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ISOLATION AND IDENTIFICATION OF PROMISING AMMONIFYING AND NITRIFYING BACTERIA FROM AGRICULTURAL FIELDS OF VISAKHAPATNAM

SUJATHA PEELA[1,2], DEEPIKA DIVYA KADIRI[2], NARESH GORLE[2] AND KRISHNAKANTH VARADA RAJU PEETALA[2]

[1]*Department of Biotechnology, Dr.B.R Ambedkar University, Srikakulam, Etcherla, INDIA*
[2]*Department of Biotechnology, GITAM University, Visakhapatnam, INDIA*

ABSTRACT

Microorganisms are indispensable components of our ecosystem. Soil microorganisms, such as bacteria and fungi, play key roles in soil fertility and promoting plant health. The improvement of soil fertility is one of the most common strategies to increase agricultural production by maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in soil. The objective of the present study is to isolate and identify promising ammonifying and nitrifying bacteria from soil samples collected from 12 different agricultural fields of Visakhapatnam. Bacteria with predominant morphological types were selected randomly from agar plates and isolated on minimal medium i.e. Yeast Extract Mannitol agar, Ashby's Mannitol Agar media for ammonifying and nitrifying bacteria. The bacterial isolates were identified using Bergey's manual of bacteriology. The bacterial isolates *Pseudomonas, Bacillus, Proteus, Enterobacter, Klebsiella*, were identified as ammonifying bacteria and *Azotobacter, Nitrobacter, Nitrococcus, Nitrosomonas*, were identified as nitrifying bacteria in the agricultural fields. Then the soil samples were tested for the presence of Ammonia, Nitrite and Nitrate by incubating the soil samples for a week in Ammonifying, Nitrite and Nitrate forming broths respectively. Based on the color developed the concentration of ammonia, nitrite, and nitrates were detected using spectrophotometer. The amount of organic nitrogen present in the broth was estimated by neutralization method using Nessler's reagent. Our work suggests that these ammonifying and nitrifying bacteria isolated from the agricultural fields were found to be promising and use of these bacteria as bio fertilizers in soils would increase the level of available nitrogen contributing substantially to improve the implantation and development of crops in various regions.

KEYWORDS: ***Soil fertility, Ammonifying bacteria, Nitrifying bacteria***

***Corresponding author**

SUJATHA PEELA
Department of Biotechnology, Dr.B.R. Ambedkar University, Srikakulam, Etcherla, INDIA

