

© 2011, TextRoad Publication

Phylogenetic and nitrite oxidoreductase activities of nitrobacteria and nitrospira isolated from shrimp pond sediment in East Java, Indonesia

Anik Martinah Hariati^{1*}, Aulanni'am², Soemarno³, Marsoedi¹ and Marc Verdegem⁴

¹Aquaculture Department, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, 65145, Indonesia ²Laboratory of Biochemistry, Department of Chemistry, Faculty of Sciences, Brawijaya University, Malang, 65145, Indonesia

³Department of Soil Science, Brawijaya University, Malang, 65145, Indonesia ⁴Aquaculture and Fisheries Group, Wageningen University, Wageningen, 6700 AH, The Netherlands

ABSTRACT

Organic accumulation, resulted in ammonia and nitrite poisoning, is the main cause of shrimp pond collapse in Indonesia and the surrounding region. Two nitrifying bacteria were isolated from shrimp-pond sediment at Pasuruan, East Java, Indonesia. The study aimed to characterize and evaluate nitrite oxidoreductase ability of the bacteria. Comparative (phylogenetic) analysis based on 16S rRNA gene sequences revealed that both isolated bacteria were moderately related (77-90%) to uncultured clones of Nitrobacteria and Nitrospira. The nitrite oxidoreductase activity of both bacteria was comparable. However, per mg of protein, the enzymatic activity of Nitrobacteria was twice than Nitrospira. Keeping pond sediment optimal for these bacteria may result in more nitrite being transferred into nitrate, that is favorable for shrimp growth.

KEEYWORDS: Nitrifying-bacteria, 16S rRNA, nitrite-degrading-enzyme, brackish water.

INTRODUCTION

Indonesia is a principal shrimp producer after China, Thailand and Vietnam [1]. With increasing export requirement, shrimp culture is getting more attention. This was done by opening new shrimp ponds or intensifying the existing ponds, and had resulted in a tremendous increase of shrimp production [2][3]. However, since the year of 2000 there was a phenomenon that more and more unproductive shrimp pond in Indonesia. Then, it was realized that intensification of shrimp culture shrimp production promotes organic matter loading ending in deterioration of water and sediment quality especially high with nitrite accumulation.

Nitrification is the process in which ammonium is oxidized to nitrate in a two-step process carried out by two different groups of chemolithoautotrophic bacteria. First, ammonium-oxidizing bacteria (AOB) oxidize ammonium to nitrite, after which it is converted to nitrate by nitrite-oxidizing bacteria (NOB) [4][5][6]. Shammas [7] reported that nitrification process was influenced by temperature and pH of the medium. Optimal pH for nitrification was reported as pH 8±0.5. Dissolved oxygen was reported to be important in nitrification when oxygen supply is below 2 mg O₂/l NH₄-N [8]. Nitrification is catalyzed by the nitrite oxidoreductase, which is an integral part of the electron transport chain [9]. Nitrite oxidoreductase, is the essential enzyme complex of nitrite oxidizing cells. This enzyme is found as a part of cells of the nitrifying bacteria Nitrobacteria and Nitrospira.

Morphological criteria are often considered inappropriate for defining natural relationships, particularly when the study concern with microorganisms. Because of such limitations, genomic methods, such as 16s rRNA oligonucleotide cataloging, have been used [10][11]. Among the genomic methods used, nucleic acid sequencing is certainly the technique which has most revolutionized microbiology; this technique has been shown to be a powerful tool for exploring the phylogeny of both prokaryotes and eukaryotes [12].

Microorganisms are now characterized not only on the basis of their phenotypic features but also with an evolutionary perspective. The phylogenetic aspect of bacterial characterization is becoming an increasingly important step, even a necessary one for any microbial taxonomic study [10]. The availability of Nitro-types bacteria is crucial in the transformation of nitrite into nitrate. This study aimed to characterize and evaluate the oxidoreductase ability of bacteria that catalyze the nitrification processes. Those bacteria were isolated from shrimp pond sediment at Pasuruan, East Java, Indonesia

MATERIALS AND METHODS

Bacterial isolation and Identification

The study was carried out in a commercial shrimp farm at Pasuruan, East Java, Indonesia. This farm using high-protein artificial feeds. The 0.5-ha ponds were stocked with 100-150 PL m⁻² on a rotational basis, and aerated with paddlewheels, the number and position of which varied from pond to pond. Samples for bacterial counts were only taken in the sediment two weeks before harvesting. Three random locations, minimum 10 m apart, were sampled using a 5-cm diameter corer and mixed into a composite sample using a manual 5-cm diameter corer. From each a 5-cm top section was taken.

