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ISOLATION AND ENUMERATION OF NITRIFYING BACTERIA FROM RHIZOSPHERE ZONE OF PADDY (ORYZA SATIVA L)

Biotechnology

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ABSTRACT

BACKGROUND: Microbial oxidation of ammonia to nitrate in the rhizosphere lead to nitrogen leaching. Nitrifying bacteria are chemolithotrophs which obtain their energy by the oxidation of reduced inorganic soil nitrogenous compounds like ammonia into nitrite and nitrate. Nitrification is the aerobic process of oxidation of ammonium ion (NH_4^+) to nitrite (NO_2^-) and subsequent nitrite oxidation to nitrate (NO_3^-). Species of *Nitrosomonas* and *Nitrosococcus* convert ammonium ion into nitrite, and species of *Nitrobacter* and related chemolithoautotrophic bacteria convert nitrite to nitrate.

AIM: To enumerate nitrifying bacteria from rhizosphere zone of *Oryza sativa*.

OBJECTIVE: To study the nitrification ability of rhizobacteria.

METHODS: In the present investigation, nitrifying bacteria from the rhizosphere soil of *Oryza sativa* were isolated and enumerated qualitatively as well as quantitatively.

RESULTS: The results revealed that the population density of *Nitrosomonas* was high, followed by *Nitrobacter*, *Nitrococcus*, *Nitrosococcus* and *Nitrospira* of the soil sample. NH_4^+ oxidising bacteria were more up to the soil depth of 1 cm (MPN 6.5 X 10^5) and minimum up to the depth of 8-10 cm (MPN 0.35 X 10^5). Similarly, NO_2^- oxidising bacteria were more up to the depth of 0-1 cm (MPN 2.5 X 10^5) which declined to 0.035 X 10^5 MPN up to the depth of 8-10 cm. Among nitrifying bacterial isolates *Nitrosomonas* had greater capacity to oxidise NH_4^+, and the amount of NO_2^- produced by the oxidation of 5.95 mg/mL of NH_4^+ was 4.70 mg/mL. *Nitrobacter*, *Nitrococcus*, *Nitrosococcus* and *Nitrospira* had lowest capacity to oxidise NH_4^+. Similarly, *Nitrobacter* had greater capacity to oxidise NO_2^-, and the amount of NO_3^- produced by the oxidation of 5.45 mg/mL of NO_2^- was 4.70 mg/mL. Other nitrifying bacterial isolates had lowest capacity to oxidise NO_2^-.

CONCLUSIONS: It can be concluded that *Nitrosomonas* is the prominent NH_4^+ oxidizer and *Nitrobacter* the prominent NO_2^- oxidizer. Others nitrifying bacteria had the ability oxidise both NH_4^+ and NO_2^-.

KEYWORDS

Rhizosphere, Nitrifying bacteria, NH4+oxidizers, NO2- oxidizers, MPN, Oryza sativa

INTRODUCTION

Nitrifying bacteria are chemolithotrophs which obtain their energy by the oxidation of reduced inorganic soil nitrogenous compounds like ammonia into nitrate and nitrite[1]. Nitrifying bacteria in the rhizosphere include species of *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, *Nitrospina*, *Nitrospira*and *Nitrococcus*[2].

Nitrification is the aerobic process of oxidation of ammonium ion (NH_4^+) to nitrite (NO_2^-) and subsequent nitrite oxidation to nitrate (NO_3^-). Species of *Nitrosomonas* and *Nitrosococcus* convert ammonium ion into nitrite, and species of *Nitrobacter* and related chemolithoautotrophic bacteria convert nitrite to nitrate.

$$NH_4^+ + 1/2O_2 \rightarrow NO_2^- + H_2O + 2H^+$$
$$NO_2^- + 1/2O_2 \rightarrow NO_3^-$$

Some nitrifying bacteria viz. *Nitrosomonas eutropha* oxidize ammonium ion anaerobically to nitrite (NO_2^-) and nitric oxide (NO) using nitrogen dioxide (NO_2) as an oxidant in a denitrification-related reaction. Heterotrophic nitrification by bacteria and fungi contributes significantly to these processes in more acidic environments. Energy released upon oxidation of both ammonia and nitrate is used to synthesize ATP by oxidative phosphorylation.

