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Screening, Identification and Immobilization of Ammonia Oxidizing Bacterial Consortium Collected from Mangrove Areas and Shrimp Farms

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ABSTRACT

The immobilization of nitrifying bacteria in alginate has been used to evaluate the performance of ammonia reduction. In this research, bacteria were screened and observed for their ability to reduce ammonia. Consortium M1, isolated from the mangrove area (Morib, Selangor) showed the most effective reduction of ammonia from an initial concentration of 2.17 ± 0.10 to 0.06 ± 0.01 mg L⁻¹ in 14 days. The consortium was then identified to consist of *Pseudomonas aeruginosa* (99%), *Pseudomonas stutzeri* (98%) and *Nocardioides albus* (98%) using the 16S rDNA gene sequences via Polymerase Chain Reaction (PCR) technique and identified by phylogenetic analysis based on their partial 16S rDNA sequences comparison in NCBI GenBank. The consortium M1 was then immobilized into alginate beads each containing 1.79×10^8 CFU mL⁻¹ bacteria cells before being tested for its efficacy in reducing ammonia under laboratory conditions using 10, 20, 30, 40, 50, 100, 150 and 200 beads. The fastest reduction rate of Total Ammonia Nitrogen (TAN) was observed in flasks containing 150 beads which on day 6, drastically reduced TAN from 2.50 ± 0.10 to 0.090 ± 0.059 mg L⁻¹ followed by treatment with 200 beads (0.104 ± 0.07 mg L⁻¹). However, at the end of experiment at day 14, the lowest TAN level (0.083 ± 0.063 mg L⁻¹) was observed in flasks with 200 beads which was not significantly different ($p > 0.05$) from flasks with 150 beads (0.106 ± 0.034 mg L⁻¹). The present study reveals that the immobilization of bacterial consortium consisting of ammonia oxidizing bacteria could be used as an alternative for reduction of high TAN concentration in shrimp or fish hatchery system.

Key words: Nitrifying bacteria, immobilization, total ammonia nitrogen, alginate, gene sequence

INTRODUCTION

The intensive culture of aquatic animals such as fish and shrimp in high stocking densities and high feeding rate increase the exposure of aquatic animals to ammonia which is harmful to the aquatic animals (Chezhian *et al.*, 2012). Nitrification is the biological process of nitrifying bacteria in the conversion of ammonia to nitrate nitrogen. Ammonia has a short lifetime as it can rapidly convert to ammonium (NH₄⁺) and ammonia (NH₃) (Bozkurt, 2006). The conversion is usually attributed to autotrophic bacteria, such as *Nitrosomonas* and *Nitrobacter* spp. However, nitrification process is not only limited to autotrophic bacteria but also numerous heterotrophic bacteria and fungi (Verstraete, 1975). From previous studies, the most well-known heterotrophic ammonia

oxidizing bacteria is *Alcaligenes* sp. which was isolated from soil (Castignetti and Gunner, 1980, 1981). Other bacteria with similar characteristics are *Arthrobacter* sp. (Brierley and Wood, 2001; Verstraete and Alexander, 1972), *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* (Pelleroni, 2005) and *Klebsiella pneumonia* (Kim *et al.*, 2002).

Immobilization is a technique in which cells are entrapped inside a support gel called alginate. The alginate material is mainly extracted from three different species of brown seaweed namely *Laminaria hyperborea*, *Macrocystis pyrifera* and *Ascophyllum nodosum* (Smidsord and Skjak-Break, 1990). Moreira *et al.* (2006) reported that the use of alginate extracted from *L. hyperborea* provide more stable beads in seawater compared to alginate extracted from other seaweed such as *M. pyrifera*. The alginate gel has been the most suitable material for cell immobilization as they provide mild and damp conditions for the cells to inhabit. Moreover, bacteria that are immobilized by alginate gel also give the most effective nitrification rate as compared with those immobilized by other types of materials such as carrageenan and agar (Kim *et al.*, 2000).

In this study, a consortium of bacteria was screened from marine environment for their ability to utilize ammonia into nitrite. The ammonia oxidizing bacteria were then immobilized using alginate gels and tested for their efficiency to reduce ammonia under controlled condition. Bacteria efficient in reducing TAN could then be used as potential isolates in managing water quality in aquaculture system.

