International Journal of Life Sciences Vol. 4. No. 1. 2015. Pp. 10-18

Full Length Research Paper

Isolation and Characterization of Nitrifying Bacteria from the Rhizospere Soil of Bt and NBt Cotton Cultivating Fields

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Abstract

Rhizosperic soil samples were collected from the Bacillus thuringiensis (Bt) and non-Bt (NBt) cotton cultivated fields for 10-12 consecutive years as a mono crop from Khammam district of Telangana. These soil sample and were used as a source material for isolation and screening of nitrifying bacteria. A total of 27 isolates, sixteen (16) from NBt and eleven (11) from Bt were isolated and were evaluated for the production of nitrites and nitrates by the primary screening. It was identified that four isolates (two from Bt and two from NBt) were common in showing a positive reaction for the production of both nitrite and nitrate and selected for the secondary screening. Out of the micro titer method there were two best (one from Bt (BtRs8) and one from NBt (NBtRs2)) nitrifying bacteria were isolated according to the highest most probable number (MPN) values. Based on the morphological, biochemical and physiological tests performed for the isolate from Bt (BtRs8) and isolate from NBt (NBtRs2), were identified as Bacillus cereus and Bacillus subtilis. The Bt isolate (BtRs8) was further sent to the IMTECH, Chandigarh for molecular characterization and gene sequence which were submitted to NCBI.

Keywords: *Rhizospheric soil, Nitrifying bacteria, Isolation, Screening, Characterization*

Introduction

Sustainable agriculture can be understood as an ecosystem approach to agriculture. It depends on replenishing the soil while minimizing the use of non-renewable resources, such as natural gas which is used in converting atmospheric nitrogen into synthetic fertilizer, or mineral ores like phosphate. Possible sources of nitrogen that would be available indefinitely include recycling of crop waste and livestock, treated human manure, growing legume crops and forages such as peanuts or alfalfa that form symbioses with nitrogen-fixing bacteria.

Plants respond to nutrient deficiency by altering root morphology, recruiting the help of microorganisms and changing the chemical environment of the rhizosphere. The nutrients most limiting to plant growth are nitrogen and phosphorus. Even though 78% of the Earth's atmosphere is composed of nitrogen (N_2 gas), it is in a form that is only utilizable by nitrogen-fixing organisms. The growth of the plant depends on the inorganic forms of N (NO_3^-, NH_4^+) that can be added to soils and used by plants. The availability of nitrogen in most soils is low because of the leaching loss of soluble nitrate (NO_3^-) with infiltrating rain water, fixation of ammonium (NH_4^+) in clays and soil organic matter and bacterial denitrification in the form of nitrogen added to the soil.

Nitrification is an important step in the nitrogen cycle in soil the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates (Focht and Verstraete, 1977). The nitrogen is the key element of the biogeochemical cycle that describes the transformation of nitrogen and nitrogen containing compounds in nature (Sun *et al.,* 2007; Sarkar *et al.,* 2009).

Methodology

Soil sampling

Rhizosperic soil samples were collected from Bt and NBt cotton growing areas of Khammam district of Telangana. From each village 3 transects across each plot were taken and the soil samples were collected at different points comprised of 10 core samples drawn from 1-10 inches deep and 3 inches wide bore made around rhizosphere of 10 randomly selected Bt cotton plant. These samples were compared with the adjoining fields where NBt cotton was growing during the same period by the same procedure.

Collection of Bt and NBt rhizosphere soils

Ten rhizosphere samples were taken along with three transects across each plot and mixed to make a representative sample. The rhizospheric soil samples were collected by shaking the roots vigorously to separate the loosely bound bulk soil. The soil samples at pre-vegetation and post harvest stage were collected from 0-15 cm depth using a 5 cm diameter soil corer (Amith Kishore Singh *et al.,* 2013). Soil samples were collected from the roots of Bt and NBt plants and was sieved using 2 mm mesh size (aggregates larger than 2 mm were broken and soil was removed as efficiently as possible from root surface so that only stones and large roots were discarded). The soil from two treatments were mixed thoroughly in separate containers, from which 100 gm of soil was collected and stored at 4°C for further study.

