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ENRICHMENT AND ISOLATION OF NITRIFYING BACTERIA AND MOLECULAR CHARACTERIZATION OF OPTIMIZED NITRIFIERS FROM MANGROVE SOIL

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ABSTRACT: In aquaculture high levels of ammonia-nitrogen excretion was observed and it should be eliminated by an efficient method for highest production rate. Nitrifying bacteria could play a vital role to remove ammonia from the aquaculture ponds. From mangrove ecosystem soil samples were collected and subjected to enrichment technique, 20 pure nitrifying bacterial colonies were obtained. Among them 16 colonies was ammonia oxidizing bacteria and 4 colonies were nitrite oxidizing bacteria. Followed by antagonistic study, nitrifying bacterial consortium was developed and its activity was optimized. Optimal nitrifying activity was observed up to 6mg/L ammonia concentration, pH 8.5, temperature 30^{o}C and 6 days incubation with dark room temperature. The data were statistically analyzed with two-way ANOVA and found to be significant. The bacterial consortium was finally sequenced with 16srRNA primers and the data were analyzed with phylodraw software. The results of the species were *Paracoccus denitrificans*, *Nitratireductor aquimarinus*, *Paenibacillus dendritiformis* and *Pseudomonas otitidis.*

KEYWORDS: Heterotrophic nitrification, AOB, NOB and *Paracoccus denitrificans.*

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1. INTRODUCTION

Aquaculture sector is becoming an emerging food industry with new technology to face the demands of peoples, but the prevailing production is not sufficient in many countries [1]. This industry sector is now equipped with new techniques with minimized resources for high production of aquaculture products. Apart from its flourishing new technologies, it is also facing

many challenges throughout the era like, disease outbreaks, lack of water-quality management and production cost [2]. Disease outbreaks are being increasingly recognized as a considerable constraint on aquaculture production and market value. So far, the use of chemicals, disinfectants and antimicrobial drugs, limited the success in the prevention of aquatic diseases. The massive use of antimicrobials for disease control increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance, which is also danger to human consumption [3]. Water quality management becomes more challenge due to highest stocking density, feed that contains high protein content and decay of biotic materials [4]. The major problem is produced from metabolism of nutrients, and then their excretion to the aquatic environment which will harm fish and other aquatic organisms to survive [5]. The most important substance among them is a highly toxic substance called ammonia, a large amount of input water requires removing it from the nurturing environment [6, 7]. Ammonia at low concentration seldom kills the aquatic animals but it stresses them causing poor growth and low survival rate. When it reaches high concentration it leads to mortality of aquatic animals [8]. Researchers have brought into being the technique of bio augmentation by nitrifying bacteria, converting toxic ammonia into non-toxic nitrate. Nitrification process is the biological oxidation of ammonia to nitrate via nitrite. The reaction is catalyzed by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and nitrite-oxidizing bacteria (NOB) [9, 10]. These nitrifying bacteria are chemolithoautotrophs that obtain energy for growth by oxidizing either ammonia or nitrite. The known ammonia-oxidizing microbes AOB and NOB are phylogenetically not closely related, and none of these organisms can oxidize both substrates. They are also found as aggregates [11]. This separation of the two nitrification steps in different organisms leads to a tight cross-feeding interaction and the frequently observed co-aggregation with NOB in nitrifying consortia [12]. AOB and NOB are found to be slow growers, their generation time takes more than 8 hours and influenced by the presence of environmental factors such as pH, temperature, C/N ratio, unsaturated fatty acids, dissolved oxygen, ammonia, and nitrite concentrations [13]. This present study was focused on isolation of nitrifying bacteria from mangrove ecosystem, and its screening, characterization and optimization of nitrifying bacterial consortium.

2. MATERIALS AND METHODS

2.1. Sample collection

Soil samples were collected in sterile containers from mangrove ecosystem and aquaculture ponds of Malipattinam, Manakudi and Sethubhavachatram in TamilNadu, India. These locations were chosen because it acts as sink for ammonia and juvenile home for shrimps and crabs. The samples were examined for the presence of NH_4+, NO_2-, NO_3-, hydrogen sulphide and pH within 6 hrs.

