AMMONICAL - NITROGEN REMOVAL BY AN AEROBIC HETEROTROPHIC BACTERIUM, *Microbacterium* sp., VCM11

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ABSTRACT   Bacterial strains were isolated from shrimp culture wastewater and assessed for ammonia reduction activity. Nine strains were selected based on nitrate reduction test. Out of nine strains, VCM11 showed significant ($p \leq 0.05$) ammonical-nitrogen (AN) removal compared to control. Based on biochemical tests, the strain was identified as *Microbacterium* sp. Strain VCM11 was inhibited in all the antibiotic tests (troleandomycin, rifamycin SV, lincomycin, and nalidixic acid). Different parameters including sodium chloride (NaCl) concentration and temperatures were tested. The results obtained indicated that VCM11 at 180rpm, 30°C, and 35 ppt of sodium chloride (NaCl) reduced 53.57 ± 3.16 NH_3^+-N to 4.83 ± 1.12 NH_3^+-N (90.98%) within 120 hours.

(**Keywords:** water quality, ammonia content (AC), *Microbacterium* sp., biochemical tests and antibiotic tests)

INTRODUCTION

The management of water quality in aquaculture and particularly in hatcheries is essential. Deterioration of water quality particularly with high total ammonia nitrogen (TAN) will affect the productivity of aquaculture. User and environmentally friendly technologies such as bioremediation are being adopted in aquaculture farms to manage water quality. Ammonia concentration is one of the crucial factors determining aquaculture water quality. Ammonia excretion rate has been used to assess the effects of various factors on total nitrogen excreted by crustaceans due to ammonotelism (Jiang *et al*., 2000). Ammonia exists in two different forms, ionized (NH_4^+) and unionized (NH_3). Unionized ammonia (NH_3) induces stress to aquatic animals and results in decreased survival of aquatic organisms (Millamena, 1990). Unionized ammonia (NH_3) is considered more toxic than ionized ammonia (NH_4^+) due to its ability to diffuse readily across the cell membrane (Emerson *et al*., 1985). Therefore, the removal of ammonia is crucial to hatcheries that produce cultured organisms.

There are three possible processes (physical, chemical, and biological) of remediation used to remove ammonia in the aquaculture system (Barik *et al*., 2011). The physical process has limits to application and is expensive. Chemical remediation involves application of chemicals to reduce the ammonia concentration. Some of the chemicals used may itself be harmful to the organisms if they accumulate in culture systems. Biological remediation is an important tool to convert toxic substance to non-toxic substance.

Biological nitrification-denitrification is one of the solutions to reduce the ammonia concentration in aquaculture. This process is technically feasible and economically favourable (Peng and Zhu, 2006). There are various methods and techniques developed for the nitrification and denitrification of aquaculture wastewater. These include sequencing batch reactor (SBR) (Fontenot *et al*., 2007; Guo *et al*., 2011), hydrogenotrophic denitrification of synthetic aquaculture wastewater using membrane reactor (Visvanathan *et al*., 2008), aerobic and anaerobic biofiltration (Rijn and Rivera, 1990), bio-electrochemical removal (Ghafari *et al*., 2008), and trickling filter using different filter media (Lekang, 2000). All these methods and techniques have varying degrees of success. Removal of TAN under conditions of continuous TAN production is not an easy task (Shan and Obbard, 2001). Further technical problems during designation, maintenance of the technology, and high capital cost restrict the efficiency in reducing ammonia concentration (Shan and Obbard, 2001).

Since extensive and sophisticated wastewater treatment methods are very expensive and unsuitable for wastewater treatment, an effective, inexpensive, and safe biological water treatment would be more beneficial. The aim of this study was to isolate and screen indigenous bacteria suitable for treating the aquaculture wastewater.

