CHAPTER VIII

8.1 DISCUSSION

Phylogenetic analysis reveals that great diversity exists among biosurfactant producing microorganisms, suggesting that biosurfactant production is an important survival tool for the producing microbes and biosurfactant production appears to have evolved in an independent yet parallel fashion (Bodour et al., 2003). Cyclic lipopeptides including surfactin, iturin, fengycin, and lichenysin, are the major classes of biosurfactant produced by Bacillus species (Deleu et al, 1999; Vater et al, 2002). It has been shown that different strains of B. subtilis can produce significantly different classes of lipopeptides in a different combinations (Ahimou et al., 2000), however the exact contribution of theses molecules to their producing species, apart from influencing the cell surface hydrophobicity is not completely understood. In the present study, the significance of diverse CLP production by two B. subtilis strains isolated from extremely different habitats, one from petroleum contaminated soil sample and the other from the cake used for the alcohol production was explored. In these diverse environment, bacteria were dependent on the utilization of specific available substrate (e.g. hydrocarbon or starch) from the environment for their natural growth and survival.

A search of the literature indicates that the ability to produce biosurfactants is wide spread in the bacterial and archeal domains, and biosurfactants producing organisms have been isolated from a wide diversity of environments including soil, seawater, marine sediments and oil fields (Maier, 2003). The biosurfactants produced by P. aeruginosa at mesophilic growth conditions (30-37 °C) have been characterized as rhamnolipids (Guerra-Santos et al. 1984; Syldatk et al. 1985; Parra et al. 1989) and are found to be excellent emulsifiers of hydrocarbons. However, with the few exceptions, efficient biosurfactants production by Pseudomonas sp. at thermophilic growth condition (45 °C) has not been reported. In the present study, two Pseudomonas aeruginosa strains were isolated by enrichment culture from hydrocarbons contaminated soil samples and they produced significant amount of biosurfactants at 45°C temperature. Infact, this was the first report describing biosurfactants production by P. aeruginosa strains at 45°C temperature. Further, the biochemical properties and biological activities of these biosurfactants were characterized with an aim to explore their possible industrial applications.
8.1.1 The bacterial isolates

In this study, three out of the four potential bacterial strains (viz., *B. subtilis* DM-04, *P. aeruginosa* M and NM) were isolated from petroleum crude oil contaminated soil sample whereas one strain (*B. subtilis* DM-03) was isolated from fermented food (Das et al., 2004). Among the environmental samples used for the isolation of microbes, 27.2%, 25% and 41.6% of the culturable microbes obtained from petroleum sludge, petroleum contaminated soil sample and petroleum contaminated water sample respective were efficient biosurfactants producers. *Pseudomonas* strains have often been isolated from soils contaminated by water-insoluble compounds such as petroleum products (Mac Elwee et al., 1990; Arino et al., 1996). In environments contaminated by unleaded gasoline, a recent study showed that they were particularly abundant since Ridgway et al. (1990) identified up to 86% of pseudomonads among 244 isolates. Exposure to hydrophobic pollutants in contaminated soils appeared to select biosurfactants producers, which could emulsify hydrophobic compounds (Francy et al., 1991; Ron and Rosenberg, 2001). Under these specific circumstances, *Pseudomonas* species appeared to exhibit the capacity to produce biosurfactants. However this property did not seem to be shared by other strains of this species which have been isolated from uncontaminated environment (Persson and Mollin, 1987). A study of polyaromatic hydrocarbons (PAHs) degrading isolates obtained from contaminated and soil sites showed that 67% of the isolates produced surfactants (Willumsen and Karlson, 1997). In a different study, twenty of the 21 soil samples (including uncontaminated, hydrocarbon-contaminated, metal contaminated and hydrocarbon-metal co-contaminated), were found to contain at least one biosurfactant producing isolate even using a very limited screening methology (Bodour et al., 2003). Hence, the association of biosurfactants production with microbial degradation of hydrocarbons is a well-established phenomenon. In fact, all the earlier reports on biosurfactant production came from the research works on hydrocarbon fermentation (Cooper and Zajic, 1980; Zajic and Steffens, 1984). So effort was directed to isolate the biosurfactants producing *P. aeruginosa* strains by enrichment culture on n-hexadecane as carbon source. The enrichment culture technique was extremely useful in selection and subsequent isolation of the hydrocarbons degrading potential bacteria. These bacteria were further characterized for biosurfactants production.

The soil borne *Bacillus* strain in the study, viz. *B. subtilis* DM-04 was isolated by crude petroleum contaminated soil sample and the food borne strain viz., *B. subtilis* DM-03 from fermented food without enrichment culture (by direct plating). Occurrence of *B. subtilis* strain
in soil has been reported earlier (Knox et al., 2000; Reva et al., 2004). However, strains of Bacillus and Pseudomonas at hydrocarbon contaminated sites have been determined to be biosurfactant producer (Jennings and Tanner, 2000). B. subtilis strains are always present in high numbers in fermented product (Sarkar et al., 1994; Nout et al., 1998; Roongsawang et al., 2000; Xia et al., 2005). This species has also been associated with incidents of food-borne disease related to unheated food such as ropy bread (Nout et al., 1998).

**8.1.2 Fatty Acid Methyl Ester (FAME) profile analysis for taxonomic identification of isolated microbes**

The analysis of fatty acid methyl ester (FAME) profiles by gas chromatography (GC) is a rapid and inexpensive technique that holds great promises in microbial identification. FAME analysis is based on the facts that there are a large number of different kinds of fatty acids in the cellular lipids of microorganisms and that different microorganisms have different combinations of these fatty acids. Because fatty acids can be readily volatized following methylation (that also prevent their oxidation during GC analysis), FAME profiles can easily be analyzed by gas chromatography following the standard protocol of Microbial Identification System. When FAME profiles study is coupled with principal component analysis, more accurate information can be obtained to identify similarities and differences in characters among microbial communities.

The analysis of whole cell FAME profiles has certain advantages over the conventional biochemical test used for strain identification (Buyer et al., 1996) like (i) Fatty acid analysis is more objective and less prone to human error. (ii) In contrast to biochemical tests, fatty acid composition is not influenced by plasmid loss or gain and is rarely influenced by organism mutation. (iii) Fatty acid analysis is based on species database (accounts for normal species variability) versus a series of yes/no answer (iv) Biochemical- based identification can take up to several days, compared to just several hours for fatty-acid-based identification.

Similarly, FAME analysis has some unique advantage over DNA based microbial identification which includes such as (i) the cost for fatty acid analysis is low as compared to microbes identification by DNA techniques (ii) technical proficiency required for DNA techniques is high (iii) fatty acid analysis can identify to the strain level versus the species level for most DNA based methods. (iv) DNA- based identification is very labor intensive (v)
DNA-based identification can take up to several days, compared to just hours for fatty-acid based identification.

Characterization of physiological and morphological properties and GC analysis of cellular FAME of two of the isolated bacterial strains viz., PS-3 and SC-1 confirmed their identity as *Bacillus subtilis*. As discussed in section 8.1.3, based on the 16s-23s rRNA PCR-RFLP analysis, these were proved to be different strains, hence subsequently designated as *B. subtilis* DM-03 and *B. subtilis* DM-04 strains respectively.

Similarly, the physiological and morphological characterization along with GC analysis of cellular FAME of bacterial strains viz., PS-1 and PS-2 leads to their identification as *Pseudomonas aeruginosa*. A minor difference in the percentage composition of the cellular FAME was observed between these strains, and one of them was identified as *Pseudomonas aeruginosa* mucoid strain. This was named as *P. aeruginosa* M where as the non-mucoid strain was designated as *P. aeruginosa* NM.

**8.1.3 Strain differentiation by 16S-23S rRNA PCR-RFLP**

The two strains of *B. subtilis* in this study viz., *B. subtilis* DM-03 and DM-04 were different at molecular level as was evident from the variation in the band patterns of the digested amplified ISR when resolved in 3% (w/v) agarose gel. However, the similar band patterns of the digested amplified ISR of both the *Pseudomonas* strains in 3% (w/v) agarose gel nullified the polymorphism in the ISR region of these strains, indicating that they probably belonged to the same phylogenetic group.

