CHAPTER 3

Physico-chemical Properties of Extracellular Polymeric Substances Produced by *Acinetobacter junii* BB1A

3.1. Introduction

Extracellular polymeric substances (EPS) are the key component of biofilms and are produced by both prokaryotic and eukaryotic microorganisms growing in natural as well as in laboratory conditions. EPS thus have been associated with many ecological functions such as protection from disinfectants and antibiotics, promotes cell attachment to medical implants, metal corrosion and other issues are of concern. In recent years microbial EPS have attracted growing interest from environmental and biotechnological point of view. Environmental applications of the EPS compounds have been focused so far on the bioremediation of industrial and municipal wastes (Czaczyk and Myszka, 2007). Being a natural polymer, EPS finds many applications in the food, pharmaceutical and other industries. The microbial exopolysaccharides such as xanthan, curdlan, pulluan and alginate, have been frequently used in food industries as thickening, gelling, or stabilizing agents (Kornmann et al., 2003). Sklodkowsaka and Matlakowska (1998) observed that the extracellular substances could also have the property to bind heavy metals from different environments. Recently biofilm EPS has been widely investigated for their flocculation, emulsification and metal binding property.

3.1.1. Bioflocculation

Flocculants are usually used to accelerate or improve the settling of suspended solids in various types of wastewater. They have been widely used in a variety of industrial processes, such as wastewater treatment, food and fermentation industries, drinking water purification, and industrial downstream processes (Shih et al., 2001; Wu and Ye, 2007).

In general, flocculants are divided into synthetic flocculants (organic and inorganic flocculants) and natural flocculants (chitosan, algin, and microbial flocculants) (Suh et al., 1997). Although synthetic flocculants, such as aluminum sulfate, polyaluminum chloride, ferric chloride and organic polymers such as polyacrylamide derivatives, are commonly used because of their low cost and high flocculating activity (He et al., 2002), many studies showed that some of the chemical/ synthetic flocculant are harmful for both human and the environment. Flocculants like aluminum is known to induce Alzheimer's disease or neurotoxic and carcinogenic in case of released acrylamide monomers from polyacrylamide (Arezoo, 2002; Matthys et al., 2005). Moreover they are non-degradable in nature

(Taniguchi et al., 2005; Ho et al., 2010). In contrast, flocculants produced by microbes known as bioflocculant are getting prominence in environmental biotechnology because they are biodegradable (Salehizadeh and Shojaosadati, 2001). Polymers from microorganisms are particularly suitable for various applications since they could be produced uniformly and reliably by fermentation processes.

Several microorganisms in nature are genetically pre-disposed to produce EPS having flocculating properties. Microbial biopolymers having flocculating activities are basically EPS containing glycoprotein, polysaccharide, protein, cellulose, lipid, glycolipid and nucleic acid (Czaczyk and Myszka, 2007; Zheng et al., 2008). Flocculants produced by a haloakalophilic *Bacillus* sp. I-471 (Kumar et al., 2004), *Bacillus subtilis* DYU1 (Wu and Ye, 2007) and *Vagococcus* sp. W31 (Gao et al, 2006) are polysaccharides. *Nocardia amarae* YK-1 (Takeda et al., 1992), *Bacillus licheniformis* (Shih et al., 2001) and *Rhodococcus erythropolis S-1*(Kurane et al., 1986) all produces protein flocculant, while *Arcuadendron* sp. TS-4 (Lee et al., 1995) and *Arathrobacter* sp. (Wang et al., 1995) produce glycoprotein bioflocculant.

The bioflocculants can be applied in various processes, such as removal of microorganisms in the fermentation industry and different industrial waste treatment of textile, cosmetic, paper, leather, pharmaceutical, and food industries (Deng, 2003; Kurane, 1986; Toeda, 1991; Meyer, 1992).

3.1.1.1. Mechanism of bioflocculation

The biopolymers have the property to anchor and bridge the adjacent cells and hence they are thought to be responsible for bioflocculation process. There are three theories which explain the mechanisms of bioflocculation: DLVO theory (or double layer theory), alginate theory, and divalent cation bridging (DCB) theory.

The DLVO theory

The DLVO theory (named after Derjaguin, Landau, Verwery and Overbeek) is a classical colloidal theory which explains that the charged particles have a double layer of counter-ions. The first layer (also known as Stern layer) is composed of a strongly associated counter-ion layer, and the second layer (known as diffuse layer) is made of loosely associated counter-ions (Adamson, 1990). The concentration of ions in the diffuse layer decreases with distance from the particle surface until the concentration of ions equals to that of the bulk solution and thus an electric potential develops around the particle. This double layer of ions surrounding the particle results in repulsion of adjacent particles and reduces aggregation (Fig.3.1). With increasing ionic concentration, repulsion between the particles gets decreased due to the compression of double layer and allows short range attractive forces (van der Waal forces) to promote aggregation (Sobeck and Higgins, 2002).

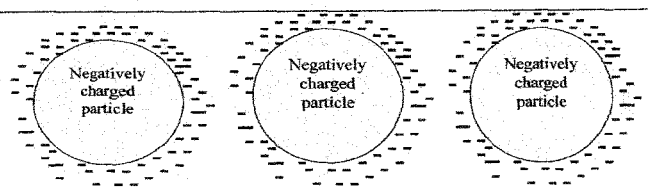
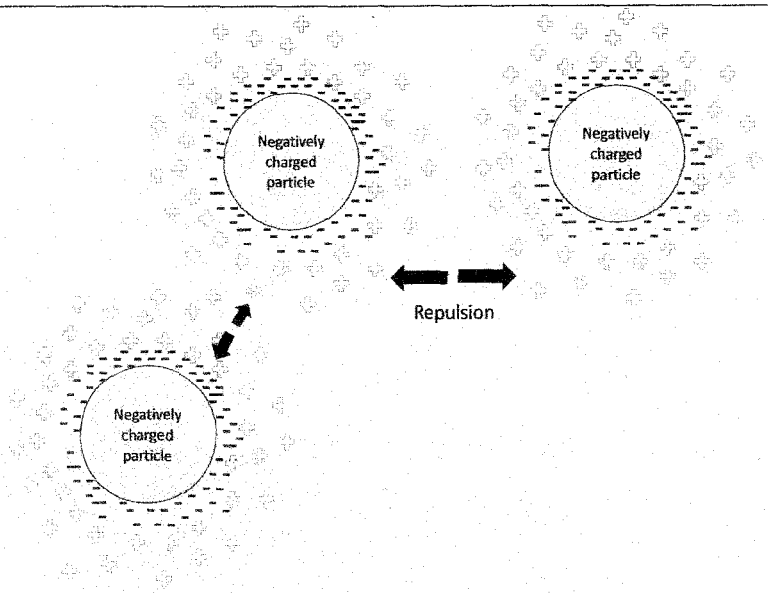
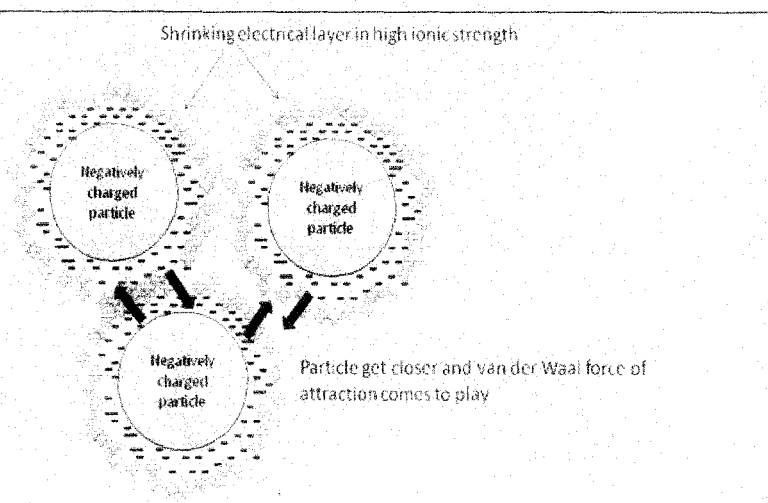
Colloidal particles gain negative electric charge when negatively charged ions of the dispersion medium are adsorbed on the particles surface.	
A negatively charged particle attracts the positive counter-ions from the surrounding medium, resulting in the formation of electrical double layer. The outer layer is diffused and causes repulsion. Because of which colloidal particle remain suspended in the medium.	
At high ionic strength the electrical double layer shrinks, allowing the particle to get closer. At such a close distance, attractive forces such as weak van der Waal forces comes to play, resulting in aggregation and flocculation/ precipitation.	

