Discussion
5.1 Microbial Characteristics of Agricultural Soil of Aligarh

Soil microorganisms can be critically used for the maintenance of soil function in both natural and managed agricultural soils because of their involvement in the key processes of soil structure formation; decomposition of organic matter, toxin removal, and the cycling of C, elements/nutrients (Van Elsas and Trevors, 1997). In addition, soil microbes play a key role in promoting plant growth and suppressing soil borne plant diseases (Doran et al., 1996). Microbial communities in root associated habitat respond with respect to density, composition, and activity to the abundance and great diversity of organic root exudates, eventually yielding plant species-specific microflora (Burdman et al., 1997; Buyer et al., 1999; Abawi and Widmer, 2000). Due in part to the scarcity of the convenient methods for exploration, our understanding of the different degrees and dynamics of microbial community variation is limited (Agrios 1997; Buyer et al., 1999; Garbeva et al., 2004). The term microbial diversity describes the number of different types and their relative abundance in a given community in a particular habitat. In molecular ecological terms, it can be defined as the number and distribution of different sequence types present in the DNA extracted from the community in the habitat. However, the term community structure implies that information is included on the numbers of individuals of different recognizable taxa (Liesack et al., 1997). These divergent terms often used interchangeably. To study microbial diversity cultivation based and cultivation independent methods are used. However, both approaches have their own advantages and limitations (Janssen et al., 2002; Garbeva et al., 2004). Though culture dependent techniques are limited for studies on the composition of natural microbial communities in soil when used alone, yet they help in understanding the growth characteristics, ecological behaviour and function of microorganisms from soil habitats (Hoitink and Boehm, 1999; Kozdroj and van Elsas, 2001).

In the present investigations, microbial viable plate counts of rhizospheric and non-rhizospheric soils of culturable microorganism’s i.e. aerobic heterotrophic bacteria, asymbiotic nitrogen fixers, actinomycetes and filamentous fungi were examined. As expected a significant increase in the microbial density of rhizospheric soil was observed compared to non rhizospheric soils which could possibly be due to the nutrient rich environment and availability of nutrients from root exudates, which
includes an array of low and high molecular weight compounds. Our observation thus demonstrated almost 10 times increase in the rhizospheric microbial density and is in close agreement with the reports of other workers. Viable plate counts of aerobic heterotrophic bacteria and asymbiotic nitrogen fixers ranged from $0.34 \times 10^7$ to $9.3 \times 10^7$ and $0.12 \times 10^5$ to $0.165 \times 10^5$ respectively. Similarly, the viable plate counts of soil actinomycetes ranged from $4.0 \times 10^5$ to $5.2 \times 10^5$, $2.5 \times 10^5$ to $3.2 \times 10^5$, $7.2 \times 10^5$ to $8.0 \times 10^5$ CFU g$^{-1}$ soil from site-A and B (Wastewater irrigated agricultural field) and site-C (Non wastewater irrigated agricultural field) respectively in the rhizosphere of different crops. The Common types of actinomycetes identified include the member of *Streptomyces*, *Nocardia* and *Micromonospora* (Alexander, 1983; Curl and Truelove, 1986).

Soil fungi are an important eukaryotic microorganism responsible for degradation of organic matter, nutrient cycling and number of activities including symbiotic relationship with plant root. In this study the quantitative estimate of free living filamentous soil fungi revealed the density as $2.5 \times 10^4$ to $3.25 \times 10^5$, $2.1 \times 10^5$ to $2.9 \times 10^5$, $6.8 \times 10^5$ to $7.9 \times 10^5$ CFU g$^{-1}$ soil in site-A, B and site-C respectively. Population density of above tropical soil microbes was in the range as found in the fertile soil. In the rhizospheric soil member of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Curvularia*, *Trichoderma*, *Trichophyton*, *Mucor*, *Monotospora*, *Verticillium*, *Alternaria*, *Chlamydospora*, *Hormodendrum*, *Trichothecium*, *Fusarium* and *Mycelia Sterillia* were more frequently encountered. The variations in the population density of above groups of culturable aerobic microorganisms from plant to plant and from sites of sample collections were observed which might be due to the several factors like age of plant, nature and types of plant root exudates, and environments like, moisture condition of field soil and field amendment (Grayston *et al.*, 1998, Ahmad *et al.*, 2006a). No significant change in microbiological characteristics was observed between both types of soils i.e. wastewater irrigated and non wastewater irrigated soils.

Similar observations were also made by Hayat *et al.* (2002) when studying the impact of long term application of oil refinery wastewater irrigation on soil health and crop productivity in the vicinity of Mathura Oil refinery, India.
The above observations were based on only small fraction of cultivable heterotrophic microorganisms which were common representative groups in the plate culture method. Therefore other culturable and non culturable microorganisms including both free living, symbiotic (VAM fungi) and endophytic organisms needs to be investigated further using modified cultural media and direct DNA extraction and PCR based molecular techniques, as adopted by many workers of the developed countries (Gomes et al., 2001; Kowalchuk et al., 1997, Smit et al., 1999, Vainio and Hantula, 2000; Van Elsas et al., 2000; Khan, 2005). Many authors are of the opinion that combination of both cultivation based and culture independent molecular approaches (polyphasic) are most appropriate way of assessing microbial diversity in any given habitat (Hill et al., 2000; Kozdroj and Van Elas, 2001; Garbeva et al., 2004.).

5.2 Metal Tolerant Microbial Population in Agricultural Soils

Long term application of wastewater containing toxic elements like heavy metals to agricultural soil may result in metal accumulation and may affect soil microbial characteristics and soil health in general. The selected sampling sites (A and B) are receiving long term (>25 years) application of city wastewater mix with industrial effluents (mainly from lock manufacturing units) as irrigant without any apparent adverse effect. This has triggered us to investigate the impact of long term use of ill treated wastewater on soil microbiological characteristics and emergence of metal tolerance in microbial population especially free living filamentous fungi. Because both types of soil samples (Wastewater irrigated and non wastewater irrigated) have almost similar properties concerning soil texture, pH and organic content, it is reasonable to assume that any change in microbial population level and metal tolerant fungi in treated soil can be attributed mainly to the effects of wastewater application. Aligarh city is famous for lock-manufacturing factories. Hundreds of small and large-scale factories are supposed to spill tremendous amount of heavy metals into sewage in the form of industrial effluents. The wastewater understudy is known to contain various potentially toxic elements. (Ajmal et al. 2003; Malik and Ahmad 1995). The heavy metal content of soil receiving wastewater was observed higher for Cr, followed by Ni, Co, Cu and Cd as compared to control site. However, it is difficult to establish a correlation between heavy metal contamination
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of wastewater and heavy metal content of soil, it needs monitoring of metal levels around the year for long period of time (Ahmad et al., 2005). Further, it is difficult to assess the fate of heavy metal contamination in soil. Various factors, such as biotic and abiotic factors are known to influence the metal speciation, solubility, and fixation and metal microbe-interactions (Babich and Stotzky, 1980; Baath 1989; Oliveira and Pampulha, 2006). Low pH in soil solution increases the metal toxicity to microbes. However, the soil samples under study showed 7.8 ±0.2 pH. This soil pH is not a major factor to influence metal toxicity. In this study, composite soil samples from different sites (wastewater irrigated and nonwastewater irrigated) were enumerated to assess the impact of long term application of wastewater on emergence of heavy metal tolerance among the indigenous population of soil fungi, bacteria and actinomycetes. The culturable fungal, bacterial and actinomycetes population were enumerated on their selected nutrient media supplemented with 50, 100, 200 and 400μg/ml concentration of individual heavy metals. No significant effect at 50μg/ml concentration of any metal was observed as compared to their respective control plate. The difference in CFU count at elevated concentration (>200μg/ml) becomes more visible and significant. The reduction in CFU was more evident against cadmium and nickel as compared to other metals. Further two sites, A and B (wastewater irrigated) showed fewer decline in CFU of tolerant fungal population as compared to the soil sample from control site- C (nonwastewater irrigated). However, such differences were less evident against Cr, Cu and Co. This has indicated that Cd and Ni might have exerted more stress in soil microbes as compared to other three metals. The metal tolerant population of actinomycetes was found relatively higher in wastewater irrigated sites as compared to nonwastewater irrigated site. Cadmium and nickel tolerant aerobic heterotrophic bacterial population in terms of CFU/g of soil were detected in low frequency at the control site, (Site-C) as compared to other metals. However, the frequency of occurrence of Cr, Cu and Ni tolerant aerobic heterotrophic bacteria was higher in wastewater irrigated sites (A and B) as compared to control (Site-C). On comparative basis, metal tolerance level among soil actinomycetes was observed lower than aerobic heterotrophs and soil fungi. This might be in part due to differences in their cell wall structures and chemical composition. We previously demonstrated concentration dependent metal toxicity in a short term study of
indigenous soil bacterial population after addition of metal in soil (Ahmad et al., 2005b). Long term exposure of oil refinery wastewater has resulted an increase level of metal tolerance in *Rhizobium* and *Bradyrhizobium* (Ansari et al., 2001; Ahmad et al., 2001). Microbial response to soil metal contamination varied from study to study (Baath, 1989). Like bacteria, fungal populations are known to develop metal tolerance by a variety of mechanisms (Gadd, 1993, Valix and Loon, 2003) and showed great capacity for adaptation against stress conditions (Baath, 1989; Frostegard et al., 1996; Oliveira and Pampulha, 2006). Baath et al. (2005) reported that fungal colony-forming units (CFUs) were ten-times lower in lead-enriched soils; the species composition was widely different from that in control soils.

It is difficult to compare our results with many other reports due to the differences in ecological conditions, soil type, organic matter content, pH, salt, vegetation and pollutant types and its levels in wastewater all will influence the results. However, in this study an attempt has been made to characterize the commonly encountered fungi, bacteria and actinomycetes on different metal amended and unamended plates.

### 5.3 Occurrence Frequency and Diversity of Metal Tolerant Soil Fungi

Resistant fungal species are mostly present at low frequencies in non-metal polluted soils, but can become dominant under toxic metal stress (Kunito et al. 1998). All nutritional groups of fungi (saprotrophs, biotrophs, and necrotrophs) can be affected by toxic metals. Toxic metal (cadmium, chromium, copper, nickel, lead and zinc) pollution of soil led to a significant decrease in the number of AM fungi and low colonization of plant roots, and as a result, to changes in the species diversity of mycorrhizal fungi (Mozafar et al. 2002; Moynahan et al. 2002).

In the present study we examined diversity and occurrence frequency of the metal tolerant fungi grown on metal amended nutrient medium. In this study fungal isolates like *Aspergillus*, *Penicillium*, *Rhizopus*, *Curvularia*, *Mucor*, *Trichoderma*, *Fusarium* and *Trichophyton* etc. were found with varied frequency at different metal concentration from wastewater irrigated soil sites. A significant difference was also observed in the occurrence frequency of fungal genera in non wastewater irrigated
soil. The frequency order of the fungal genera at 100µg/ml was found similar for chromium and nickel metals. Other metals like Cd, Cu and Co showed different pattern of occurrence for fungal genera.

However, in both of wastewater irrigated and nonwastewater irrigated soil, there was no significant difference in the diversity of metal tolerant soil fungal isolates but their occurrence frequency varied at varying concentration of the heavy metals. This difference in occurrence frequency is expected due to variation in the metal resistance. Occurrence of various different fungal genera or morphological forms varied with different metal concentrations.

Reduction in morphological diversity of fungi was more evident against Cd, and Ni followed by Co, Cu and Cr from site-A. However, it was higher against Ni, and Cu followed by Co, Cd and chromium from site B samples. The observations from wastewater irrigated soil samples were comparable with control site which have demonstrated some differences.

Our findings may be correlated with the observations made by other workers where reduction in the diversity of soil fungi in metal polluted environment are reported (Gadd, 1993; Kunito et al.1998). The frequency of tolerant microbial population may increase with an increase in toxic metal levels (Olson and Thornton 1982; Huysman et al. 1994; Kunito et al. 1997). Plaza et al. (1998) reported zygomycetes are more tolerant to cadmium than ascomycetes and conidial fungi. Similarly, the most frequent soil saprotrophic microfungi isolated from heavily metal polluted habitats in Argentina, Czech Republic and Ukraine were species of Penicillium, Aspergillus, Trichoderma, Fusarium, Rhizopus, and Mucor, as well as Paecilomyces lilacinus, Acrostalagmus luteo-albus (as ‘Nectria inventa’), Cladosporium cladosporioides, Alternaria alternata, and Phoma fimeti (Kubatova et al. 2002; Massaccesi et al. 2002). Soil pollution causes a pressure on sensitive microorganisms and so changes the diversity of soil microflora (Zaguralskaya 1997), the decrease in microbial density caused by a high level of heavy metal contamination found at the sites (A and B) we also examined is in agreement with Kikovic (1997) and Smajkalova et al. (2003).

In Indian soil characterization of fungal diversity from metal contaminated soil of the Wazirpur industrial area is reported by Sexena et al (2006). In this area highly
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acidic hazardous solid waste produced high concentration of heavy metals (Ni, Cu, Cr, Fe and Mn). Nickel toxicity is a major environmental concern. More than 20 strains were isolated, most of them belonging to species of *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor* genera. Seasonal variation in fungal diversity was significant. To establish the exact effect on fungal diversity, the genetic and molecular tools are now available to some laboratory which could provide more definite information (Rajapaksha *et al.*, 2004).

5.4 Metal Tolerance Limits of Fungal Isolates

Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions, or modifying the active conformations of biological molecules (Doelman *et al.*, 1994). However, at relatively low concentrations some metals (Co, Cu, and Zn) are essential for microorganisms since they provide vital cofactors for metallo proteins and enzymes (Doelman, *et al.*, 1994). In natural environment metal-microbe interaction is complex and influence by multiple factors.

Environmental factors may exert some selective pressure influencing the community structure and dominance of individual species. Soil pollutants such as heavy metals might develop the conditions that enable some of fungi in their community to become dominant. Very little information is, however, available on the type of interspecific interactions which might occur between fungi in heavy metal polluted habitats or soils (Peciulyte, 2002).

In this study different morphological forms of fungi developed on different metal amended plates, indicated that metal tolerance is a common trait of fungi isolated from soil. Therefore, to assess the tolerance level of individual fungal isolates, 73 common representatives of fungal isolates belonging to 18 distinct genera were selected from metal amended plates of 100μg/ml concentration indicating that all selected isolates could grow at this concentration of Cd, Ni, Cr, Co and Cu when amended individually.

The selected fungi were tested for the tolerance limits in terms of minimum inhibitory concentration (MIC) of metals. The tolerance limit of different fungi against individual metal was found as the characteristics of the isolates and nature of
metals. Maximum level of tolerance was recorded up to 2000μg/ml against Cr, Co, Ni, and Cu among the fungal isolates. However, none of the isolate could grow above 1200μg/ml of cadmium. The tolerance level of fungi against individual metals varied from isolate to isolates. Majority of the fungi showed resistance to heavy metals tested. The tolerance to metals might be due to the various physiological adaptation and genetic modifications of these isolates over a period of metal exposure time. Presence of the efflux pump mechanism may be common in these isolates providing multi-metal resistance (Mehra and Winge, 1991; Gadd, 1993).

Some of the fungal isolates might be indigenous to soil while other might had added through wastewater irrigation. Thus, different isolates might have different level of heavy metals exposure. Variation in metal tolerance among different isolates of the same genus had become more evident when 20 isolates of Aspergillus, showed significant variations in metal tolerance from 200-2000μg/ml against one or other metals. Various workers have reported the metal tolerance by fungi to varied extent (Gadd et al., 2001; Moynahan et al. 2002; Baldrian, 2003).