The sequencing of 16S rRNA alone cannot discriminate among closely related species because of the highly conserved nature of this region (Stackebrandt and Goebel, 1994). However, the ISR of 16S-23S was reported to possess much greater variability in the DNA sequence and proved to be much more useful for the differentiation of closely related bacterial strains (Jensen et al, 1993; Garcia- Martinez et al, 1999, 2001; Johnson et al., 2000; Shaver et al., 2002). Moreover, PCR- RFLP of the 16S-23S ISR was reported to be comparatively much cheaper and simpler technique than sequencing the whole ISR (Gurtler and Stanisich, 1996; Guasp et al., 2000; Kabadjov et al., 2002) and therefore advantage of this technique was exploited to differentiate the *B. subtilis* DM-03 and DM-04 and *P. aeruginosa* M and NM strains at molecular level.
8.1.4 Can the isolated *B. subtilis* or *P. aeruginosa* strains be referred to as thermophilic bacteria?

Both the *B. subtilis* strains in the present study displayed optimum growth at 55°C temperature, demonstrating their thermophilic nature (Sharp and Munster 1986; Weigel and Ljungdht 1986). However, maximum biosurfactants production was observed at 45 °C and 55 °C for *B. subtilis* DM-03 and DM-04 strains respectively, showing that optimum temperature for growth and biosurfactants production may not be the same for a particular *B. subtilis* strain and hence it was concluded that temperature requirement was strain specific. Many of the previous reports supported this observation (Cooper et al., 1981; Makkar and Cameotra, 1997a; Kowall, 1998; Vater et al., 2002).

Both the *P. aeruginosa* strains in the present study displayed optimum growth and biosurfactants production at 45°C temperature, revealing moderate thermophilic nature of the strains (Sharp and Munster, 1986; Weigel and Ljungdht, 1986; Alcamo, 1997). However, due to poor growth of these strains at 50 °C, it would be better to refer them as thermotolerant, instead of stating them as thermophilic strains. Although there existed few reports on biosurfactants production by the *Bacillus* sp. under thermophilic condition (Banat 1993; Makkar and Cameotra 1997a,b, 1998); however not a single report existed showing the optimum temperature requirement for biosurfactants production by *Pseudomonas* sp. exceeds 45 °C. The optimum growth temperature for the *P. aeruginosa* M and NM strains was higher than that reported by Yuste et al., (2002). While studying rhamnolipid production by *Pseudomonas aeruginosa* 44T1 on glucose, Robert et al., (1991) noticed that the best temperature for the product formation was 37°C.

8.1.5 The choice of the nitrogen and carbon sources has major influence on the yield of biosurfactant production by the microbial community

The choice of the nitrogen and carbon source has a major influence on the yield and biosurfactant production by bacteria under study (Suzuki et al., 1974; Desai et al., 1994). The role of nitrogen in influencing biosurfactant production was quite evident in our study, because the microbes growing on nitrogen depleted medium showed poor surface activities (biosurfactant production) as well as least growth, which was in good agreement with the findings of Makkar and Cameotra (1997a, 1998). However, our observation as well as the
findings from other laboratories suggested that different strains of *B. subtilis* had different preferences for either organic or inorganic nitrogen for growth and biosurfactants production. For example, *B. subtilis* DM-03 preferred ammonium nitrate but *B. subtilis* DM-04 was grown well when medium was supplemented with tryptone; *B. subtilis* MTCC 1427 strain failed to utilize ammonium ions but exhibited a preference for nitrate ions (Makkar and Cameotra, 1998); *B. subtilis* MTCC 2423 was not able to utilize ammonium sulphate, but exhibited preference for nitrate ions (Makkar and Cameotra, 1997).

It has been well established that ammonium chloride served as the best source of nitrogen for growth and biosurfactants production for various *Pseudomonas* sp., like *Pseudomonas aeruginosa* 50.3 (Turkovskaya et al., 2001), *Pseudomonas aeruginosa* ATCC 10145 (Chayabutra and Ju, 2001). *Pseudomonas aeruginosa* PTCC 1637 (Tahzibi et al., 2004) etc. Present work also agreed with these findings and further, dose dependent study had proved that among the tested nitrogen sources, 0.1% (w/v) concentration of ammonium chloride served as the best nitrogen source maximum for growth of and biosurfactants production by *P. aeruginosa* M and NM strains. However, the observation that a further increase in the concentration of the ammonium chloride (>0.1 %) resulted in a decrease in the biosurfactants production may be related to the fact that an optimum C/N ratio was required by *P. aeruginosa* for maximum biosurfactants production. An increase in the nitrogen concentration resulted in a lower rhamnose production as well as higher interfacial tension values (Guerra-Santos et al., 1984). Syldatk et al. (1985) reported that nitrogen limitation in the medium caused an overproduction of rhamnolipids by *Pseudomonas* sp.

Haferberg et al., (1986) and Guerra Santos et al., (1984) reported that the majority of known biosurfactants are synthesized by microorganisms grown on water- immiscible hydrocarbons, but some have been produced on water soluble substrates such as glucose, glycerol and ethanol (Cooper et al., 1981; Palejwala and Desai, 1989). In the present study it was observed that *B. subtilis* strains preferred glucose (2% w/v) whereas, the *P. aeruginosa* strains had a preference for glycerol (2% v/v) for maximum growth and biosurfactants production, which reflected the difference in the choice of carbon source for growth and energy production by bacteria at the genus level.

The fact that glucose was the best carbon source for biosurfactants production by both the *Bacillus* strains in this study was in good agreement reports from many other laboratories (Nakano et al., 1988; Sandrin et al., 1990; Roongsawang et al., 2002; Vater et al., 2002).
Discussion

Saccharose and fructose had also been mentioned as efficient carbon sources while the presence of glycerol severely decreased surfactin production. In contrast to other biosurfactants, surfactin biosynthesis did not follow stimulation by hexadecane (Cooper et al., 1981; Sandrin et al., 1990). Makkar and Cameotra (1997a, 1998, and 2001) described the ability of Bacillus strain in their study to use starch and sucrose as the preferred carbon sources for maximum growth and biosurfactants production. Utilization of starch as the carbon source for biosurfactants production was observed in case of B. subtilis DM-03 strain in the present study.

The Pseudomonas strains in present investigation exhibited maximum growth and biosurfactants production when the mineral salt medium (M9) was supplemented with glycerol. Glycerol was also used by some earlier investigators like Turkovskaya et al., (2001) for biosurfactants production. Although growth on glucose had resulted in the maximum decrease in the surface tension of the medium, but the emulsifying activity was only 40%, whereas glycerol was the best source for surfactant synthesis and exhibited a better emulsifying property of 60% (Turkovskaya et al., 2001). We have demonstrated that glucose was the second best carbon source for growth and biosurfactants production by both the Pseudomonas strains which is in good agreement with the findings of many other groups (Guerra- Santos et al., 1984; Reiling et al., 1986; Schenk et al., 1995; Turkovskaya et al., 2001).

8.1.6 Optimization of culture conditions is an important criteria for maximization of bacterial growth along with biosurfactants production.

Various key factors such as choice of carbon and nitrogen sources, growth temperature, growth period, NaCl and mineral-salts concentrations, agitation of culture medium etc, influenced the bacterial growth along with yield of biosurfactants. Therefore, it is utmost important to optimize the various culture conditions in an order to improve the biosurfactants yield or to get the best product.

M9 mineral salt medium was best for growth and biosurfactants production by both the B. subtilis and P. aeruginosa strains. The yield of biosurfactants (6.5g/l) from P. aeruginosa M strain in presence of glycerol as sole source of carbon was appreciably higher than the reported biosurfactants production by other Pseudomonas sp. (Jarvis and Johnson, 1949; Pruthi and Cameotra 1995). Pseudomonas aeruginosa LBI produced rhamnolipids during the
whole cell cycle. It began to accumulate in the medium soon after incubation and followed, in some cases, a diauxic pattern (Benincasa et al., 2002). They had also reported the effect of aeration on rhamnolipid formation and observed that aeration rate (K_a) of 169.9 h^{-1} was optimum for rhamnolipid production by *Pseudomonas aeruginosa* LBI. A batch feed process often enhances the production yield (Linhardt et al., 1989). Benincasa et al. (2002) reported a product conversion yield of 70% and the production was 0.20 g/l per hour, which was higher than that reported by Reiling et al. (1986) while cultivating *P. aeruginosa* DSM 2659 on glucose under continuous culture conditions. NaNO_3 optimum for rhamnolipid production by *Pseudomonas aeruginosa* UW-1 (Sim et al., 1997), *Pseudomonas aeruginosa* 47T2 NCBIM 40044 (Haba et al., 2003), *Pseudomonas aeruginosa* LBI (Benincasa et al., 2002), *Pseudomonas aeruginosa* AT 10 (Abalos et al., 2002), *Pseudomonas aeruginosa* 47T2 NCBIM 40044 (Haba et al., 2003).