Fig. 3.1: The DLVO theory of bioflocculation.

The alginate theory

Alginate theory was first proposed by Bruus et al. (1992) in order to describe the role of cations in bioflocculation. Alginate is a polysaccharide produced by bacteria and is made up of repeating mannuronic and gluronic acids. In presence of calcium ions this polysaccharide forms gel. They concluded that the biopolymers have high affinity for Ca^{2+}, and this support their role in bioflocculation.

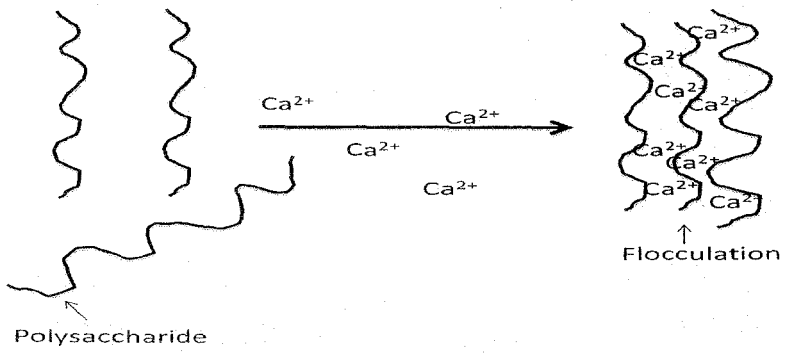


Fig. 3.2: Alginate theory of bioflocculation.

The DCB theory

The divalent cation bridging (DCB) theory was first proposed by McKinney (1952) and Tezuka (1969). According to this theory, divalent cations such as Ca^{2+} amd Mg^{2+} play an important role in bioflocculation by forming bridges (Fig. 3.3) between the negatively charged functional groups within the EPS and this bridging helps to aggregate and stabilize the matrix of biopolymer and microbes and therefore promote bioflocculation.

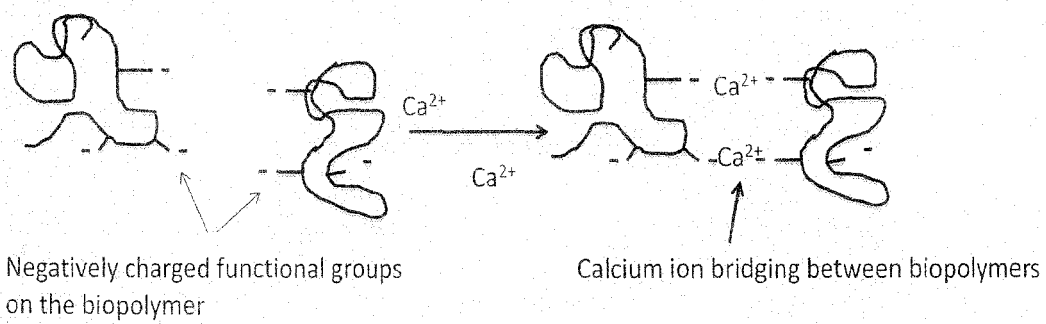


Fig. 3.3: Divalent cation bridging (DCB) theory of bioflocculation.

3.1.2.Emulsification

Surfactants and emulsifiers are widely used in the pharmaceutical, cosmetic, detergents in various industrial sectors, petroleum and food industries (Makkar and Cameotra, 1998; Lang, 2002). Most of these compounds are synthetic and are not easily biodegradable and their manufacturing processes and by-products can be environmentally hazardous. Bio-emulsifiers are microbial products that have the property of reducing surface tension and various advantages over synthetic emulsifiers. As they are microbial origin, they are also biodegradable and hence their use can prevent toxicity problems and accumulation in natural ecosystems (Leahy and Colwell, 1990). Besides possessing biodegradable property, microbial emulsifiers are more effective over a wide range of pH, temperature and salinity (Banat et al., 2000). Microbial emulsifiers are composed of low-molecular-weight glycolipids,

lipopeptides and high-molecular-weight lipid-containing polymers such as lipoproteins, lipopolyssacharide-protein complexes and polysaccharide-protein-fatty acid complexes (Ron and Rosenberg, 2001). A large number of microbial species from different genera produces emulsifiers which are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers (Table 3.1).

Table 3.1: Bio-emulsan/ Surfactant producing bacteria (Data source: Rosenberg and Ron, 1999)

Emulsan/ Surfactant	**Producing microorganisms**
BD4 emulsan	*Acinetobacter calcoaceticus BD413*
RAG-1 emulsan	*A. calcoaceticus RAG-1*
Alasan	*A. radioresistens KA53*
Biodispersan	*A. calcoaceticus A2*
Liposan	*Candida lipolytica*
Emulsan 378	*Pseudomonas fluorescens*
Protein complex	*M. thermoautotrophicum*
Thermophilic emulsifier	*Bacillus stearothermophilus*
Sulfated polysaccharide	*H. eurihalinia*
Glycolipid (Extracellular polysaccharide)	*Penicillium citrinum*
Exopolysaccharide	*Enterobacter cloacae*
Biosurfactant	*Yarrowia lipolytica* IMUFRJ50682

Bio-emulsifiers containing a polysaccharide component attached to lipid and/or protein has been widely studied. The best-studied are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron, 1998; Kaplan and Rosenberg, 1982; Navon-Venezia et al., 1995). Among the bacterial emulsifiers, emulsan obtained from from *Acinetobacter calcoaceticus* known as RAG-1 is the only commercialized one (Rosenberg et al., 2002). RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (Rosenberg et al., 1979). Bio-emulsifier has the property to emulsify wide variety of hydrocarbon and thus can be used for bioremediation of oil pollutant (Calvo et al., 2009; Rosenberg and Ron, 1999).

