CHAPTER-VII

General Discussion
As a result of many industrial and waste disposal operations, our environment is becoming increasingly contaminated by heavy metals, pesticides and phenolics. Harmful effects of such pollutants have been established on plants, animals and humans, living in close proximity to industrial plant outfalls and hazardous waste sites (Schneider, 1979; Martirani et al., 1996; Kamaludeen et al., 2003). A large amount of pesticides used for agricultural purposes are known to enter the soil and water ecosystems (Hill and Wright, 1978; SGCI, 1999). The effluents from leather tanning industry and solid wastes from other industries especially contain various types and large amounts of heavy metals (Errasquin and Vazquez, 2003; Megharaj et al., 2003; Pandey et al., 2003).

Soil is the major habitat for diverse microorganisms of importance to soil fertility which serve as a major sink for various organic and inorganic substances including pesticides, phenols and heavy metals. For instance soil borne microbes *Phanerochaete chrysosporium*, *Aspergillus spp.* and *Pleurotus ostreatus* are well documented to detoxify phenolic compounds from olive mill liquid waste and industrial waste water (Sayadi and Ellouz, 1992; Sayadi and Ellouz, 1995; Martirani et al., 1996; Garcia et al., 2000).

Removal of toxic metal ions from polluted waters by using microbial biomass has been studied extensively (Akhtar and Mohan, 1995; Akhtar et al., 1995). Several bacterial strains were found resistant to or capable of detoxifying heavy metals. Among those certain strains of *E.coli* (Shen and Wang, 1994; Rouch et al., 1995), *Enterococcus hirae* (Wunderli-Ye and Selioz, 2001), *Xanthomonas campestris* (Stall et al., 1986), *Pseudomonas pickettii* (Gilotra and Srivastava, 1997), and *Pseudomonas putida* (Saxena and Srivastava, 1998) were resistant to copper. Some species of *Pseudomonas* and *Arthrobacter* were
able to detoxify cadmium (Scott and Palmer, 1990; Wang et al., 1997; Roane and Pepper, 2000; Lee et al., 2001). Chromium bioremediation in various bacteria, algae and fungi has also been reported (Cervantes et al., 2001; Viti et al., 2003). Moreover, the acidophilic heterotroph Acidiphilium symbioticum was found remarkably resistant to Cd and Zn (Mahapatra et al., 2002). Besides heavy metal tolerant and bioremediating microbes, several pesticides degrading bacteria utilizing these toxicants as their sole carbon source, have also been reported (Johnson and Talbot, 1983; Nagata et al., 1993, Fulthorpe et al., 1995, 1996; Nawab et al., 2003).

The present study deals with the isolation and characterization of P. fluorescens strain capable of tolerating the major pollutants in Indian waters viz. heavy metals, pesticides and phenolics (Agnihotri et al., 1994; CPCB, 1995; Datta 1999). According to Duxbury and Bicknell (1983), the majority of the highly metal tolerant organisms were Gram-negative. Moreover, Pseudomonas was the predominant genus among majority of metal tolerant bacteria (Austin et al., 1977; Houba and Remacle, 1980). Pseudomonas was also the most common genus used for phenol degradation studies (Hinteregger et al., 1992; Allsop et al., 1993; Lee et al., 1998). The ability of the test Pseudomonas fluorescens SM1 strain to reduce the concentrations of heavy metals, pesticides and phenolics has been assessed vis-a-vis evaluating its detoxification potential in model water containing as high as 4x concentrations of these toxicants usually present in the heavily polluted sites of Northern India (Malik and Ahmad, 1995; Rehana et al., 1995; Athar, 1999).

Literature searched till date led us to suggest that the microorganisms so far isolated by various investigators have not shown to detoxify all these three
classes of pollutants in a single strain. However, some microorganisms are known to be capable of degrading only two classes of these toxicants or showed multiple metal tolerance. For instance, a *Flavobacterium* sp. was capable of degrading three pesticides, and also imparting resistance to Hg (Chaudhry and Huang, 1988). Moreover, Neuman et al. (2004) have recently reported simultaneous degradation of atrazine and phenol by *Pseudomonas* sp.