Further, it could be assumed that the ability of both the *Bacillus* strains to tolerate high salt (NaCl) concentration (5%, w/v) for growth and biosurfactants production makes them suitable candidates for their field application in saline environment (Bryant, 1987; Banat, 1995; Wilson and Bradley, 1997; Mulligan, 2005).

A biosurfactants yield of 15.6 g/l was reported by Sim et al., (1997), by cultivating *P. aeruginosa* ATCC 10145 on corn oil as the carbon source. *P. aeruginosa* UW-1 produced 24.3 g/l of biosurfactants using 6% (v/v) canola oil after 9 days of incubation at 30 °C (Sim et al., 1997).

As shown by many workers agitation of the Erlenmeyer flask has a major role in the bacterial growth and biosurfactants production (Syldatk and Wagner, 1987; Turkovskaya et al, 2001) Agitation helped in the mixing of mineral salts components of the medium. Intense aeration was crucial for the culture growth and biosurfactant synthesis. Besides supplying oxygen, aeration also helped in mixing the inoculum with the media composition. The agitation at 200 rpm was observed as optimum for all the four bacterial strains in the present study which was in a good agreement with the findings from many other laboratories (Makkar and Cameotra, 1997a,b, 1998; Sim et al, 1997). However, Turkovskaya et al, (2001) reported that agitation at 160 rpm was optimum for maximum biosurfactant production by *Pseudomonas aeruginosa* 50.3. An increase of agitation speed from 250 to 500 rpm caused a decrease in surfactant production by *Nocardia erythropolis* due to a shear rate effect on the growth kinetics of the microorganism (Syldatk and Wagner, 1987). We also observed a similar result.
Chayabutra and Ju, (2001) reported that *P. aeruginosa* ATCC 10145 could grow optimally at a hexadecane concentration of 8% (v/v). The findings of the optimum amount of NaCl requirement and pH, NH₄Cl for the optimum growth and biosurfactants production by the *Pseudomonas* strains in the present study was in good agreement with the findings of Chayabutra and Ju, (2001). Rhamnolipid production is influenced by the nutrients used in the culture media and also on the applied culture parameters. Guerra-Santos et al (1986) reported better yields of rhamnolipids, produce by *P. aeruginosa*, when the concentration of magnesium, calcium potassium, sodium salts and trace elements were minimized. Syldatk and Wagner (1987) reported a similar findings for a *Pseudomonas* sp. Guerra- Santos et al. (1986) also have found many operational problems such as foam formation and wall growth during continuous cultivation.

8.1.7 Correlation between diverse biosurfactants production and survival of producing bacteria in a particular habitat

Lipopeptide profile and bacterial cell surface hydrophobicity varies greatly within the strains. Although the ability of *Bacillus subtilis* to synthesize lipopeptides is independent of bacterial hydrophobicity, but the accumulation of extracellular lipopeptides in the culture medium induce changes in the cell surface hydrophobicity of the producing strain (Ahimou et al., 2000). The hydrophobic alterations suggested the contribution of lipopeptide molecules in adhering *B. subtilis* strains by hydrophobic interaction onto the surface of various hydrophobic substrates found in that habitat, for the easy uptake of the substrate(s), presumable by increasing the surface area of substrate and increasing their apparent solubility (Ahimou et al., 2000; Ron and Rosenberg, 2001). Therefore, it may be concluded that major CLP isoforms secreted by a specific *B. subtilis* strain, may help in the better utilization of specific hydrophobic substrate(s) (eg. starch in case of DM-03 where as hydrocarbons for DM-04 strain) available in the habitat of the lipopeptides producing strain. The following observations supported this hypothesis-

(a) *B. subtilis* DM-04 strain could utilize starch more efficiently (p< 0.001) only when growth medium was supplemented with crude CLPs secreted by *B. subtilis* DM-03 strain.

(b) Although *B. subtilis* DM-03 failed to utilize hexadecane or pyrene carbon source, but significant growth was achieved in the same hydrocarbon source(s) when incubated along with crude CLP isolated from *B. subtilis* DM-04 strain.
(c) The cell surface hydrophobicity of *B. subtilis* DM-03 strain was enhanced when incubated with lipopeptide from *B. subtilis* DM-04 strain, documenting that lipopeptides from the latter strain induce changes in the cell surface hydrophobicity of the former strain. This results in enhanced interaction of *B. subtilis* DM-03 with hydrocarbons and their subsequent utilization.

(d) Chemical analysis of the petroleum oil contaminated soil sample (from where DM-04 strain was isolated) revealed that it contains higher proportions of aliphatic hydrocarbons than the aromatic hydrocarbons and *B. subtilis* DM-04 was shown to be more efficient in utilization of aliphatic hydrocarbon viz. hexadecane as compared to aromatic hydrocarbons for growth and energy production. This finding reinforced that the type or amount(s) of the hydrophobic substrate(s) available in the parent habitat of the bacteria influences the biosurfactants production and subsequent enhance utilization of a specific group of substrate.

The surfactin molecules, owing to their larger molecular size and hence more space occupation, exhibit higher cell surface hydrophobicity than that of iturin molecules (Maget-Dana et al., 1992). This fact can be well correlated with findings from present study that *B. subtilis* DM-04 strain, producing higher amount of surfactin molecules exhibited greater cell surface hydrophobicity as compared to the *B. subtilis* DM-03 strain producing iturins as dominated forms of CLP.

Therefore in the present study, biosurfactants produced by two different strains of *B. subtilis*, isolated from extreme habitats were characterization, in an order to understand their natural role in the growth of the producing strain. The study supported the hypothesis that one group of biosurfactants would have an advantage in a specific ecological niche, whereas another group of emulsifiers would be more appropriate for a different niche (Ron and Rosenberg, 2001; Maier, 2003).

8.1.8 Surface activities (surface tension and CMC values) of biosurfactants are indicators of their efficiency

Both the *B. subtilis* strains were found to be efficient biosurfactant producers based on surface tension measurements of culture supernatants obtained from fermentation performed at various temperatures as well as pH. The biosurfactants produced by *B. subtilis* DM-04
exhibited better surface tension reducing ability in comparison to *B. subtilis* DM-04 strain. This observation supported that *B. subtilis* DM-04 strain produced higher amount of surface active compounds as compared to *B. subtilis* DM-03 strain. Production of higher amounts of surfactin by the former strain supported this observation. These surface tension reduction values were quite consistent with the reported values for the most efficient biosurfactants which had been isolated and studied so far (Cooper and Zajic, 1980; Cooper et al., 1981,1989; Desai and Banat, 1997).

