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Isolation, Identification and Characterization of Nitrate Reducing Bacteria from the Estuarine Waters of Selected Sampling Stations of Rajakkamangalam Estuary

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Abstract

Nitrate reduction by bacteria is a crucial step in nitrogen cycle making the Nitrogen available for organisms. Nitrate reductase enzyme and the bacteria as a denitrifying organism find important in applications such as waste treatment, aquaculture pond restoration, to improve crop growth. Aimed at good nitrate reducers, bacteria were isolated from the estuarine waters of Rajakamangalam, Kanyakumari, Southwest Coast of India. A total of 19 dissimilar bacterial strains were isolated and screened for nitrate reducing activity. Four isolates showed significant nitrate reducing ability and are subsequently characterised phenotypically and genotypically. The 16S rRNA sequences of the isolates were deposited in the Genbank with their respective names as *Marinobacter hydrocarbonoclasticus, Bacillus subtilis, Denitrovibrio acetiphilus,* and *Pseudomonas citronellolis.*

Keywords
Denitrification, estuarine, bacteria, 16S rRNA

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INTRODUCTION

Denitrification as a part of biological nitrogen cycle can be simply put as reduction of nitrates into the molecular and inert nitrogen gas. The process is essentially facilitated by bacteria completing the nitrogen cycle. After reduction to ammonia, the nitrogen is still in the environment, changed into a lesser mobile form. The reduction of nitrate has a crucial function in preventing leaching and pollution of the groundwater. Nitrogen fluxes from terrestrial and marine environments into the atmosphere directed by microbial denitrification is about 90 × 10^{12} to 243 × 10^{12} g per year for terrestrial ecosystems and 25 × 10^{12} to 179 × 10^{12} g every year for marine ecosystems (Averill & Tiedje, 1981; Purvaja et al., 2008).

Reduction of nitrate to nitrite is the first step of denitrification involving the enzyme nitrate reductase. The specific enzymes, dissimilatory nitrate reductases, which mediate the reduction of

nitrate to nitrite in both denitrifiers and bacteria that dissimilate nitrate in nitrate reducing bacteria, have been the focus of the physiological and biochemical studies (Gonzalez et al., 2006). Nitrate reductase thus plays the crucial role in initiating the breakdown of nitrate and making it available for other organisms. This potential of denitrifying bacteria is used in waste treatment and recycling processes.

Most attention has been given to the membrane-bound nitrate reductase within the nitrate-reducing bacterium. In this decade, there has been a significant increase in the quantity of research works in search for novel classes of respiratory nitrate reductases. With the rising demand for various bacteria and their enzymes involved in nitrogen cycle especially on nitrate reductase in the field of aquaculture, agriculture and bioremediation to use nitrate and nitrite as the final electron acceptor in the respiratory cycle in marine as well as freshwater environments, an attempt was made to investigate the estuarine coastal sediment bacteria for its potential to produce nitrate reductase. This enzyme will be on focus in the coming years for its merits over environment and its pristine.

MATERIALS AND METHODS

Media: 0.15 g of beef extract, 0.15 g of yeast extract, 0.5 g of sodium chloride and 0.5 g of peptone were weighed and dissolved in 100 ml of distilled water in a conical flask. To this dehydrated broth 1.5 g of agar powder was added and boiled for 5 mints, till the agar became dissolved.

Isolation of estuarine sediment bacteria

The sediment samples were collected from the selected stations of Rajakamangalam estuarine, South west coast of Kanyakumari district, Tamilnadu India and were brought to the laoboratory in an ice box. Serially diluted samples were plated on to the plates prepared with the media. Morphologically dissimilar, well-isolated colonies were randomly selected and streaked on the nutrient agar slants. The selected colonies were sub-cultured in nutrient agar slants. The cultured slants were then stored at 4° C in refrigerator.

Screening of nitrate reducing bacteria - Nitrate reduction test

A loop full of culture was taken in the inoculation needle and streaked on the nitrate broth present in the test tubes. After inoculation the tubes were incubated for 48 to 96 hours at 37ºC in the incubator. Then 1 ml of test reagent A (8.0 g sulphuric acid in 1 litre of acetic acid) and test reagent B (5.0 g alpha naphthalamine in 1 litre of acetic acid) were added. The formation of red colour indicated positive result. If there is no colour change, a small amount of Zn powder was added. The formation of red colour after adding Zn powder indicated the negative result. If there is no colour change after Zn powder was added, it indicated a positive result.