2.2. Isolation of Nitrifying Bacteria by Enrichment Method [14]

All the collected soil samples were subjected to primary enrichment culture technique in mineral

salt media (MS media). One gram of each soil sample was inoculated in 100ml of MS media with initial ammonia concentration of 6mg/L and incubated in dark for 28 days under agitation 60 rpm at 30.0 ± 1.0˚C. At an interval of 7 days samples were obtained for analyzing the concentration of NH_4+, NO_2- and NO_3-. After 28 days 10 ml of each aliquot from primary enrichment was transferred to 90ml of fresh MS media, with initial ammonia concentration of 6mg/L and kept under agitation 60 rpm at 30.0 ± 1.0˚C. Secondary enrichment culture was analyzed for concentration of NH_4+, NO_2- and NO_3- at an interval of 7 days until ammonia was completely depleted. Again 10 ml of each aliquot from secondary enrichment was transferred to 90ml of fresh MS media, with initial ammonia concentration of 8mg/L and kept under agitation 60 rpm at 30.0 ± 1.0˚C. Tertiary enrichment culture was analyzed for concentration of NH_4+, NO_2- and NO_3- at an interval of 7 days until ammonia was oxidized. This was done to enrich the population of ammonia oxidizing bacteria. 0.1ml of the aliquot from tertiary enrichment culture was spread plated on MS agar plates. Visibly distinct colonies were, again sub cultured on MS agar medium. The streaking techniques were followed until pure bacterial colonies were isolated. The isolated pure colonies were subjected for screening of ammonia and nitrite oxidation.

2.3. Screening of Nitrifying Bacteria

Pure isolates were screened for ammonia and nitrite oxidation. The isolated bacterial colonies were inoculated in 100 ml of MS media with initial ammonia and nitrite concentration of 10mg/L in separate conical flask and kept under agitation for 60 rpm at 30.0 ± 1.0˚C. At regular interval of 7 days, samples were withdrawn and analyzed for concentration of NH_4+, NO_2- and NO_3-.

2.4. Development of Nitrifying Bacterial Consortium

Isolated pure colonies were subjected for antagonistic study to identify which are capable to co-culture. The study was carried out by disc diffusion method. The colonies which showed absence of inhibition was selected for consortium development. Based on the results of antagonistic study, bacterial consortium was formulated and grouped. 10 ml of each selected isolates for consortium were mixed well. This bacterial suspension was taken as seed culture and inoculated in MS medium with ammonia concentration of 10mg/L and kept in a shaker at 30^{o}C for 14 days. At regular interval of 7 days, samples were withdrawn and analyzed for the concentrations NH_4+, NO_2- and NO_3-.

2.5. Optimization of nitrifying bacterial consortium

Mineral salt media was prepared with different concentration of ammonia in two range to study the effect of nitrifying bacteria with high ammonia concentration (2mg/L, 4mg/L, 6mg/L, 8mg/L and 10mg/L) and low ammonia concentration (0.5mg/ L, 1mg/L, 1.5mg/ L and 2mg/L). Bacterial consortium was added to each concentration and kept in a shaker at 30^{o}C for 14 days. At regular interval of 7 days, samples were withdrawn and analyzed for the concentrations NH_4+, NO_2- and NO_3-. Optimal ammonia concentration was subjected for further studies. Parameters such as pH

(5.5, 6.5, 7.5, 8.5 and 9.5), temperature range (30, 35, 40 and 45 degree Celsius), Comparison of light vs. dark and incubation period were optimized.

2.6. Molecular Characterization

Isolates involved in consortium development were molecular characterized. All the single isolates were subjected for DNA isolation. The isolated DNA was subjected to amplification by PCR technique followed by 16srRNA sequencing and phylogenetic analysis.

3. RESULTS AND DISCUSSION

In this study, 11 soil samples were collected from mangrove ecosystem and aquaculture pond. Among them 4 soil samples from mangrove ecosystem of Manakudi, 4 soil samples from mangrove ecosystem and aquaculture ponds of Malipattinam and 3 soil samples from Sethubhavachatram mangrove ecosystem of Tamil Nadu, India (Table I).

Table I: Analysis of soil samples collected

S.No	Soil Sample	Source	pH	Conc. of NH_4^+ (mg/l)	Conc. of NO_2^- (mg/l)	Conc. of NO_3^- (mg/l)	Conc. of H_2S (mg/l)
1	A	AQ	8.2	4.3	0.7	3.8	0.2
2	B	AQ	8.1	5.5	1	0.9	0.2
3	C	AQ	8.2	6	0.4	1.8	0.2
4	D	ME	7.3	2.8	0.3	1.7	0.5
5	E	ME	7.5	2.6	0.2	1.5	0.3
6	F	ME	6.9	4	0.72	0.5	0.3
7	G	ME	6.8	6.2	0.5	2.8	0.2
8	H	ME	7.6	6.6	0.7	2.6	0.1
9	I	ME	7.4	5.6	0.5	1.9	0.1
10	J	ME	7.2	6.3	1	5.5	0.1
11	K	ME	6.9	6.9	0.1	3	0.1

Table1: Denotes the area of soil collected and initial concentration of ammonia, nitrite, nitrate, hydrogen sulphide and pH in samples. AQ- Aquaculture pond; ME–Mangrove ecosystem.

