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# ***CHAPTER 4***

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## **Exploring Biotechnological Prospect of Exopolysaccharide Produced by *K. pneumoniae* PB12: Flocculation Studies**

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### **4.1. Introduction**

Flocculants 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). They are typically used to accelerate or improve the settling of suspended solids in various types of wastewater. Water pollution is one of the major problems these days and researchers are trying to solve this problem. Flocculants used in water treatment can be classified into three main groups: (i) inorganic flocculants such as alum, ferric flocculants or polyaluminum chloride; (ii) synthetic organic flocculants like polyacrylamide derivatives or polyethylene imine; and (iii) naturally occurring flocculants like sodium alginate or microbial flocculants. Some inorganic and synthetic organic flocculants are carcinogenic and neurotoxic. Polyferric sulfate flocculant can be costly and the resultant excess iron may cause unpleasant taste, odor, color etc. Although synthetic flocculants are used because of their cost effectiveness, they are not biodegradable and are some of their degraded monomers such as acrylamide are neurotoxic and even show strong carcinogen (Yokoi et al., 1995). Bioflocculants are generally non-toxic and biodegradable. Most of the reported bioflocculants are principally comprised of exopolysaccharide (EPS). 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 & Myszk, 2007). Flocculants produced by a haloalkalophilic *Bacillus* sp. I-471 (Kumar et al., 2004), *Bacillus subtilis* DYU1 (Wu & 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 produce 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 et al., 2005).

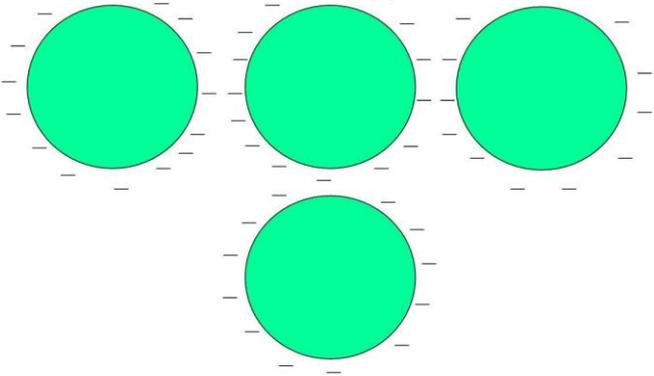
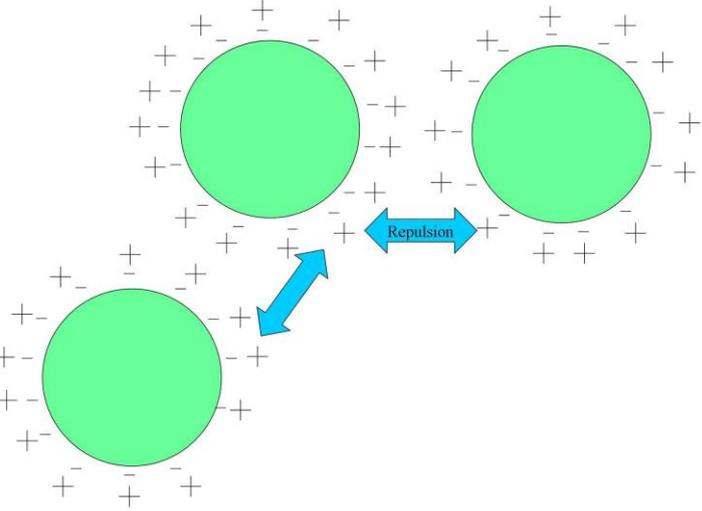
#### *4.1.1. Mechanism of bioflocculation*

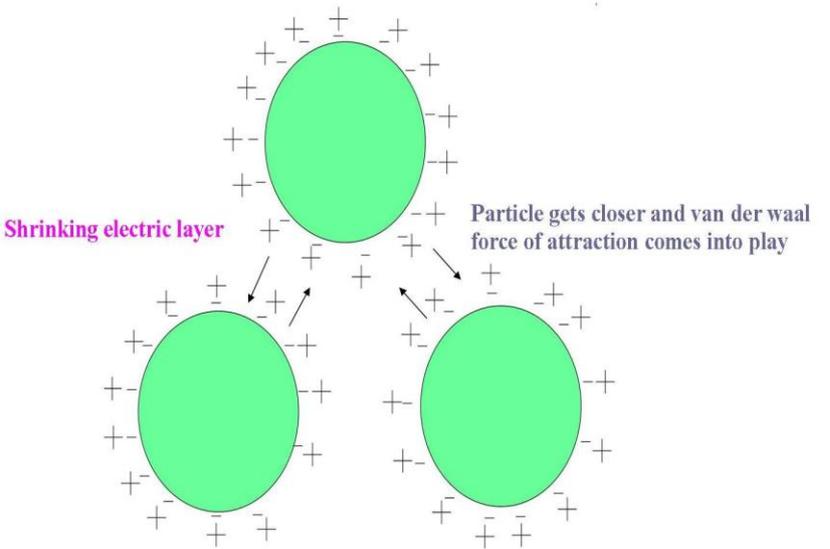
The biopolymers have the property to anchor and bridge the adjacent cells and hence they are thought to be responsible for the bioflocculation process. There are three theories which explain the mechanisms of bioflocculation: DLVO theory (or double layer theory), alginate theory, and divalent cation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