MATERIALS AND METHODS

Isolation and screening of potential ammonia oxidizing bacteria: Soil and water samples were collected from several sampling sites along the west coast of Peninsular Malaysia such as mangrove areas of Kuala Gula, Perak, Kuala Juru, Pulau Pinang, Morib, Selangor and Kuala Langat shrimp farms. Samples were subcultured in Skinner and Walker (1961) medium four times and nitrite test was carried out to all the consortia samples using nitrite strips (Merckoquant® Merck, Germany) to detect the accumulation of nitrite in the culture.

Screening for total ammonia nitrogen (TAN) reduction: Total ammonia nitrogen analysis (Parsons *et al.*, 1984) was carried out for the bacterial consortia KJ4, KJ6, M1, M2, M3 and M4 that showed positive results with nitrite strip. To test for TAN reduction, initial ammonia concentration of $2.0 \pm 0.1 \text{ mg L}^{-1}$ was prepared in 300 mL flask. To prepare 2.0 mg L^{-1} of ammonia, 10 mL of $100 \text{ mg L}^{-1} (\text{NH}_4)_2\text{SO}_4$ stock solution was diluted in 90 mL seawater (10.0 mg L^{-1}). Then 20 mL of this stock solution (10.0 mg L^{-1}) was diluted again in 80 mL of seawater to form the final value of 2.0 mg L^{-1} . Each consortium was added to a flask in triplicate at 10^6 CFU mL^{-1} bacteria per flask. Analysis of TAN was conducted on alternate days for 14 days.

Identification of selected bacterial consortium: Consortium M1 was selected for identification using 16S rDNA sequence as it showed the best rate in reduction of ammonia. The DNA was extracted using conventional method. The extracted genomic DNA was used as template to perform PCR amplification for 16S rDNA identification. Universal primers as previously published were used (Kim *et al.*, 2003): Forward: 5' GAT TAG ATA CCC TGG TAG TCC AC 3' and Reverse: 5' CCC GGG AAC GTA TTC ACC G 3'. The PCR was carried out in 50 μL of reaction mixture containing 1 X PCR buffer with $1.5 \text{ mmol L}^{-1} \text{ MgCl}_2$, $200 \mu\text{mol L}^{-1}$ deoxynucleotide, 2.5 units *Taq* DNA Polymerase, $0.2 \mu\text{mol L}^{-1}$ of each forward and reverse primers and 10 μL genomic DNA as a template. Reaction was carried out in a thermal cycler (DNA Engine PTC-200; Bio-Rad) beginning with initial denaturation at 98°C for 2 min and 35 cycles of denaturation at 96°C for 1 min,

annealing at 60°C for 1 min, elongation at 72°C for 1 min and terminated by extension step at 72°C for 10 min. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The PCR products were further purified before sending for sequencing. Partial sequences of 16S rDNA were compared by BLAST application against representative prokaryote of 16S rDNA gene sequences in NCBI GenBank (National Centre of Biotechnology Information) database.

Immobilization of nitrifying bacterial consortium: Alginate solution was prepared as described by Smidsord and Skjak-Break (1990). Alginate beads were formed by dissolving 3% (w/v) alginate (FMC Biopolymer, Protanal LF 10/60, Drammen, Norway) in autoclaved seawater for 1-2 h. Bacterial culture (1.2×10^6 CFU mL⁻¹) was centrifuged at 10000 rpm for 1 min before the bacteria suspension was mixed with 3% (w/v) of alginate solution. The mixture of suspension cells and alginate was dropped using a syringe (0.2 mm internal diameter) into a cation solution of 0.1 M strontium chloride (SrCl₂, Merck, Germany) from a height of approximately 15-20 cm and at a rate of approximately one drop per second. Beads were immediately formed in the SrCl₂ with gentle stirring and left in the cation solution for 45-60 min to allow complete hardening of the alginate beads. Beads were then washed several times using distilled water and a final wash with seawater. The beads were stored in Skinner and Walker medium at 4°C prior to use. For counting the bacteria in the entrapped cells, the bacteria were recovered by dissolving the beads in 0.5 M of trisodium citrate solution (pH 6.5). The released bacteria were then counted on the basis of the viable cell number on Skinner and Walker agar plate medium.

Experimental design

Ammonia reduction using immobilized nitrifying bacteria: The immobilized bacterial consortium in alginate at various numbers of beads (10, 20, 30, 40, 50, 100, 150 and 200) was tested (triplicates) in 500 mL flasks for their efficacy in TAN reduction. About 300 mL of sterilized seawater with an initial TAN concentration of 2.50 ± 0.10 mg L⁻¹ was prepared from ammonia stock solution (100 mg L⁻¹ of ammonia) in 500 mL flasks. No beads were used for the control flasks. Total ammonia nitrogen analysis (Parsons *et al.*, 1984) was conducted to observe the effectiveness of immobilized beads in removing TAN. Analyses were carried out on alternate days for 14 days.