Identification of nitrate reducing bacteria

Morphological and biochemical characterisation

Morphological and biochemical characteristics of the bacterial strains were studied as per the the Bergey's manual of determinative bacteriology (Prescott, 2002).

Molecular identification using 16S rRNA gene sequencing and analysis

Isolation of Genomic DNA

All the denitrifying positive bacterial isolates were individually cultivated in tryptone soya broth. After 48 h of incubation, bacterial cultures were centrifuged for 10 min at 5000 rpm, then the supernatants were discarded carefully, and the pellets were used for the isolation of genomic DNA. The pellets of individual strains were collected and resuspended in 330 µl GTE (Glucose Tris EDTA) buffer and incubated for 1 h at 37°C. After incubation, a pinch of lysozyme was added into the pellet and further incubated for 1 h at room temperature. Further the pellets were added with 10 µl SDS (20%) and incubated for 3 h, after that 18 µl of RNAse was added and incubated overnight at 37°C. After overnight incubation, they were added with 17 µl of 0.5 M EDTA and incubated for 10 min at 50°C, then mixed with 10 µl of proteinase K and incubated for 3 h at 37°C. After incubation, 200 µl of phenol: chloroform was added to the mixture and centrifuged at 10000 rpm for 15 min. Then the aqueous layer was collected carefully and added with equal volume of isopropanol and incubated for 3 min and centrifuged individually for 15 min at 10000 rpm. Then the pellets were collected and washed with 95% ethanol and centrifuged for 10 min at 10000 rpm and once more the pellets were collected and washed with 70% ice cold ethanol.

Amplification of 16S rRNA gene

Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG3' and reverse primer 5' ACGGCTACCT TGTTACGACTT3'. Polymerase chain reaction was performed in a 50 µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5µM, 1.5 mM $MgCl_2$, and each deoxy nucleoside triphosphate at a concentration of 50 µM, as wellas 1 U of *Taq* polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial

denaturation for 3 minat 95°C, there were 40 cycles consisting of denaturation at 95°Cfor 1 min, followed by annealing at 55°C for 1 min, extension at 72°C for 2 min and then a final extension step consisting of 5 minat 72°C; Master cycler Personal (Eppendorf, Germany) was used. The amplification of 16S rDNA and the amplified products were confirmed by running in 1% agarose gel in 1X TAE.

Cloning and sequencing of 16S rRNA gene

The amplified products (1,500-bp) were individually purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, U.K) according to manufacturer's instruction. The 16S rDNA amplicons of each strain was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria were carried out in Macrogen (Seoul, South Korea).

Nucleotide sequence analysis

The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST. Multiple sequence analysis was carried out using CLUSTALX and further NJ plot (Perrière and Gouy, 1996) and MEGA 7.0 were employed for constructing phylogenetic tree (Saitou & Nei, 1987).

RESULTS AND DISCUSSION

A total of 19 morphologically different bacterial strains were isolated from the sediments of selected stations around Rajakamangalam estuary, South west coast of Kanyakumari district, Tamilnadu India. Among the 19 bacterial isolates, four strains showed good nitrate reductase activity. The nitrate reducing isolates were designated as MANS1, MANS2, MANS3 and MANS4 and the strains were isolated from the respective stations as follows: Bar mouth region (Station – I), Near the highway bridge (Station- 2), Effluent Mixing zone (Station-III), Fresh water zone (Station – 4).

All the four bacterial strains were subsequently characterised phenotypically and genotypically. Phenotypic and genotypic characterisation of all the four nitrate reducing bacteria revealed their identity as *Marinobacter hydrocarbonoclasticus, Bacillus subtilis, Denitrovibrio acetiphilus,* and *Pseudomonas citronellolis* respectively. All the four strains were Gram negative rod shaped (*Denitrovibrio acetiphilus* was curved rod shaped) and non-spore forming. Among the four strains only *M. Hydrocarbonoclasticus* was non motile. All the four were able to utilize citrate and were catalase positive. Morphological and biochemical characteristics of all the four strains are as given in the Table.1

Table 1. Morphological and biochemical characteristics of the four-nitrate reducing bacterial isolates

