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Molecular biological characterization and biostimulation of ammonia-oxidizing bacteria in brackishwater aquaculture

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In the present study, molecular methods based on sequencing of clone libraries have been used to provide sequence and the phylogenetic information of ammonia oxidizing bacteria (AOB). *Ammonia monooxygenase* (*amoA*) gene, which catalyzed the oxidation of ammonia to hydroxyl amine in the initial rate-determining step of nitrification was targeted for detection and characterization of AOB using gene-specific primers. The *amoA* genes obtained through the clone library construction are closely affiliated with *Nitrosomonas* sp. and other uncultured beta proteobacteria. The levels of nucleotide similarity and amino acid similarity ranged from 85–99% and 83–88%, respectively. The level of conservation of the amino acid sequences is 73%. The use of a matrix prepared from abundantly available lignocellulosic agrowaste-bagasse has successfully been demonstrated for biostimulation of AOB in aquaculture environment by supplementing nutritional requirement facilitating the biofilm mode of growth of the autotrophic consortia. Present study is useful in predictability and reliability of the treatment of ammonia in brackishwater aquaculture.

Keywords: Ammonia-oxidizing bacteria, *ammonia monooxygenase*, bagasse, biostimulation, brackishwater aquaculture.

Introduction

Zero-water exchange brackishwater aquaculture system generates toxic ammonia.[1] Total ammonia nitrogen (TAN, compromising NH_4^+ and NH_3) concentration often is the key limiting water quality parameter in aquaculture systems. This metabolite increases blood pH, reduce the oxygen content in the blood, affect gills, creating stress, resulting in reduced feeding and strong potential for viral disease. There is a need for effective and economical management method for treating ammonia. A packed bed bioreactor have been developed by immobilizing nitrifying bacterial consortia for ammonia removal in aquaculture systems.[2,3]

However, these treatment methods make water management costs prohibitive. An emerging field of interests is employing certain plant based products, that stimulate microbial activity and biochemical transformation, which subsequently increase the detoxification potential. This process is often referred to as plant-assisted bioremediation or biostimulation.[4–8] Bagasse, which is a complex native cellulosic fibrous waste left after extraction of juice from cane sugar, is an attractive lignocellulosic agricultural by-product for a pond supplement because of its low cost and general availability in shrimp-growing latitudes.[9] Biostimulation involves the addition of electron donors, acceptors, or nutrients to increase the numbers or stimulate the activity of indigenous autotrophic bacteria, which play a significant role in the reclamation of the aquaculture system and waste processing.

However, cultivation of AOB, is especially challenging because of their slow growth rates and the frequent occurrence of culture contamination by heterotrophic bacteria.[10,11] This makes them difficult to detect in environmental samples by traditional methods.[12] This causes failure of the performance of aquaculture treatment processes. Alternatively, molecular techniques that do not depend on traditional cultivation have therefore been used to monitor bacterial populations in a variety of environmental samples at concentrations previously considered undetectable.[13,14]

A better knowledge of diversity of these organisms is prerequisite for bioremediation studies. To our knowledge, the information on the application of molecular tools for detection and characterization of AOB for biostimulation strategy in aquaculture systems is not available. In the present study, *ammonia monooxygenase* (*amoA*), which catalyzes the oxidation of ammonia to hydroxylamine is used for detection and characterization of AOB and their biostimulation using partially delignified bagasse for bioremediation of ammonia.

Materials and methods

Preparation of partially delignified material

Native bagasse (lignocellulosic substrate) was obtained from agro-industries, Chennai, India. This was then subjected to treatment with 1.5% NaOH and autoclaved for 30 minutes in order to remove the low molecular weight lignin compounds. After filtration with nylon sieve, this material was washed with deionized water until the pH reached a constant value close to neutrality. The solid fraction was filtered off and dried in an oven at 50°C. This was cut into small pieces (fibres) and also powdered (0.2 mm).

Microscopic and spectroscopic analyses of partially delignified bagasse (PB)

Surface morphology of PB was studied with the scanning electron microscope. Micrographs of PB were obtained using double sided carbon tape. X-ray photo electron spectroscopy (PHI model 5400axis Ultra Kratos Analytical instrument, Manchester UK) was used to characterize the surface chemistry of PB. In order to determine the functional groups present, PB was analysed using a Fourier transform infrared spectrometer-FT/IR 4100 (Jasco Corp., Tokyo, Japan) in the range of 500–4000 cm^{-1}.

Collection of environmental samples

Composite soil and water samples were collected from three different sampling coastal sites ie. (i). Brackishwater lagoon (BWL), near Chennai (Tamil Nadu), (ii). shrimp pond (BWP1) from Mahabalipuram (Tamil Nadu) and (iii). shrimp pond (BWP2) from Bhimavaram (Andhra Pradesh). pH and electrical conductivity were measured with a pH meter and EC meter, respectively, using standard method.[15] Soil water ratio for pH and EC measurement was 1:2.5. Organic carbon was determined using the chromic acid digestion method.[16] The pH, turbidity, salinity, alkalinity, dissolved oxygen, total ammonia nitrogen (TAN), nitrite-nitrogen, phosphate, COD and BOD of composite water samples were determined using standard methods.[17,18] UV-visible spectrophotometer (Shimadzu, Japan) was employed for analyses.

DNA Extraction from environmental sediment samples

The meta-genomic DNA of the bacterial population was extracted from approximately 0.7g of soil sample with a FastDNA spin kit for sediment (UltaClean Soil DNA Kit, MO Bio laboratories, Carlsbad, California) using bead beating according to the manufacturer's instructions. DNA extraction procedures can miss entire groups that are difficult to lyse, such as gram positive organisms. Therefore, the genomic DNA was also extracted from composite sediment samples using modified CTAB standard method. Briefly, 0.7 g of sample was suspended in 0.5 mL of extraction buffer (with 5% CTAB), 25μL of lysozyme and 0.5 mL of phenol: chloroform; isoamyl alcohol (25: 24:1) was added before cell lysis.