Almost similar level of tolerance among soil fungi have been reported by some other workers (Ahmad et al., 2005; Zafar et al., 2006). Aspergillus strains were more cadmium tolerant than strains of Penicillium (Plaza et al., 1998), but Penicillium species are often reported to be dominant in copper-contaminated environments. Tolerance of fungi in soil microcosm was studied in Brazilian soils with copper concentrations 25– 11500 mg kg⁻¹ the predominance of a Penicillium species tolerant to a copper concentration of 750 mg kg⁻¹ in soil was found (Ribeiro et al., 1972). A very high predominance of Penicillium species (80 % of isolations) was observed in freshly excavated archeological soil, containing 500 mg kg⁻¹ copper and 500 mg kg⁻¹ lead, at the site of a Bronze Age ancient Greek copper-smelting furnace (Olviya, Ukraine) (Gadd, 2007).

5.5 Biochemical and Potential Plant Growth Promoting Traits of Soil Fungi

Soil is a major reservoir of almost all types of fungi including pathogenic, symbiotic, as well as free living non pathogenic fungi. Role of soil fungi in plant growth stimulation and plant health protection is well established among certain
symbiotic fungi such as mycorrhizal fungi. However, with few exceptions, free living filamentous rhizospheric fungi are less scrutinized for their biochemical activities relevant to plant growth promotion. In this screening programme an attempt has been made to detect the traits like phosphate solubilization, production of indole acetic acid, organic acid and metal solubilization, siderophore, ammonia and antibiotic as well as production of extra cellular enzymes (lipase, amylase, chitinase, cellulase etc.) by soil fungi. Such traits are relevant to plant growth promotion and soil fertility (Gaur, 1990; Arshad et al., 1991 and 1993).

5.5.1 Extracellular Enzymes

Soil microbial communities are important contributors to the decomposition of organic matter. Saprophytic fungi play a major role in decomposition because they must rely on dead organic matter as their source of carbon and energy. A wide variety of microbes including bacteria, fungi, actinomycetes and protozoa are involved in the decomposition of cellulose, fungi have generally considered to be the main organisms responsible (Alexender, 1986). Role of various extra cellular fungal enzymes in soil are known to degrade complex carbonaceous and nitrogenous substances and synthesis of humus leading to increased soil fertility and nutrient availability to producing as well as other organisms including plants (Paul and Clark, 1989). These enzymes have been commonly studied and well exploited for industrial applications. However, little attention has been given towards the role of such free living rhizospheric fungi producing the extra cellular enzymes and their influence on the plant growth directly or indirectly.

Extra cellular enzymatic activities among isolated fungal strains was maximum (95.52%) for lipase followed by amylase (61.11%), cellulase (41.79%) and chitinase (35.82%). The production of above enzymes varied among different genera and even among the isolates of the same genus like Aspergillus, Curvularia, and Mycelium Sterilia group. Such variations might be due to many reasons including the differences in their genetic make up as well as nature and regulation mechanisms involved in enzyme synthesis (Crueger and Crueger, 1990).

Lipase production was observed most common among fungal isolates under study. Aspergillus sp-04 (ASF-04) showed highest lipase production (SI=2.80) among
all the *Aspergillus* isolates. Similarly, *Curvularia* sp-05, *Hormodendrum* sp-01, *Penicillium* sp-03, and Unidentified sp-08 showed good efficiency in lipase production. However, *Penicillium* sp-01 showed maximum lipase producing capacity. Similarly, among amylase producing fungi, *Alternaria* sp-02 (SI=2.47), *Penicillium* sp-01 (SI=2.41), *Hormodendrum* sp-01 (SI=2.25), and *Curvularia* sp-04 (SI=2.21) and some other isolates from unidentified genera also demonstrated strong amylase production capability. *Aspergillus* sp-13 (ASF-13) isolate showed the maximum amylase production (SI=2.07) among all fungal isolates tested.

Fungi are preferable lipase sources because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media (Arima et al., 1972).

Production of lipases is well known and expected from heterotrophic microorganism like fungi. On the other hand, fungal pathogens secrete various extracellular enzymes hypothesized to be involved in virulence (Wu et al., 1999). Positive effects were shown for pectinolytic enzymes from *Aspergillus flavus*, *Botrytis cinerea* (ten Have et al., 1998), and *Claviceps purpurea* (Voigt et al., 2005). The role of lipases in plant growth promotion is suspected as these enzymes producing and acts upon organic substrates in the rhizosphere to make minerals available both for other rhizospheric microorganisms as well as for plants (Dodd et al., 1987). The precise role of most other extracellular enzymes is still controversial (Wu et al., 1999; Voigt et al., 2005).

Amylase activity was next to lipase and frequently demonstrated by representative of the most of the genera except some isolates like *Fusarium*, *Chlamydospora*, *Mucor*, *Rhizopus*, and *Verticillium*. Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000).

α-Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2002). Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and to only one species of *Penicillium*, *P. brunneum* (Haska and Ohta, 1994; Pandey et al., 2000).
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Production of amylases is widely distributed among the filamentous fungi (Oberoi and Kalra, 2006). Such fungi are expected to solubilize starch and utilize it and will also lead to co-mineralization of nitrogenous compounds enriching the availability of plant nutrient in the rhizosphere. Different types of amylases are common in fungi, and *Aspergillus* sp and *Rhizopus* sp are often used as sources of industrial amylases (Boyce, 1986; Moreira et al., 1999). However, only few micro-organism including *Aspergillus* species have been reported to possess ability to produce raw starch degrading amylase (Abe et al., 1988; Hayashida et al., 1988; Okolo et al., 1995 Alves et al., 2002; Guimaraes et al., 2006). Certain species of *Aspergillus* and *Bacillus* are almost exclusively used for the commercial production of a-amylases (Scriban, 1993).

In this present study cellulolytic activities were observed among fungal isolates belonging to *Aspergillus*, *Curvularia*, *Fusarium*, *Monotospora*, *Tricophyton*, *Trichoderma*, *Mycelia sterillia*, *Monillia* fungal genera and some other isolates belong to unidentified fungal strains. Fungi with cellulolytic activity are known to play important role in degradation of carbonaceous plant materials, enrichment and acceleration of compost process (Gaur, 1987). A wide variety of microbes including bacteria, fungi, actinomycetes and protozoa are involved in the decomposition of cellulose, fungi have generally considered being the main organisms responsible (Alexenderk, 1986; Cowling, 1958; Gas-coigne and Gascoigne, 1958; Siu, 1951).

Several fungi were isolated and screened for high total cellulase activity in an attempt to develop a practical process for the enzymatic conversion of cellulose into glucose (Mandels, 1975; Mandels and Sternberg, 1976). Mandels (1975) observed that some species of thermophilic fungi degraded cellulose rapidly but that their culture filtrates had low cellulase activity. This was contradicted by reports that the thermophilic fungi *Sporotrichum thermophile* (Coutts and Smith, 1976) and *Talaromyces emersonii* (Folan and Coughlan, 1978) produced cellulase activity nearly comparable to that of the mesophilic fungus *Trichoderma reesei*, regarded as the best source of fungal cellulase. Although cellulase productivity varies among strains (Oberson et al., 1992), using uniform procedures for the measurement of cellulase activity. Fungi with such activities are expected to bring about accelerated rate of mineralization in the rhizosphere and may modulate the activity of microbes in the rhizosphere.
Chitinase production capability of soil filamentous fungi was found in *Aspergillus* isolates, *Curvularia* and some isolates from *Mycelia Sterilia* and unidentified fungal groups. It is interesting to note that these isolates could produce both cellulase and chitinase. Fungal isolate *Curvularia* sp-09 showed relatively higher production of cellulase as compared to other fungal isolates tested. On the other hand *Aspergillus* sp-17 (ASF-17), *Mycelia sterilia* sp-05, Unidentified sp-05 and Unidentified sp-11, isolates demonstrated relatively higher chitinolytic activity as compared to *Monotospora* sp-01, *Microsporum* sp-01 and Unidentified sp-09.

Chitin is one of the most important structural polysaccharides and provides mechanical strength to organisms containing it. Chitin is produced by the members of both plants and animals including certain microbes except bacteria and actinomycetes. Chitin degrading activity is predominant among the soil actinomycetes, some fungi (*Mortierella, Trichoderma, Verticillium, Paecilomyces* etc.) and few bacteria like *Bacillus and Pseudomonas* (Alexender, 1985; Paul, 1990). It is proved in many cases that chitinase production may have lytic effect on phytopathogens like *Fusarium*, which have chitin in their cell wall constituents. However, the actual mechanisms of phytopathogen suppression by a biocontrol organism through chitinase production are not yet well established (Nagraj Kumar *et al.*, 2004). However, the role of chitinases in carbon cycle of biosphere is well known (Mukherjee and Sen, 2004).

Secreted chitinases have been reported from a number of filamentous fungal species, and roles in hyphal branching and autolysis have been proposed (Gooday *et al.*, 1992). Secreted chitinases also are considered to be important in the mycoparasitic and entomopathogenic processes of some fungi (Garcia *et al.*, 1994; St. Leger *et al.*, 1996). Fungal chitinases are important enzymes required for hyphal growth (Takaya *et al.*, 1998). Chitinases from *T. harzianum* are more effective against fungus pathogens than those from plant origin (Lorito *et al.*, 1998).

In our study among the *Aspergillus* isolates tested, production of these four extra cellular enzymes varied. Lipase and amylase activities were common in majority of the isolates. Majority of the isolates demonstrated three enzymatic activities. Only one isolate could produce all four extracellular enzymes. This is probably the first report of screening of rhizospheric soil fungi of Aligarh soil for extra cellular enzymatic
activities. These potential isolates of different PGP traits and extracellular activities might be exploited both for industrial and agricultural purposes.

5.5.2 Phosphate solubilization and Organic acid production

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. A greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and cannot be utilized directly by the plants (Vassileva et al., 1998). To increase the availability of phosphorus for plants, large amounts of fertilizer are being applied to soil (Omar, 1998). Many bacterial, fungal, yeast, and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied (Goldstein, 1986; Halder et al., 1991; Abd-Alla, 1994; Whitelaw, 2000). Species of Aspergillus, Penicillium and yeast have been widely reported solubilizing various forms of inorganic phosphates (Asea et al., 1988; Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). Phosphorus absorption by plants can be increased by the presence of symbiotic organisms such as mycorrhizal fungi (Azcon-Aguilar et al., 1986) or by the inoculation with the soil mineral phosphate solubilizing fungi particularly black Aspergilli (Vassilev et al., 1997; Narsian and Patel, 2000; Goenadi et al., 2000; Reddy et al., 2002) and some species of Penicillium (Asea and Kucey, 1988; Cunningham and Kuiack, 1992; Whitelaw et al., 1999). The black Aspergilli include Aspergillus tubingensis, Aspergillus niger, Aspergillus awamori and Aspergillus aculateus.

Phosphate solubilization by various fungal isolates (42.46%) belonging to Aspergillus, Curvularia, Alternaria, Trichophyton, Hormodendrum, Monotospora, Mucor, Microsporum, Penicillium, Trichoderma, and Verticillium were detected on nutrient medium amended with tricalcium phosphate (TCP). Many unidentified fungal isolates were also found as phosphate solubilizer.

On the basis of phosphate solubilisation index the activity was found high among the isolates of Mucor sp-01, all Aspergillus isolates, Alternaria sp-01, 02, Curvularia sp-02, Fusarium sp-01, Monotospora sp-01, Penicillium sp-02, Trichophyton sp-02, and certain other isolates. Phosphate solubilization in liquid medium was also assessed in 09 isolates of Aspergillus spp., and one isolate from
each genus (*Penicillium* spp., *Rhizopus* spp., *Curvularia* spp, *Alternaria* spp and *Fusarium* spp.). Amount of phosphate solubilised varied from 188 to 465 μg/ml. *Alternaria* sp-01 could solubilised maximum amount of phosphate among all isolates tested while *Aspergillus* sp-07 (ASF-07) was observed most efficient phosphate solubiliser among all other *Aspergillus* isolates. No direct correlation was observed between biomass produced by various fungi and the amount of phosphate solubilised. Our findings are in agreement with the reports of other workers who have reported phosphate solubilization among isolates of *Aspergillus, Curvularia, Alternaria, Trichophyton, Hormodendrum, Monotospora, Mucor, Microsporum, Penicillium, Trichoderma, Verticillium* and other fungi (Agnihotri, 1970; Asea *et al.*, 1989; Gaur, 1990; Whitelaw, 2000; Zhao *et al.*, 2002; Reddy *et al.*, 2002; Xu *et al.*, 2004; Souchie *et al.*, 2006; Barroso, *et al.*, 2006; Vyas *et al.*, 2007). The variation from study to study might be due to different nature of fungal isolates and ecological conditions of the source of fungal isolation. However, in this study many new indigenous soil fungi are found phosphate solubilizer.

Low molecular weight organic acids (LMWOA) are recognized to be of major importance in a number of soils and plant processes (Ryan *et al.*, 2001). Within plants and soil microorganisms organic acids perform a number of pivotal metabolic roles including the provision of C for respiration and biomass production, making nutrients available (e.g. Fe-citrate) and maintaining internal charge balance (Jones, 1998). Several organic acids are found in soil solution with the most commonly identified ones being oxalate, citrate, acetate and formate (Fox, 1995; Jones, 1998). Normally, soil solution concentrations are in the micromolar range (Jones, 1998). Moreover, it is well documented that bacteria and saprophytic fungi can produce significant amounts of organic acids in soils (Fox, 1995). Likewise, mycorrhizal fungi may be an important source particularly in forest soils.

Fungi classified as Zygomycetes, including *R. oryzae*, have developed a different strategy for acidifying the environment by producing lactic acid to compete with fungi unable to metabolize lactic acid. These fungi often produce both ethanol and lactic acid, a combination that would discourage many competitors (Jan and Lene Lange, 2004).
Strasser et al. (1994) observed that the complex-forming compound oxalic acid can effectively solubilize metals such as aluminium, iron, lithium and manganese. In order to produce high amounts of oxalic acid for biohydrometallurgical processes, Aspergillus niger, a fungus well known for its ability to produce oxalic acid was used to optimize oxalic acid production. In sucrose medium, Aspergillus niger produced high amount of gluconic acid and oxalic acid, whereas in lactose permeate medium only oxalic acid was produced.

In this study organic acid production was detected among 28.35% fungal isolates belonging to different fungal genera like Aspergillus, Alternaria, Penicillium, Mucor, Fusarium, Trichoderma, Verticillium and many other unidentified isolates. Among 25 phosphate solubilizing fungal isolates belonging to Aspergillus, Penicillium, Alternaria, Fusarium, Monotospora, Mycelia Sterilia, Rhizopus, Trichothecium, Trichoderma, Verticillium and many other isolates of unidentified fungi could produce organic acids. It was observed that at least in some fungi, phosphate solubilization was occurred due to the organic acid production.

Similarly, our findings on production of organic acid by Aspergillus, Rhizopus, Alternaria, Penicillium, Monotospora, and Verticillium are in agreement with other workers (Cunningham and Kuiack, 1992; Denevre et al., 1996; Ahonen-Jonnarth et al., 2000).

Singal et al. (1994) found Aspergillus japonicus and A. foetidus to solubilize five types of Indian rock phosphates at pH 8 and 9. Solubilization was higher in the presence of pyrite than in controls lacking either pyrite or fungal inoculum. Both the aspergilli were found to be good pyrite solubilizers and could grow over a wide pH range. Solubilization of rock phosphates was the result of organic acid release and pyrite oxidation. Species of Aspergillus, Penicillium and yeast have been widely reported solubilizing various forms of inorganic phosphates (Asea et al., 1988; Cunningham and Kuiack, 1992; Whitelaw, 2000; Bojnova et al., 2008; Wakelin et al., 2007; Ahuja et al., 2007).

Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by PSMs (Asea et al., 1989; Gaur, 1990; Halder et al., 1991; Abd-Alla, 1994; Zaidi and Khan, 2003 and 2005).
5.5.3 Solubilization of insoluble metals by fungal isolates

In this study we examined the metal tolerant soil fungal isolates for their ability to solubilize insoluble metals specially cadmium sulfide (CdS) and manganese carbonate (MnCO₃). Fungal isolates belonging to Aspergillus, Penicillium, Nigrospora, Hormodendrum, Trichoderma, Trichothecium, Fusarium, Curvularia, Mucor and some other fungal genera could solubilize one or more metals. Isolate unidentified sp-13 demonstrated the highest solubilization (SI=2.73) of (MnCO₃). On the other hand Mucor sp-01 showed the maximum solubilization of the metal (CdS) followed by other fungi such as Fusarium sp-01, Monilia sp-01, and Aspergillus sp-16. The percentage of cadmium sulfide solubilization was observed lower as compared to manganese carbonate solubilization by the fungal isolates.

Several workers have reported the metal solubilization activity by soil fungi and their possible mechanisms are described. Organic acid anions are frequently capable of soluble complex formation with metal cations, thereby increasing mobility (White et al., 1997). Such complexation is dependent on the relative concentrations of the anions and metals in solution, pH, and the stability constant of the various complexes (Denevre et al., 1996). A further mechanism of metal solubilization is the production of low molecular weight iron-chelating siderophores, which solubilize iron (III). Siderophores are the most common means of acquisition of iron by bacteria and fungi and are effective in a wide range of soils, including calcareous soil. The most common fungal siderophore is ferrichrome (Crichton, 1991).

Oxalic acid can also act as a leaching agent for those metals that form soluble oxalate complexes, including Al and Fe (Strasser et al., 1994). In many fungi an important leaching mechanism occurs through the production of organic acids (e.g. oxalic acid, citric acid) (Gadd, 1999 and 2000b; Sayer and Gadd, 2001; Jarosz Wilkolazka and Gadd, 2003; Fomina et al. 2005a). Organic acid excretion by fungi is inter- and intraspecific, and can be strongly influenced by the presence of toxic metal minerals (Sayer and Gadd, 2001; Fomina et al. 2004 and 2005c). Notably, oxalic acid was the only tested agent to give a clear solubilization zone for pyromorphite (Fomina and Gadd, 2007).

Sayer et al. (1997) reported the ability of soil fungus Aspergillus niger to tolerate and solubilize seven naturally occurring metal bearing minerals, limescale
and lead phosphate was investigated. *Aspergillus niger* was able to solubilize four of the test insoluble compounds when incorporated into solid medium.

Martino *et al.* (2003) reported that fungal strains derived from polluted and unpolluted soils mobilize insoluble inorganic zinc compounds to different extents. Strains from polluted soils showed in fact little ability to solubilize Zn from both ZnO and Zn₃(PO₄)₂, whereas strains from unpolluted soils showed a higher solubilization potential. Induction of organic acids (malate and citrate) by the metal compounds was at least in part responsible for metal solubilization. In the case of ericoid mycorrhizal fungi, Fomina *et al.* (2005) investigated the ability of these fungi to solubilize different toxic metal (Cd, Cu, Pb, Zn)-containing minerals. Minerals were incorporated into solidified agar media and solubilization assessed by measuring clearing of the agar after fungal growth.

### 5.5.4 Indole acetic acid production (IAA)

Production of plant growth regulators is considered an important PGP trait of plant associated microbes. It is expected that rhizospheric fungi capable of producing plant growth hormones such as indole acetic acid (IAA) may have direct influence on plant growth promotion. Five major types of hormones regulate plant development namely: auxins, gibberellin, cytokinin, ethylene and abscisic acid. Indole acetic acid (IAA) has been found to be of universal occurrence and is a major plant hormone. It is one of the most common and extensively used auxin (Wilkins, 1972). Most microbial species use tryptophan to produce indole-3-acetic acid (IAA), mainly through the indole-3-pyruvic acid and tryptamine pathways (Tudzynski and Sharon, 2002).

Role of various plant growth regulators in promoting plant growth is well established in plant growth promoting bacteria and mycorrhizal fungi. In the present investigations 73 metal tolerant free living rhizofungi were screened for IAA or IAA like compounds *in vitro* in the presence of tryptophan. Our findings are supported by limited number of reports available on IAA production by common soil fungi like *Aspergillus, Curvularia, Alternaria, Trichophyton, Penicillium*, etc (Arshad and Frankenberger, 1992).

Further, quantitative estimation of IAA production in the presence of fixed concentration of tryptophan (500 µg/ml) in broth varied from 11.50 µg/ml to 31.80
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μg/ml among 13 Aspergillus isolates. Fusarium sp-01 produced maximum amount (42.41 μg/ml) of IAA followed by Penicillium sp-03 and Rhizopus sp-02 after one week of incubation period. Our findings are in agreement with the reports of Hasan (2002), who found that the fungi like Aspergillus flavus, A. niger, Fusarium oxysporum, Penicillium coryophilum, P. cyclopium, P. funiculosum and Rhizopus stolonifer for their capability to produce gibberellin and IAA.

Tuomi et al. (1993) reported the molds Botrytis cinerea, Cladosporium cladosporioides, and the yeast Aureobasidium pullulans, isolated from the leaves of three short-rotation Salix clones, to producer of indole-3-acetic acid (IAA). Robinson et al. (1998) characterized the biosynthesis of indole-3-acetic acid by the mycoherbicide Colletotrichum gloeosporioides f. sp. aescyphonem. Auxin production was tryptophan dependent. Compounds from the indole-3-acetamide and indole-3-pyruvic acid pathways were detected in culture filtrates. In a study of various phytopathogenic fungi, Furukawa et al. (1996) found that fungi that belong to the genus Rhizoctonia produce IAA efficiently from tryptophan.

5.5.5 Siderophore production

Siderophore production is considered as another indirect plant growth promoting activity of rhizospheric microorganisms. To sequester and solubilize ferric iron, many microbes utilize an efficient system consisting of low molecular weight (≥ 1000 da) compounds with high iron affinity, siderophore (Neilands, 1995). Siderophores are typically produced by bacteria, fungi, and monocotyledonous plants in response to iron stress (Ratledge and Dover, 2000). Siderophore studies have recently received much attention in rhizospheric bacteria as an indirect mechanism of plant health promotion. However, this property is considered as virulence factor in pathogenic microorganism (Neilands 1993 and 1995). The production siderophores is repressed in higher concentration of iron. Therefore, a certain variation can be expected depending on the particular species under investigation (Neilands 1984; Guerinot 1994).

Our study revealed that 52.05% isolates showed siderophore production among 73 fungal isolates of Aspergillus, Curvularia, Alternaria, Monotospora, Penicillium, Trichoderma, Chlamydospora, Mucor and Trichothecium etc. Certain
isolates of mycelia sterilia and unidentified fungi could also produce siderophore. However, such activity was not detected among many other fungal isolates such as *Trichophyton* and *Verticillium* etc.

Our findings are comparable with a number of reports available on the production of siderophores by fungi. Hydroxamate type siderophores production in fungi belonging to zygomycetes, basidiomycetes, and ascomycetes has been reported (Gupta and Satyanarayan, 2002; Neilands, 1974) while the members of mucorales produced carboxylates (Korat *et al*., 2001). Most species of the genus *Aspergillus* are known to produce several hydroxamate-type siderophores and many reports on the isolation and characterization of siderophores have been published (Dube *et al*., 2000). Moreover, *Aspergillus* fungi are producers of organic acids such as citric and oxalic acids which have been reported to act as siderophores in other microorganisms (Guerinot *et al*., 1990; Carson *et al*., 1992; Winkelmann, 1992; Dutton and Evans, 1996; Machuca and Milagres, 2003).

Most fungi excrete at least one type of hydroxamate siderophore (Winkelmann 2002; Baakza *et al*., 2004) with the exception of some fungi belonging to zygomycota class (Drechsel *et al*., 1995) which produce carboxylatetype siderophores and wood decaying fungi, specially brown-rot fungi, that produce in addition to hydroxamates, catecholate-type compounds (Milagres *et al*., 2002; Arantes and Milagres, 2006). In filamentous fungi, the siderophore mediated iron uptake pathway plays an important role in iron metabolism, and the gene products which are involved in iron metabolism are regulated by GATA-factor (Haas, 2003). Korat *et al.* (2001) examined 18 filamentous fungi belonging to Zygomycotina (10 mucorales) and Ascomycotina (4 Aspergillus spp. and 4 penicillium spp.), all produced siderophores in iron deficient M9 and grimm-allen media except *Rhizopus* sp. and *Aspergillus flavus*. The siderophore produced by the nine mucorales were carboxylates, while those produced by three aspergilli and four penicillia were hydroxamates. They found highest (72.2×10⁻⁴ gml⁻¹) and lowest (14.9×10⁻⁴ gml⁻¹) siderophore producers *Rhizopus oryzae* and *Synchephalastrum* sp. respectively.

Baakza *et al.* (2004) examined siderophore producing potential of 20 fungal isolates (same 10 species from each marine and terrestrial habitat) and compared. Except marine *Aspergillus flavus*, all isolates produced siderophores. They reported

5.5.6 Ammonia and antibiotic production

Production of ammonia by fungi (89.04%) demonstrated their role in ammonification which is the key compound for further cycling of nitrogen elements (Alexander, 1985). Although no direct role of ammonia produced by rhizospheric fungi have not yet been described in plant growth promotion. On the other hand *Aspergillus* sp-09, *Curvularia* sp-05 *Nigrospora* sp-01, *Chlamydospora* sp-01 *Trichothecium* sp-01 and three unidentified isolates showed antibacterial activity. Production of antibiotics by soil microorganism mainly actinomycetes, certain bacteria and fungi are well known (Alexander, 1986). However, such activity by rhizospheric fungi might be helpful in suppression of phytopathogenic bacteria. Such activity should be viewed with care as it can also inhibit other useful bacteria. On the other hand the role of antibiotic production by test fungi is of ecological significance.

5.6 *In vitro*, Toxicity of Heavy Metals to Spore Germination and Growth of Soil Fungi

Toxic metals are known to inhibit growth and spore germination of fungi, affect reproduction and metabolic activity, and reduce the ability of mycorrhizal fungi to colonize host plant roots (Gadd, 1993a; Fay and Mitchell, 1999; Hartley-Whitaker *et al.*, 2000; Mozafar *et al.*, 2002; Baldrian, 2003). Effects of toxic metals on fungal growth have shown intra and interspecific variability and dependence on metal species and speciation (Gadd, 1993a; Plaza *et al.* 1998). Depending upon the nature of fungi various authors have reported heavy metal toxicity to different levels. For example nickel was reported to be more toxic to fungal growth and ectomycorrhiza formation than chromium (Aggangan *et al.*, 1998). Copper was found to be toxic and cadmium very toxic to cultures of 15 decomposer basidiomycetes (Holland, 1995). However, a similar toxicity of both metals was shown on a solid medium with
cadmium and copper reducing radial growth of most strains of aquatic hyphomycetes by 50% at concentrations between 150 and 400 mM (Miersch et al., 1997). Radial extension rates of *Trichoderma virens* did not significantly differ during growth on tap water agar containing glucose and 0.1mM copper or cadmium (Ramsay et al. 1999).

In our present investigations, we examined the heavy metals (Cr$^{+6}$, Cd$^{+2}$, Ni$^{+2}$, Co$^{+2}$, and Cu$^{+2}$) responses of fungal growth in liquid medium in terms of spore germination time, mat formation and total biomass formation. Varying level of heavy metal concentrations showed different mode of toxicity to test fungi. Spore germination was delayed in almost all test fungi irrespective of heavy metals (At higher metal concentration) as compared to control. Similarly, development of mycelium leading to pellet/mat formations was affected by heavy metal concentration and period of incubation. In general heavy metal toxicity was found to be dependent upon nature of test strain, concentration of heavy metals and incubation time. Fungi at their tube emergence stage were observed most susceptible to the metals as compared to other periods of their development. We have also observed that the fungal isolates were more metal sensitive in liquid as compared to solid medium. Spore germination of fungal isolates was recorded more sensitive to the metals as compared to mycelial growth. *In vitro*, toxicity of selected heavy metals among the five Aspergillus isolates, the spore germination of isolate *Aspergillus niger* (ASF-07) was delayed at higher concentration of Cd as compared to control. Cd inhibited the growth of *Aspergillus niger* at 800 μg/ml up to 120 hours and after that the spore germination was occurred. A delay was observed in spore germination of *Aspergillus niger* at higher concentration (>400 μg/ml) of Cr, Ni and Cu as compared to control. It was observed minimum (2.6 mg/ml) biomass against Cd and maximum against Ni (13.3 mg/ml) at 50 μg/ml concentration as compared to control (14.5 mg/ml) after seven days of growth. The spore germination of *Aspergillus sydowi* (ASF-01) was recorded after 20, 60 and 80 hours at 800 μg/ml concentration against Co, Ni and Cr respectively as compared to control while no growth was observed against Cd at the same concentration. *Aspergillus sydowi* showed Cd>Co>Cu>Cr>Ni order for biomass production at 100 μg/ml concentration. Spore germination of isolate *Aspergillus* sp-02 (ASF-02) was recorded after 80 and 120 hours of incubation at 400 μg/ml concentration against Cr and Cd respectively. *Aspergillus* sp-02 exhibited maximum
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reduction in its biomass against Cd (68.70%) followed by Ni (40.87%), Cr (32.18%), Co (27.87%) and Cu (27.87%) as compared to control. *Aspergillus* sp-03 (ASF-03) also showed delay in spore germination time against cadmium and chromium as compared to other metals tested. *Aspergillus* sp-03 also demonstrated diverse spore germination time against Co (20hrs), Ni (60hrs) and Cu (80hrs) at 800 µg/ml concentration. *Aspergillus* sp-03 showed maximum (92.86%) reduction in its biomass against Cd and minimum (9.19%) against Cu as compared to control. Similar to other *Aspergillus* isolates, *Aspergillus* sp-05 (ASF-05) also demonstrated a varying trend of metal toxicity on its spore germination and biomass production.

*Rhizopus oryzae* (RZF-02) was also recorded most sensitive against Cd. Its spore germination time was found extremely slow in the presence of higher concentrations (>100 µg/ml) of Cd. The biomass production was less affected by Co and Cu as compared to Cd, Ni and Cr.

In the case of isolates of *Penicillium* spp., diverse spore germination time of isolate *Penicillium* sp-01 (PNF-01) was recorded at 800 µg/ml against Cr (80 hrs). Cd (140 hrs), Ni (100 hrs), Co (100 hrs) and Cu (120 hrs) in respect to control. Isolate *Penicillium* sp-03 (PNF-03) showed extremely slow mat formation and spore germination at 100 µg/ml concentration against Cu and Cd. An increase in biomass of *Penicillium* sp-03 (PNF-03) was recorded 35.71% against Ni at 100 µg/ml concentration. All four isolates of *Curvularia* spp. exhibited variations in their spore germination time due to the presence of metals. Isolate *Curvularia* sp-03 (CVF-03) showed maximum reduction in biomass against Cd (64.52%) and minimum against Co (32.26%) as compared to control.

Spore germination of isolate *Fusarium* sp-01 (FUF-01) was found slow against Cr and Cd as compared to Ni, Co and Cu. The biomass production of *Fusarium* sp-01 was found similar against Cr and Cu but it was 4.1, 1.7 and 3.9mg/ml against Ni, Cd and Co respectively at 100 µg/ml concentration after one week of incubation.