3.1. Isolation and Screening of AOB and NOB

3.1.1. Enrichment Cultures

Nitrification activity has been increased from primary enrichment culture to tertiary enrichment culture with series of incubation days. This confirms the enrichment of ammonia and nitrite oxidizing bacterial population. 95 percentage of ammonia oxidation was observed in all tertiary enrichment cultures except soil sample G, (Figure I). From tertiary enrichment culture 0.1ml of each sample was spread plated on MS agar medium plates. 20 Pure isolates were obtained and

screened for ammonia oxidation. 16 strains were found to be ammonia oxidizers (AOB) and 4 strains (NOB) were found to be nitrite oxidation (Figure II a and II b).

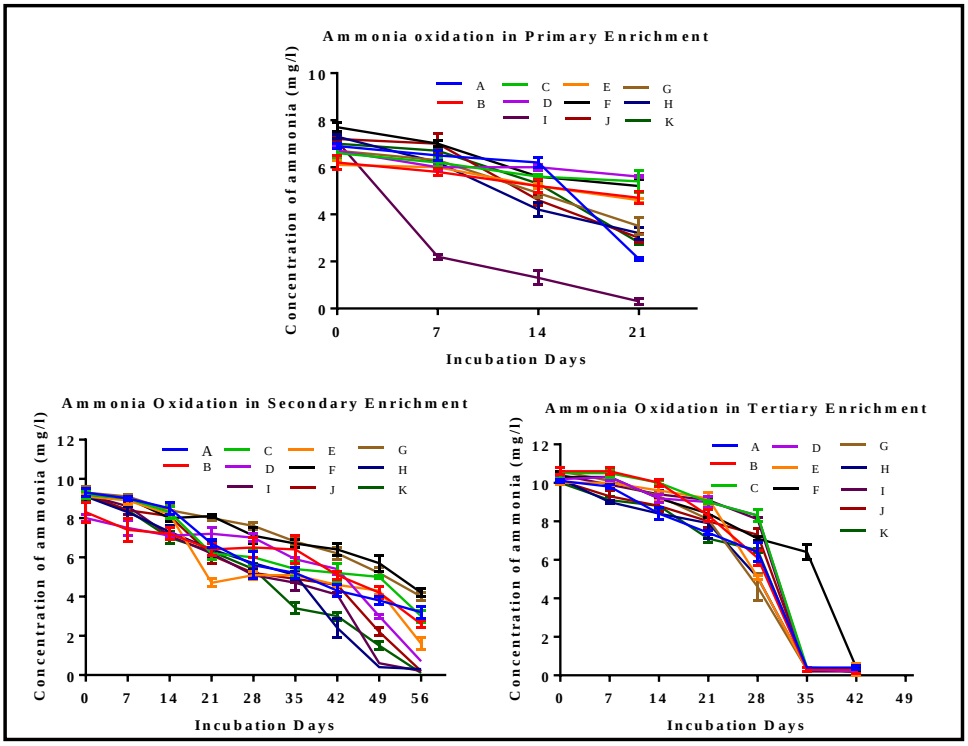

Figure I: Denotes the ammonia oxidation of soil samples in each enrichment cultures with varying incubation period. In tertiary enrichment culture ammonia concentration has been eliminated on 35th day, which was found faster than secondary enrichment culture.