After centrifugation the aqueous supernatant was extracted with chloroform : isoamyl alcohol (24:1) and nucleic acids were precipitated with polyethylene glycol/NaCl. DNA obtained were washed with 70% ethanol and dried. Finally, the DNA were dissolved in water and then stored at −20°C. Composite brackishwater samples (10L) collected from the soil–water interface, in which the superficial soil was also mixed, was passed through a clean sterile coarse filter paper to remove soil particles and then transferred to ultra filtration unit to filter the bacteria onto the membrane (0.2 μm). The membrane filter containing bacteria was used for genomic DNA isolation using modified phenol chloroform method.[19] Genomic DNA extracted from environmental samples was quantified using Nanodrop (Agilent) at 260 nm, and purity was determined by measuring the 260/280 nm absorbance ratio.

PCR Amplification of amoA

The polymerase chain reaction was performed on the samples along with negative control (water) with a 40 μl reaction mixture using Eppendorf thermal cycler (Master cycler gradient). The following composition was used for a single reaction: (1X) 40 μL: water 27.4 μL; buffer (10 × Tris with 15 mM MgCl$_2$): 4 μL, 10mM dNTP (2.5 mM): 2 μL, forward primer (30 pM) 2 μL, reverse primer (30 pM) 2 μL, *Taq* (5U/μL) 0.2 μL, BSA (20 mg/mL) 0.4 μL, DNA template 2 μl. The amplification programs were as follows: one cycle consisting of 94°C for 2 min, followed by 35 cycles consisting of denaturation (94°C for 30 sec), annealing (49.2 to 57.7°C for 30 sec) and elongation (72°C for 30 sec) and a final extension step consisting of 72°C for 5 min. Aliquots (8 μL) of the PCR products were electrophoresed and visualized in 1% agarose gels by using standard electrophoresis procedures.

Forward primers (amoA-1F 5′-GGGGTTTCTACTG GTGGT-3′) originally designed by Rotthauwe et al.[13] and reverse primer (A682 5′-GAASGCNGAGAAGAASGC-3′) originally designed by Holmes et al.[20] were used for amplification of 349 bp fragment of *ammonia monooxygenase (amoA)* gene in brackishwater aquaculture environment.

Cloning and sequence analysis

The amplified *amoA* gene (349 bp) was purified with a gel extraction kit (QIAquick) by following the manufacture's instructions. The purified PCR product were ligated by using the pGEM-T Easy vector system (Promega) as recommended by the manufacturer and were transformed into high efficiency competent cells (*E. coli* DH5-α). Clones were confirmed by release of insert using *EcoRI* restriction endonuclease. Sequencing was done in an ABI 3100

Genetic Analyzer. Primary sequences were analyzed by using BLAST programmes (nucleotide blast, BLASTN and BLASTP).

Effect of initial TAN concentration, partially delignified bagasse (PB) and biofilm on ammonia removal

An experiment was conducted in 200 mL brackishwater containing indigenous ammonia-oxidizing bacteria, where an appropriate amount of stock solution of ammonium sulfate was added asceptically to attain TAN concentration of 5.1, 24.8, 50.0 and 73.4 mg/L. A total of four runs in batch mode were performed in triplicate: (1) brackishwater media without soil (Control); (2) brackishwater media with 1 g soil samples from BWL, BWP1 and BWP2; (3) brackishwater medium (24.8, 50.0 and 73.4 mg/L) with 1 g soil from BWP2 and 20 mg of partially delignified bagasse powder (0.2 mm); and (4) brackishwater medium with 1 g soil from BWP2 and 20 mg partially delignified bagasse fibres.

Samples were drawn from the flasks on weekly intervals for the measurement of ammonia concentration using the standard method.[17] After 1 week of incubation, bagasse with biofilm from the run-4 was transferred to another sterile jar containing brackishwater culture media with ammonia concentrations of 2.5, 5.1 and 10.4 mg/L. Further, ammonia concentrations were measured at 48-h intervals up to 192 hours. In order to demonstrate PB (10 g) as biostimulator for natural AOB, a field experiment was also conducted in 1 ton capacity tanks containing 1000 L discharge water (0.72–1.62 mg/L TAN) from a shrimp farm, where water samples were analyzed at 24-h intervals up to 96 hours.

Results and discussion

Characterization of partially delignified bagasse

Scanning electron microscopy, X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy were performed onto the partially delignified bagasse (PB), which indicated the physical integrity of the material and there are no surface deformations due to the chemical treatment (Fig. 1a). PB is composed of C, O and cations such as Ca^{2+}, Mg^{2+} and Na^+ as evidenced from XPS spectra (Fig. 1b). The outer surface of the PB is charged due to the presence of carbohydrate contents bearing hydroxyl and carboxylic functional groups as shown by FTIR (Fig. 1c).

Soil and water characteristics

Analysis results of physicochemical parameters of soil and water samples collected from brackishwater lagoon and shrimp ponds are given in Table 1, which indicated that soil parameters such as pH, electrical conductivity and or-

Table 1. Physicochemical parameters of soil and water samples collected from brackishwater environment.

	Brackishwater lagoon	Shrimp pond-1	Shrimp pond-2
Soil characteristics			
pH	7.7	8.4	8.3
EC (dS/m)	6.2	8.8	10.2
Organic C (%)	0.22	0.54	0.71
Water quality parameters			
pH	7.6	8.01	7.91
Salinity (g/L)	27	33	29
Turbidity (NTU)	3.4	7.2	8.3
Alkalinity (mg/L)	105	112	111
Dissolved oxygen (mg/L)	6.8	6.2	5.8
Nitrite-N (mg/L)	0.03	0.06	0.048
Total ammonia N (mg/L)	0.5	1.0	0.97
Phosphates-P (mg/L)	0.053	0.045	0.04
Chemical oxygen demand (mg/L)	61	69	75
Biochemical oxygen demand (mg/L)	6.8	8.8	9.4

ganic carbon and water quality parameters such as pH, turbidity, alkalinity, nitrite-N, total ammonia N, phosphates-P, chemical oxygen demand and biochemical oxygen demand were higher in the samples collected from shrimp ponds (BWP1 and BWP2) as compared to brackishwater lagoon.

