Summary

Phenanthrene degradation

Four polycyclic aromatic hydrocarbon (PAH) degrading bacteria namely *Arthrobacter sulphureus* RKJ4, *Acidovorax delafieldii* P4-1, *Brevibacterium* sp. HL4 and *Pseudomonas* sp. DLC-P11, capable of utilizing phenanthrene as sole source of carbon and energy, were tested for its degradation using radiolabelled phenanthrene. [9-^{14}C]Phenanthrene was incubated with microorganisms containing 100 mg/l unlabelled phenanthrene and evolution of $^{14}CO_2$ was monitored. Within 18 h of incubation, 30.1, 35.6, 26.5 and 2.1% of the recovered radiolabelled carbon were degraded to $^{14}CO_2$ by RKJ4, P4-1, HL4 and DLC-P11, respectively. When mixtures of other PAHs such as fluorene, fluoranthene and pyrene in addition to phenanthrene were added as additional carbon sources, there was a 36.1 and 20.6% increase of $^{14}CO_2$ production from [9-^{14}C]phenanthrene in case of RKJ4 and HL4, respectively, whereas P4-1 and DLC-P11 did not show any enhancement in $^{14}CO_2$ production. Although many a times combination of bacteria enhance the degradation of organic compound(s), no enhancement in the degradation of [9-^{14}C]phenanthrene was observed by mixed cultures involving all four microorganisms together. However, when different PAHs as indicated above were used in mixed cultures, there was 68.2% increase in $^{14}CO_2$ production. In another experiment, the overall growth rate of P4-1 on phenanthrene could be enhanced by adding non-ionic surfactant Triton X-100 whereas RKJ4, HL4 and DLC-P11 did not show any enhancement in growth.

Phenanthrene degradation pathways

Phenanthrene degradation pathways were analyzed in above mentioned four different bacterial isolates. From thin layer chromatography, gas chromatography and gas chromatography-mass spectrometry analyses, common intermediates, *o*-phthalic acid and protocatechuic acid were detected in case of RKJ4 and *o*-phthalic acid in case of P4-1 in phenanthrene degradation. A new intermediate, 1-naphthol, was detected in case of HL4 and DLC-P11. HL4 degrades phenanthrene through 1-hydroxy-2-naphthoic acid, 1-naphthol and salicylic acid which is a novel transformation sequence. In case of DLC-P11, phenanthrene

degradation takes place through another novel transformation sequence via the formation of 1-hydroxy-2-naphthoic acid, 1-naphthol and *o*-phthalic acid.

Cloning of phenanthrene degrading genes

All the four phenanthrene-degrading strains (RKJ4, P4-1, HL4 and DLC-P11) were used to locate the phenanthrene degrading genes. Since, many a times PAH degrading genes are plasmid encoded, the presence of plasmid in these four strains were tested by various methods. Mega plasmids were found to be present in RKJ4, HL4 and DLC-P11. To check the possibility of the presence of phenanthrene degrading genes on the plasmid, curing of the plasmids from the strains were attempted using different agents. However, no such derivatives were obtained which were incapable of growth on phenanthrene. Therefore, the involvement of these plasmids in phenanthrene degradation could not be established. In order to locate the genes for phenanthrene degradation, chromosomal and plasmid DNA from all the strains were subjected to Southern hybridization using *nah* (containing naphthalene dioxygenase genes) and *pah* (containing phenanthrene dioxygenase gene) as probes. Results indicated that although *nah* gene did not have any homology with the DNA samples tested, *pah* showed homology with the chromosomal DNA of P4-1. Partially digested *Eco*RI fragments from P4-1 were cloned in pGEM-3Z vector and one of the putative clone (pSJ16) having 1.8 kb and 3.7 kb *Eco*RI fragments could produce $^{14}CO_2$ from [9-^{14}C]phenanthrene and apparently formed new intermediate(s) as indicated by GC-MS studies suggesting the presence of phenanthrene dioxygenase.