*Pseudomonas fluorescens* is a non-pathogenic soil bacterium. It exhibits nutritional versatility and produces greenish fluorescent pigment. *Pseudomonas fluorescens* is also used for the control of soil borne plant diseases (Ryder and Rovira, 1993). Several strains of *P. fluorescens* were capable of adapting to multiple metal stress for Mn, Co, Ni and Cs and also detoxified aluminium (Appanna et al., 1995, 1996; Appanna and Hamel, 1996; Hamel and Appanna, 2003). Moreover, some strains of *Pseudomonas fluorescens* displayed resistance to chromium (Bopp et al., 1983; Bopp and Ehrlich, 1988; DeLeo and Ehrlich, 1994; Appanna et al., 1996). These strains could also biodegrade citrate complexes of Zn, Ni and Fe (Joshi-Tope and Francis, 1995).

Bioremediation is the most promising and cost effective technology widely used nowadays to clean up both soils and waste water containing organic or inorganic contaminants. Soils contaminated with both metals and organics are considered difficult to remediate because of the mixed nature of the contaminants (Roane and Pepper, 2001). The issue of cocontamination is a serious one, since approximately 37% of all contaminated sites in the United States alone contain both metals and organic contaminants (Riley et al., 1992).
*P. fluorescens* SM1 strain was isolated from polluted soil by enrichment method (Hinteregger et al., 1992). This strain was found to tolerate pesticides, phenolics and heavy metals even in combination. Treatment of the isolated strain with industrial waste water as well as with high doses of the combination of pesticides, heavy metals and phenolics not only resulted in the detoxification of contaminated water and bioremediation of these pollutants but also in the biotransformation of certain toxicants with the concomitant growth of the test isolate (Chapter III, V and VI).

Unlike organics, metals cannot be degraded, and thus most biological metal remediation approaches rely on the detoxification and immobilization of the metal both to reduce the biological toxicity and to retard metal transport. *Pseudomonas fluorescens* SM1 strain could withstand 4 times higher concentration of the Pb, Ni, Cu, Cr and Cd than the normal levels whilst *Pseudomonas putida* S4 strain isolated by Saxena et al. (2001) exhibited resistance to fairly smaller concentrations of Zn, Ni, Co, Cu and Al. This isolate was found to be more resistant than other bacterial strains reported by earlier workers (Babich and Stotzky, 1983; Kawai et al., 1990; Baldrian et al., 1996; Errasquin and Vásquez, 2003). Moreover, our strain also seemed to utilize BIIC and phenolics as the carbon sources, especially the utilization of phenols was more efficient as a source of carbon and energy as compared to BHC (Tables 7,8, Chapter III). The test *Pseudomonas fluorescens* SM1 strain thus was not only tolerant to phenols it could also utilize them as the sole source of carbon and energy. Similar results were reported by Hinteregger et al. (1992) and Yap et al. (1999).
Our *Pseudomonas fluorescens* SM1 strain showed resistance to five heavy metals (i.e. Cu, Cd, Pb, Ni, Cr) whereas studies conducted on *P. putida* S by Saxena et al. (2001) also reported resistance to five metals (i.e. Zn, Ni, Co, Cu, Al). Moreover, Cu and Ni resistance markers are also common in both studies. Interestingly enough, *P. putida* S was able to grow in the presence of 1mM Cu$^{2+}$, 2 mM Zn$^{2+}$ and 1mM each of Al$^{3+}$, Co$^{2+}$ and Ni$^{2+}$ only, the test *P. fluorescens* SM1 could grow comfortably in the presence of as high as 2.7 mM Cd$^{2+}$, 11.7 mM Cu$^{2+}$, 2.3 mM Pb$^{2+}$, 5.1 mM Ni$^{2+}$ and 1.0 mM Cr(VI), besides acquiring the appreciable degree of phenolics and pesticides resistances also (Table 5 of Chapter III, Table 4 of Chapter IV).