Detection of ammonia oxidizing bacteria

Knowledge of ammonia oxidizing bacteria is required to analyze the fate of toxic ammonia. Rotthauwe et al.[13] and Holmes et al.[20] reported that the PCR amplification of *amoA* is a very powerful tool for analyzing indigenous ammonia-oxidizing communities because this gene is specific for AOB's, represents a functional trait rather than a phylogenetic trait. To detect AOB in brackishwater aquaculture environment, we used PCR-gel electrophoresis targeting the *amoA* gene with a set of primers originally constructed by Rotthauwe et al.[13] and Holmes et al.[20] We report amplification of 349 bp fragment (Fig. 2) of *amoA* gene using *amoA*-1F + A682 in different environmental samples, for which, optimum annealing temperature was found to be 57.7°C (Fig. 3). The primers reported here allow specific amplification of homologous genes from ammonia oxidizing bacteria representatives of beta-proteobacteria. The original forward primer devised by Rotthauwe et al.[13] contained no degeneracy. In this study, a forward primer with degeneracy at position 5th nucleotide-*AmoA*-1F-w (W= A or T) (5′-GGGGWTTCTACTGGTGGT-3′) amplified the templates with less efficiency, as shown in Figure 4.
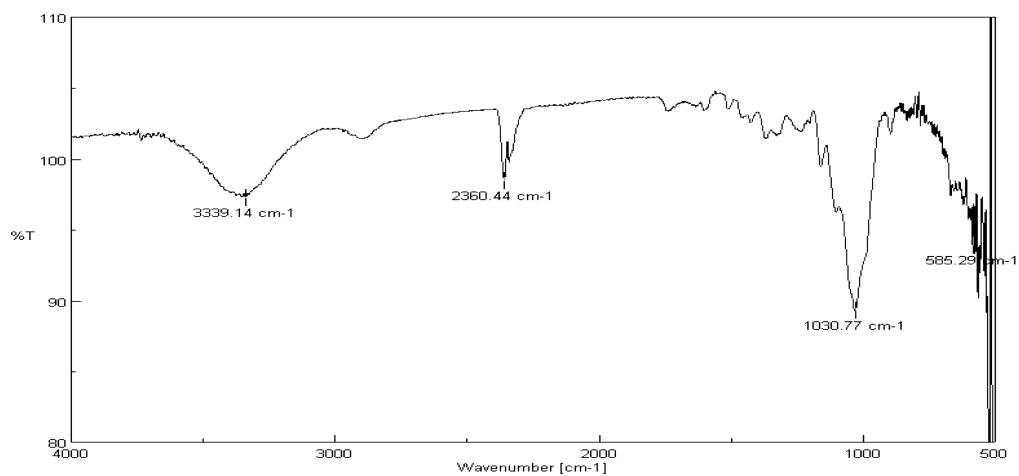
(a)
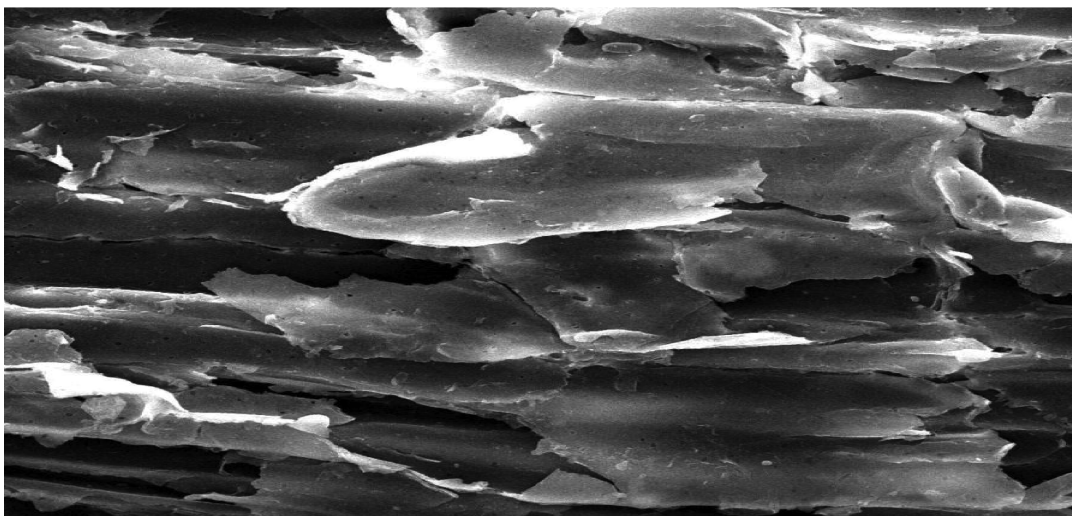
(b)
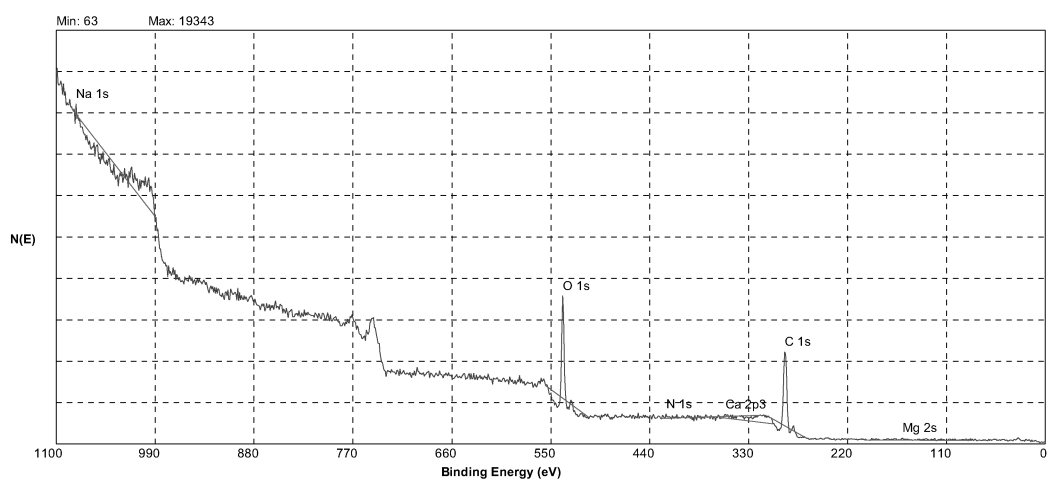
(c)