Isolation of nitrifying bacteria from the soil samples

Soil samples were serially diluted and plated on Nutrient Agar (NA) and incubated at 37°C for 24 hrs for 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). A total of twenty seven bacterial strains were isolated as pure cultures. Eleven from the Bt and sixteen strains from Non Bt soil samples.

Primary screening of the isolates for the ability of nitrification

Primary screening was done individually for all the twenty seven isolates and tested for the production of nitrites and nitrates. This production was measured according to the method of (Cappucino and Sherman, 2008).

Determination of nitrite production

Ammonium sulfate broth was inoculated with the twenty seven isolates separately and incubated for 3 weeks at 37°C. Trommsdorfs reagent and sulfuric acids were used to test the presence of Nitrites in the broth, at weekly intervals.

Determination of nitrate production

Nitrite broth was inoculated with the twenty seven isolates separately and incubated for 3 weeks at 37°C. Diphenylamine reagent and sulfuric acid were used to test for the presence of Nitrates in the broth at weekly intervals.

Secondary screening of the isolates for the ability of nitrification

Secondary screening was performed on the positive isolates to obtain from the primary screening to identify the ability of nitrification by a micro titre plate technique based on the Most Probable Number (MPN) method, developed for the enumeration of ammonia and nitrite oxidizing microorganisms as described by (Rowe *et al.,* 1977). The MPN values were calculated according to the table provided by de Man (1975) and Parnow (1972).

A aliquot of 0.05 ml Ammonium Calcium Carbonate medium was placed into each of the 8 by 12 wells of sterile microplate as described by (Alexander and Clark, 1965). The suspension of 0.5 ml of bacterial isolates was inoculated into the first eight wells and were serially diluted to other wells and rotated rapidly. The plates were covered with polypropylene tape and incubated for 3 weeks. Replicates were maintained for all isolates and samples were observed at weekly intervals.
A blue colour reaction, by the addition of Diphenylamine reagent (0.2 g in 100 ml of Conc. H_2SO_4) indicated the end products (nitrite and/ nitrates) which were formed and such well was scored as positive. The absence of a blue color was scored as negative. The MPN values were calculated according to the table provided by de Man (1975) and Parnow (1972).

Identification and molecular characterization of bacterial isolates NBtRs2 and BtRs8

Identification of bacterial isolate

Morphological, cultural and biochemical characterization of the selected bacterial isolates (NBtRs2 and BtRs8) were carried out according to the guidelines of Bergey's Manual of Systemic Bacteriology (Volume II) 2005 and Manual of Medical Microbiology (Mackie and Mac Cartney, 1996; 9th Edition)

Morphological characterization

Morphological characterization of the selected bacterial isolates (NBtRs2, BtRs8) was done by inoculating on NA and incubating for 24-48 hrs at 37°C. Colony morphology was observed and isolated colonies were selected for staining techniques.

Results

Isolation of bacteria from Bt and NBt cotton rhizospheric soils

The representative soil samples for the isolation of nitrifying bacteria from Bt and NBt fields were collected at the growth stage -3 i.e. 90 days crop. The nitrifying bacteria were isolated by serial dilution plate method by using nutrient agar maintaining triplicates and incubate at 37°C for 24 hrs for both Bt and NBt isolates. A total of sixteen (16) distinctly variable colonies in NBt and eleven (11) in Bt based on their morphology were sub cultured and preserved for further studies.

Primary screening of Bt and NBt isolates for the ability of nitrification

All the twenty seven (27) selected isolates were evaluated for nitrifying property by primary screening from Bt and NBt cotton rhizospheric soil and tested for the production of nitrites and nitrates.

Determination of Nitrite production

After 3 weeks of incubation, inoculated broth was tested for the presence of nitrite using Trommsdorf's reagent and sulfuric acid. Among the eleven (11) Bt isolates four (4) had shown positive reaction. Among the sixteen (16) NBt isolates seven (7) had shown positive results with the appearance of blue-black colour indicating the presence of nitrite in the medium.

Fig. 1. Nitrite positives in Bt and NBt isolates

Determination of Nitrate production

After 3 weeks of incubation, the inoculated broth culture was tested for the presence of nitrate using Diphenylamine reagent and sulfuric acid. From the total eleven (11) isolates tested (Four from Bt and Seven from NBt), six (6) isolates (Two from Bt and Four from NBt) had shown positive reaction with the appearance of deep blue colour indicating the presence of nitrate in the medium. Among from the six (6) isolates four (4) isolates were common in showing a positive reaction for the production of both nitrite and nitrate hence selected for the secondary screening.