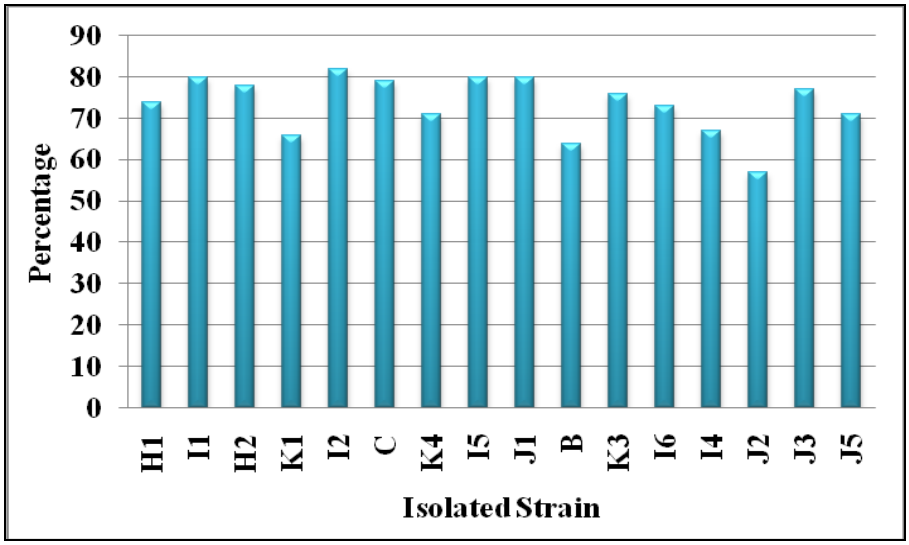

Figure II a: Denotes the screening of ammonia oxidation. 16 colonies were found to oxidize ammonia and their percentage of oxidation is shown the figure.

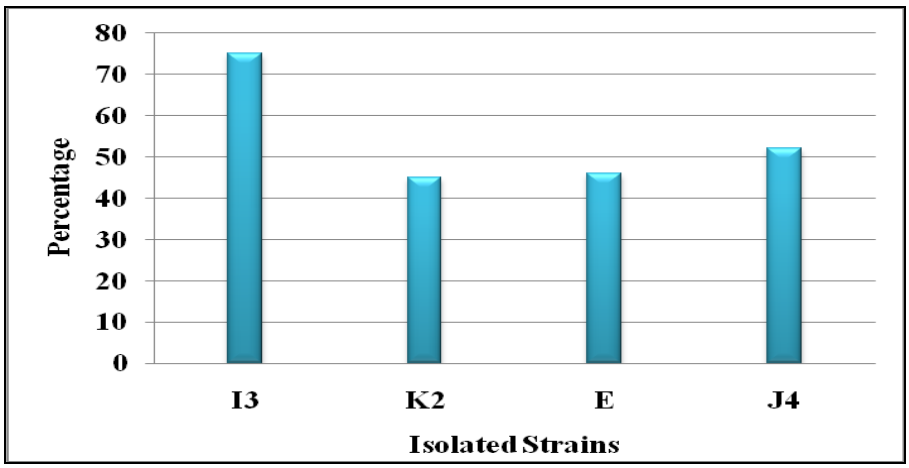

Figure II b: Denotes the screening of nitrite oxidizing pure isolates. 4 colonies were found to oxidize nitrite and their percentage of oxidation is shown the figure.

3.2. Antagonistic study

Among the 20 isolated bacterial strains, 6 were found to have the ability of co-culture (Table II). The bacterial strains were J1, J4, I3, I4, I1 and J5. These colonies were subjected for consortium studies and numerically renamed as 1, 2, 3, 4, 5 & 6. Among the six isolates, J4 and I3 were nitrite oxidizers and J1, I4, I1 and J5 were ammonia oxidizer.