Fig. 1. (a) Scanning electron microscopy, (b) X-ray photoelectron spectroscopy, and (c) Fourier transform infrared spectroscopy of partially delignified bagasse.
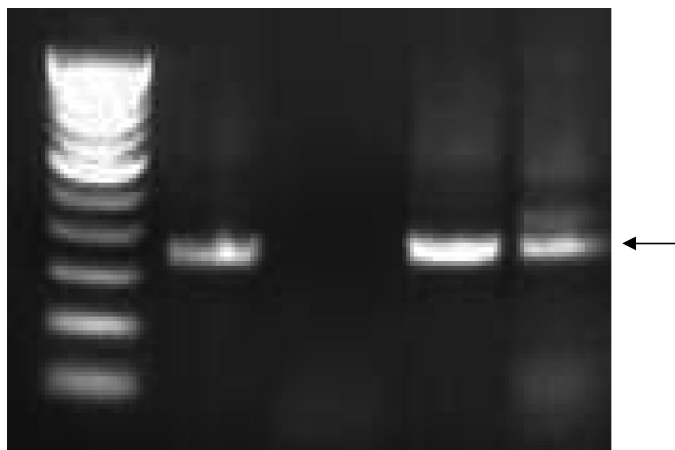
Fig. 2. PCR amplification of 349 bp fragment of the *amoA* gene in different environmental samples.

Fig. 4. Effect of primer degeneracy on amplification of *amoA*: Lane-1 Marker 2. *amoA*-1F + A682 3. *amoA*-1F-w + A682.

Diversity of amoA in brackishwater aquaculture

The nucleotide sequences of *amoA* (349 bp) determined in this study have been deposited in the GenBank database. The accession numbers for the gene sequences are EU104363 through EU104365. The level of similarity between different pairs of *amoA* genes at nucleotide and amino acids levels were 81–99% and 87–97%, respectively. This revealed that sequence difference has been resulted from actual diversity in the sample and there was no PCR artifact.

The phylogenetic tree of *amoA* genes, generated by using the maximum likelihood method, based on the alignment of 349 bp of *amoA* gene fragments (Fig. 5) revealed 99% homology with *Nitrosomonas sp.*, and other uncultured *beta-proteobacteria* available in the GenBank. Nucleotides and
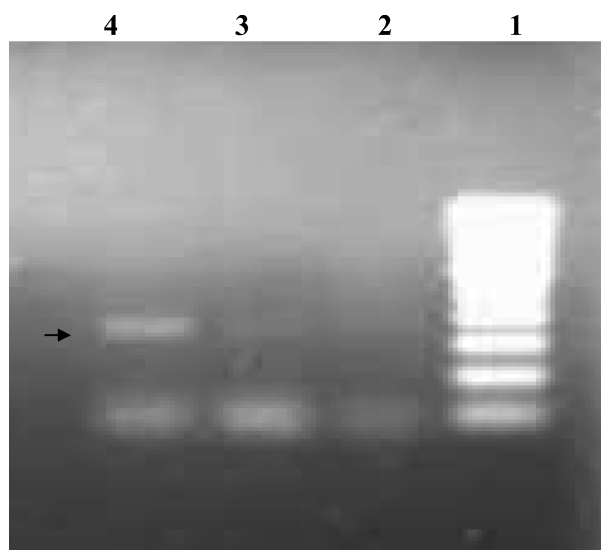
predicted amino acids sequences of the *amoA* genes identified in this study are compared with the sequences available in the GenBank. The levels of nucleotide similarity and amino acid similarity ranged from 85–99% and 83–88%, respectively. Partial alignment of the predicted amino acids encoded by *amoA* is shown in Figure 6. Residues conserved in the sequences (ABU94723 to ABU94725) are highlighted. The level of conservation of amino acid sequences is 73%. As shown in Figure 6, a total of 84 of 115 amino acid residues are conserved.

Removal of ammonia using AOB

The decrease in ammonia concentration in culture medium containing four different initial TAN concentrations of 5.1, 24.8, 50.0 and 73.4 mg/L using AOB population inhabiting aggregate soils from brackishwater lagoon (BWL) and shrimp ponds (BWP1 and BWP2) is shown in Figure 7 (a,b,c,d). For all initial ammonia levels, the rate of decrease in ammonia concentration in the treatments such as BWL and BWP1 was slower, as compared to BWP2. In the case of BWP1 and BWP2 culture media containing 24.8 mg/L TAN (Fig. 7b), ammonia concentration decreased from 24.8 mg/L to 17.3 (30%), 12.5(50%) and 7.9(68%) and 12.2(51%), 5.4(78%), and 1.3(95%) mg/L after 2, 4 and 6 weeks, respectively. Similarly, there was a continuous decrease in TAN concentration in the case of higher initial ammonia concentrations of 50.0 (Fig. 7c) and 73.4 mg/L (Fig. 7d). However, effect of initial ammonia concentrations on the ammonia removal showed that percentage ammonia removal was decreasing with an increase in initial ammonia concentration.

Biostimulation of AOB using partially delignified bagasse (PB)

Effect of partially delignified bagasse (PB) on the decrease in TAN concentration is shown in Figure 8. PB decreased ammonia from 24.8 mg/L to 6.9(72%), 0.67(97%) mg/L and nil (100) (Fig. 8a) and from 50 mg/L to 19.2(62%),