1. INTRODUCTION

Plants are surrounded by the nitrogen (N) in our atmosphere. Every acre of the earth's surface is covered by thousands of pounds of this essential nutrient, but because atmospheric gaseous nitrogen is present as almost inert nitrogen (N_2) molecules, this nitrogen is not directly available to the plants that need it to grow, develop and reproduce. Despite nitrogen being one of the most abundant elements on earth, nitrogen deficiency is probably the most common nutritional problem affecting plants worldwide. Healthy plants often contain 3- 4% nitrogen in their aboveground tissues. These are much higher concentrations than those of any other nutrient except carbon, hydrogen and oxygen. Soil nitrogen exists in three general forms - organic nitrogen compounds, ammonium (NH_4^+) ions, and nitrate (NO_3^-) ions. 95-99% of the potentially available nitrogen in the soil is in organic forms, either in plant and animal residues, in the relatively stable soil organic matter or in living soil organisms, mainly microbes such as bacteria. As nitrogen is not directly available to plants, but some can be converted to available forms by microorganisms[1]. A very small amount of organic nitrogen may exist in soluble organic compounds, such as urea, that may be slightly available to plants .The majority of plant-available nitrogen is in the inorganic (sometimes called mineral nitrogen) NH_4^+ and NO_3^-forms. Ammonium ions bind to the soil's negatively-charged cation exchange complex (CEC) and behave much like other cations in the soil. Nitrate ions do not bind to the soil solids because they carry negative charges, but exist dissolved in the soil water, or precipitated as soluble salts under dry conditions. Some NH_4^+ and NO_3^- may also exist in the crystal structure of certain soil minerals, and may be quite available[2]. In most agricultural soils, ammonium (NH_4^+) from fertilizer is quickly converted to nitrate (NO_3^-) by the process of nitrification. Nitrification occurs in two steps: NH_4^+ is first converted to nitrite (NO_2^-), and the NO_2^- is then converted to NO_3^-. Both reactions are carried out by bacteria present in the soil. Nitrifying bacteria are chemoautotrophic, in that they produce energy by chemical oxidation of NH_4^+ or NO_2^- and utilize CO_2 as a source of C[2]. Different groups of bacteria are responsible for the two steps involved in nitrification. First step of nitrification is carried out by ammonia oxidizing bacteria include species from five genera i.e. *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus* and *Nitrosovibrio,* which oxidize ammonia into nitrite. Second step of nitrification is carried out by nitrite oxidizing bacteria i.e. *Nitrobacter, Nitrosococcus*, *Nitrospina* and *Nitrospira*, which oxidize nitrite into nitrate[3].Some microorganisms can utilize atmospheric N_2 to manufacture nitrogenous compounds for use in their own cells. This process, called biological nitrogen fixation, requires a great deal of energy; therefore, free-living organisms that perform the reaction, such as *Azotobacter* and *Rhizobia* [4, 5]. Microbes in the soil cause the breakdown and decay of dead organisms, a process that in turn adds more nutrients to the soil. Soil bacteria play pivotal roles in various biogeochemical cycles (BGC) [6, 7] and are responsible for the cycling of organic compounds like carbon, nitrogen, phosphorus, and sulphur. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition [8] plant health [9] soil structure[6,] and soil fertility, decomposition of organic matter and toxin removal. In addition, microorganisms play key roles in suppressing soil borne plant diseases, in promoting plant growth, and in changes in vegetation[10]. Nitrification in agricultural soils is important because it affects the distributions of the different forms of inorganic nitrogen in the soil and there with nitrogen availability; the nitrate that is formed is both more readily available to plants and more easily leached from soil than ammonium. In addition, nitrification is a soil-acidifying process[7]. Because there are only a few species of nitrifying bacteria, nitrification is much more sensitive to environmental conditions .All of the nitrifiers are obligate aerobes, which means that they require atmospheric O_2 so nitrification is especially sensitive to soil moisture content and does not occur in waterlogged soils. The rate of nitrification increases with soil temperature up to about 35°C (95°F); below 5°C (40°F) very little NO_3^- is formed. Soil pH is also important. Below a pH of 6.0, nitrification is inhibited by acidity, and the process virtually ceases at a pH of 4.5 to 5.0. Under alkaline conditions, production of NO_3^- is markedly enhanced. The optimum pH is normally between 7.0 and 8.0, but NO_3^- may be formed at a pH of 9.0 or even higher[11, 12]. In the present study isolation and characterization of ammonifying and nitrifying bacteria (Qualitative estimation), Determining the amount of ammonia, nitrite and nitrate in the soil samples and Estimation of organic nitrogen (i.e. the ability of bacteria to convert inorganic nitrogen to organic form) (Quantitative estimation) and evaluation of the potential of Ammonifying and nitrifying bacteria as fertilizer were undertaken.

2. MATERIALS AND METHODS

2.1 Collection of sample

Surface (0-15cm) soil samples were collected randomly from different regions. All samples were kept in plastic bags and transported to the laboratory and stored at 4ºC prior to be analyzed. These samples were air-dried and ground to pass through 2mm sieve before the chemical analyses. The samples were analyzed for pH, soil chemistry and texture.

2.2 Microbial count

Microbial population was estimated by plate count method[13, 14]. Ten grams soil was suspended in 90 ml sterile distilled water in an Erlenmeyer flask and mixed thoroughly for 30 minutes using a mechanical shaker at 110 rpm. Then 1ml an aliquot transferred with sterile pipettes to 9 ml sterile distilled water in test tube. This suspension was stir for 10second. A subsequent serial dilution was prepared as above to 10^{-7}. From each serial dilution, 0.2 ml of aliquot was transferred to sterile petri plate and over poured and dispersed swirling with agar media.