Test	*Marinobacter hydrocarbonoclasticus*	*Bacillus subtilis*	*Denitrovibrio acetiphilus*	*Pseudomonas citronellolis*
Gram Staining	Negative	Positive	Negative	Negative
Simple staining	Rod	Rod	Curved Rod	Rod
Spore	Non spore former	Non spore former	Non spore former	Non spore former
Motility	Non-Motile	Motile	Motile	Motile
Casein hydrolysis	Negative	Positive	Positive	Negative
Gelatin hydrolysis	Negative	Positive	Positive	Negative
Starch Hydrolysis	Negative	Negative	Negative	Negative
Nitrate Reduction	Positive	Positive	Positive	Positive
Indole	Negative	Negative	Negative	Negative
MR/VP	Negative	Positive	Negative	Negative
Citrate	Positive	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Positive
Urease	Negative	Positive	Positive	Negative
TSI agar	Negative	Positive	Negative	Negative
Oxidase	Negative	Positive	Positive	Positive
Lipase	Negative	Negative	Positive	Negative

Table 2. Carbohydrate fermentation pattern of the nitrate reducing bacterial isolates

Test	*Marinobacter hydrocarbonoclasticus*	*Bacillus subtilis*	*Denitrovibrio acetiphilus*	*Pseudomonas citronellolis*
Gluocse	Negative	Positive	Positive	Negative
Sucrose	Negative	Positive	Positive	Negative
Lactose	Negative	Positive	Negative	Negative
Maltose	Negative	Negative	Positive	Negative
Fructose	Negative	Negative	Positive	Negative
Xylose	Negative	Positive	Negative	Positive
Trehalose	Negative	-	Positive	Negative
Manitol	Negative	-	Positive	Negative
Cellobiose	Negative	-	Positive	Negative

Table. 3. Gene bank accession numbers of nitrate reducing bacterial strains isolated from Rajakamangalam estuarine coast of kanyakumari district

Bacterial isolates	Gene bank accession number	Isolation source	Isolation site
Marinobacter hydrocarbonoclasticus MANS1	KR270727	Water	Station 3
Bacillus subtilis MANS2	KR270728	Water	Station 1
Denitrovibrio acetiphilus MANS3	KR270729	Water	Station 2
Pseudomonas citronellolis strain MANS4	KR270730	Water	Station 4

Fig.1. Study area

Fig.2. Phylogenetic tree showing the evolutionary positions of the isolates with maximum likelihood method.

