Chapter – III

MATERIALS AND METHODS
3.1. Physico – Chemical properties of Rhizospheric, Non-Rhizospheric soils and Vermicompost:

3.1.1 Collection of the Rhizospheric, Non-Rhizospheric soils and Vermicompost samples:

3.1.1a. Collection of Rhizosphere soil suspension:

Soil samples were collected from the roots of groundnut plants grown in S.V. Agricultural college, Tirupati, Chittoor Dt. Roots were carefully washed under tap water to remove large soil aggregates. The soil adhering to the roots after washing was suspended by shaking the roots in sterilized distilled water, and was used as the rhizosphere soil suspension (Ramakrishna and Sethunathan, 1982).

3.1.1b. Collection of Non – rhizosphere soil suspension:

Soil sample away from the plant root region was collected, air-dried and sieved through a 2 mm mesh. One gram of this was added to 100 ml of distilled water, and was used as the non-rhizosphere soil suspension (Ramakrishna and Sethunathan, 1982).

3.1.1c. Collection of Vermicompost samples:

Triplicate samples from Vermicompost pits were withdrawn at weekly intervals from S.V. Agricultural college, Tirupati Region, Chittoor Dt. and extraction was done by shaking the samples with 2 M KCl (10:1 extraction solution to the sample ratio) for 1 h in a wrist - action shaker (Keeney and Nelson, 1982).

All the three sample suspensions were then filtered through Whatmann No. 1 filter paper. Appropriate aliquots of the filtrate were used and triplicates were maintained for further studies.
3.1.2. Chemical characterization of Rhizospheric, Non-Rhizospheric soils and Vermicompost

pH of the samples was measured at 1:5 sample (W) : water (V) using ELICO digital pH meter with a Calomel glass electrode assembly. Organic carbon content in compost samples was oxidized with excess acidified potassium dichromate according to Walkley and Black method (Jackson, 1971) and unutilized dichromate was titrated against ferrous ammonium sulphate. The organic matter was calculated by multiplying the values with 1.72 (Jackson, 1971). Total nitrogen content in samples was digested with sulphuric acid and a pinch of digestion mixture (K$_2$SO$_4$ + CuSO$_4$ + Selenium powder) and distilled with NaOH into boric acid and titrated with H$_2$SO$_4$ in accordance with the method of Micro – Kjehldahl (Jackson, 1971). Content of inorganic ammonium nitrogen extracted, in 2 M KCl from samples was analyzed by Nesslerisation method. To suitable aliquots of the sample extracts, 0.5 ml of the Nessler's reagent was added and the volume was made to 7 ml (Jackson, 1971). The yellow colour developed was read at 495 nm in a UV-Vis spectrophotometer 117 (Systronics). The amount of ammonium was calculated by referring to a calibration curve prepared with standard solution of known ammonium concentrations. Content of nitrate nitrogen in sample extracts (2 M KCl) was measured by Brucine method (Ranney and Bartlet, 1972). One tenth of ml of brucine reagent (4 % brucine in ethanol) was added to suitable aliquots of the sample extracts followed by 2 ml of Conc. H$_2$SO$_4$. The solution was mixed by vortexing and placed in dark for 30 minutes to ensure full colour development, after which volume was made up to 10 ml with distilled water, and yellow colour was read at 410 nm in a UV-Vis spectrophotometer 117. The amount of nitrate was calculated by referring to a calibration curve prepared with the standard solution of known nitrate concentrations. The total phosphorous
(P%) and potassium (K%) were determined by volumetric Ammonium Phospho Molybdate method and Cobaltinitrite method respectively following the procedures of Gupta (1999). The available micronutrients were determined by method of Lindsay and Narvell (1978). The method consists of use of DTPA (Diethylene tri amine penta acetic acid) as an extractant which has been widely accepted for the simultaneous extraction of micronutrient cations viz., Zinc (Zn), Copper (Cu), Iron (Fe) and Manganese (Mn). The content of these cations in the extract was determined on an Atomic Absorption Spectrophotometer (AAS). The amount of available Boron (B) was determined by the hot water extraction method developed by Berger and Truog (1939).

3.2. Isolation, Identification and Molecular characterization of nitrifying bacteria from earthworm (Eisenia foetida) gut:

3.2.1. Collection of Earthworm gut homogenate:

Earthworms (Eisenia foetida) were collected from top ploughed horizons (0-20 cm) of vermicompost pits from S.V.Agricultural University, Tirupati region. Homogenates were prepared under aseptic conditions. Earthworms were washed with sterile distilled water and placed on a petridish moistened with filter paper and subjected to starvation for 24 hrs to exclude the microbes associated with soil and other organic content of earthworm gut. After starvation the earthworms were washed thrice with double distilled water, weighed and sedated with 70% ethanol and gut was dissected out. The content was weighed (1 gm wet weight) in sterile 0.85% NaCl solution and homogenized (for 5 minutes in a vortex mixer). Solid matter was separated after centrifugation at 10,000xg rpm for 15mins, supernatant was filtered (pore size 0.2 µm) and used for isolation of indigenous microflora of earthworm gut.
(Prasanna et al., 2010., Cappucino and Sherman, 2008 and Modified method of Toyoto and Kimura, 2000).

3.2.2. Isolation of bacteria from earthworm gut homogenate by dilution plate method:

Gut homogenate was 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 subcultured for the isolation of pure culture (Cappucino and Sherman, 2008).

3.2.3. Primary screening of the isolates for the ability of nitrification:

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

3.2.3a. Determination of Nitrite production:

Ammonium sulfate broth was inoculated with the ten isolates separately and were incubated for 3 weeks at 37°C. Trommsdorf’s reagent and sulfuric acids were used to test the presence of Nitrites in the broth, at frequent intervals (Cappucino and Sherman, 2008).

3.2.3b. Determination of Nitrate production:

Nitrite broth was inoculated with the ten isolates separately and incubated for 3 weeks at 37°C. Diphenylamine reagent and sulfuric acids were used to test the presence of Nitrates in the broth at frequent intervals (Cappucino and Sherman, 2008).
3.2.4. Secondary screening of the isolates for the ability of nitrification:

Secondary screening was done to the isolates which were positive in primary screening, to select the best nitrifier by a microtitre plate technique based on the Most Probable Number (MPN) method, developed for the enumeration of ammonia and nitrite oxidizing microorganisms by Rowe et al., (1977).

An 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). Aliquots of 0.05 ml of the bacterial isolates were 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. A blue color reaction, by the addition of Diphenylamine reagent (0.2 g in 100 ml of Conc. H$_2$SO$_4$) had indicated the end products i.e., nitrite and/or 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).

3.3. Identification of bacterial isolate:

Morphological, Cultural and Biochemical characterization of the selected bacterial isolate was carried out according to the guidelines of Bergey’s Manual of Systemic Bacteriology (Volume II) and Manual of Medical Microbiology (Mackie and MacCartney, 1989).