3.1.3.Metal Binding

During the last two decades, extensive attention has been paid on the use of microorganisms for environmental restoration. It is well known that several microbial biomasses are able to bind and accumulate heavy metals from solution through the process of biosorption (Baldrian and Gabriel, 2003; Gadd, 1990). These relatively simple and inexpensive technologies try to exploit the cationic and anionic functional groups present on the surface of the cell which form a stable, non-toxic complex with the metal ion. Several studies have been undertaken to disclose the type of these different functional groups. Their result revealed the participation of carboxyl, sulfhydryl, hydroxyl sulfonate, phosphonate, amine, and amide groups in metal binding (Maier et al., 2009). In *Pseudomonas fluorescens*, the carboxyl groups in the cell envelop has been found to be associated with binding to Ni, Cu and Zn (Falla and Block, 1993)

Extracellular polymeric substances (EPS) produced by different microorganisms are of particular importance to the bioremediation process because of their involvement in the flocculation and binding of metal ions from solutions (Salehizadeh and Shojaosadati, 2003). The binding of cations to bacterial EPS generally occurs through electrostatic interaction with negatively charged functional groups such as uronic acids, phosphoryl groups and carboxylic groups. In addition protein component of EPS also plays a major role in complexation of metal ions (Mejare and Bulow, 2001). Proteins rich in acidic amino acids, including aspartic and glutamic acid, also provide anionic properties to the EPS.

The application of EPS for biosorption seems to be more economical, effective and safe alternative to chemical methods such as precipitation, coagulation, ion exchange, electrochemical and membrane processes. As the EPS is a non-living sorbent (thus avoids the concern for pathogenecity), its potential application in the treatment process has been widely acknowledged (Gavrilescu, 2004).

Table 3.2: Metal binding potential of EPS produced by different bacteria. (Data source: Pal and Paul, 2008)

EPS producing bacteria	Metal biosorbed
Marine sulphate reducing bacteria	Mo (VI), Ni (II), Cr (III)
Methylobacterium organophilum	Pb (II), Cu (II)
Alteromonas macleodii subsp. *fijiensis*	Pb (II), Cd (II), Zn (II)
Pseudomonas aeruginosa Cu^r	Cu (II)
Rhizobium etli M4	Mn (II)
Enterobacter cloaceae AKI-MB-71a	Cr (VI)
Chryseomonas luteola TEM05	Cd(II), Co(II)
Paenibacillus polymyxa P13	Cu(II)
Paenibacillus jamilae CECT 5266	Pb (II), Cd (II), Zn (II), Cu(II), Ni (II), Co(II)

3.2. Materials and Methods

EPS (Extracellular Polymeric Substances): The EPS used in this study was extracted from 144 h old culture of *A. junii* BB1A. 144 h old culture of *A. junii* BB1A produces maximum EPS (1.3 g/l) (Chapter 2). EPS was purified by dialysis and was lyophilized. The lyophilized EPS was stored at room temperature for further use.

Kaolin clay solution: 5gm of Kaolin clay (Purchased from LOBA CHEMEI, Mumbai, India) was suspended in 1000ml of distilled water by continuous stirring.

Activated charcoal: Activated charcoal powder was purchased from E Merck, India.

Metal salt solutions: De-ionized double distilled water and analytical grades of metal salts [$ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $HgCl_2$, $CdCl_2.H_2O$ and $NiCl_2.6H_2O$ (E. Merck, India)] were used to prepare 100 mM stock solutions.

3.2.1. Determination of flocculating rate of EPS

The flocculating rate of the EPS was measured using the method described earlier (Kurane et al., 1986) in which kaolin clay (5 g/l) was used as the solid phase suspension. In a test tube, 9 ml of kaolin suspension was taken and to this appropriate volume of EPS solution (3mg/ml) was added. The test tube was vortexed for 2 min and then kept standing for 5 min. After 5 min the absorbance of upper phase was immediately determined by using a digital spectrophotometer (Electronics India model 302) at 550 nm (A). In the control experiment, water instead of test sample was added and the absorbance was determined (B).

The flocculating rate (%) was calculated according to following equation:

$$\text{Flocculating rate (\%)} = [(B-A)/B] \times 100$$

3.2.1.1. *Effects of EPS dosage, $CaCl_2$ concentration, pH, temperature and metal ions on flocculating rate*

EPS dosage and $CaCl_2$ concentration were varied from 5-70 mg/l and 0-5 mM respectively to ascertain cost effective dosages. The pH of the kaolin suspension was varied from 1-10 using HCl or NaOH and flocculating rate was measured with or without using optimum $CaCl_2$ concentration. To determine the effect of temperature on flocculating rate, the temperature of the kaolin suspension was varied in water bath in the range of 10-100 °C. Finally, different salts like $MnCl_2$, $MgCl_2.6H_2O$, $K_2Cr_2O_7$, $CuSO_4.5H_2O$,

$CoCl_2.6H_2O$, $HgCl_2$, $FeCl_3$, $CdCl_2.H_2O$, $ZnSO_4.7H_2O$, NaCl and KCl (final concentration 0.7 mM) were added instead of $CaCl_2.2H_2O$ in order to determine their effects on flocculating rate.

3.2.1.2. *Flocculation of activated charcoal powder*

In order to determine the potential application of EPS in the treatment of waste water particularly released from the coal washeries, the flocculating ability of EPS obtained from *A. junii* BB1A was tested with activated charcoal powder (purchased from E Merck, India). In the experiment activated charcoal powder (fine powder) in the concentration of 5 gm/l was used instead of kaolin clay. The flocculation test was carried out using optimized concentration of EPS and $CaCl_2$.

3.2.2. Determination of emulsifying activity of EPS

The emulsifying activity was evaluated by an emulsification index (E_{24}). Emulsification index of EPS was determined by the procedure described earlier (Cooper and Goldenberg, 1987). To 5 ml aqueous solution of EPS (0.5% w/v), 5 ml of hydrocarbon or oil was added and agitated vigorously for 2 min on vortex. Toluene (E merck, India), n-hexadecane (E merck, India), hexane (E merck, India), olive oil (commercial brands), kerosene oil and diesel were used as hydrophobic substrate to study the emulsifying activity of EPS. The emulsion and aqueous layers were measured after 24 h and emulsification index (E_{24}) was calculated by the following formula:

E_{24} = Height of the emulsion layer/ total height x100

In order to understand the contribution of protein component of EPS in flocculation and emulsification, de-proteinised EPS, obtained by chemical {trichloroacetic acid [30%; TCA] (Zhang et al., 2002)} as well as enzymatic {Proteinase K [20 mg ml^{-1}] (Mata et al., 2006)} methods, was subjected to both the assays.

3.2.2.1. *Emulsifying stability*

Stability of emulsion produced by n-hexadecane was studied by measuring emulsification index (E) at every 24 h interval. The emulsification index was also studied with respect to concentration of EPS (final concentration 2, 2.5, 3 and 3.5 mg/ml).

3.2.3. Demonstration of metal binding property of EPS by Energy dispersive X-ray spectroscopy (EDS)

An aliquot of 400 µl each of $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $HgCl_2$, $CdCl_2$. H_2O stock solution (100 mM) was mixed separately with EPS solution (1mg /ml) in a test tube with a final volume of 4ml (initially 2ml of EPS solution containing 4 mg EPS was mixed with 1.6 ml of distilled water). The mixture was then incubated for equilibration at room temperature for 30 min. Double volume of ice-cold ethanol (95%) was added to metal-EPS solution to precipitate the EPS. The precipitate was washed 2 times with 8 ml ethanol (95%) to remove unbound metal, thereafter dried under vacuum. Preparation of the control EPS was identical as described except addition of metal salt solution was excluded. Elemental analysis of both dried precipitate(s) (control and metal salt treated EPS) was done using a scanning electron microscope coupled with energy dispersive X-ray spectroscopy (SEM-EDX) (FEI Quanta 200MK2).