The biosurfactants isolated from all the bacterial strains in the present study have lower CMC values as compared to the tested synthetic surfactant e.g. SDS. CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant (Desai and Banat, 1997). Biosurfactants possess low critical CMC values as compared to synthetic surfactants and thus have an advantage over chemical surfactants (Lin et al., 1998). Rhamnolipids (Steinbuechel, 1991) and lipopeptides are (Morikawa et al., 2000; Vater et al., 2002) the most efficient biosurfactants known till date. Their critical micelle concentrations (CMCs) in water can be as low as 10-20 mg/l, and the corresponding minimal surface tension (MST) ranges from 25 to 30 mN/m. Both properties compared very favorably to those of the synthetic surfactants, eg., CMC at 2023-2890 mg/l and MST at 37 mN/m for sodium dodecyl sulfate, and CMC at 590 mg/l and MST at 47 mN/m for alkylate dodecyl benzene (Lenz et al., 1992; Brandl et al.,1990; Margaert et al., 1992). The CMC values and the MST of biosurfactant from *Pseudomonas* strains in the present investigation are in close proximity with that reported by Haba et al. (2003), Syldatk et al. (1985), Mata-Sandoval et al. (1999). A mutant strain of *P. aeruginosa*, obtained by mutagenesis with N-Methyl-N'-nitro-N-nitrosoguanide designated as *P. aeruginosa* PTCC 1637 produced rhamnolipid biosurfactant having a CMC value of 9 mg/l (Tahzibi et al., 2004). The characteristic of rhamnolipids produced by the mutant strain was identical to that produced by the wild type bacteria. Moreover, biosurfactant from *P. aeruginosa* M strain was found to be excellent emulsifier and the stability of the emulsion was for a much higher period of time as compared to stability of emulsion reported by Turkovskaya et al. (2001). Biosurfactants from *Nocardia* was found to have 4.5 times more emulsion stability in comparison to the chemical surfactant SDS (Kim et al., 2000). In this study it was observed that emulsion stability of biosurfactants from *P. aeruginosa* M and NM stains is 5.4 times and 4.5 respectively more stable than SDS, as was evident from the calculation of decay constant ($K_d$).
8.1.9 MALDI-TOF MS analysis is a useful tool in determining the molecular mass of biosurfactants

It is well known that MALDI-TOF mass spectrometry is an innovative, highly efficient technique to characterize the molecular structures of the microbial biosurfactants or their secondary metabolites (Leenders et al., 1999; Vater et al., 2002; Bonmatin et al., 2003). The [M+H]\(^+\) or [M+Na]\(^+\) ions generated by this technique are very stable, thus leading to intense signals which are very useful for determining rapidly the homogeneity or heterogeneity of the samples by MS analysis (Bonmatin et al., 2003) including the molecular masses of samples with an accuracy of 0.01 to 0.02%. Therefore in this study, MALDI-TOF mass spectrometry was used as a tool to check the homogeneity of preparation and determining the molecular mass. By comparing the mass data obtained for individual fractions and by comparing the mass numbers reported for the lipopeptide complexes from other *B. subtilis* strains (Kakinuma et al., 1969; Kowall et al., 1998; Leenders et al, 1999; Peypoux et al., 1976, 1978, 1984; Schneider et al., 1999; Vater et al., 2002) the lipopeptide products of *B. subtilis* DM-03 could be identified as iturins (C\(_{16}\) - C\(_{19}\)) and surfactins (C\(_{13}\) - C\(_{15}\)) whereas the major lipopeptide isoforms of *B. subtilis* DM-04 were iturins (C\(_{17}\) - C\(_{18}\)) and surfactins (C\(_{13}\) - C\(_{15}\)). The isoforms of these CLP were resolved on the basis of their hydrophobicities which in turn were determined mainly by the length of the fatty acid chains (Vater et al., 2002); therefore iturin isoforms were eluted first from the C18-\(R\) Nova pack reverse phase HPLC column followed by surfactin isoforms. The *B. subtilis* DM-03 produced higher quantity of iturins as compared to surfactins (1.55 Vs 0.546 % of total lipopeptide) but the reverse was true for the *B. subtilis* DM-04 strain, where surfactin isoforms predominated over iturins. *B. subtilis* RB14, YB8 and ATCC6633 have been reported to co-produce surfactin and Iturin A, surfactin and plipastatin B1, and surfactin and mycosubtilin, respectively (Huang et al., 1993; Tsuge et al., 1996; Duitman et al., 1999). Therefore it seems that diverse CLP production by different strains of *B. subtilis* is rather a common ability and must confer some kind of competitive advantage to the producing microorganism in a particular environment (Maier, 2003). It appears that this ability has evolved across the bacterial domain in a parallel and non-convergent fashion with respect to biosynthesis and regulation (Maier, 2003).

In this study, the biosurfactants produced by both the *P. aeruginosa* strains were nearly identical demonstrating only minor difference exists in the biosurfactant isomers of these strains. In general, molecular mass of active biosurfactants from *P. aeruginosa* strains have been detected in the range from m/z 331 to 677 (Jarvis and Johnson, 1949; Chayabutra and
Ju, 2001; Haba et al., 2003; Benincasa et al., 2004), but very interestingly the m/z of most active biosurfactant isoforms secreted by P. aeruginosa M and NM strains were detected in the range from m/z 1348 to 1832.

8.1.10 Thermostability assay of biosurfactants: a helpful method in assessing the potency of biosurfactants before their industrial applications

Biochemical characterization of biosurfactants secreted either from B. subtilis DM-03 or DM-04 demonstrated their lipopeptide nature, whereas the secreted biosurfactants P. aeruginosa M and NM strains composed of mixture of carbohydrate, protein and lipid. High thermostable nature of these biosurfactants was evident when heating at 100 °C for 60 min did not effect their tested biochemical or surface active properties. This was in good agreement with the earlier reports demonstrating thermostable nature of biosurfactants from other B. subtilis strains (Makkar and Cameotra, 1998) as well as from Pseudomonas strains (Johnson and Boese-Marrazzo, 1980; Turkovskaya et al., 2001). However, the crude lipopeptide from DM-04 strain preferred higher pH as compared to DM-03 strain for exerting maximum surface activity. This phenomena may be related to the presence of higher amount of surfactin isomers in the crude lipopeptide secreted by B. subtilis DM-04 strain because it is well established that surfactin molecules have a preference for higher pH (Morikawa et al., 2000).

8.1.11 Pharmacological characterization and biosafety assessment are important criteria before the field trial of biosurfactants

The iturin and not the surfactins isoforms, of both the Bacillus strains exhibited direct hemolytic activity on washed human erythrocytes, crude lipopeptide mixture from either strain showed higher hemolytic activity as compared to individual CLPs. However, because of the limited recovery of the HPLC purified iturins and surfactins molecules, the finding of Delue et al., (2003), who reported nearly 30% hemolysis by 10μM of surfactin, could not be confirmed. It seems that very low concentration of surfactins (0.05 μM in the present study) may not induce hemolysis and therefore hemolytic effect of these molecules are due to detergent like action rather than presence of any specific receptor(s) in the surface of RBC. Infact, Deleu at al., (2003) on the basis of computer stimulation modeling also suggested that hemolysis activity of surfactin results from the their detergent effect. The higher hemolytic activity of crude lipopeptide as compared to individual fractions was due to the synergistic
interaction between the surfactin and iturin molecules. The presence of surfactin, at a concentration at which alone, it is inactive, increases to a very large extent the haemolysis percent induced by iturin A (Maget-Dana et al., 1992).

The crude lipopeptide from *Bacillus subtilis* DM-03 and DM-04 exerted anticoagulant activity. Arima et al., (1968), first reported the anticoagulant property of surfactants from *Bacillus subtilis* strain. The concentration of surfactin needed to bring about 50% inhibition was about 30 μg/ml (Arima et al., 1968). The inhibition site of surfactin in Ca-clotting system is the polymerization step from fibrin monomer to fibrin polymer. The ability of surfactin to inhibit clot formation may be derived from the strong surface-active properties of surfactin (Arima et al., 1968).

The crude lipopeptide (100μg) from *Bacillus subtilis* DM-03 and DM-04 displayed in vitro liver tissue damaging activity, but lipopeptide biosurfactants from either source under the identical experimental condition, failed to show any detrimental effect on heart and lungs tissues showing a tissue specificity for these lipopeptides. Surfactant exerted its cellular effect by altering membrane integrity (Bernheimer and Avigad, 1970). The alteration in the membrane topography could be attributed to the ability of lipopeptide to interact with phospholipids, along with an interference with ion chelation systems (Peypoux et al, 1999). The cation membrane topography facilitated the lipopeptides to penetration into the membrane (Maget-Dana and Ptak, 1995). At a very low concentration the lipopeptide is miscible with phospholipids, the mechanism of interaction being assisted by conformational changes displayed by the peptide cycle when it collides with a lipid with which it has affinity. At high concentrations, the detergent effect was prominent and led to the membrane disruption (Carrillo et al., 2003). Carrillo et al., 2003 reported dose dependent surfactin induced vesicle-content leakage, finally leading to the destabilization of the membrane. The onset for membrane solubilization occurred at a surfactin / lipid ratio of 0.92, which was termed as $R_{sol}$ (Carrillo et al., 2003). It was also previously reported that in mixtures with dimyristoylphosphatidylcholine, surfactin displays fluid-phase immiscibility, suggesting the formation of surfactin-rich domains within the membrane (Grau et al., 1999). Sheppard et al., (1991) proposed the formation of ionic channels induce by surfactin in planar lipid bilayer membrane.

