GENERAL DISCUSSION AND SUMMARY

For many years, microorganisms such as bacteria has been isolated, cultured and studied as a free floating cells (in planktonic form) and are used to study many of the their activities. Recent advances in microscopy and molecular technologies have contributed largely to the study of surface attached bacterial communities within biofilm. Biofilm may be defined as surface attached microbial aggregates, encased in a self produced matrix primarily made from polysaccharide material (Donlan, 2002). In nature, majority of the microbes exist as surface attached structure within a biofilm ecosystem and not as a free floating organism (Costerton et al., 1995). Biofilm is now the major focus area of research with respect to human health, bioremediation, waste water treatment and biotechnological applications. Among various ecological advantages of biofilm mode of growth, protection from adverse environmental conditions particularly from the presence of antibiotics (Costerton et al., 2003; Patel, 2005) or toxic metals (Teitzel and Parsek, 2003; Harrison et al., 2007) is an important aspect needed to be studied.

The genus *Acinetobacter* is composed of diverse aerobic, gram negative, non-fermenting bacteria (Juni, 2005). Some of which are medically relevant species such as *A. baumannii* (Vidal et al., 1996) and *A. calcoaceticus* (Habimana et al., 2010) that have been studied for their biofilm forming abilities on medical devices. Very few studies have been focused on the environmental species such as *A. junii*. In natural environment, especially in polluted environment, the survival and stability of biofilm depends on many factors including their tolerance to heavy metals, since the polluted water bodies may have high concentration of heavy metals. The metal-sorption property of biofilm leading to protection to the bacteria against the onslaught of metal toxicity is yet another aspect needed to be studied. In natural environments such as river water, biofilm forms the key element in the self purification process as they harbor a heterogeneous microbial community that degrades various organic compounds. In the present work, biofilm formation by a metal tolerant bacterium *A. junii* BB1A isolated from river water (Bhadra et al., 2006), was investigated. This strain is heavy metal tolerant and produces abundant EPS, characteristics which are advantageous to the growth and formation of stable biofilms in metal polluted water bodies.

In chapter one, one of the methods for assaying biofilm was standardized and influence of various environmental factors on the biofilm formation by *A. junii* BB1A was studied. Although many methods to quantify biofilm have been developed (Deighton et al., 2001; Arciola et al., 2002; Harraghy et al., 2006), no universal method for the evaluation of biofilm applicable to all biofilm-forming bacteria

is either available or standardized. However, microtiter plate method or tissue culture plate assay is the most frequently used method for the investigation of biofilms. During the standardization of microtiter plate method (or tissue culture plate method), it was observed that fixing with methanol, staining with 0.1% crystal violet and de-staining with 33% glacial acetic acid gave most optimum result. The strain was found to adhere most efficiently to the plastic surfaces such as polystyrene or polypropylene compared to glass surface. This may be due to the higher cell surface hydrophobicity of *A.junii* BB1A as reflected by the MATHS (microbial-adhesion-to-hydrocarbon) test. Previous studies have already shown the correlation between affinity of bacteria to polystyrene and cell surface hydrophobicity (Rosenberg, 1981). Among various environmental factors tested, nutrient excess and salt concentration has a profound effect on biofilm formation. The growth in BHI (nutrient rich media) resulted in more biofilm formation compared to LB media (comparatively nutrient poor). Diluted medium was found to be least effective in promoting biofilm formation. The process of biofilm formation is found to be associated with the synthesis of extracellular polymers, which is energetically demanding and carbon-expensive (Chakrabarty, 1996). The strain was also found to tolerate high concentration of NaCl (3.5%, w/v) and form biofilm. The results suggest that the strain has the potential to form biofilm under stressful conditions (high NaCl concentration of 3 to 3.5 % and pH 8).

Chapter two dealt with the production and characterization of structural component of biofilm; extracellular polymeric substance (EPS) produced by *A. junii* BB1A. Prior to characterization, protocol for EPS extraction from the culture broth was optimized. Centrifugation along with alcoholic precipitation was found to be convenient and satisfactory method compared to other extraction methods such as heating, NaOH , sonication, formaldehyde, and EDTA treatment. Centrifugation along with ethanol precipitation is also the most common and widely used method for EPS extraction (Underwood et al., 1995; Decho et al., 2005), because it is less degradative technique and do not involve addition of chemicals (Comte et al., 2006) like EDTA which may interfere with the protein or carbohydrate analysis (Peterson, 1979; Underwood and Paterson, 1995).