Isolation of nitrifying bacteria has successfully been performed by enrichment of batch culture[3] or by serial dilution methods[4].

Nitrifying bacteria are widely distributed in soil and are responsible for the maintenance of soil fertility and a part of nitrogen cycle[5]. The enumeration of nitrifying bacteria is normally carried out employing the Most Probable Number (MPN) technique[6-9]. This is based on the production of nitrite and nitrate and disappearance of ammonium (NH_4^+) or Nitrite (NO_2^-) from the medium. The aim of the present investigation was to isolate and enumerate the nitrifying bacteria from rhizosphere soil of paddy (*Oryza sativa*).

MATERIALS AND METHODS

Nitrification ability of Rhizobacteria

The ability of rhizobacteria to convert ammonium ion into nitrite and nitrate was demonstrated qualitatively in ammonium sulphate broth. After inoculation of bacterial isolates, production of nitrite was estimated after 7-days of incubation. The presence of ammonia was tested by use of Nessler's reagent. Appearance of yellow colour indicated that ammonia was not oxidized to nitrite. No change in colour indicated the presence of nitrite. The presence of nitrite was also tested by use of Trommsdorf's reagent and sulfuric acid. The presence of a blue-black colour indicated the presence of nitrite; no change in colour indicated the absence of nitrite.

Isolation and enumeration of nitrifying bacteria from rhizosphere soil of paddy field

The nitrifying bacteria from rhizosphere soil of paddy field were isolated by batch culture enrichment methods followed by plating onto solid media[3] or by repeated serial dilution methods[4]. For this purpose, soil samples from rhizosphere zone of paddy field were taken and sliced into one-centimetre sections aseptically and the slurries were prepared by serially diluting the samples with 0.9 % (W/V) sterile saline. Nitrifying bacteria were then enumerated by most probable number (MPN)[10] using 8 X 12 microtiter plates. Ammonium bicarbonate medium (NH_4^+) of Alexander and Clark[11] with the concentration of $(NH_4)_2SO_4$ reduced to 0.5 g/L and NO_2^- medium of Aleem and Alexander[12] were used separately in the microtiter plates for the detection of NH_4^+-oxidizers and NO_2^- oxidizers. The microtiter plates were incubated at 30^{0}C for 21 days for the detection of NH_4^+ oxidizers and for 28 days for the detection of NO_2^- oxidizers. At the end of the incubation period, NO_2^- production by NH_4^+oxidizing bacteria and NO_2^- utilization by NO_2^- oxidizing bacteria were analysed using Griess-L-Losovay reagents.

First medium (Winogradsky I) consisted of $(NH_4)_2SO_4$- 0.5g; K_2HPO_4-1.0g; $FeSO_4.7H_2O$- 0.03g; NaCl-0.3g; $MgSO_4.7H_2O$-0.3g; $CaCO_3$-7.5g; Distilled water-1000.0mL. Similarly, Second medium (Winogradsky II) consisted of (ingredients in g/L) $NaNO_2$-0.006; K_2HPO_4-1.0; NaCl-0.3; $MgSO_4.7H_2O$-0.1; $FeSO_4.7H_2O$-0.03; $CaCl_2$-0.3; $CaCO_3$-1.0 g; Distilled water-1000.0 mL.

The medium first was used for the cultivation and enumeration of all the nitrifying bacteria viz., ammonium and nitrite oxidizers. The

second medium was used for the cultivation and enumeration of nitrite oxidizers only.

Batch culture enrichments

Enrichments of batch culture were carried out in 250 mL conical flasks containing 100 mL of the medium first and medium second separately. The enriched media were regularly sub-cultured and samples were removed at the intervals of two week and plated onto medium plates, gelled by the addition of 1.2 % purified agar. Both the media were amended with 50 mg/L chloramphenicol and cycloheximide, and then incubated at 30^{0}C for 5 weeks in plastic bags to prevent desiccation.