There was hardly any report available describing the pharmacological properties of biosurfactant produced by *P. aeruginosa*. The RBC hemolytic properties of biosurfactants
produced by both the *P. aeruginosa* strains could be exploited for rapid screening of the biosurfactant producing microbes in blood agar plates. Capability of rhamnolipids to cause hemolysis of erythrocytes was also reported earlier (Johnson and Boese-Marazzo, 1980; Tuleva et al., 2002). Since, the *P. aeruginosa* biosurfactants did not have any detrimental effects on tissues or living system, therefore the coagulant biosurfactants from both the *P. aeruginosa* strains can be exploited pharmaceutically for the development of topical (local) coagulant drugs to prevent the bleeding during minor surgical operations.

8.1.12 Plasmid DNA does not play any role in biosurfactant production by *P. aeruginosa* M strains.

Among the four bacterial strains, plasmid DNA was found on only on *P. aeruginosa* M strain. The present study revealed that *P. aeruginosa* M strain plasmid DNA had no role to play in biosurfactant production, since no significant change in the surface activity of culture supernatants as well as biosurfactant production by wild and mutants strains of *P. aeruginosa* M strain were observed. This finding reinforced the observations from other laboratories that major biosurfactant producing genes of *P. aeruginosa* are located disparately on the chromosome and not on plasmids (Maier, 2003).

8.1.13 Production of biosurfactants by utilizing non-conventional and cheaper carbon sources is cost effective and can further enhance the industrial application of biosurfactants

The economic of biosurfactants production is one of the factors, which determines its better acceptability for industrial applications and future prospect. Different ways should be explored to reduce the production costs through better yields of biosurfactants and product accumulation, economic engineering processes and use of cost-free or cost-credit feedstock for microbial growth and biosurfactant production (Makkar and Cameotra, 1997b). Currently microbial surfactants price ranges between 2 to 3 USD per 1 kg and are 20-30% more expensive than their synthetic equivalents. The choice of the medium components is very important since they constitute 50% of the total production costs. So, food industry by products or other wastes can be exploited from this point of view (Daniel and Otto, 1999; Haba et al., 2000). In recent year, researchers have targeted to use cheaper material for the production of biosurfactants.
Since *B. subtilis* secrete significant α- amylase enzymes in the culture supernatant (Das et al., 2004) and also produce lipopeptide biosurfactants, it was thought that biosurfactant production could be feasible from potato peels, a cheaper source of carbon. Present study vouch for the production of lipopeptide biosurfactants by thermophilic *Bacillus* strains using potato peels from kitchen wastes as the carbon source. There are numerous reports existed on biosurfactants production using industrial by products or waste. Mulligan and Cooper (1985) used water collected during drying of fuel-grade peat for biosurfactant production by a *Bacillus subtilis* strain. Sheppard and Mulligan (1987) used peat hydrolysate for biosurfactant production. Rhamnolipid production by a *Pseudomonas* sp. was reported when the organism was grown on olive oil mill effluent (Mercede et al., 1993), soap stick oil has (Mercede and Manersa, 1994), soapstock and waste-water from sunflower oil (Benincasa et al., 2002). Daniel et al., (1998) reported sophorolipids by *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509 from deproteinized whey and rapeseed oil in a two stage fed batch process. Bednarski et al., (2004) reported glycolipid production by *Candida antarctica* ATCC 20509 and *Candida apicola* ATCC 96134 grown on oil refinery waste. *B. subtilis* stains are known for utilization of waste substrates for biosurfactants production. For example, Ohno et al., (1996) reported the ability of *B. subtilis* NB22 strain to produce lipopeptide biosurfactant by utilizing soybeand curd residue. Makkar and Cameotra (1997b) reported biosurfactant production by two *Bacillus* strain viz., *Bacillus subtilis* MTCC2423 and *Bacillus subtilis* MTCC 1427 using molasses (a by-product of sugar cane industry) as carbon source supplement in mineral medium, under thermophilic growth conditions. Hence, the utilization of potato peels in biosurfactants production by the *B. subtilis* strains could be expected to cut the production cost and subsequently commercialization of the products.

8.1.14 The isolated biosurfactants from *Pseudomonas* and *Bacillus* strains may be useful in MEOR application

The potential use of biosurfactant in Microbial Enhanced Oil Recovery (MEOR) was evaluated using the sand pack column technique. Among the four bacterial strains under study, biosurfactant from the *P. aeruginosa* were comparatively more efficient than *B. subtilis* strains in releasing appreciable amount of oil (kerosene) from sand pack column. The percent oil release from both the *P. aeruginosa* strains was in good agreement with the report by Pruthi and Cameotra (1997) using biosurfactant produced by a strain of *Serratia marcescens*. This recovery of oil from saturated sand pack column by either biosurfactant in the present study is much higher than that of the biosurfactants from *Bacillus subtilis* strains
as reported by Makkar and Cameotra, (1998), but lower than the biosurfactant from a thermophilic *Bacillus* sp. (Banat, 1993). The ability of both the stains to grow in high salt concentration, wide range of temperature (30-55°C) and pH (4-12) could be exploited for such industrial application, such as for *in situ* oil recovery from oil wells with moderate well temperature.

8.1.15 Application of biosurfactant producing bacteria and isolated biosurfactants in bioremediation of oil contaminated environmental samples: an eco-friendly strategy to degrade the hydrocarbon components

Oil pollution accidents are now a days become a common phenomenon and have caused ecological and social catastrophes (Shaw, 1992; Burger, 1993; Burns et al., 1993; Mishra et al., 2001). The ability of biosurfactants to emulsify hydrocarbon-water mixtures has been widely reported. These emulsification properties has also been demonstrated to enhance hydrocarbons degradation in the environment, hence making them potentially useful tools for oil spill pollution-control (Atlas and Bartha, 1992; Atlas, 1993b; Bertrand et al., 1994).

Many of the standard treatment processes used to decontaminate soil and groundwater have been limited in their application, are prohibitively expensive, or may be only partially effective (Nicholas, 1987). Problems associated with the clean up of leaking disposal sites and spills of toxic substances have demonstrated the need to develop remediation and waste reduction technologies that are effective, economical and rapidly deployable in a wide range of physical setting (Catallo and Partier, 1992).

Traditional methods of treating soil and groundwater contamination have relied upon removal or containment (Brown et al., 1986). Most of these treatment schemes are not completely effective and do not offer permanent solutions for containment or remediation. Some methods might even create additional uncontrolled hazardous waste. Therefore, this is the urgent demand by the society to greatly reduce the volume and toxicity of waste and development of safe, effective and economic alternatives for its disposal (Nicholas, 1987).

Both *in situ* and on-site treatment processes by involving the use of microorganisms to break down hazardous organic environmental contaminants, avoid the economic and technical disadvantages, as well as environmental risks, incurred by transport of hazardous wastes to alternative treatment facilities (Ahlert and Kosson, 1983; Lee and Ward, 1985).
An assessment and comparison of the capability of *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains was made to explore biodegradation efficiency of oily sludge, a hazardous hydrocarbon waste generated by the petroleum industry. Survival of the microorganisms in the soil after their seeding/ inoculation is a key deciding factor in the rate of biodegradation of hydrocarbons (Ramos et al., 1991). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, therefore they survived and adopted the oil-contaminated soil environment very easily (Sugiura et al., 1997; Mishra et al., 2001; Rahman et al., 2003).

Natural soil bacteria present in this oily sludge may be in a dormant or slow-growing state, did not show appreciable biodegradation of hydrocarbons even when supplied with glucose or mineral salts media (control experiment). It has been reported that bioremediation is negligible, if the population of hydrocarbon-degrading microorganisms is less than $10^5$ cfu/ g in soil (Forsyth et al., 1995; Mishra et al., 2001). These reports have supported our view that a specific group of bacteria or a bacterial consortium capable of degrading the petroleum hydrocarbons must be seeded in the soil at a population greater than $1\times10^5$ cfu/ g in soil for the effective bioremediation of contaminated soil (Mishra et al., 2001). Further, it has been shown in the present study that supplying of glucose as co-carbon source along with pyrene (a major carbon source) enhanced the rate of degradation of pyrene by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains along with a concomitant increase in bacterial biomass. This leads to prompt us to supply glucose in the soil after a regular interval to increase the rate of biodegradation of TPH. However, the results are in contradiction to report of Chhatre et al., (1996) describing addition of nutrients in the soil was unlikely to have dramatic effect on the microbial degradation of crude oil.

