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ISOLATION AND CHARACTERIZATION OF NITRIFYING BACTERIA FROM THE GUT OF EARTHWORM (*EISENIA FOETIDA*)

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ABSTRACT
Atmospheric nitrogen is thought to be a major source of nitrogen in soils. Isolation of nitrifying bacteria from the Earthworm gut (*Eisenia foetida*) by a microtiter system based on the most probable number (MPN) method has been discussed in this study, with an increased accuracy as compared to standard MPN tube technique. Our studies have shown that there is higher number of nitrifying bacteria associated with earthworm gut and their nitrifying ability was detected through qualitative analysis.

INTRODUCTION

Nitrogen gas comprises about 78% of the earth's atmosphere. Growing plants, animals and microbial populations need a continual source of nitrogen, as it is an essential component of the proteins that build cell material and plant tissue. Plants need at least 17 elements to grow. Among them carbon, oxygen and hydrogen are the building blocks where as nitrogen, potassium and phosphorus are referred to as nutrients. Nitrogen has traditionally been considered one of the most important plant nutrients (Sridhar, 2000). 95-99% of the potentially available nitrogen in the soil is in organic forms either in plant or animal residues, which is not directly available to plants and can be converted to available forms by microorganisms.

Earthworms play key role in soil biology by providing ideal conditions for the growth of microorganisms. They are considered to be farmer's best friends. Soil consumption for *Eisenia foetida*, an earthworm is estimated to be 16 mg soil/ individual/day. The nitrogen excretion rate of *E. foetida* has been estimated at 0.4 mg/g/day, which is very high relative to other earthworm species (Stafford and Edwards, 1985). Experiments have proven that crops grown in earthworm inhabitant soils had increased the yields from 25% over 300% than in earthworm free soils (Barley, 1961). Literature provides sufficient evidence of the presence of heterotrophs like bacteria, fungi, actinomycetes, etc., in the gut of soil earthworms (Kalsen *et al*., 1992; Fischer *et al*., 1995; Vinceslas – Akpa and Loquet, 1995; Karsten and Drake, 1995).

Nitrogen (N) is one of the nutrients essential to living organisms. Available nitrogen in the soil is the nutrient most strongly limiting the growth of the trees (Malkonen, 1990). It is generally accepted that nitrogen mineralization plays a decisive role in supplying nitrogen to plants. Inorganic forms of nitrogen are the available sources for plants. Agricultural crops assimilate large quantities of nitrates, so that the production of this plant nutrient in soil is of considerable importance. In addition to the variety of autotrophic microbes capable of nitrification there are heterotrophs known to be capable of nitrification, most of which appear to be soil organisms (Verstraete and Alexander, 1973, Pennington and Ellis, 1993). The significance of nitrification is that it converts the positive charge on the ammonia into the negative charge on the nitrite and nitrate ions (Shetty and Magu, 2000). This effectively polarizes the molecules, makes them soluble in water and can be taken up by plant roots and used for assimilation into organic compounds. A much more heterogeneous group of bacteria and fungi are involved in heterotrophic nitrification (Kuenen and Robertson, 1988). Focht and Verstraete, (1977) suggested that heterotrophic nitrifiers could utilize certain intermediates of nitrogen oxidation as growth factors or as biocidal factors to assist in their competition and survival.

MATERIALS AND METHODS

Collection of Earthworm gut homogenate

Earthworms were collected from S. V. Agricultural University, Tirupati region, washed with sterile tap water and subjected to starvation for 24hrs. After starvation earthworms were then disinfected with 70% ethanol and gut content was dissected out, weighed and homogenized (for 5 minutes in a vortex mixer) in sterile 0.85% NaCl solution for the isolation of gut bacteria (Modified method of Toyota and Kimura, 2000).

Isolation of bacteria from earthworm gut homogenate by dilution plate method

Gut homogenate was serially diluted and were plated on Nutrient Agar (NA) and incubated at 37ºC for 24 hr for the isolation of bacteria. Replicates were maintained for each dilution. Single isolated colonies were sub cultured for the isolation of pure culture (Cappucino and Sherman, 2008).

Primary screening of gut bacteria for nitrification

A total of ten bacterial strains were isolated from the gut of earthworm (*Eisenia foetida*). Primary screening was done individually for all the ten isolates and determined for the production of nitrites and nitrates (Cappucino and Sherman, 2008).

1) Determination of Nitrite production

Ammonium sulfate broth was inoculated with the gut isolates and were incubated for 3weeks. Trommsdorf's reagent and sulfuric acid were used to test the presence of Nitrites (Cappucino and Sherman, 2008).

2) Determination of Nitrate production

Nitrite broth was inoculated with the gut isolates and incubated for 3weeks. Diphenylamine reagent and sulfuric acids were used to test the presence of Nitrates (Cappucino and Sherman, 2008).

Secondary screening of gut bacteria for nitrification

Bacterial isolates which were positive in the primary screening were screened out for the best nitrifying bacteria by a microtechnique based on the Most Probable Number (MPN) method, developed for the enumeration of ammonia and nitrite oxidizing microorganisms by Rowe *et al*. (1977). A blue color reaction indicates that these end products have been formed and the well was scored as positive. The absence of a blue colour was scored as negative. The MPN values were calculated according to the table provided by De Man, (1975) and Parnow, (1972).