*Corresponding Author: Anik Martinah Hariati, ¹Aquaculture Department, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, 65145, Indonesia. E mail: amhariati09@gmail.com

A serial dilution method was used [13]. The core was 10fold diluted in inorganic media, in order to eliminate organic matter and heterotrophic bacteria. Nitriteoxidizing bacteria were enriched in mycotrophic medium containing 55 mg/l sodium pyruvate, 150 mg/l yeast extract, 150 mg/l peptone, and 2 g/l NaNO₂. The pH was 7.9. The tubes were incubated at 28°C in the dark for a period of 1-2 weeks, and periodically checked for nitrification by measuring the decrease in nitrite concentrations.

DNA Extraction

DNA extraction was performed using DNA Extraction Kit Nucleospin [14]. The extracted DNA obtained was confirmed by 1% agarose gel and visualized under UV light.

Amplification of 16S rDNA using PCR

PCR was done by mixing the ingredients in a thin wall. These ingredients are 5 µl of PCR mix, 1 µl (10 pmol) primer 11F (5'-GTT TGA TCC TGG CTC AG-3'), and 1 µl (10 pmol) 1512AR (5'-ACG GYT ACC TTG TTA CGA CTT-3'), 1 mL DNA template, and 10 µl ddH2O as 2 ml. Subsequently included in the Gene Cycler PCR, with PCR program used in one cycle is a hot start at 94[°]C for two minutes, denaturation at 94[°]C for 30 seconds, annealing at a temperature of 56°C for 45 seconds, extension at 72°C for one minute. Cycle performed 35 times. After 35 completed cycles of PCR, it is visualized by agarose gel electrophoresis. Bands were extracted from the gel and re-amplified and the partial 16S rRNA gene sequences were compared with those available on publicly accessible databases by using the Basic Local Alignment Search Tool program (BLAST, NCBI).

Nitrite oxidoreductase Isolation

10 ml of each NOB (Nitrobacteria and Nitrospira) concentration of 10⁸ cell ml⁻¹ were harvested by centrifugation, washed with 0.9% NaCl, and sonicated on ice for 15 to 30 min using a Biorupter apparatus for collecting protein. The protein concentrations of the crude extracts were measured by using the method of Bradford. Sample crude extract of 15 μ l were diluted (1:1) with 10 mM Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 20% glycerol, 1% 2-mercaptoethanol, and 0.001% bromophenol blue and boiled for 8 min. Samples (30 µl) were loaded onto lanes of 1-mm-thick polyacrylamide gels. The stacking and separating gels contained 4.5 and 10% polyacrylamide, respectively. Electrophoresis was performed at 28 mA, 200 V by using a Minigel Tetra apparatus. The molecular weight of the protein in the gel was determined by comparing those with protein marker.

Western Blot

Western blot was used for confirming nitrite oxidoreductase (NOR) recognized by monoclonal anti α and β -NOR. The protein isolated from the SDS-PAGE was transferred for 12 h at 25 V, temperature 4°C to Nitrocellulose (NC) membrane. The protein was then

blocked 1 h in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The proteins on the NC were incubated overnight at 4°C with mouse monoclonal to α and β -NOR as a primary antibody [15] diluted 1:1,000 in PBS containing 0.05% BSA and 0.025% Tween 20. Subsequently, they were incubated in goat anti mouse IgG phosphatase labeled (diluted 1:2500 in TBS for 1 h in room temperature). The NC membrane was washed twice with 10 mM Tris-HCl (pH 8.6) containing 0.05% Tween 20 and incubated with a substrate solution of Western Blue in darkness. The reaction was stopped by adding distilled water. The positive reaction was confirmed by appearance of a blue color band