Table II: Antagonistic effect of isolated pure colonies

Isolates	I2	J1	I1	J5	I3	H1	I4	J4	B	J2	J3	H2	K1	I5	K2	E	K3	C	I6	K4
I2	_	**NI**	2	1	2.7	2	**NI**	1	2	1	1	**NI**	2	1.2	2.5	0.9	1.4	1.5	2	1
J1	**NI**	_	**NI**	**NI**	**NI**	2	**NI**	**NI**	1.4	1.3	1.6	1	1	2	1.3	0.8	1	1.3	2	1.4
I1	1.8	**NI**	_	**NI**	**NI**	1	**NI**	**NI**	2	2.2	**NI**	1.2	1	1.5	1	0.9	1	2	1	0.8
J5	1	**NI**	**NI**	_	**NI**	2.2	**NI**	**NI**	1	2.5	1	**NI**	1.5	2	1	0.9	1	1.3	2	1.5
I3	2.5	**NI**	**NI**	**NI**	_	0.9	**NI**	**NI**	1.6	2	2.5	1.5	**NI**	2	1.2	0.8	1	1	1.7	1.4
H1	2.1	**NI**	1	2.5	1.1	_	1.4	0.7	1	0.9	1	1.7	1	2	1.5	1	0.7	1	1.2	1
I4	**NI**	**NI**	**NI**	**NI**	**NI**	1.2	_	**NI**	1.8	1	0.8	1	2	0.9	1	0.7	1.1	1	1.8	2
J4	1.4	**NI**	**NI**	**NI**	**NI**	1	**NI**	_	2	1.6	1.5	1.7	1	0.9	1.3	0.8	1.1	1	2	2.2
B	2	1.4	1.8	1.4	1.9	1	1.8	1.8	_	1.4	1.1	1.3	1	2.5	0.9	1	2	1.4	1.8	1
J2	1	1.4	2.1	2.4	1.8	0.8	1	1.5	1.2	_	1	1.2	2	1.5	2	1	0.9	1	1.5	1
J3	1.2	1.5	**NI**	1.2	2.4	1	0.9	1.2	1	1.2	_	2.1	**NI**	**NI**	1.1	1	1.5	1	**NI**	1.8
H2	**NI**	1.2	1.2	**NI**	1.6	1.5	1	1.5	1.1	1	2	_	**NI**	1	1.3	1.5	1	2	**NI**	1
K1	2	1	1	1.5	**NI**	1	1.8	1	0.8	1.9	**NI**	**NI**	_	1	**NI**	1.4	**NI**	1.8	**NI**	**NI**
I5	1.4	2.1	1.4	1.9	2	1.8	1	1c	2.3	1.6	**NI**	0.8	**NI**	_	1.1	1.2	2.5	1.6	1.2	1
K2	2.6	1.2	1	0.9	1.7	1.5	0.9	1.2	1	2.1	1.1	1.4	**NI**	0.9	_	0.9	1.8	1.2	0.9	1.1
E	0.9	0.8	1	1	0.9	0.9	1	0.9	0.8	1.2	0.9	1	1.4	1.1	1.1	_	1.9	1.5	1.1	2

K3	1.2	1	1	1	1	0.9	1	1	2.1	1.1	1.4	0.8	**NI**	2	2	1.7	_	2.4	1	1.7
C	1.5	1.2	1.8	1.2	1	1	1.1	1.2	1.6	0.9	1	1.7	1.4	1.5	1.2	1.3	2.1	_	1	1
I6	1.9	2	1	2	1.2	1	1.5	1.2	1.6	1.4	**NI**	**NI**	**NI**	1.4	1.2	0.9	1	0.7	_	1.9
K4	0.8	1.3	0.9	1.6	1.4	1	2.2	2	1	1	1.5	1.2	**NI**	1.2	1.3	1.6	1.5	1	2	_

* **NI**- No Inhibition; **Zone of Inhibition** was measured in cm.

3.3. Formulation of nitrifying bacterial consortium

The six isolates, which oxidized ammonia and nitrite above 60% was selected for double consortium. Double consortium possessing ammonia oxidation above 60% was selected for triple consortium and analyzed for above 90% of ammonia oxidation. The double consortium oxidized ammonia above 60% were 1+5 (96%); 4+6 (64%); 5+6 (70%); 5+3 (87%) and 6+3 (63%). In triple consortium the combinations 6+3+1 and 4+6+5 possessed 92% and 90% of nitrification respectively. Triple consortium was again subjected to quad combination. The percentage of ammonia oxidation above 95 in quad combination (6+3+1+5) was 98%. Highest nitrification was observed with quad combination (6+3+1+5), the bacterial strains involved in consortium development were J1, I3, I1 and J5 (Figure III). This bacterial consortium was subjected to optimization studies.

3.4. Optimization of nitrifying bacterial consortium

The bacterial consortium J1, I3, I1 and J5 were selected for optimization with following parameters, concentration of ammonia, pH, temperature, light vs. dark and incubation time. The optimal nitrifying activity was observed with ammonia concentration upto 6mg/l, pH 8.5, temperature 30^{o}C and incubation at dark within 6 days. The results were statistically analyzed by Two-Way ANOVA in PRISM software and found to be significant at 0.05% level of significance (Figure IV).

3.5. Molecular Characterization of nitrifying bacterial consortium

The bacterial consortium was subjected for 16srRNA sequencing and it was identified as *Paracoccus denitrificans* – sample J1, *Nitratireductor aquimarinus* – sample I3, *Paenibacillus dendritiformis* - sample I1 and *Pseudomonas otitidis* – sample J5. Nucleotide sequence was submitted in NCBI Gen Bank and the accession number were MH027385 - *Paracoccus denitrificans*, MH027386 - *Paenibacillus dendritiformis*, MH774572.1- *Nitratireductor aquimarinus* and MH569468 - *Pseudomonas otitidis.*

