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Research Article

Characterization of Enriched cultures of Nitrifying bacteria from Black cotton soil of *Purna* Basin

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Abstract

To arrest the increasing nitrate pollution in *Purna* river basin due to indiscriminate anthropogenic commercial nitrogenous chemical fertilisation, the regional study of soil microbial nitrification was found to be important and in this regard, the causal soil chemoautotrophs were attempted for enrichment and characterisation from the black cotton soil by adopting standard logical microbiological techniques. The species of *Nitrosomonas* and *Nitrobacter* were traced to be behind nitrosification and nitrification in this heavily fertilized soil zone. These soils have shown higher nitrification potentials due to the presence of nitrifying bacteria. These bacteria were enriched in liquid broths and characterised morphologically and biochemically for confirmation. The related details are discussed herewith.

Key words: Soil microbial nitrification, *Nitrosomonas, Nitrobacter*, black cotton soil, *Purna* river basin, nitrification potential.

INTRODUCTION

The north-eastern Maharashtra region majorly covers the areas of *Purna* river basin and forest covers on the southern *Satpura* range with the alluvial to black cotton soils. Among the chemical fertilizers used for maximizing crop production, nitrogenous fertilizers like amide (most preferred was urea), ammonium (NH_4^+) and nitrate (NO_3^-) containing fertilizers are widely used due to the poor nitrogenous fertilizing capacity of these soils[1]. Thus, maximal reliance on the bulky doses of the fertilizers for the past five decades (since 1970s) have been resulted into (i) nitrate contamination in water reservoirs, (ii) erosion through nitrate loss by leaching and floods, (iii) nitrogen enrichment in water aquifers causing threat of eutrophication and (vi) stagnation in crop yield non-commensurate with inputs[2]. The two steps of oxidative nitrification, the formation of nitrite (NO_2^-) from ammonium (NH_4^+) (called nitrosification) and the formation of nitrate (NO_3^-) from nitrite (NO_2^-) (called nitrification), are carried out by different soil chemolithoautotrophic microbial populations, dominated by bacterial genus *Nitrosomonas* involved in nitrosification and another *Nitrobacter* for nitrification[3,4]. The transfer of nitrate and nitrite ions from surface soil to groundwater, pose a serious human health hazard. Nitrate contamination in the groundwater was a particular problem in agricultural areas of Purna and Tapi river basin, especially banana bowl in *Khandesh*, *Citrus* zone in *Varhad* and cotton belt in all north eastern Maharashtra, where high concentrations of nitrogenous fertilizers are applied to soil[5]. On this background, it was attempted to isolate, characterise and study these nitrifying bacteria from the black cotton soils around Amravati, Maharashtra, India for its subsequent study in the role of the nitrate pollution. The details in this regards are discussed herewith.

MATERIALS AND METHODS

The five soil samples were collected from the different agricultural fields after Monsoon, around Amravati District, Maharashtra, India. Soil samples from upper layer of soil were collected directly into the sterile polythene zip lock bags, labelled and stored in dark until analysis.