MATERIAL AND METHODS

Isolation and screening of bacteria

Ammonium oxidizing bacteria (AOB) media was prepared by dissolving 5 g of peptone, 3 g of beef extract, 1 g of potassium nitrate (KNO_3), and 20 g of sodium chloride (NaCl) in 1L of distilled water. The AOB medium was autoclaved for 15 min at 121°C. Sterile medium (100 mL) in 250-mL conical flasks (n = 3) was inoculated with 5 mL of aquaculture wastewater (7 days without water exchange) collected from Marine Culture Unit, University Malaya, and incubated at 30°C on a rotary shaker at 180 rotations per minute (rpm). After 7 days of incubation, 1 µL of sample spread on fresh agar plates. After 48 hours, single colonies were picked and streaked on fresh AOB agar plates. Pure isolates were obtained by repeated streaking on fresh agar plates. The resulting bacterial isolates were tested for their ability to produce nitrite by using nitrate reduction test kit (Sigma-Aldrich Chemie GmbH, Switzerland). Nine grams of nitrate broth powder were dissolved in 1 L of distilled water. Ten mL of the broth was dispensed into 20 mL sample tubes and autoclaved at 121°C for 15 minutes and cooled. One mL of bacterial sample was then inoculated into three sample tubes. A negative control (autoclaved wastewater) was set up. All the tubes were incubated at room temperature for 48 h. Five drops of reagent A (sulfanilic acid solution) and 5 drops of reagent B (α – naphthylamine solution) were added into all the tubes.

Treatment of wastewater by selected strain

Out of 21 strains, nine strains were screened for on their ability to produce nitrite. All nine strains were screened for ammonia reduction in aquaculture wastewater collected from Marine Culture Unit, University Malaya. Ammonia concentration was analysed by photometric determination (Hach spectrophotometer DR2400, USA) with ammonia salicylate reagent, ammonia cyanurate reagent and AmVer™ high range ammonia test 'N tube™ reagent (Hach, Loveland, USA). The strain that showed better reduction of ammonia was selected for further analysis.

Biochemical Identification

Strain VCM11 was selected due to its significant reduction (p ≤0.05) of ammonia concentration compared to the rest. Biochemical test for strain VCM11 was based on morphological, physiological, and biochemical characteristics. Gram staining of strain VCM11 was carried out to determine whether it was Gram negative or Gram positive. The carbon utilization test was performed with 15 different carbons. The bacterium was identified according to Bergey's Manual of Determinative Bacteriology.

Effect of temperature on growth

Temperature plays an important role for the optimum growth of a bacterium. The effect of temperature on growth of strain VCM11 was studied by incubating the culture media at temperatures 25°C, 30°C, 37°C, and 40°C. The optical density (OD) read at 600nm was recorded after 48 h for all temperatures tested.

Effect of Sodium Chloride on growth

The effect of NaCl for best growth of strain VCM11 was studied by supplying various concentrations of NaCl to the production medium. The experiment was carried out separately for various concentrations of NaCl namely 10ppt, 35ppt, 50ppt, and 65ppt. The optical density (OD) reading at 600nm was recorded at 48 h after incubation for all the NaCl concentrations.

Statistical Methods

All tests were conducted in triplicates. Two-way analysis of variance (ANOVA) was applied to assess the difference in each parameter among treatments using the SPSS 17.0 statistical software package. P ≤ 0.05 was taken to indicate statistical significance at 95.0% confidence level.

RESULTS

Isolation, selection, and treatment of strain VCM11

Out of 21 isolates, 9 strains gave good results. Strain VCM11 was selected based on its excellent reduction of ammonia concentration in the aquaculture wastewater and detection of nitrite. Appearance of distinct red colour from the nitrite indicates nitrate reduction. **Figure 1** shows the ammonia concentration of control (wastewater without autoclaving) and wastewater inoculated with bacteria (VCM11) for 120 h. Strain VCM11 reduced 90.98% of ammonia concentration within 120 h compared to control (24.91%).