In this study, *Pseudomonas* consortium was shown to possess significantly higher potential for the biodegradation of TPH as well as various fractions of crude petroleum oil compared to *B. subtilis* DM-04 strain. It is to be mentioned that *B. subtilis* DM-03 strain was not included in biodegradation study owing to its poor growth in hydrocarbons. An organic chemical may be subjected to non-enzymatic reactions brought about by microorganisms in the soil (Alexander, 1980); however, it is the enzymatic reactions that bring about the catabolism of organic compounds (Kanaly and Harayama, 2000). Key factors such as presence of a specific and or higher amount of inducible enzyme(s), substrate specificity of hydrocarbon degrading enzymes (Gibson et al., 1984; Kanaly and Harayama, 2000; Sharnagouda and Karegoudar, 2001) etc., may be responsible for higher metabolism of TPH by *Pseudomonas*
strains. Although the biochemical pathways for the biodegradation of various PAHs by microbes have been well established (Gibson et al., 1984), however the pathways for PAHs degradation in *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains are yet to be discovered. Apart from the biodegradative enzymes, presence of sphingolipids or other specific molecule in the outer membrane structure of bacteria, enabling them to adhere to a specific substrate, may also be responsible for higher bio-degradation of TPH or a specific fraction of petroleum hydrocarbon by a specific group of bacteria (Sugiura et al., 1997). Possibility of presence of these kinds of molecules in the outer membrane of *B. subtilis* DM-04 or *P. aeruginosa* strains should be investigated in near future.

It has been stated that biosurfactant production is an important survival tool for the producing microbes (Ahimou, 2000; Ron and Rosenberg, 2001; Maier, 2003) production of biosurfactant is related to the utilization of available hydrophobic substrate(s) by the producing microbes from their natural habitat, presumably by increasing the surface area of hydrophobic substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). This hypothesis supports our present observation, where biosurfactants from *B. subtilis* DM-04 or *P. aeruginosa* M and NM strains were shown to enhance the apparent solubility of PAHs in dose-dependent manner. Hence for efficient and complete biodegradation, solubilization of contaminating hydrocarbons with biosurfactants prior to bioremediation is advantageous (Rahman et al., 2003). To achieve this goal, the oil-sludge was treated with biosurfactants prior to inoculating the known *Bacillus* or *Pseudomonas* cultures with an aim to enhance the apparent solubility of hydrophobic hydrocarbons with a corresponding increase in their bioavailability for higher biodegradation by bacteria. Moreover, use of biosurfactant producing, hydrocarbon degrading, microorganisms for bioaugmentation to enhance hydrocarbon degradation offer the advantage of a continuous supply of no-toxic and biodegradable surfactant at low cost (Moran et al., 2000; Rahman et al., 2002).

GC/FID analyses and other study demonstrated that n-alkanes were preferentially degraded compared to PAHs by all the bacteria used in this study. The outer membrane permeability of bacteria may be one of the major factors to determine the biodegradability (Sugiura et al., 1997). Solubility may be another factor to influence biodegradability, it is being known that the solubility, and hence the accessibility to catabolic enzymes, of a hydrocarbon molecule decreases as the number of its carbon atom and cyclization increases (Sugiura et al., 1997).
In conclusion, biodegradation as a treatment alternative generally consists of optimizing various physical and chemical factors of soil including optimization of nutrient and biosurfactants concentrations to stimulate the growth of the organisms that will metabolize the particular contaminants present. Therefore it is necessary to establish the optimum environmental conditions, nutrient and biosurfactants application rates in the laboratory bench-scale studies prior to practical field application of microbes for effective bioremediation.

8.1.16 Comparative study of pyrene utilization by \textit{B. subtilis} DM-04, \textit{P. aeruginosa} M and NM strains: Role of biosurfactants in enhancing the apparent solubility and metabolism of pyrene

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants occurring mostly as a result of fossil fuel combustion and as by-products of industrial activities. Since many of them are either known or suspected carcinogens and mutagens, exposure to PAHs may result in a significant health risk to human populations (White, 1986), and therefore their fate in nature is of great environmental and medical concern.

In this paper, we have presented the data demonstrating differences in utilization of pyrene as sole source of carbon and energy by two non-actinomycetes groups of bacteria viz. \textit{B. subtilis} DM-04 and \textit{P. aeruginosa} N and NM strains, which were isolated from a petroleum contaminated soil sample. This difference in pyrene metabolism resulted in a significantly different growth of these bacteria. Further, we studied the role of biosurfactants produced by respective bacterial strains in enhancing the apparent solubility of pyrene that leads to a significant increase in pyrene metabolism by bacteria for growth and energy production.

There are many reports on bacterial degradation of pyrene, a high-molecular weight precondensed PAH, mainly by actinomycetes groups of bacteria such as \textit{Mycobacterium} and \textit{Rhodococcus} (Kanaly and Harayama, 2000; Vila et al., 2001). Besides, a variety of non-actinomycetes bacteria such as \textit{P. aeruginosa}, \textit{P. pudita}, \textit{Flavobacterium} sp. were reported to utilize pyrene, when supplemented with other forms of organic carbons (Trzesicka-Mlynarz et al., 1995). But subsequently it was demonstrated the possibility of utilization of pyrene or other PAHs as sole source of carbon and energy by \textit{B. subtilis} DM-04 and \textit{P. aeruginosa} strains owing to their ability to produce biosurfactants in the culture medium (Mueller et al., 1990; Deziel et al., 1996). In the present study, it was provided further evidence of utilization
of pyrene by *Bacillus* and *Pseudomonas* bacteria as sole source of carbon by demonstrating the correlation between increase in bacterial growth (dry biomass, protein concentration and O.D. of culture medium at 600 nm) and a concomitant decrease (degradation) in pyrene content from the culture medium with respect to time. However, there are also reports on soil *Pseudomonas* sp. capable of degrading PAHs, but fail to utilize them as sole source of carbon and energy (Foght et al., 1988).

The growth (dry biomass yield) of *B. subtilis* DM-04 and *P. aeruginosa* N and NM strains at the expense of pyrene as sole carbon source after 96 h of inoculation suggested an assimilation of about 35.6%, 32.0% and 28.0 % of pyrene carbon respectively, showing differences in metabolism and utilization of pyrene by these three bacterial strains, isolated from the same environmental sample. Guo et al., (2005) demonstrated that PAH- degrading bacteria isolated from mangrove sediments possess different potential to degrade PAHs, and degradation percentages were not related to levels of PAHs contaminated in mangrove sediments. Moreover, the result of the study was also in good agreement with the findings of many previous researchers, who observe that microbial growth on pyrene did not result in complete removal of the substrate (Mulder et al., 1998; Vila et al., 2001). Observed attenuation in pyrene degradation occurred presumable by biofilm formation on pyrene crystals and consequent prevention of pyrene dissolution, while previously accumulated intermediates would allow further cell growth (Vila et al., 2001.)

There may be several key factors such as presence of a specific and or higher amount of inducible enzyme(s), substrate specificity of PAH degrading enzymes (Sharanagouda and Karegoudar, 2001, Gibson et al., 1984), are responsible for higher metabolism of pyrene by *B. subtilis* DM-04 strain compared to *P. aeruginosa* M and NM strains. The biochemical pathways for the biodegradation of aromatic compounds by microbes have been well documented (Gibson et al., 1984), and initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of PAH to a dihydriodiol by a multi-component enzyme system (Kanaly and Harayama, 2000). The identification of metabolites accumulating during the growth of *Mycobacterium* sp. strain AP1 in pyrene suggested that this stain initiates its attack on pyrene by either monoxygenation or dioxygenation at its C-5, C-5 positions (Vila et al., 2001). However, the pathway(s) for pyrene degradation in *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains are yet to be discovered.
Phylogenetic analysis revealed that great diversity exists among biosurfactant producing microbes suggesting that biosurfactant production is an important survival tool for the producing microbes and appears to have evolved in an independent yet parallel fashion (Bodour et al., 2003). It has been reported that biosurfactant producing bacteria are found in higher concentration in hydrocarbon contaminated soils (Bodour and Maier, 2003), however there are only few reports of microorganisms producing surface-active compounds while growing on PAHs (Deziel et al., 1996). In the present study, all the three bacteria were found to be efficient biosurfactant producers based on (i) surface tension measurement of culture supernatants obtained after 24 h of bacterial growth and (ii) by determining the yield of biosurfactants. The production of biosurfactant by all the three bacterial strains increased significantly when the medium was supplied with glucose (as a co-substrate) along with pyrene. Glucose, which is a versatile precursor for many biomolecules, acts as inducer and co-source of carbon, energy and reducing power for microbes that leads to substantial increase in bacterial biomass with a corresponding increase in biosurfactant production.