3.3.1 Morphological characterization:

Morphological characterization of the selected bacterial isolate was done by inoculating on Nutrient Agar (NA) and incubating for 24 – 48 hrs at 37°C. Colony morphology was observed and isolated colonies were selected for staining techniques.
3.3.1a. Gram staining:

Gram staining was performed for the selected isolate by Jensen’s modification of Gram’s Method.

3.3.1b. Motility of the organism:

Motility of the isolate was determined by hanging drop method (Mackie and MacCartney, 1989). A small drop of 24 hrs fresh culture, grown in nutrient broth was placed on a cover slip. Petroleum jelly was applied to the corners of the cover slip and the cavity slide was inverted over the cover slip. The slide was observed under the microscope (45x) for motility of the organism.

3.3.1c. Spore staining:

Culture of the isolate from 48 hrs old nutrient broth was smeared on a thin clean glass slide and placed on a boiling waterbath. Smear was flooded with aqueous solution of (5%) malachite green reagent and left for 1 min. then the smear was washed with cold water and treated with saffranine for 30 sec. Smear was rinsed with tap water, air dried and observed under microscope for the pink vegetative cell with green coloured spores.

3.3.2 Biochemical characterization:

Further characterization of the isolate was carried out by performing various biochemical tests adopting standard protocols (Mackie and MacCartney, 1989).

3.3.2a. Oxidase Test:

The method of Stainer et al., (1966) was employed to test oxidase-producing isolates, which catalyse the transport of electrons between electron donors in bacteria and a redox dye, tetra-methyl-P-phenylene diamine dihydrochloride. Strips of Whatmann No. 1 filter paper soaked in a freshly made 1% solution of the redox dye,
were spread on the petridish. Single isolated colony from the plate was picked up with a sterile small glass rod and spread on the moistened area of the filter paper. Appearance of intense purple within 5-10 seconds indicates a positive reaction. *Pseudomonas aeruginosa* and *Escherichia coli* were used as positive and negative controls respectively.

3.3.2b. Catalase Test:

The production of catalase enzyme, which mediates the release of oxygen from hydrogen peroxide, was tested by picking up single colony from 24 hrs culture plate and introducing into 1 ml of 3% of hydrogen peroxide. The appearance of effervescence / gas bubbles from the surface of the liquid indicates a positive test. *Staphylococcus aureus* and *Streptococcus pneumoniae* were used as positive and negative controls.

3.3.2c. Indole test:

The ability of the isolate to convert the amino acid Tryptophan to Indole was tested by adding 0.5 ml of Kovac’s reagent to 48 hrs culture broth. The production of red color in the alcohol layer indicates a positive indole reaction. *E. coli* and *Klebsiella pneumoniae* were used as positive and negative controls.

3.3.2d. Methyl Red test:

The ability of the isolate to ferment glucose and produce acid was determined using Glucose Phosphate Peptone (GPP) medium. Methyl red indicator of 2-3 drops was added to 48 hrs culture tubes and results were recorded immediately. Appearance of bright red color in the medium indicates a positive reaction. *E. coli* and *K. pneumoniae* were used as positive and negative controls.
3.3.2e. Voges – Proskauer test:

The ability of the isolate to produce Acetoin as the end product was detected by adding 5% solution of α – naphthol in ethanol and 0.2 ml of 40% KOH to 1ml of GPP broth cultures after incubating at 37°C for 48 hrs. The development of eosin pink color indicates a positive reaction. *K. pneumoniae* and *E. coli* and were used as positive and negative controls.

3.3.2f. Citrate utilization test:

The ability of the isolate to utilize citrate as the sole carbon and energy source for growth and an ammonium salt as the sole source of nitrogen, was determined by streaking the isolate on Simmon’s citrate agar slant (pH 6.8) and incubating for 24 hrs at 37°C. A positive test indicated change in the colour of the medium from green to intense deep purple hue with an appearance of a streak of growth. *K. pneumoniae* and *E. coli* were used as positive and negative controls.

3.3.2g. Urease test:

The production of urease enzyme by the isolate was tested by inoculating the culture on Christensen’s urease medium and was incubated at 37°C for 48 hrs. The appearance of purple pink color growth indicated a positive reaction. *Proteus vulgaris* and *E. coli* were used as positive and negative controls.

3.3.2h. Nitrate reduction test:

The production of the nitrate reductase enzyme was tested by inoculating the culture into nitrate broth and incubating for 48 hrs at 37°C. The freshly prepared 0.1ml of the test reagent with equally mixed sulphanilic acid and α - naphthylamine in acetic acid was added. Development of red colour within few minutes indicated the
presence of nitrite resulting from reduction of nitrate. *E. coli* and *P. aeruginosa* were used as positive and negative controls.

### 3.3.2i. H₂S production test:

Production of hydrogen sulphide was tested by inoculating 24 hrs culture of test organism into Nutrient broth having lead acetate strip inserted from the top of the test tube. The tubes were incubated at 37°C for 24-48 hrs. Blackening of the strip was taken as positive reaction. *Proteus vulgaris* and *E.coli* were used as positive and negative controls.

### 3.3.2j. Sugar fermentation tests:

The ability of the isolate to ferment different carbohydrates was tested. Different carbohydrates used in the test were monosaccharides like glucose, galactose, fructose and arabinose; disaccharides like lactose, sucrose and maltose and polysaccharides like soluble starch and glycogen. Peptone water was supplemented with 0.5% of each sugar, Andrade’s indicator (0.005%) was added to the medium and Durham tubes were inserted upside down into the medium tubes. Medium was sterilized at 10 lbs pressure for 10 minutes and were inoculated and incubated at 37°C for 24-48 hrs. Production of acid/gas was observed.

### 3.3.3. Enzyme Assays:

#### 3.3.3a. Hydrolysis of gelatin:

The hydrolysis of gelatin by the isolate was tested by inoculating the culture onto a Nutrient Gelatin Agar medium. The plates were incubated at 37°C for 3-5 days followed by flooding the plates with 1% tannic acid solution. Excess of solution was drained out after few minutes and the plates were observed immediately for the reduction of relative opacity around gelatin liquefying colonies.
3.3.3b. Hydrolysis of starch:

The ability of the bacterial isolate to hydrolyze starch was tested on Starch agar plates. The plates were streaked with the isolate and incubated at 37°C for 24 hrs. After incubation, the plates were flooded with 1% Gram’s iodine and observed for zone of clearance around amylase producers.

3.3.3c. Hydrolysis of casein:

Milk agar was prepared by adding 1% sterile Skimmed milk nutrient agar medium (autoclaved at 10 lbs for 10 min.) cooled upto 50°C. The plates prepared were inoculated with single isolated colony and incubated for 48 hrs at 37°C. Hydrolysis of milk protein was seen as a zone of clearing around colony.