Several studies have been conducted on different isolates for bioremediation of heavy metals. Roane and Pepper (2000) isolated three isolates which were identified as *Arthrobacter*, *Bacillus*, and *P. fluorescens* and further observed that two of the isolates were exhibiting maximum resistance to cadmium up to a level of 275 mg/L while our *P. fluorescens* SM1 strain could tolerate cadmium up to a level of 496 mg/L.

Errasquin and Vazquez (2003) isolated a fungus, *Trichoderma atroviride* from a sample polluted with heavy metals including Cu, Zn and Cd. They observed that in case of copper *T. atroviride* survived upto a concentration of 300 mg/L, while for Zn the concentration was higher that is up to 750 mg/L. Among these heavy metals cadmium was the most toxic metal and it showed 50% reduction in biomass at 125 mg/L.

Compared with the above findings our isolate seems to be far superior in terms of resistance to copper and cadmium, since it could be able to resist copper and cadmium up to 2942 mg/L and 496 mg/L respectively.
*Pseudomonas marginalis* and *Bacillus megaterium* strains exhibiting lead resistance were isolated by Roane (1999), but the degrees of lead tolerance in those strains were significantly lower as compared to that in the test *P. fluorescens* SM1 isolate.

Six known metal resistance mechanisms in microorganisms reported by several workers were exclusion by permeability barrier, intra and extra-cellular sequestration, active transport or efflux system, enzymatic detoxification and reduction in the sensitivity of cellular targets to metal ions (Silver, 1992; Rouch et al., 1995; Bruins et al., 2000). Among all the six known mechanisms of metal resistance in microorganisms, Megharaj et al. (2003) working on *Arthrobacter* and *Bacillus* species, and Ganguli and Tripathy (2002) working on *P. aeruginosa* reported that their isolates had the ability to reduce Cr(VI) to Cr(III). In our case it was found that the test isolate also had the ability to reduce Cr$_{6+}$ to Cr$_{3+}$ (Tables 5, 6 and Fig. 5 of Chapter IV). Moreover, the resistance level of Cr(VI) in our case was much higher than that found by both of these workers.

Besides heavy metal resistance, our strain also showed resistance to BHC and phenolics and their tolerance levels were also comparable to those obtained by other workers (Sahu et al., 1990; Yap et al., 1999).

Among several bacteria having the resistance to toxic concentrations of heavy metals, many of them carry plasmids (Silver and Misra, 1988; Mergeay, 1991). The Role of plasmids in *Pseudomonas* species in the biotransformation of certain heavy metals, pesticides and phenolics is also well documented (Sayler et al., 1990; Rani and Mahadevan, 1992; Deshpande et al., 2001; Thakur et al., 2001).

151
Interestingly, Vargas et al. (1995) isolated nine toxicant tolerant *Pseudomonas* strains. All of them possessed large plasmids but the results suggested that the transformation character was conferred by chromosomal genes. These findings necessitated to carry out the transformation and curing experiments (Deshpande et al., 2001). The test *P. fluorescens* strain was found to contain a 44 Kb plasmid. This plasmid was responsible for imparting the multiple resistance character and most probably harbouring some genes for the degradation of toxic pesticides and phenols (Tables 1, 2, 3 and 9 of Chapter IV). This conclusion was obviously based on the transformation and/or curing experiments vis-a-vis other experiments conducted in support (Tables 1-6 and 9 of Chapter IV).