Fig. 2 Nitrate positives in Bt and NBt isolates

Secondary screening of the isolates for the ability of nitrification

The four isolates selected through the primary screening were subjected to secondary screening to determine the best isolates based on their ability to nitrification through microtiter plate method. Ammonium–Calcium Carbonate medium was placed into each of the 8 by 12 wells of a sterile microplate. Aliquots of the four test isolates were pipetted into each of the first eight wells. Serial dilutions were then performed by using sterile micropipettes calibrated to deliver Aliquots of the four test isolates 0.05ml and were rotated rapidly. Then the dilutions were further moved to the next eight wells where they were again rapidly rotated. This process was continued until serial dilutions have been carried out across the plate. The result obtained was 12 twofold serial dilutions, with eight replicates at each dilution.

After inoculation and the performance of serial dilutions, the plates were covered with polypropylene tape and incubated for 3 weeks. Three replicates were maintained in order to note the weekly reports. This is because conflicting reports existed concerning the optimum incubation time for ammonia and nitrite oxidizing microorganisms (Curtis, *et al*., 1975 and Matulewich, *et al*., 1975).

At the end of the incubation period each plate was scored by adding an indicator (0.2 gm of diphenylamine in 100 ml of concentrated H_2SO_4) to test for the presence of nitrate and/or nitrite at room temperature (Morgan, 1930).

A blue color reaction indicated that these end products had been formed and the well was scored as positive. All the four isolates tested had shown a positive result. The absence of a blue color was scored as negative. The MPN values were calculated according to the table provided by de Man (1975) and Parnow (1972) where the table represents MPN values and standard errors, for a two fold dilution series with eight tubes per dilution.

The codes P_1, P_2, P_3 represents the number of positive wells in three successive dilutions, where P_1 corresponded to the highest dilution at which all wells gave positive readings or to the dilution showing the highest number of positive wells and results were mentioned in Table .

Table 1. Ability of nitrification by the four isolates 2 from Bt and 2 from NBt organisms labeled as BtRs7, BtRs8 and NBtRs2, NBtRs4

Sample	Incubation time (in weeks)	No. of positive wells in each dilution			MPN value	MPN value in the original inoculum
		*P_1 (32)**	*P_2 (64)**	*P_3 (128)**		
Isolate-1 BtRs7	After 3 weeks	8	6	4	1.565	2003.2
Isolate-2 BtRs8	After 3 weeks	8	7	4	1.855	2374.4
Isolate- 3 NBtRs2	After 3 weeks	8	7	6	2.465	3155.2
Isolate- 4 NBtRs4	After 3 weeks	8	6	6	1.993	2551.04

**Dilution code* ***Dilution factor*

Bt rhizospheric soil BtRs8 was positive with in 3 weeks of incubation period, indicating the results in P_1, P_2, P_3 dilutions of positive wells 8, 7, 4 respectively and the value obtained was 1.855 according to the MPN table. NBt rhizospheric soil NBtRs2 was positive in the 3 weeks of incubation period, indicating the results in P_1, P_2, P_3 dilutions in the positive wells 8, 7, 6 respectively and the value obtained was 2.465.

The MPN value was then multiplied by the dilution factor for P_2, 64 hence a value of 118.72 for BtRs8 and 157.76 for NBtRs2 were obtained. To calculate the MPN in 1 ml of the original inoculums, this number was multiplied by 20 (i.e., 20 x 118.72 = 2374.4 for isolate 2 (BtRs8), 20x 157.76=3155.2 isolate 3(NBtRs2)). Hence, the MPN for 1 ml of the original inoculums were 2374.4 and 3155.2 (for isolate 2 and isolate 3). Among the tested isolates BtRs8 and NBtRs2 had exhibited the highest positive values.

Depending on the results obtained from primary and secondary screening from BtRs8 environment and NBtRs2 were found to have good nitrifying property hence these were further characterized for their taxonomic position. The isolates were labeled as BtRs8, and NBtRs2 to facilitate the description of the organisms in further studies.

Identification of bacterial isolates

Morphological, Cultural and Biochemical characterization of BtRs8 and NBtRs2 was carried out according to the guidelines of Bergeys Manual of Systemic Bacteriology (Volume II) 2005 and Manual of Medical Microbiology (Mackie Mac Cartney, 1996).