Similarly, spore germination and biomass production of *Monilia* sp-01 (MOF-01) *Trichophyton* sp-01 (TPF-01), *Microsporum* sp-01 (MIF-01), *Alternaria* sp-01 (ALF-01), *Mucor* sp-01 (MUF-01) and *Trichoderma* sp-01 (TRF-01) varied in response to different metal ions concentrations.
In our present investigations, we also examined the toxicity of different heavy metals (Cr$^{6+}$, Cd$^{2+}$, Ni$^{2+}$, Co$^{2+}$, and Cu$^{2+}$) on the radial growth of the soil fungi on solid medium at different time intervals. Fungal isolates showed their diverse sensitivity to the metals at varying concentrations and time intervals. We observed that in most of the cases at lower concentration, the growth of the fungal isolates was promoted but at higher concentration it was decreased in varying trends. Most of the fungal isolates showed maximum toxicity against cadmium among the metals tested. Among the aspergillus isolates, isolate Aspergillus niger (ASF-07) demonstrated a decrease by 21.18%, 72.95%, 42.36%, 15.30%, and 4.71% in radial extension against Cr, Cd, Ni, Co, and Cu respectively at 100 µg/ml concentration after 96 hours of growth as compared to control. Aspergillus sydowi (ASF-01) also showed varying trends of radial growth extension against the toxicity of tested metals. The isolate Aspergillus sp-03 (ASF-03) showed an increase by 4.83% in radial extension at 100 µg/ml concentration against cobalt after 96 hours of incubation period as compared to control. No growth of isolate Aspergillus sp-03 was observed at 800 µg/ml concentration against chromium and cadmium throughout the incubation period. The isolate Aspergillus sp-05 (ASF-05) exerted a decrease by 82.97%, 76.93%, 59.62%, and 57.70% in its radial growth extension against nickel, chromium, cobalt and copper respectively at 800 µg/ml concentration after 4 days of incubation. But no growth was observed at the same condition against cadmium. Among the Penicillium isolates, the isolate Penicillium sp-01 (PNF-01) exhibited maximum negative effect on its radial growth extension against cadmium and minimum against chromium at 100 µg/ml concentrations after 96 hours. However, isolate Penicillium sp-03 (PNF-03) showed no radial extension against chromium (800 µg/ml), cadmium (800 µg/ml), and copper (≥400 µg/ml) during the incubation period.

Among the four isolates of Curvularia, it was observed a varying pattern of metal toxicity. Maximum reduction in radial growth of Curvularia sp-01 (CVF-01) was observed against Cd followed by Co, Cu, Cr and Ni at 100 µg/ml (96 hours) over control. The other isolates of Curvularia sp. like Curvularia clavata (CVF-02), Curvularia sp-03 (CVF-03) and Curvularia sp-04 also demonstrated metal toxicity differently.

Isolate Mucor sp-01 (MUF-01) demonstrated maximum metal toxicity against copper followed by chromium and cadmium but an increase against nickel and cobalt at 100
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μg/ml after 96 hours of growth. *Trichoderma* sp-01 (TRF-01) demonstrated the maximum growth reduction against Cd and minimum against Cu at 100 μg/ml after one week incubation. Isolate *Fusarium* sp-01. (FUF-01) exhibited maximum decrease by 70.74% in its radial growth extension against Cd at 800 μg/ml and minimum by 52.44% against Cu. *Alternaria* sp-01(ALF-01) showed an increase by 10.90% (96 hours) and 5.45% (168 hours) at 100 μg/ml concentration against Ni and Co respectively over control. Isolate *Monilia* sp-01 (MOF-01) also showed 8.97% increase against Cr, Ni and Co while 66.42% and 1.29% decrease in its radial growth against Cd and Cu respectively at 100 μg/ml in 96 hours of growth. The mycelial radial growth of isolate *Monilia* sp-01 was completely reduced at ≥200 μg/ml concentration against cadmium throughout its incubation period. *Trichophyton* sp-01 (TPF-01) demonstrated complete reduction in its growth against chromium at 400 and 800 μg/ml after one week of incubation. We have also observed the heavy metals toxicity on the morphology of all fungal isolates tested in different manners such as color, texture and size etc (Plate -9).

This is probably the first systemic study on indigenous isolates of free living soil fungi from Northern India on metal concentration dependent effects on spore germination and growth of fungi. Our findings on cadmium toxicity to sporulation and growth inhibition and as most toxic metal are in agreement with the reports of Babich and Stotizky (1977). They reported a variety of microorganisms, including gram-negative and gram-positive eubacteria, actinomycetes, yeasts, and filamentous fungi, for their sensitivity to cadmium (Cd). They noted wide extremes in sensitivity to Cd among the fungi; there was no correlation between the class of fungus and tolerance to Cd. Fungal sporulation was more sensitive to Cd than was mycelial growth, as spore formation was inhibited at Cd concentrations that were noninhibitory to mycelial proliferation.

Our findings are also comparable to thereports of Baldrian and Gabriel (2002). They found intraspecific variability in growth response to cadmium (Cd) on agar media and in liquid culture among fourteen strains of a wood-rotting fungus *Piptoporus betulinus*. The variability of Cd tolerance was found to be very high. On agar media the addition of Cd to nutrient media resulted in reduction of relative growth rate and increased lag time. While the reduction of growth rate was already apparent at 10 mM Cd, the lag time was significantly increased in higher Cd
concentrations. Biomass production in liquid culture was less sensitive to addition of Cd than the growth rate on solid media. At 100 mM Cd the radial growth rate of the mycelium was reduced to 27%, whereas the dry mass of mycelium was 77% of the respective control value. A group of four Cd-sensitive strains was found, showing low metal tolerance both on solid media and in liquid cultures. The growth rate of fourteen tested strains displayed lower variability than biomass production, showing that radial growth rate is more species-specific and therefore more valuable for interspecific comparisons of growth response.

Similar observation on *Penicillium* was made by Levinskaite (2001). He investigated the influence of the cadmium, nickel and zinc on the development of fungi *Penicillium atramentosum* 25SL and *P. funiculosum* 6AL. All tested strains of fungal development (spore germination, germ tube growth, radial growth rate and conidiogenesis) were affected. The both fungi at their tube emergence stage were most susceptible to the metals than at the other periods of their development.

Gadd *et al.* (2001) examined the fungal colony growth and biomass distribution in response to toxic metals. In solid medium, 0.1mM Cd and Zn caused a decrease in radial expansion of both *Trichoderma viride* and *Rhizopus arrhizus*. These metals also affected the overall length of the fungal mycelium and branching patterns. *T. viride* showed a decrease in overall mycelial length and number of branches in response to Cu, resulting in an extremely sparsely branched colony. Conversely, although, Cd also reduced overall mycelial length to about one third of the control length, the number of branches decreased only slightly which resulted in a highly branched colony with many aberrant features. Cu and Cd induced similar morphological changes in *R. arrhizus*. A large scale mycelial mapping technique showed that disruption of normal growth by Cu and Cd resulted in altered biomass distribution within the colony when grown on metal free medium. Valix and Loon (2003) observed the heavy tolerance development of *Aspergillus niger*, *Penicillium simplicissimum*, *Aspergillus foetidus* and *Aspergillus carbonarius* strains in the presence of Ni, Co, Fe, Mg and Mn up to concentrations of 2000 ppm. The results indicated that these initial adaptive behaviours were reflections of the strains tolerance development with increasing metal concentration.
Plaza et al. (1998) investigated the effect of Cd on the mycelial growth of some potentially pathogenic soil fungi. Final colony diameter, radial growth rate and final mycelial dry mass (for Cd: 1-200ppm) were measured. Among the keratinolytic fungal group, the genera *Arthrographis*, *Trichophyton* and *Chrysosporium* were the most resistant to Cd. *T. mentagrophytes* was the most sensitive of all *Trichophyton* species tested. Among the nonkeratinolytic fungal group, the genera *Pseudallescheria*, *Absida* and *Rhizopus* were highly resistant to Cd than strains of *Penicillium*. Zygamycetes were more resistant to Cd than Ascomycetes with fungi *Imperfecti*. Non keratinolytic fungi showed higher resistant to Cd than keratinolytic fungi.

Levinskaite (2002) tested soil fungi for their resistance to 0.2-2mM chromium (VI), using K₂Cr₂O₇. They found most sensitive were *Acremonium sp.* and *Penicillium decumbens* whose growth was inhibited completely at 1 mM chromium in the medium. More tolerant fungi were not affected up to 0.5 mM Cr concentration, and the most resistant *Trichoderma viride* and *Penicillium chrysogenum* were able to develop in the presence of 2mM chromium in the medium. Our work is further supported by other workers (Gadd and Griffiths, 1980b; Baldrian and Gabriel, 2002; Baldrian, 2003; Fomina et al. 2003) where a decrease in growth rate in the presence of toxic metals is sometimes accompanied by an increase in the lag phase (or growth delay). A considerable increase in the lag period was observed for *T. virens* and *C. rosea* grown with copper and cadmium. Mercury and cadmium exhibited also the highest toxicity towards *S. hirsutum* (Baldrian and Gabriel, 1997). The essential metals are relatively less toxic. Growth of biomass of *P. chrysosporium* was limited in the presence of 50 ppm Ni, Cd, and Pb, whereas in the case of Co and Cu, decrease of the growth rate was apparent in 150 ppm and in the case of Mn in 300 ppm (Falih, 1998 and 1997). In *Ganoderma lucidum*, toxicity of heavy metals decreased in the order Hg > Cd > Cu > U > Pb > Mn = Zn (Tham et al., 1999).

The decrease of fungal growth rate is sometimes accompanied with the increase of the lag phase. The presence of metals also interferes with the colonization of soil (Baldrian et al., 2000). Changes in mycelial morphology have also been observed in Stereum hirsutum and Trametes versicolor cultivated with cadmium and mercury (Baldrian and Gabriel, 1997; Fomina et al., 2000 and 2003), in *Mucor rouxii* in the presence of a high copper concentration (Gardea-Torresdey et al., 1997), and in
ectomycorrhizal fungi during growth in metal-containing (copper, aluminium, zinc) media (Jones and Muehlchen, 1994). It was also found that biomass distribution within Trichoderma viride colonies was altered by toxic metals, with biomass concentrated in the periphery of the colonies in the presence of copper and towards the interior of the colonies in the presence of cadmium (Ramsay et al. 1999; Gadd et al. 2001).

Spore germination was found to be more sensitive to Ni$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$ than mycelial growth (Amir and Pineau, 1998). However, a proportion of the spores of metal-sensitive strains of Curvularia sp. and Fusarium sp. were able to germinate and grow moderately well in the presence of relatively high metal concentrations (Amir and Pineau, 1998).

5.7 Effect of Heavy Metal Ions on Phosphate Solubilization and IAA Production by Selected Plant Growth Promoting Fungi

Many metals are essential for fungal growth and metabolism (e.g. sodium, potassium, copper, zinc, cobalt, calcium, magnesium, manganese, Fe), but all can exert toxicity when present above certain threshold concentrations. Other metals (e.g. cadmium, mercury, lead) have no known biological function but can still be accumulated by fungi (Gadd, 1993). In the case of a toxic metal-containing domain, aggregated mycelia could produce high local concentrations of extracellular products such as complexing agents (e.g. organic acids, siderophores, polyphenolic compounds), metal precipitating agents (e.g. oxalate), and polysaccharides and pigments with metal-binding abilities (Gadd, 1993a; Dutton and Evans, 1996; Morley et al., 1996; Baldrian, 2003).

It has also been suggested that cations increase membrane stability and that they may also play specific roles in nucleic acid structure, functions and metabolism (Dedyukhina and Eroshin, 1991). However, when the concentrations of beneficial metals in the environment are excessively high, or when metals with no known essential biological functions are present, for instance, mercury, lead, or cadmium, can become toxic (Gadd, 1986; Dedyukhina and Eroshin, 1991, Errasquin and Vazquez, 2003).
After heavy metals enter the fungal cell, they affect both individual reactions and complex metabolic processes. Cd and Hg are in general the most toxic metals almost for all Fungi (Lilly et al., 1992).

Toxicity of heavy metals to fungi is determined in terms of their minimum inhibitory concentration (MIC) or minimum fungicidal concentrations (MFC) or inhibition in radial growth and activities of the organisms. Biomass and activity of the organism in vitro could be more precisely studied at sub MIC concentrations. In the present study we assessed the effect of heavy metal ions (50 to 300 μg/ml) on phosphate solubilization, IAA production and growth of some selected fungal isolates (Aspergillus, Penicillium, Rhizopus, Fusarium and Curvularia) in liquid medium.

In our present investigations of phosphate solubilization and IAA production, we observed that at lower concentration (50 μg/ml) of the metals tested, the activity and the growth of fungal isolates were increased significantly as well as insignificantly, while at higher concentrations, reduction in activity and growth of fungal isolates was observed as compared to control. On the basis of heavy metal interference, a comparison between activity and fungal growth was carried out and as a result in many cases at higher concentrations, it was observed insignificant reduction in activity while the fungal growth reduced significantly and vice versa among the most of the fungal isolates tested. On the basis of this investigation, we can say that the effect of toxic metals on the activity and growth of the test fungi may be parallel or different both.

In the case of phosphate solubilisation by Aspergillus niger (ASF-07) at 50 and 100 μg/ml of Cr, Ni, and Cu showed either marginal increase or at par with control indicating no significant inhibition of activity. However, Cd showed significant inhibition at above concentration. At 100 μg/ml concentration, Aspergillus niger solubilized maximum amount (453 μg/ml) of TCP after 7 days of incubation against Ni followed by 448, 441 and 165 μg/ml in the presence of Cr or Co, Cu and Cd respectively as compared to control (435 μg/ml). On the other hand, isolate of Aspergillus sydowi (ASF-01) exhibited increase in TCP solubilization only against Ni (419μg/ml) as compared to control (405 μg/ml) at 100 μg/ml concentration after one week of incubation time. At higher concentration >200 μg/ml both activity and biomass production of Aspergillus niger and Aspergillus sydowi was decreased over
control. The decreasing trend of activity against metal was in order of Cd>Cu>Ni>Cr for *Aspergillus niger* and Cd>Co>Cu>Cr>Ni for *Aspergillus sydowi* at 300 μg/ml concentration.

It is not surprising to note that Cd was found toxic in affecting the phosphate solubilising activity and biomass production. However, the effect of different metal varies with fungal cultures. *Alternaria* sp-01(ALF-01) showed maximum increase in TCP solubilization against Co (495μg/ml) and reduction against Cd (224 μg/ml) at 100 μg/ml concentration. At higher concentration of all metals tested, the *Alternaria* sp-01 exhibited significant amount of TCP reduction. In the case of *Penicillium* sp-03 (PNF-03), the TCP solubilization was decreased only against Cr (256 μg/ml ) and Cd (232 μg/ml ) at 100 μg/ml concentration But it was observed a significant increase (9.12%) against Ni (100 μg/ml ) over control. The order of toxicity on activity against *Alternaria* sp-01 was Cd>Cr>Cu>Co>Ni at 300 μg/ml. *Curvularia clavata* (CVF-02) also showed similar trend of metal ions effect on TCP solubilization in order of Cd>Co>Cu>Cr>Ni. Similarly, *Fusarium* sp-01(FUF-01) activity was affected in order of Cd>Cu>Cr>Co at higher concentration.

*Rhizopus oryzae* (RZF-02) showed the highest TCP solubilization against cobalt (356 μg/ml) and minimum against cadmium (225 μg/ml) at 100 μg/ml concentrations after 7 days of incubation time over control. *Rhizopus oryzae* also exhibited reduction of TCP solubilization against cadmium at 50μg/ml concentration.