3.3.3d. Screening for the Phosphate Solubilization:

A pin point inoculation of the isolate on solid formulations, was made under aseptic conditions for its ability to solubilize phosphate using Pikovskay’s medium, PKVS (Sundar Rao and Sinha 1963). The plates were incubated at 28°C ±1°C for 7 days and observed regularly for solubilization zone (Edi-Premono et al., 1996).

3.3.3e. Determination of antibiotic sensitivity profile:

The susceptibility of the isolate to various antibiotics was tested by disc diffusion method (Romanenko et al., 2003). Commercial discs of antibiotics procured from Hi-Media, Bombay; with the following concentrations per disc namely Penicillin G (10 IU/disc), Ampicillin (10μg), Chloramphenicol (30μg), Erythromycin (15μg), Streptomycin (10μg), Tetracycline (30μg), Gentamycin (10μg), Tobramycin (10μg), Rifampicin (15μg) and Polymyxin B (50IU/disc) were used. NA agar plates were swabbed with 36 hrs culture (~10^5 CFU/ml) of the isolate and 4-5 discs were placed per plate. The plates were incubated for 24 hrs at 37°C and the formation of
zones were measured. Appropriate antibiotic sensitive strains were included as controls (Palleroni, 1984).

3.3.4. Cultural characterization of the isolate:

The cultural characterization of the isolate was carried out by studying the growth at different temperatures, salt concentrations, pH and oxygen requirement.

3.3.4a Growth at different temperatures:

The ability of the isolate was determined to grow at different temperatures ranging from 4⁰C to 45⁰C with an intervals of 5⁰C. Tubes with nutrient broth were inoculated with 0.1 ml of over night cultures of the isolate and incubated at different temperatures for 48 hours. After incubation the tubes were observed for visible turbidity. The optimum temperature for the growth of the isolate was recorded.

3.3.4b Growth at different salt concentrations:

The ability of the isolate to grow at different salt concentrations between 1-10 % was studied. Nutrient broth tubes with respective salt concentrations were prepared and inoculated with 0.1 ml overnight culture and incubated at 37⁰C for 48 hrs and observed for visible turbidity. The ability of organism to grow under different salt concentrations was recorded.

3.3.4c Growth at different pH:

The ability of the isolate to grow at pH ranging from 4.5 - 8.5 was tested in nutrient broth. The isolate was inoculated and incubated at 37⁰C for 48 hrs and observed for the growth at different pH ranges and optimum pH for growth of the isolate was recorded.
3.3.4d Oxygen requirement:

Oxygen requirement for the isolate was determined by stabbing the culture in nutrient agar tubes overlaid with glycerol and incubated at 37°C for 48 hrs. Growth and location of growth in the tubes were noted and the isolate was characterized as aerobe/anaerobe/or facultative form.

3.3.4e Arginine dihydrolase Test:

The production of ammonia from arginine was catalyzed by arginine dihydrolase enzyme. This test was performed by growing the isolate for 48 hrs at 37°C, in nutrient broth supplemented with 1% L-arginine hydrochloride and 0.002% phenol red.

3.3.4f. Lecithinase test:

This test was performed by using the egg yolk – nutrient agar plates inoculated with the isolate and incubating at 37°C for 24 hrs. The Lecithinase producing colonies will be surrounded by zone of opalescence.

3.3.4g. Denitrification:

Denitrification ability of the isolate was tested by inoculating 0.5 ml of overnight culture of the test organism into 5 ml nitrate broth with a Durham’s tube (Cappucino and Sharman, 2008). The tubes were incubated at 37°C for 48 hrs and tested for nitrate reduction as described earlier. To those tubes that did not develop red color, a pinch of zinc dust was added. Development of red colour indicated that nitrate was gradually reduced to nitrite state, ammonia or nitrogen gas, which is also evidenced by the presence of air bubble in the Durham’s tube.
3.3.4h. **Levan formation from sucrose:**

Nutrient agar supplemented with 5% sucrose (w/v) was sterilized and poured into plates, inoculated, incubated for 7 days and observed for the development of domed mucoid colonies (Dorthy and Collins, 1986).

3.3.4i. **Utilization of amino acids:**

Ammonium chloride was substituted with amino acids such as L-arginine to test the ability of the isolate to utilize them as nitrogen source (Mackie and McCartney, 1989). The tubes with minimal medium were incubated at 37°C for 24-48 hrs.

3.3.4j. **Utilization of Sugars:**

Utilization of different sugars by the isolate was tested by inoculating the minimal medium in which glucose was substituted with sucrose, maltose, xylose, starch, galactose and arabinose. The tubes were incubated at 37°C for 24-48 hrs.

3.4 **Molecular Characterization of the isolate:**

3.4.1 **Genomic DNA Extraction of the isolate:**

Genomic DNA extraction was done using the isolate, following the method described by Sambrook *et al.*, (1989). The isolate was grown in Luria broth for 24 hrs at 37°C. The cells were harvested by centrifugation at 10,000Xg rpm for 5 min. The pellet was suspended in STE buffer –I and centrifuged at 10,000Xg rpm for 10 min. The pellet was resuspended in STE buffer –II and 50 µl of 10% SDS. The cells were left at -80°C for 30 min. To the cell suspension 500 µl of phenol-chloroform was added and spinned for 10 min. The supernatant was collected and 100 µl of chloroform: isoamyl alcohol (1:1) was added. To the supernatant obtained by centrifuging at 10,000Xg rpm, 1/10<sup>th</sup> volume of sodium acetate and 2.5 volumes of
ice cold 100% ethanol were added and centrifuged for 10 min. at 10,000Xg rpm. The supernatant was removed and pellet was dried for 3 hrs. DNA was resuspended in 20 µl of distilled water.

3.4.2 Agarose gel electrophoresis:

Purity of the isolated DNA was checked by electrophoresis in 0.7% agarose gel. Agarose gel was prepared by dissolving 0.7% of agarose in IX Tris-borate buffer. 20 µl of ethidium bromide (10 mg/ml) was added to agarose gel and placed in the electrophoresis tank consisted of IX Tris-borate buffer. The DNA samples and 10 kb DNA marker were loaded into agarose wells and run for 30 min at 100 V. The gel was observed under UV transilluminator (Sadasivam and Manickyam, 1996).

3.4.3 DNA sequencing:

Phylogenetic analysis of 16S ribosomal gene is an important tool to differentiate *Bacillus* and other related genera from each other.

3.4.3a. Amplification of 16S ribosomal DNA:

To identify bacterial isolate of interest, the genomic DNA was extracted followed by amplification of 16S ribosomal DNA by PCR. The PCR product was purified and sequenced. The gene sequence obtained was blast searched to get homologous sequences followed by phylogenetic analysis of the isolate. The 16S rDNA of the isolate was sequenced with the assistance of BIOSERVE, Hyderabad.

