GENERAL DISCUSSION AND SUMMARY

Bacteria are social when famished. They are slippery and mucous when they are social. Bacteria are attractive model of current research, as methodologies behind social organization have started getting revealed. Determining the costs and benefits of any bacterial behavior in a particular setting of an ecosystem enables one to understand the evolution of that behavior. In natural conditions, however, there is no scope for an experimenter to provide a readily available food source; but in laboratory we can create condition of famine or feast. When bacteria were forced to starve, natural instincts of finding each other could be demonstrated. Once the bacteria clumped together, they take part in a collective effort to find the nutrients they need. After they aggregate, the bacteria actually change their physiology to form a protective, adhesive gel-like biofilm. The spatial relationships between cells in micro-meter scale spectacularly gets altered when environmental circumstances of biofilm growth is changed that determines the quanta of benefits received by the bacteria out of the secretion of extra-cellular polymeric substances (EPS). Consequentially, in varied growth conditions a single behavior can yield reverse effect on competition. For example, certain mutants of *Vibrio cholera,* that produce EPS constitutively, outcompete cells which fails to secrete EPS within biofilms grown on solid surfaces. In real world situation, secretion of EPS is an important determinant of survival or in other words, EPS production is a competitive behavior. In the lungs of people with cystic fibrosis, mutant strains of *Pseudomonas aeruginosa*, that produce EPS constitutively, overpopulates and cause fatal lung infections.

Social life of bacteria when living in scarcity (oligotrophic conditions)

Oligotrophic environments usually deficient in exogenous supply of nutrients and are defined by a low nutrient flux, <1 mg carbon per litre per day (Schut et al., 1993) as well as by low absolute concentrations of nutrients (Morita, 1997). The aquatic environment is the

largest habitat on Earth, accounting for >90% of the biosphere by volume and harbouring microorganisms responsible for ~50% of total global primary production. Despite of highest cellular production rate of any ecosystem on the planet, aquatic environments has vast oligotrophic (e.g., nutrient-limited open ocean water) situation.

Numerous reports have shown that most marine bacteria adapt to oligotrophic environments and prefer lower nutrient conditions (<10 mg C 1^{-1}) to higher nutrient conditions (Carlucci & Shimp 1976, Akagi et al. 1977, Ishida et al. 1986). An oligotrophic bacterium growing slowly in nutrient-poor condition may have a life strategy in which dispersal is promoted to optimize cell access to substrates. In the latter scenario colony formation is possibly not adaptive. While aquatic ecologists have had an interest in oligotrophic bacteria (Yokoi et al., 1995; Tago and Aida 1977; Nguyen and Schiller 1989; Costerton et al., 1981), these organisms are still relatively unknown to many microbiologists, especially clinical microbiologists. Growth and continued existence of bacteria are often influenced by EPS produced by them.

Oligotrophic bacteria can tentatively be divided into 2 groups: obligate oligotrophs and facultative oligotrophs (Ishida et al. 1980, 1986). Facultative oligotrophs can grow in a relatively wide range of nutrient concentration, hence; can be used as model organisms to understand the various strategy used by oligotrophs for their survibility under nutrient deprived condition. In this thesis, an attempt was made to understand the physiology of a model facultative oligotrophic bacterium *K. pneimoniae* PB12. Since sustenance of bacteria is often influenced by EPS, structural investigation of the exopolysaccharide produced by *K. pneimoniae* PB12 was explored in details. In addition to the emphasis on the EPS structure, immunological and biotechnological prospects were also revealed.

Chapter 1 dealt with screening and characterization of the test strain. On the basis of the phenotypic and phylogenetic analyses, the strain PB12 was identified as *Klebsiella*

pneumoniae. It was observed that PB12 cells were able to grow in 0.001x LB broth as well as sterile river water without supplementation of any carbon or nitrogen source or growth factors. An increment of nearly 13 times the initial cell number was noted in a span of 3 days in 0.001x LB. In our earlier reports, facultative oligotrophic strains, *Acinetobacter johnsonii* MB52 and *Klebsiella pneumoniae* MB45 showed an increase of 2.5 and 4.6 times the initial cell number when grown in 0.001x LB in the span of 8 and 2 days, respectively (Kumar et al., 2010; Kumar et al., 2011). The ability of PB12 to survive without any reduction in viable cell number from the input cells establishes its oligotrophic nature. After the strain characterization, protocol for EPS extraction from the culture broth was optimized. It was noted that centrifugation along with alcoholic precipitation was found to be convenient and satisfactory method compared to other extraction methods reported in the literature (Comte et al., 2006; Peterson, 1979; Underwood & Paterson, 1995). This method is also the most common and widely used method for EPS extraction (Underwood et al., 1995). Yield of EPS in R2A varied with time and was maximal (1.3 g l^{-1}) at 48 h of incubation at 30 °C. Above 30 ºC, the productivity of EPS was reduced. Furthermore, maximum growth in terms of optical density was also noticed at 30 ºC, below and above this temperature, both growth and EPS production decreases. Moreover, the effect of various carbon sources on EPS production was checked and it was obtain that both the growth and production of EPS in R2A was enhanced maximally when supplemented with 1% glucose. The high productivity of EPS (1.3 g l^{-1}) was observed at pH 7. The yield of EPS as well as growth decreased below and above pH 7. There is reduction in the amount of EPS after 48 h which was probably due to utilization of the EPS by the bacterium. Similar utilization of EPS as carbon source was also observed by earlier authors (Gauri et al., 2009). In the present study *Klebsiella pnemoniae* PB12 showed decrease in approximately 31% of EPS during further incubation from 48 h to 72 h in the same batch culture was associated with two times increment in viable cell number. This

