crustaceans and acquires economic importance with increasing demand, but its production shows a decrease. From 2000 to 2010, production of mud crab increased up to 183.5%, which its live distribution reaches 2.1% (Food and Agriculture Organization-FAO 2011). On the contrary, the technology of mud crab culture was rather scarce, which was unable to meet its high demand (FAO 2016). Recirculation Aquaculture System (RAS) was proposed to increase the mud crab production. RAS was applicable to maintain culture condition and to deal with water limitation (Metaxa et al. 2006). Presence of integrated recirculation in RAS could promote reduction-oxidation reaction of toxic compounds, which provided an answer to increase crab production.

Waste of environmental aquaculture is a source of Dissolved Organic Nitrogen (DIN) is used by microorganisms to produce DIN products. Bacteria degrading organic materials would produce NH_4^+, which is, in turn, transform into NH_3. Nitrifying bacteria enabled to produce NO_2^-(nitrite) from NH_4^+ and NO_3^-(nitrate) from NO_2^-under aerobic condition. This suggested that the bacteria could improve nitrification and denitrification of nitrogen available in shrimp culture (Hastuti, 2011). Camargo and Alonso (2006) found that toxicity of inorganic nitrogen compounds including ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) at a particular concentration could promote mortality for aquatic animals. In addition, these compounds also adversely affected their physiological, neurological, and cytological functions, thereby inducing stress condition and less feed consumption (Constable et al. 2003).

Identification of nitrifying bacteria is critical for the process of cultivation environment aquaculture, one of the approaches is by using molecular technique. The identification of particular nitrification bacteria for mud crab environment culture is considered as an initial stage for the analysis of nitrifier diversity in a controlled environmental.

The presence of nitrifying bacteria in aquaculture environments can balance the ecosystem of aquaculture by degrading ammonia waste from ammonification of organic feed waste and metabolic waste. The nitrifying bacteria can degrade ammonia into nitrite and subsequently to become nitrate with the assistance of ammonia oxidase (Amoa) and nitrite bioxidase (NoB) enzyme. The nitrifying bacteria can be identified using DNA sequence of 16S rDNA (Stackebrandt et al. 1991).

During culture, the concentration of ammonia, nitrite, and nitrate was increased with the increasing age of mud crab, which affected its survival (Hastuti et al. 2015). The concentration of nitrite in day 45 reached 1.02 mg L^{-1} and continuously increased up to 1.72±0,57 mg L^{-1} in day 100. In the case of *vannamei* culture, a remarkable increase of nitrite and ammonium-producing bacteria was recorded up to 7.03 log CFU L^{-1} in day 30 (Hastuti et al. 2010). In the aerobic system, ammonium removal could be performed by oxidizing ammonium (NH_4^+) through enhanced nitrification activities induced by nitrite-forming bacteria (ammonium oxidizer) and nitrate-forming bacteria (nitrite oxidizer). Nitrite (NO_2^-) served as electron acceptor with the presence of denitrifying bacteria under an anaerobic system, which was then reduced into N_2O and N_2 gas (Zumft 1997; Richardson 2000). Nitrification allows reduction of harmful compounds (ammonia and nitrite) into inorganic nitrogen compound (nitrate) that are not toxic. Therefore, this current work aimed to identify and characterize nitrifying bacteria present in mud crab recirculation aquaculture system.

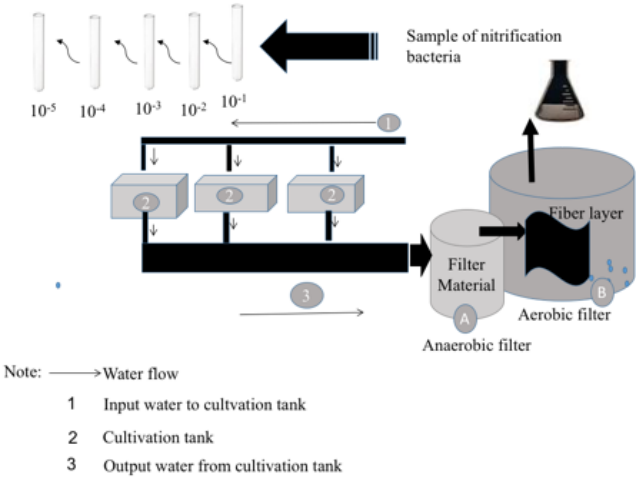

Figure 1. Installation of Recirculated Aquaculture System for mud crab culture and sample collection