Nitrite oxidoreductase activities and Kinetics

Nitrite oxidoreductase activity was determined spectrophotometric ally with ferricyanide as an artificial electron acceptor [9]. The system contained 30 mM Tris-HCl, 10 mM MgCl₂, 13 mM K₃ [Fe(CN)6], 0.8 - 3.2 mM NO₂ in 1.15 total volume, pH 8. The reaction was started by the addition of 50 µl NOR. The system without NOR addition was used as a standard curve. The mixture was incubated at 30°C for 30 min. Denaturized protein (1 min, 100 °C) served as a control. Nitrite consumption was measured spectrophotometric ally at 420 nm. The enzyme activity can be expressed as nitrite degraded (nmol) per minute per milligram of enzyme. The kinetic enzyme was measured based on the Michaelis-Menten equation:

$$V = V \max \frac{S}{S + Km}$$

where V is the NO₂-N oxidation rate (mM/ h), V_{max} is the maximum NO₂-N oxidation rate, K_m is the half-saturation constant (mM) and S is the substrate (NO₂-N) concentration (mM). Determination of K_m and V_{max} values are calculated at several levels of substrate (0.8-3.2 mM) with the addition of 50 µl NOR. The reaction between enzyme and substrate was carried out at an enzyme optimum temperature of 30°C and pH of 8 for 60 min.

Statistical Analysis

DNA sequence analysis conducted in the laboratory of Biomolecular, University of Brawijaya. Database is similarity search for nucleotide sequence performed by BLAST program from the National Center of Biotechnology Information (www.ncbi.nim.nih.com). 16S rRNA- is based phylogenetic tree using the MEGA 5.03 program with the least sequence similarity of 80%. While the nitrite oxidoreductase activities of Nitrobacteria and Nitrospira by curvy-linear.

RESULTS

Isolation and identification of Nitrobacter and Nitrospira

The observations showed that Nitrobacteria and Nitrospira were the most dominant genera. Both genera were gram negative, mostly rod shaped; motile and un-

motile (Figure 1). Nitrobacteria and Nitrospira were identified in the nitrifying source by a comparative 16S rRNA sequence analysis. Result of the sequences from isolated Nitrobacteria and Nitrospira were 708 and 759 nucleotides, respectively (Figure 2). Based on Alignment test it was identified that the Isolated Nitrobacteria has 8

clones affiliated and the Similarity matrix of clones affiliated with Nitrobacteria spp. The similarity of isolated Nitrobacteria with 8 uncultured Nitrobacteria sp 16S ribosomal RNA gene, partial sequence was identical ranging from 77 to 83%. While for Nitrospira 74-90% is similarities from 23 uncultured Nitrospira clone.



Figure 1. Nitrite-oxidizing bacteria, A. Nitrobacter and B. Nitrospira isolated from shrimp pond sediment



Figure 2. (M) Marker ;Total DNA of (1) Nitrobacteria (2) Nitrospira; 16S rRNA of (3) Nitrobacteria and (4) Nitrospira

Phylogenetic analyses

Figure 3 illustrate their affiliation in a phylogenetic tree. The remaining partial sequence was related to the 16S rRNA gene of uncultured Nitrobacteria sp. and Nitrospira clones. Partial 16s rRNA sequences were determined for all of the bacteria. The resulting dendrogram clustered in 8 clone of uncultured

Nitrobacteria and 23 clones for Nitrospira. This finding led us to determine the partial 16s rRNA sequences. The phylogenetic tree deduced from the partial sequences are shown in Figure 3,. The average level of similarity determined for the isolated Nitrobacteria strains was between 77-83%.