In this present study, seven selected fungal isolates were also tested for the production of IAA in the presence of different metal concentrations (50-300 μg/ml). In general, fungal cultures at lower concentrations of Cr, Ni and Cu showed maximum variation in response to IAA production but at higher concentration (300 μg/ml) IAA production was decreased along with reduction in biomass over control. However, cadmium could decrease the IAA and biomass production at all tested concentrations. The order of toxicity of metal on IAA production was Cd>Cu>Co>Cr>Ni at 300 μg/ml for all fungal isolates (*Aspergillus niger*, *Aspergillus sydowi*, *Alternaria* sp-01, *Penicillium* sp-03, *Fusarium* sp-01, *Curvularia clavata*) except *Rhizopus oryzae* where the order of metal adversely affected on IAA production was Cd>Cu=Co=Cr>Ni. At lower concentration of Cr, Ni and Co IAA production increased to different extent in certain fungi including *Alternaria* sp-01. It
is interesting to note that certain metals like Cr exerted more percent reduction in biomass as compared to percent reduction in IAA production with their respective controls at the same concentration of metal. For example: Cr\(^{6+}\) exerted 37.28% reduction in biomass and 28.11% reduction in IAA at 300 \(\mu\text{g/ml}\) against *Aspergillus niger* (ASF-07). Similar effect was also observed against Cd. However, it was observed a slight decrease (45.76%) in biomass production and more decrease (68.33%) in IAA activity against Cu over their respective controls. This has indicated that biomass and activity was affected differently by heavy metals in fungi. This behavior of metal effect on IAA and biomass production differ at different concentration of metals. This is probably due to the different physiological response of fungi to different metals and its different concentrations. The toxic effect of both essential and non-essential metals is known at higher concentration (Gadd, 1986; Dedyukhina and Eroshin, 1991, Errasquín and Vazquez, 2003). However, their specific effect on IAA production in fungi is not known to best of our knowledge.

It is expected that at least in some fungi, fungal metabolites like siderophores and organic acids may able to bind metals (magnesium, manganese, chromium) or macromolecules such as polysaccharides, (Gadd and Griffiths, 1978; Birch and Bachofen, 1990) that may affect metal bioavailability and toxicity. In the recent year, Dimkpa *et al.* (2008) investigated streptomyces strains which can produce auxin and siderophores simultaneously. However, Al\(^{3+}\), Cd\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\) and Ni\(^{2+}\), or a combination of Fe and Cd, and Fe and Ni affected auxin production negatively as revealed by spectrophotometry and gas chromatography-mass spectrometry. This effect was more dramatic in a siderophore-deficient mutant. They also found that siderophores promote auxin synthesis in the presence of Al\(^{3+}\), Cd\(^{2+}\), Cu\(^{2+}\) and Ni\(^{2+}\) by chelating these metals. Chelation makes the metals less able to inhibit the synthesis of auxins, and potentially increases the plant growth-promoting effects of auxins, which in turn enhances the phytoremediation potential of plants.

The influence of heavy metal addition on total, bacterial, and fungal activities was studied by Rajapaksha *et al.* (2004). Fungal activity (acetate-in-ergosterol incorporation rate) initially increased with the level of metal contamination, being up to 3 and 7 times higher than that in the control samples during the first week at the highest levels of Zn and Cu addition, respectively. The positive effect of metal addition on fungal activity then decreased.
Lorenz et al. (2006) studied the As and Cd effect on microbial function and community composition in soils and they observed that Arsenic (As) and cadmium (Cd) in soils can affect soil microbial function and community composition and, therefore, may have effects on soil ecosystem functioning. Application of biosolid on land has been widespread in numerous countries for last several decades. Kao et al. (2006) reported about the effects of metal containing biosolid on soil microorganisms. The supplemented Cu, Pb and Zn in biosolid reduced the mineralized C by roughly 36%. This phenomenon was probably caused by a portion of the Cu, Pb and Zn being complexed with organic matter to prevent decomposition of organic carbon by microorganisms. Equally, soil treated with biosolid increased the quantity of mineralized N by approximately five-fold and accelerated the rate of N mineralization by about one-fold compared to untreated soil.

The addition of heavy metals in the soil–biosolid mixture clearly reduced the microbial biomasses C (MBC) and N (MBN), indicating that the microbial activities had been disrupted by the heavy metals. The microbial biomass C/N ratio had changed initially from 8 to 13 at the end of incubation period, owing to various groups of microbes expressing different mechanisms of metabolism. However, when the concentrations of beneficial metals in the environment are excessively high, or when metals with no known essential biological functions are present, for instance, mercury, lead, or cadmium, can become toxic (Gadd, 1986; Dedyukhina and Eroshin, 1991; Errasquin, and Vazquez, 2003).

Gibson and Mitchell (2005) reported the heavy metal effect on phosphatase enzyme activity of Ericoid endomycorrhizal fungi (two isolates of Hymenoscyphus ericae obtained from unpolluted heathlands and two H. ericae-type endophytes isolated from Calluna vulgaris growing on Cu-contaminated mine spoil). The elevated levels of Cu in the medium had no effect on the growth of the two H. ericae-type endophytes from mine spoil sites but caused a significant reduction in growth of the two H. ericae isolates from unpolluted sites. Wall, cytoplasmic and extracellular fractions were assayed for phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activity. K (m) and V (max) values varied between the different endophytes and both were highest in the wall fractions. Wall-bound phosphatase activity, excluding PDEase of one H. ericae-type endophyte, was generally unaffected after the isolates had been grown on medium containing 0.25 mM Cu. Extracellular PDEase of
the two H. ericae-type endophytes from mine spoil sites was stimulated by 0.25 mM Cu in the growth medium. Cu concentrations up to 5.0 mM in the assay medium did not inhibit wall-bound phosphatase activity whereas three of the isolates showed a stimulation of extracellular activity with increasing Cu. Zhau et al. (2009) also reported the effect of zinc ions on soil microbiological biomass and activities.

Some people also reported the similar metal toxicity on bacterial activity like our findings of soil filamentous fungi for example-

Similar findings were also observed in agreement of our observations by Wani et al., 2007. They reported that the production of IAA by the strain of bradyrhizobium decrease progressively with increase in nickel and zinc concentration but did not differ significantly among the treatments.

Gupta et al. (2001) examined the heavy metal effect on siderophore production by Pseudomonas aeruginosa. Different metal ion compounds (ZnSO$_4$, MnCl$_2$, FeCl$_3$, and MnSO$_4$) of different ions concentrations were used and observed their effect. Individually ZnSO$_4$ (12μm) promoted siderophore production but suppressed the growth and protein content of test organism, MnCl$_2$ and FeCl$_3$ (12μm) enhanced the growth, whereas MnCl$_2$ and MnSO$_4$ (12μm) induced protein contents of test organism.

5.8 Biosorption of Heavy Metals (Cr$^{6+}$, Cd$^{2+}$, Ni$^{2+}$) by Fungal Biomass

5.8.1 Effect of pH on biosorption

Fungal metal uptake is essentially a biphasic process consisting of a metabolism-independent and metabolism-dependent step. The initial biosorption step is rapid and independent of temperature metabolic energy, the presence of a metabolisable energy source and the presence of metabolic inhibitors (Mowll and Gadd, 1984; de Rome and Gadd, 1987; White and Gadd, 1987). Often this initial binding is thought to involve the microbial cell wall, although extracellular polymers may be responsible in some cases. Binding is attributed to ion-exchange, adsorption, complexation, precipitation and crystallisation within the multilaminate, microfibrillar cell wall structure (Remacle, 1990; Volesky, 1990). Biosorption is exclusively responsible for metal accumulation by non-viable biomass (Avery and Tobin, 1992) owing to the absence of metabolic activity necessary for intracellular metal
accumulation. However, it is known that various factors like treatment of biomass and pH has a significant effect on the metal removal from solutions. Therefore it is prerequisite to determine the optimum pH for biosorption of individual metal by fungal biomass. In this study, the biosorption experiment of heavy metals by fungal biomass was carried out at different range of pH (2.5 to 6.0), maintaining the constant biomass (0.1g), initial metal ions concentration (2 mM), and temperature (25°C). Optimum pH for maximum biosorption was found in the range of 2.5 to 4.5 for different metals and fungal biomass. Maximum biosorption/adsorption of Cr\(^{6+}\) ions were observed at pH 2.5 for *Aspergillus niger*, *Rhizopus oryzae*, *Penicillium* sp-03, *Curvularia* sp-06, *Mucor* sp-01, *Fusarium* sp-01, *Curvularia clavata* while *Aspergillus sydowi* (ASF-01), *Aspergillus* sp-02 (ASF-02) and *Aspergillus* sp-03 (ASF-03) exhibited maximum Cr\(^{6+}\) ions biosorption at pH 3.5, 3.0 and 4.0 respectively. The biosorption was decreased by increasing the pH value above the optimum pH. For Cd ions biosorption the optimum pH value for the fungal biomass were recorded 4.0 or 4.5 for one or more than one fungi tested. Isolate *Aspergillus* sp-03 showed optimum pH 3.5 for Cd. The optimum pH value for Ni ions biosorption was also varied from 3.5 to 4.5 among different fungal isolates. Our findings are comparable with the reports of other workers. Negligible sorption at pH values lower than 4.0 was reported by Tien and Huang, (1987), Delgado et al. (1998) and Wang et al. (1999). Leung et al. (2001) stated that Zn uptake was negligible at pH 2.0 and then increased rapidly with increasing pH. A trend of increasing metal ion binding with increasing pH by nonliving biomass of the *Fusarium flocciferum* was observed for three metal ions copper, cadmium and nickel (Delgado et al., 1998).

Our findings and results of above workers may be explained by competition between hydrogen ions and metal ions for the sorption sites of cells. At very low pH values, metal cations and protons compete for binding sites on the cell walls, which results in lower metal uptake. As pH levels are increased, more ligands with negative charge would be exposed with a subsequent increase in attraction for positively charged metal ions. It has been recognized by Crist et al. (1994) that the main effect of pH on metal ion binding consists of a reduction in the number of binding sites available with decreasing pH. In addition to this competition between protons and metal ions for binding sites, there are also some other ways in which pH influences sorption. Since adsorption depends not only on the attraction of the sorbate to the
solid surface but also on its lyophobic behavior (sorption increases with decreasing solubility), for most metals that means adsorption increases with increasing pH (Schiewer and Volesky, 1995). Also, the solution pH affects the surface charge of the adsorbent, the degree of ionization, and the speciation of the surface functional groups like carboxylate, phosphate and amino groups of the cell wall. Moreover, pH variations can modify the speciation and the availability of the metal cations in solution as well as the chemical state of the biomass functional groups responsible for metal binding (Fourest et al., 1994; Aksu and Akpynar 2000; Reddad et al., 2002).

Gonzalez et al. (2001) stated that the ionization constants for different carboxyl groups were around 3.0-4.0. This means at pH values less than 3, the carboxyl groups become protonated and thus no longer available to attract metal ions from solution. On the contrary, when the pH is higher than 4, the carboxyl groups are deprotonated and therefore negatively charged and able to bind positively charged metal ions. On the other hand, too high pH values, which cause precipitation of metal complexes where distinguishing between sorption and precipitation metal removal becomes difficult, should be avoided during sorption experiments. Holan and Volesky (1994) found that the danger of microprecipitation starts at pH above 5.0 for lead and at pH 6.0-7.0 for nickel. Similarly, Stumm and Morgan (1996) reported that hydroxyl species are formed above pH 8 for cadmium. On the other hand Bai and Abraham (2001) reported that the optimum pH for biosorption of Cr (IV) was found to be 2.0. Higher adsorption percentage was noted at lower initial concentrations of Cr ions.

5.8.2 Effect of Initial concentration on heavy metal biosorption

After the screening of optimum pH value for maximum biosorption for specific fungal biomass, the biosorption process was carried out at varying concentrations of heavy metal (Cr, Cd and Ni) with constant temperature, time, rpm speed, optimum pH values and fungal biomass. The biosorption process was conducted by the dead and living biomass of the fungi in single and multi-metal ions solution.
5.8.3 Biosorption by dead and living fungal biomass in single and multi-metal ions solution

Dead fungal biomass demonstrated varying biosorption depending upon the initial concentration of the metal. All fungal isolates showed maximum sorption of metal ions (Cr, Cd and Ni) at 4 mM and 6 mM concentration. If concentration was increased above the limit of optimum concentration, the biosorption was decreased in the case of all fungal biomass tested. The maximum biosorption of chromium metal from ionic solution was recorded 26.3 mg/g by *Rhizopus oryzae* followed by *Penicillium* sp-03, *Fusarium* sp-01, *Aspergillus niger* or *Aspergillus* sp-02, *Mucor* sp-01, *Aspergillus* sp-03, *Aspergillus sydowi*, *Curvularia clavata* and *Curvularia* sp-06. For cadmium ions biosorption, the order of the efficient fungal biomass was observed as *Mucor* sp-01 > *Aspergillus sydowi* > *Aspergillus niger* > *Penicillium* sp-03 > *Rhizopus oryzae* > *Curvularia clavata* > *Aspergillus* sp-03 > *Fusarium* sp-01 > *Aspergillus* sp-02 > *Curvularia* sp-06. The maximum Cd ions were bioadsorbed 22.5 mg/g and minimum was 18.3 mg/g by *Mucor* sp-01 and *Curvularia* sp-06 biomass respectively. The order of fungal biomass for Ni ions sorption was recorded *Rhizopus oryzae* > *Aspergillus niger* > *Aspergillus* sp-03 > *Mucor* sp-01 > *Fusarium* sp-01 > *Curvularia* sp-06 > *Aspergillus* sp-02 > *Penicillium* sp-03 > *Aspergillus sydowi* and *Curvularia clavata*.

For bi- or multi-metal ions adsorption, biosorption capacity of individual metal ions was reduced in the presence of other metal ions, but total biosorption capacity might be increased or decreased, indicating the capability of fungal biomass in adsorbing multi-metal ions.

Fungal biomass showed sorption of metal ions in lower amount in multi-metal ions solution as compared to single metal ion solution. The sorption of chromium metal ions was observed highest (7.1 mg/g) by *Aspergillus sydowi* and least (2.1mg/g) biosorption was recorded by the biomass of *Penicillium* sp-03. At higher concentration, the isolates exhibited sorption less than lower metal ions concentrations. Maximum sorption of cadmium was recorded 10.5mg/g for *Aspergillus sydowi*. *Rhizopus oryzae* demonstrated highest sorption while *Aspergillus* sp-03 showed minimum sorption for nickel among all the fungal isolates.
In the case of living biomass of the metal tolerant fungal isolates it was observed lesser biosorption in single and multi-metal ions solution both as compared to dead fungal biomass. Similar biosorption trend was observed in single and multi-metal ions solutions by living as well as dead fungal biomass.

Our findings are almost agreement with the reports of some other workers. Yakup et al. (2004) investigated effect of pH and maximum adsorption of metal ions on the calcium alginate and both live and inactivated immobilized fungal preparations of *Fumalia trogii* at pH 6.0. The biosorption of Hg$^{2+}$, Cd$^{2+}$ and Zn$^{2+}$ ions on the Ca-alginate beads and on both immobilized forms was studied in aqueous solution in the concentration range of 30-600 mg/l. The metal biosorption capacities of the heat inactivated immobilized *F. trogii* for Hg$^{2+}$, Cd$^{2+}$ and Zn$^{2+}$ were 403.2, 191.6, and 54.0 mg/g respectively, while biosorption capacities of the immobilized live form were 333.0, 164.8, and 42.1 mg/g respectively. The same affinity order on a molar basis was observed for single or multi-metal ions. Our findings are also similar or contradictory with other workers for example:

Niu et al. (1993) studied the removal of lead ions from aqueous solutions by adsorption on nonliving *Penicillium chrysogenum* biomass. Biosorption of the Pb$^{2+}$ ion was strongly affected by pH. Within a pH range of 4 to 5, the saturated sorption uptake of Pb$^{2+}$ was higher than that of some other microorganisms. At pH 4.5, *P. chrysogenum* biomass exhibited selectivity for Pb$^{2+}$ over other metal ions such as Cd$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and As$^{3+}$ Sorption preference for metals decreased in the following order: Pb > Cd > Cu > Zn > As.