3.4.3b. Primers for 16S rDNA PCR:

PCR primer pair designed using consensus degenerate sequence was used in the study, which was capable of amplifying a wide variety of bacterial taxa.
3.4.3c. PCR of 16S rDNA:

PCR parameters were standardized initially for amplifying 16S rDNA sequence of the isolate at 30 μg / ml concentration of genomic DNA in a total volume of 50 μl reaction mixture at a gradient temperature of 50 to 60⁰C. As positive control of PCR, a single colony of *Bacillus* sps was picked up from the plate and resuspended in 20 μl of water in PCR tube. The tubes were heated at 98⁰C for 5 min and the sample was used as template. In negative control reaction mixture the Taq polymerase was omitted. The reaction mixture concentrations and PCR programme used for amplification are given below:

3.4.3c. (i) PCR- Reagent per reaction (μl)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Buffer</td>
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</tr>
<tr>
<td>20 mM MgCl₂</td>
<td>4.0</td>
</tr>
<tr>
<td>20 mM dNTPs</td>
<td>0.25</td>
</tr>
<tr>
<td>5’ primer (10 μM) Forward</td>
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</tr>
<tr>
<td>3’ primer (10 μM)</td>
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</tr>
<tr>
<td>DNA template</td>
<td>1.0</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5</td>
</tr>
<tr>
<td>MQ H₂O</td>
<td>40.5</td>
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</tbody>
</table>

**Total** 50.00

PCR programme consisted of the following steps that were run for 40 cycles.

**Programme**

Initial denaturation : 94⁰C for 3 min.

Denaturation : 94⁰C for 1 min.
Annealing : 51°C for 1min.

Extension : 72°C for 1min.

Final extension : 72°C for 5min.

**Agarose gel electrophoresis:**

After initial standardization of PCR, the optimum temperature was used for further amplification with 100 µl of amplification reaction. The PCR product was visualized on 1.5 % (w/v) agarose preparative gel using standard DNA markers and run at 50V for 45 minutes. The gel was visualized under UV Transilluminator.

**3.4.3d Purification of PCR product:**

The excised agarose slice with DNA fragment gel in an eppendorff tube was weighed and 2.5 volume of sodium iodide was added. The tubes were incubated at 55°C for 5 min. 15 µl of silica solution was added and spun for 30 seconds. This was incubated at 55°C for 5 mins. Pellet was collected by centrifugation at 12,000Xg for 30 seconds and supernatant was discarded. Pellet was washed with wash buffer for 3 times and air dried for 15 minutes. 25 µl of TE buffer was added to elute DNA from the silica matrix. Eluted DNA was checked again using 1.5 % agarose gel electrophoresis and sequencing was attempted.

**3.4.3f. Sequencing of the 16S rDNA:**

Purified DNA product was adjusted to 100 mg/µl concentration in MQ water (pH 8) and sequencing was carried out using primers. Sequencing was carried out in a 313 OXL capillary DNA sequencer utilizing thermocycling reaction Big Dye termination version 3:1. The 16S rRNA gene was sequenced in both directions by primer walking using primers directed to the conserved regions within the gene.
3.4.4 Phylogenetic analysis:

The DNA sequences of the 16S rRNA gene from the isolate of interest were edited manually and blast searched individually to fish out sequences of homology. The sequences were aligned using the programme CLUSTAL W (Wisconsin, 1994). The aligned sequences were applied to genetic distance by using Neighbor-Joining method for phylogenetic inference. Phylogenetic tree was visualized using tree programme.

3.5 Physiological conditions, Carbon and Nitrogen sources influencing the growth of the isolate:

In order to study the different parameters, influencing the growth of the isolate, 24 hrs culture was inoculated into Nutrient broth and was used for growth experiments. All these experiments were in triplicates. The turbidity of the culture was measured at 600 nm.

3.5.1 Effect of temperature:

The ability of the isolates to grow at different temperatures ranging from 25°C to 50°C with intervals of 5°C, was determined. Tubes with nutrient broth were inoculated with 0.1 ml of over night cultures of the isolate and incubated at different temperatures for 48 hours. After incubation the tubes were observed for visible turbidity. The optimum temperature for the growth of the isolate was recorded.

3.5.2 Growth at different salt concentrations:

The ability of the isolate to grow at different salt concentrations between 2-10 % was studied. Nutrient broth tubes with respective salt concentrations were prepared and inoculated with 0.1 ml overnight culture and incubated at 37°C for 48 hrs and
observed for visible turbidity. The ability of organism to grow under different salt concentrations was recorded.

3.5.3 Growth at different pH:

The ability of the isolate to grow at pH ranging from 4.5 - 8.5 was tested in nutrient broth. The isolate was inoculated and incubated at 37°C for 48 hrs and observed for the growth at different pH ranges and optimum pH for growth of the isolate was recorded.

3.5.4 Effect of different Carbon sources:

Different carbon sources (1% w/v) like glucose, starch, sucrose, maltose, lactose, raffinose, D-mannitol, and arabinose were amended in 250 ml of Basal mineral salts medium to find out suitable carbon source for optimum growth of the organism and incubated at 37°C. The growth after 24 hrs was determined by the turbidity. Culture flask with out carbon source was the control.

3.5.5 Effect of different Nitrogen sources:

Different nitrogen sources (1% w/v) like L-Aspargine, L-Alanine, L-Histidine, L-Proline, L-Arginine, Soya bean meal, KN03, CaN03, Peptone and Yeast extract were amended in 250 ml of Basal mineral salts medium to find out suitable nitrogen source for optimum growth of the organism and incubated at 37°C. The growth after 24 hrs was determined. Culture flask with out nitrogen source was the control.

3.5.6 Effect of different C/N ratios:

The carbon and nitrogen sources in different ratios (0.5:0.5, 0.5:1, 1:1 % w/v) were amended in 250 ml of Basal mineral salts medium to find out suitable ratio of
carbon and nitrogen source for optimum growth of the organism and incubated at 37ºC±2 for 24 hrs. Culture flask with out carbon and nitrogen source was the control.

3.6. Selection of suitable medium for the growth of nitrifying Bacillus sps.:

Different media for both the ammonia oxidizers and nitrite oxidizers were tested for selecting a suitable and efficient medium.

3.6.1. Media for Ammonium oxidation:

3.6.1a. Medium suggested by IMTECH Chandigarh:

(NH₄)₂SO₄ - 235 mg
KH₂PO₄ - 200 mg
CaCl₂.2H₂O - 40 mg
MgSO₄.7H₂O - 40 mg
Dis.H₂O - 1 L

Following stock solution was prepared, 2ml of aliquots per 1 litre medium was added and adjusted to pH 7 with Na₂CO₃.