MATERIALS AND METHODS

Samples were taken from the environmental recirculation of mud crab (*Scylla serrata)* culture. Mud crab (*S. serrata*) as test biota was sampled from the coast of Banjarmasin in South Kalimantan and Pasuruan in East Java, and reared in Laboratory of Environmental, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB), Bogor, Indonesia. Bacteria were isolated and characterized in Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB. Seawater used in this study was taken from Ancol beach, Jakarta, Indonesia.

Installation of mud crab RAS

Mud crab (10 individuals) was parallelly reared in a culture tank containing 60 L of seawater. Recirculation system was made by using six shelters (total area of 220 cm^2) at a density of 10 crabs, using seawater with a salinity of 25 g L^{-1} (obtained by dilution) oxygen level of >5 mg L^{-1} in culture tank, the temperature of 27-31 °C, and pH of 6.5-7.5. For each treatment, two filter chambers contained (Hastuti et al. 2017). Close Filter System (operated as an anaerobic filter) and an Open Filter System (as aerobic filter) were installed with the capacity of 220 L per chamber (water debit of 0.125 L sec^{-1}, water pump capacity of 200 V) (Figure 1). Water sample for specific nitrification bacteria was taken from the open filter system or aerobic condition filter. Each filter system was equipped with inlet and outlet. Practically, water from the culture tank was flowed into anaerobic filter chamber (consisting of 250 bio balls, 40 kg zeolite, 90 kg grains of sand, 800-1200 bio balls, respectively from bottom to top, and covered by gauze), while waved fiber was set in aerobic filter chamber. For bacterial isolation, water was sampled from the inlet of the aerobic filter.

Isolation of nitrifying bacteria from mud crab RAS

The water sample was diluted at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. The bacteria were isolated in heterotroph specific media containing succinate as carbon source and ammonium as a single source of nitrogen and incubated for five days (White 2000). The composition of nitrifying bacteria medium consisted of KH_2PO_4, Na_2HPO_4, $FeCl_3$, $CaCl_2$ and yeast extract. The resulting pure isolate was observed for colony morphology (form, color, margin, and size). Gram staining was used to distinguish bacterial species (Hadioetomo 1983).

DNA extraction, amplification, and identification of the 16S rRNA genes in the isolated cells

The DNA of selected pure isolates was extracted using the protocol of Presto™ Mini gDNA Kit (Geneid). The concentration and purity of extracted DNA were then determined using Nanodrop 2000 (Thermo Scientific, Wilmington DE, USA). Genomic DNA from the extraction of nitrification bacteria in aquaculture system was used for PCR amplification of the 16S rRNA primer pair 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). The final concentration of each reagent PCR reaction was 25 µL, consisting of 12.5 µL from GoTag Green Master Mix 2X (Promega, Madison, W1, USA) or Red mix my tag (Bioline), 2.5 µL primer 63F and 1387R (concentration of 10 pmol); 6.5 µL Nuclease-Free Water, and 1 µL genomic DNA templates. PCR experiment consisted of 35 cycles and was performed by following conditions: pre-denaturation (95 °C; 5 min), denaturation (95 °C; 15 min), annealing (55 °C; 1 min), elongation (72 °C; 1.5 min), and extension (72 °C; 10 min). The DNA sequences of 16S rRNA were subjected to BLAST-N in NCBI website (blast.ncbi.nlm.nih.gov) to search for the homologous sequence alignment. A phylogenetic tree was constructed using Mega 6.00 *Neighbour Joining* Boostrap

2000× replicates (Tamura *et al*. 2013). The alignment process of the results is made phylogenetic tree construction by method of Neighbor-Joining. Diversity and uniformity of indexes are estimated based on interpretations that were carried out by CLIQS ID software and processed with Past3 software.