RESULTS

Isolation and screening of potential ammonia oxidizing bacteria: From a total of 46 consortium cultures, 11 consortia showed positive results for the conversion of ammonia to nitrite using nitrite strip. These 11 consortium cultures were then tested for their ability to reduce TAN for 14 days. At the end of experiment, only three consortia showed reduction in TAN levels from an initial concentration of 2.00 ± 0.10 to 0.01 ± 0.01 mg L⁻¹ (Fig. 1). Consortium M1 showed the most effective reduction of TAN (0.06 ± 0.01 mg L⁻¹) compared to consortium M2 (0.15 ± 0.03 mg L⁻¹) and consortium M4 (0.13 ± 0.05 mg L⁻¹) within 14 days period.

Identification of selected bacterial consortium: Consortium M1 produced three bacterial colonies that were selected for identification using 16S rDNA sequence. After bacterial DNA was extracted using the conventional method, the amplification of 16S rRNA genes was determined using universal primer of 16S rRNA. The PCR products were sent for sequencing before being analyzed in NCBI GenBank using BLAST. Based on the BLAST results, sequences showed high homology with three different types of bacterial species namely *P. aeruginosa* (99%), *P. stutzeri* (98%) and *N. albus* (98%).

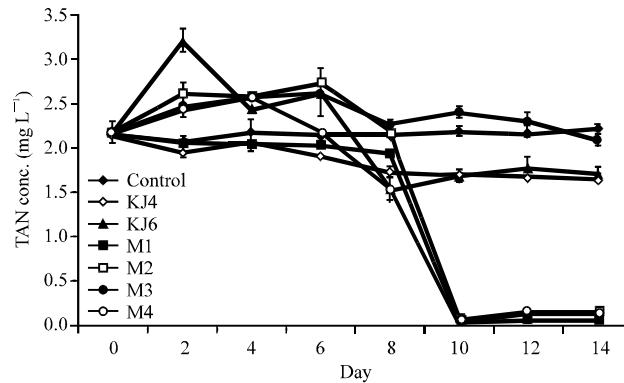


Fig. 1: Reduction of ammonia by bacterial consortia, vertical bars are standard error of the means

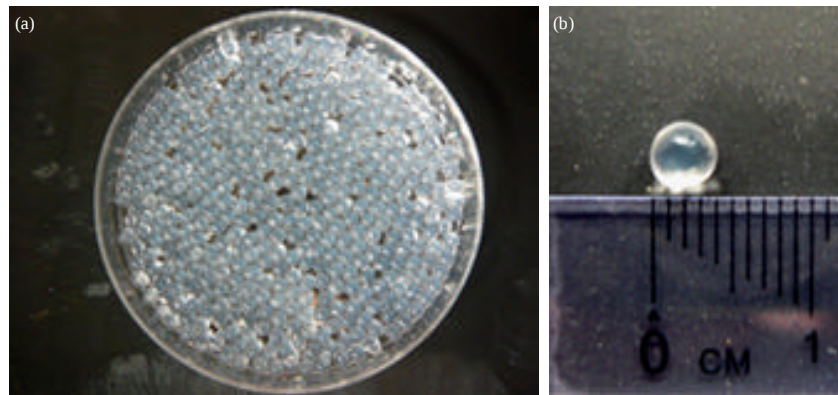


Fig. 2(a-b): Bacterial consortium, (a) immobilized in beads and (b) bead size

Immobilization of nitrifying bacteria consortium: Bacterial consortium immobilized in alginate gel was 4 mm in diameter (Fig. 2). The bacteria cells in each alginate bead showed an average of 1.79×10^8 CFU mL⁻¹ bacteria cells after plate count.

Ammonia reduction using immobilized nitrifying bacteria: Throughout the experiment, all treatments except for the control flasks showed an increased level of TAN after the addition of immobilized bacteria (10, 20, 30, 40, 50, 100, 150 and 200 beads) to each flask (Fig. 3). The initial increase in TAN levels in all flasks with beads was followed by a gradual decrease of TAN in all the treatments after day 4. Based on the preliminary experiment, all the different number of beads (i.e., 10, 20, 30, 40, 50, 100, 150 and 200 beads) were able to reduce TAN. Total ammonia nitrogen reduction took 10 or more days in flasks containing less than 50 beads. However, it took 6-8 days for reduction of TAN in flasks containing more than 100 beads. The fastest reduction rate of TAN was observed in flasks containing 150 beads which drastically reduced TAN to 0.090 ± 0.059 mg L⁻¹ on day 6 followed by treatment with 200 beads (0.104 ± 0.07 mg L⁻¹). However, at the end of the experiment (14 days), the lowest TAN level was observed in flasks with 200 beads (0.083 ± 0.063 mg L⁻¹) which was however not significantly different from flasks with 150 beads (0.106 ± 0.034 mg L⁻¹).