Identification of strain VCM 11

Strain VCM11 was characterized by plating on nitrate agar. It was found to be aerobic, Gram-positive, and rod-shaped bacterium. The physiological and biochemical characteristics are summarized in **Table 1**. Based on morphological, physiological, and biochemical characteristics, strain VCM11 was identified as *Microbacterium* sp.
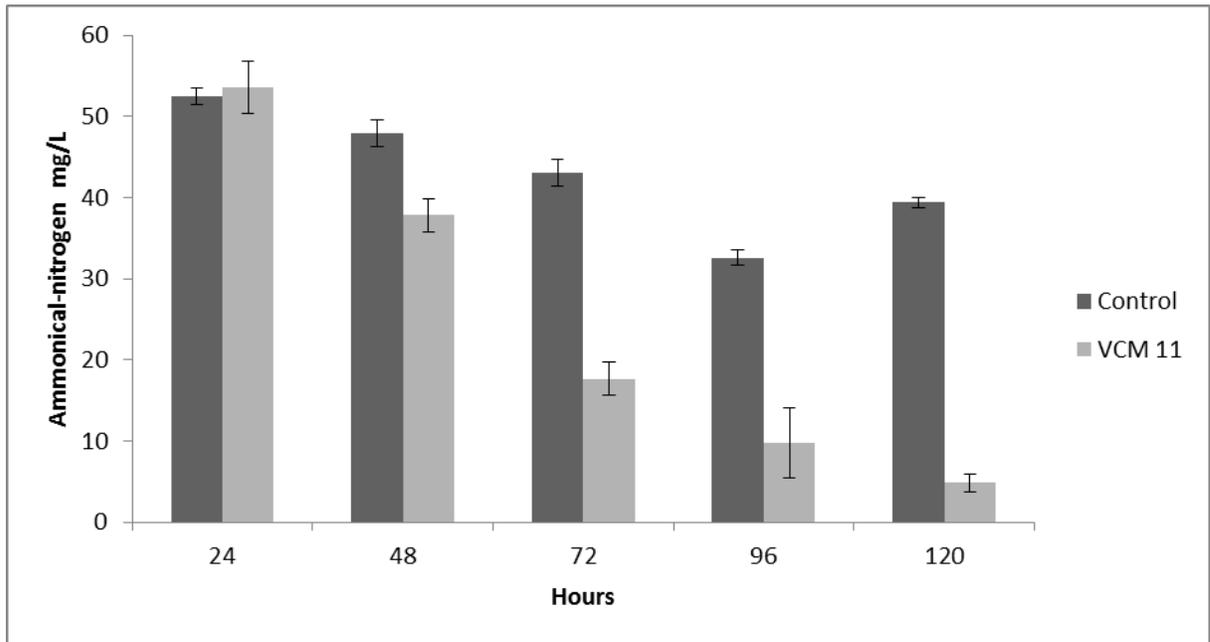
Figure 1. Concentrations of ammonical-nitrogen (NH_3^+-N) mg/L in control and treatment (autoclaved wastewater with bacterium VCM11). Number of replicates (n) =3.

Table 1: Morphological and biochemical characteristics of strain VCM 11

| Biochemical test	Results
Gram's staining	Positive
Carbon source:	
D-Maltose	+
D-Trehalose	+
Sucroce	+
D-Raffinose	-
α – D-Lactose	+
D-Melibiose	+
α – D-Glucose	+
D-Mannose	+
D-Fructose	+
D-Galactose	+
L-Rhamnose	+
Inosine	+
D-Sorbitol	-
D-Mannitol	-
Glycerol	+
Antibiotic resistance test:	
Troleandomycin	-
Rifamycin SV	-
Lincomycin	-
Nalidixic Acid	-

+   Positive results, - Negative results

Effect of Sodium Chloride (NaCl) on growth

Figure 2, shows the effect of NaCl on the growth of strain VCM11 based on optical density (OD), after an incubation period of 48 h at room temperature. Its halotolerant nature was observed. Among the tested concentrations of NaCl, the optimum growth (OD_{600nm} = 0.316) was observed at 35ppt NaCl supplemented AOB medium.

