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Study of Nitrifying Bacteria from Arid Soil of Purna Basin of Vidarbha Region Maharashtra, India

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Abstract

In the basin of river Purna, main crops cultivated are cotton, sorghum, pigeon pea, black gram, green gram, wheat, gram etc. The nitrogen is essential element for growth and development of plants. In soils, organic nitrogen (N2) is converted to ammonia through microbial decomposition. Ammonium already formed in the soil, added as fertilizer or in precipitation is rapidly oxidized to nitrate in the nitrification process carried out by specific bacteria like *Nitrobacter* and *Nitrosomonas*. This region has with stressed the accountable changes in climate, rise in nitrate content in underground waters and least nitrogenous fertilizers use effectively. The present study involves a review over the existing knowledge of microscopic data and processes that are relevant for the isolation and characterization of nitrifying bacteria from soil, mainly to isolate nitrifying bacteria by a less time consuming method. All the isolates showed morphological and biochemical criterion similar to species of the genera *Nitrosomonas* and *Nitrobacter*.

Keywords arid Soil, fertilizer, *Nitrobacter*, *Nitrosomonas*, purna basin

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Introduction

Nitrogen is an incredibly versatile element, existing in both inorganic and organic forms as well as in many different oxidation states. The movement of nitrogen in the atmosphere, biosphere and geosphere in different forms is described by nitrogen cycle, one of the major biogeochemical cycles, which involves five main processes: nitrogen fixation, nitrogen uptake, nitrogen mineralization, nitrification and denitrification. Micro-organisms particularly bacteria, play major roles in all the principle nitrogen transformations [1]. Nitrification comprises the oxidation of ammonia to nitrite and subsequent oxidation of nitrite to nitrate and is a key process of biogeochemical nitrogen cycle. Nitrifiers are ubiquitous in soil, freshwater and marine environment and have been found in unusual habitats like building sandstone [2-3].

They play a key role for nitrogen turnover in this ecosystem. All nitrifiers are closely related by their specialized biochemical reactions and oxidize reduced nitrogenous compound for energy and fix carbon dioxide as their carbon source. The primary taxonomic difference at the genus level is based on cellular morphology. Ammonia and nitrite are toxic to aquatic life and release of these pollutants from sewage into receiving water must be minimized.

Excess water input from the wastewater contributes to the eutrophication of natural waters, which causes incalculable ecological damage

and nitrifying activity also contributes to nitrogen losses from agricultural soils. As nitrate is relatively mobile it can be readily leached, which represents not only a loss from the ecosystem, but also a potential environmental problem [4]. Therefore, a detailed insight into the isolation, biodiversity and eco-physiology of nitrifiers is urgently needed for improving the efficiency of soil. Biochemistry of nitrifiers is less explored as compared to many heterotrophs, both because nitrifiers grow slowly and due to difficulties in growing them in in-vitro conditions [5].

Studies revealed that most plant prefers nitrate more readily as a source of nitrogen as compared to ammonia, means nitrate is more preferred source which is provided by nitrifying bacteria [6]. The primary organisms responsible for ammonium oxidation to nitrate in soil are chemolithotrophs. The organism derives their energy from the oxidation of the nitrogen atom. Conversion of ammonium to nitrite yields 66 kcal per mole; whereas nitrite oxidation to nitrate produces 20 kcal. The recovery of this energy by the microbes- ammonium oxidizers oxidizes typically 14 and 70 ammonium–nitrogen per carbon incorporated into cellular biomass; whereas, between 76 to 135 nitrite-nitrogen must be oxidized for the comparable task [7].

The study of nitrifying bacteria has been done from wetland soil such as wet tundra, paddy soil, rain fed and Roth Amsted soil, but no research for the isolation is reported particularly from the black clay soil. The soil of Purna basin has been developed on the hilly and undulating topography. Therefore, the soil shows a wide variation in depth. So, the present study was undertaken to isolate and characterize nitrifiers from this particular soil.