3.3. Results

3.3.1. Flocculating activity

Effect of EPS dosage on flocculating rate

Figure 3.4 shows the relationship between the EPS dosage and the flocculating activity. When the EPS in kaolin suspension was tested in the dosage range of 5-70 mg/l, the flocculating activity increased proportionally and reached a maximum at 30 mg/l, after that it decreased with further rise in EPS dosage.

Effect of $CaCl_2$ concentration on flocculating rate

While observing the effect of $CaCl_2$ concentration on flocculation, it was observed that 0.7 mM $CaCl_2$ concentration was optimum for flocculation and higher or lower salt concentration reduced flocculation (Fig. 3.5).

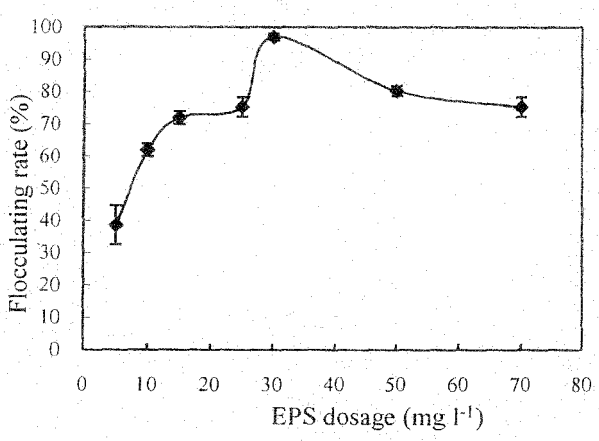


Fig. 3.4: Effect of EPS dosage on the flocculating rate.

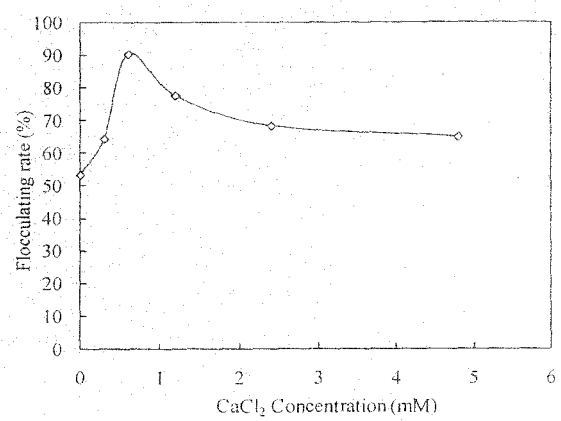


Fig. 3.5: Effect of $CaCl_2$ concentration on the flocculating rate (EPS dosage remained constant at 30 mg/l).

Effect of pH on flocculating rate

The effect of pH on flocculating rate was investigated in presence or absence of $CaCl_2$. Results showed that in absence of $CaCl_2$ flocculating rate was above 94% in the pH range of 4-5, while the rate dropped down to 21% when pH was raised from 6 to 10 (Fig. 3.6). The flocculating activity in presence of $CaCl_2$ remained above 90%.

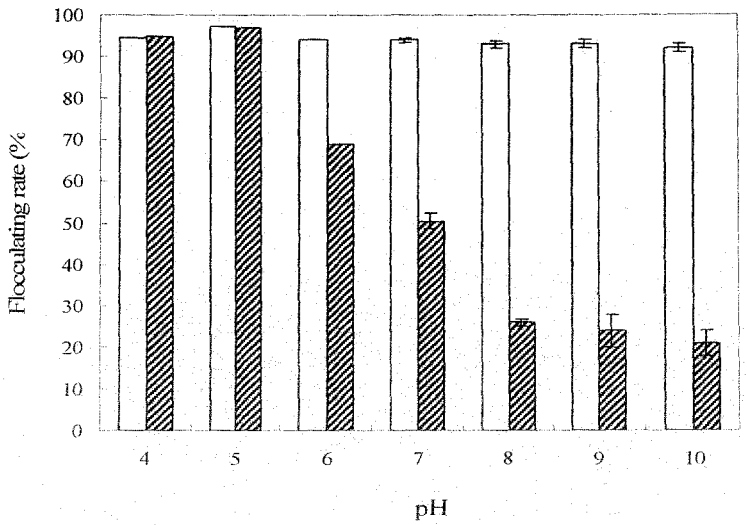


Fig. 3.6: Flocculating rate of EPS (30mg/l) in different pH in presence and absence of $CaCl_2$. Open column, flocculating rate in presence of $CaCl_2$ (0.7 mM); grid column, flocculating rate in absence of $CaCl_2$.

Effect of temperature on flocculating rate

While observing the flocculating rate of EPS at different temperatures ranging from 10-100 °C, highest flocculation was observed at 20 °C. When the temperature was dropped down to 10 °C or raised up to 40 °C, decrease in flocculating rate was observed (Fig. 3.7). While further increasing the temperature from 40 °C to 50 °C caused no change in flocculation whereas decrease of 15% in flocculating rate was noted when temperature was increased from 50 °C up to 80 °C; finally when the temperature was increased from 80-100 °C, a further 10% loss of flocculating rate was noted.

Effect of different metal ions on flocculating rate

Additionally, the effects of various metal ions (Cr^{6+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, and K^{+}) on flocculation were observed (Fig. 3.8). The monovalent cations such as K^{+} and Na^{+} were less effective than divalent cations in enhancing flocculation while the trivalent cation, Fe^{3+} showed the least activity (15%).

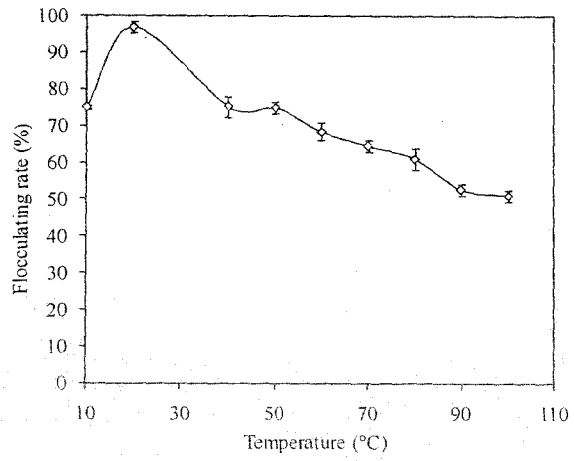


Fig. 3.7: Effect of Temperature on flocculating rate. (EPS 30mg/l, $CaCl_2$ 0.7 mM, pH 7.0)

