Chapter – V

DISCUSSION
Earthworms are important and much neglected components of ecosystem. By understanding the key role of earthworms in many biogeochemical cycles and in soil development, it is essential to understand the gut microbial diversity and their influences on soil and plant growth. The search for bioactive metabolites especially from bacteria requires screening of a large number of isolates. The physico-chemical factors in the earthworm gut gives an evidence that the gut environment has certain beneficial bacterial taxonomic groups (Schonholzer et al., 2002; Furlong et al., 2002; Egert et al., 2004). Earthworms have a significant influence on N dynamics and contribute for change of N through time. Hence, the present study had focused upon the isolation of nitrifying bacteria from the gut of earthworm (*Eisenia foetida*) and their role in nitrification.

The interaction of microorganisms in rhizospheric, non-rhizospheric soils and vermicompost and their chemical, biological, physiological dynamics at the molecular level are of fundamental and practical importance for sustained food security. The rhizosphere is the “bottle neck” in the supply of nutrients. It is influenced by the ecotoxicological effects of organic and inorganic contaminants supplied to plants (Brown and Funk, 2008). In the present study for chemical characterization, the rhizospheric, non-rhizospheric and vermicompost samples were collected and analysed. In our study macronutrient analysis indicated that vermicompost consisted of 1.30 % N; 1.0 % P; 1.5 % K; 6.2 % Ca and 3.4 % Mg. These values were higher than those of rhizospheric and non-rhizospheric values. On the other hand non – rhizospheric soil consisted of 54.2 % organic matter with pH 6.4 while it was 37.8% with pH 7.4. Theses values indicated that in vermicompost bioconversions had taken place effectively than that of rhizospheric soil and non rhizospheric soils. Further vermicompost showed a narrow range of C:N ratio (22:1) when compared to
rhizospheric (37:1) and non-rhizospheric (31:1) soils. Vermicomposting resulted in faster reduction of C:N ratio as compared to normal compost. The vermicompost had higher amounts of micronutrients such as Fe, Mn, Cu, Zn and B than in rhizospheric and non-rhizospheric soils. The same observations were also made by Kale et al., (1988). In the present study, the significant reduction of organic matter, organic carbon, C/N and C/P ratio in vermicompost fall in line with the already reported results (Kale et al., 1994; Ramalingam, 1997; Karmegum and Daniel, 2000).

The reduction in carbon and lowering of C/N ratio in the vermicompost could be achieved by the respiratory activity of earthworms and bioconversions of gut microorganisms (Edwards and Bohlen, 1996). The increase of nitrogen by microbial mineralization of organic matter combined with the addition of worm’s nitrogenous wastes through excretion had been published earlier (Syers et al., 1979; Curry et al., 1995). The observed narrow range of C:N ratio in the vermicompost reflected the efficient worm activity, leading to accelerated rate of decomposition and mineralization of organic waste, there by resulting in nutrient rich vermicompost. According to Vincelas – Akpa and Loquet (1997) there was a great reduction of total organic matter and organic carbon in vemicompost than the farmyard compost up to 7 months. Edwards and Heath (1963) had reported that there was more than 50% loss of organic carbon from oak leaves when inoculated with L. rubellus.

The significant increase in the levels of N, P, K, Ca and Mg in the vermicompost worked by E. foetida confirms the enhanced microbial and enzyme activity in the gut of E. foetida as suggested by Parthasarathi and Ranganathan (2000). It seems an enhancement occur in pH towards neutrality which is an important factor in retaining the stability of N to promote its availability to the plant (Brady, 1990). In
the present study the pH was brought to around neutral pH and similar was recorded by many researchers. Hence, the neutral pH recorded in the vermicompost obtained by the action of *E. foetida* is the optimum pH for the availability of nutrients to the plants.