RESULTS AND DISCUSSION

Isolation of nitrifying bacteria

Selection of nitrifying bacteria was performed through the following steps: purification, biochemical test for nitrifying bacteria, OF test, and metabolic activity test (Table 1). From 169 initial isolates of nitrifying bacteria, 68 isolates were obtained through further selection based on their stability and growth rate. All of 68 isolates were observed for their capability in oxidizing NH_4^+, $NO_2^-$and ultimately forming NO_3^-. According to bacterial metabolic activity, 20 isolates were obtained, and a further test was carried out including biochemical test and quantitative analysis. Partial performance of nitrification bacteria has been produced with a relatively higher source of ammonium (Dong et al. 2017). The results of abundance nitrifying bacteria from different container showed a significant difference (Hastuti et al. 2018).

Based on the morphological examination of five isolates of nitrifying bacteria, it showed that most isolates were classified as Gram-negative (Table 2). Previous studies reported that strains of nitrifying bacteria with heterotrophic and aerobic denitrification activity were from *Paracoccus* sp., *Bacillus licheniformis* and *Pseudomonas stutzeri* (Takenaka et al. 2007; Miyahara et al. 2010). Also, Zhang et al. (2011) reported that YZN-001 strain with nitrification activity has features such as rod-shaped, Gram-negative, motile, catalase-positive and oxidase-positive. Identification of nitrifying bacteria in the RAS culture of mud crabs has been identified in some ways which have motility, catalase and oxidase capabilities. From the colors that are produced, there is a color of the bacteria that appears that is yellow, white and a tone that has large colonies and there are small colonies.

Pseudomonas putida strain Y-9 is known to be a heterotrophic bacterium that has nitrification activity in wastewater management (Xu et al. 2016). *Pseudomonas putida* as heterotrophic nitrifying bacteria including as mesophilic and is capable of low-temperature activity. Even pseudomonas putoda Y-9 is capable of active denitrification at low temperatures. With the identification of nitrifying bacteria in a culture environment, it can be interpreted that there is a group of bacteria capable of degrading ammonium in high quantities. The treatment of dark containers has a significant effect on growth of mud crabs so that this study can provide important information in the development of mud crab cultivation technology (Hastuti et al. 2018) and based on the results, *S. serrata* culture using light container at high light intensity demonstrated the better result in comparison with using dark container.

Several environmental factors such as substrate concentration, temperature, pH, salinity, dissolved oxygen (DO), C/N ratio and organic carbon (C) availability strongly influenced the growth of nitrifying bacteria and nitrification activity in these bacteria. Reported by Isnansetyo et al. (2014) that nitrifying bacteria (NR code) from Ariake seawater can grow a temperature between 15 ° C to 29.5 ° C Kim et al. (2008) reported a similar finding in that nitrification activity is constant value at a temperature range 10-30 ° C, while in this study, the pH of the FB filter during maintenance is 5.68-7.98.

Table 1 Isolation of nitrifying bacteria from mud crab recirculation aquaculture system

Type of bacteria	The initial number of isolate	Well growth isolate	Isolates by their metabolic activity	The selected isolates
Nitrifying bacteria	169	68	20	3

Table 2 Morphological characteristics of the ammonium oxidation bacteria (AOB) colony and cell in mud crab recirculation aquaculture system

Isolate code	Colony morphology	Cell morphology	Motility	Catalase	Oxidase
HIB_b	Large, yellowish, slimy colony	Rod/Gram-	+	+	+
HIB_d	A small, pale yellowish white colony	Rod/Gram-	+	+	+
HIB_e	Large, yellowish, slimy colony	Rod/Gram-	+	+	+
HIB_f	Large, yellowish, slimy colony	Rod/Gram-	-	+	+
HIB_g	Small, clear transparent, slimy colony	Rod/Gram-	+	+	+