Stability of a recombinant plasmid

It is essential to study the stability of recombinant plasmid(s) for the application of "genetically-engineered microorganisms" (GEM) in treatment of toxic organic compounds before releasing them into the environment. In the present study, attempts were made to determine the stability of a recombinant plasmid carrying genes for naphthalene mineralization. A strain of *P. putida* RKJ1 capable of mineralizing naphthalene (Nap^+) via salicylate (Sal^+) was isolated and the genes for naphthalene mineralization resided on an approximately 83 kb plasmid. All regulatory and structural genes for the whole naphthalene-degrading pathway were encoded on a 25 kb *Eco*RI fragment and this fragment was cloned into a tetracycline-resistant (Tc^R) broad-host-range plasmid vector pLAFR3. This

recombinant plasmid, designated as pRKJ3 (Nap^+ Sal^+ Tc^R), was transferred into a plasmid-free strain *P. putida* KT2442 (Nap^- Sal^- Tc^S) in order to test the stability of the plasmid. The recombinant plasmid pRKJ3 was found to be segregationally and/or structurally unstable depending on the growth conditions. Two types of novel derivative strains having the phenotypes Nap^- Sal^+ Tc^R and Nap^- Sal^- Tc^R were obtained. Specific deletions of approximately 2 kb and approximately 18 kb, respectively, were apparent in the above mentioned derivatives. Further, no other kinds of deletions in the derivatives screened were detected indicating the specificity of the deletions in the recombinant plasmid pRKJ3.

Chemotaxis of *P. putida*

A naphthalene and salicylate-degrading microorganism *P. putida* RKJ1 is chemotactic towards these compounds. As mentioned above this strain carries a 83 kb plasmid and a 25 kb *Eco*RI fragment of the plasmid contains the genes responsible for naphthalene degradation through salicylate. The plasmid-cured derivative of RKJ1, RKJ5, is neither capable of degradation nor is chemotactic towards naphthalene or salicylate. The recombinant plasmid pRKJ3, which contained 25 kb *Eco*RI fragment, was transferred back into plasmid-free strain RKJ5 and the transconjugant showed both the degradation and chemotaxis. The recombinant plasmid pRKJ3 was also transferred into another plasmid-free *P. putida* KT2442 strain and was tested for chemotaxis. The transconjugant thus obtained (RKJ15) showed chemotaxis towards both naphthalene and salicylate. Two mutant strains carrying deletions in pRKJ3 (in *P. putida* KT2442) with phenotypes Nap^- Sal^+ and Nap^- Sal^- were also tested for chemotaxis. It was found that Nap^- Sal^+ mutant strain showed chemotaxis towards salicylate only, whereas Nap^- Sal^- mutant strain was non-chemotactic towards both the compounds. These results suggest that the metabolism of naphthalene and salicylate may be required for the chemotactic activity.

Metabolic engineering

Metabolically-engineered microorganisms may have tremendous potential in removing toxic compounds from nature. In general, microorganisms may prefer to utilize simpler carbon source over toxic compounds when both are present in an environment and, therefore, the presence of simpler carbon sources may greatly reduce the efficiency of a microorganism towards toxic compound. If a microorganism is prevented to utilize simpler carbon sources

and thereby making it totally dependent upon the toxic compounds, it should increase the specificity and efficiency of degradation of the toxic compounds in the presence of other simpler carbon sources. To test this hypothesis, in a model laboratory system, the efficiency of naphthalene and salicylate degradation in presence of glucose by a recombinant *P. putida* strain, which was mutated in glucose metabolism, was determined in comparison to the non-mutated strain. Results obtained indicate that the impairment of glucose metabolism leads to better degradation of naphthalene and salicylate in presence of glucose.

Indigo production

A wild-type naphthalene degrading strain *P. putida* RKJ1 and a wild-type phenanthrene degrading strain *A. delafieldii* P4-1 and other *P. putida* and *Escherichia coli* strains containing the genes for naphthalene degradation on a recombinant plasmid pRKJ3 produced indigo and indirubin pigments from indole.