Our *P. fluorescens* SM1 strain has an additional advantage of exhibiting a high level of tolerance to chromium (VI), a very toxic metal species (Table 4 of Chapter IV). It was, therefore, of interest to extend the studies on the biotransformation of Cr(VI) to Cr(III). Several workers reported the detoxification of Cr(VI) via oxidation-reduction pathway (Horitsu et al., 1987; Fuji et al., 1990; Ishibashi et al., 1990). The test SM1 strain also seems to have such type of reduction pathway available for its chromium detoxification (Tables 5, 6 and Fig. 5 of Chapter IV).

Similarity in the patterns of reduction of Cr(VI) in the transformed *E. coli* and *P. fluorescens* SM1 cells also suggested for the plasmidial nature of the chromium reduction machinery which was further confirmed by various experiments (Tables 5, 6 and 9 of Chapter IV). Such a biotransformation of Cr(VI) to Cr(III) is consistent with the earlier reports (McLean and Beveridge, 2001; Ganguli and Tripathi, 2002; Megharaj et al., 2003; Sultan and Hasnain, 2003).
A remarkably high efficiency of bioremediation in the viable as well as heat killed SM1 cells as evident from the presence of test metals in pellet strongly suggests for a functional biosorption mechanism involved in our system for Cd, Cu, Ni and Pb but such biosorption was not observed in case of Cr(VI) because the efficiency of chromium uptake was only 18% (Table 7 of Chapter IV). A significant rather strong inhibition in the efficiency of removal of Cr(VI) in the presence of sodium azide and 2,4-DNP, the well known metabolic inhibitors also led us to suggest at least one mechanism other than biosorption that presumably made use of ATP hydrolysis and/or active transport system involved in the detoxification of Cr⁶⁺ (Table 8 of Chapter IV). However, we did not find any evidence in favour of efflux pump for the exclusion of Cr(VI) operating in the SM1 cells as proposed by some investigators in bacterial system (Silver et al., 1989; Nies and Silver, 1995) since the efficient removal of this metal species (actually Cr⁷⁺O₂⁻) from the supernatant (Table 5 of Chapter IV) goes against such process. Therefore, other possibilities in support of energy requiring transport process under our experimental conditions could have been the active transport of the reduction machinery out of the cell and sulfate mediated Cr⁶⁺ influx for inside transport. Similarly, the presence of metallothionein like proteins for the bioremediation of internalized chromium cannot be ruled out because such proteins have been isolated from the Pseudomonas putida, Cyanobacterium and Synechococcus spp. as well as from E. coli (Gupta et al., 1993). Further details on the mechanistic aspects of Cr⁶⁺ bioremediation are given in the Discussion section of Chapter IV.

The experiment conducted in the presence of chloramphenicol, an inhibitor of protein biosynthesis further revealed the constitutive rather than inducible
nature of the metal bioremediation mechanisms which further affirms the role of structural component of the cell to be involved in the metal bioremediation or detoxification.

The major mechanism operating for the bioremediation cum detoxification of Cd, Cu, Ni and Pb in our isolate seems to be the biosorption presumably involving the non-specific binding of cationic species of metals only (Synder et al., 1978; Flemming et al., 1990). It is further suggested that such biosorption process could not be a plasmid mediated character (Table 9 of Chapter IV). However, the oxidation reduction pathway for the Cr(VI) bioremediation cum detoxification has been found to be plasmid mediated (Tables 4, 5 and 6 of Chapter IV). Bruins et al. (2000) have given the summary of various mechanisms of metal resistance in microorganisms. In view of the known mechanisms of tolerance, metal exclusion by permeability barrier and active transport of metal away from the cell/organism was not possible in our system. It seems that intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets may be some of the plausible mechanisms in our system (Fig. 1 of Summary)

Immobilized microbial cells have been commonly used for the bioremediation processes (Linko and Linko, 1983; Hietkamp et al., 1990; Ignatov et al., 2002). The immobilization of the intact microbial cells contrary to that of enzymes derived from them is more beneficial due to its higher operational stability, ease of use, high cell density required for immobilization and ability to scale up the process (Hunik and Tramper, 1993; Bickerstaff, 1997; Srinath et al., 2003). It is also noteworthy that sometimes enzyme immobilization contrary
to the immobilization of intact parents cells, did not reduce the toxicity of the system (Martirani et al., 1996). Because of this and other reasons we included the detoxification studies along with the bioremediation status of the immobilized system.