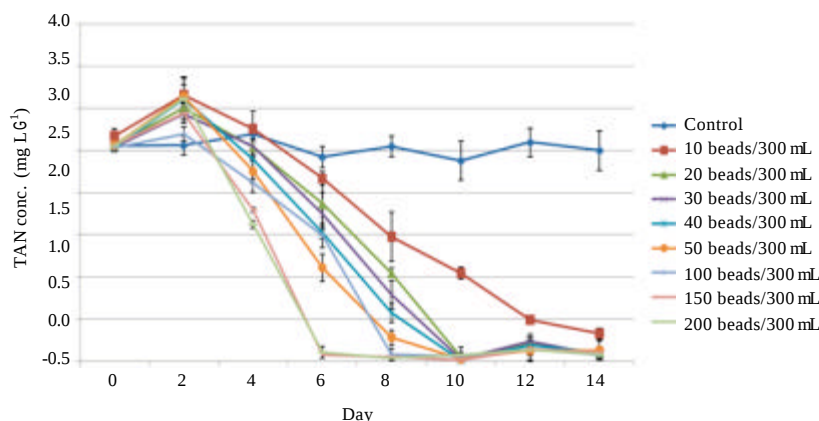


Fig. 3: Total ammonia nitrogen concentration with the use of different number of beads over a period of 14 days, vertical bars are standard error of the means

DISCUSSION

Liao (1989) reported that poor soil and water quality can be the main cause of shrimp mortality in hatcheries or ponds. In the past, water exchange was the only way to overcome the accumulation of ammonia. However, due to the risk of infectious diseases as a result of frequent water exchange, bioremediation techniques have been used in shrimp pond management (Thimmalapura *et al.*, 2002; Antony and Philip, 2006). The use of other processes involving physical or chemical treatments for ammonia reduction can be costly for large scale aquaculture industry as well as the risk of toxic by-products formation at the end of the reaction. Hence, the use of microorganisms to clean up polluted areas has provided opportunities to expand the technologies in providing not only low cost aquaculture management but also the potential for reducing contaminant in different aquaculture systems (Jones and Hood, 1998). By utilizing biological nitrification process, water quality problems can be solved, as biological nitrification process not only controls the levels of nitrogenous compounds in the water but it is also an essential process of the nitrogen cycle in aquatic system (Ambrose *et al.*, 1993). Nitrification process involves the conversion of ammonia to nitrite and nitrite to nitrate which is a crucial process in many wastewater and aquaculture treatment schemes (Blasiola and Vriends, 1991). In the present study, isolation of nitrifying bacteria was difficult due to the slow growth of these bacteria. This has also been substantiated by Soriano and Walker (1968) who reported similar problems in their study on nitrifying bacteria. Aakra *et al.* (1999) reported the difficulty to obtain pure culture of nitrifying bacteria on solid medium. Meanwhile, Schmidt and Belser (1994) reported that bacterial colonies were hardly visible in agar plates and suggested that nitrifying bacteria should be cultured in liquid culture media. After BLAST analysis in GenBank, three species of bacteria; *P. aeruginosa*, *P. stutzeri* and *N. albus* were identified in the consortium. Previous studies by Zhoua *et al.* (2007) reported that *P. aeruginosa* bacterium can utilize ammonium (NH_4^+) and nitrate (NO_3^-) under controlled conditions. Zhoua *et al.* (2007) also reported that the ammonium that was utilized by the bacterium was used as a nitrogen source for its cell components and for cell growth of the bacterium. For *P. stutzeri*, Kester *et al.* (1997) and Mahne and Tiedje (1995) mentioned that this bacterium is a denitrifying bacteria which has the ability to convert nitrite or nitrate to nitrogen gases. Meanwhile, for *N. albus*, there is not much information available to classify the