Fig. 3. Gradient PCR amplification of 349 bp fragment of the *amoA* gene Lane-1 Marker, (2) 49.2°C, (3) 51.6°C, (4) 57.7°C.
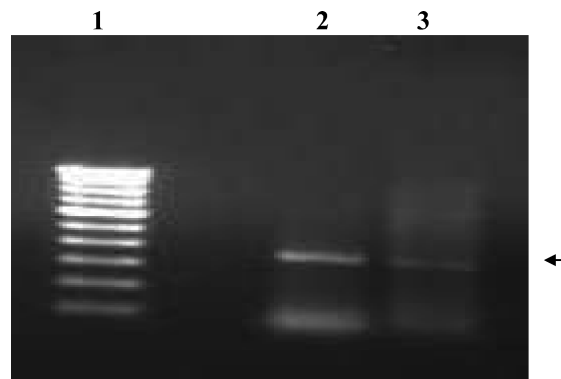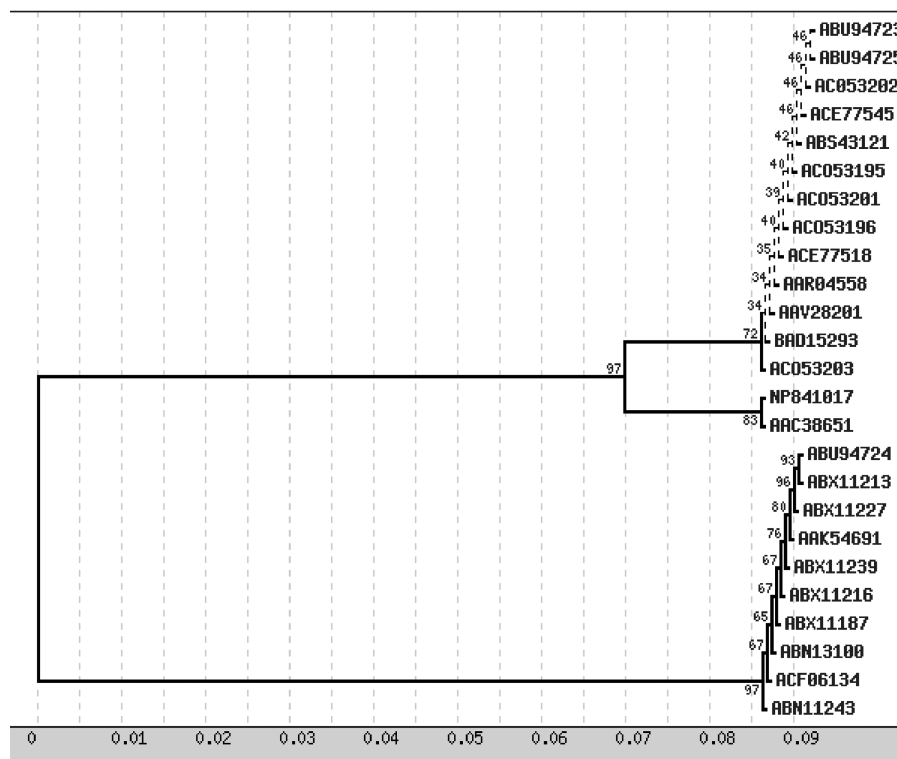
Fig. 5. Phylogenetic tree based on *amoA* gene products (ABU94723, ABU94724, ABU94725) as determined by maximum likelihood method.

5.3(89%) mg/L and 0.5(99%) mg/L (Fig. 8b) and from 73.4 mg/L to 39.4 (46%), 18.7 (74%) and 6.8 (91%) (Fig 8c) in 2, 4 and 6 weeks, respectively. Percent ammonia removal is higher with PB treatment as compared to AOB alone, which can mainly be attributed to the enhancement of autotrophic AOB. PB as biostimulator (10 g) for natural AOB has also been demonstrated in 1 ton capacity tanks containing 1000 L discharge water (0.72–1.62 mg/L TAN) from shrimp farm in the field condition. PB was found to be efficient to remove ammonia from 0.72 to 0.14 (80%) and nil (100%) mg/L and from 1.62 to 0.42 (74%) and 0.095 (94%) mg/L in 48 and 96 hours, respectively.

This by-product amounts to 25 to 30% of the cane weight and contains approximately cellulose, hemicellulose, lignin, and cell contents. Lignin is the main component, which add to the recalcitrance of this material, mainly due to covalent bonds between lignin and carbohydrates. Lignin shields the carbohydrate (cellulose) from any kind of microbial attack. Hence, prior to incorporation into an aquatic system, the lignocellulosics have to be processed to reduce recalcitrance to the maximum possible extent and to render themselves manually more efficient[9]. Ammonia-oxidizing bacteria are delicate organisms and extremely susceptible to a variety of inhibitors. In the present study, partial delignification reduced the recalcitrance of bagasse, which enhanced the growth of AOB.

Ramirez-Lopez et al.[21] found that bagasse has the high water holding capacity (WHC), which was able to absorb up to 9 times its dry weight in water. High WHC may also be one of the factors responsible for the autotrophic periphytic growth, which is stimulated by partially delignified bagasse for the removal of ammonia in the present study. Other reports on the use of lignocellulosic materials for enhancing growth of microorganisms suggest that bagasse could be used as the source of carbon in bioprocess techniques[22,23] Ammonia oxidizing bacteria are obligate chemolithoautotrophs that derive their energy through oxidation of ammonia and fix inorganic carbon dioxide (CO_2) to fulfill their carbon requirements. Furukawa et al.[24] conducted a study of nitrification of synthetic polluted river water using zeolite-coated nonwovens and reported that there was a reduction in ammonium-nitrogen removal performance because of an inhibition of the nitrification reaction due to the lack of an inorganic carbon source. In the present study, partially delignified bagasse as the source of carbon biostimulate ammonia oxidizing bacteria.