Note: HIB: Indigenous bacteria with isolate code of b, d, e, f and g

Molecular identification of nitrifying bacteria isolates

The DNA purity of the nitrifying bacteria was relatively high, with A260/280 of 1.83-2.36 and A260/230 of 1.09-2.27. The purity from each isolate was high, ranging from 1.94±0.01 to 2.34±0.02 (Table 3) A260 shows the values of DNA and A230 for phenol and humic acid, A280 value is the wavelength for protein. The A260 / 230 is considered good if the value more than 2.0, indicating that no humic acid-like molecules are detected in A230 wavelength absorption. Humic acid is a type of contaminant which enables to adversely affect the quantification of DNA since it is detected at A230 nm and A260 nm (Yeates et al. 1998), The RAS environment culture can produce a good nitrifying bacterial community for ammonia waste degradation. Ammonia can be degraded by a group of nitrifying bacteria into nitrite and then becomes nitrate.

Results from BLAST-N search showed that the DNA sequences of both nitrifying bacteria from this study and those from the NCBI database have similarity value of 99% and 100%. The phylogenetic tree of nitrification bacteria showed that HIB_b, HIB_e, and HIB_g was amplified as a group *Pantoea calida*, *Pantoea* sp, while HIB_d was recognized as *Pseudomonas stutzeri*, and HIB_f was recorded as *Halomonas* sp, *Salinicola salaries* (Figure 2).

Table 3. The quantification of nitrification isolates in the mud crab (*Scylla serrata)* with recirculation system

Isolate Code	Concentration DNA (ng µL^{-1})	A260/280	A260/230
HIB_b	162.35±3.25	1.90±1.34	2.05±0.02
HIB_d	53.15±0.15	1.94±0.01	2.03±0.01
HIB_e	133.50±1.00	1.94±0.01	2.25±0.01
HIB_f	168.35±1.45	1.99±0.01	2.26±0.01
HIB_g	16.25±0.25	2.34±0.02	1.10±0.01