2.3. Isolation and characterization of Ammonifying and nitrifying bacteria (Qualitative estimation)

Bacteria representative of the predominant morphological types present on the plates was selected at random and purified on Yeast extract mannitol agar[15] and Ashby's Mannitol Agar media for isolation and identification of Ammonifying and nitrifying bacteria. The composition of the media was shown in the Table 2 & 3. Ammonifying and nitrifying bacteria are screened qualitatively by plating the bacteria on YEMA and Ashby's Mannitol Agar media, diluted in 1 l distilled water. The pH of the media was adjusted to 7.4±0.2 before autoclaving. Then the plates were incubated for 7 days at 28ºC. Growth of bacterial colonies was observed after 7 days of incubation. Later the isolates were identified following Bergey's manual for bacteriology [16].

2.4 Biochemical detection tests for ammonia, nitrite, and nitrate

Quantitative estimation to demonstrate the presence of ammonia, Nitrite and Nitrate were carried out in 30 ml boiling tubes containing 10ml of respective broths. Peptone Broth (Ammonia Forming Broth) for ammonia detection, Ammonium Sulfate Broth (Nitrite Forming Broth) for Nitrite detection, Nitrite Broth (Nitrate Forming broth) for nitrate detection is prepared. Add 0.1 gm of soil samples to the tubes and are labeled with the soil description. The tubes are incubated for a week at room temperature and tested for ammonia, nitrite and nitrate production. Sterile uninoculated broths served as control. After 7 days of incubation the test tubes were tested for ammonia by adding Nessler's reagent, nitrite by adding three drops of Trommsdorf's Solution and H_2SO_4 reagent, nitrate by adding three drops of concentrated H_2SO_4 alone. The intensity of color developed was detected using spectrophotometer. The results are compared with uninoculated broths (control) and the results are recorded

2.5 Quantitative estimation of organic nitrogen by neutralization method using Nessler's reagent

0.4 ml aliquots of working standard is pipette into series of test tubes and volume in each tube is made to 4ml with distilled water. For blank 4ml of distilled water is pipette out into a test tube. Add 1ml of $ZnSo_4$ solution and 0.4-0.5ml of 6N NaOH solution to maintain alkaline pH. To this mixture add 1 drop of EDTA to precipitate any calcium, magnesium or any other ions. Filter this mixture and add 2ml of Nessler's reagent, well mix well and measure the O.D at 420nm using Spectrophotometer. These standard O.D values are plotted on a graph. The reddish brown color of ammonical nitrogen present in the sample can be measured at the wavelength of 420 nm using spectrophotometer and the O.D values of test samples are plotted on the standard graph [17].