Effect of bagasse biofilm on the ammonia removal

The effect of biofilm formed over partially delignified bagasse on ammonia removal is presented in Figure 9. This shows that biofilm decreased initial ammonia

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ACO53196    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTTLIGGGFFGLLFYPGNWPIFGP 60
AAV28201    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
ACO53201    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
ACO53195    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
AC053202    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
ABU94725    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
ABU94723    GFYWWSHYPINFVTPSNMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
BAD15293    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALIGGGFFGLLFYPGNWPIFGP 60
ACE77545    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
ABS43121    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
ACO53203    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALVGGGSFGLLFYPGNWPIFGP 60
AAR04558    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
ACE77518    GFYWWSHYPINFVTPSIMIPGALMLDITLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
NP841017    GFYWWSHYPINFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
AAC38651    GFYWWSHYPINFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGP 60
ABX11213    GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLITALLGGGFWGLFFYPGNWPIFGP 60
ABX11227    GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLITALLGGGFWGLFFYPGNWPIFGP 60
ACF06134    GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLITALLGGAFWGLFFYPGNWPIFGP 60
ABX11239    GFYWWSHYPLNFVLPSTMIPGALMLDTILLLTRNWLITALVGGGFWGLFFYPGNWPIFGP 60
ABX11187    GFYWWSHYPLNFVLPSTMIPGALMLDTILLLTRNWLITALVGGGFWGLFFYPGNWPIFGP 60
ABX11216    GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLITALVGGGFWGLFFYPGNWPIFGP 60
AAK54691    GFYWWSHYPINFVLPSTMIPGALMLDTIMLLTGNWLITALLGGGFWGLFFYPGNWPIFGP 60
ABN13100    GFYWWSHYPINFVLPSTMIPGALMLDTIMLLTGNWLVTALLGGGFWGLFFYPGNWPIFGP 60
ABN11243    GFYWWSHYPVNFVLPSTMIPGALMLDAILLLTGNWLVTALLGGGFWGLFFYPGNWPIFGP 60
ABU94724    GFYWWSHYPINFVLPSTMIPGALMLDTILLLTRNWLVTALIGGGFWGLFFYPGNWPIFGP 60
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ACO53196    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
AAV28201    THLPVVAEGVLLPMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
ACO53201    THLPVVAEGVLLSMTDYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
ACO53195    THLPVAAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
AC053202    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
ABU94725    THLPVVAEGVLLSMADYMGLLYIRTGTPEYVRLIEQDSLRTFGGHTTVIAAFFAA 115
ABU94723    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
BAD15293    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLGTFGGHTTVIAAFFAA 115
ACE77545    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
ABS43121    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
ACO53203    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
AAR04558    THLPVVAEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLHTFGGHTTVIAAFFAA 115
ACE77518    THLPVVTEGVLLSMADYMGHLYIRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFAA 115
NP841017    THLPIVVEGTLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFSA 115
AAC38651    THLPIVVEGTLLSMADYMGHLYVRTGTPEYVRHIEQGSLRTFGGHTTVIAAFFSA 115
ABX11213    THLPIVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
ABX11227    THLPIVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
ACF06134    THLPLVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
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ABX11187    THLPLVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
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ABN11243    THLPLVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
ABU94724    THLPVVVEGVLLSVADYTGFLYVRTGTPEYVRLIEQGSLRTFGGHTTVIAAFFSA 115
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Fig. 6. Partial alignment of the predicted amino acids encoded by *amoA*. (The residues conserved in amino acid sequences are highlighted by * mark).

concentration of 2.5, 5.1 and 10.4 mg/L to 1.1(56%), 3.48(32%) and 7.36(29%) mg/L in 48 h and 0.46 (82)%, 1.47(71%), and 5.12(51%) mg/L in 96h, there was further removal and after 144 h, ammonia levels decreased up to nil (100%), 0.78(85%) and 3.87(63%) mg/L, respectively.

Bacterial biofilm cells are generally resistant to environmental stresses.[25] Biofilms bear great potential for the simultaneous and efficient removal of organic carbon and nutrients like N and P in wastewater treatments.[26,27] Adoption of microbial biofilm—based on agrowaste-periphyton aquaculture[28−30] and artificial substrates-periphyton aquaculture[31−33] has the potential to not only increase the productivity by conversion of nutrients into harvestable products but also remediate water quality. The improved growth has been attributed to the enhancement of the colonization of epiphytic biota by added surface area created by the substrates, which in turn provides a natural food supplement for the shrimp.[31−33] Ammonia-oxidizing bacteria are largely non-motile and must colonize a surface such as gravel, sand, synthetic biomedia, etc. for optimum growth. They secrete a sticky slime matrix, which they use to attach themselves. The present study reveals the role of partially delignified bagasse in supporting the biofilm mode of growth of the autotrophic consortia. PB partially supplement bacterial nutritional requirement thus facilitating better biofilm formation.
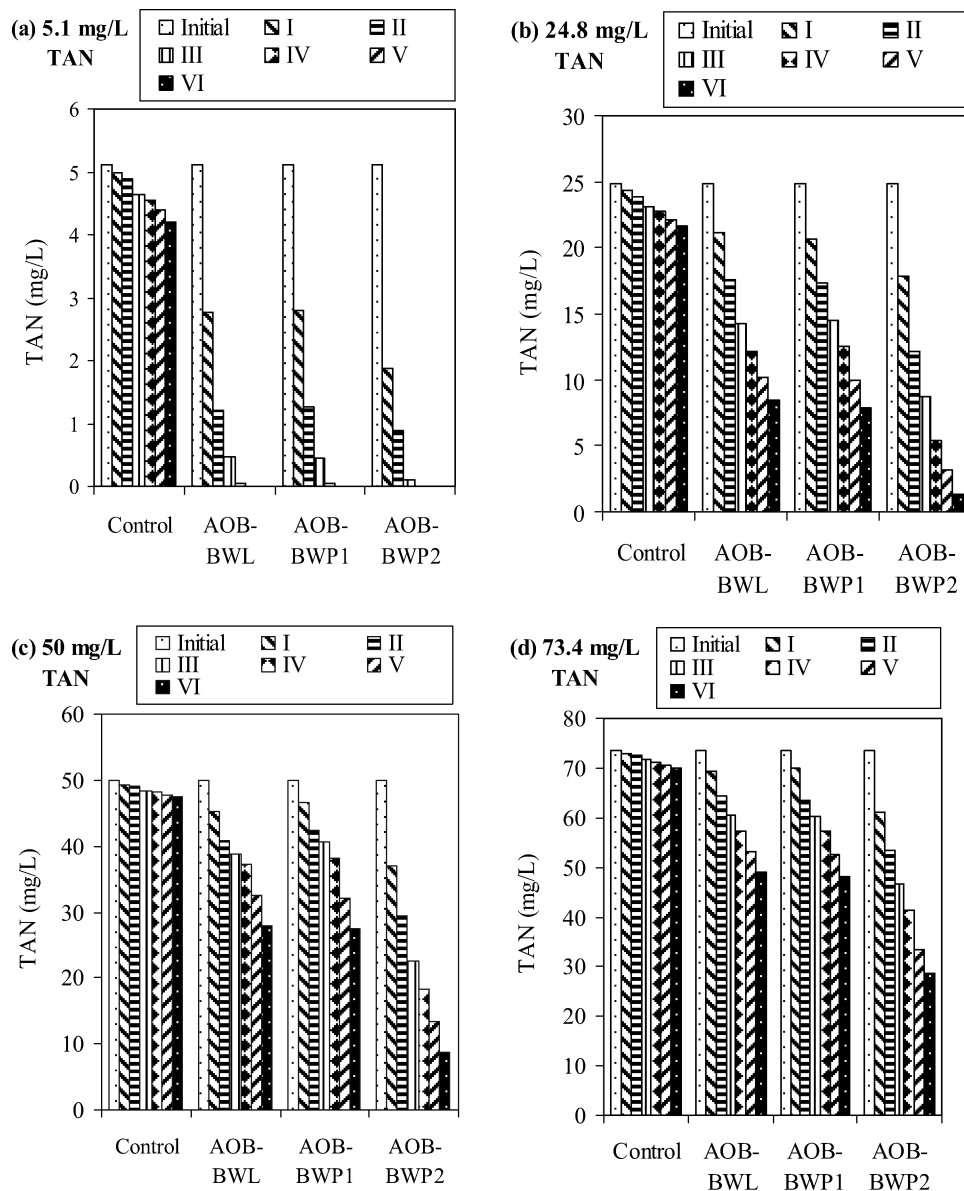
Fig. 7. Bioremediation of ammonia using AOB from brackishwater lagoon (BWL), brackishwater shrimp pond 1 (BWP1) and pond 2 (BWP2) at weekly intervals from I to VI.