Methodology

Total five soil samples were collected from arid soil of Purna Basin. The samples were analyzed and two types of isolates were isolated by using different media. Winogradsky media used for isolation of *Nitrobacter* and *Nitrosomonas* spp. [8]. The media for isolation and culture of *Nitrosomonas* in Phase-I consisting of,

$(NH_4)_2SO_4$	-	2.0 g
K_2HPO_4	-	1 g
$MgSO_4.7H_2O$	-	0.5 g
NaCl	-	2.0 g
$FeSO_4.7H_2O$	-	0.4 g
$CaCO_3$	-	0.01 g
Agar	-	15.0 g
Distilled water	-	1,000 ml

While *Nitrobacter* was isolated using medium B for nitrification in Phase-II consisting of,

KNO_2	-	0.1 g
Na_2CO_3	-	1 g
NaCl	-	0.5 g
$FeSO_4.7H_2O$	-	0.4 g
Agar	-	5.0 g
Distilled water	-	1,000 ml

The media were dispersed into sterile Petri dishes after cooling to about 45°C. The petri dishes were then inoculated and incubated aerobically for 7 days at room temperature (28 ± 2°C) for both spp. Further, identification and characterization of pure cultures of isolates were undertaken using the protocol provided by Holt et al. [9].

Sample collection and processing

Five soil samples were collected from different agricultural fields in Purna basin. Soil samples were collected from the upper layer of soil and kept directly into sterile zip lock polythene bags. The samples were labeled and stored in dark, until analysis. Approximately, one gram of separate soil samples was taken into 9 ml sterile saline solution in different test tubes. The tubes were shaken well and samples were allowed to settle down and supernatant was discarded and samples were used for streaking.

Inoculation on winogradsky medium-A and medium-B

The suspensions were then inoculated on solidified, Winogradsky medium A and medium B with the help of sterilized inoculating needle by four way streaking method. The inoculated plates were then incubated at 280C for seven days and observed for the development of colonies.

Identification of bacteria

Identification of isolates was based on morphological and biochemical characteristics. The decreased concentration of ammonia and increased concentration of nitrite and nitrate suggested growth and activity of nitrifying bacteria. Slides were examined for cell morphology, Gram's staining, motility, flagellation and capsulation (Figure 1). Standard biochemical tests such as catalase, oxidase, urease, nitrate reduction and ammonia utilization tests were performed [10].

Results and Discussion

In the present study, from five soil samples, twelve isolates showing resemblance to *Nitrosomonas* spp. and *Nitrobacter* spp. were isolated. Both the types of isolates were found to be Gram negative. Out of twelve isolates, nine were found to be motile and three were non-motile. Eight isolates were found to be spore forming and four were non spore forming.