Volesky and May-Phillips (1995) observed that live and non-living biomass of *Saccharomyces cerevisiae* differed in the uptake of uranium, zinc and copper at the optimum pH 4-5. Non-living brewer's yeast biomass accumulated 0.58 mmol U/g. The best biosorbent of zinc was non-living baker's yeast (approximately 0.56 mmol Zn/g). Dead cells of *S. cerevisiae* removed approximately 40% more uranium or zinc than the corresponding live cultures.

Mogollon et al. (1998) reported that the metal uptake of a *Rhizopus sp.* strain was corroborated by electron microscopy. Nickel accumulation by the selected strains is fast, occurring in less than 30 min, and did not require a microorganism's active metabolism to take place.
Bai and Abraham (2001) reported that the optimum pH for biosorption of Cr (IV) was found to be 2.0. Higher adsorption percentage was noted at lower initial concentrations of Cr ions. Yan and Viraraghavan (2001) reported *Mucor rouxii* biomass to remove metal ions such as Pb, Cd, Ni and Zn not only from single component metal solutions but also from multi-component metal solutions. They observed that the metal removal capacities of the biomass were higher in single-component metal solutions, than multi-metal ions solution individually. In single-component metal solutions, Pb, Cd, Ni and Zn were 4.06, 3.76, 0.36, and 1.36 mg/g respectively and in multi-component metal solution containing Cd, Ni and Zn, the capacities were 0.36, 0.31 and 0.40 mg/g Cd, Ni and Zn respectively.

Pradhan and Rai (2001) reported that the biosorption of Cu, Zn and Cd by *Microcystis sp.* in single, bi and trimetallic combination. Highest biosorption of Cu followed by Zn and Cd in single as well as in mixture containing two or three metals was noticed. The order of inhibition of Cu, Zn and Cd biosorption in bi and trimetallic combinations was suggestive of screening or competition of the binding sites on the cell surfaces. Tsekova and Petrov (2002) also proved the decreased metal sorption in multi-metal system in the comparison of single metal system by fungal biomass which is agreement with our findings. Yan and Viraraghavan (2003) reported that the dead biomass of *Mucor rouxii* adsorbed metal ions in the order of Pb$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ and Ni$^{2+}$, with the biosorption capability of 25.22, 16.62, 8.36 and 6.34 mg/g at pH 5.0 respectively. At pH 6.0, the capacity of the dead biomass increased to 53.75, 53.85, 20.31 and 20.49 mg/g, respectively. For bi- or multi-metalions adsorption, biosorption capacity of individual metal ions was reduced in the presence of other metal ions, but total biosorption capacity increased, indicating the capability of *Mucor rouxii* biomass in adsorbing multi-metal ions.

Bai and Abraham (2003) reported biosorption of Cr(VI) from aqueous solution, by many immobilized biomass of *Rhizopus nigricans*. The Cr sorption capacity (mg Cr/g sorbent) of all immobilized biomass was lesser than the native powdered biomass. The CR sorption capacity decreased in the order of free biomass (119.2) > polysulfone entrapped (101.5) > polyisoprene immobilized (98.76) > PVA immobilized (96.69) > calcium alginate (84.29) > polyacrylamide (45.56), at 500 mg/l concentration of Cr (VI). Dong (2004) studied entrapped with alginate live and inactivated spores of *Cladosporium* sp. for biosorption capacity from aqueous
solutions. He reported that the Ca-alginate beads containing live spores of Cladosporium sp. had the maximum biosorptive capacity. The maximum biosorption of Cu (II) on Ca-alginate entrapping spores and no spores were obtained between pH 4.0 and 3.5. The biosorptive capacity increased with initial concentrations in the concentration range of 30-800mg/l.

Ahmad et al. (2006) studied metal tolerant fungal isolates for biosorption capacity. Fungi including Aspergillus and Penicillium, resistant to Ni^{2+}, Cd^{2+}, and Cr^{6+} were Two isolates, Aspergillus niger and Penicillium sp. were tested for their Cr, Ni and Cd biosorption potential using alkali treated, dried and powdered mycelium. Biosorption experiment was conducted in 100 ml of solution at three initial metal concentrations i.e., 2, 4 and 6 mM with contact time (18 hr) and pretreated fungal biomass (0.1g) at 25 degrees C. At 4 mM initial metal concentration, chromium biosorption was 18.05 and 19.3 mg/g of Aspergillus and Penicillium biomass, respectively. Similarly, biosorption of Cd and Ni ions was also high at 4 mM initial metal concentration by Aspergillus (19.4 mg/g for Cd and 25.05 mg/g of biomass for Ni) and Penicillium (18.6 mg/g for Cd and 17.9 mg/g of biomass for Ni). In general, biosorption of metal was influenced by initial metal concentration and type of the test fungi.

Eleven out of total 38 isolated fungi which tolerated > 6.0 mM Co (II) were evaluated by Pal et al. (2006) for cobalt biosorption using dried mycelial biomass. Maximum Co(II)-loading (1036.5μM/g, 60 min) was achieved with Mortierella SPS 403 biomass, which removed almost 50% of 4.0 mM cobalt from the aqueous solution. The optimum pH was 7.0. However, Co (II)-uptake was inhibited in the presence of other metals (Pb, Cd, Cu, Ni, Cr and Zn). Naeem et al. (2006) studied Proton, Cd, Pb, Sr, and Zn adsorption onto the fungal species Saccharomyces cerevisiae. The affinity of the fungal cells for the metal ions follows the following trend: Pb > Zn > Cd > Sr. they used the metal adsorption data to determine site-specific stability constants for the important metal fungal surface complexes.Melgar et al. (2007) investigated biosorption of the metals like zinc, copper, mercury, cadmium or lead by living or non-living biomass of A. macrosporus from an acid solution, acid solution supplemented with potassium and phosphorus, and an alkaline solution. Results of metal uptake showed the maximum percentage uptake of all metals was found to occur at alkaline pH (Cu 96%, Pb 89%). With living biomass,
metal biosorption was greater and faster in K/P-supplemented acid medium than in non-supplemented acid medium. Zafar et al. (2007) reported biosorption of Cr and Cd by *Aspergillus* and *Rhizopus* isolates at initial metal concentrations of 2, 4, 6 and 8 mM. Maximum biosorption of Cr and Cd ions was found at 6 mM initial metal concentration.

Tsekova et al. (2007) reported the biosorption of copper and cobalt, both singly and in combination (in equimolar concentrations), by the resting cells of *Penicillium brevicompactum*. He observed that the adsorption of binary mixtures of heavy metal solutions on the fungal biomass was found to be of competitive type where the adsorption capacity for any single metal decreased in the presence of the other. Bishnoi et al. (2007) reported pH dependent biosorption of Cr (VI). He observed the maximum adsorption at pH 2.0 by using the powdered biomass of *Trichoderma viride*. Mukhopadhyay et al. (2007) studied Copper sorption by *A. niger* was influenced by the biomass dose, initial metal ions concentration, and pH of the solution. The retention capacity of the biomass was determined at pH 6.0 to be equal to 23.62 mg/g of biomass. The untreated, heat- and alkali-treated *Lentinus sajor-caju* mycelia were used for the recovery of uranium from aqueous solutions by Bayramoğlu (2006). The alkali treated form had a high biosorption capacity (378 mg/g) than those of the untreated (268 mg/g) and heat-treated fungal mycelia (342 mg/g). Optimum biosorption was observed at pH 4.5 for all the tested fungal preparations and was independent of temperature (5-35 degrees C). Akar et al. (2007) found the maximum lead (II) biosorption capacity of the fungal biosorbent (*Aspergillus parasiticus*) was found as $4.02 \times 10^{-4}$ mol g$^{-1}$ at pH 5.0 and 20 degrees C.

Das and Guha (2007) found the sorption of hexavalent chromium by live *Termitomyces clypeatus* (TCB) depends on the pH of the solution, the optimum pH value being 3.0. The amino, carboxyl, hydroxyl, and phosphate groups of the biomass were involved in chemical interaction with the chromate ion forming a cage like structure depicted by scanning electron microscopic (SEM) and Fourier transform infrared spectroscopic (FTIR) results. Bhainsa and D'Souza (2008) studied the treated biomass of *Rhizopus oryzae* for biosorption. Investigations on effect of pH indicated improved performance in the range of pH 4-6 in alkali treated biomass. The maximum copper loading capacity of the viable and pretreated biomass according to Langmuir isotherm was 19.4 and 43.7 mg/g, respectively. Similarly, others workers also
reported the biosorption capacity of living and nonliving biomass of fungi differently (Pan et al., 2009; Amini et al., 2009; Pakshirajan and Swaminathan 2009)

5.8.4 Biosorption by metal trained and metal untrained dead and living fungal biomass in single and multi-metal ions solution

In the recent years, use of metal tolerant fungal biomass for metal removal from aqueous solution has been documented. Using actively growing metal tolerant fungi will be an added advantage to overcome metal toxicity. However, for dead biomass resistant traits seems to be of a little importance. Although there is an assumption among workers that metal tolerant fungal strain may be more efficient in metal removal. Probably in some tolerance mechanisms, high level metal binding may also be involved on outer surface of cell. Therefore, we have selected fungal isolates showing certain level of tolerance to metal like Cd, Cr and Ni (parent strain). These cultures were sequentially exposed to higher concentration and more tolerant cultures were obtained on metal supplemented plates. Such trained fungal cultures derived from less tolerant parent were subjected to metal biosorption to assess their difference if any.

In our present study we also observed that dead fungal biomass showed a little difference between sorption capacities of metal untrained and trained. Dead biomass of untrained Aspergillus niger demonstrated maximum sorption of 22.3, 20.4 and 25.0 mg/g while metal trained biomass showed 24.1, 21.5 and 25.9 mg/g at 4mM concentration of chromium, cadmium and nickel respectively. Dead biomass of metal untrained Penicillium sp-03 also exhibited less sorption of metal ions of chromium, cadmium and nickel as compared to metal trained Penicillium sp-03. Cadmium and nickel metal ions sorption from aqueous solution was also increased by metal trained dead biomass of Rhizopus oryzae as compared to metal untrained isolate but in the case of chromium ions sorption it was observed a decrease as compared to metal untrained isolate of Rhizopus oryzae at 2mm, 4mm and 6mm metal concentrations.

In the multi-metal ions solution the sorption of metal ions was also recorded less than single metal ions solution, the trend of metal ions sorption by metal trained and untrained dead fungal isolates was observed almost similar to the single metal ions solution. In the case of living biomass of metal trained and untrained fungal
isolates it was found almost similar trend of biosorption in single and multi-metal ions solution.

Our findings are found in the agreement of other workers. Magyarosy et al. (2002) reported the biosorption capacity of metal tolerant *Aspergillus niger* isolated from a metal contaminated soil which was able to grow in the presence of cadmium, chromium, cobalt, copper and usually high levels of nickel on solid (8.0 mM) and in liquid (6.5mM) media. They observed that the fungus removed >98% of the nickel from liquid medium after 100 hrs of growth but did not remove the other metals, as determined by inductively coupled plasma spectroscopy.

Zafar et al. (2007) reported biosorption of Cr and Cd metal tolerant and sensitive or less tolerant *Aspergillus* sp.2 and *Aspergillus* sp.1 Accumulation of these two metals by very tolerant *Aspergillus* sp.2 isolate was at par with relatively less tolerant *Aspergillus* sp.1 isolate. There is little, if any, correlation between metal tolerance and biosorption properties of the test fungi. Gardea-Torresdey et al. (1997) reported the biosorption capacity of the metal tolerant and metal sensitive strain of *Mucor rouxii* that metal tolerant strain exhibited high adsorption of metal ions than metal sensitive strain.

The fungal isolates used by the above described workers might be different in respect to their metal biosorption potential as compared to our isolates studied.

5.8.5 Adsorption isotherm

In our investigations, we observed that dead biomass with $Q_{max}$ value was found to have a higher adsorption capacity as compared to other live fungal biomasses tested. Dead biomass of *Aspergillus* sp-03 showed maximum $Q_{max}$ value of 13.35mg/g for Cr ions followed by dead biomass of *Rhizopus oryzae*, *Mucor* sp-01, *Fusarium* sp-01, *Penicillium* sp-03, *Aspergillus niger*, *Aspergillus sydowi*, *Aspergillus* sp-02, *Curvularia clavata* and *Curvularia* sp-06. Dead biomass of *Aspergillus niger*, *Aspergillus sydowi*, *Aspergillus* sp-02 and *Aspergillus* sp-03 demonstrated higher $Q_{max}$ value of 9.04, 8.50, 8.40 and 13.35 mg/g as compared to 8.65, 8.04, 8.27 and 12.23 mg/g of Cr ions respectively by the living biomass of the same fungal isolates. Maximum $Q_{max}$ value of Cd ions sorption was observed by dead biomass of *Mucor* sp-01 and minimum $Q_{max}$ value was recorded by the dead biomass of *Aspergillus*
Discussion

Q\textsubscript{max} value of Cd ions sorption was observed 8.87, 7.10 and 9.42 mg/g by dead biomass of *Aspergillus sydowi*, *Aspergillus* sp-02, and *Aspergillus* sp-03 while Q\textsubscript{max} value was recorded 8.56, 6.45 and 8.14 mg/g respectively by living biomass of the same fungal isolates. Dead biomass of *Rhizopus oryzae*, *Penicillium* sp-03, *Fusarium* sp-01, *Curvularia* sp-06 and *Curvularia clavata* demonstrated Q\textsubscript{max} value of 9.28, 8.25, 8.79, 7.65, and 9.55 mg/g for Cd ions sorption respectively. Dead biomass of *Rhizopus Oryzae* showed maximum Q\textsubscript{max} value for nickel ions sorption followed by dead biomass of *Aspergillus* sp-03, *Mucor* sp-01, *Aspergillus niger*, *Aspergillus sydowi*, *Aspergillus* sp-02, *Curvularia* sp-06, *Penicillium* sp-03 and *Curvularia clavata*.

Dead biomass of *Penicillium* sp-03 isolate demonstrated Q\textsubscript{max} value of 7.27 mg/g for nickel ions sorption as compared to 6.86 mg/g by living biomass of *Penicillium* sp-03 isolate.

Some other workers also reported a different range of Q\textsubscript{max} value by different fungal biomasses. For example Deepa *et al.* (2006) reported Q\textsubscript{max} value with 0.335 mg/g for cr adsorption by autoclaved *Aspergillus niger* biomass as compared to other pretreated biomass of the same fungal isolate. Cell surfaces of fungi are mainly anionic due to the presence of ionized groups such as carboxylate, hydroxyl and phosphate in various cell wall polymers. Presence of these ionized groups is highly responsible for adsorption, which varies widely between various fungi (Hughes and Poole, 1989). Sathishkumar (2003) reported a wide variation in the presence of ionic groups among *Aspergillus* sp.

Q\textsubscript{max} values of some fungal sorbents like amino propyl trimethoxy silane treated biomass of *Rhizopus nigricans*, unmodified biomass of *R. nigricans*, formaldehyde treated *R. arrhizus*, autoclaved *R. nigricans* biomass and *Aspergillus niger* biomass were reported to be 200, 123.45, 62.5, 43.47 and 5.1 mg g\textsuperscript{-1} (Bai and Abraham, 2001 and 2002; Dursun *et al.*, 2003; Sag *et al.*, 2001). Presence of less number of these ionized groups could be the possible reason for low adsorption in the present study.