Several immobilization methods are available in literature (Mattiasong, 1983; Chibata et al., 1986). Keeping in view of the relevance of immobilized microorganisms, \textit{P. fluorescens} SM1 cells were immobilized in calcium alginate beads (Chapter V) because the use of alginate in the cell immobilization is well documented (Keweloh et al., 1989; Lo et al., 2003; Pandey et al., 2003). From the comparison of the data on growth and viability of \textit{P. fluorescens} SM1 strain, it is obvious that the immobilized cells could withstand the environmental stress more easily and efficiently than in the free state (Table 1 of Chapter V). It was further observed that immobilized cells were able to produce more number of colonies than the free cells under identical conditions (Table 1 of Chapter V). These results are consistent with those of several workers (Bandyopadhyaya et al., 1993; Zezza et al., 1993; Vilchez and Vaga, 1994) who demonstrated that microbial cells entrapped in alginate gel can remain physiologically active. It was further established that microbial cells could be able to grow within the alginate beads (Frioni et al., 1994). The efficiency of bioremediation of phenolics, heavy metals and pesticides was much higher in case of the immobilized \textit{P. fluorescens} SM1 cells compared with free cells (Tables 2,3,4 of Chapter V). These findings are also in conformity with several investigations carried out earlier (Bettman and Rehm, 1984; Saxena et al., 2001; Tsekova and Ilieva, 2001).
In the last three chapters, our focus was on the detailed studies related to bioremediation potential of the test *P. fluorescens* SM1 isolate towards the organic and inorganic toxicants, i.e. heavy metals, pesticides and phenolics. To gain an insight into the mechanisms of tolerance of the test *P. fluorescens* SM1 strain towards the organic and inorganic toxicants, the biodegradation and biotransformation studies were carried out vis-a-vis detoxification of the model water under the free and immobilized conditions. Bioremediation and biotransformation processes sometimes do not result in the detoxification of the sample. For instance, Aggelis et al. (2002) observed that the phytotoxicity of green olive waste water treated with *Pleurotus ostreatus* strain did not reduce while large reduction of phenolic content took place. Several workers reported similar findings (Tsioulpas et al., 2002; Aggelis et al., 2003).

Toxicity tests are necessary in water pollution assessment because chemical and physical tests of the pollutants alone are not sufficient to evaluate potential effects of toxicants on aquatic biota (USEPA, 1987, 1991). For instance, the effects of chemical interaction and the influence of complex matrices on toxicity can not be determined from chemical tests alone. Different species of aquatic organisms are not equally susceptible to the same toxic substances nor are organisms equally susceptible throughout the life cycle (APHA, 1985, 1995).

The present state of pollution and its multidimensional hazardous effects have led to the developments of a number of biological assays for assessing the toxicity of pollutants in the living system (APHA, 1985, 1995). Moreover, it is also emphasized that a single genotoxicity/toxicity testing system does not reflect the actual behaviour of the test sample. A battery of test is thus necessary for the toxicity evaluation and risk assessment procedures and has been used as
an established procedure for the complex toxicants systems (Malik and Ahmad, 1995; Rehana et al., 1995, 1996; Dutka, 1996; Siddiqui, 2002).

A survey of the research work undertaken in the field of toxicity assessment shows that plant systems have gained more popularity as part of test batteries for monitoring effects of various chemicals in the environment and also as bioindicators of aquatic environment (Constantin and Owens, 1982; Fiskesjo, 1993; Gopalan, 1999; Ma, 1999a, 1999b; DeLima and Jordaq, 2001; Aggelis et al., 2003). \textit{Allium cepa} toxicity/ genotoxicity test is widely carried out for the toxicity determination of various pollutants/mutagens/clastogens etc. (Grover and Kaur, 1999; DeLima and Jordaq, 2001). It is one of the tests that has also been recommended by the USEPA's Gene Tox Prog. (Ma, 1999b). The advantages of this test include its simplicity, reliability and sensitivity as well as a strong inter laboratory relationship.

