SUMMARY AND CONCLUSIONS

Oil contamination with petroleum hydrocarbons has caused several environmental and health defects and increasing attention has been paid for developing and implementing innovative bioremediation technology for cleaning up this contamination. The ability of many microorganisms including *P. aeruginosa* to grow on these hydrocarbons is attributed to its synthesis and excretion of surface active compounds, biosurfactants, extracellular rhamnolipids in case of *Pseudomonas aeruginosa*. The biosurfactants solubilize and emulsify the hydrocarbons, thus enhancing their water solubility, decreasing surface tension and increasing the displacement of oily substrates from soil particles making them available to the degrading microbes. This work was carried out with the objective to develop a consortium for use in hydrocarbon pollution remediation in soil under natural environmental conditions. One of the members of the consortium, SSC2, was used for isolation of biosurfactant for use in the remediation process. Structural, physicochemical and biological characterization of the isolated biosurfactant was also done.

On the basis of increase in biomass (indicating good growth on hydrocarbons), maximum reduction in surface tension (indicating efficient and effective biosurfactant solution) and maximum hydrophobicity (indicating efficient affinity of the organisms towards hydrocarbons), three organisms from among the 38 oil field isolates were selected as members of the consortium for use in crude oil sludge degradation. These organisms were characterized to be members of *Pseudomonas* group (SSC2: *P. aeruginosa*; P2: *P. aeruginosa*) and *Rhodococcus* group (*R. equi*). Different combination of these organisms were tried for crude oil sludge remediation in laboratory and a combination of all three acting together was found to be most effective against crude oil sludge hydrocarbons as compared to any two members, bringing about a complete reduction the hydrocarbon content.

The ability of seed cultures to function effectively under natural soil conditions signifies the success of any consortium for use in bioremediation. To ascertain the nature of our developed consortium, two field studies on two different crude oil sludges were

performed in soil under natural environmental conditions. Crude oil sludge from Ratnagiri and from IOCL, Faridabad was used for the study. Effect of two amendments was analyzed, a nutrient mixture (acting mainly as a source of nitrogen) and biosurfactant solution (rhamnolipid mixture produced by one of the consortium member, SSC2). Contrary to earlier works, we used crude biosurfactant solution, in the form of culture supernatant, thus testing a strategy for reduction of the overall bioremediation technology cost. Indigenous population in the soil was not sufficient to bring about any substantial reduction in hydrocarbon content as was evident by negligible reduction in the peak area of residual hydrocarbon in control (uninoculated and untreated) plot after the analyses time. Enhancement of biodegradation was achieved by both bioaugmentation and biostimulation. Addition of biosurfactant or nutrient mixture alone reduced the hydrocarbon content to 50-60% while addition of seed organisms along with the additives increased the degradation upto 90%. Although biosurfactant was more effective in reducing the hydrocarbon content of oil polluted soil as compared to nutrient mixture alone, use of both the additives together was the most effective remediation stimulation strategy with a complete reduction being observed by the action of the consortium in the presence of nutrient mixture and biosurfactant solution. These findings establish the success of the developed consortium, devised nutrient mixture and also successful use of biosurfactant mixture in its crude form. This work hence holds great scope for crude oil sludge contaminated site cleanup.

Rhamnolipid are among the best known biosurfactants produced by many members of *Pseudomonas aeruginosa* sp. from a wide variety of carbon sources. Production of rhamnolipid from *Pseudomonas aeruginosa* SSC2, an oil field isolate was studied with respect to its structural, physicochemical and biological activities. SSC2 was found to produce biosurfactant from a number of carbon sources (both hydrophilic and hydrophobic): Glucose in minimal media, Peptone rich PPGAS media, and a number of water immiscible compounds in minimal media, n-hexadecane, n-dodecane, pristane and n-pentadecane. Minimum surface tension was reached within 2 days on most of the substrates and was indicative of effective biosurfactant production. Maximum reduction was achieved upon growth on PPGAS media (29dynescm^{-}) followed by growth on hydrocarbon, n-hexadecane (30dynescm^{-1}).

Biosurfactant was isolated from two chosen media: PPGAS medium and hexadecane in minimal media by acid precipitation method, purified and used for structural, biochemical and biological characterization. In contrast to earlier reported fact that maximum reduction in surface tension indicates maximum biosurfactant production, we observed a higher amount of biosurfactant (1.4gm/l) from hexadecane than from peptone medium (0.8g/l) although latter brought about more reduction in surface tension. Biosurfactant from PPGAS medium was however more efficient as was evident by a low CMC ($2mgl^{-1}$ as compared to 6 mgl^{-1} for biosurfactant from n-hexadecane). HPLC-ES-MS revealed both the mixtures to be made up of around 13 rhamnolipid congeners each differing from the other on the basis of the number of rhamnose moieties and carbon chain length of the fatty acid. 3 of the rhamnolipid congeners could be separated and structurally characterized with mono and dirhamnolipid being prominent. Also found was a new species (Rh-Rh-C_{10}-C14). These compounds were used further for biological characterization. Satisfactory antimicrobial activity was observed for the rhamnolipid mixture as well as the congeners studied against a number of bacteria and actinomycetes. No activity was observed against yeasts for the mixture. However, contrary to any of the earlier reports, two of the compounds showed satisfactory activity against one of the test organism (*Kluveromyces marxianus*). Limited literature is available on the antimicrobial activity of rhamnolipids and even limited literature is there on the use of separated compound as antimicrobial agent. This is also first report on the antiyeast effect of rhamnolipid. Therefore on the basis of its biological and physico-chemical properties, the rhamnolipid mixture has the potential be used as an antimicrobial agent in the pharmaceutical industry and also holds potential for use in bioremediation applications.