Hariati et al., 2011



Figure 3 Phylogenetic tree inferred from comparative analysis of 16S rRNA sequences (A). Nitrobacteria and (B) Nitrospira. The tree is based on the results of maximum-likelihood analysis of sequences of .708 and 759 nucleotides

Isolation and activities of nitrite oxidoreductase

The protein isolated both from Nitrobacteria and Nitrosprira were separated by SDS-PAGE. Nitrobacteria had more bands than Nitrospira. However they shared 3 bands with molecular masses of 98.4, 70.7 and 26.8 kDa. In order to compare the nitrite oxidoreductase from isolated nitrite-oxidizing bacteria with the nitrite oxidoreductase of Nitrobacteria (marker standard), western blotting experiments were performed using two monoclonal antibodies that recognize the α - and β -sub unit of the nitrite oxidoreductase as shown in Figure 4.



Figure 4 Nitrite oxidoreductase isolated from Nitrobacteria and Nitrospira

Nitrite Oxidoredoctase activities and kinetics

Isolated nitrite oxidoreductase oxidized nitrite to nitrate in the present of the artificial electron acceptor ferricyanide. The specific nitrite oxidase activity amounted to 79.6156. And 41.5012 nM NO₂

consumed/mg protein per minute (Table 1). The apparent Km value of the nitrite oxidoreductase for nitrite amounted to 0.6334 and 0.6227 mM (Figure 5). The highest enzyme activity of all bacteria was at 31°C and the pH of 8 (Figure 6).

 Table 1
 The Kinetically character and enzyme activity of Nitrite oxidoreductase isolated from Nitrobacteria and Nitrospira

Bacteria	Total Protein	Total activity	Specific activity	Vmax	Km
	(mg)	(U)	(U/mg/ min)	(mM/ mg/ min)	(mM)
Nitrobacteria	152.50	0.01214	7.96156	0.03498	0.6334
Nitrospira	300.75	0.01248	4.15012	0.0354	0.6227
U = mM NO2 consumed per minute					



Figure 5 Kinetics of nitrite oxidoreductase of (A) Nitrobacteria and (B) Nitrospira



Figure 6. The effect of (A) temperature and (B) pH on enzyme activity of Nitrobacteria and Nitrospira

DISCUSSION

The genera Nitrobacteria and Nitrospira have been dominantly isolated from shrimp sediment, although another studies show that there might be a greater variation in the nitrifying population [16]. They are obligate aerobes and cannot multiply or convert nitrites in the absence of oxygen [17]. However, in accordance with

their eco-physiological characteristics, several species have been observed to occur either predominantly or exclusively at special sites, e.g., in rivers, freshwater lakes, salt lakes, oceans, brackish waters, sewage disposal plants, rocks or natural stone of historical buildings, and acid soils.

Nitrite oxidizers are phylogenetic ally more distinct. Among the nitrite oxidizers, the genera Nitrobacteria were assigned to the α -subclass of Proteobacteria. The species have a typical Gram-negative multilayered cell wall. Alternatively, nitrifies can be detected in environmental samples by using a variety of different PCR-techniques, which in general use 16S rRNA [18] as well as key enzyme sequence information.

The moderate level of sequence homology found within the genus Nitrobacter confirms the suggestion of Seewaldt et al. [19]. These results also establish the fact that the genus Nitrobacteria is an evolutionarily coherent group as the phylogenetic relationships agree with the relationships determined in phenotypic and genomic studies.

Eight genetically different populations were distinguished in this study. The main population was more distantly related to Uncultured Nitrobacteria sp. The smaller population had a higher level of 16S rRNA similarity to Nitrobacteria. It was restricted to the active nitrifying zone. The genotypic differences obviously coincide with different physiological adaptations leading to these distinct spatial distributions. The exact description of the differing physiological properties of the new nitrite oxidizers, however, will require their isolation. A directed isolation can be monitored by the oligonucleotide probes developed in this study. The detection of Nitrobacteria spp. also yields independent support for a recent molecular study reporting that isolated Nitrobacteria were the main nitrite-oxidizing population in brackish and marine water while Nitrospira dominantly in freshwater aquaria [20][21][22]. From the distant matrix calculation showed that the Nitrobacteria isolated from shrimp pond sediment was closed to uncultured Nitrobacteria sp clone Sb-05-40, while for Nitrospira one group with uncultured Nitrospira clone RUGL6-220.