Microorganisms have been evaluated as a source of enzymes based on their distribution in various habitats (Huck *et al*., 1991; Crawford *et al*., 1993; Gerhardson, 2002). This study had highlighted nitrifying activity of the Earthworm Gut Bacteria which emphasized their importance as candidates for further investigation in nitrification and as plant growth promoters. It is anticipated that the isolation, characterization and the study on bacteria can be useful in the discovery of selective nitrifying bacteria. The natural roles played by earthworm to protect soil health may be replaced by earthworm gut flora.

Bacteria usually inhabit in various distributions and are important decomposers. They are able to metabolize many different compounds including sugars, alcohols, amino acids and aromatic compounds by producing extracellular hydrolytic enzymes. In modern agriculture, application of chemical fertilizers is still an invaluable and effective method. However, since use of agrochemicals is falling into disfavor because of environmental pollution and detrimental effects on variety of nontarget organisms. The potential use of microbe based agents as replacement or supplements for agrochemicals has been addressed in many recent reports.

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. Bacteria are the most widely distributed groups of microorganisms involved in nitrification in nature.
In the present study the nitrifying bacteria were isolated from the gut of Eisenia foetida by Most Probable Number (MPN) method. MPN method has been the traditional approach to study 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 oxidizers were observed by Matulewich et al., (1975). Four isolates showing nitrification activity on secondary screening and among them one of the best nitrifier was 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). The occurrence of B. cereus in higher proportion than other species may be due to the fact that B. cereus is more adaptable to a wider varying environment than other species. It was identified and confirmed as Bacillus cereus by 16S rRNA sequencing.

The physiological conditions play an important role in the growth of the organisms. Incubation temperature, pH, NaCl concentration, Carbon, Nitrogen sources and C/N ratio influences the metabolic reactions through enzymatic activities thereby effecting the growth of the organisms. So, in the present study, the influence of all the above physiological conditions on the growth of Bacillus cereus was determined. Provision of utilizable nitrogen source to organism is the basic requirement to be fulfilled for the optimum growth, hence nutrient broth was supplemented with different carbon and nitrogen sources and growth was measured by turbidity using O.D values at 600 nm.
The optimum growth of *Bacillus cereus* isolate was observed at the temperature of 37°C, pH 7, NaCl 2%, glucose as a carbon source, peptone as nitrogen source and C/N ratio of 0.5% glucose and 1% of peptone. The similar reports were observed by Goepfert *et al.*, (1972), Marrku and Constantin, (1975), Okanlawon *et al.*, (2010).

Number of selective media have been used for studying the metabolic activities of nitrifying bacteria. However, there has been confusion and contradiction about the effects of various stimulating and inhibiting factors on the growth of nitrifying bacteria. One of the problems that often obscure the interpretation of results is the lack of a standard test medium. To serve as a research tool, a successful test medium must be capable of furnishing results consistent under given experimental conditions. A better enriched medium, therefore is to be employed in order to obtain maximum metabolic activity of the nitrifiers (Satto *et al.*, 1985).

The present observation of maximum activity of ammonium oxidizers associated with ACC medium could be ascribed to the presence of calcium carbonate. The Calcium carbonate has been shown to neutralize the nitrous acid produced during the oxidation of ammonical nitrogen (Engel and Alexander, 1958b). Also, adsorption of cells to particulate ingredients such as calcium carbonate in the medium is necessary for active multiplication of bacteria to occur (Imshenetsky and Ruban, 1954; Lees, 1955). The reason for the significantly lowered activity of ammonia oxidizers in other three media could be due to the lowering of pH of the medium as a consequence to the release of H⁺ and due to the absence of a neutralizing agent (Azam and Farooq, 2003). When compared to the ACC medium the level of the calcium carbonate in the modified Winogradsky’s medium is low. Since ACC and NCC media
have been identified as a best available media for the growth of earthworm gut bacteria, they were employed in the further studies in characterization of nitrifiers.