It was reported 4.5 and 8.8 mg/g value of Q\textsubscript{max} for Cr adsorption by using the biomass of *R. arrhizus* by Nourbakhsh *et al.* (1994) and Prakasham *et al.* (1999) respectively. Cd adsorption was also reported by many workers using the fungal
biomass as a biosorbent. The adsorption capacity of Cd was also reported by other workers, i.e. 26.8 mg/g for *R. arrhizus* (Fourest and Roux, 1992) and 18.8 mg/g for *Aspergillus niger* (Malik, 2004) free biomass. Chen *et al.* (2007) used waste biomass of Saccharomyces as biosorbent to adsorb 10 kinds of metal ions, and their maximum biosorption capacity (q (max)) was determined by the Langmuir isotherm model. They reported the values of q (max) decreased in the following order (in millimole per gram): Pb^{2+} (0.413) > Ag^{+} (0.385) > Cr^{3+} (0.247) > Cu^{2+} (0.161) > Zn^{2+} (0.148) > Cd^{2+} (0.137) > Co^{2+} (0.128) > Sr^{2+} (0.114) > Ni^{2+} (0.108) > Cs^{+} (0.092). It suggested that the greater the covalent index value of metal ions was, the greater the potential to form covalent bonds with biological ligands, such as sulphydryl, amino, carboxyl, hydroxyl groups, etc. on the biomass surface, and the higher the metal ions biosorption capacity was. Classification of metal ions, for divalent ion or for soft-hard ion could improve the linear relationship (R (2) = 0.89). Kumar *et al.* (2008) reported Cr (VI) uptake Q_{max} was higher 17.61 mg/g with *A. Niger* than 9.07 mg/g with *A. sydoni* and 9.35 mg/g with *P. janthinellum* biomass. The adsorption capacity of Cr (VI) was also reported by other workers, i.e. 2.66 mg/g for *A. niger* (Kapoor *et al.*, 1999), 12.06 mg/g for *R. nigricans* (Bai and Abraham, 2001), 11.10 mg/g for *R. arrhizus* free biomass.

5.8.6 Removal of heavy metals (Cr, Cd and Ni) from wastewater by soil fungi

In our present investigations, removal of the heavy metals from wastewater was observed significantly less than the metal ions sorption from pure metal ions systems (single or multi). A large number of inorganic and organic substances were found in the wastewater which may create the hindrance of proper binding among functional groups of fungal biomass and metal ions. Due to the presence of different types of heavy metals in wastewater, possibility of competition among the metals ions is increased for binding sites present at functional groups of fungal biomass that is why the adsorption of metals ions from wastewater is decreased individually. The fungal biomass of *Aspergillus niger*, *Penicillium* sp-03, *Aspergillus sydowi*, *Aspergillus* sp-02, *Curvularia* sp-06, *Rhizopus oryzae*, *Aspergillus* sp-03, *Mucor* sp-01, *Fusarium* sp-01, and *Curvularia clavata* demonstrated high level of metal ions sorption at optimum pH 3.5 as compared to biosorption from wastewater at its natural
pH 8.2. This investigation has revealed the importance of optimum pH value for the metal ions removal from wastewater or industrial effluents.

It was recorded almost similar trend of biosorption of particular heavy metals (Cr, Cd and Ni). Similar competition among the metal ions for binding sites present at functional groups of fungal biomass is also reported by many workers (Yan and Viraraghavan, 2001 and 2003; Pradhan and Rai, 2001; Tsekova and Petrov, 2002; Malik, 2004; Pal et al., 2006; Tsekova et al., 2007).

5.9 Interactive Effect of *Mesorhizobium* and Plant Growth Promoting Soil Fungi (PGPF) on Chickpea

Indian soils are poor in nitrogen and about 46 percent of them are low, 52 percent medium and only 2% are high in their phosphorus content (Ghosh and Hasan, 1979). Therefore to supplement these two essential nutrients in order to achieve optimum crop yield chemical fertilizers have widely been used world over. Most developing countries however import these chemical fertilizers, which are often in limited supply and represent an outlay for resource poor farmers. Moreover, the use of chemical fertilizers is reaching the theoretical maximum use beyond which there will be no further increase in crop yield (Ahmad, 1995). However, it is becoming increasingly clear that conventional crop practices can not sustain the production base, a healthy plant soil system, for too long: while, to augment crop productivity agronomists heavily depends on chemical fertilizers. Since the indiscriminate and excessive application of chemical fertilizers affects severely the soil health and causes environmental hazards, agronomists are therefore desperate to find an alternative strategy that can ensure competitive yield while protecting the health of soils. This new approach to farming, often referred to as sustainable agriculture, require agricultural practices that are the friendlier to the environment and that maintain the long term ecological balance of the soil ecosystem. The dependence of fertilizer production on fossil energy source and prospects of diminishing availability costly input of fertilizer in years to come have thus obviously brought the subject of mineral phosphate solubilization (mps) and biological nitrogen fixation (BNF) in the forefront. The microbial systems can siphon out but appreciable amounts of nutrients from the natural reservoirs and enrich the soil with important but scares nutrients. The
crop microbial ecosystem can thus be energized in sustainable agriculture with considerable ecological stability and environmental quality. In this context, the use of microbial inoculants (biofertilizers) in agriculture represents an environment friendly alternative to further applications of mineral fertilizers. A continued exploration of natural biodiversity of soil microorganisms and the optimization/ manipulations of microbial population represent a prerequisite step to developing more efficient microbial inoculants with sustainable plant growth promoting activities. Among the beneficial rhizospheric microorganisms including both symbiotic nitrogen fixers (that forms symbiosis with legume plants) and non symbiotic (associative and free living) have substantially proved then potential in nitrogen contribution of the crop plants (Zakhia and Lajudie, 2001; Dakora, 2002; Sahgal and Johri, 2003; Dobbelaere, et al., 2003; Vessey, 2003). Similarly, phosphorus (P) is one of the major nutrients that restrict the growth of plants severely. Most of the soils throughout the world are phosphorus deficient (Omar, 1998) and therefore require P to replenish the P demand by crop plants. To circumvent the P deficiency in soils phosphate fertilizers are applied. However, after application a considerable amount of P is rapidly transformed into less available form by forming a complex with Al and Fe in acid soils in acid soils or Ca in calcareous soils (Lindsay, et al., 1979) before plant roots have had enhance to absorb it. In this context, the phosphate solubilizing microorganisms (PSM) provides a cheap source of phosphate fertilizer.

Many rhizobacteria and rhizofungi are able to solubilize sparingly soluble phosphates, usually by releasing chelating organic acids (Kucey et al., 1989; Whitelaw, 2000; Richardson, 2001; Vessey et al., 2004). Phosphatesolubilizing bacteria (PSB) have been identified, but their effectiveness in the soil–plant system is still unclear (Barea et al., 2002a).

Many microbial interactions, which are regulated by specific molecules/signals (Pace, 1997), are responsible for key environmental processes, such as the biogeochemical cycling of nutrients and matter and the maintenance of plant health and soil quality (Barea et al., 2004). Many studies have demonstrated that soil-borne microbes interact with plant roots and soil constituents at the root–soil interface (Lynch, 1990; Linderman, 1992; Glick, 1995; Kennedy, 1998; Bowen and Rovira, 1999; Barea et al., 2002b).
Microbial activity in the rhizosphere affects rooting patterns and the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates (Bowen and Rovira, 1999; Gryndler, 2000; Barea, 2000).

\[ \text{Discussion} \]

\[ \text{N}_2\text{-fixation is the first step for cycling N to the biosphere from the atmosphere, a key input of N for plant productivity (Vance, 2001). The bacteria responsible belong to the genera } \text{Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium, and Azorhizobium}, \text{ collectively termed rhizobia. These bacteria interact with legume roots leading to the formation of } \text{N}_2\text{-fixing nodules (Spaink } \text{et al., 1998; Sprent, 2002).} \]

The other major groups of microbial plant mutualistic symbionts are the fungi which establish a (mycorrhizal) symbiosis with the roots of most plant species. The soilborne mycorrhizal fungi colonize the root cortex biotrophycally, then develop an external mycelium which is a bridge connecting the root with the surrounding soil microhabitats.

The AM symbiosis influences nutrient cycling in soil–plant systems, and improves plant health through increased protection against biotic and abiotic stresses, and soil structure through aggregate formation (Bethlenfalvay and Linderman, 1992; Gianinazzi and Schu‘epp, 1994; Smith and Read, 1997; Kapulnik and Douds, 2000; Gianinazzi \textit{et al.}, 2002; Turnau and Haselwandter, 2002; van der Heijden and Sanders, 2002; Jeffries \textit{et al.}, 2003; Barea \textit{et al.}, 2005a; Turnau \textit{et al.}, 2005).

The physiological and biochemical basis of AM fungal \textit{3 Rhizobium} interactions in improving legume productivity indicated that the main effect of AM in enhancing Rhizobium activity is through a generalized stimulation of host nutrition, but some localized effects may also occur at the root or nodule level (Barea \textit{et al.}, 1992). Multi-microbial interactions, including not only AM fungi and \textit{Rhizobium} spp. but also PGPR, have also been tested (Requena \textit{et al.}, 1997).

In our present investigations, the results observed on plant growth promoting fungi in culture medium has led to investigations on the interaction of these organisms in soils using chickpea and wheat as test crops. However, the complexity of inoculation effects of rhizotrophic organisms on legume crops arise from variations in the specific functionality of microorganisms, differences in plant microbe interaction and to variations in microbe-microbe interaction and soil which in turn has led to many contradiction in literature. Yet the increase in plant vitality, symbiotic
traits and yield in crop plants following inoculation with nitrogen fixing and phosphate solubilizing microorganisms either alone or in combination has been reported (Tilak et al., 2005). Indeed, results from our study had clearly indicated the enhancement of the plant growth, nodulation and yield of chickpea grown for consecutively two years in response to plant growth promoting fungi inoculation. In the present study when symbiotic nitrogen fixing bacteria (*Mesorhizobium* sp. in the case of chickpea), and PGPF (*Aspergillus niger, Penicillium* sp-03 and *Rhizopus oryzae*) were used in different combinations, a significant level of plant growth promotion was recorded in certain treatments with the added benefit of greater yield under field soil conditions during the two growing seasons.

The dry matter accumulation was recorded maximum in chickpea in the treatment receiving the consortia of microorganisms viz. *Mesorhizobium* + *Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae* in the year 2006 and 2007. Such an effect can be attributed to the enhanced supply of nitrogen by *Mesorhizobium* and phosphorus by PGPF to host plants. However, when this organism was used with other PGPF, the measured parameters were increased even further as compared to the dual inoculation treatments.

It is believed that PGP traits including IAA and siderophore produced by the PGPF of this study might have accounted for the over all improvement in the growth of chickpea and wheat plants. Similar results have also been observed where Arshad and Frankenberger (1991) and Barazani and Friedman (1999 and 2001) demonstrated the production of auxins and gibberellins and consequently augmented the crop yield. Augmentation in growth parameter may be due to the production of IAA by fungi which promotes plant growth directly by stimulating plant cell elongation and cell division. Low levels of IAA stimulate root elongation; whereas high levels of IAA secreted from high density inocula may stimulate formation of lateral or adventitious roots (Patten and Glick, 2002). Almost similar results have been observed where single and composite application of phosphate solubilizing microorganisms (*Ps. striata* and *Penicillium variable*) have shown considerably positive effect on growth of chickpea (Zaidi et al., 2003 and 2005). However, more direct evidence are needed to indicate that the crop has benifited from PGP functions like IAA production and phosphate solubilisation under field conditions.
Nitrogen content

In the present study, the nitrogen content in chickpea plants (roots + shoots) at vegetative, flowering and harvest stage were greater in majority of the inoculation treatments. Generally, ‘N’ content in chickpea plants was significantly higher at the vegetative stage of plant growth as compared to the flowering (90 DAS) and at harvest (145 DAS). Among all the treatments, composite application of *Mesorhizobium* + *Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae* showed the largest effect on ‘N’ content in chickpea crop at the measured stages of plant growth which was followed by the triple inoculation treatments. However, the ‘N’ content in the crop decreased consistently with the increase of plant age with all inoculation treatments during both the growing seasons. Thus the ‘N’ concentration decreased in the following order: vegetative < flowering < fruiting. The increase in nitrogen contents in chickpea plants treated with microbial inoculation could be due to increase in translocation of soil ‘N’ into the plants or as a result of ‘N’ fixation by *Mesorhizobium*. The increased uptake of nitrogen suggest that a positive interaction between root colonization and ‘N’ uptake derived from ‘N’ fixation to be substantially enhanced above those of un-inoculated control plants.

A linear regression was recorded between the chlorophyll content and N content in chickpea (Fig. 53a and b) which has indicated that with the increase in photosynthetic rates nitrogen content also increases. Dashti *et al.* (1998) reported a similar increase in ‘N’ content in soyabean following the combined application of *Bradyrhizobium* and PGPR (Gupta, 2004)

Symbiotic traits

Infection of the roots of legumes is undoubtedly linked to the expansion of the various parts of the root systems. Therefore, it is possible that *rhizobia* which induces the establishment of an effective symbiosis may increase the number of nodules formed per plant by accelerating the root growth and/or formation of lateral roots, and hence the number of root sites available for infection. Generally, the mixtures of *Mesorhizobium* + *Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae* used in the present study improved the nodulation considerably in chickpea above all treatments, When phosphate solubilizing and plant growth promoting soil fungi (*Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae*) were used in
combination with nitrogen fixing bacterium *Mesorhizobium* have shown stimulatory effect on the nodulation in the chickpea crop. However, among all the treatments, the four cultures i.e. *Mesorhizobium* + *Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae* when used together had the largest effect on the symbiotic properties of the test crop during the two growing seasons. From the results of a two years trial, it seems that the synthesis and release of phytohormones and expression of other PGP traits into the soil environment by the PGPF and enhanced availability of essential nutrients to the plants might have accounted for the synergistic effect on the symbiosis of chickpea. In contrast, the single inoculation of *Mesorhizobium* or fertilized or unfertilized treatment (control) was not sufficient to raise yield of chickpea possibly due to the insufficient availability of the essential nutrients required for the proper growth and development of the crop. These results thus consolidate the involvement of microbial interaction subsequently leading to the enhancement in the productivity of chickpea. Furthermore, the better nodulation in the case of composite inoculation at flowering stage appears to be a result of favorable effects of phosphate solubilizing activity in making more phosphorus soluble and available to the legume crop (Saber, et al., 2005). The phosphate solubilizing activity results in solubilization of the inorganic P (Kang, et al., 2002) through various mechanisms eg, organic acid production (Maliha et al., 2004) and hence have become a valid alternative to chemical phosphatic fertilizers. On the other hand, plant growth promoting microorganisms can improve the extent and quality of plant growth directly through the production of phytohormones or indirectly by facilitating the uptake of certain plant nutrients such as iron from the soil environment (Crowley et al., 1988, Wang et 1993). The crop essentially requires key elements for optimum growth and hence alternative approach is to use these organisms together so that the nutrients can be provided to the crop resulting in enhanced crop productivity. In this regard, the simultaneous application of nitrogen fixing and PGPR (Chanway et al., 1989; Gupta et al., 1998; Gautam and Pant, 2002; Tilak et al., 2005) has been shown to stimulate plant growth more than inoculation of each microorganism alone. However, the information on such interaction and their effect on crop plants, particularly under Indian conditions using fungi, are scarce. To address this problem, the interactive effect of symbiotic nitrogen fixer (*Mesorhizobium*) and PGPF (*Penicillium* sp-03 +
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Aspergillus niger and Rhizopus oryzae) on growth and yield commonly grown Indian crop chickpea, was undertaken.

A linear correlation was also observed between dry nodule weight and nodule number (Fig. 54a and b), total dry weight of plant with dry nodule weight, in chickpea in year 2006 and 2007 (Fig. 55a and b).

It is generally believed that leghaemoglobin plays no active role in symbiotic nitrogen fixation but functions as a biological valve in regulating the supply of oxygen to bacteroids at optimum levels which becomes conducive for proper functioning of nitrogen fixing system. Leghaemoglobin content in fresh nodules collected from root systems of legume recorded at 60 and 90 DAS (chickpea) displayed a maximum level of in the treatment having consortium of four cultures (Mesorhizobium + Penicillium sp-03 + Aspergillus niger and Rhizopus oryzae) which was followed by the triple combination of Mesorhizobium + Aspergillus niger + Rhizopus oryzae. The leghemoglobin thus decreased in the following order: quadruplet > tripartite > dual > single inoculation. Furthermore a strong co-relation was observed between leghaemoglobin content and nitrogenase in chickpea crop (Fig. 56a and b).