The band with molecular weight of 98.4 kDa and 70.7 kDa confirmed as α and β -subunit of the nitrite oxidoreductase. This result confirmed that both Nitrobacteria and Nitrospira produced protein with molecular weight of of 98.4 kDa and 70.7 kDa, respectivesly, as an enzyme of at the nitrite oxidoreductase. It was relatively lower than that of *Nitrobacteria hamburgensis* of 115 and 65 kDa [9][23]. The enzyme activities of both Nitrobacteria and Nitrospira were comparable. Meanwhile Nitrobacteria had higher rate of specific activity of enzyme than Nitrospira as there was high protein content in Nitrospira.

Temperature optimum of the highest nitrification rate is in line with Kim et al. [24] who concluded at 30°C. However, former research Groeneweg et al. [25] should optimum temperature was a bit higher (33°C). As for the nitrite oxidation rate, it increases by 5 times from 0.002 to 0.012 Unit as the temperature increase from 20 to 30°C. At temperature higher than 31 enzymatic activities will decrease. This was mainly due to denaturation process. The optimum pH of Nitrospira little bit higher than that of Nitrobacter at a value of 8 and 7.7 respectively. In wastewater treatment sludge, the optimum pH for nitrification was approximately 7.8 over a pH range of 6.4 - 8.2. For culturing pure strains of nitrifying bacteria, it was recommend a media pH of 7.5 - 7.8. Both NH₃ and NH₄⁺ occur within an environment with relative proportions being dependent upon pH and temperature. The relative NH₃ concentration increases with increasing pH. Above the optimum pH for Nitrification, the advantages of increased availability of free NH3 may be counterbalanced by the energy required to maintain the cytoplasmic pH below that of the external environment. As pH falls below the optimum, evidence exists that enzyme activity is negatively affected, thereby reducing nitrification [26].

CONCLUSIONS

The sequences from isolated Nitrobacter and Nitrospira were 708 and 759 nucleotides and have 8 and 23 clones affiliated, respectively. Comparative analysis of 16S rRNA gene sequences revealed a moderate (77-90%) phylogenetic relationship to uncultured Nitrobacteria and Nitrospira clone. Nitrobacteria isolated from shrimp pond sediment was closed to uncultured Nitrobacter sp clone Sb-05-40 16S ribosomal RNA gene, partial sequence while for Nitrospira close to RUGL6-220. The optimum condition for Nitrite oxidoreductase isolated from both Nitrobacteria and Nitorspira are pH of 8; temperature 31°C , and unit activity of 0.01214 and 0.01248, respectively.

ACKOWLEDGEMENTS

The Cooperation and Planning Bureaus Abroad, Indonesian Ministry of National Education provided scholarship to do Sandwich Program at Wageningen University. Thank to the Wageningen University for providing the facilities during the author did a sandwich program.

REFERENCES

- 1. FAO. 2010. The State of the World Fisheries and Aquaculture 2010. Food and Agriculture Organisation, Rome. 197p.
- 2. Hariati AM, Wiadnya DGR, Tanck MW, Boon JH, Verdegem MCJ (1996). Pond production of Penaeus monodon (Fabricus) in relation stocking density, survival rate and mean weight at haverst in East Java. Aquaculture 27: 277-282.
- 3. Hariati AM, Wiadnya DGR, Rini RK, Boon JH, Verdegem MCJ (1998). Penaeus monodon (Fabricus) and Penaeus merguensis (de Man)