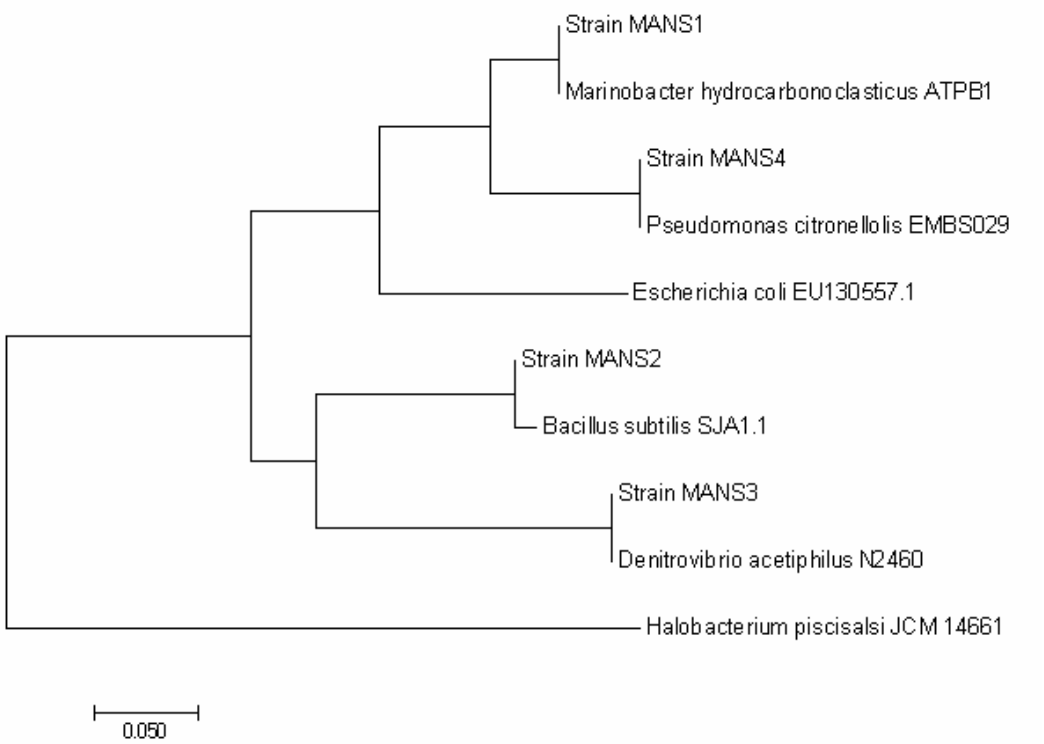
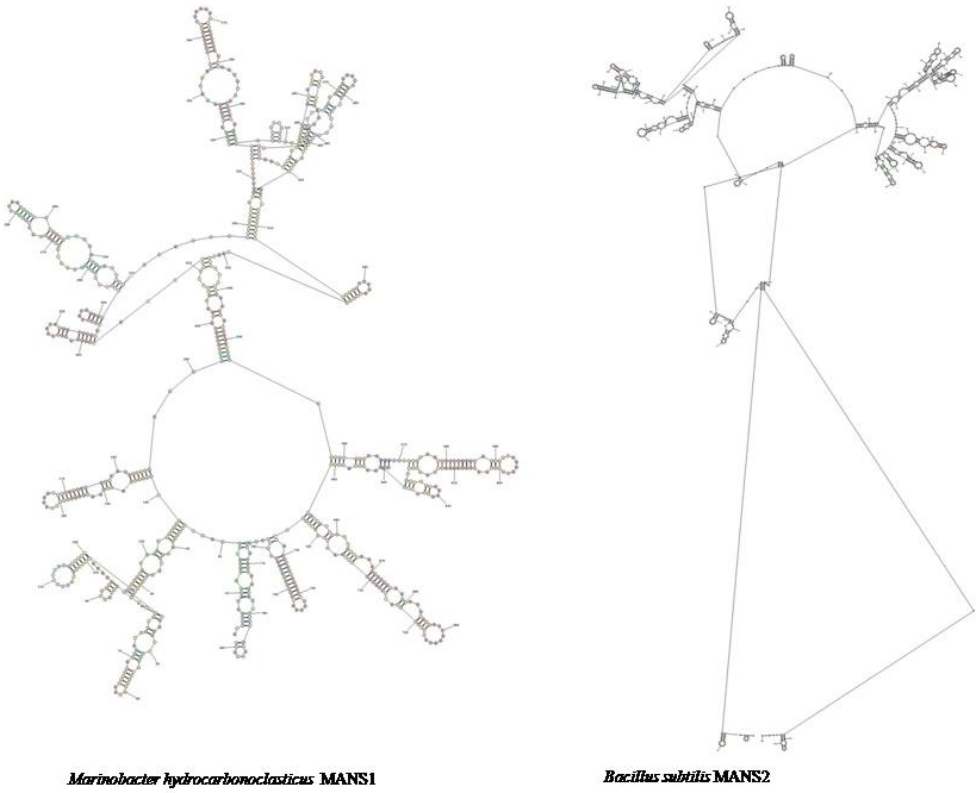
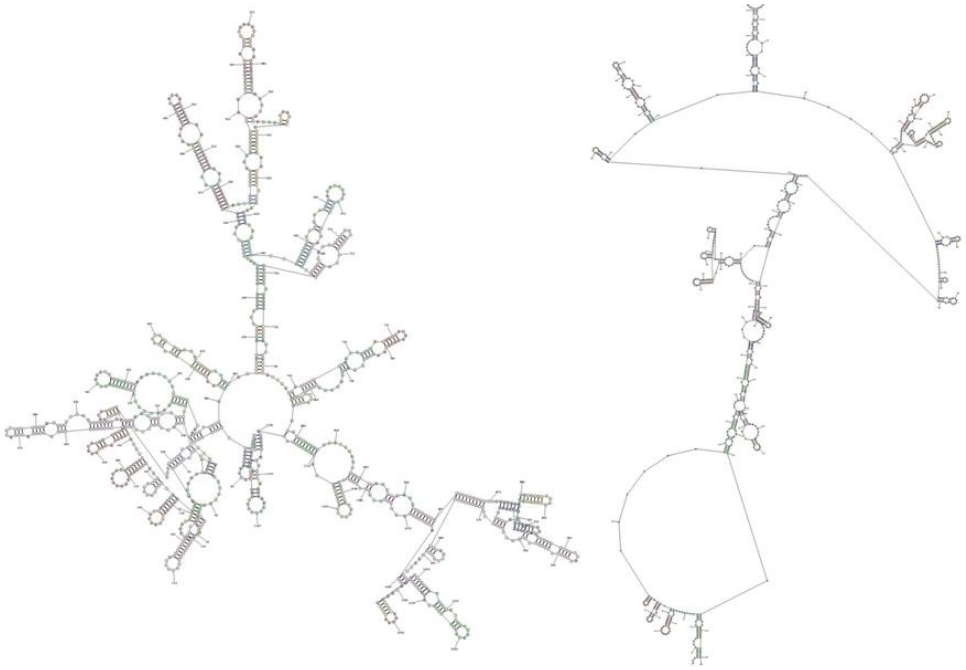