established that EPS somehow help this particular bacterium to survive under nutrient deprived condition via providing as an alternative carbon source.

Chapter 2 dealt with basic physiology of the test strain, *K. pneumoniae* PB12. It was noted that the PB12 cells can carry out reproduction along with the macromolecular synthesis with whatever in their milieu even at 10 times diluted LB i.e., 0.01x LB. When allowed to grow on LB or 10 times diluted LB agar plates (0.01x LB agar) it was noted that colony diameter of PB12 grown on LB agar after 24 h was found to be 1.5 mm, whereas, colony diameter of PB12 on 0.01x LB after 24 h of incubation was 0.5 mm. Interestingly, after assessing the number of cells presents in this colony it was noted that colonies obtained on LB agar (d=1.5 mm) there are 1.8 x 10^8 cells present. Whereas, 5.4 x 10^8 (approximately 3 times more) numbers of cells was obtained from the colony grown on diluted LB agar plates. SEM and FACS analysis of the PB12 cells grown in either LB or 0.01x LB showed that cells grown in diluted LB have reduced their size in order to increase its surface area to volume ratio to cope nutrient scarcity. It was established earlier that nutrient availability is a primary determinant of cell size for single-celled organisms. In 1958, Schaechter, Maaløe, and Kjeldgaard determined that *Salmonella* cell size is coupled to growth rate, which is itself a function of nutrient availability. PB12 cells population grown in LB showed increment in FSC (forward- scattered light), indicating that the cells were possibly larger than PB12 cells grown in 0.01x LB. Earlier researcher used flow cytometry to detect and quantify cell shape differences between the rod-shaped cells of *E. coli*: CS109 and the seriously deformed cells of *E. coli*: CS315-1K, a mutant lacking PBPs 4, 5, and 7 (Meberg et al., 2004). Organisms that grow aerobically are regularly exposed to unavoidable by-products of aerobic respiration like reactive oxygen species (ROS) [e.g. peroxide, superoxide (SO)] (Storz & Imlay, 1999) ROS damages cellular macromolecules and thus elicit adaptive oxidative stress responses in bacteria intended to permit survival in the presence of this stressor. Using an oxidant-sensing

fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) intracellular ROS can be measured (Barzegar & Moosavi-Movahedi, 2011). Results showed that both intracellular ROS and NO production is more in LB grown cells compare to 0.01x LB grown cells. Whereas, both Cu,ZnSOD and catalase activity was higher in 0.01x LB grown PB12 cells compared to LB grown PB12 cells. To validate this observation a model ROS generator, ZnO-PEI NPs were used to study its effect on PB12 cells grown in either LB or 0.01x LB. Results showed that LD_{50} dosage of ZnO-PEI NPs cause only 35% reduction in bacterial growth of 0.01x LB grown cells whereas, approximately 50% reduction in bacterial growth was noted when LB grown cells were treated with same dosage of NPs. After NPs exposure it was further analysed using Raman spectroscopy to get more insight.

Chapter 3 dealt with the structure and functional aspects of the purified EPS (KNPS). The KNPS was found to be an average molecular weight of $\sim 1.8 \times 10^5$ Da. Structural characterization of KNPS was carried out using sugar and methylation analysis, Smith degradation and 1D/2D NMR experiments. Sugar analysis showed that the KNPS composed of arabinose, galactose, 3-O-methyl-galctose and glucose in a molar ratio of nearly 4:3:1:1. The GC–MS analysis of partially methylated alditol acetates revealed the presence of 3,5-linked-arabinofuranosyl, 2,6-linked-galactopyranosyl, 6-linked-galactopyranosyl, 6-linked-glucopyranosyl, 2-linked-galactopyranosyl, 5-linked-arabinofuranosyl, 3-linked-arbinopyranosyl, terminal galactopyranosyl and arabinofuranosyl residues in a relative proportion of approximately 1:1:1:1:1:1:1:1:1. The ^{1}H NMR spectrum (500 MHz) of KNPS recorded in D_2O at 30 ºC showed the presence of eight signals in the anomeric region at δ 5.20 (**A**), 5.17 (**B**), 5.16 (**C** and **D**), 5.10 (**E**), 5.05 (**F**), 5.02 (**G**), 4.46 (**H**), and 4.42 (**I**) as evidenced from HSQC couplings. In ^{13}C NMR spectrum (125 MHz; Fig. 3d) at the same temperature, eight signals were observed in the anomeric region at δ 109.4, 106.8, 103.3, 102.7, 102.2, 101.5, 99.5, and 97.7. These eight anomeric carbon signals were correlated to