Ramesh et al.[34] compared dried raw sugarcane bagasse, paddy straw, and water hyacinth (*Eichhornia spp.*) leaves as substrates and observed the best growth of common carp with bagasse. They also reported that ponds with substrates had lower total ammonia levels than control ponds and concluded that enhanced bacterial biofilms on the raw substrates might reduce ammonia levels through promotion of nitrification. Previously, we also reported[8] the use of higher dose (1–6 g/L) of raw bagasse for the removal of lower range of ammonia concentration (1.015–5.64 mg/L) from shrimp farm wastewater, however, removal efficiency was very low due to the presence of low molecular weight lignin molecules, which inhibited the optimum growth of ammonia-oxidizing bacteria. In the present paper, use of very low dose of partially delignified bagasse (0.1 g/L and 0.01 g/L) has successfully been demonstrated for the removal of very high concentrations (24.8–73.4 mg/L) of ammonia from brackishwater and also removal of ammonia (0.72–1.62 mg/L) from shrimp farm discharge water in the field condition through biostimulation of natural AOB.

Effect on other water quality parameters

During the course of the experiment, salinity did not show any change with all the treatments. However, in the experiments (as shown in Figs. 8 and 9) on the effect of PB and biofilm on the ammonia removal, there was a slight
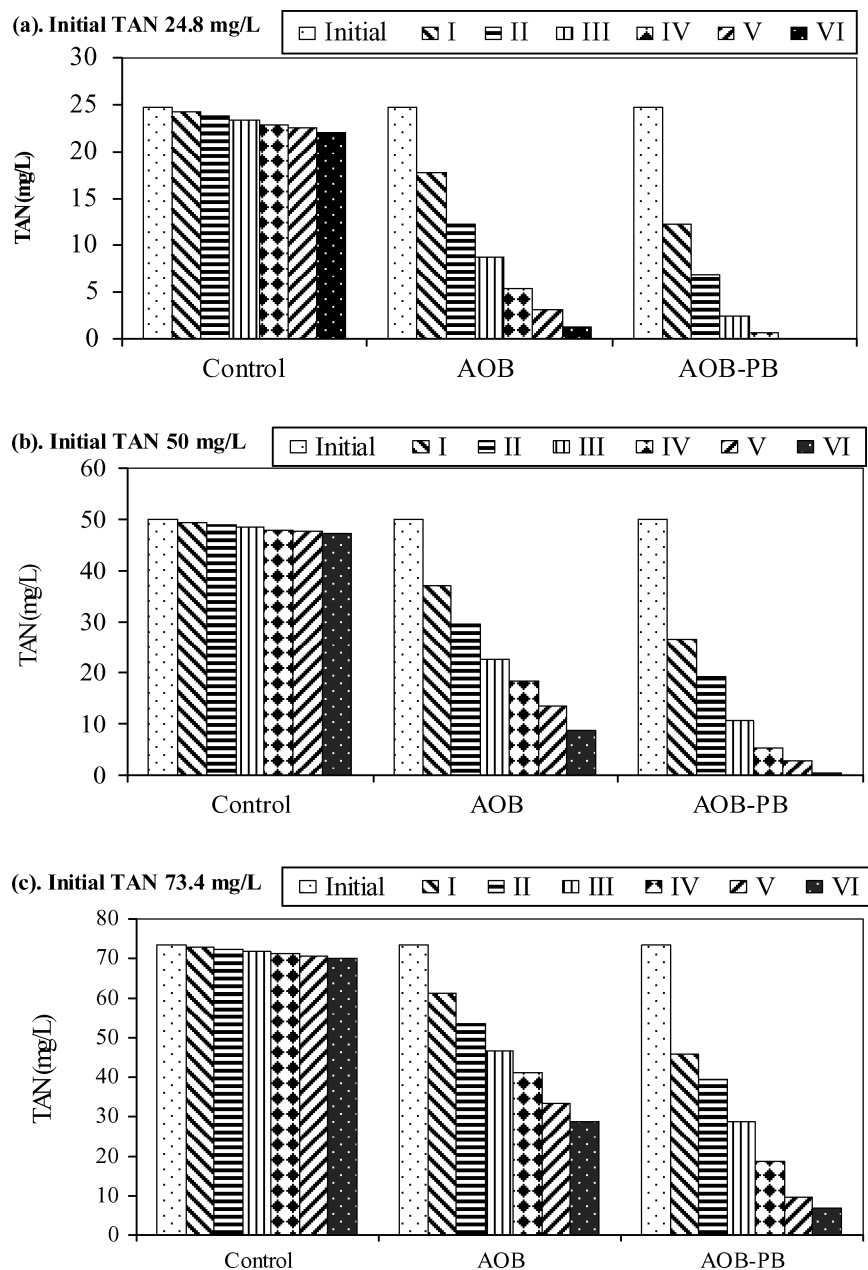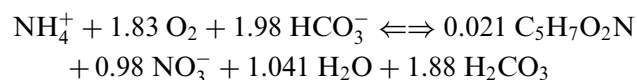
Fig. 8. Biostimulation of ammonia-oxidizing bacteria (AOB) using partially delignified bagasse (PB) for ammonia removal at weekly intervals I to VI: (a) Initial 24.8 mg/L; (b) 50 mg/L (c) 73.4 mg/L.