Optimization of growth conditions for maximum EPS production was studied. EPS production in BHI media was found to be maximum (1.3 g/l) during the stationary phase (at 144 h incubation). The yield of EPS was higher at 30°C (optimum growth temperature), pH 7 to 8, in presence of glucose and yeast extract as carbon and nitrogen source respectively and at higher C/N ratio of 4. The production was also found to be stimulated by the addition of 3% NaCl or 0.2 % Na_2HPO_4.

The important sugar for cell to cell adherence, a pre-requisite for cell-cell interaction within biofilm community; N-acetyl-D-glucosamine has been detected from the cell pellet. The extracellular polymeric substances (EPS) released by the cells was separated from the cell-free supernatant/ culture filtrate. Chemical analysis revealed that the carbohydrate to protein ratio of EPS extracted from *A. junii* BB1A was 3.4. The molecular weight of the purified polysaccharide component of EPS was estimated as ~2 x 10^5 Da and composed of three main sugar residues, namely mannose, galactose and arabinose in molar ratio of 3:1:1. This is the first report showing mannose rich and arabinose containing EPS obtained from *Acinetobacter* sp. and thus it is novel so far. The EPS was found to be anionic in nature. The presence of uronic acids in the EPS established the anionic properties of EPS. FT-IR spectra of the EPS showed the presence of carboxyl, hydroxyl and amide groups. The carboxyl group in the EPS confers various important features such as binding to divalent cations (Bramhachari et al., 2007). The presence of hydroxyl groups within the polymer favored the possibility of hydrogen bonding with one or more water molecules; as a result the EPS extracted from *A.junii* BB1A exhibited high solubility in aqueous solutions. Thermogram of EPS, obtained from differential scanning calorimetry (DSC), revealed high thermal stability up to 363°C, thus making it a promising additive for industrial applications. DSC as well as X-ray diffraction analysis depicted the amorphous nature of EPS. Rheological analysis of EPS, showed the pseodoplastic non-Newtonian fluid nature.

The EPS obtained from various microorganisms have been reported for their potential application in bioremediation or waste water treatment such as metal binding (Iyer et al., 2005; Wu and Ye, 2007; Gong et al., 2008; Lin and Harichund, 2011), bioflocculation (Katja and Mika, 2007; Kumar et al., 2004), and emulsification(Ashtaputre and Shah, 1995; Iyer et al., 2006). The important role of EPS in the removal of heavy metals from the environment has been associated with its flocculation and metal binding properties (Pal and Paul, 2008). In chapter 3, the physico-chemical properties of EPS from *A. junii* BB1A were investigated such as bioflocculation, emulsification and metal binding properties. EPS from *A. junii* BB1A was shown to flocculate (flocculating rate >90%, EPS dosage 30 mg/l) either kaolin clay or activated charcoal at wide range of pH (pH 4-10). The flocculation required only 0.7 mM of divalent cation (Ca^{2+}) above pH 5 (no cation addition was required at pH 4-5) minimizing the excess input of $CaCl_2$ which at higher concentration may be costly or contribute to the secondary pollutant. The EPS harvested from *A. junii* BB1A has efficient emulsification properties comparable with some of the standard gums. The stability of emulsion was studied using n-Hexadecane and found to be stable (Emulsifying index between 60-55%) up to one month. High and stable emulsification property of EPS from *A. junii* BB1A renders its potential application in enhanced oil recovery. Apart from flocculation and emulsification properties, the EPS was also shown to be a good sorbent for various heavy metal ions

including Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+} and Cd^{2+}, this has been confirmed by the EDS analysis of metal treated EPS.