Effect of temperature on bacterium density

Temperature is one of the most critical parameters that have to be controlled in bioremediation. **Figure 3** shows the effect of temperature on optical density (OD) of strain VCM11 at 35ppt NaCl after 48 h. Strain VCM11 showed optimum growth at 30°C with 0.943 OD at 600nm.
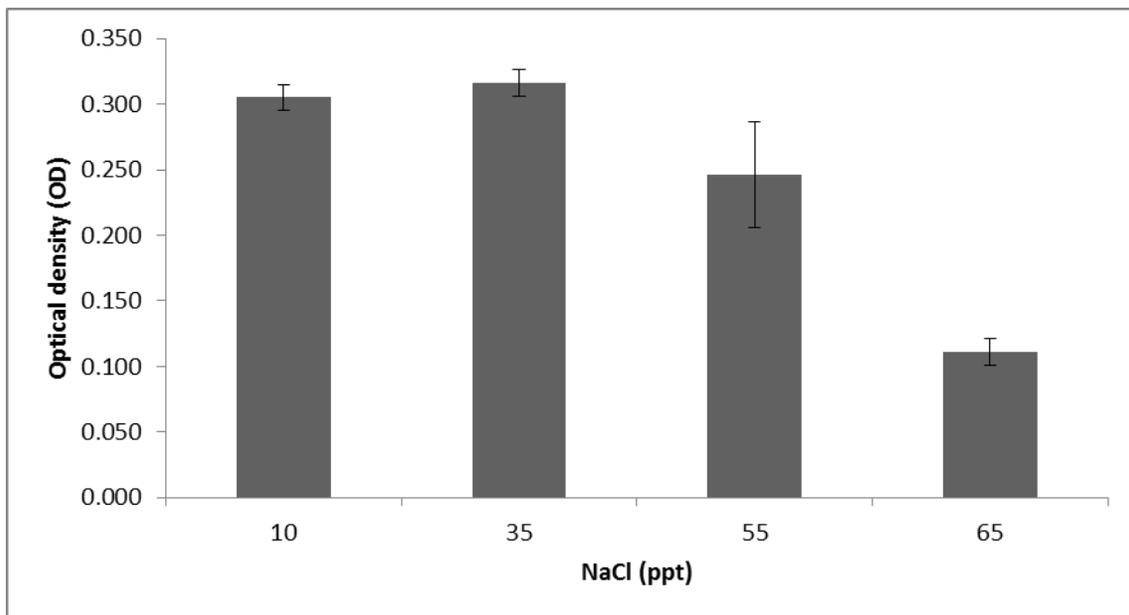
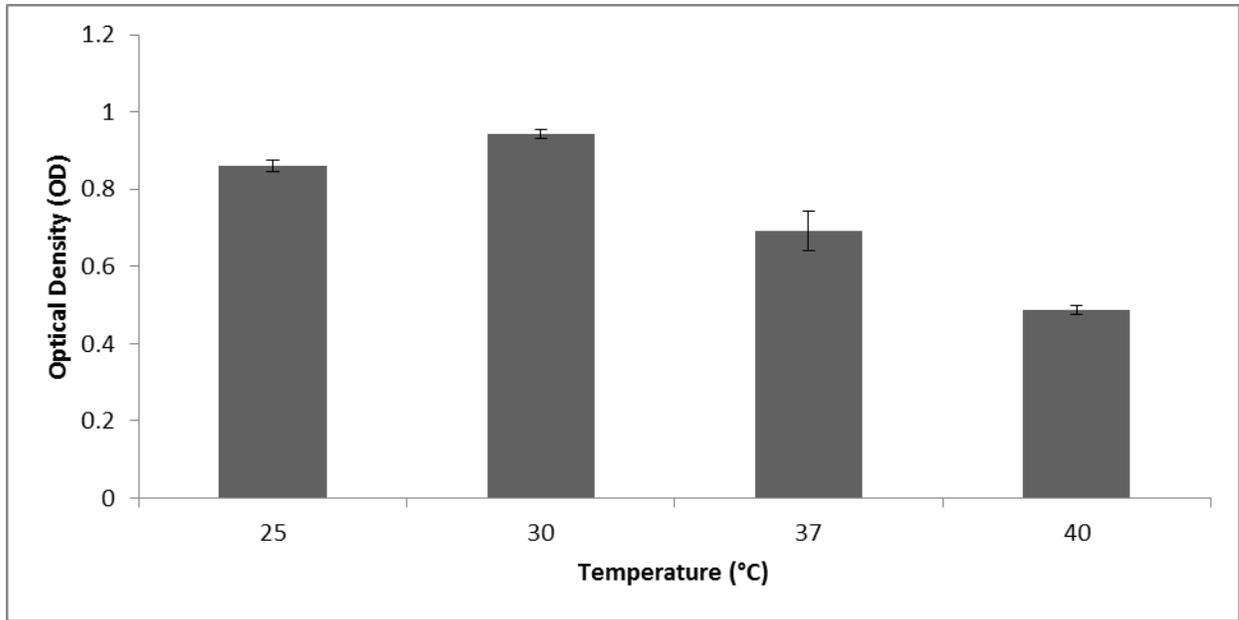**Figure 2.** Effect of NaCl concentration (ppt) on growth. Number of replicates (n) =3.