the anomeric proton signals of residue (**A**), (**B**), (**I**), (**H**), (**G**), (**F**), (**E**), (**C** and **D**), respectively as assigned from the HSQC spectrum. The residues **A** and **B** were assigned as (1→3,5)-α-L-Ara*f* and (1→5)-α-L-Ara*f* residues respectively. The α-configuration of both **A** and **B** residues were assigned from the chemical shift values of anomeric carbon and proton (δ 109.4/5.20 and δ 106.8/5.17) respectively. The downfield shift of C-3 (δ 81.8) and C-5 (δ 65.4) with respect to the standard methyl glycosides (Rinaudo & Vincendon, 1982; Agarwal, 1992; Mandal et al., 2011) indicated that the residue **A** was (1→3,5)-linked-α-L-arabinofuranose. The downfield shift of C-5 (δ 65.4) with respect to the standard methyl glycosides indicated that the residue **B** was (1→5)-linked α-L-arabinofuranosyl moiety. The linkage at C-5 of the both residues **A** and **B** were further confirmed from DEPT-135 spectrum. Hence, these observation confirmed that the residue **A** was (1→3,5)-α-L-Ara*f* and the residue **B** was (1→5)-α-L-Ara*f*. The proposed repeating unit of the KNPS has a backbone chain consisting of two(1→6)-galactopyranosyl residues, two (1→5)-arabinofuranosyl residues, one (1→6)-glucopyranosylresidue and one (1→3)-arabinopyranosyl residue, out of which one (1→6)-galactopyranosyl residue was branched at O-2 position with a (1→2)-linked-galactopyranosyl residue terminated with non reducing arabinofuranosyl residue and one (1→5)-arabinofuranosyl residue branched at O-3 position with nonreducing end 3-O-Me-galactopyranosyl residue. KNPS enhanced malondialdehyde (MDA), reactive oxygen species (ROS), and have the potential to alter the ratio of oxidized glutathione (GSSG) and reduced glutathione (GSH) levels in the cellular system.

Chapter 4 dealt with the biotechnological aspects of EPS. It was noted that the optimal dosages for flocculation of activated carbon suspension were 17 mg l^{-1} EPS and 4 mM $CaCl_2$. However, higher or lower EPS dosages cause reduction in flocculating rate(s). When the dosage of EPS was inadequate, the effective bridging phenomenon gets hindered causing reduction in flocculation. This relationship between EPS dosage and flocculating rate was

similar to earlier authors (Suh et al., 1997; Zheng et al., 2008). In the absence of Ca^{2+} ion no effective flocculation was observed at pH 7 which dictates the requirements of $CaCl_2$ for effective flocculation by forming Ca^{2+} mediated complexes of the EPS and activated carbon (Kurane et al., 1986). The presence of C=O group as revealed in FT-IR may play an important role in flocculation and can serve as binding sites for divalent cations. Results showed that the divalent cations like Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} were better than other tested cations. The flocculating rate in presence of Fe^{3+} was 53% lesser than that of Ca^{2+} induced flocculation. Monovalent cations like K^+ and Na^+ are less effective for flocculation due to weaker electrostatic force of attraction between monovalent cations and EPS (Li et al., 2008). EPS produced by *C. daeguense* W6 in nutrient poor medium have shown flocculating rates above 90% in the temperature range of 0-45 °C with maximum of 97% at 15 °C (Liu et al., 2010). Maximum flocculation of 98% was observed by EPS produced by PB12 in both 10 °C and 20 °C. When temperature was increased to 100 °C, a decrease of approximately 26% from the maximum flocculation rate was observed; such decrease (26%) at 100 °C from the maximum value was observed with EPS produced by *Bacillus mojavensis* 32A (Elkady et al., 2011). Decrease in flocculating rate at higher temperature (above 60 °C) could be explained by simultaneously rise in kinetic energy of activated carbon particles due to heating effect. The EPS produced by the oligotrophic bacterium PB12 could be an effective replacement of a commercial polymer with regard to flocculation. The high flocculation rate of PB12-EPS over a wide range of temperature (10 °C-50 °C) and pH (3-10) possess a promise for application in water treatment and other biotechnological applications including metal processing industries. Moreover, the physiological and structural studies conducted in this thesis may help to put on more insights on the survival strategy of oligotrophic bacteria under nutrient deprived conditions along with the mechanisms of septic shock and KNPS-induced immunosuppression and autoimmunity.