Previous study have documented that production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). Therefore, use of biosurfactants has been reported as a mechanism to enhance the bioavailability of hydrophobic pollutants and PAHs for microbial degradation (Thiem, 1994; Guerin and Jones, 1988). It has been demonstrated that growth of Mycobacterium sp. strain LB 50 IT was directly related to substrate dissolution from crystals and to the uptake of substrate from the solution by microorganisms (Wick et al., 2001). Low molecular weight biosurfactant like lipopeptides that have low critical micelle concentrations (CMCs) increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities off micelles (Miller and Zhang, 1997), whereas alasan, a high molecular weight bioemulsifier complex produced by Acinetobacter radioresistens KA 53 enhanced the aqueous solubility of PAHs by a physical interaction most likely of a hydrophobic nature and increases the biodegradation rate of PAHs (Barkay et al., 1999).

In this study, convincing data was presented showing that B. subtilis DM-04, P. aeruginosa M and NM biosurfactants at a concentration of 0.5 mg/ml subsequently enhance the apparent solubility of pyrene by factors 5 to 7 resulting in its higher uptake and metabolism by bacteria compared to non-solubilized pyrene. The difference in pyrene solubilization effect of biosurfactants from different bacterial strains in this study may be related to the chemical
nature as well as surface properties of biosurfactants. For example, major biosurfactants secreted by \textit{B. subtilis} DM-04 were lipopeptide in nature containing higher amount of surfactins compared to iturins, whereas biosurfactants secreted by \textit{P. aeruginosa} M and NM were found to be a complex mixture of lipopeptides and glycoproteins. Moreover, the significantly higher pyrene solubilization effect of biosurfactants from \textit{P. aeruginosa} NM strain compared to \textit{P. aeruginosa} M strain reinforces the hypothesis that a minor variation in biosurfactant isoforms between these two strains may result in a large variation of the emulsification property and specificity of biosurfactants. It may be concluded that higher pyrene solubilization effect of biosurfactants from \textit{P. aeruginosa} NM strain dramatically enhanced the metabolism of pyrene, that sustained the growth of this bacteria in pyrene, otherwise it would not be able to grow on pyrene. Further studies to understand the microbial ecology of PAHs degrading communities and their application for the development of bioremediation strategies for PAHs are in progress.

### 8.1.17 Potential use of the isolated biosurfactants as antimicrobial agents

Several lipopeptide surfactants including the cyclic lipopeptides of \textit{B. subtilis} are potent antibiotics (Ron and Rosenberg, 2001; Maier, 2003, Singh and Cameotra, 2004). Interestingly, the antibiotic potency and microbial specificity of the crude lipopeptides from \textit{B. subtilis} DM-03 and DM-04 strains as well as between \textit{P. aeruginosa} N and NM strains differed which may be due to the production of diverse biosurfactants isoforms. It is reasonable to assume that this antibiotic specificity of lipopeptides may have a natural role in enhancing the growth of the producing bacteria by inhibiting the other interfering microbes present in their original habitat. Differences in the antimicrobial potency of crude lipopeptide produced by \textit{B. subtilis} DM-03 and DM-04 strains against \textit{Saccharomyces cerevisiae} and \textit{Pichia anamala} yeast strains, which were co-isolated along with \textit{B. subtilis} DM-03 strain from fermented food, supported the hypothesis. These yeast strains, present in the natural habitat of the \textit{B. subtilis} DM-03 strain, compete with the bacteria for the uptake of glucose formed by the hydrolysis of starch by the action of \textalpha-amylase secreted by \textit{B. subtilis} strains. Therefore, to prevent these yeast strains from substrate (glucose) utilization and to favor the growth of producing bacteria, higher amounts of iturin isomers produced by \textit{B. subtilis} DM-03 strain inhibits the yeast cells probably by disrupting the plasma membrane of yeast cells by formation of small vesicles and the aggregation of their intra membranous particles (Thimon et al., 1995).
Most work on biosurfactant applications has been limited to their use in pollution control (Banat, 1995) and enhancing the availability of various hydrophobic compounds for microbial degradation (Mulligan, 2005). In addition, biosurfactants might have other applications, particularly as antimicrobial agents. Antagonistic activity of Pseudomonas sp. biosurfactant was reported against pathogenic fungi Pythium ultimum and Rhizoctonia solani at a concentration of 100 μg (Nielsen et al., 2002). Rhamnolipids from Pseudomonas aeruginosa were reported to have antagonistic activity against a wide range of microorganisms (Jarvis and Johnson, 1949; Abalos et al., 2001; Haba et al., 2003; Benincasa et al., 2004), and bacteriostatic effect on Mycobacterium tuberculosis, a tuberculosis causing agent. Very recently, the algicidal potency of rhamnolipid biosurfactants produced by Pseudomonas aeruginosa against some harmful algae was demonstrated (Wang et al., 2005). In addition, rhamnolipids from P. aeruginosa have potential for biological control of zoosporic plant pathogens (Staghellini and Miller, 1997). The biosurfactants from P. aeruginosa M strains exhibited significant anti-microbial activity on all the tested microorganisms, except Candida glabrata and therefore their use as broad-spectrum antibiotics is highly promising. The current investigation provided the evidence that only a minor variation in the biosurfactant isoforms can cause a huge difference in their antimicrobial specificity. However, the antibiotic potency and specificity of the biosurfactants from these strains against the medically important microbes remains to be elucidated. Moreover, in order to develop a safe drug, it is essential to explore the pharmacological properties and toxicity of the biosurfactants. Neither of the biosurfactants in this study displayed any appreciable toxicity or pharmacological properties such as hemolysis, tissue damaging activity etc. implying their biosafety in humans.

8.1.18 Application of crude biosurfactants from B. subtilis DM-03 and DM-04 strains as potential mosquito larvicidal agent

Culex quinquefasciatus is the dominant vector of lymphatic filariasis, which infected some 120 million people worldwide and can also lead to genital damage and elephantiasis. More recently Culex quinquefasciatus is regarded as a potential vector of West Nile virus (Zinser et al., 2004). Although, lipopeptides particularly iturin are known to possess antimicrobial activities (Ohno et al, 1996; Cameotra and Makkar, 2004; Singh and Cameotra, 2004), but there has not been a single reports demonstration the anti-insecticidal (against mosquito larvae) potential of the lipopeptide.
Lipopeptide biosurfactants from *Bacillus subtilis* DM-03 exhibited better antimosquitocidal activity than *Bacillus subtilis* DM-04 in the tested 3rd instar *Culex quinquefasciatus* larvae. The difference in the larvicidal potency between the crude CLPs from *B. subtilis* DM-03 and DM-04 strains was due to presence of quantitatively as well as qualitatively different proportions of iturins and surfactins in the crude lipopeptides. The crude lipopeptides of *B. subtilis* DM-03 contained more iturin molecules whereas *B. subtilis* DM-04 strain contained higher amount of surfactins as revealed by MALDI-TOF mass spectral analyses in the present study. However, our results are in contradiction to the report of Assie et al. (2002), who had showed that surfactin molecules and not the iturins displayed a dose dependent insecticide activity. Therefore, the mosquito larvicidal activity of individual lipopeptides from *B. subtilis* and their exact mode of action remain to be elucidated. Mortality of the *culex* larvae may have occur due to the disruption of the cell membrane by detergent effect of the lipopeptides (MageBDana and Ptak 1995) with the formation of ionic channels on the membrane (Sheppard et al, 1991).