Figure 3. Effect of temperature (°C) on optical density (OD) at 600nm. Number of replicates (n) =3.

DISCUSSION

Microbacterium sp., VCM11 isolated from aquaculture wastewater showed significant reduction of ammonia concentration (p≤0.05) compared to control. The control in this experiment represents the untreated aquaculture wastewater without the addition of any bacteria or adjustment in parameters. Treatment experiments were carried out with 25 percent inoculums of *Microbacterium* sp., VCM11 into the autoclaved aquaculture wastewater. The ammonia content in the treated wastewater (90.98% within 120 h) gradually decreased as compared to the control (24.91%). Since high ammonia concentration is toxic to aquatic organisms, the pathway which lead to the formation of gaseous nitrogen are ideal in culture systems (Barik *et al*., 2011). In the aquaculture system, the nitrification process takes a longer period to oxidize ammonia to nitrite due to the high nutrient load resulting in hypoxia (Jørgensen, 1996). According to Boyd and Ahmad, 1987, denitrifying bacteria such as *Pseudomonas* and *Acinetobacter* utilize the nitrate molecules as electron acceptor during reduced oxygen tension. *Microbacterium* sp., VCM11 worked well to reduce ammonia.

Microbacterium sp. VCM11 has its own merit with respect to reduced interspecific competition and lesser growth inhibition. The use of indigenous bacteria reduces the inter-specific competition and takes shorter acclimation period (Shan and Obbard, 2001). Non-indigenous bacteria are not recommended for bioaugmentation as they will be edge out by the competing native microorganisms (Stephenson and Stephenson, 1992)Hence, increasing the cell density of such beneficial bacteria in wastewater reduces the toxic ammonia level safely and effectively.

Biochemical tests were performed to identify the species. *Microbacterium* sp. VCM11 is not resistant to troleandomycin, rifamycin SV, lincomycin, and nalidixic acid. Antibiotic resistance tests are important to ensure that the microorganisms used do not contain transferable antimicrobial resistance determinants (Matto *et al*., 2007). Carbon utilization tests (CUS), as used for the classification and identification of bacteria (Koser, 1923), have shown that *Microbacterium* sp. VCM11 can utilize D-maltose, D-trehalose, sucrose, α – D-lactose, D-melibiose, α – D-glucose, D-mannose, D-fructose, D-galactose, L-rhamnose, inosine, and glycerol.

Temperature and salinity are two major environmental factors in aquaculture systems (Jiang et al, 2000). *Microbacterium* sp. VCM11 shows optimum growth at 30°C and 35ppt of NaCl. The optical density value at 600nm for 35ppt NaCl was 0.316. Better growth was observed (0.943 optical density at 600nm) when the temperature was optimized with 35ppt NaCl. However, there is no significant difference

(p≥0.05) within the range of 10-35ppt NaCl. Therefore, this strain shows a good range of tolerance towards sodium chloride concentration (seawater). Temperature of 25°C-30°C shows similar correlation with sodium chloride concentration, resulting in no significant difference (p≥0.05) for this range. Since this strain can withstand such ranges (10-35ppt of NaCl and 25°C-30°C), for cost effectiveness, natural concentration of seawater and room temperature would be sufficient to provide an optimum results.

CONCLUSION

Microbacterium sp. VCM11 can be used as a biological tool to reduce ammonia concentration in aquaculture wastewater. The bacterium is able to remove 91% of NH_3^+ -N concentration after 120 h of growth.

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