Chapter 4 provides an insight into the heavy metal resistance and quorum sensing phenomena in *A. junii* BB1A. Although microorganism possess different mechanisms to combat heavy metal stress, among them extracellular sequestration of heavy metals was found to be an important mechanism in *A. junii* BB1A. The EPS component of the biofilm in BB1A was shown to bind heavy metal ions including Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+} and Cd^{2+}. The strain was capable of growing in presence of heavy metal (Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}) at a concentration greater then 1mM in both liquid and solid medium, an ability that may be important with regard to the capacity of this bacterium to survive in polluted environment with elevated heavy metal levels. The resistance to the metal was found to be dependent on the type of growth media used; the minimum inhibitory concentration (MIC) was higher when determined in nutrient rich BHI as compared to AB minimal media. Differences in MIC values on agar and liquid medium are probably caused by entirely different environment. It was observed that the production of EPSs by *A. junii* BB1A increased with increasing concentration of added metal ions (Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}); attaining maximum (two- fold increase) at the tolerable concentration in both AB and BHI medium. This phenomenon of enhanced production of bacterial EPS in the presence of toxic metals was ascribed to a defense mechanism against toxicity (Decho, 1994; Fang et al., 2002). Role of EPS in the resistance against toxic metal ions, was further confirmed by creating EPS deficient condition in BB1A strain with the threshold concentration (0.1mM) of 4-NPO (a quorum sensing inhibitor) that prevented production of EPS but allowed normal growth. In presence of 0.1 mM 4-NPO, the sensitivity of BB1A cells against all metals tested was significantly increased. The phenomenon of quorum sensing in *A. junii* BB1A was demonstrated by using AHL-Biosensor strain *A. tumefaciens* NTL4 (pZLR4).

In Chapter 5, mutational and biofinformatic studies were carried out in order to understand the genetics of biofilm formation in *A. junii* BB1A. Transposon mutagenesis with *E. coli* transposon Tn5 carried by plasmid pSUP5011, was done and a single isolate of BB1A (BB1A:Tn5) carrying a Tn5 insertion was obtained. This mutant was biofilm deficient and was found to be more sensitive to metals/metalloids compared to the wild type. The DNA sequence interrupted by the Tn5 transposon, was amplified using inverse PCR, cloned and sequenced, however, similarity search using BLAST revealed no significant result. Further, degenerate primer(s) was designed to fish-out the auto-inducer synthase gene(s) (homologues to *luxI* gene) of the quorum sensing system and wet lab amplification was performed using *A. junii* BB1A DNA. *In silico* amplification with the degenerate primer successfully amplified *luxI* homologues sequences from several bacterial species. In the wet lab, PCR amplification

of *A. junii* BB1A DNA with degenerate primer generated a single 223 bp amplicon. The protein-protein BLAST analysis of the amplicon revealed 94% identity with the predicted protein of *A. junii* SH205. It is now predicted that in *A. junii* BB1A, the AHL based quorum sensing phenomena was controlled by genes dissimilar to *luxI*. Protein-protein BLAST search in the protein data bank of *A. junii* SH205 (the strain whose protein database is available on NCBI site) revealed the presences of a cluster of four genes, similar to the *pgaABCD* locus in *Acinetobacter baumannii*. The biofilm formation in many bacteria has been reported to be under the regulation of pga*ABCD* operon. In *Acinetobacter baumannii*, *pgaABCD* locus is known to encode proteins involved in the production of poly-β-1-6-*N*-acetylglucosamine, which was found to be critical for biofilm formation (Choi et al., 2009). PCR amplification of *A. junii* BB1A genome with the primer derived from the *pgaC* gene of *A. junii* SH205 generated a single amplicon of expected size, revealing the probability of similar *pga*ABCD locus in the test strain. Structure and functional prediction of all the four proteins from *A. junii* SH205 revealed their 'membrane protein' nature. Among the four proteins, the structure of protein glycosyl transferase (similar to PgaC) was modeled and docked with its substrate UDP-N-acetyl-D-glucosamine. BLAST search in protein data bank at NCBI revealed the presence of all necessary enzymes involved in the biosynthesis of UDP-N-acetyl-D-glucosamine. Finally, based on the results of various bioinformatics tools used in the study, a model for the biosynthesis of poly-β-1-6-*N*-Acetylglucosamine in *A. junii* BB1A was predicted.

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