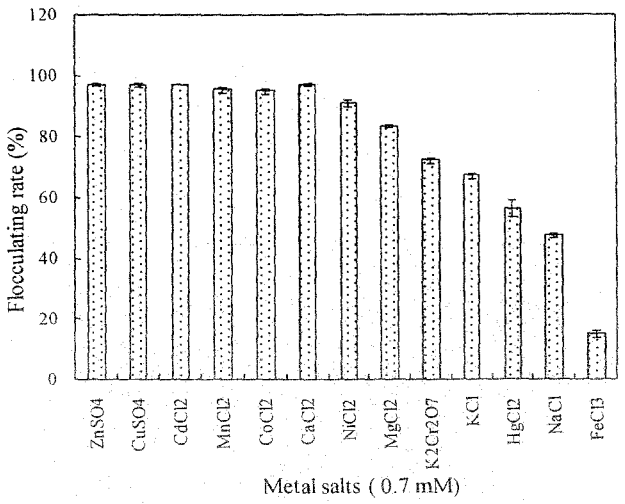
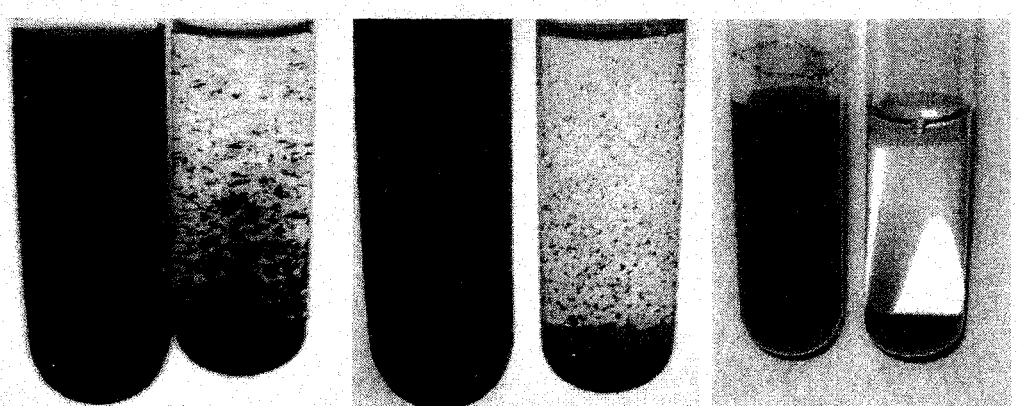


Fig. 3.8: Effect of different metal salts on flocculating rate. (EPS 30mg/l, pH 7.0)

Flocculation of activated charcoal

The EPS obtained from *A. junii* BB1A, showed an excellent flocculation of activated charcoal powder (Fig. 3.9 a-c). The flocculating rate was more than 90% without addition of $CaCl_2$ at pH 4 and 5. However, similar to flocculation of kaolin clay, above pH 5 the flocculation of charcoal required 0.7 mM $CaCl_2$.



(a) (b) (c)

Fig. 3.9: :(a to c) Flocculation of activated charcoal with EPS (30 mg/l) in presence of $CaCl_2$ (0.7 mM at pH 7) at different time interval of reaction; a, 0 min; b, 30 sec; c, 5 min.

3.3.2. Emulsifying activity of EPS

EPS showed characteristic emulsifying activity with toluene, n-hexadecane, olive oil and least activity with kerosene and diesel (Table 3.3 and Fig. 3.10). E_{24} values were superior for n-hexadecane, toluene and xylene. The relationship between the EPS concentration and emulsifying activity was tested using n-Hexadecane as substrates (Fig 3.11). 2.5 mg/ml of EPS was found to be optimal as there was no further improvement in the degree of emulsification at higher concentration. The emulsion was found to be stable with emulsification index remained unchanged in the range of 55-60 even after one month of incubation at room temperature (Fig 3.12).

In order to understand the possible role of protein fraction of EPS in flocculation and emulsification, the experiments were performed with de-proteinized EPS. It was observed that, both flocculating and emulsifying activity was negatively affected, when EPS was deproteinized (Table 3.4). This revealed the role and necessity of protein fraction in the flocculation and emulsification process.

Table 3.3: Emulsification of different hydrocarbons and oils by EPS (data are the mean of triplicates).

Hydrocarbon/oil	EPS mg/ml	Emulsifying Index (E24)
Diesel	1	6
	2	13
	3	23
Kerosene	1	6
	2	27
	3	53
n-Hexadecane	1	55
	2	45
	3	60
Toluene	1	63
	2	64
	3	70
Xylene	1	63
	2	65
	3	73
Hexane	1	6
	2	66
	3	66
Olive oil	1	53
	2	55
	3	54

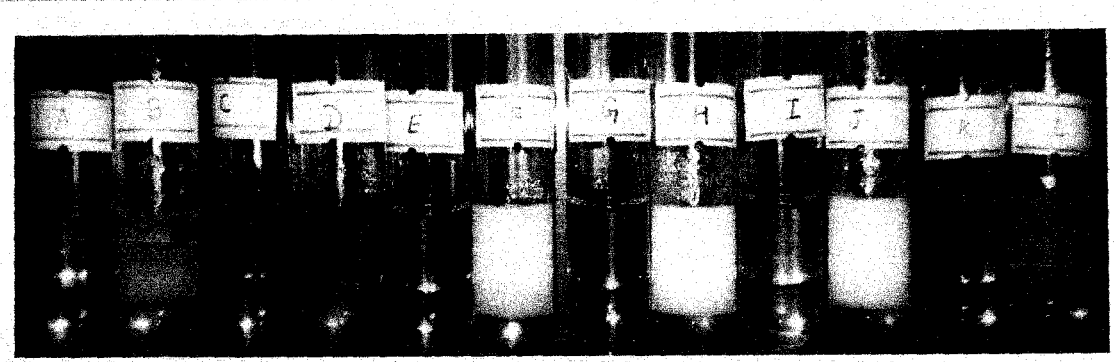


Fig. 3.10: Photographs of hydrocarbon/oil-water emulsion (E24) without and with EPS after 24 h at room temperature. (B) Diesel+EPS, (D) Kerosene+EPS, (F) n-Hexadecane+EPS, (H) Toluene+EPS, (J) Xylene+ EPS, (L) Hexane + EPS. A, C, E, G, I, K are the controls for diesel, kerosene, n-Hexadecane, Toluene, Xylene and Hexane respectively with water instead of EPS solution.

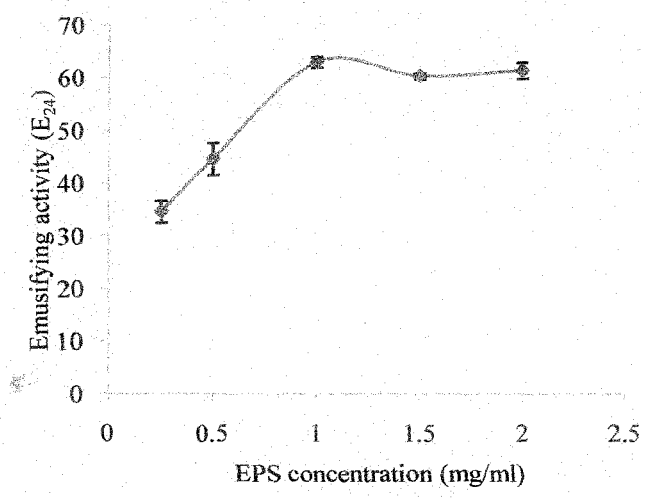


Fig. 3.11: Emulsifying activity at different concentration of EPS with n-Hexadecane.