Studies on hexadecane uptake was carried out to gain insight in the uptake of hydrocarbon by *Pseudomonas aeruginosa* SSC2. Involvement of biosurfactant was substantiated by pseudosolubilization studies revealed by presence of droplets smaller than 0.22µm. Electron microscopic studies revealed numerous surface and ultrastructural changes upon growth of *Pseudomonas aeruginosa* on hexadecane. Surfactant formed a complex with hydrocarbon providing an anchorage site for cells. Surfactant layered hexadecane droplet was observed to be internalized for assimilation by a process similar in appearance to pinocytosis. Large reserves of unmodified substrate was build up inside

cell before being broken down into numerous smaller inclusions for breakdown. The overall picture for hexadecane uptake would provide valuable information for the process of hydrocarbon uptake.

Biodegradation of atrazine

Pesticides are also one of the extremely hydrophobic compounds finding extensive use in modern agricultural practice for enhanced crop yields. Atrazine is a triazine herbicide used extensively for a variety of crops. Given the large scale use and extreme toxicity of the molecule there is a need for finding new and unexploited organisms capable of degrading atrazine not only at the lab scale but also in the environment. A member of *Acinetobacter* species, characterized to be *A. radioresistance*, was isolated from soil rich in atrazine and was found to be capable of degrading atrazine as high as 250ppm. No exclusive report is there on the use of this member of the soil community for remediation of atrazine contamination. A6 was capable of utilizing other members of the triazine compound family and hence could be very efficient in remediation of soil, heavily contaminated with not only atrazine but also other related compounds which are equally toxic. Using this isolate, a fluorescence based method was developed for fast and easy monitoring of the uptake of atrazine from the media. This method was based on the change in fluorescence with time in the culture supernatant and cell biomass of the organism growing upon fluorescent atrazine as the substrate. FITC tagged atrazine was used for the assay. This change was observed only when A6 utilized the fluorescent molecule which was confirmed by the rise in the fluorescence inside cells of the test organism around the same time (18 to 20hr.) when the fluorescence in the supernatant decreased. No such change was observed when only FITC or ethanol was used for the assay. Neither was this change observed in the absence of A6 confirming the uptake of atrazine by A6. This is a new application of the fluorescence technique for use in pollution remediation. This method could be used for fast screening of potential organisms for use in atrazine pollution remediation. Also, it could be applied on other contaminants using other environmentally relevant microorganisms. It is safer, easy and faster than the existing methods of screening for degrading organisms and also pollutant

uptake by the same, and has immense potential to be used in the field of environmental biotechnology.

To summarize the entire work:

1. A consortium made up of 3 naturally occurring soil isolated bacterial species was developed for use in hydrocarbon pollution remediation.
2. Consortium was capable of acting on hydrocarbons under both lab and field conditions.
3. Consortium members were effective biosurfactant producers, thus reducing the cost of technology by eliminating the use of hydrocarbon degrader and biosurfactant separately.
4. Effectiveness of the use of crude biosurfactant mixture as compared to use of pure compounds used earlier was successfully established.
5. SSC2, one of the consortium member was found to produce rhamnolipid mixture upon growth on different carbon sources. A low surface tension (29-30dynescm^{-1}) and a low CMC (2-6mgl^{-1}) was achieved by the isolated biosurfactant signifying its effectiveness and efficiency.
6. Structural characterization of rhamnolipid mixture revealed the presence of 13 different congeners differing slightly upon change in growth media, RL1 and RL2 being the most prominent members.
7. A new rhamnolipid homolog was reported: Rh-Rh-C_{10}-C_{14}
8. Some of the separated individual compounds exhibited excellent antimicrobial activity against a number of different organisms signifying their potential application in the field of biomedical science. Earlier, use of rhamnolipid mixture has been analyzed as a whole and no report is there on the specific biological activity of individual compounds. Also two of the isolated compounds exhibited activity against yeast, a fact not earlier reported for rhamnolipid.
9. Hydrocarbon uptake studies revealed pseudosolubilization as an active mechanism adopted by the studied *Pseudomonas aeruginosa* strain.
10. Role of biosurfactant in hydrocarbon (n-hexadecane) uptake was substantiated by electron microscopic studies which revealed the formation of biosurfactant-hexadecane complex as an anchorage site for feeding bacteria.

11. Contrary to earlier held view, we propose the internalization of biosurfactant layered hydrocarbon droplet inside cells and accumulation of large amounts of unmodified hydrocarbon before their breakdown into smaller inclusions for degradation.

12. A new atrazine degrading *Acinetobacter radioresistance* sp was isolated for potential remediation of triazine contaminated soil. No report is there on the degradation of atrazine by any member of this genus.

13. A new fluorescent based method was developed for potential application in the field of environmental biotechnology for fast screening of atrazine or other pesticide degrading organism (s) and uptake of the toxic pollutant by the same.