Morphological studies of the two isolates

BtRs8 was found aerobic endospore forming, non pigmented colonies and wrinkled with concentric rings. The organism was also positive for growth under anaerobic conditions. 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 6% (w/v). Good growth was observed for BtRs8 at the temperature ranging from 10°C - 45°C with an optimum growth around 37°C. BtRs8 showed positive results for catalase, methylred, VogesProskauer, citrate utilization, urease production, nitrate reduction, H_2S production, casein hydrolysis, starch hydrolysis; degradation of tyrosine, production of lecithinase, gelatin liquifaction, arginine dihydrolysis, production of lipase, and chitinase and phosphate solubilisation reactions. BtRs8 was also positive for the utilization of sugars and similarly positive utilization of aminoacids like, glucose, mannitol, fructose, arabinose, maltose, succinate and starch. β-alanine, L-histidine, L-lucine and D-alanine. It was negative for oxidase, indole, and utilization of xylose, arabinose, lactose and mannitol

NBtRs2 was found aerobic, endospore forming, non pigmented, having wrinkled columns with concentric rings. The growing cells were Gram positive, motile with rod shape. NBtRs2 showed positive results for oxidase production, citrate utilization, lipase, and urease production, starch hydrolysis, lecithinase production, arginine dihydrolysis, phosphate solubilization, chitinase production, denitrification casein hydrolysis and degradation of tyrosine. Negative towards indole production, catalase H_2S production, and gelatin liquifacation. The NBtRs2 was also positive for the utilization of sugars like starch, maltose, glucose, mannitol, fructose, arabinose, succinate and glycerol and aminoacids like β-alanine, L-histidine, L-lucine and D-alanine. The isolate grew well in nutrient broth at pH range of 7.0 to 9.0 and showed salt tolerance at NaCl concentration from 1-6 (w/v). Bacterial growth was observed in the temperature ranging from 30°C – 55°C with an optimum growth around 37°C. The NBtRs2 was sensitive to antibiotics like penicillin G, ampicillin, tetracycline, gentamycin, and showed resistance to streptomycin, chloramphenicol, erythromycin, tobramycin, rifampicin and polymyxin.

Table 2. Morphological/physiological and biochemical tests for identification of (BtRs8 and NBtRs2)

Identification tests		Bacterial isolate BtRs8	Bacterial isolate NBtRs2
Colony morphology			
Configuration		Round, Concentric, Cream, Wrinkled	Round, Concentric, Cream, Wrinkled
Margins		Smooth	Entire
Surface		Butyraceous	Butyraceous
Elevation		Slightly Raised	Slightly Raised
Pigmentation		-	-
Opacity		Translucent	Opaque
Gram's reaction		Positive	Positive
Cell shape		Rods	Rods
Size (µm)		3-5 µm in length, width 1.0 -1.2 µm	2-4 µm in length, width 1.0 -1.2 µm
Spores		+	+
Motility		+	+
Physiological tests			
Growth at temperature			
4°C		-	-
10°C		+	-
30°C		+	+
37°C		+	+
40°C		+	+
45°C		+	+
50°C		-	+
55°C		-	-
Growth in NaCl (%)			
	1	+	+
	2	+	+
	4	+	+
	6	+	+
	8	-	-
Growth at pH			
	5	+	-
	6	+	-
	7	+	+
	8	+	+
	9	+	+
Growth under aerobic condition		+	+
Biochemical tests			
Indole test		-	-
Methyl red test		+	-
Voges Proskauer test		+	+
Citrate utilization test		+	+
H_2S production		+	-
Gelatin hydrolysis		+	-
Urea hydrolysis		+	+
Starch hydrolysis		+	+
Lectinase		+	+
Lipase (Tween 80 hydrolysis)		+	+
Catalase test		+	-
Oxidase test		-	-
Denitrification		+	+
Arginine dihydrolase		+	-
Phosphate solubilization		+	-
Chitinase		+	-