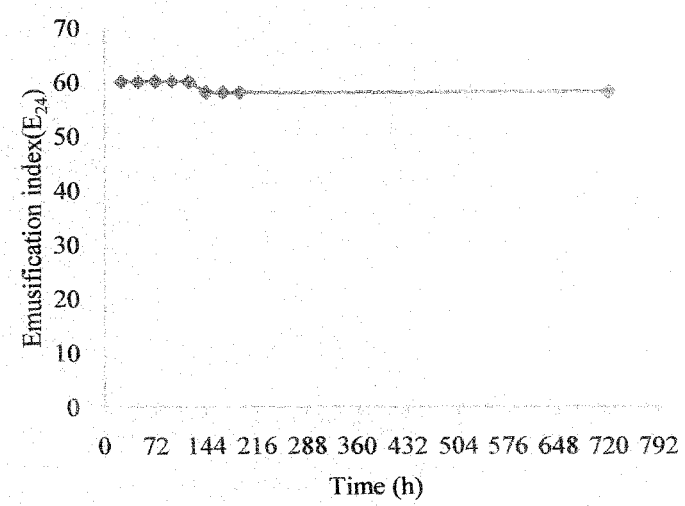


Fig. 3.12: Stability of EPS and n-Hexadecane emulsion over time.

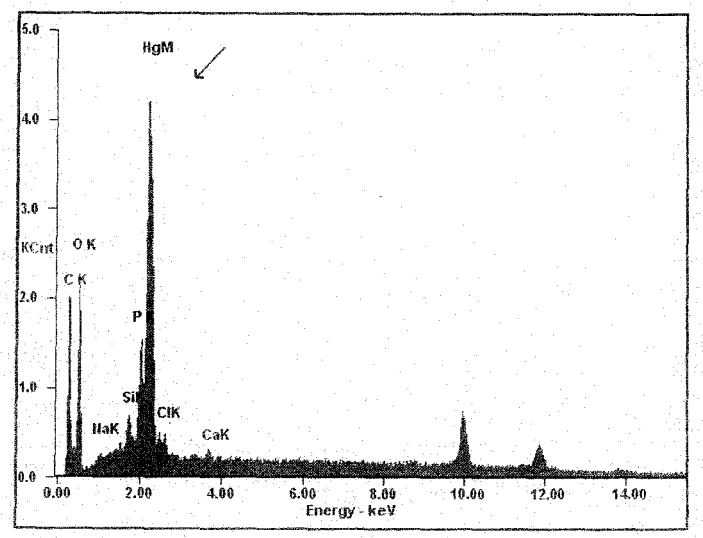
Table 3.4: Flocculating and emulsifying activity of deproteinised EPS.

Tested item	**Flocculating rate (%)**	**Emulsifying activity (E_{24}) with n-hexadecane**
EPS	94 ± 0.9	60.0 ± 0.2
EPS+Proteinase K[a]	68 ± 0.4	35.0 ± 0.5
EPS + TCA[b]	62 ± 1.3	31.0 ± 0.4

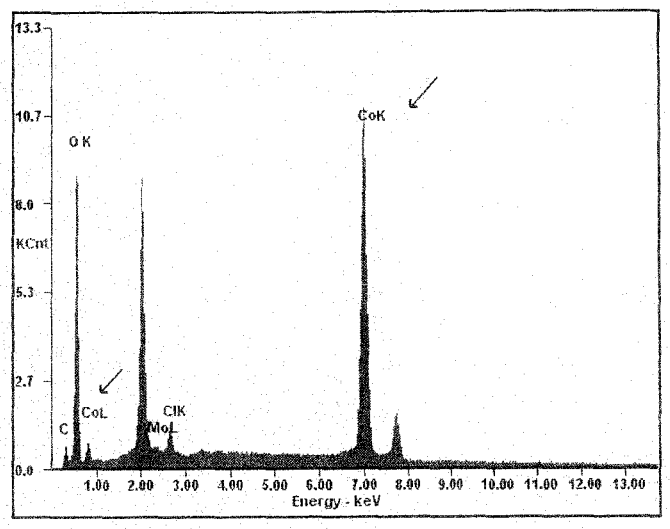
[a] EPS (1mg/ml) treated with proteinase K (20 mg/ml) at 37 °C for 30 min.
[b] EPS (1mg/ml) treated with 30% TCA.
Data are the mean of triplicates.

3.3.3. Demonstration of metal ion binding property of EPS

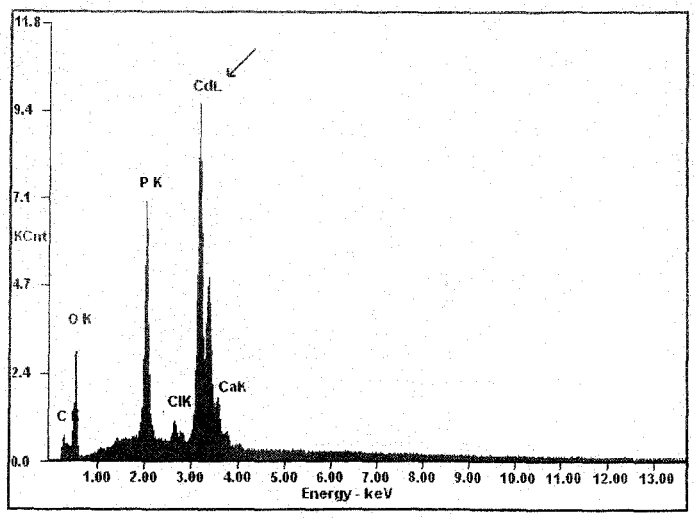
EDS spectra of EPS exposed to 10 mM of each metal salt solution ($ZnSO_4.7H_2O$ or $CuSO_4.5H_2O$ or $CoCl_2.6H_2O$ or $HgCl_2$ or $CdCl_2$. H_2O), showed the presence of prominent corresponding peaks (Fig. 3.13 a to f), which were absent in the control (Fig. 3.13 g) and have confirmed the sorption of different metal ions in the EPS of *A. junii* BB1A.



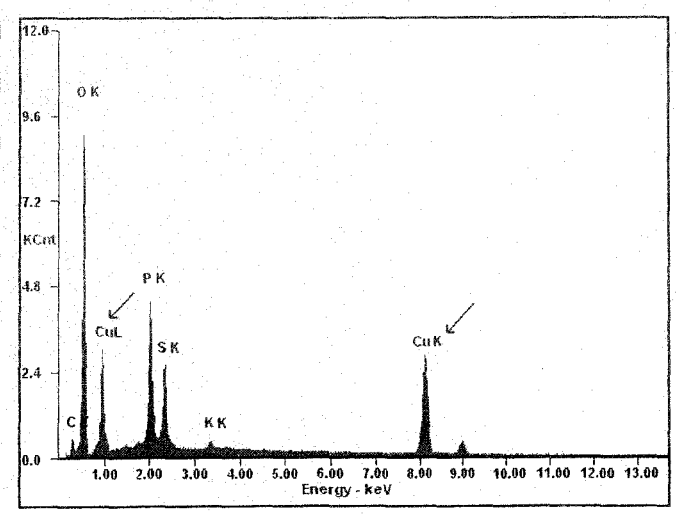
a)