morphology and characteristics of the bacteria. Utilization of nitrogenous compounds such as ammonia is not necessarily specific by autotrophic nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* but could also be done by heterotrophic nitrifying bacteria (Focht and Verstraete, 1977). Previous studies by Castignetti and Gunner (1980, 1981), Brierley and Wood (2001), Verstraete and Alexander (1972), Castignetti and Hollocher (1984), Dalsgaard *et al.* (1995) and Hooijmans *et al.* (1990) have successfully isolated and identified heterotrophic nitrifying bacteria such as *Pseudomonas* sp., *Alcaligenes* sp., *Arthrobacter* sp. and *Thiosphaera* sp. that are capable of utilizing nitrogenous compounds. Unlike autotrophic nitrifying bacteria that require oxygen, many heterotrophic nitrifying bacteria can survive in the presence or absence of oxygen. Apart from that, autotrophic nitrifying bacteria do not form spores or known as resting stages (Rothfuss *et al.*, 1997). However, for heterotrophic nitrifying bacteria, they can form spores in adverse condition and moreover can be practically used for commercial purpose as they can be dried packed and sold as viable culture (FI, 2012). The effectiveness to reduce TAN levels depends on the ability of ammonia oxidizing bacteria to oxidize ammonia to nitrate which could be affected by certain environmental conditions. In addition, the ability of nitrifying bacteria is limited in cases where there are bacteria washouts with frequent water exchanges (Sumino *et al.*, 1992). Thus, a rational approach would be to use immobilized bacteria which will ensure that the bacteria are retained within the treatment system and are also protected from adverse conditions (Wijffels and Tramperl, 1995) without affecting their nitrifying ability. Therefore, in the present experiment the bacterial consortium was immobilized in alginate extracted from *L. hyperborea*. According to Smidsord and Skjak-Break (1990), alginate extracted from *L. hyperborea* contains high guluronic acid residues which strengthen the stability of the beads in the seawater. The present study found that alginate extract from *L. hyperborea* (FMC Biopolymer, Protanal LF 10/60, Drammen, Norway) was stable in seawater and most of them can be maintained in immobilized form throughout the experimental period of 14 days. This study also revealed that the use of sodium alginate from other brown algae, disintegrated after 1-2 days in seawater. Other polymers like cellulose, chitin and chitosan, their rigorous preparations with the extensive chemical processes are not suitable in aquaculture system practices as they become resistant to biodegradation. In previous studies, Shan and Obbard (2001), Kim *et al.* (2000) and Chen (2001) have tested the use of immobilization technology for water quality control in aquaculture systems. The advantages of using immobilized cells are because bacteria can be maintained for longer period (Parvanova-Mancheva and Beschkov, 2009) and have high rate of nitrogenous compound uptake when compared with the free suspended cells (Garbayo *et al.*, 2000). Focht and Verstraete (1977) reported that unlike autotrophic nitrifying bacteria, heterotrophic nitrifying bacteria have lower nitrification activities as they require organic compounds for their energy sources in nitrification. By immobilizing heterotrophic nitrifying bacteria in alginate gel, the nitrification activities will improve as well as the ability of alginate gel to maintain the entrapped bacteria during the long operation time (Tramper *et al.*, 1985). According to Rostron *et al.* (2001), there are not many studies conducted on mixed cultures or consortium because many research on bacteria immobilization focused on pure bacterial culture. The consortium M1 used in the present study consisted of three isolates, i.e., *P. aeruginosa*, *P. stutzeri* and *N. albus*. Since, the pure isolates could not grow well on their own, they could not be studied separately for their efficiency of reducing TAN. Nevertheless, when used as a consortium, they successfully reduced TAN. Although, further studies on the efficiency of the pure isolates to reduce TAN would provide valuable information, Tramper *et al.* (1985), has reported that the reduction trends of consortium were largely similar

with that of the pure culture. The sudden increase in TAN levels after the addition of immobilized bacteria in the flasks was due to the mineralization of proteinaceous matter to inorganic nitrogen as has been described by Rodina (1972). However, after the mineralization process, TAN were immediately reduced in all the flask treatments except for control flasks.

CONCLUSION

According to the results of this study, consortium M1 collected from mangrove area of Morib, Selangor showed the highest reduction of TAN concentration after 14 days compared to other bacterial consortium from shrimp ponds and other mangrove areas. Therefore, after the immobilization of bacterial consortium, the presence of these ammonia oxidizing bacteria could be used as alternative for reduction of high TAN concentration in shrimp or fish hatchery system. Further studies need to be conducted to optimize the use of immobilized ammonia oxidizing bacteria in maintaining a good water quality for aquatic animals in aquaculture system.

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