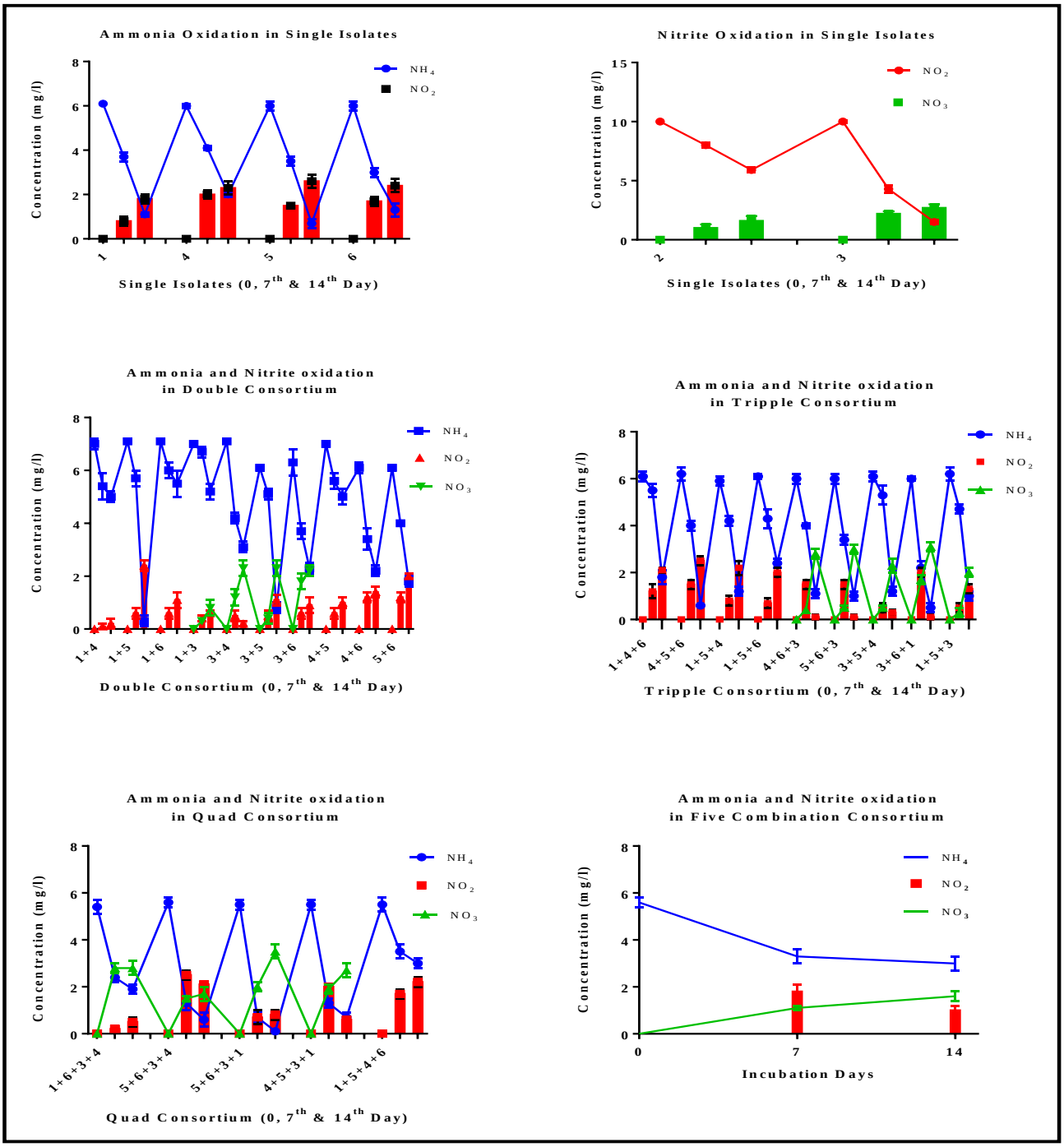

Figure III: Denotes the development of bacterial consortium. Ammonia and nitrite oxidation in each consortium with respect to incubation intervals initial, 7th and 14th day was shown in the figure. In quad consortium 5+6+3+1 was observed to perform efficient nitrification.