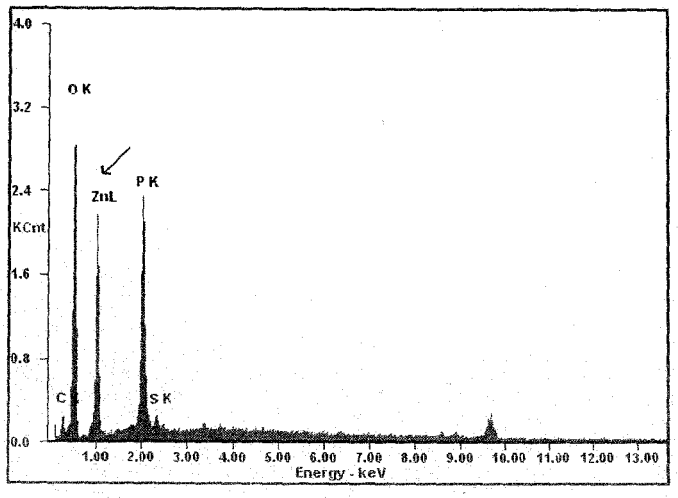
b)



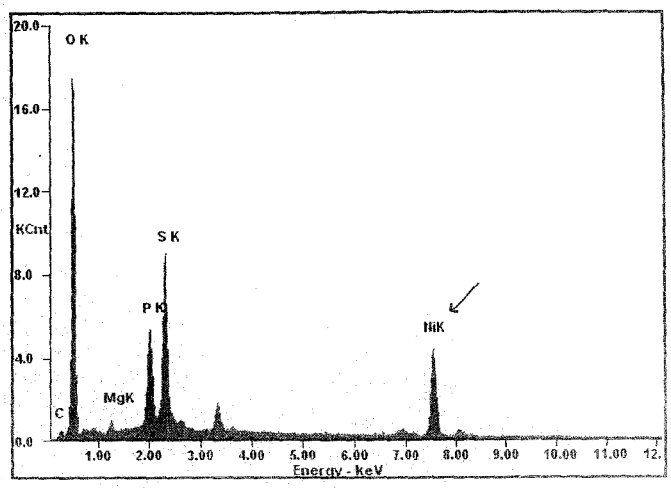
c)



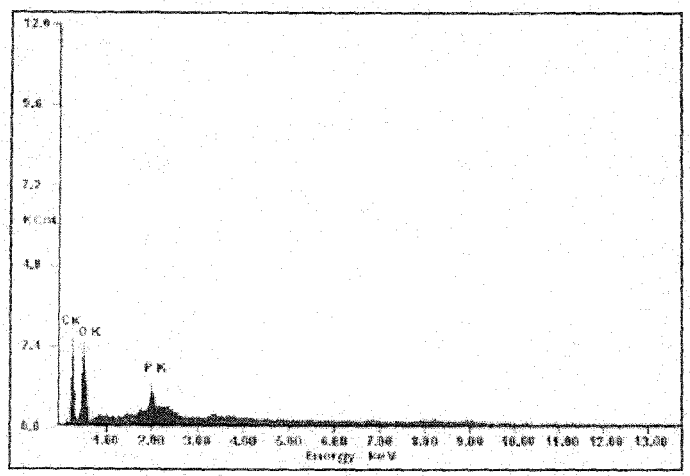
d)



e)



f)



g)

Fig. 3.13: Energy dispersive X-Ray spectroscopy (EDS) spectra of EPS obtained from *A. junii* BB1A. a) Mercury signal (HgM) measured by EDS, b) Cobalt signal (CoL and CoK) measured by EDS, c) Cadmium signal (CdL) measured by EDS, d) Copper signal (CuL and CuK) measured by EDS, e) Zinc signal (ZnL) measured by EDS, f) Nickel signal (NiK) measured by EDS and g) EDS spectra of control EPS (unexposed to metal ions) shows absence of specific signal. The horizontal axis is energy in KeV and the vertical axis is intensity in Kilo counts (Kcnt).

3.4. Discussion

Among various microorganisms found in the different ecosystems such as soil, fresh water and waste water, several strains from the genera *Acinetobacter* have been drawing much interest from medical, environmental and biotechnological point of view. Bacteria from this genus have been studied for various applications such as degradation of xenobiotics, degradation of oils, removal of phosphate and heavy metals, and for the production of bio-emulsifier (Abdel-El-Haleem, 2003).

Flocculating activity: Presently there is growing demand for biodegradable and renewable flocculants instead of chemical flocculants. In this study, flocculating property of EPS obtained from *A. junii* BB1A was studied. The flocculating activity was determined by Kaolin assay. Flocculation of kaolin was more than 90% with 30 mg/l EPS dosage. However, higher or lower dosages of EPS showed reduction in flocculating rate(s). When the dosage of EPS was inadequate, the effective bridging phenomenon gets hindered causing reduction in flocculation. The relationship between EPS dosage and flocculating rate was similar to the results described by earlier authors (Suh et al., 1997; Zheng et al., 2008). Table 3.5 summarizes the bioflocculant producing microorganisms and their optimum dosage for flocculation on kaolin suspension reported in the literature. On comparing the reported data with the data obtained with bioflocculant (EPS) produced by *A. junii* BB1A, it can be concluded that BB1A EPS is a promising flocculating agent.

Table 3.5: Comparison of the flocculating rates at optimum dosage of different EPS/bioflocculants.

Microorganism	Optimum concentration (mg/l)	Optimum pH	Cations required for flocculation	Maximum flocculating rate (%)	Reference
Rhodovulum sp.	71	8	Ca^{2+}(25 mM)	-	Watanabe et al., 1999.
Bacillus megaterium TF10	30.2	-	Ca^{2+} (5.6 mM)	95.5%	Yuan et al., 2011.
Bacillus subtilis	200	7-10	Al^{3+}(10 mg/l)	85%	Patil et al., 2009.
Staphylococcus cohnii ssp	0.3	7	Ca^{2+} (0.2g/l)	70.3%	Wong et al., 2012.
Bacillus sp. F19	2	2	No cations	97%	Zheng et al., 2008.
Citrobacter sp. TKF04	1 to10	2-8	No cations	Above 90%	Fujita et al., 2000.
Bacillus coagulants As-101	30	3.7	Ca^{2+} (8 mM)	92%	Salehizadeh et al., 2000.
***Acinetobacter junii* BB1A**	**30**	**4-10**	**Ca^{2+} (0.7 mM)**	**Above 90%**	**This study**
		4-5	**No cation**	**Above 90%**	

The flocculating mechanism of EPS can be described by bridging effect. Bridging is formed when the biopolymer attached to the particle extends its arm into the solution for a distance that is greater than the effective distance of inter-particle repulsion. Due to bridging, the biopolymers adsorbed to particles surface help to form flocs (Yim et al., 2007). High EPS dosage generally causes stabilization of particle

(Kaolin clay) resulting in no further binding of particle. Also, the excess of EPS causes increase in the viscosity of solution; as a result the settling of floc is negatively affected (Li et al., 2008). According to Bala Subramanian et al. (2008) the Kaolin clay mimics wastewater sludge in terms of their charge (−32 mV), which is more similar to the surface charge of sludge and can provide more reproducible results than the sludge that is found to vary every time it is sampled.