3 RESULTS AND DISCUSSION

The soil samples collected from 12 regions of Visakhapatnam were analyzed for the bacterial isolation. The isolated bacteria were identified using Bergey's Manual of Determinative Bacteriology. Most of the bacteria were found to be aerobic and few isolates are spore forming bacteria. Totally, 30 isolates obtained from 12 Soil samples collected randomly, and the samples were tested for nitrogen reducing ability of these 15 isolates are identified as Ammonifying and Nitrifying bacteria. Isolated bacteria from different soil samples were identified as species from eight genera i.e. *Bacillus, Nitrobacter, Proteus, Nitrosomanas, Klebsiella, Pseudomonas, Proteus, and Streptomyces.* Among these *Bacillus, Nitrobacter, Proteus, Nitrosomanas, Klebsiella , Pseudomonas* are the most predominant Ammonifying and nitrifying bacteria found in all of soils tested (S-1,3,4,6,8,8,9,11) followed by *Proteus, Streptomyces* were in soil samples S-2,5,10,12, respectively (Table-1). As shown in Table 4 among the 15 bacterial isolates *Pseudomonas, Bacillus, Proteus, Enterobacter, Klebiella*, were identified as ammonifying bacteria and *Azotobacter, Nitrobacter, Nitrococcus, Nitrosomonas*, were identified as nitrifying bacteria in the agricultural fields of Visakhapatnam. The Qualitative estimation of Ammonia, nitrite and nitrate were tabulates and results were shown in Table-5. Among these Ammonia levels were high in sample No1, 3, 5 &9 (Figure 1) whereas Nitrite levels were high in 1 &5 (Figure 2) while Nitrate levels were high in 1, 4 &5 (Figure 1). Among all the samples, sample no-1 is rich with Ammonia, nitrite and Nitrate. Figure 2 states that from the biochemical test results sample no.1.3, 4, 5, 6, 9, 10 showed the large amounts of ammonia, nitrates and nitrates in the soil when compared to other samples. Therefore, the soil samples at these fields are said to be fertile in nature, as it contained adequate amount of N. Ammonifying and Nitrifying bacteria isolated from these fields could serve as bacteria as bio fertilizers in soils would increase the level of available nitrogen contributing substantially to improve the implantation and development of crops in various regions. The quantitative estimation of ammonical nitrogen by neutralization method using Nessler's reagent has done and the results were presented in Table-6. As shown in the Table-6 among all the isolates *Nitrobacter* show highest concentration of organic ammonical nitrogen i.e. 21.8µg. As shown in Figure the graphical representation of the data indicates that most of the isolated bacteria have the ability to convert inorganic nitrogen to organic form of which bacterial isolates *Nitrosomonas, Nitrobacter, B.cereus, B.megaterium* are found to be more promising ammonifying and nitrifying bacteria. As shown in Figure 4 from the Quantitative estimations, the Bacterial isolates *Nitrobacter*, and *B.cereus* showed high ability in converting the inorganic nitrogen to organic form when compared to control. The present investigation was carried out to study the occurrence of ammonifying and nitrifying bacteria. The isolated microbes were identified, screened and characterized. Most of the bacteria were isolated from soil samples with pH values close to 7 and 8. This suggests that availability of organic form of N could be the result of organic acids released from bacterial metabolism, as reported in literature. The bacterial isolates. *Nitrosomonas,* *Nitrobacter, B.cereus B.megaterium* strain used in this study have the capacity to fix atmospheric nitrogen[18]. It is also known that N availability in soils is important in soils for the plants to grow and reproduce. Therefore the application of these bacteria could be considered as an appropriate substitute for chemical nitrogenous fertilizers in organic and sustainable agricultural systems[19]. Different soil nutrient status and vegetation type in the investigated sites resulted in the different bacterial population and bacterial type. The difference was caused by releasing organic and inorganic root exudates that can be used by surrounding organism. Jha et al.[1] and Setiadi[20, 21, 22] found that biological activity and composition of soil microbes are generally affected by many factors including physico-chemical properties. Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes.

Table 1
List of identified isolates from 12 different regions soil samples of Visakhapatnam

Soil Samples	Bacterial isolates
S-1	*Proteus vulgaris,Pseudomonas flourescens*
S-2	*Bacillus megateriym, Micrococcus bovis*
S-3	*Bacillus cereus,Proteus vulgaris*
S-4	*Proteus mirabilis ,Nitrobacter , Nitrococcus*
S-5	*Streptococcus species, Nitrosomonas ,B.subtilis*
S-6	*Pseudomonas aeruginosa*
S-7	*Streptococcus species,Proteus inconstans*
S-8	*Corynebacterium Xerosis, Neisseria mucosa,Klebsiella aerogens*
S-9	*Klebsiella pneumonia, Azotobaccter species*
S-10	*Nitrosomonas , Streptococcus species*
S-11	*Bacillus megaterium, Bacillus thermodenitrificans, Streptococcus species*
S-12	*Pseudomonas stutzeri Pseudomonas aeruginosa, Micrococcus bovis*

Table 2
Composition of YEMA (yeast extract mannitol agar) media

S.No	Components	gm/l
1.	Mannitol	10gm
2.	K_2HPO_4	0.5gm
3.	NaCl	0.1gm
4.	$MgSO_4.7H_2O$	0.2gm
5.	Yeast extract	1gm
6.	Congo red	2.5gm
7.	Agar	20gm
8.	Distilled water	1000ml