Three replicates of 100 ml of a stock medium A [AOB, Ammonia oxidation broth with composition in gL^{-1} Na_2HPO_4 13.5, KH_2PO_4 0.7, $MgSO_4.7H_2O$ 0.1, $NaHCO_3$ 0.5, $FeCl_3.6H_2O$ 0.014, $CaCl_2.2H_2O$ 0.18, $(NH_4)_2SO_4$ 0.5, pH 7.2 $\pm$ 0.2 at 28 ºC adjusted using 0.5% w/v aqueous sodium bicarbonate] and medium B [NOB, Nitrite oxidation broth with composition in gL^{-1} Na_2HPO_4 13.5, KH_2PO_4 0.7, $MgSO_4.7H_2O$ 0.1, $NaHCO_3$ 0.5, $FeCl_3.6H_2O$ 0.014, $CaCl_2.2H_2O$ 0.18, $NaNO_2$ 0.5, pH 7.2 $\pm$ 0.2 at 28 ºC adjusted using 0.5% w/v aqueous sodium bicarbonate] each for each soil sample inoculation were prepared[6] in six 500 ml conical flasks. These were wrapped with aluminium foil to ensure darkness and avoid direct exposure to light, since nitrifiers are photosensitive. For the isolation of nitrifying bacteria, repeated enrichment and subsequent subculture method was employed. A 20 g of fresh soil was first inoculated in 200 ml of sterile saline [0.85% w/v $NaCl_{(aq)}$] in a 500 ml conical flask and agitated on a rotary shaker for half an hour for proper homogenization. Then, flask was allowed to settle for 10 minutes and from it, 10 ml supernatant was pipetted out aseptically and added to the conical flasks containing culture media replicates. Most of nitrifiers are obligate aerobes and have huge oxygen demand; therefore, incubation was on vigorous agitation at 120 rpm on rotary shaker. Incubation temperature was maintained at 28ºC. All the flasks were incubated for about 28 days and the same process was again repeated to have second and subsequently third enrichment of nitrifiers during which it was expected to remove most of the nitrifying heterotrophs from the culture. After third enrichment, the resultant culture was subjected for microscopic morphological and biochemical analysis according to standard microbiological and biochemical techniques[7, 8, 9, 10, 11]. Slides were examined for cell morphology, cell grouping, Gram staining, and motility, flagellation and capsulation etc. Standard biochemical tests such as catalase, oxidase, urease, nitrate reduction and ammonia utilization tests were performed. Also, the decreased concentration of ammonia and increased concentration of nitrite and nitrate in the culture media suggested the growth and activity of nitrifying bacteria.

The collected soil samples were analysed for (a) Quantitative estimation of ammonia through Nesslerisation method, (b) Quantitative estimation of nitrite, (c) Quantitative estimation of nitrate and (d) Nitrification potential rate etc. for nitrification studies[12, 13].

The nitrification potentials of soils were determined as a measure of the maximal nitrifying activity under optimized conditions[14] by conducting short-term experiments within 24h.

To confirm if nitrification has occurred by the enriched chemoautolithotrophic bacteria in the culture broth, nitrite analysis was conducted after every three days by withdrawing adequate aliquots throughout the incubation period to ensure the detection of nitrite generated from metabolized ammonia in AOB and before being further metabolized to nitrate in NOB. Ammonium sulphate may be added intermittently to maintain ammonium concentrations at 0.5 gL^{-1}; to lengthen the experiment's duration for more accuracy[15]. Aliquots of the culture broth were treated with 2-3 drops of nitrite detecting reagents N,N-dimethyl-alpha-napthylamine and sulphanilic acid in a test tube and the development of pink colour was observed as a presence of nitrite.

The all observations and results were taken and recorded and compared with that of standards given in Bergey's Manual of Determinative Bacteriology and accordingly interpreted[9, 10].

RESULTS AND DISCUSSIONS

From the collected samples of black cotton soils from different agricultural areas around Amravati, Maharashtra, India; twelve enriched cultures showing morphological and biochemical resemblance to the genera *Nitrosomonas* and *Nitrobacter* as per Bergey's Manual of Determinative Bacteriology[10] were obtained in almost highly enriched and less contaminated form (Table 1). Both these types were found to be Gram negative. Out of twelve types, nine were found to be motile and three were non-motile. Eight cultures were found to be sporulating and four were non-sporulating.

Primary goal of the present investigation was to isolate Nitrifying bacteria from particularly the soil of Amravati (black clay soil of dry land region) using suitable culture media and a simple technique. Two different medium (A and B) were used for the enrichment of nitrifying bacteria (AOB and NOB). Both medium A and B favoured the growth of several heterotrophic nitrifiers first which were considered as contaminant[16,17,18]. Several heterotrophic microorganisms oxidize either ammonium or organic nitrogen to nitrite or nitrate. Heterotrophic nitrifiers include both fungi and bacteria, therefore, a repeated enrichment and subculture method was employed to reduce heterotrophic load i.e. contaminants and attempted to obtain most pure form/ isolate of the necessary chemoautolithotrophic bacterial culture.