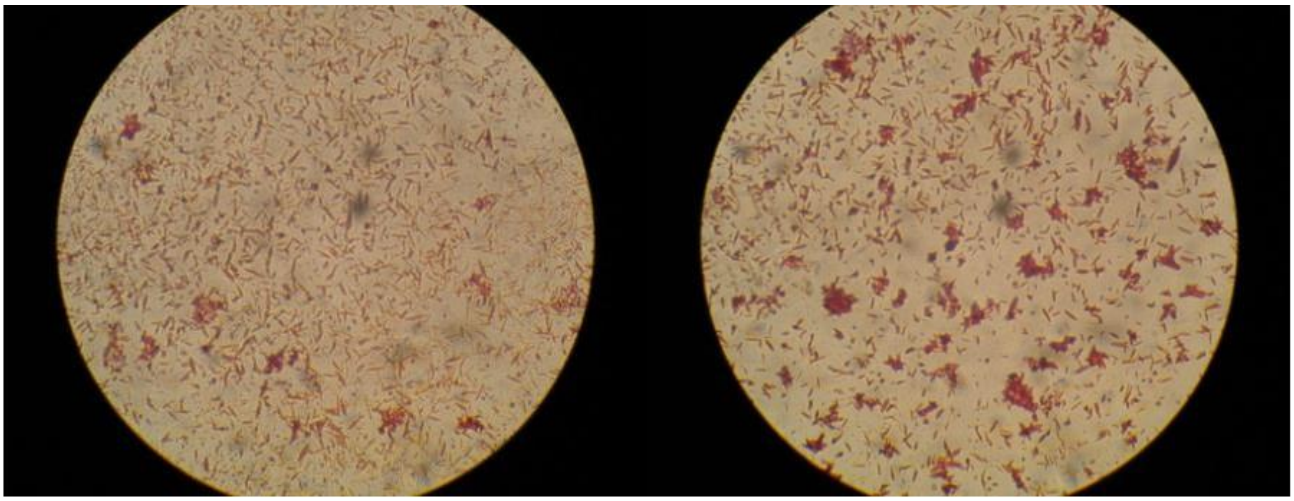

Figure 1. Gram staining microscopic observation

Out of twelve isolates, seven isolates showed positive response innitrate reduction test and five isolates showed negative nitrate reduction test. Five isolates showed positive ammonia utilization test and seven isolates showed negative ammonia utilization test. The positive isolates for nitrate reduction test indicated that they were capable of utilizing nitrate as a source of nitrogen, while positive isolates for ammonia utilization test indicated that they were capable of utilizing ammonia as a source of nitrogen (Table 1).

Table 1. Characterization of nitrifying bacteria from purna basin soils

Sr.No	Sample	Media Used	Isolation code	Gm Reaction	Shape of Bacteria	Motility	Spore	Capsule	Flagella	Catalase	Oxidase	Urease	Ammonia utilization	Nitrate Reductase	Isolates
1	Soil	A	NS(1)	-	SR	+	+	-	P to S	-	-	+	+	-	*Nitrosomonas* spp.
		A	NS(2)	-	LR	-	-	-	-	-	-	-	-	-	-
		B	NB(1)	-	PL	+	-	-	P to L	-	-	+	-	+	*Nitrobacter* spp.
		B	NB(2)	+	LR	-	-	-	-	-	-	-	-	-	-
2	Soil	A	NS(3)	-	SR	+	+	-	Tuft	-	-	+	+	-	*Nitrosomonas* spp.
		A	NS(4)	-	PL	-	-	-	-	-	-	-	-	-	-
		B	NB(3)	-	SP	-	-	-	P to L	-	-	-	-	+	*Nitrobacter* spp.
		B	NB(4)	-	SR	+	-	-	P to L	-	-	+	-	+	*Nitrobacter* spp.
3	Soil	A	NS(5)	+	SR	-	-	-	-	-	-	-	-	-	-
		A	NS(6)	-	SE	+	+	-	Tuft	-	-	-	+	-	*Nitrosomonas* spp.
		B	NB(5)	-	PL	+	-	-	-	-	-	+	-	+	*Nitrobacter* spp.
		B	NB(6)	+	Cocci	-	-	-	-	-	-	-	-	-	-
4	Soil	A	NS(7)	+	SP	-	-	-	-	-	-	-	-	-	-
		A	NS(8)	-	SE	-	+	-	P to S	-	-	-	+	-	*Nitrosomonas* spp.
		B	NB(7)	-	SP	-	-	-	P to L	-	-	-	-	+	*Nitrobacter* spp.
		B	NB(8)	+	LR	-	-	-	-	-	-	-	-	-	-
5	Soil	A	NS(9)	-	SR	+	+	-	-	-	-	+	+	-	*Nitrosomonas* spp.
		A	NS(10)	+	Cocci	-	-	-	-	-	-	-	-	-	-
		B	NB(9)	-	PL	+	+	-	-	-	-	+	-	+	*Nitrobacter* spp.
		B	NB(10)	-	PL	+	+	-	P to L	-	-	-	-	+	*Nitrobacter* spp.