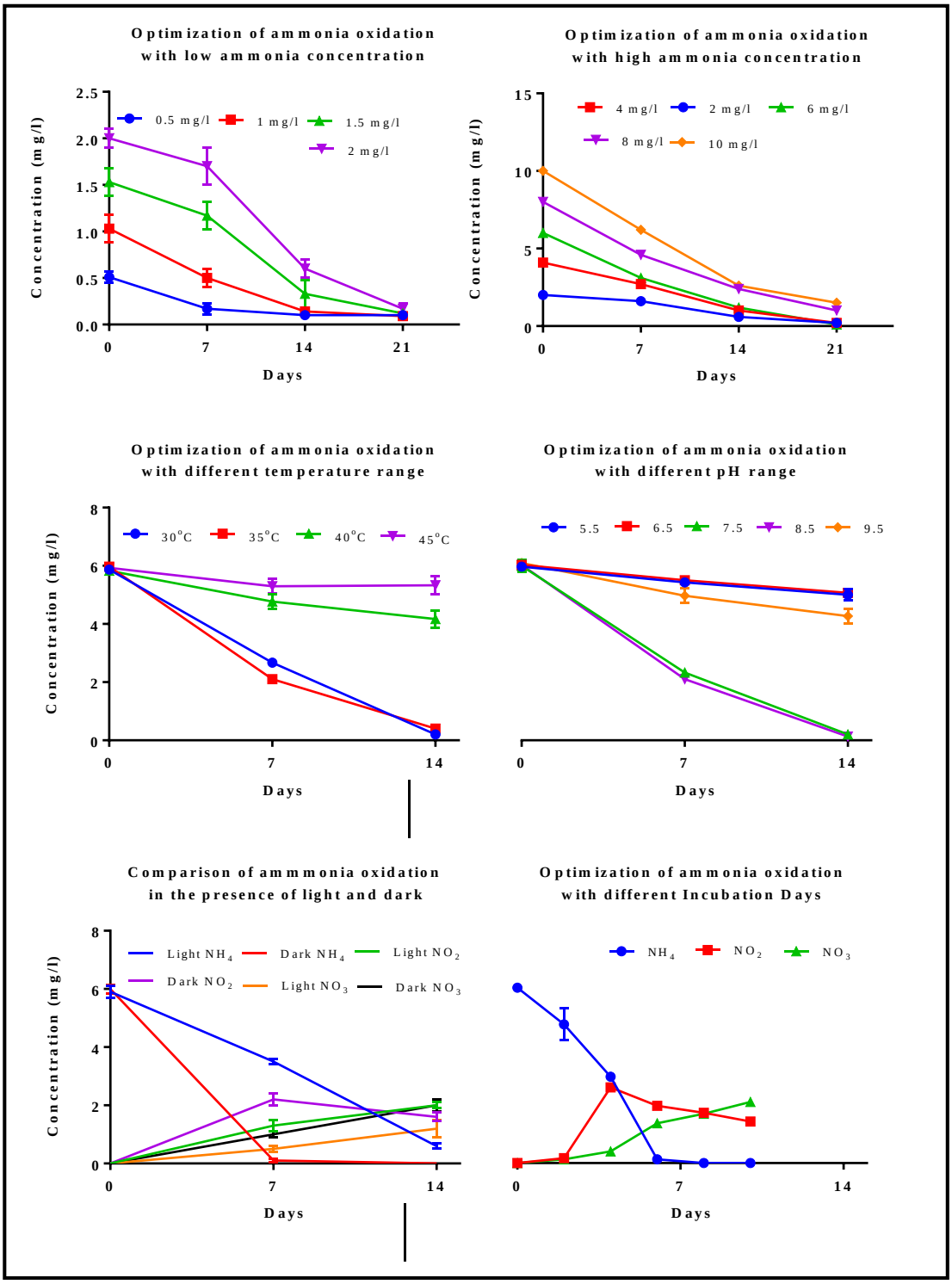

Figure IV: Denotes the optimization of nitrification with various parameters (Substrate concentration- low and high, temperature, pH, light vs. dark and incubation days).

DISCUSSION

The aim of the study was to enrich and isolate the nitrifying bacteria from mangrove ecosystem. Autotrophic nitrifying bacteria have been discovered more than one decade. But research on nitrifying bacteria has been limited until advance molecular techniques have been formulated and culture-independent studies have been carried out. Erna *et al* [15] have stated that isolation of