decrease in alkalinity from 111 to 96 and 94 mg/L as CaCO₃, pH from 7.91 to 7.76 and 7.69 and a substantial decline in DO from 5.8 to 2.4 and 1.8 mg/L, respectively.

Ammonia-oxidizing bacteria are extremely slow growing, have a very narrow pH tolerance, preferring a range between 7.5 and 8.6. They use substantial amounts of oxygen and hydrogen carbonates and reduce hardness and buffering capacity, and have a mild acidifying effect. The carbonate buffering system ($H_2CO_3^-$, HCO_3^- and CO_3^-), controls the pH in aquaculture systems. In nitrification[35], H^+ ions are produced that consume alkalinity in the form of HCO_3^- ($HCO_3^- + H^+ \Longleftrightarrow H_2CO_3$) and lower the pH of

the system:

$$NH_4^+ + 1.83\,O_2 + 1.98\,HCO_3^- \Longleftrightarrow 0.021\,C_5H_7O_2N$$
$$+ 0.98\,NO_3^- + 1.041\,H_2O + 1.88\,H_2CO_3$$

Declines in pH and alkalinity, as observed for the bagasse-biofilm based treatment in the present study, indicate high oxygen and bicarbonate consumption by the autotrophic microbial population. Hence, the two most important considerations in managing the alkalinity and pH of aquaculture system are the rate of alkalinity destruction (rate of nitrification) and the type of supplement to be
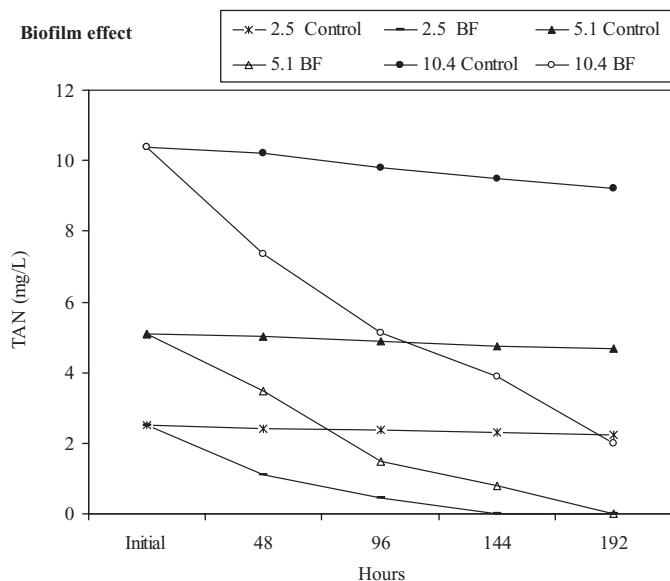
Fig. 9. Biostimulation of ammonia-oxidizing bacteria (AOB) using partially delignified bagasse (PB) biofilm at 48-h intervals.

used to replace lost alkalinity. Brackishwater is a very good buffer system and also the treatment with partially delignified bagasse, naturally buffered the pH of the system.

In the present study, experiment conducted on the effect of initial ammonia concentration on ammonia removal revealed that the percent of ammonia removal decreased with higher initial ammonia concentrations (Fig. 7), which can mainly be attributed to the competition for dissolved oxygen (DO) as the ammonia-oxidizing bacteria are aerobic and have a high requirement for oxygen as an electron acceptor and need a minimum of 1 mg/L dissolved oxygen. Significant declines in dissolved oxygen concentration from 5.8 to 1.8 mg/L, as observed for the PB-biofilm based treatment in the present study, indicate high oxygen consumption by the autotrophic microbial population.

Conclusion

Ammonia-oxidizing bacteria (AOB) are extremely slow growing organism, which causes failure of the performance and stability of treatment processes in aquaculture systems. Therefore, the further understanding of the microbial ecology of AOB in aquaculture treatment processes is essential for enhancing nitrification predictability and control of process stability. The work reported here, described the characterization of ammonia-oxidizing bacteria in Indian brackishwater aquaculture environment through analyses of *amoA* and biostimulation of these indigenous chemolithoautotrophs using partially delignified bagasse. The present study based on DNA sequencing of clone library of *amoA* supply novel sequence information and allow the phylogenetic identification of individual clones.

Three unique *amoA* genes each were identified and sequenced, which were most closely related to *amoA* genes of *Nitrosomonas sp.* and other uncultured *beta-proteobacteria*.

The present study has a potential for making strategy for ammonia oxidation, and also for predictability and reliability of the treatment based on biostimulation in shrimp aquaculture. Biofilms are increasing in popularity as a means of remediating wastewater. However, little is known about the bacterial population structure and how organic source affect nitrifying bacteria. Partially delignified bagasse is a biodegradable substrate that has no residue problem or adverse effect on other water quality parameters. Successful studies on abundantly available bagasse could be beneficial for the immobilization of AOB onto the partially delignified bagasse, which have an applications in the development of environment friendly products based on biostimulation and indigenous bioaugmentation for *in situ* treatment of aquaculture water, where factors of low cost and ease of application are paramount. Therefore, the present study has a potential effect in making biological oxidation of ammonia and biostimulation strategies for aquaculture water and wastewater treatment.

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