FeSO₄.7H₂O - 90 mg
Na EDTA.7H₂O - 50 mg
Phenol red - 50 mg
Dis.H₂O - 200 ml

3.6.1b. Modified Winogradsky’s Medium (Kannan, 2003)

(NH₄)₂SO₄ - 0.5 g
KH₂PO₄ - 1.0 g
FeSO₄.7H₂O - 0.03 g
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<tr>
<th>Ingredient</th>
<th>Quantity</th>
<th>Notes</th>
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<tbody>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
<td></td>
</tr>
<tr>
<td>CaCO₃</td>
<td>5.0 g</td>
<td></td>
</tr>
<tr>
<td>Dis.H₂O</td>
<td>1 L</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7</td>
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**3.6.1c. Stephenson’s medium (Kannan, 2003)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2.0 g</td>
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<tr>
<td>K₂HPO₄</td>
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</tr>
<tr>
<td>KH₂PO₄</td>
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<tr>
<td>FeSO₄·7H₂O</td>
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<tr>
<td>MnSO₄·7H₂O</td>
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</tr>
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<td>CaCl₂·2H₂O</td>
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**3.6.1d. Lewis and Pramer’s Medium (Lewis and Pramer, 1958)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2.5 g</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.35 g</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.7 g</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.1 g</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.5 g</td>
<td></td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>14.4 mg</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>18.4 mg</td>
<td></td>
</tr>
</tbody>
</table>
3.6.1. Ammonium Calcium Carbonate Medium (Subba Rao 1999)

(NH$_4$)$_2$SO$_4$ - 0.5 g
K$_2$HPO$_4$ - 1.0 g
FeSO$_4$·7H$_2$O - 0.03 g
NaCl - 0.03 g
MgSO$_4$·7H$_2$O - 0.3 g
CaCO$_3$ - 7.5 g
Dis.H$_2$O - 1 L
pH - 7

3.6.2. Media for the Nitrite Oxidation:

For the enrichment and/or growth of Nitrite oxidizer, the sources of ammonia were replaced with nitrite sources for the oxidation of nitrite (NH$_4$)$_2$SO$_4$ with NaNO$_2$ at 247 mg/l, 200 mg/l and 50 mg respectively in IMTECH medium, Modified Winogradsky’s medium and Ammonium Calcium Carbonate medium (Nitrite Calcium Carbonate medium).

Aliquots of sterilized media were inoculated with 1 ml of bacterial suspension and incubated at 37$^\circ$C in an Orbitol shaker at 120 rpm. Increase or decrease in nitrite nitrogen was recorded regularly by diazotization method to check the activity of ammonium oxidation or nitrite oxidation respectively (Barnes and Folkard, 1951).
3.7. Estimation of Nitrogen mineralization by nitrifying *Bacillus* sps. in Ammonium Calcium Carbonate (ACC) medium:

Nitrogen mineralization was detected by estimating the initial ammonium and oxidised forms, the nitrite and nitrate levels as per the following procedures.

3.7.1. Estimation of ammonium in ACC:

The ammonium ion (NH$_4^+$-N) in the ACC medium was analysed by Nesslerisation method (Jackson, 1971). To suitable aliquots of the medium, 0.5 ml of the Nessler's reagent was added and the volume was made upto 7 ml. The developed yellow colour was read at 495 nm in a UV-Vis spectrophotometer - 117 (Systronics). The amount of ammonium was calculated by referring to a calibration curve prepared with standard solution of known ammonium concentrations.

3.7.2 Estimation of nitrite in ACC:

Nitrite (NO$_2^-$-N) was estimated by diazotization following the method of Barnes and Folkard (1951). Suitable aliquots from the culture was pipetted into test tubes and 1 ml of 1 % Sulphanilamide in 1N HCl was added and shaken thoroughly. To the so formed coloured diazo compound 1 ml of 0.12 % N-(1-naphthyl) ethylene diamine dihydrochloride in distilled water was added. Absorbance of pink coloured solution was read at 520 nm in a UV-Vis spectrophotometer - 117 (Systronics). The amount of nitrite was calculated by referring to a calibration curve prepared with the standard solution of known nitrite concentrations.

3.7.3. Estimation of nitrate in Nitrite Calcium Carbonate (NCC) medium:

The nitrate (NO$_3^-$ -N) produced due to the activity of microorganisms in NCC medium was determined by the method of Ranney and Bartlett (1972). Three drops of brucine reagent (2 g brucine dissolved in 50 ml ethanol) were added to
suitable aliquots of the culture tubes followed by 2 ml of concentrated Sulphuric acid. The solution was mixed by vortexing and placed in the dark for 30 minutes to ensure full colour development, after which the volume was made upto 10 ml with distilled water and the developed yellow colour was read at 410 nm in a UV-Vis spectrophotometer - 117 (Systronics). The amount of nitrate was calculated by referring to a calibration curve prepared with the standard solution of known nitrate concentrations.

3.8. Factors effecting nitrification activity:

Different physiological factors like Carbon sources, nitrogen sources, C/N ratio, Temperature, pH, metal ions, inhibitors, chealating agents and pesticides on nitrification were observed.

3.8.1. Effect of carbon sources:

Different carbon sources (1% w/v) like glucose, sodium acetate, L-lysine, malate and citrate were amended in 250 ml of Basal mineral salts medium to find out suitable carbon source for the production of nitrite and nitrate and was determined. Culture flask with out carbon source was the control (Eric Allen Strauss, 2000).

3.8.2 Effect of different Nitrogen sources :

3.8.2a. Effect of Nitrogen sources for production of Nitrite:

The effect of various nitrogen sources (1% w/v) such as Tryptone, Beef extract, Ammonium sulphate, Urea, Soya bean meal, and Peptone were amended in 250 ml of Basal mineral salts medium to find out the suitable nitrogen source for the production of nitrite was determined. The Culture flask with out nitrogen source was the control (Karl-Paul Witzel and Hans Jirgen Overbeck. 1979).
3.8.2b. Effect of Nitrogen sources for production of Nitrate:

The effect of various nitrogen sources (1% w/v) such as Tryptone, Beef extract, Ammonium sulphate, Ammonium chloride, Urea, Soya bean meal, Peptone, Sodium nitrite, KN0₂, and CaN0₂, were amended in 250 ml of Basal mineral salt medium to find out the suitable nitrogen source for the production of nitrate was determined. The Culture flask with out nitrogen source was the control (Karl-Paul Witzel and Hans Jirgen Overbeck. 1979).

3.8.3. Effect of pH on nitrification activity:

The effect of pH on nitrification activity was determined by using (NH₄)₂SO₄ and NaNO₂ as substrates in the basal medium. The pH was adjusted using the following buffers (0.05M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 37°C for 24 hrs, and the activity was estimated. The basal medium without substrates was the control (Mulvaney, 1994).

3.8.4. Effect of temperature on nitrification activity:

The effect of temperature on nitrification activity was determined by using (NH₄)₂SO₄ and NaNO₂ as the substrate in the basal medium with incubation at the various temperatures from 30-60 °C. Media was incubated at respective temperatures for 24 hrs, and the activity was estimated. The basal medium without substrates was the control (Milton and Saidu, 2009).