Table 3
Composition of Ashby's Mannitol Agar media

S.No	Components	gm/l
1.	Mannitol	20gm
2.	K_2HPO_4	0.2gm
3.	$MgSO_4.7H_2O$	0.2gm
4.	NaCl	0.2gm
5.	K_2SO_4	0.1gm
6.	$CaCO_3$	5gm
7.	Agar	15gm

Table4
List of Ammonifying and Nitrifying bacteria from soil samples of agricultural fields of Visakhapatnam

S.No	Ammonifying bacteria	Nitrifying bacteria
1.	*Proteus vulgaris*	*Nitrobacter*
2.	*Proteus mirabilis*	*Nitrococcus*
3.	*Proteus inconstans*	*Nitrosomonas*
4.	*Bacillus cereus*	*Azotobacter*
5.	*Bacillus megaterium*	
6.	*Bacillus subtilis*	
7.	*Pseudomonas fluorescens*	
8.	*Pseudomonas mallei*	
9.	*Klebsiella pneumonia*	
10.	*Streptomyces sp*	
11.	*Clostridium sp*	

Table 5
Biochemical detection of ammonia, nitrite, and nitrate from soil

Sample No	Ammonia	Nitrite	Nitrate
1.(DVD)	+++	+++	+++
2.(STP)	++	+	+
3.(PVD)	+++	+	++
4.(YLM)	++	+++	++
5.(AKP)	+++	++	+++
6.(KMNP)	++	+	--
7.(GANG)	+	++	+
8.(SML)	++	+	+
9.(GPT)	+++	++	++
10.(PDT)	+	+	+

The amount of ammonia present is interpreted as follows:

a. No color-no ammonia -
b. Pale Orange color-small amount of ammonia **+**
c. Orange-more ammonia **++**
d. Brown precipitate-large amount of ammonia **+++**

The amount of nitrite present is interpreted as follows:

a. Clear – Nitrite is absent
b. Pale blue+-- Small amount of Nitrite is present.
c. Blue++--More amount of Nitrite is present.
d. Blue/black+++--Large amount of Nitrite is present.

The amount of nitrate present is interpreted as follows:

a. Red ++ (nitrates present)
b. Orange + (nitrates and nitrites present)
c. Yellow - (nitrites present)

Table 6
Quantitative estimation of organic ammonical nitrogen shown by the bacterial isolates

S.No	Name of the bacteria	Concentration of organic ammonical nitrogen (µg)
1.	*Bacillus cereus*	17.4
2.	*Proteus vulgaris*	13.4
3.	*Nitro bacter*	21.8
4.	*Nitrosomonas*	17.4
5.	*Klebsiella pneumoniae*	11.4
6.	*Bacillus megaterium*	15.8
7.	*Pseudomonas stutzeri*	10.0
8.	*Streptococcus species*	9.0
9.	*Pseudomonas flourescens*	10.0
10.	*Micrococcus bovis*	12.0
11.	*Azotobacter species*	14.0
12.	*Proteus inconstans*	13.5

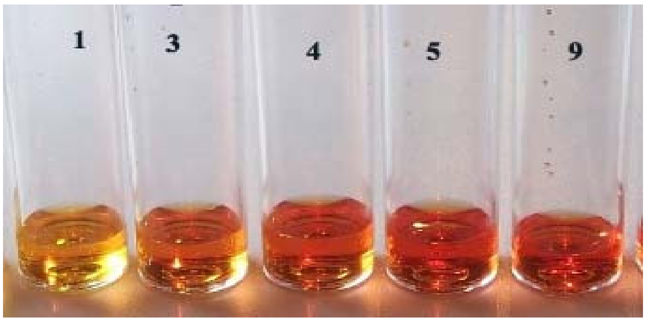


Figure 1
NITRATE AND AMMONIA DETECTION TEST

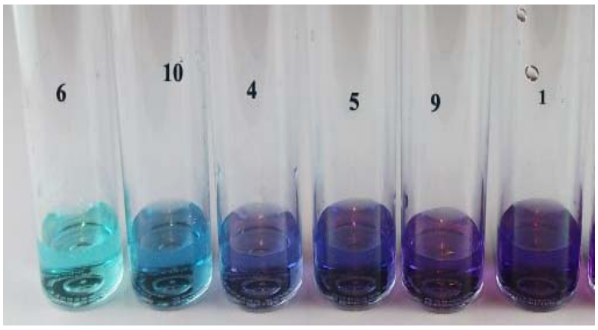


Figure 2
NITRITE DETECTION TEST

Figure 3
Graphical representation of concentration of organic ammonical nitrogen by neutralization method using Nessler's reagent.