Casein hydrolysis	+	+
Degradation of Tyrosine	+	+
Nutritional characteristics		
Starch	+	+
Maltose	+	+
Glucose	+	+
Mannitol	-	+
Sucrose	+	+
Fructose	+	+
Glycerol	+	-
Succinate	+	-
β-alanine	+	+
L-histidine	+	+
L-lucine	-	-
D-alanine	+	+
Antibiotic resistance		
Penicillin G	-	-
Ampicillin	+	-
Chloramphenicol	+	+
Erythromycin	+	+
Streptomycin	-	+
Tetracycline	-	-
Gentamycin	+	-
Tobramycin	+	+
Rifampicin	+	+
Polymyxin	+	+

Molecular characterization of the isolate

Based on the morphological, physiological and biochemical tests performed for BtRs8 and NBtRs2, these were identified as *Bacillus cereus* and *Bacillus subtilis* respectively. BtRs8 was further subjected with the assistance of IMTECH, Chandigarh for molecular characterization

PCR amplification of 16S rRNA for BtRs8

PCR amplification of 16S rRNA was carried out for BtRs8 using universal primers as described in the materials and methods. A product of 495 bp was obtained from the isolate. The optimum temperature for amplification of the gene was 55°C and maximum product was 30µg/ml. PCR product was purified and sequenced by using DNA sequencer as already described in the previous chapter. The BLAST search analysis of the 16S rRNA gene sequence of the isolate was done against the *Bacillus sps*. The BLAST results revealed 99.77 % homology with *Bacillus cereus*. Multiple sequence alignment of the isolate with closely related homologous species was done with CLUSTAL W. The 16S rRNA sequence of the isolate was genotypically similar to *Bacillus cereus*.

BLAST search results of the 16S rRNA sequence of *Bacillus cereus*

>Cul 2
GCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTA
TGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAA
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CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
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GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACC

Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
Bacillus cereus	Frankland and Frankland 1887	ATCC 14579(T)	AE016877	99.791	3/1437	2791	2791
Bacillus anthracis	Cohn 1872	Ames	AE016879	99.791	3/1437	2791	2791
Bacillus thuringiensis	Berliner 1915	ATCC 10792(T)	ACNF01000156	99.513	7/1437	2759	2759
Bacillus pseudomycoides	Nakamura 1998	DSM 12442(T)	ACMX01000133	99.513	7/1437	2759	2759
Bacillus mycoides	Flügge 1886	ATCC 6462(T)	AF155956	99.513	7/1437	2759	2759
Bacillus mycoides	Flügge 1886	DSM 2048(T)	ACMU01000002	99.304	10/1437	2736	2736
Bacillus weihenstephanensis	Lechner et al. 1998	WSBC 10204(T)	Z84578	99.210	11/1409	2664	2658
Bacillus weihenstephanensis	Lechner et al. 1998	KBAB4	CP000903	99.165	12/1437	2720	2720
Bacillus gaemokensis	Jung et al. 2010 (invalid)	BL3-6 KCTC 13318(T)	FJ418489	98.818	16/1351	2517	2518
Bacillus cytotoxicus	Lapidus et al. 2008 (invalid)	NVH 391-98(T)	CP000764	97.982	29/1437	2585	2585
Bacillus marisflavi	Yoon et al. 2003	TF-11(T)	AF483624	96.521	54/1429	2282	2238

BtRs8 gene sequence was deposited in NCBI

LOCUS 1443 bp rRNA linear BCT 05-SEP-2013
DEFINITION Bacillus cereus strain
ACCESSION Cul.
SOURCE Bacillus cereus
ORGANISM Bacillus cereus
Bacteria; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
REFERENCE 1 (bases 1 to 1443)
AUTHORS polakula,S.
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1443)
AUTHORS polakula,S.
TITLE Direct Submission
JOURNAL Submitted (05-SEP-2013) Department of Microbiology,
Sri Padmavathi Mahila Vishwavidyalayam, Tirupati.
Andhra Pradesh 517502, India
COMMENT Bankit Comment: TOTAL # OF SEQS:1.
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/organism="Bacillus cereus"
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/product="16S ribosomal RNA"
BASE COUNT 371 a 330 c 441 g 301 t

ORIGIN

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 121 actgggataa ctccgggaaa ccggggctaa taccggataa cattttgaac cgcatggttc
 181 gaaattgaaa ggcggcttcg gctgtcactt atggatggac ccgcgtcgca ttagctagtt
 241 ggtgaggtaa cggctcacca aggcaacgat gcgtagccga cctgagaggg tgatcggcca
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1441 acc
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Discussion