As far as the toxicity evaluation of the model water was concerned, we employed both the \textit{in vitro} and \textit{in vivo} tests. These tests included \textit{Allium cepa} test, Ames fluctuation test and plasmid nicking assay.

A remarkably high reduction in toxicity to the tune of 12-31 fold was obtained by \textit{Allium cepa} after the treatment of sample with immobilized cells of \textit{P. fluorescens} SM1. The efficiency of detoxification for single group of toxicants was greater than that for the combination of all toxicants. The efficiency of detoxification of phenols by \textit{Allium cepa} test was maximum in our case i.e. 31 fold. This finding is contrary to the findings of Aggelis et al. (2002) who could not find any toxicity reduction concomitant with removal of phenols.
Ames fluctuation test was also carried out to determine the detoxification potential of the test model water before and after treatment with *P. fluorescens* SM1 strain. We employed *S. typhimurium* TA98 and TA100 strains because their validity in detecting the mutagens among inorganic and organic pollutants has been established by several workers (Loper, 1980; Harington et al., 1983; Meier, 1988; Le Curieux et al., 1995). The toxicity/genotoxicity was reduced 4-7 fold by Ames fluctuation test after the sample was passed through the immobilized cells of the test isolate. These findings simply suggested that Ames fluctuation test was not a better index of toxicity/genotoxicity evaluation under our experimental doses of toxicants presumably because of very high toxicity thus rendering it less sensitive in this case (Green et al., 1977; Bridges, 1980; Venitt et al., 1984; Le Curieux et al., 1995).

The conversion of the supercoiled plasmid DNA molecules to nicked (open circle) and linear form is evident in our plasmid nicking assay (Fig.7 of Chapter VI), thereby establishing the DNA damaging character of the model test water samples before exposure to the test *Pseudomonas* SM1 strain. Following treatment of model water with the test *P. fluorescens* SM1 strain, the DNA damaging potential of samples reduced to insignificant level which is evident from the absence of linear form of plasmid DNA. Nobody seems to have used plasmid nicking assay for detoxification studies. However, in our lab this system has been developed to estimate the genotoxic potential of various water bodies (Siddiqui. 2002). The reactive oxygen species (ROS) have been implicated in the causation of DNA damage in the industrial waste water especially impregnated with heavy metals in our previous studies (Malik and Ahmad, 1995; Siddiqui, 2002). It therefore, seems quite logical to propose that the test
*P. fluorescens* SM1 strain is capable of efficiently reducing the genotoxicity of pollutants supposedly generating the ROS.

Schematic representation of the integrated pathways for the bioremediation of test pesticides and phenolics are summarized in Fig. 1.
Fig. 1: A common pathway for the bioremediation of pesticides and phenolics proposed to be followed by *Pseudomonas fluorescens* SM1 strain

1. Pathway proposed by Miyauchi et al. (2002) for the degradation of hexachlorocyclohexane

2. Pathway proposed by De Lipthay et al. (1999) for the degradation of catechol by meta cleavage pathway

3. Pathway proposed by De Lipthay et al. (1999) for the degradation of phenoxyacetic acid, phenol and benzoic acid to catechol by ortho cleavage pathway and further degradation to substrates of the tricarboxylic acid cycle

4. Pathway proposed by O'Reilly and Crawford (1989) for the degradation of cresol

5. Pathway proposed by Vyas and Somani (1996) for the degradation of protochatechuate to succinic acid

6. Pathway proposed by us involving the dechlorination of 2,4-dichlorophenoxyacetic acid

*F = First step, **L = Last step