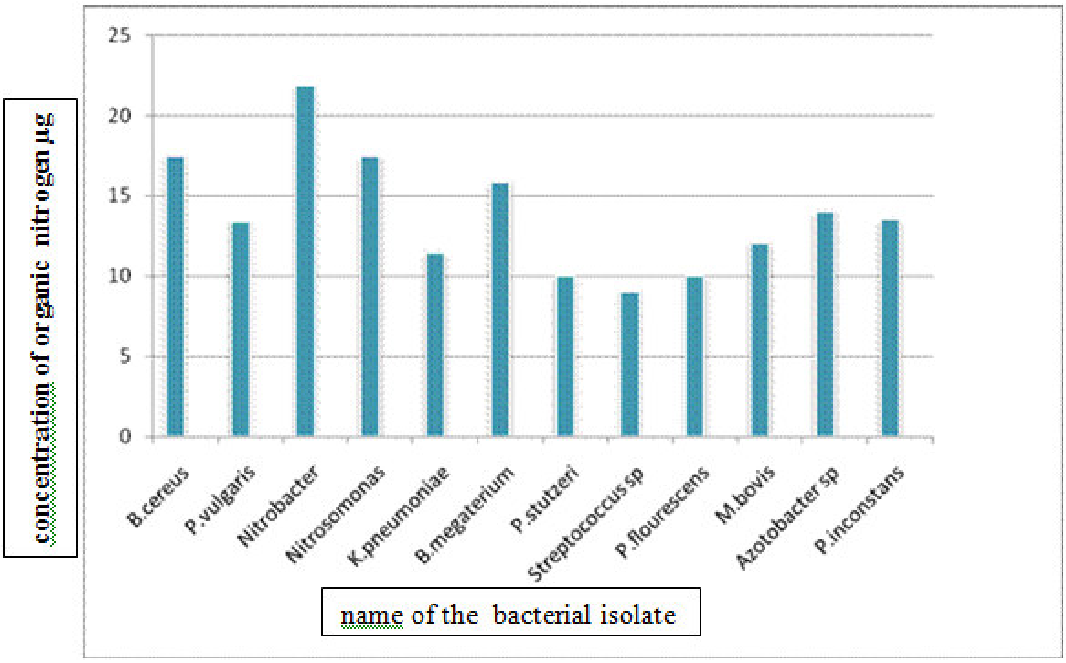
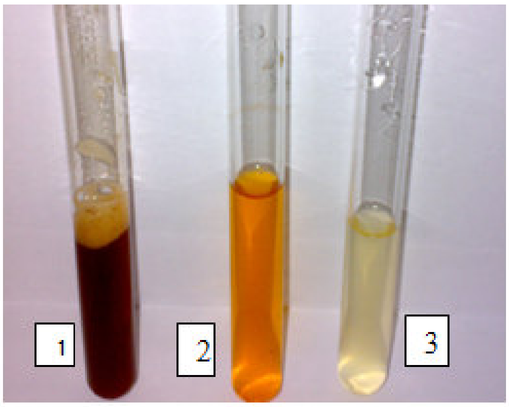


Figure 4
Quantitative estimations of the Bacterial isolates in converting the inorganic nitrogen to organic. (1-Nitrobacter; 2- B.cereus; 3- control).



4 CONCLUSION

The Ammonifying and Nitrifying bacteria isolated from the agricultural fields of Visakhapatnam were found to be promising and use of these bacteria as bio fertilizers in the soils would increase the level of available nitrogen contributing substantially to improve the implantation and physiological development of various crops in different regions.

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