3.8.5 Effect of metal ions on nitrification activity

The effects of metal ions (eg, Ca²⁺, Mg²⁺, Hg²⁺,Co⁺², Cd⁺², Fe⁺³, Cu⁺², and Zn⁺² [5mM]) were investigated by adding them to the basal medium having (NH₄)₂SO₄ and NaNO₂ as substrate and incubated at 37°C for 24 hrs, and the activity
was estimated. The basal medium without substrates was the control (Karl-Paul Witzel and Hans Jürgen Overbeck, 1979).

3.8.6. Effect of inhibitors and chelating agents on nitrification activity

The effect of various inhibitors (5 mM) such as Nitrapyrin, Dicyandiamide, Thiourea, Potassium cyanide, Sodium diethyl dithiocarbonate and L- Histidine (Subba Rao et al., 2006) in the basal medium having (NH$_4$)$_2$SO$_4$ and NaNO$_2$ as substrates was incubated at 37°C for 24 hrs, and the activity was estimated. The basal medium without inhibitors/Chelators was the control (Ian Fillery, 2007).

3.8.7 Effect of pesticides on nitrification activity

In order to determine the effect of pesticides on nitrification, a chlorinated organic insecticide Endosulfan, a contact fungicide, Dithane M-45 (Mancozeb) and Neem oil (Azadirachtin), a biological insecticide were selected in the present study. Stock solutions of pesticides were prepared by dissolving in distilled water (Martinez – Toledo et al., 1992).

50 ml of ACC and NCC media were dispensed into 100 ml conical flasks and sterilized respectively. After cooling, 2 ml of 24 hrs culture was inoculated into the media. Different concentrations of pesticides 10, 25 and 50 µg/ml were added to the flasks. The flask containing medium and inoculum, but without a pesticide served as control. All the flasks were incubated at 37°C in an orbital shaker at 120 rpm. Nitrite and nitrate were estimated daily for a period of two weeks.

3.9. Purification and Characterization of Nitrifying Enzymes:

The nitrifying enzymes (Ammonium Monooxygenase and Nitrite Oxidoreductase) were purified and characterized.
3.9.1. Partial purification and confirmation of AMO enzyme:

3.9.1a. Extraction of crude enzyme:

The organism was grown in 100 ml of Ammonium Calcium Carbonate broth in 250 ml of conical flask at 37°C, shaken at 200 rpm. This 100 ml of culture was grown to a turbidity of absorbance $A_{650} = 1$ and subsequently used as inoculum for minimal medium. The inoculated minimal medium was aerated while maintaining at 30°C. The culture was harvested at $A_{650} = 0.4$ by centrifugation at 5000 X g at 4°C for 30 mins and the pellet was resuspended in 40 ml of 10mM Tris HCl (pH 8.0). The cells were broken by sonication at 4°C for 4 min. The cell free culture filtrate was used as crude enzyme source (Moir et al., 1996).

3.9.1b. Estimation of protein content

The protein content was estimated by Bronsted and Lowry method (Sadasivam and Manickam, 1996). Protein concentration ranging from 0.2-1 mg i.e. 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard solution was taken into a series of test tubes and 10 µl of the above crude was taken in another tube. The volume was made up to 1 ml with distilled water and 5 ml of reagent C was added to all the tubes.

A tube with 1 ml water and 5 ml of reagent C served as blank. The tubes were incubated at 37°C for 10 minutes. Reagent D of 0.5 ml was added to all the tubes along with blank. The tubes were incubated at 37°C for 30 minutes. The developed blue colour was measured at 660 nm in the colorimeter. The amount of protein present in the test sample was calculated by the following formula:

$$\text{Protein in test sample} = \frac{\text{O.D of the test}}{\text{O.D of the standard}} \times \text{Conc. of standard}$$
3.9.1b. (i) Composition of reagents

**Reagent A:** 2 % Sodium carbonate in 0.1 N NaOH

**Reagent B:** 0.5 % Copper sulphate in 1% Sodium Potassium Tartarate

**Reagent C:** 50 ml of reagent A and 1 ml of reagent B was mixed prior to use

**Reagent D:** (Folin Ciocalteau) The commercial reagent was diluted with an equal volume of water on the day of use. This reagent consists of Sodium tungstate and Sodium molybdate in Phosphoric acid and Hydrochloric acid.

3.9.1b. (ii) Protein solution:

**Standard:** Stock of the standard solution was prepared by dissolving 100 mg of bovine serum albumin in 100 ml of distilled water (1 mg/ml).

3.9.1c. Ammonium sulphate precipitation

The crude was concentrated by precipitation with solid ammonium sulphate (80% W/V) and kept overnight at 4 °C. The resulting precipitate was collected by centrifugation at 10,000X g for 30 minutes at 4°C in a refrigerated centrifuge. The precipitate was dissolved in 20 mM Tris HCl buffer (pH 8.0) and dialysed (in dialysis bag, sigma 10 -12 KDa) overnight against the same buffer. During the course of dialysis, the buffer was frequently changed with the fresh buffer until no traces of ammonium were found in the buffer upon testing with Nessler’s reagent. The dialysed fraction was considered as ammonium sulphate precipitated fraction.
3.9.1d. Ion exchange separation

The ammonium sulphate precipitation fraction of 40 ml was applied to a DEAE Sepharose CL6B which had been previously equilibrated with the same buffer i.e., Tris HCl buffer. Elution was performed by the 0 to 250 M NaCl in 10 mM Tris HCl (pH 8.0) linear gradient method and fractions of 3 ml were collected at a flow rate of 15 ml / h. All the fractions were assayed for nitrification activity. The fractions were pooled showing nitrification activity under a peak.

3.9.1e. Gel filtration

The fractions collected from DEAE – Sepharose column were pooled, precipitated with (NH$_4$)$_2$SO$_4$ (80% W/V) and concentrated to 2-3 ml by dialysis against the 20 mM Tris HCl buffer (pH 8.0). The dialysed and concentrated fraction was subjected to gel filtration on Sephadex G-75 column. Five grams of Sephadex G-75 (Sigma, USA) was suspended in 50 ml distilled water and kept for swelling in overnight with intermittent stirring at shorter intervals to prevent formation of clumps. The swelling gel bead solution was poured into a column tube (2.5 X 50 cm) which was previously inserted with glass wool at the bottom. The gel beads were allowed to settle gently without trapping of air bubbles. In this fashion, the prepared column was pre-equilibrated with 10 mM Tris HCl buffer (pH 8.0).

The dialysed and concentrated fraction was loaded on Sephadex G-75 column pre-equilibrated with 10 mM Tris HCl buffer (pH 8.0). The loaded column was eluted with the same buffer at a flow rate of 15 ml/h and fractions of one milliliter were collected and measured at 280 nm in a UV-Visible Spectrophotometer for protein content. Proteins in the sample were resolved into peaks on the column. Aliquots from one milliliter fractions were used for finding activity of AMO (Barnes and Folkard, 1951) and protein content in accordance with methods as specified in section (5.2).
The specific activity of the purified enzyme was compared with that of the crude enzyme and purification fold was calculated.