In the present study after selection of suitable media, the isolate was tested for nitrite and nitrate estimation by using standard protocols (Barnes and Folkard, 1951; Jackson, 1971; Ranney and Bartlett, 1972). The change in concentration of exchangeable forms of $\text{NH}_4^+$-N, $\text{NO}_2^-$-N and $\text{NO}_3^-$-N during the nitrification processes was studied up to 14 days. It was observed that nitrite production by \textit{B. cereus} increased with incubation time up to 9 days. A gradual decrease in the nitrite production was observed in ACC medium after 9th day. The nitrate production was observed to increase up to 12th day and later there was a gradual decrease in NCC medium. Similar results were observed by Ramakrishna and Sethunathan (1983).

The effect of temperature, pH, nitrogen (N), carbon source, metal ions inhibitor/ chelators and pesticides on the nitrification was studied in the present study. Similar study was done in a sandy, moderately acidic soil.

Autotrophic or heterotrophic bacteria may be responsible for the nitrification resulting in decrease of ammonia. In the present study nitrification by \textit{B. cereus} was studied. Nitrification by heterotrophic microorganisms seem to differ in four main points from autotrophic nitrification: (1) a broad spectrum of organisms, N-sources and products involved in heterotrophic nitrification (Hirsch \textit{et al.}, 1961; Focht and Verstraete, 1977); (2) an organic carbon source necessary not only for growth of the organisms but in addition for nitrification of the inorganic N-sources (Hylin and Matsumoto, 1960; Verstraete and Alexander, 1972 b); (3) heterotrophic nitrifiers are much less active in their nitrification ability than autotrophic ones (Gode and Overbeck, 1972; Focht and Verstraete, 1977); (4) the heterotrophs mostly accumulate
nitrite or nitrate when active growth of the cells has ceased (Alexander et al., 1960; Doxtader and Alexander, 1966c; Obaton et al., 1968; Verstraete and Alexander, 1972a).

In the present study optimum pH for the nitrification lies at pH 7. These results were supported by the reports of Mulvaney, 1994; Paavolainen, 2000; Metcalf and Eddy, 2003 and Leiningher et al., 2006. It was reported that the nitrifiers can only carry out efficient nitrification with in a pH range of 7.0 – 9.0 and the ideal pH for the nitrification was lying at an optimum value in the range of 7.2 – 8.4 (Kanchana Maguluri, 2007).

Temperature is an important factor which markedly affects certain biochemical activities. Focht and Verstreate, 1977; Arunachalam and Arunachalam, 1999; Tate, 2000 and Kanchana Maguluri, 2007 had observed that the rate of nitrification increases with increasing temperature. Similar results were observed in our present study as nitrification process was increased upto 55\(^0\)C.

The results presented here, however, showed that nitrite excretion is a distinct metabolic feature of \textit{B. cereus}. The clear dependency of nitrite accumulation, on certain carbon sources, nitrogen sources and metal ions is strong evidence that metabolically active cells are required for nitrification. So, it can be readily assumed that nitrite is formed from ammonium by an oxidative sequence involved in some way with the metabolism of a special carbon source and that at least, one step is Mg\(^{2+}\) dependent. Similar study was conducted by Loveless and Painter, (1968), where some heterotrophic nitrifiers are stimulated by Fe\(^{2+}\) and Fe\(^{3+}\) which may be replaced by several other metal ions (Aleem et al., 1964; Verstraete and Alexander, 1972a). The function of the carbon source up to now is not quite clear. But, assuming an organic
pathway of nitrogen oxidation, a specific carbon source could provide the acceptor molecules for the synthesis of intermediate organic N-compounds (Alexander et al., 1960; Verstraete and Alexander, 1972b). If the carbon skeleton is not recycled, there should be a relationship between the amount of carbon utilized and the nitrite/nitrate released. The amount of nitrite accumulated was proportional to the initial acetate concentration. However, up to now there is no convincing explanation why nitrite is produced only with acetate, citrate, malate, and ethanol while several other C-sources supported growth but not nitrification. Similar carbon dependencies of heterotrophic nitrification have been reported (Hylin and Matsumoto, 1960; Verstraete and Alexander, 1972b), but the carbon sources favoring nitrification are not unique among different organisms. This may be explained by the regulative properties of the key enzymes in the glyoxylate cycle (Kornberg, 1966). These enzymes may be induced by acetate and repressed by glucose, PEP or pyruvate (Donawa and Inniss, 1970; Kleber and Mtiller, 1970; Hanozet and Guerrirote, 1972; Wolfson and Grulwich, 1972).