Similar enhancement in leghaemoglobin content in legume nodules of greengram and pigeon pea is reported (Gupta et al., 1998; Tilak et al., 2006). The composite application of PGPR in general increased the number of nodules on the roots of inoculated plants possibly due to the because of the production of siderophore (Parmar and Dadarwal, 1999). In other studies rhizobacteria capable of producing siderophores have been found to induce phytoalexins (a class of fluorescent compounds, closely related to flavonoids and isoflavonoids) in the roots of several plants (Ramamorthy et al., 2001).

Moreover, the nitrogenase activity of nodules recorded at 90 DAS was found to be positively affected by the tested fungal cultures. Again, the nitrogenase activity was influenced by microbial combination in the same way as it did the leghaemoglobin and protein content (Fig 57a and b), the linear regression between nitrogenase activity and protein content of seed in chickpea was determined strongly corelated (Fig 58a and b). A lenear correlation between nitrogen and protein content was also observed strongly (Fig. 59a and b). On the other hand the nitrogenase activity
was strongly correlated with seed protein in chickpea. This study thus clearly indicated that the symbiotic properties greatly influenced the productivity of chickpea.

Similar reports on the effect of single or composite applications of rhizospheric microorganisms on nitrogenase or acetylene reduction activity (ARA) is reported (Gupta, et al., 1998; Tilak et al., 2006).

Yield attributes of Chickpea

Seed microbial attachment temporarily changes the balance of the rhizosphere population and such changes may some times enhance the plant growth and consequently the yield of the crops depending upon the establishment of the introduced cultures. Accordingly, the nitrogen fixers (*Mesorhizobium*), phosphate solubilizers and plant growth promoting harmones (IAA and siderophores) producing fungal strains used in the present study were found as good competitors since the growth and yield of chickpea crop was increased synergistically to a greater extent. The plant growth and effective symbiosis observed in this study suggested a strong synergistic relationship between root colonization, nutrient uptake and growth promotion. Simultaneous application of ‘P’ soublizing, nitrogen fixing and PGPF strains has been shown to stimulate plant growth and yield more than inoculation of either organism alone in certain situations when soil is ‘P’ deficient. Accordingly, the combined application of PSF (*Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae*) and (*Mesorhizobium*) substantially increased the yield parameters of the crop. Moreover, the seed production in chickpea was increased even further when three cultures or four cultures were used together. For instance, the triple inoculation of *Mesorhizobium* + *Aspergillus niger* and *Rhizopus oryzae* significantly enhanced the number of pods per plant by 22.22% and consequently the dry biomass of seeds/ plant by 63.99% during both the years.

Furthermore, the consortium of four cultures i.e. *Mesorhizobium* + *Penicillium* sp-03 + *Aspergillus niger* and *Rhizopus oryzae* increased the seed yield by 75.95% in chickpea 2007 as compared to other treatments. In comparison, some of the dual or triple inoculation treatments marginally augmented the dry matter production or yield of chickpea crop relative to the un-inoculated and unfertilized or fertilized control. The variation in the effectiveness of certain microbial inoculations in the present study could probably be due to the variation in the functionality of the tested
microbial strains, differences in the survivalibility and colonization efficiency of the introduced cultures in the soil or strong competition among introduced bioinoculants for limited nutrients leading to the exclusion of organisms from the rhizosphere. Moreover, the differential rhizosphere effect of legume crop in harbouring a target microbial strain (Paul and clark, 1996) or even modulation of the ‘P’ solubilizing capacity by specific root exudates (Goldstein, et al., 1999) may account for the observed differences among treatments. Similar evidence of single or composite application of bioinoculants on legume crops have been reported (Dashti et al., 1997; Tilak et al., 2005). Many studies have shown an increase in growth and P-uptake by plants through the inoculation of PSMs in pot experiments (Omar, 1998; Vassilev et al., 2006, Mittal et al., 2008; Varsha and Patel, 2009) and under field conditions (De Freitas et al., 1997; Duponnois et al., 2005; Valverde et al., 2006). These PSMs can also increase the growth of plants by other mechanisms i.e. production of phytohormones such as Indole acetic acid (IAA) (Patten and Glick, 2002; Mehnaz and Lazarovits, 2006) which are plant growth promoters.

In our study we have also observed the positive effect of the phosphate solubilizing and growth harmones (IAA and Siderophores) producer fungi on chickpea crop. In our findings we observed Aspergillus niger as a better inoculant than Penicillium sp-03 and Rhizopus oryzae in vitro and field condition both but it is not necessary to exhibit good results in field condition as shown in vitro. It may be possible that any inoculant which shows poor performance in vitro, can exhibit better performance in pot/field condition than the inoculant which exhibiting better performance in vitro.

The beneficial effects of PSF on various crops has been demonstrated with Aspergillus (Omar, 1998; Babana and Antoun, 2006), Penicillium (Kucey, 1987; Reyes et al., 2002) and Trichoderma (Zayed and Motaal, 2005; Rudresh et al., 2005). There are also a few reports on the effect of PSF on chickpea plants. Dudeja et al. (1981) reported stimulatory effect of A. awamori strain on chickpea variety H208 in terms of nodules number, nodule dry weight, nitrogenase activity, P/N-uptake, and grain yield. Rudresh et al. (2005) demonstrated positive effect of Trichoderma spp. inoculation on chickpea variety Annegeri-1 in terms of plant height, number of branches, biomass, and P/Nuptake by shoots/roots. However, Zaidi et al. (2003)
reported decline in nodule number, less increase in grain and straw yield of chickpea variety T3 using a PSF, *Penicillium* variable.

Some other workers also reported increase plant growth and yield by using microbial inoculants specially fungi. De Freitas *et al.* (1997) reported that the stimulatory effect on chickpea plants was more pronounced with *A. awamori* than *P. citrinum*, though *Penicillium* strains exhibited more P-solubilization activity under *in vitro* conditions as compared to *Aspergillus* strains. They also reported that growth and yield in pot experiments. Like P-solubilizing activity, the IAA production was also found to be more in *Penicillium* strains than *Aspergillus* strains under *in vitro* conditions.

Many others workers also reported the composite effect of bacteria and fungi on crops like chick pea, moon bean, maize etc, (Zaidi *et al.*, 2003, Hameeda, *et al.*, 2008). Mittal *et al.* (2008) carried out various pot experiments in green house, maximum stimulatory effect on chickpea plants growth was observed by inoculation of two *A. awamori* strains. They reported 7–12% increase in shoot height, nearly three-fold increase in seed number and two-fold increase in seeds weight as compared to the control (un-inoculated) plants. Inoculation of four strains of *P. citrinum* exhibited lesser stimulatory effect. It showed 7% increase in shoot height, two-fold increase in seed number and 87% increase in seeds weight as compared to the control plants.

Varsha and Patel (2009) reported that after the various rhizosphere soil properties studied, a highly significant positive correlation was established between PSF and soil available P as well as pH. A significant positive association observed between total fungal population and organic matter as well as soil available P. Both abundance and number of PSF were more pronounced in descending order in plant covers: oilseeds, flowers, orchards, vegetables, pulses and cereals.

### 5.10 Effect of Fungal Inoculation on Growth and Yield of Wheat Crop

In our findings similar to chickpea, we also observed the stimulatory effect of PGPF on yield attributes of wheat crop like shoot length as well as weight, spikelet, ear and grain number, seed yield and protein content etc. Generally, the inoculation of
three cultures (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) together was observed most effective as compared to dual and single inoculation treatments (*Aspergillus niger* + *Penicillium* sp-03, NPK+ *Aspergillus niger*, *Aspergillus niger*, and *Penicillium* sp-03) and showed a significant increase in yield attributes of the wheat plants. The highest grain yield was obtained in the treatment of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* 64.3g 1000 seeds\(^{-1}\) which was greater than those obtained with best performing combination of NPK + *Aspergillus niger*. Triple inoculation of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* which enhanced the seed yield by 19.7 g/plant as compared to control. This triple inoculation factor had significantly greater effect on seed yield as compared to dual culture treatments.

Among the dual culture inoculations, the combination of *Aspergillus niger* + *Penicillium* sp-03 significantly increased the shoot growth by 3.57, 5.22 and 87.9 cm at 50, 70 and 145 DAS respectively, as compared to control in the year 2007. Combination of *Aspergillus niger* + *Penicillium* sp-03 was also superior and resulted in significant increase in fresh mass of shoots at 70 DAS during growing season. Inoculation of *Aspergillus niger* combination with plant growth promoting *Penicillium* sp-03 significantly increased the grain number 75%/Ear as compared to inoculation with unfertilized treatment or control. Recently, there has been a growing interest in the application of phosphate-solubilizing microorganisms, mainly filamentous fungi and bacteria, as inoculants for solubilizing the insoluble RP to soluble phosphorus in fermentation and soil conditions (Rodriguez and Fraga, 1999; Kumar, *et al.*, 2001; Babana and Antoun 2006; Harrise/et al., 2006). Many others workers also reported the stimulatory effect of the fungi on different crops like wheat, lentil and maize etc.

Wahid and Mehana (2002) reported about the consortium effect of three phosphate solubilizer fungal isolates, *Aspergillus niger*, *A. fumigatus* and *Penicillium pinophilum* on faba beeb and wheat. They observed the yield components of wheat and faba bean plants increased as a result of soil inoculation with the isolated fungi. *Penicillium pinophilum* was the most efficient isolate. It increased the yield of wheat grains by 28.9 and 32.8% in the soil treated with rock phosphate and superphosphate, respectively. Similarly, it increased the production of faba bean seeds by 14.7 and
29.4% with the same treatments. The uptake of phosphorus by both crops significantly increased due to inoculation of the soil with the tested fungi.

Babana and Antoun (2006a) reported that the bacterial isolate *Pseudomonas* sp. BR2, which appeared to be a mycorrhiza helper bacterium, significantly enhanced wheat seedling emergence very early (5 days after planting) under field condition, and caused 128% increase in root dry matter yield. The two TPR-solubilizing fungal isolates *Aspergillus awamori* Nakazawa C1 and *Penicillium chrysogenum* Thom C13 also caused respectively 60 and 44% increases in root dry matter yields.

Wakelin *et al.* (2007) tested three phosphate solubilizing fungi *Penicillium radicum, Penicillium bilalae* for their ability to increase the growth and phosphorus (P) nutrition of wheat, medic, and lentil. *Penicillium* sp. KC6-W2 was found the strongest plant growth promoting (PGP) strain which stimulated significant increases in shoot growth and dry mass in seven of the nine experiments conducted.

Xiao *et al.* (2009) reported the effect of three phosphate-solubilizing fungi, identified as *Penicillium expansum, Mucor ramosissimus*, and *Candida krissii*, on seedling growth of wheat. All the isolates promoted growth, soil available phosphorus, phosphorus, and nitrogen uptake of wheat seedling in field soil containing rock phosphate under pot culture condition.
Conclusions

1. Aligarh soil was found rich in microbial diversity in terms of heterotrophic aerobic bacteria, asymbiotic nitrogen fixers, actinomycetes and free living filamentous fungi. Their population diversity and density was variable and influenced by plant roots and other environmental factors.

2. Metal tolerant microbial population in wastewater irrigated soil was observed higher than non wastewater irrigated soils. It might be due to the resistance development against the heavy metals occurred in wastewater. Metal tolerant population of soil actinomycetes was lower than aerobic heterotrophs and soil fungi. This might be in part due to differences in their cell wall structures and chemical composition. A significant number of metal tolerant microbial population was also detected in the wastewater used for crops irrigation. Thus metal tolerant microbial population in soil is also contributed by wastewater.

3. No significant difference was observed in the diversity of metal tolerant soil fungal genera between wastewater irrigated and nonwastewater irrigated agricultural fields but their occurrence frequency varied at varying concentration of the heavy metals. This difference in occurrence frequency is expected due to the variation in metal resistance.

4. The tolerance limit of selected 73 metal tolerant fungal isolates against individual metal was recorded up to 2000µg/ml against Cr, Co, Ni, and Cu among fungal isolates. However, none of the isolate could grow above 1600µg/ml of cadmium. Variation in metal tolerance among different isolates of the same genus had become more evident when 20 isolates of *Aspergillus* showed significant variations in metal tolerance from 200-2000µg/ml against the metals tested.

5. Seventy three metal tolerant fungal isolates of 19 distinct fungal genera were observed to produce extracellular enzymes, siderophore, IAA, organic acid, antibiotic, ammonia and also as phosphate and metal solubilizers which are relevant to human health and plant growth directly or indirectly. So they could be exploited to increase the crop productivity by improving soil health especially in metal polluted agricultural fields and in pharmaceutical industries.
Discussion

6. Among the metal tolerant 73 various fungal isolates belonging to *Aspergillus*, *Curvularia*, *Alternaria*, *Trichophyton*, *Hormodendrum*, *Monotospora*, *Mucor*, *Microsporum*, *Penicillium*, *Trichoderma*, *Verticillium* genera etc, many were determined for having multiple plant growth promoting traits but among them the isolates of *Aspergillus* spp. were observed more efficient with multiple traits for plant growth promotion than others.

7. In the assessment of metal toxicity to soil fungi in liquid and solid medium it was observed that at lower concentrations the spore germination and radial growth promoted significantly or insignificantly but as the concentration increased, a delay in spore germination and radial growth extension of the test fungi was recorded which varied with the time and the nature of fungal isolate. The fungal isolates showed more metal sensitivity in liquid as compared to solid medium. Diversity was observed in response to spore germination and mycelial proliferation of the same fungal isolate against the metal concentrations. All fungal isolates showed higher sensitivity against Cd as compared to other metals like Cr, Ni, Co and Cu.

8. Effect of metal ions on activity and fungal growth may be parallel or different depending upon the nature of the test fungi and metals. At lower concentrations of metal ions except Cd, an increase in activity and growth of the test fungal isolates was recorded. In our present investigations in the case of phosphate solubilization and IAA production, an insignificant effect of metal ions was observed on fungal activity while the growth was significantly effected and vice versa at different metal ions concentrations.

9. Maximum removal of metal ions from single and multiple metal ions solution by using the fungal biomass is dependent upon the pH and metal ions concentration. The optimum pH and metal ions concentration depend upon the nature of the test fungi differently. We observed in our investigations that the removal of metal ions of Cr, Ni and Cd from aqueous metal ions solution was observed in acidic pH range (2.5-4.5) at 4 and 6mM metal ions concentrations. Dead fungal biomass of our test fungi was recorded to be a better biosorbent as compared to living biomass of same isolates. Better removal of metal ions from single metal ions solution was determined by the test fungal biomass in
comparison to multiple metal ions solution. It may be possible due to the competition among the different metal ions for binding sites present on limited area of biosorbent. An insignificant increase in Cr, Cd and Ni ions sorption by metal trained fungal biomass was found as compared to untrained fungal biomass among all the fungal isolates tested but in the case of chromium ions sorption it was observed a decrease as compared to metal untrained isolate of *Rhizopus oryzae* at 2mM, 4mM and 6mM metal ions concentrations. Sorption of metal ions of Cr, Cd and Ni was observed higher at pH 3.5 as compared to its natural pH 8.2 due to the presence of large number of organic and inorganic matter.

10. Composite stimulatory effect of *Mesorhizobium* and fungal isolates having multiple plant growth promoting traits was observed in the form of increase in productivity of chickpea and wheat crops. The microbial combination of *Mesorhizobium* + *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* and *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* were determined to be the best for chickpea and wheat crops respectively as compared to other inoculants combinations used.