3.9.1f. Molecular weight determination of the purified enzyme by SDS-Polyacrylamide gel electrophoresis (Laemmli, 1979)

(i) Preparation of solutions for SDS-Polyacrylamide gel electrophoresis

**Monomer solution**

Acrylamide 58.4 g  
Bis acrylamide 1.6 g  
Dissolved in 200 ml of distilled water (Stored at 4°C in dark)

**4X Running gel buffer**

Tris 36.3 g  
Dissolved in 200 ml of distilled water, adjusted to pH 8.8 with HCl and stored at 4°C.

**4X Stacking gel buffer**

Tris 3.0 g  
Dissolved in 50 ml of distilled water, adjusted to pH 6.8 with HCl and stored at 4°C.

**10% Ammonium per sulphate**

Ammonium per Sulphate 0.5 g  
Dissolved in 5 ml of distilled water.
**12% SDS**

SDS  12 g

Dissolved in 100 ml of distilled water and stored at room temperature.

**Sample buffer**

Tris  2.5 ml of 4X stacking gel buffer

SDS  4.0 ml of 10% SDS

Glycerol  2.0 ml

Mercaptoethanol  1 ml

Bromophenol blue  0.5% (w/v)

Made up to 10 ml with distilled water and stored at room temperature.

**Tank Buffer**

Tris  12 g

Glycine  57.6 g

SDS  40 ml of 10% SDS

Dissolved in 4.0 liters of distilled water.

**Staining solution**

**Formaldehyde fixing solution:**

40% Methanol  : 20 ml

37% Formaldehyde  : 25µl

Distilled water  : 30 ml
Sodium Thiosulphate

Dissolved 0.01 gms in 50 ml of distilled water

Thiosulphate Developer

Sodium Carbonate : 1.5 gms
Sodium Thiosulphate : 0.0002 gms
37% Formaldehyde : 25µl

Dissolved in 50 ml of distilled water

Drying Solution

Ethanol : 5ml
Glycerol : 2ml
Distilled water: 43ml

Silver nitrate

0.05 gms in 50 ml of distilled water

Citric Acid Solution

Dissolved 24.16 gms in 50 ml of distilled water.

(ii) Preparation of Gel (12% SDS- PAGE)

The vertical mini slab gel unit was assembled using 1.5 mm spacers. In a flask, 12 ml of acrylamide stock, 7.5 ml of 4X Running gel buffer; 0.3 ml of 10 % SDS; 0.3 ml of 10% Ammonium per sulphate; 10 ml of distilled water and 20 µl of TEMED were added. The solutions were mixed thoroughly and poured between the plates. The solution was overlaid with a thin water layer and was allowed to polymerize simultaneously. The stacking gel solution was prepared by adding 1.3 ml
of acrylamide stock; 2.5 ml of 4X stacking gel buffer; 6 ml of distilled water; 0.1 ml of 12% SDS; 0.1 ml of 10% Ammonium per sulfate and 5µl of TEMED. The solutions were mixed thoroughly and poured over the polymerized separating gel. The comb was inserted and care was taken to avoid trapping of any air bubbles below the teeth of the comb. The stacking gel was allowed to polymerize. After the gel polymerization, the comb was removed and the slabs were assembled in the electrophoresis unit. The upper and the lower tank of the unit were filled with tank buffer.

Protein samples were boiled for 3 min in 10 mM Tris-HCl buffer (pH 6.5) containing 1% SDS, 1% β-mercapto ethanol and 20% glycerol and loaded onto the wells and electrophoresis was carried out under constant supply of 35 mA current.

(iii) Staining of the SDS-PAGE gel

Gel was stained with silver nitrate solution.

(iv) Determination of molecular weight of AMO

SDS-PAGE is the most frequently applied method for determination of molecular weight of enzymes / proteins. The purified enzyme sample and protein standard markers were run side by side on polyacrylamide gel. The molecular weight of the enzyme was determined based on the relative mobilities of markers. Protein standard markers used were (Fermentos): β-galactosidase- 116.0 KDa (E.coli), BSA-66.2 KDa (bovine plasma), Ovalbumin- 45 KDa (chicken egg white), Lactate dehydrogenase- 35 KDa (porcine muscle), REase Bsp- 25KDa (E.coli), β-Lactoglobulin- 18.4 KDa (bovine milk) and Lysozyme- 14.4 KDa (chicken egg white).
3.9.2. Partial purification and confirmation of Nitrite Oxidoreductase (NOR) enzyme:

3.9.2a. Extraction of crude enzyme:

To purify the nitrite oxidizing enzyme the culture was grown in Nitrite Calcium Carbonate medium containing 5mM NaNO₂ and incubated at 30°C for two days. The cells were harvested by centrifugation at 8,000 X g for 20 mins at 4°C and washed with 10mM potassium phosphate buffer (KPB) at pH 7.5. The cells suspended in KPB were disrupted by ultrasonication at 4°C for 4 mins. A crude extract was obtained by centrifugation at 10,000 X g for 20 mins.

3.9.2b. Estimation of Protein content:

As above in the section 7.2

3.9.2c. Ammonium sulphate precipitation

As above in the section 7.3

3.9.2d. Ion exchange separation

A 40 ml of sample filtrate which was concentrated and dialysed against 10 mM KPB (pH 8.0) in a dialysis bag (Sigma 10-12 KDa) was applied to a DEAE Cellulose column (2.5 X 50 cm) which had been previously equilibrated with the same buffer. Elution was performed by the 0 M to 0.13 M NaCl linear gradient method and fractions of 3 ml were collected at a flow rate of 15 ml / h. The fractions showing nitrite oxidizing activity under a peak were pooled.

3.9.2e. Gel filtration

The fractions collected from DEAE – Cellulose column were pooled, precipitated with (NH₄)₂SO₄ (80% W/V) and concentrated to 2-3 ml by dialysis against the 10 mM KPB (pH 8.0). The dialysed and concentrated fraction was subjected to gel filtration on Sephadex G-75 column. Five grams of Sephadex G-75
(Sigma, USA) was suspended in 50 ml distilled water and kept overnight for swelling with intermittent stirring at shorter intervals to prevent formation of clumps. The swelling gel bead solution was poured into a column tube (2.5 X 50 cm) which was previously inserted with glass wool at the bottom. The gel beads were allowed to settle gently without trapping of air bubbles. In this fashion, the prepared column was pre–equilibrated with 10 mM KPB (pH 8.0).

The dialysed and concentrated fraction was loaded on Sephadex G-75 column pre-equilibrated with 10 mM KPB (pH 8.0). The loaded column was eluted with the same buffer at a flow rate of 15 ml/h and fractions of one milliliter were collected and measured at 280 nm in a UV-Visible Spectrophotometer for protein content. Proteins in the sample were resolved into peaks on the column. Aliquots from one milliliter fractions were used for finding activity of NOR (Ranney and Bartlett, 1972) and protein content in accordance with methods as specified in section 5.3. The specific activity of the purified enzyme was compared with that of the crude enzyme and purification fold was calculated.