Several physico-chemical and biotic factors such as pH of water, temperature, sunlight, larval age etc. have been reported to influence the efficacy of bacterial formulation or toxins against the target mosquito larvae (Mulla, 1985; Becker et al, 1992; Mittal et al., 1995). For example, the efficacy of spherix (*B. sphaericus*) and bactoculicide (Bti) formulations against anopheline larvae were reduced to about 10 fold in laboratory bioassay at 21 °C as compared to 31 °C. But in the present study, larvicidal potencies (LC₅₀) of crude lipopeptides from *B. subtilis* DM-03 and DM-04 strains were reduced to 4.2 and 4.5 folds of their original activity respectively at 23 °C as compared to 35°C. Moreover, heating the crude lipopeptide solutions at 100°C up to 60 min does not influence their larvicidal potency, showing extremely thermostable nature of these lipopeptides, this property makes them suitable for application in tropical countries.

It has been reported that protein toxins of Bti and *B. sphaericus* are highly sensitive to sunlight (UV radiation). For example, exposure to sunlight for 6 h reduces the biolarvicidal potency of Bti and Bs to about 50% and 75 % respectively of their original activity (Mittal, 2003). Sunlight irradiation brings about widespread destruction of indole residues of protein crystals from *B. thuringiensis var kurstaki* (Pozsgay et al., 1987). This study showed that the crude lipopeptides from either *B. subtilis* strain was insensitive to UV/ sunlight exposure as sunlight irradiated and non-irradiated (control) lipopeptides were equally effective in killing
the larvae. This clearly demonstrated the greater UV radiation stability of B. subtilis lipopeptides as compared to Bti and Bs toxins.

Previous studies have shown that stability, solubility and insecticidal activity of the crystal toxins of B. thuringiensis var aizawai and B. thuringiensis var kurstaki are effected by pH of the medium (Nishiitsusui-Uwo et al., 1977; Gringorten et al., 1992). In the present study, there was significant (p< 0.05) decrease in the larvicidal potency (LC₅₀) of B. subtilis CLPs at pH 11.0, as compared to pH 7.0. The optimum range of pH at which B. subtilis CLPs exhibited maximum larvicidal efficacy was observed in between 5 to 10. The results of this study revealed that although the CLPs from B. subtilis strains possess lower mosquito larvicidal potency as compared to the currently available larvicidal preparations of the other bacteria, the higher stability of B. subtilis lipopeptides against heating, sunlight exposure and different pH render this bacterium to be considered as a potential candidate for mosquito larvicidal control.

In conclusion, the crude CLPs secreted by B. subtilis strain had shown larvicidal activity against Culex mosquito, can withstand many environmental stresses like extreme pH, sunlight/UV radiation etc., and they did not impart toxicity to the tested aquatic vertebrate Labeo rohita up to a concentration that induced mortality in the mosquito larvae. These properties can be exploited for the formulation of a safer, novel biopesticide for effective control of mosquito larvae. Further studies to assess the mosquitocidal activities of purified CLPs and their mode of action are in progress.

8.2 Conclusion

In the present study, biochemical and pharmacological properties of biosurfactants produced by two B. subtilis (DM-04 and DM-04) strains and two P. aeruginosa (M and NM) strains growing under thermophilic growth condition (45°C) were characterized. Further some of the potential industrial applications crude natural roles of the biosurfactants to their producing bacteria were also explored. Three out of the four bacteria, viz., B. subtilis DM-04, P. aeruginosa M and NM were isolated from petroleum oil contaminated soil samples whereas the remaining strain, viz. B. subtilis DM-03 was isolated from traditional fermented food.

The RFLP pattern of 16S-23S ISR of B. subtilis DM-03 and DM-04 strains showed clear difference as was evident from the different banding patterns in a 3% agarose gel.
Restriction digested amplified products of the 16S–23S ISR of *P. aeruginosa* mucoid (M) and non-mucoid (NM) strains were resolved into similar banding patterns in 3% agarose gel, documenting that these strains belong to same phylogenetic trait.

Both the *Bacillus* strains exhibited distinct preferences for the nitrogen source, pH and temperature optima for bacterial growth and biosurfactants production. For example, *B. subtilis* DM-03 had a preference for ammonium nitrate, whereas *B. subtilis* DM-04 had a preference for tryptone for maximum biosurfactants production. However, both the strains had a common preference for glucose as sole source of carbon for maximum biosurfactants production. The optimum pH for maximum growth of and biosurfactants production by *B. subtilis* DM-03 and DM-04 strains was observed at pH 8.0 and pH 7.0 respectively. Comparative study showed that *B. subtilis* DM-04 had a better salt tolerance as compared to *B. subtilis* DM-03 strain.

Maximum growth of and biosurfactants production (6.5g/l Vs 5.0 g/l) by *P. aeruginosa* M and NM strains were observed at the stationary phase (96 h post inoculation) with (2.0% v/v) glycerol and (0.1% w/v) ammonium chloride as respective carbon and nitrogen sources and at an incubation temperature of 45°C and agitation set at 200 rpm.

In this study, least growth or biosurfactants production by *B. subtilis* DM-03 was observed when hydrocarbons were used as sole carbon sources. But the growth rate of DM-03 strain in hydrocarbons (eg. hexadecane and pyrene) was enhanced significantly (p<0.001) when crude lipopeptide isolated from *B. subtilis* DM-04 strain was added in the growth medium. Similarly, *B. subtilis* DM-04 exhibited poor growth on starch, but excellent growth was observed in the same medium, when exogenously supplemented with crude lipopeptide isolated from *B. subtilis* DM-03 strain.

Biochemical compositions of crude biosurfactants from *B. subtilis* DM-03 and DM-04 strains revealed the lipopeptide nature whereas, the secreted biosurfactants by the *P. aeruginosa* strains composed of mixture of carbohydrate, lipid and protein. The crude biosurfactants from both the *Bacillus* and *Pseudomonas* strains retained their surface-active properties in a wide range of pH as well as post heating at 100°C for 60 min.

MALDI-TOF mass spectra analyses showed that iturins and surfactins isoforms represented the major lipopeptide biosurfactants produced by *B. subtilis* DM-03 and DM-04 strains.
respectively. The molecular mass of the biosurfactants present in these active peaks of *P. aeruginosa* M and NM strains were detected in the range from m/z 1348 to m/z 1832. The molecular mass of active biosurfactants from *P. aeruginosa* strains have been detected in the range from m/z 331 to 677, showing they were novel biosurfactants reported till date.

Pharmacological characterization of the crude biosurfactants from all the bacterial strains demonstrated that they induced dosed dependent lysis of washed human erythrocytes, but to different extent. Biosurfactants from both the *B. subtilis* strains demonstrated their anti-coagulant nature on platelet poor plasma, but biosurfactants from *P. aeruginosa* strains decreased the clotting time of platelet poor plasma. As the isolated crude biosurfactants from the *P. aeruginosa* strains failed to exhibit any detrimental effects on chicken heart, lungs, liver and kidney tissue, there is enough scope to develop topical (local) coagulant drugs to prevent the bleeding during minor surgical operations.

Present study shows that other industrial applications of biosurfactants and their producing microbes are highly promising. Laboratory scale study on bioremediation demonstrated the capacity of the *P. aeruginosa* strains and *B. subtilis* DM-04 strain to appreciable reduced the TPH of petroleum contaminated soil sample in a time period of 4 months. These strains were also shown to be efficient degraders of pyrene and *B. subtilis* DM-04 strain possessed significantly higher pyrene utilizing capacity compared to *P. aeruginosa* M and NM strains. Crude biosurfactants from all the bacterial strains exhibited antimicrobial activity against certain tested microorganisms, biosurfactants from *B. subtilis* DM-03 and *P. aeruginosa* M strains showed better anti-microbial potency compared to other two strains.

The lipopeptide biosurfactants from the *B. subtilis* strains exhibited dose dependent larvicidal activity against 3rd instar *C. quinquefasciatus* larvae and among the four strains, crude biosurfactants from *B. subtilis* DM-03 strain exhibited best larvicidal activity. Further, these lipopeptides were shown to be non-harmful to the aquatic non-target organism (*Labeo rohita*) and therefore, biosurfactants from *B. subtilis* DM-03 strain could be exploited for the development of eco-friendly biopesticides.