Fig.3. Secondary structure prediction of 16S rRNA sequences of the nitrate reducing isolates by Minimum free energy model



Marinobacter hydrocarbonoclasticus **MANS1** *Bacillus subtilis* **MANS2**



Denitrovibrio acetiphilus MANS3 *Pseudomonas citronellolis* MANS4

Carbohydrate fermentation pattern and acid production ability of all the four nitrate reducing bacterial strains were studied and the results are as given in Table.2. Among the four isolates studied all of them showed no ability to produce indole. Only *D. acetiphilus* proved to produce lipase enzyme. All the four isolates were not able to hydrolyse starch and two strains viz. *B. subtilis* and *D. acetiphilus* were able to hydrolyse gelatine. Nitrate reducing bacteria *Achromobacter sp.* isolated from an Egyptian lake was able to ferment glucose, mannitol and xylose. The strain Achromobacter sp. was described to be oxidase +ve, catalase +ve, Indole –ve and able to utilize citrate (Marwar, et al., 2015)

Molecular characterisation of all the four strains revealed their identity in line with the phenotypic characters. 16S rRNA sequences of all the four bacterial isolates were blasted and submitted to Genbank with the respective species names and the accession numbers are as given in Table.3. Phylogenetic relationship and position of the isolates are plotted using maimum likelihood method in MEGA 7.0 (Fig.2.). Secondary structure predictions of the 16S rRNA sequences of all the isolates were drawn in RNAstructure software using Minimum free energy model (Fig.3.). The prediction of the secondary structure of the 16S rRNA sequences of the isolates revealed their varying and unique nature. All the four strains differed in the RNA folding pattern, in terms of the pattern of branching and number of loops.

Out of the 19 isolates all the four nitrate reducers belonged to different genus and species. Fan et al (2018) reported isolation of five nitrate reducing bacteria that produce surfactants through enrichment from offshore petroleum-reservoir brines. All the five strains reported were belonged to the species *Pseudomonas stutzeri.* Marwa et al (2015) isolated 25 different strains from different Egyptian ecosystems and identified a single hyper-active nitrate reducer strain as *Achromobacter* sp.

In the present study among the four nitrate reducing isolates two viz., *M. hydrocarbonoclasticus* and *P. citronellolis* come under the Phylum: Proteobacteria and Class: Gammaproteobacteria. While the *B.subtilis* and *D. Acetiphilus* differ and fall within Phylum: Firmicutes, Class: Bacilli; and Phylum: Deferribacteres, Class: Deferribacteres respectively. Seenivasagan et al (2014) reported isolation of 74 nitrate reducing bacterial isolates from nitrate contaminated water and soils of Tamilnadu and among the isolates 64% were *Bacillus.* Canion et al (2013) studied the nitrate reducing bacteria from arctic sediments and reported psycrophilic nitrate reducing bacterial isolates. All the fifteen nitrate reducing isolates reported in the study belonged to *Proteobacteria*. A crucial report by Laufer et al (2016) provide evidence for the existence of autotrophic nitrate-reducing Fe (II) oxidizers in coastal marine

sediments. In a recent study a sediment isoalte *Pseudomonas mendocina*, identified as a potential denitrifier was found to be potential and effective in effluent denitrification process in waste water treatment plant (Zhang et al., 2019).

Recent reports emphasise the importance of studies on nitrate reducing bacteria and their potential applications. Molecular characterisation of 16S rRNA, phylogeetic analysis of the sequence and analysis of rRNA folding patterns and their predicted secondary structure's topology together contributed effectively in better phylogentic understanding and differentiaton of the bacterial species. The present study has documented isolation, identification, characterisation of four nitrate reducing bacterial strains from a novel estuarine environment of Tamilnadu coast, providing the potential bacterial candidates for nitrate reductase enzyme production and denitrification applications.

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