AOB was difficult due to its slow growth. Aakra *et al* [16] reported it difficult to obtain a pure culture of AOB on solid medium. Coci *et al* [10] have reported that ammonia oxidizer grow better in natural environment compared to mineral media. Population of nitrifying bacteria present in natural system depends on the nitrogen sources available. Due to the anthropology activity and litters of mangrove trees, mangrove ecosystems were observed to be base for ammonia. Hence to isolate AOB and NOB from mangrove ecosystem, enrichment culture technique was performed by fed-batch cultivation. Kim *et al* [17] have enriched AOB until nitrite concentration reached 300mg/l in culture media. Nakagawa and Takahashi [18] have undergone enrichment culture from primary culture to tertiary cultures by detecting the nitrite production after 35 days in each enrichment cultures. Achuthan *et al* [19] have reported that ammonia oxidizers frequently occur as cell aggregates and its nitrification activity were much faster in mixed cultures than in pure form. In our studies also similar results were obtained. In enrichment cultures ammonia oxidation increased from primary enrichment culture to tertiary enrichment culture. All the enrichment cultures oxidized ammonia above 90% and the nitrate produced was also high. But when isolated as pure strain and screened for ammonia and nitrite oxidizer, the percentage of oxidation was observed to low than enrichment cultures. Hence to increase the nitrification rate, bacterial consortium was developed. The nitrification rate of the bacterial consortium was higher than enrichment cultures (*ie*) complete removal of ammonia within 14 days in developed bacterial consortium whereas in enrichment culture it was 42 days. Similarly Erna *et al* [15] have stated bacterial consortium (M1) collected from mangrove area of morris Selangor, Malaysia showed highest reduction of TAN concentration after 14 days. Further nitrification rate was increased by optimization of nitrifying bacterial consortium. It was observed that higher level of nitrification at ammonia concentration of 6mg/l, at a pH range of 7.5 -8.5, temperature 30^{o}C, with an incubation period of 6 days at dark. Growth studies of autotrophic nitrifiers have been carried out with different parameters by researchers. Nakagawa and Takahashi [18] have isolated a strain KYUHI, which oxidized ammonia at 1000mM concentration and found to have optimum growth at 25^{o}C and pH 8. Wang *et al* [20] reported that the isolated strain DIFHS oxidized ammonia at a concentration of 50mM, pH 7.5 to 8 and 37^{o}C in dark. Satoh *et al* [21] found *N. Multiformis* grown at optimum temperature 30^{o}C. French *et al* [22] reported AOB grow faster in high ammonia concentration at pH 7 to 7.5, also suggested AOB very sensitive to blue near UV light. Hence higher nitrification was observed in dark incubation. Shears and Wood [23] discussed that inhibition of nitrification by light may be due to absorption of light by oxygenated state of copper containing ammonia monooxygenase enzyme. Apart from autotrophic nitrifiers heterotrophic bacteria and fungi can also oxidize ammonia to hydroxylamine, nitrite and nitrate [11, 24, 25, 26, 27 and 28]. Daum *et al* [29] have stated that many soil nitrifiers are not restricted to an autotrophic mode of life but can catabolise organic soil compounds. In our results, 16srRNA sequence and

phylogenetic analysis revealed that the heterotrophic bacterial species present in the optimized nitrifying bacterial consortium are *Paracoccus denitrificans, Nitratireductor aquimarinus, Paenibacillus dendritiformis and Pseudomonas otitidis.* Kumar *et al* [9] reported that the isolated nitrifiers possessed majority of hits with the sequences of uncultured bacteria and heterotrophs like *Alcanivorax sp., Paenibacillus sp., Flavobacterium sp*., and *Gramella sp*., *Paenibacillus* and *Beijerinckia sp.* From marine recirculating aquaculture system, Tal *et al* [30] characterized a nitrifying microbial consortium and found ammonia oxidizer *Nitrosomonas cryotolerans* and nitrite oxidizer *Nitrospira marina* associated with the system as well as a number of heterotrophic bacteria, including *Pseudomonas sp.* and *Sphingomonas sp*. and two Planctomycetes sp. Bothe *et al* [11] have reported purification of ammonia monooxygenase enzyme was achieved only from the hetertrophic nitrifier *Paracoccus denitrificans* Our report supports the heterotrophic nitrifying bacteria *Paracoccus denitrificans, Paenibacillus dendritiformis.* In addition we report *Nitratireductor aquimarinus* and *Pseudomonas otitidis* also posses nitrifying activity.

4. CONCLUSION

This present work concludes, nitrifying bacterial consortium (*Paracoccus denitrificans, Nitratireductor aquimarinus, Paenibacillus dendritiformis and Pseudomonas otitidis*) enriched and isolated from mangrove soil sediments, possess efficient nitrification activity. This developed nitrifying consortium further could undergo lab trials and employed in aquaculture system for proficient nitrification activity.

CONFLICT OF INTEREST

Authors have no any conflict of interest.

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