Abbreviations: NB-Nitrobacter, NS-Nitrosomonas, SR-Straight rods, PL-Pleomorphic, SP-Spherical, SE-Ellipsoidal, P to S- Polar to sub-polar, P to L- Polar to lateral, (+) Positive reaction, (-) Negative reaction.

Out of twelve isolates, seven isolates formed spores and five isolates did not form the spores. Nine isolates were flagellated and three isolates were non-flagellated. Out of twelve isolates, none of the isolates was capsule former (Table 2). Out of twelve isolates, five isolates, belonged to the genera *Nitrosomonas*, were gram negative and none of the isolates belonged to this genera was gram positive. Seven isolates, belonged to the genera *Nitrobacter*, were gram negative and none of the isolates belonged to this genera was gram positive (Table 3).

Table 2. Number of isolate and their morphological character

Source	Gram Positive		Gram Negative		Motile		Non- motile		Spore		Non Spore		Flagellated		Non flagellated		Capsulated		Non capsulated	
Soil	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*
	0	0	5	7	4	5	1	2	5	2	0	5	4	5	1	2	0	0	0	0
Total	**0**		**12**		**9**		**3**		**7**		**5**		**9**		**3**		**0**		**0**	

Out of twelve isolates, all the isolates showed negative results for catalase and oxidase tests; thus, isolates were incapable of producing catalase and cytochrome oxidase enzymes. Seven isolates were urease positive and five isolates were negative. Urease positive isolates indicated that they were capable of splitting urea and releasing ammonia, which could be utilized by them as a growth substrate (Table 4).

Primary goal of the present investigation was to isolate Nitrifying bacteria from particularly, the soil of Purna Basin (black clay soil of dry land region) using different culture media and a simple technique. Two different mediums (A and B) were used for the isolation of nitrifying bacteria. The used media composition was similar to that provided by Colwell and Zambruski [8]. The most efficient isolation method was to use the enrichment culture followed by plating on agar or silica-gel [5].

The great problem with the isolation of nitrifying bacteria on solid media was heterotrophic microorganisms. This was also suggested by Berg [11] that heterotrophic microorganisms easily outgrow nitrifiers, before they can reach detectable numbers on the plates. This occurs even if mineral selective media is used, since heterotrophs can metabolize organic substances in the inoculums.

The biochemistry of nitrifying bacteria is less explored than that of many of the heterotrophs, both because nitrifiers grow slowly and also due to difficulties to grow them in in-vitro environment [5]. Not much detail on the specific biochemical test for nitrifying bacteria is available in the literature. Only a few species shows positive response to Urease test. Biochemical characterization is possible only by ammonia utilization and nitrate reductase test.

Table 3. Number of isolated organism with their Gram Reaction

Source	Gram Positive		Gram Negative	
Soil	*Nitrosomonas*		*Nitrobacter*	
	+	-	+	-
	0	5	0	7
Total	**0**	**5**	**0**	**7**

Table 4. Number of isolated organism with their Catalase, oxidase and urease test

Source	Catalase				Oxidase				Urease			
Soil	*Nitrosomonas*		*Nitrobacter*		*Nitrosomonas*		*Nitrobacter*		*Nitrosomonas*		*Nitrobacter*	
	+	-	+	-	+	-	+	-	+	-	+	-
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**	**0**	**4**	**0**

In the present study, similar test were performed which were not sufficient for characterization. Hence, more research is needed to be conducted on this issue, so that the identification of nitrifiers on the basis of biochemical characterization would be possible.

Conclusion

All the twelve isolates showed morphological and biochemical criterion similar to species of the genera *Nitrosomonas* and *Nitrobacter*. Further identification of the isolates up to species level required 16S rRNA identification.

Further study on nitrification would explore the way through which these nitrifiers could be used in various fields as well as its major environmental impact, which is the depletion of stratospheric ozone layer could be minimized.

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