Growth of gram negative isolates related to genera *Nitrosomonas and Nitrobacter* was supported by medium A and B respectively. Identification of

isolates was based on morphological and biochemical characteristics.

Out of twelve isolates (Table 2), five isolates, belonged to the genera *Nitrosomonas*, were Gram negative and none of the isolate belonged to this genera was Gram positive. Seven isolates, belonged to the genera *Nitrobacter*, were Gram negative and none of the isolate belonged to this genera was Gram positive.

Out of twelve isolates (Table 3), regarding spore production seven isolates were spore former and five isolates were non-spore former. Nine isolates were flagellated and three isolates were non-flagellated. Out of twelve isolates regarding capsule production none of the isolates was capsule former.

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

Out of twelve isolates (Table 5), five isolates were showing nitrate reductase test negative and seven isolates were showing it positive. Five isolates were showing ammonia utilization test positive and seven isolates were ammonia utilization test negative. The nitrate reduction test negative isolates indicated that they were incapable of reducing the nitrate, while those showing it positive indicated that those are also capable of reducing the nitrate. The isolates showing ammonia utilization test positive, indicated that they were capable of utilizing ammonia as a source of nitrogen.

The nitrification potential rate of the collected soil samples was determined (Table 6). The nitrification potential rate showed the maximum nitrifying activity of collected soil sample.

The above figure showed that, nitrification potential rate of the collected soil sample was 0.762 $\mu g g^{-1} h^{-1}$ N dw. The nitrification potential rate of the sample showed significant higher amount. This was because; soil from an agricultural field was continuously leached with added ammonium fertilizers. Thus such soil had growth substrate continuously supplied for the nitrifying bacteria. The potential nitrification rate was the maximum nitrification rate of a sample under optimal conditions during the incubation period. Measurement of potential nitrification rate gave a good insight into the quality of soil.

The collected soil samples were tested for nitrification by estimating the concentration of ammonia, nitrite and nitrate in it. Following results were obtained as per Table 7.

The above figures (Table 7) showed concentration of ammonia, nitrite and nitrate in the five soil samples collected from different agricultural field from Amravati. The concentration of nitrite was higher than that of ammonia and nitrate. It was probably due to the fact that, in an agricultural field soil was leached continuously with ammonium fertilizers which get subsequently utilized by nitrifying bacteria. In the soil, the nitrite and nitrate content were present as a result of the activity of nitrifying bacteria. The relatively low concentration of nitrite and nitrate was probably due to less number of nitrifying bacteria in the soil. Since nitrifying bacteria were slow growers. By the twelfth day of incubation, all five test tubes turned pink, indicating nitrification by bacteria. These confirmed nitrifying bacteria were present in the culture broth.

In the present study, culture dependent approach was applied to isolate nitrifying bacteria from soil samples by using enrichment media. Morphological and biochemical examination of the four isolates clearly indicated that these belong to the genera *Nitrosomonas and Nitrobacter*. The media composition used was similar to that which was previously given[6]. Although the method used in this study shows slight variation from the method given by the prior.

In the present method, serial sub-culturing was done to minimize the time period of isolation of nitrifying bacteria to 6 weeks[19,20]. Later on further improvement in the methodology were made to minimize the time period of isolation[21], the total time period needed was 40 days, they have suggested an improved media for the isolation of nitrifying bacteria from soil and the methodology adopted also showed sub culturing and enrichment method. The most efficient isolation method was to use enrichment culture followed by plating on agar or silica-gel media[22]. So, after enrichment an attempt will be made to obtain an isolated colony on plates containing the media having same composition (used for enrichment) with agar. The great problem with the isolation of nitrifying bacteria on solid media was heterotrophic microorganisms. It was also suggested that heterotrophic microorganisms easily outgrow nitrifiers before they can reach detectable numbers on the plates[23]. This occurs even if mineral selective media used, since heterotrophs can metabolize organic substances in the inoculums and even dead nitrifiers cells.