A gel-model system was constructed which consisted of a solid agar base layer in a sterile glass beaker overlain with a semi-solid agar layer[13]. In this system the effects of opposing solute gradients on bacterial growth could be studied. A similar gel-model system was used to enrich for NH_4^+ oxidizing bacteria. The gel-model system was constructed for both the solid and semi-solid basal medium layer. The basal medium consisted of following ingredients: $MgSO_4.7H_2O$-0.15 g; $CaCl_2.6H_2O$-10mg; $NaHCO_3$-0.5g;$FeSO_4.7H_2O$-14mg; KH_2PO_4-0.4 g; K_2HPO_4-4.2 g; Cycloheximide-50 mg; Distilled water-1000.0 mL.

Prior to autoclaving 100 mL of this medium was amended with $(NH_4)_2SO_4$ and purified agar and pH was adjusted to 7.6. The medium was then poured in to Quick fit 11 reaction vessel and 100 mL of the sediment was layered on the top of this solid layer. The cooled semi-solid layer containing mineral salts medium plus 0.28 % purified agar was then gently poured into the vessel. The batch culture system was then covered with aluminium foil and incubated at ambient temperature (20 ±2^{0}C). Production of nitrite was detected in the top 1 cm of the gel after four weeks of incubation. After eight weeks of incubation, the samples were removed using a sterile Pasteur pipette and streaked onto the mineral medium plates (second medium).

Chemostat enrichments

The continuous culture enrichments were carried out in a single stage without pH control in a glass chemostat[14] at 300C using NH_4^+ and NO_2^- media. Due to the high buffering capacity of the NH_4^+ oxidizer medium there was no requirement for pH control in these enrichments. The chemostats were covered with aluminium foil to prevent light inhibition of the enrichment cultures. The chemostats were then inoculated by addition of 50 g of soil sample. Dilution rates of either 0.02 or 0.04/hour were employed throughout the enrichment period. After inoculation, the chemostats were allowed to equilibrate for 48 hours before the pumps were switched on. NO_2^- production or utilisation was detected after 24 hours of incubation. The samples were then plated out onto the mineral medium plates (second medium). The colonies of nitrifying bacteria were removed using a sterile Pasteur pipette and then transferred into the appropriate enrichment medium.

Nitrifying activity of bacteria

The purified nitrifying bacteria were grown in 20 mL of Winogradsky medium in incubator shaker at 200 rpm and at 30^{0}C. Starting concentration of nitrogenous compounds was 5 mg/L (N-NH_4^+) or 5 mg/L (N-NO_2^-). After five days of incubation, the culture media were withdrawn for activity test. For this purpose, culture media were centrifuged at 10000 rpm for five minutes. The cell pellets were removed, and the sediment was used to determine the activity of ammonium ion oxidising bacteria. The NH4+ oxidising activity of bacteria was determined by the amount of N-NH_4^+ lost and N-NO_2^- formed by Nessler and Griss methods. Nitrite oxidising activity of bacteria was determined by the amount of N-NO_2^- lost and N-NO_3^- formed by Griss and Salicylate methods. The results obtained have been presented in Table-1, 2 and 3.

Table-1: Nitrifying bacteria isolated from rhizosphere of *Oryza sativa*

Nitrifying bacteria	Population density (N X 10^5/ g of soil)
Nitrosomonas	20.0 X 10^5
Nitrobacter	15.0 X 10^5
Nitrococcus	9.0 X 10^5
Nitrosococcus	8.5 X 10^5
Nitrospira	7.5 X 10^5

Table-2: Distribution of nitrifyingbacteria in rhizosphere soil of paddy (*Oryza sativa*)

Depth of soil in cm	$NH4^+$-oxidizing bacteria (MPN)	NO_2^--oxidizing bacteria (MPN)
0-1	6.5 X 10^5	2.5 X 10^5
2-3	1.5 X 10^5	0.21 X 10^5
4-5	1.4 X 10^5	0.25 X 10^5
6-7	0.45 X 10^5	0.15 X 10^5
8-10	0.35 X 10^5	0.035 X 10^5

