Summary

The work presented in the thesis mainly involved biochemical and molecular studies on bacterial degradation of *p*-nitrophenol and its degradation products. It also focuses on the study of biodiversity of a *p*-nitrophenol contaminated soil and the diversity and phylogeny of benzenetriol dioxygenase, an enzyme involved in *p*-nitrophenol degradation. Monitoring of an exogenous microorganism of a *p*-nitrophenol contaminated soil for bioaugmentation via plate counting and molecular techniques is another aspect of this work.

p-Nitrophenol is known to be degraded two pathways by bacteria. In the first case *p*-nitrophenol is degraded via the formation of 4-nitrocatechol and 1,2,4-benzenetriol, whereas, the other pathway proceeds through the formation of benzoquinone and hydroquinone. However, there are only few reports related to the gene(s) involved in degradation and their regulation. Attempts were made to elucidate and characterize some of the gene(s) involved in *p*-nitrophenol degradation in the laboratory and some of the gene(s) of the lower degradation pathway encoded on a ~6.8 kb fragment of the genomic DNA of strain SJ98 were cloned in pBluescript vector. The insert was completely sequenced, annotated and submitted to GenBank (Accession no. AY574278). The fragment showed the presence of seven ORFs namely, aldehyde dehydrogenase, ferredoxin component of ring hydroxylating dioxygenases, a conserved protein, regulatory protein belonging to the GntR family, ATP binding transporter protein, benzenetriol dioxygenase and maleylacetate reductase. Genomic DNA library of strain SJ98 was constructed in two different vectors, pUC18 and SupercosI.. Using the gene(s) for benzenetriol dioxygenase and aldehyde dehydrogenase as probe the libraries were screened in order to elucidate the other gene(s) involved in *p*-nitrophenol degradation. The library constructed in pUC18 did not contain any positive clones, however, the library constructed in SupercosI showed the presence of nine clones encoding benzenetriol dioxygenase and aldehyde dehydrogenase gene(s) as determined by colony and slot blot hybridization, polymerase chain reaction (PCR) and sequencing results. Since gene(s) involved in degradation are known to exist in clusters, the upper pathway genes are expected to be located downstream or upstream of the lower pathway genes encoded by the clones.

In order to assess community functions researchers have developed several PCR-based techniques wherein catabolic/ functional gene(s) are amplified and sequenced from environmental isolates or nucleic acids. *p*-Nitrophenol degradation proceeds via the formation of benzenetriol followed by maleylacetate in several organisms and therefore benzenetriol dioxygenase, the enzyme that converts benzenetriol to maleylacetate, was targeted for studying the diversity of this gene amongst various *p*-nitrophenol degrading organisms. Some bacteria were isolated from pesticide contaminated agricultural fields using enrichment technique. In addition, degenerate primers were designed for the amplification of a partial sequence of benzenetriol dioxygenase from the *p*-nitrophenol degrading strains. Using these primers nine different strains showed positive amplification and these were thereafter cloned and sequenced. Two of the amplicons was found to be spurious and was left out from analyses. 16S rRNA gene was amplified and sequenced from all the seven different strains using degenerate primers. In addition, sequences of benzenetriol dioxygenase gene involved in other pathways and also that of other intradiol aromatic ring-hydroxylating dioxygenases were obtained from the database in order to compare with the sequences obtained for this study. Using various programs dendrograms were constructed to study the phylogenetic relationship of this gene amplified from *p*-nitrophenol degrading organisms and other sources and/or other dioxygenases. It was shown that this enzyme had two distinct sources of evolution and was phylogenetically closer to catechol/ chloro-catechol dioxygenases. The partial 16S rRNA gene sequences and the partial benzenetriol dioxygenase gene sequences were submitted to GenBank under the accession nos. DQ282187-DQ282193 and AY86618-AY86624, DQ286583-DQ286584 respectively.