CONCLUSION

From the above experiment it was concluded that enrichment-isolation, characterization and study of the nitrifying bacteria from the black cotton soil of a

dry and wet land of purely rain-fed region was possible. All the twelve isolates showed morphological and biochemical criterion similar to the species of the genera *Nitrosomonas and Nitrobacter* as well as the variation in the concentration of nitrate and ammonia confirmed the activity of nitrifying bacteria and thus, the presence of the isolates.

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Table 1
Characterization of Nitrifying Bacteria From Amravati Soils

Sr. No	Sample	Sample collection site	Date of sampling	Media Used	Isolation code	Gram's Reaction	Shape Of Bacteria	Motility	Sporulation	Capsulation	Flagellation	Catalase test	Oxidase test	Urease test	Ammonia utilization	Nitrate Reductase	Isolates
1	Soil	SKU	5.9.10	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	PKV	8.9.10	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	R	29.1.11	A	NS(5)	+	SR	-	-	-	-	-	-	-	-	-	-
				A	NS(6)	-	SE	+	+	-	Tuft	+	+	-	+	-	*Nitrosomonas* spp.
				B	NB(5)	-	PL	+	-	-	-	+	+	+	-	+	*Nitrobacter* spp.
				B	NB(6)	+	Cocci	-	-	-	-	-	-	-	-	-	-
4	Soil	SGBAU	8.2.11	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	V	15.2.11	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.

SKU- Shivaji krishi udyan, PKV- Panjabrao Deshmukh Krishi Vidyapeeth, R- Rahatgaon, SGBAU- Sant Gadge Baba Amravati University, V- Valgaon. SR- Straight rods, PL- Pleomorphic, SP-Spherical, SE- Ellipsoidal, P to S- Polar to subpolar, P to L- Polar to lateral, (+) Positive reaction, (-) Negative reaction.

Table 2
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 3
Number of isolate and their morphological character:

Source	Gram Positive		Gram Negative		Motile		Non- motile		Sporulating		Non-Sporulating		Flagellated		Non-flagellated		Capsulated		Non-capsulated	
	Nitrosomonas	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*	*Nitrosomonas*	*Nitrobacter*
Soil	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	

Table 4
Catalase, oxidase and urease test of Organism

Source	Catalase test				Oxidase test				Urease test			
Soil	*Nitrosomonas*		*Nitrobacter*		*Nitrosomonas*		*Nitrobacter*		*Nitrosomonas*		*Nitrobacter*	
	+	-	+	-	+	-	+	-	+	-	+	-
Total	5	0	7	0	5	0	7	0	3	2	4	3

Table 5
Nitrate reduction and Ammonia utilization test of Organisms

Source	Nitrate reductase test				Ammonia utilization			
Soil	*Nitrosomonas*		*Nitrobacter*		*Nitrosomonas*		*Nitrobacter*	
	+	-	+	-	+	-	+	-
	0	5	7	0	5	0	0	7
Total	0	5	7	0	5	0	0	7

Table 6
Average soil nitrification potential

Time (h)	Nitrite concentration (μgg^{-1} N dw) NO_2^-	Nitrate concentration (μgg^{-1} N dw) NO_3^-	Nitrification potential (μgg^{-1} N dw) ($NO_x = NO_2^- + NO_3^-$)
0	-	-	0
1	2.35	0.01237	2.36
3	10.83	0.01428	10.84
6	15.07	0.01713	15.08
22	18.84	0.02189	18.86
26	19.79	0.03046	19.82

Table 7
Quantitative estimation of ammonia, nitrite and nitrate

Tube no.	Quantitative estimation of ammonia		Quantitative estimation of nitrite		Quantitative estimation of nitrate	
	Absorbance (at 525 nm)	Concentration of ammonia ($mgml^{-1}$)	Absorbance (at 493 nm)	Concentration of nitrite ($mgml^{-1}$)	Absorbance (at 530 nm)	Concentration of nitrate
Sample 1	0.12	0.036	0.03	0.05	0.02	0.03
Sample 2	0.14	0.045	0.04	0.065	0.05	0.06
Sample 3	0.12	0.036	0.06	0.09	0.06	0.08
Sample 4	0.09	0.026	0.025	0.035	0.08	0.1
Sample 5	0.10	0.04	0.07	0.11	0.05	0.06

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