The flocculating activity of EPS is often influenced by the addition of cations. The cations can neutralize negatively charged functional groups of both EPS molecules and suspended particles, thus they can increase the adsorption of bioflocculant (EPS) to suspended particles (Li et al., 2008). The stimulatory effect of salts such as $CaCl_2$ on flocculation can be explained by DLVO theory, according to which high ionic strength causes compression of electrical double layer over kaolin clay particles, thus minimizes repulsion and promote EPS to form floc with kaolin particle. While observing the effect of $CaCl_2$ concentration on flocculation, maximum flocculation by the EPS obtained from BB1A was observed in presence of 0.7 mM $CaCl_2$, while higher or lower salt concentration reduced flocculation. In this study, the amounts of $CaCl_2$ required for effective flocculation was comparatively less compared with other bioflocculants (Table 3.5). This low dose requirement of $CaCl_2$ avoids the introduction of secondary pollutants (salt addition at higher dose, for waste water treatment, may subsequently lead to secondary pollution).

Flocculating rate without addition of $CaCl_2$ was above 94% in the pH range of 4-5, while the rate dropped down to 21% when pH was raised from 6 to 10. In the pH range of 1-3, EPS was insoluble; therefore no experiments were performed in this pH range. The negative charges of the clay particles in pH 4-5 was decreased due to adsorption of H^+ resulting in reduction of distance between kaolin particles which in turn enhanced the bridging effect of the bioflocculant (Elkady et al., 2011). Moreover, amino and amide groups present in the EPS as revealed by FT-IR spectra were protonated at acidic pH, and thus the positively charged EPS gets attracted towards negatively charged kaolin clay particles. Therefore, electrostatic interaction played an important role in the adsorption of negatively charged clay particles (Deng et al., 2005). Whereas in alkaline condition, increase in pH increases the OH^-, which may further increase the charge density on clay suspension causing inhibition of charge neutralization effect of $CaCl_2$ (Elkady et al. 2011). Furthermore, increasing the pH of the kaolin suspension decreased the electrostatic attraction. Under such condition, EPS would exhibit negative charge upon complete deprotonation of the functional groups (amino and amide). Electrostatic repulsion would then prevent the clay particles from approaching towards EPS, thus flocculating rate decreases (Deng et al., 2005). Above pH 5, addition of 0.7 mM $CaCl_2$ was required for effective flocculation (Fig. 3.6) and the flocculating rate was >90% in the pH range of 6-10. At higher pH condition, addition of Ca^{2+} ions

increased the flocculation rate by forming Ca^{2+} mediated complexes of bioflocculant and kaolin clay. So, increase or decrease in pH from 5 shall lead to lowering of flocculating rate in absence of $CaCl_2$ (Liu et al., 2010). EPS from *A. junni* BB1A was found to retain high flocculant activity (over 90% removal rates for the Kaolin suspension) at either acidic or basic pH (Fig. 3.6). This characteristic of EPS is favorable for its use under extreme pH conditions.

The effect of various other cations (Cr^{6+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, and K^{+}) on the flocculating activity of EPS was also studied (Fig. 3.8). The monovalent cations such as K^{+} and Na^{+} were less effective than divalent cations in enhancing flocculation while the trivalent cation, Fe^{3+} showed the least activity (15%). Similar results have been reported very recently in case of *Bacillus mojavensis* bioflocculant (Elkady et al., 2011) where presence of monovalent and trivalent cations showed reduction in biopolymers efficiency. Trivalent cation strongly inhibits flocculation which may be explained by the effect on changing the surface charge of kaolin clay particles and coverage of the adsorbing sites on the biopolymer (Elkady et al., 2011).

While observing the flocculating rate of EPS at different temperatures ranging from 10-100 °C, highest flocculation was observed at 20 °C. Reduction in flocculating rate at higher temperature may be explained by denaturation of protein component in EPS and simultaneously increase in kinetic energy of kaolin particles due to rise in temperature (Liu et al., 2010). Chemical characterization of the EPS showed that it is a protein-polysaccharide complex (Chapter 2). As revealed from table 3.4, the protein part of EPS, obtained from BB1A, was indispensible for displaying its full activity of the flocculant. Many bioflocculants have been reported to be constituted of protein–polysaccharide complex (Nakamura et al. 1976a; Yokoi et al. 1997; He et al. 2002; Kobayashi et al. 2002; Zhang et al. 2002; Xia et al. 2008; Zheng et al. 2008; Li et al. 2009b; Patil et al. 2009; Liu et al. 2010b; Xiong et al. 2010) and in many cases, the native protein portions were required for maximum flocculant activity (Kurane et al. 1986b; He et al. 2002; Zhang et al. 2002).

The flocculation of activated charcoal by the EPS (from strain BB1A) shows a potential application in the treatment and reuse of waste water particularly from coal washeries or tailings, which are often problematic in terms of solid-liquid separation and dewatering. The settling rate of fine coal particles by interaction with proper coagulants and flocculants is important in the dewatering process. Generally, the discharged water from coal tailing or coal washeries itself contains high concentration of metal ions such as Ca^{2+}, Mg^{2+} along with some trace metal ions such as Fe, Co, Ni, Mn, etc.(Das et al.,2006; Sabah and Cengiz, 2004; Volcich, 2007). This therefore avoids the addition of salt to enhance the

flocculation. Thus the EPS obtained from *A. junii* BB1A can be suitably used in place of synthetic flocculants.

Emulsifying activity: The EPS harvested from *A. junii* BB1A has efficient emulsification properties comparable with some of the standard gums. The emulsifying activity (E_{24}) of *A. junii* EPS against n-hexadecane (E24=60 at 2.5 mg/l dose) when compared with published data derived with 3.0 mg/l dosages of some commercial emulsifiers like xanthan (E_{24}=40), alginate (E_{24}=10), pectin (E_{24}=20), and Triton X 100 (E_{24}=80) (Freitas et al., 2011). The stability of emulsion was studied using n-hexadecane and found to be stable (Emulsifying index between 60-55%) up to one month (Fig. 3.12). The emulsifying property of exopolysaccharide from *Sphingomonas paucimobilis* is reported to be due to presence of hydrophobic lipid portion (Ashtaputre and Shah, 1995) whereas, the protein portion, in acacia gum is known to be responsible for emulsifying activity (Dickinson et al., 1990). The ability of EPS obtained from BB1A, to form stable emulsion may be attributed to the concentration and nature of protein present in the EPS similar to as observed in *Enterobacter cloaceae* (Iyer et al., 2006). High and stable emulsification property of EPS from *A. junii* BB1A renders its potential application in enhanced oil recovery.

Metal ion binding property: Bioflocculants have been considered as an alternative choice to the chemical flocculants, in various industrial waste treatments including heavy metal removal (Iyer et al., 2005; Wu and Ye, 2007; Gong et al., 2008; Lin and Harichund, 2011). They have extensive metal binding capacity and are therefore recommended as surface-active agents for the removal of heavy metals (Morillo et al., 2006). According to Pal and Paul (2008) the important role of EPS in the sorption and removal of heavy metals from the environment is due to their participation in flocculation and ability to bind metal ions from solutions. The flocculating activity of EPS extracted from BB1A was found to be stimulated by the addition of various metals ions (Fig. 3.8), indicating its potential to bind these metals from the solution. The metal binding property of the EPS was demonstrated by EDS analysis (Fig. 3.13)

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