3.9.2f. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) of protein fractions

Separation of protein based on the molecular weight was demonstrated by PAGE technique developed by Laemmli (1979). The reagents and the solutions required for SDS-PAGE were prepared as follows:

i) Acrylamide stock solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30.0 g</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>800 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Stock solution was prepared by taking acrylamide and bis-acrylamide in small quantity of water and was made up to 100 ml with distilled water.

ii) Separating gel buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>18.15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>75 ml</td>
</tr>
</tbody>
</table>

By dissolving Tris in 75 ml of distilled water, pH was adjusted to 8.8 with 0.1 M HCl and the volume was made up to 100 ml with distilled water.

iii) Stacking gel buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

Tris was dissolved in 20 ml of distilled water, pH was adjusted to 6.8 with 1 M HCl and the final volume was made up to 25 ml with distilled water.

iv) 10% Sodium Dodecyl Sulphate (SDS)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.0 ml</td>
</tr>
</tbody>
</table>

SDS was first dissolved in small quantity of water and the final volume was adjusted to 10 ml.

v) 10% Ammonium per sulphate (APS)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>
100 mg of APS was dissolved in 1 ml of distilled water and prepared freshly each time.

vi) Gel overlay solution (pH 8.8)

- Separating gel buffer: 25 ml
- 10 % SDS solution: 1.0 ml
- Distilled water: 74 ml

All the solutions were mixed and final volume was adjusted to 100 ml.

vii) Sample buffer

- Stacking gel buffer: 2.5 ml
- 10 % SDS solution: 4.0 ml
- 20 % Glycerol: 2.0 ml
- 0.02 % Bromophenol Blue: 2.0 mg
- 0.2 M β-Mercapto ethanol: 0.5 ml

All the above were mixed, pH was adjusted to 6.8 and the final volume was made to 10 ml. Aliquots of 0.5 ml quantity were stored at -20°C.

viii) Tank buffer

- Tris: 3.28 g
- Glycine: 14.41 g
- SDS: 1.0 g

Tris, glycine and SDS were dissolved in small volume of distilled water, the final volume was made up to 1000 ml with distilled water and pH was adjusted to 8.3.
viii) Water saturated n-butanol

n-Butanol 100 ml
Distilled water 10 ml

n-Butanol and distilled water were mixed. Water saturated n-butanol was layered over the gel to avoid further drying of gels.

The separating gel consisted of 10 % (W/V) acrylamide, N, N-methylene bis acrylamide (Sigma, USA) at concentrations such that the ratio of monomer to bis was 30:0.8, 0.375 M, Tris–HCl (pH 8.8) and 0.1 % SDS. It was chemically polymerized with 0.05 % (W/V) TEMED. The solution was cast into slabs and was overlaid with n-butanol to exclude contact with air. The stacking gel containing 4 % (W/V) acrylamide 0.12 M, Tris–HCl (pH 6.8), 0.1 % SDS, 0.05 % (W/V) ammonium per sulphate, 0.05 % (V/V) TEMED. Samples of 50-200 µg of protein were digested with an equal volume of sample buffer (0.0625 M Tris–HCl, pH 6.8); 10 % (v/v) glycerol; 5% β-Mercapto ethanol; 2 % SDS and 0.02 % bromophenol blue by heating in a boiling water bath for 3 minutes. After cooling, the samples along with protein markers were loaded into the slots. The samples were stacked and run at 120 V for about 6 hrs using 0.025 M Tris, 0.192 M glycine buffer (pH 8.3) containing 0.1 % SDS as electrode buffer. The protein bands separated on acrylamide gel were visualized by staining with Comassie Brilliant Blue R-250, 0.25 % in 40 % methanol and 7 % acetic acid, followed by destaining in a solution of methanol : acetic acid : water (40:5:55) (V/V).

3.9.2g. Determination of molecular weight of NOR

SDS-PAGE is the most frequently applied method for determination of molecular weight of enzymes / proteins. The purified NOR sample and protein
standard markers (GENEI) were run side by side on polyacrylamide gel. The molecular weight of the separated NOR was calculated on the basis of relative mobilities of the markers.

3.10. Effect of Nitrifying *Bacillus cereus* on growth of groundnut variety JL-24

Efficient nitrifying *Bacillus cereus* was selected to study the effect of nitrification on growth of groundnut variety JL-24, based on the ability to oxidize unavailable forms of ammonium ions into available forms in liquid media.

3.10.1. Inoculant preparation and seed inoculation:

100 ml of sterile ACC broth was inoculated with a loopful of 24 hrs culture of efficient nitrifying *Bacillus cereus* isolate. The flasks were incubated at 37°C for three days on rotary shaker. The bacterial count of the isolate ranged between 36-37 X 10^8 ml (Gaind and Gaur, 1991). Groundnut JL- 24 seeds were surface sterilized with acidified 0.01% HgCl_2 for 5 mins and after washed several times with sterilized water. Seed inoculation was done by soaking 4 days old liquid culture of *Bacillus* strain for 2 hrs, air dried and sown immediately (modified method of Sudhansu, 1998).

3.10.2. Experimental design:

The soil characters used in this study were presented in the table 1. The soil selected for the study is poor in available nitrogen content and it was steam sterilized at 100°C and distributed in pots (1Kg/ pot). For basic N, P and K fertilization (NH_4)_2SO_4 – 180 mg/Kg of soil, P- Superphosphate – 65.5 mg, K-KCl – 167 mg/Kg of soil were added to the soil respectively. The N – rates applied were 0, 1 and 2 times to the basic N- fertilization (0, 180 and 360 mg N Kg⁻¹), (modified method of Rock Chabot *et al.*, 1998).
Bacteria inoculated and combined with N P K along included the following treatments.

T0: Water (negative control)

T1: Nitrogen (positive control)

T2: NPK (Fertilizer control)

T3: NPK (N-0, P-65.5 mg, K-167 mg) + Bacterial inoculum

T4: NPK (N-180 mg, P-65.5 mg, K-167 mg) + Bacterial inoculum

T5: NPK (N-360 mg, P-65.5 mg, K-167 mg) + Bacterial inoculum

Thus the pot assays with 5 treatments with triplicates were conducted, average for the following variables were calculated after 120 days. Percentage of seed germination was recorded. Height and weight of the plants were measured. Seedling Vigor Index (SVI) (Agarwal, 1980) was calculated by the formula described by Abdul Baki and Anderson (1973).

\[
\text{Seedling Vigor Index} = \left( \frac{\text{Root length} + \text{Shoot length}}{\text{Seed Germination}} \right) \times 100
\]

3.11. Statistical analysis:

The effects of different factors on nitrification were statistically compared and the significant difference between values of each samples were performed using Duncan’s Multiple Range (DMR) test.