Screening of microorganisms for the conversion of ammonia to nitrite and nitrate is of importance and it has been intensively pursued for many years by scientists. In the present study the nitrifying bacteria were isolated from Bt and NBt cotton soil by Most Probable Number (MPN) method to understand the dynamics of the populations of nitrifiers in soils Belser and Schmidt, (1978). Various MPN methods were used for the enumeration of autotrophic nitrifiers Alexander, (1965) Schmidt and Belser, (1982), Alexander and clark, (1965). Maximum MPN counts of ammonia oxidixers were observed by Matulewich et al., (1975). Four isolates obtained through primary and secondary screening were showing the best nitrification activity, and among them one of the best nitrifier from Bt and one from NBt were characterized up to species level. The cultural and biochemical characteristics of the *Bacillus sps*, isolated in this work were confirmed as per the Bergey's Manual of Systematic Bacteriology Sneath et al., (1986). From Bt cotton soil the occurrence of *Bacillus cereus* in higher proportion than other species may be due to the fact that *Bacillus cereus* is more adaptable to a wider varying environment than other species. It was identified and confirmed as *Bacillus cereus* by 16S rRNA sequencing. *Bacillus subtillus* from NBt cotton soil.

References

Alexander, M and Clark, F.E., 1965. Nitrifying bacteria. In C.A. Black *et al.,* (ed), Methods of soil analysis, part 2. American society Agronomy, Madison, Wis. pp: 1477-1483.

Amit Kishore Singh, Major Singh and Suresh Kumar Dubey., 2013. Changes in Actinomycetes community structure under the influence of Bt transgenic brinjal crop in a tropical agroecosystem, BMC Microbiol, 10.1186/1471-2180-13-122.

Belser, L.W. and Schmidt, E.L. 1978. Diversity in the ammonia- oxidizing nitrifier population of a soil. *Appl. Environ. Microbiol.* 36: 584-588.

Cappucino, J.G. and Sherman, N., 2008. Nitrification. In Microbiology a Laboratory Manual. International student edition. 7th edition, Addison Wesely Publication. pp: 343-344.

Curtis, S. E., C. R. Anderson, J. Simon, A. H. Jensen, D. L. Day and K. W. Kelley. 1975. Effects of aerial ammonia, hydrogen sulfide and swine-house dust on rate of gain and respiratory-tract structure in swine. *J. Anim. Sci.* 41:735.

De Man, J.C., 1975. The probability of most probable numbers. *Euro. J. Appl. Microbiol.* 1: 67-78.

Focht, D.D. and Verstraete, W., 1977. Biochemical ecology of nitrification and denitrification. Adv. *Microb.Ecol*., 1:135-214.

Mackie and McCartney, 2008 Practical Medical Microbiology (Collee J G / fraser A G / Marmion B P and Simmons A) 14th edition. Elsevier private limited India. New York.

Matulewich, V.A., Strom, P.F. and Flnstein, M.S. 1975. Length of incubation of enumerating nitrifying bacteria present in various environments. *Appl. Microbiol.* 29: 265-268.

Morgan, M.F., 1930. A simple spot-plate test for nitrate nitrogen in soil and other extracts. *Science.* 71: 343-344.

Rowe, R., Todd, R. and Waide, J. 1977. Microtechnique for most probable number Analysis. *Appl.Environ.Microbiol.* No.3 p. 675-680.

Schmidt, E.L. and Belser, L.W. 1982. Nitrifying bacteria. In Methods of Soil Analysis. Part 2. Chemical and Microbiological properties (A.L. Page, R.H. Miller and D. R. Keeney, Eds.). Second edition, *Agronomy Monograph.* No.9, *American Society of Agronomy*, Madison, Wisconsin, USA, pp: 1027-1042.

Sneath, P.A., Mair N.S., Sharpe M.E, *et al.,* 1986. Bergey's Manual of Systematic Bacteriology, Vol. 2, Baltimore MD: Willam and Wilkins.

Sun, C.X., Chen, L.J., Wu, Z.J., *et al.,* 2007. Soil persistence of *Bacillus thuringiensis* (Bt) toxin from transgenic Bt cotton tissues and its effect on soil enzyme activities. *Biol. Fertil. Soils* 43, 617–620.