biculture in East Java, Indonesia. Aquaculture Research 29:1-8

- 4. Koops HP, Purkhold U, Pommerening-Roser A, Timmermann G, Wagner M (2003). The lithoautotrophic ammonia-oxdizing bacteria. in Dworkin et al., eds., The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community.
- Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437: 543-546.
- Alawi M, Lipski A, Sanders T, Pfeiffer EM, Spieck E, (2007). Cultivation of a novel cold-adapted nitrite oxidizing beta proteobacterium from the Siberian Arctic. ISME J 1:256-264
- Schramm A, De Beer D, van den Heuvel JC, Ottengraf S, Amann R (1999). Microscale Distribution of Populations and Activities of *Nitrosospira* and *Nitrospira* spp. along a Macroscale Gradient in a Nitrifying Bioreactor: Quantification by In Situ Hybridization and the Use of Microsensors. *Appl. Environ. Microbiol.* 65 (8): 3690-3696
- 8. Nogueira R, Lazarova V, Manem J, Melo LF (1998). Influence of dissolved oxygen on the nitrification kinetics in a circulating bed biofilm reactor. Bioprocess Eng. 19:441–9.
- Sundermeyer-Klinger H, Meyer W, Warninghoff B, Bock E (1984). Membrane-bound nitrite oxidoreductase of Nitrobacter : evidence for a nitrate reductase system. Arch Microbiol. 140:153– 158
- Navarro E, Fernandez MP, Grimont F, Clays-Josserand A, Bardin R (1992). Genomic heterogeneity of the genus Nitrobacter. Int. J. Syst. Bacteriol. 42: 554-560.
- Seewaldt E, Schleifer KH, Bock E, Stackebrandt E (1982). The close Dhvlogenetic relationshit, of Nitrobacter and Rhodopseudomonas palustris. Arch. Microbiol. 131:287-290.
- Woese CR (1987). Bacterial evolution. Microbiol. Rev. 51:221-271
- Schmidt EL, Belser LW (1982). Nitrifying bacteria. Methods of soil analyses, Part 2. Chemical and Development of nitri®cation inhibition assays 439 microbiological properties. Agronomy Monograph, 2nd ed., Vol. 9: 1027-1041.
- Vogelstein B, Gillespie D (1979). Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 76: 615-619
- 15. Litman MR, Chiu NHL, Wang J. 2006. Specific recognition of non-denaturated nitrite-oxidizing

system of Nitrospira moscoviensis by monoclonal antbody Hyb 153-3.

- 16. Koops HP, Purkhold U, Pommerening-Roser A, Timmermann G, Wagner M (2003). The lithoautotrophic ammonia-oxdizing bacteria. in Dworkin et al., eds., The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community
- Grunditz C, Dalhammar. 2001. Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter. Wat. Res. 35. 2: 433-440
- Vanparys B, Spieck E, Heylen K, Wittebolle L, Geets J, N. Boon N, De Vos P (2007) The phylogeny of the genus Nitrobacter based on comparative rep-PCR, 16S rRNA and nitrite oxidoreductase gene sequence analysis. Syst Appl Microbiol 30: 297-308.
- Seewaldt E, Schleifer KH, Bock E, Stackebrandt E (1982). The close Dhylogenetic relationshit, of Nitrobacter and Rhodopseudomonas palustris. Arch. Microbiol. 131:287-290.
- Prosser JL, Cox DJ (1982). Nitrification, p. 178-193. In Burns RG, Slater JH (ed.), Experimental microbial ecology. Blackwell Scientific Publications, Oxford.
- Orso S, Gouy M, Navarro E, Normand P (1994). Molecular phylogenetic analysis of Nitrobacter spp. Int. J. Syst. Bacteriol. 44:83–86.
- 22. Lebedeva EV, Alawi M, Maixner F, P. G. Jozsa PG, Daims H, Spieck E (2008) Physiological and phylogenetic characterization of a novel lithoautotrophic nitrite oxidizing bacterium, 'Candidatus Nitrospira bockiana'. Int J Syst Evol Microbiol 58:242-250
- 23. Meincke M, Bock E, Kastrau D, Kroneck PMH (2004). Nitrite oxidoreductase from Nitrobacter hamburgensis: redox centers and their catalytic role. Arch Microbiol 158: 127-131
- 24. Kim JH, Guo X, Park HS (2008). Comparison study of the effects of temperature and free ammonia concentration on nitrification and nitrite accumulation. Process Biochemistry 43: 154-160.
- Groeneweg J, Sellner B, Tappe W (1994). Ammonia oxidation in Nitrosomonas at NH3 concentration near Km: effect of pH and temperature. Water Res. 28:2561-2566.
- Prosser JL, Cox DJ (1982). Nitrification, p. 178-193. In Burns RG, Slater JH (ed.), Experimental microbial ecology. Blackwell Scientific Publications, Oxford.