Table-3: Activities of isolated nitrifying bacteria

Bacterial isolates	Ammonia oxidizing bacteria		Nitrite oxidizing bacteria	
	NH_4^+-N remaining in mg/mL	NO_2^--N formed in mg/mL	NO_2^--N remaining in mg/mL	NO_3^--N formed in mg/mL
Negative control	5.95	Free	5.45	Free
Nitrosomonas	1.25	4.70	5.25	0.20
Nitrobacter	3.85	2.10	0.75	4.70
Nitrococcus	4.55	1.10	2.75	2.50
Nitrosococcus	4.15	1.83	2.85	2.40
Nitrospira	4.25	1.70	2.65	2.85

RESULTS AND DISCUSSION

The nitrifying bacteria isolated from the rhizosphere soil of *Oryza sativa* were species of *Nitrosomonas, Nitrobacter, Nitrococcus, Nitrosococcus* and *Nitrospira*. The population density of *Nitrosomonas* was high (20 X 10^5), followed by *Nitrobacter* (15 X 10^5), *Nitrosococcus* (9 X 10^5), *Nitrosococcus* (8.5 X 10^5) and *Nitrospira* (7.0 X 10^5) per gram of the soil sample (Table-1). NH_4^+ oxidising bacteria were more up to the soil depth of 1 cm (MPN 6.5 X 10^5) and minimum up to the depth of 8-10 cm (MPN 0.35 X 10^5). Similarly, NO_2^- oxidising bacteria were more up to the depth of 0-1 cm (MPN 2.5 X 10^5) which declined to 0.035 X 10^5 MPN up to the depth of 8-10 cm (Table-2). Among nitrifying bacterial isolates *Nitrosomonas* had greater capacity to oxidise NH_4^+, and the amount of NO_2^- produced by the oxidation of 5.95 mg/mL of NH_4^+ was 4.70 mg/mL. *Nitrobacter*, *Nitrococcus, Nitrosococcus* and *Nitrospira* had lowest capacity to oxidise NH_4^+. Similarly, *Nitrobacter* had greater capacity to oxidise NO_2^-, and the amount of NO_3^- produced by the oxidation of 5.45 mg/mL of NO_2^- was 4.70 mg/mL. Other nitrifying bacterial isolates had lowest capacity to oxidise NO_2^- (Table-3). From the results it is evident that *Nitrosomponas* is the prominent NH_4^+ oxidizer and *Nitrobacter* the prominent NO_2^- oxidizer. Others nitrifying bacteria had the ability oxidise both NH_4^+ and No_2^-.

Microbial population density of both NH_4^+ and NO_2^- oxidizing nitrifying bacteria declined with depth which might be due to reduced O_2 concentrations. The present findings gain support from the works of Mac Farlance and Herbert[15] who also recorded the decline in density of nitrifying bacteria with increasing depth of soil.

Alexander[7] reported that if autotrophic NH_4^+ oxidation provided the sole source of NO_2^- for NO_2^- oxidation, the population densities of NH_4^+ oxidizing bacteria should be approximately three times greater than those of NO_2^- oxidizing bacteria. Mac Farlane and Herbert[16] reported that an additional source of NO_2^- may be derived from the dissimilatory reduction of NO_3^- to NO_2^-, and that this supported larger populations of NO_2^- oxidizing bacteria within the rhizosphere soil. The present findings are in agreement with those of Voltz *et al*[17] and Belser[18] who also reported that NO_3^- dissimilation to NO_2^- supported large populations of NO_2^- oxidizing bacteria in soil.

CONCLUSIONS:

It can be concluded that the dominant nitrifying bacteria in the rhizosphere of paddy field were *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* was proved to be NH_4^+ oxidiser and *Nitrobacter* the NO_2^- oxidizer. The chemostat enrichment methods proved to be the most reproducible and efficient method for the rapid isolation of nitrifying bacteria particularly NH_4^+ oxidizing bacteria.

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