Identification of bacterial isolate

Further identification of the bacterial isolate was carried out based on the morphological, cultural and biochemical characteristics according to the guidelines of Bergey's Mannual of Systemic Bacteriology.

The bacterial isolate was identified as a strain of the genus *Bacillus* based on the morphological and biochemical characteristics. For further identification, the isolate was submitted to Vimta Labs, Hyderabad for 16S rDNA sequencing and phylogenetic analysis of the isolate.

Table 1: Ability of nitrification by the earthworm gut isolates based on MPN value

Sample	Incubation time(in weeks)	No.of positive wells in each dilution			MPN value
		*P_1 (32)**	*P_2 (64)**	*P_3 (128)**	
ALS 1	After 1 week	8	7	7	2.921
ALS 2	After 2 weeks	8	4	7	1.607
ALS 3	After 3 weeks	7	6	5	1.376
ALS 4	After 3 weeks	8	4	3	1.054

*Dilution code; **Dilution factor

Table 2: Morphological and biochemical tests for identification of bacterial isolate

Identification tests	Bacterial isolate
Colony morphology	
Configuration	Wrinkled, cream, round, concentric
Margins	Smooth
Surface	Butyraceous
Pigmentation	-
Turbidity	+
Opacity	Translucent
Gram's reaction	Positive
Cell shape	Rods
Size(μm)	3-5μm in length, width 1.0 -1.2 μm in width
	+
Spores	+
Motility	
Physiological tests	
Growth at temperature	-
5ºC	+
10ºC	+
30ºC	+
37ºC	+/W
40ºC	+
45ºC	-
50ºC	
Growth in NaCl (%)	w
2	w
5	+
7	-
10	
Growth at pH	W
4	+
5	+
6	+
7	+
8	+
Growth under anaerobic condition	
Biochemical testsIndole test	-
Methyl red test	+
Voges proskauer test	+
Citrate utilization test	+
H_2S production	+
Gelatin hydrolysis	+
Urea hydrolysis	+
Starch hydrolysis	+
LectinaseLipase (Tween 80 hydrolysis)	+
Catalase test	-
Oxidase test	+
Denitrification	+
Arginine dihydrolase	+
Phosphate solubilization	+
Chitinase	+
Casein hydrolysis	+
Degradation of Tyrosine	
Nutritional characteristics	+
Starch	+
Maltose	+
Glucose	+
Glycerol	-
suucinate	-
β-Alanine	-
L-histidine	-
L-Arginine	-
L-lucine	-
D-alanine	-

RESULTS AND DISCUSSION

A total of ten bacterial isolates were isolated from the gut of Earthworm. All the ten isolates were subjected to primary screening to determine their ability to nitrification. All the ten isolates were incubated in Ammonium sulphate and Nitrite broths. Out of the ten only four isolates had exhibited blue-black colour in Ammonium sulphate broth upon the addition of Tromsdorff's reagent and deep blue colour in Nitrite broth with the diphenyleamine reagents, indicating the positive reactions.

Four isolates which were positive in primary screening were further subjected to secondary screening to isolate the best nitrifier through microtitre plate method. As the four isolates were inoculated in Ammonium Calcium Carbonate medium and incubated for three weeks. Blue colouration upon the addition of diphenyleamine reagent indicated that the end products were formed and the wells were scored as positive. The MPN values were calculated according to the table provided by De Man, (1975) and Parnow, (1972). According to the MPN value achieved one of the best isolate among the four was found and labelled as ALS 1 (Table 1). Hence the ALS 1 strain isolated from the eight was selected for the morphological, physiological, Biochemical and molecular characterization.

Morphological studies revealed that the isolate ALS 1 was aerobic endospore forming, non pigmented and wrinkled with concentric colonies. The growing cells were Gram positive, motile with rod shape. The isolate grew well in nutrient broth at pH range of 5.7 to 8.0 and showed salt tolerance at Nacl concentration upto 10% (w/v). Bacterial growth was observed in the temperature ranging from 10ºC – 45ºC with an optimum growth around 37ºC.

The isolate was positive for the utilization of glucose, glycerol, maltose. ALS 1 was shown positive results for catalase, methyl red, Voges proskauer, Citrate utilization, Urease, Nitrate reduction, H_2S production casein hydrolysis, starch hydrolysis, Degradation of tyrosine, Lecithinase, Gelatin liquefaction, Arginine hydrolysis, Lipase and phoaphate solubilisation reactions. Negative towards oxidase, indole, utilization of arabinose, xylose, lactose and mannitol (Table 2).

Morphological, biochemical, physiological characteristics and also by 16S rDNA sequence analysis the ALS 1 isolate was confirmed as *Bacillus cereus*. 16S rDNA sequencing was done by Vimta labs, Hyderabad and the phylogenetic relationship revealed that 99.9%identity was with *Bacillus cereus*.

Based on the above characteristics *Bacillus cereus* was able to oxidize ammonia and nitrite resulting in the nitrate formation there by showing the ability to nitrification.

The finding of the present study reinforces the general concept that the gut and casts of earthworms tend to be much more microbiologically active than the surrounding soil. Enhancing the growth of these soil organisms can serve as a basis for the development of living soils by optimizing the potentials of the beneficial biotic populations. The most significant effect of earthworms was to increase the amounts of extractable NO_3^- in the upper layer of the soil. Amounts of extractable NH_4^+ were an order of magnitude lower than the amounts of extractable NO_3^- indicating the high nitrification potential of the soil.

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