Extracts of *Arthrobacter sp.* contained malate synthase (MS) and isocitrate lyase (IL), the activity of which was induced by incubation in a medium containing acetate.

A perusal of the literature clearly indicates that the information related to the effects of pesticides, which are commonly used to combat various major pests in recent agriculture, is far from complete. The farmers from Chittoor district (A.P) heavily rely on cultivation of groundnut as the regular crop and practice the application of pesticides to control important pests and diseases. In the present study, a comparison of the data on the effect of pesticides towards nitrification at the end of 14 days clearly suggested that endosulfan, mancozeb and neem oil when applied even at low doses would greatly inhibit nitrification. Similar results were reported by earlier scientists (Hauck, 1980; Bronson *et al.*, 1992; Guiraud *et al.*, 1992; McTaggart...
et al., 1997). Also, a large number of chemicals especially, pesticides have been used in recent years to inhibit nitrification (Bremner and Bundy, 1974; Feng and Barker, 1990; Lodhi et al., 1996; Rice, 1984; Doneche et al., 1983; Sudhakar et al., 2000; Saxena and Rai., 1999; Jana et al., 1998). The effect of various Inhibitors/ Chealating agents (5mM) such as Nitrapyrin, Dicyanodiamide, Thiourea, Potassium cyanide, Sodium diethyl dithiocarbonate and L- Histidine was studied on Nitrification, out of which the Nitrapyrin had shown profound effect. Similar reports on the effect of inhibitors on nitrification process were observed (Aulakh et al., 1984 and Merino et al., 2001).

In the present experiment the molecular weight was determined for the enzyme, Ammonium monooxygenase having two subunits, with 36 and 42 kDa. Nitrite oxidoreductase molecular weight was determined as 63 kDa. Similarly, the Ammonium monooxygenase from Paracoccus denitrificans was 38 and 46 kDa (James Moir et al., 1996) and for Nitrite oxidoreductase was 65 kDa from Bacillus badius I-73 (Sakai et al., 2000).

Most plant growth promoting strains consists of Gram-negative genera. The greatest numbers of strains are the members of pseudomonads (Kloepper,1993; Glick,1995). They exert beneficial effect on plants in both direct and indirect ways. Direct methods include the solubilization of inorganic NPK and mineralization of nitrogen and phosphate, which make them available to plants (Krasilnikov,1961; Gaur,1972; Subba Rao, 1982). The plant growth promotion was through the synthesis of phytohormones (Xie et al., 1996), Nitrogen fixation (Christiaensen – Wenger, 1992), reduction of membrane potential of roots (Bashan and Levanony, 1991) and synthesis of some enzymes that modulate the level of plant hormones (Glick et
Indirect growth promotion by means of decrease or prevention of deleterious effect of pathogenic microorganisms is due to the synthesis of antibiotics or siderophores (Sivan and Chet, 1992; Leong, 1986). In our present study, *B. cereus* is promoting a significantly high level of growth in ground nut plants in terms of shoot length, root length and total dry weight. Interaction between plants and microorganisms in the rhizosphere can effect plant growth, nutrient management and yield (Whitelaw, 2000, Rodriguez and Fraga, 1999, Leggett *et al.*, 2001). When these bacteria are inoculated they promote plant growth via different mechanisms including enhanced nutrient uptake (Jakobsen *et al.*, 2005). *B. subtilis* increased root length and yield in rice crop significantly from the control in both pot and field experiments in an Himalayan soils (Trivedi *et al.*, 2003). In the present study the ability of *B. cereus* to mineralize nitrogen was assessed under controlled growth conditions in the designed pot trails which had shown a positive influence on plant growth.