Note: HIB: Indigenous bacteria with isolate code of b, d, e, f and g

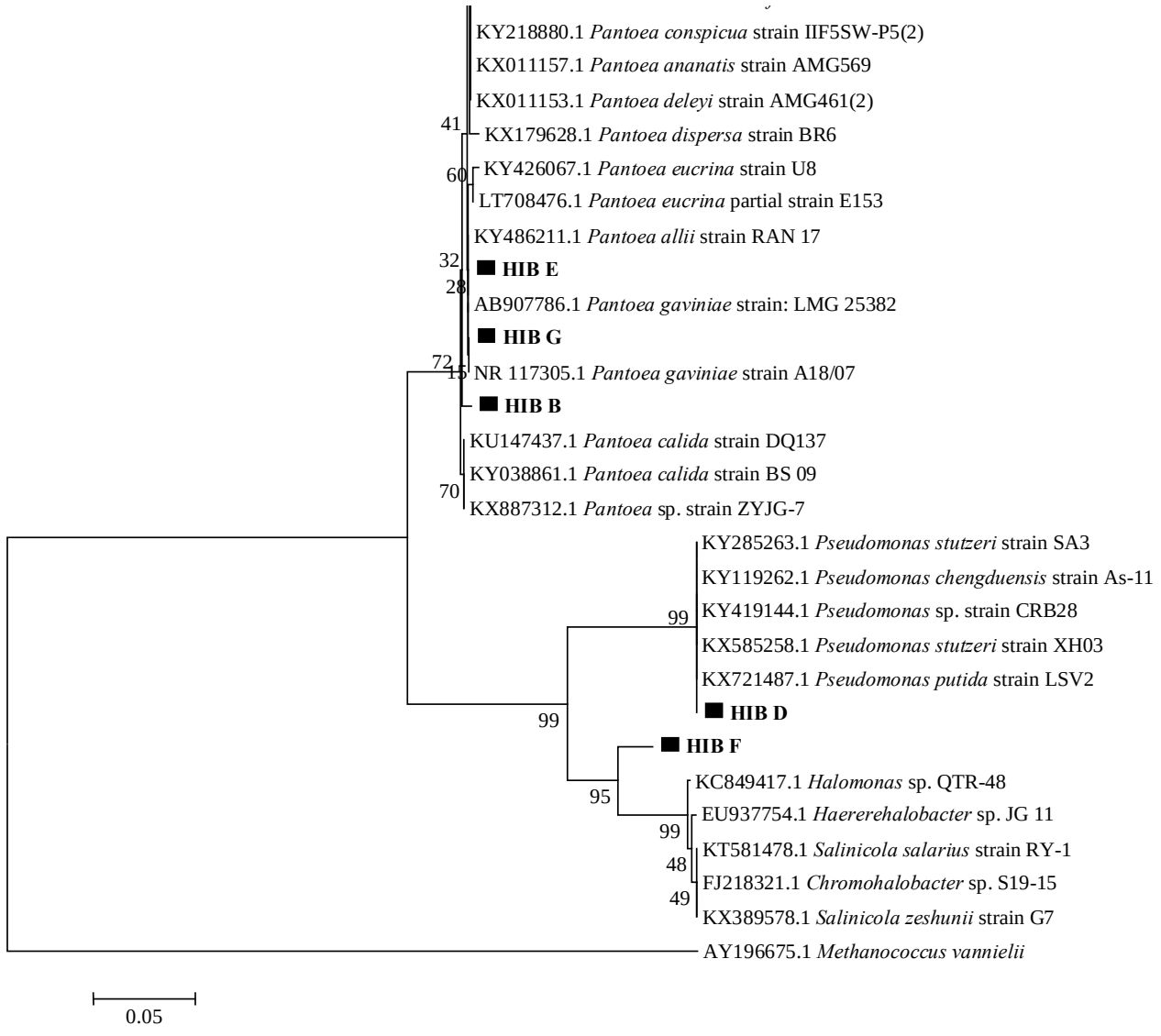

Figure 2 Phylogenetic tree of nitrification bacteria isolated from mud crab (*Scylla serrata)* recirculation aquaculture system. Tamura-3 parameters, 2000× Bootstrap replicates using Mega 6.00 *Neighbour Joining* Boostrap 2000× replicates (Tamura et al. 2013).

Halomonas spp. is potential nitrifying bacteria for saline ammonium wastewater (Sangnoi et al. 2017). Pseudomonas can grow well at high ammonium concentrations in Jogjakarta farms (Fitriyanto et al. 2017). Ammonium is used by the bacteria *Pseudomonas* to become nitrite and then nitrate. All nitrifying bacteria that have the character of couple nitrification-denitrification can oxidize ammonium and reduce nitrate. Microbial communities can be applied for the management of biological, industrial waste, including the archaea group (Calderon et al. 2013). In addition to its role in the process of nitrifying, *Thauera, Pseudomonas,* and *Denitromonas* also have a function as denitrification microbes, which are commonly used in activated sludge and form biofilms in the marine environment (Gao et al. 2012; Babatsouli et al. 2015; Wang et al. 2017). *Halomonas campisalis* is a halophilic strain has the ability as heterotrophic nitrification and aerobic denitrification bacteria that can remove inorganic nitrogen compounds (e.g., NH_4^+, NO_2^- and NO_3^-) and related the enzyme napA, nirS, norB and nosZ (Guo et al. 2013).

In conclusion, based on an identification of 16S rRNA genes, nitrifying bacteria isolated from mud crab recirculation aquaculture system showed a similarity of 99 % to *Pantoea calida*, *Pseudomonas stutzerii* and *Halomonas* sp. Based on the characterization of colony morphology, the bacteria showed a big colony, appeared cream in color, and produced mucoid. Also, their cells were recognized as basil and Gram-negative.

ACKNOWLEDGEMENTS

The authors would like to thank you to the Bogor Agricultural University, Indonesia for the research support and the Ministry of Research, Technology and Higher Education for the research funding given.

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