Bioaugmentation is the addition of exogenous microorganisms to a contaminated site to increase the rate of degradation of pollutants. It is through the introduction of actively degrading microorganism(s) that the pollutant is mineralized, thereby improving degradation rates as compared to that achieved by indigenous microorganisms. Bioaugmentation under natural field conditions was conducted to determine the potential of a *p*-nitrophenol degrader *Arthrobacter protophormiae* RKJ100 in a parathion/ methyl parathion contaminated soil. For this purpose 4 plots (1mX 1mX 30 cm) were packed with soil sprayed with pesticides; immobilized bacterial cells were added to one plot and free cells to another. Uninoculated soil and soil mixed with corncob powder alone (carrier) served as controls. The efficiency of the bioremediation process was assessed by

monitoring the depletion of the pollutant and the effect of strain RKJ100 on the existing soil community structure. High performance liquid chromatography analysis of samples withdrawn at different time intervals revealed that *p*-nitrophenol could be depleted within 5 days from soil containing immobilized cells, whereas free cells degraded only 75% of *p*-nitrophenol in this time; in control fields *p*-nitrophenol persisted even after 30 days. The survival of the introduced organism was monitored at various time intervals during the bioremediation process using different techniques such as (i) CFU counting, (ii) colony hybridization, (iii) slot blot hybridization and (iv) real time polymerase chain reaction. Results unequivocally showed that immobilized cells survived in the soil throughout the bioremediation process without showing significant changes in population. However, free cells showed a decrease in population by 2 log units in 30 days indicating that immobilization of strain RKJ100 enhanced their stability.

The community structure of the *p*-nitrophenol contaminated field was also studied and thereafter changes in community structure due to introduction of the exogenous strain were analyzed. The 16S rRNA gene pool amplified from the soil metagenome, taken from the control plot at the beginning of the experiment, was cloned and restriction fragment length polymorphism studies revealed 46 different phylotypes on the basis of similar banding patterns. Partial sequencing of representative clones of each phylotype showed that the community structure of the pesticide-contaminated soil mainly constituted of Proteobacteria and Actinobacteria. Members of these taxa are known to be important degraders of toxic compounds and, therefore, their presence in the form of significant populations in such a pesticide-contaminated site was expected. The sequences have been annotated and submitted to GenBank (Accession no. AY596117-AY596163).

Another aspect of research focused on bacterial chemotaxis toward nitroaromatic compounds. Till date, there are no reports of bacterial chemotaxis in a heterogenous medium such as soil, however, for bioremediation purposes it is important to know whether bacteria actually migrate through the heterogenous soil medium towards a gradient of a particular chemoattractant. A laboratory isolate, *Ralstonia* sp. SJ98, was previously reported to be chemotactic toward various nitroaromatic compounds such as *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoate, *p*-nitrobenzoate, 3-methyl-4-nitrophenol, etc. Another *p*-nitrophenol degrading strain RKJ200 was used as negative control for the study as it was shown to be non-chemotactic toward *p*-nitrophenol via drop and swarm

plate assays in semi solid medium as standardized in the laboratory previously. In the present study bacterial chemotaxis of strain SJ98 was successfully demonstrated in soil microcosm using a qualitative assay, i.e. plate assay, in which chemotaxis of strain SJ98 through soil was demonstrated in two days. In order to reconfirm the results and monitor the migration of bacteria through soil, a quantitative 'tray assay' was developed. The remaining amount of *p*-nitrophenol in soil at various distances from the point of inoculation at different time intervals was estimated. Also, the migration of bacteria was established using several methods including (i) plate counting, (ii) vital staining and flow cytometry, (iii) slot blot hybridization. The results of all the studies suggested that SJ98 cells depleted *p*-nitrophenol as it traveled through soil. The study clearly substantiated the hypothesis that chemotactic bacteria have a potential for *in situ* bioremediation as they are capable of migrating through soil. The assays developed for this study may also be used to study the chemotactic behavior of other organisms in soil microcosm and the results may be extrapolated for conducting field scale studies under natural conditions.