##### *4.1.1.1. The DLVO theory*

The DLVO theory (named after Derjaguin, Landau, Verwey 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 4.1). 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 & Higgins, 2002).

<p>Negatively charged ions of the dispersion medium when adsorbed on the colloidal particles surface renders them negative electric charge.</p>	
<p>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</p>	

<p>causes repulsion. Because of which colloidal particles remain suspended in the medium.</p>	
<p>At high ionic strength the electrical double layer shrinks, allowing the particles to get closer. At such a close distance, attractive forces such as weak van der Waal forces come into play, resulting in aggregation and flocculation/precipitation.</p>	

**Fig. 4.1.** The DLVO theory of bioflocculation.

*4.1.1.2. The alginate theory*

This theory was first proposed by Bruus et al. (1992) for describing 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  $\text{Ca}^{2+}$ , and this support their role in bioflocculation.

#### *4.1.1.3. 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  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  play an important role in bioflocculation by forming bridges 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.

#### *4.1.2. Emulsification*

Surfactants and emulsifiers are widely used in the pharmaceutical, cosmetic, detergents in various industrial sectors, petroleum and food industries (Makkar & Cameotra, 1998). 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 & 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, lipopolysaccharide-protein complexes and polysaccharide-protein-fatty

acid complexes (Ron & 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. 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* (Kaplan & Rosenberg, 1982; Navon-Venezia et al., 1995). Among the bacterial emulsifiers, emulsan obtained from *Acinetobacter calcoaceticus* known as RAG-1 is the only commercialized one (Rosenberg et al., 1998). RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (Rosenberg et al., 2002). 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).

#### 4.1.3. Metal Chelating property

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 & Gabriel, 2003). 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 envelope has been found to be associated with binding to Ni, Cu and Zn (Falla & Block, 1993).

EPSs 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 & Shojaosadati, 2003). The binding of cations to bacterial EPSs generally occurs through electrostatic interaction with negatively charged functional groups such as uronic acids, phosphoryl groups and carboxylic groups. In addition protein component of EPSs also plays a major role in complexation of metal ions (Mejare & Bulow, 2001). Proteins rich in acidic amino acids, including aspartic and glutamic acid, also provide anionic properties to the EPSs.

The application of EPSs 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 EPSs is a non-living sorbent, its potential application in the treatment process has been widely acknowledged (Gavrilescu, 2004). In this chapter the potential biotechnological application of the EPS has been explored.

## **4.2. Materials and methods**

### *4.2.1. Flocculating rate measurement*

Activated carbon was suspended in distilled water at a concentration of 5 g l<sup>-1</sup> at pH 7 and used as a stock solution for the subsequent assays. After the pH of the suspension was adjusted, 0.1 ml of culture supernatant was added and stirred for 2 min. The solutions were allowed to settle for 5 min at room temperature and the optical density (OD) of the clarifying upper phase solution was measured at 550 nm with a UV-vis spectrophotometer. A control tube in which the culture supernatant was

replaced with distilled water was also included and measured under the same conditions. The flocculating rate was determined according to following equation:

$$[B-A]/B \times 100\% \quad (1)$$

Where A and B are optical densities at 550 nm of the sample and control, respectively. Mean % flocculating rate of three independent experiments was considered.

#### *4.2.2. Emulsifying activity measurement*

Toluene, n-hexadecane, olive oil, and kerosene oil were used to study the emulsifying activity of the purified EPS according to the procedure described earlier (Ashtaputre & Shah, 1995). To 3 ml aqueous solution of EPS ( $1 \text{ mg ml}^{-1}$ ), 3 ml of hydrocarbon or oil was added and agitated vigorously for 2 min on vortex. The emulsion and aqueous layers were measured after 24 h and emulsification index (E24) was calculated by the following formula (Cooper & Goldenberg, 1987):

$$E24 = \text{volume of the emulsion layer} / \text{total volume} \times 100 \quad (2)$$

#### *4.2.3. Effect of dosage, temperature, pH and metal cations on the flocculating rate*

The effects of EPS and  $\text{CaCl}_2$  dosage, temperature, pH and metal ions on flocculating rate were examined. The dosage of EPS and  $\text{CaCl}_2$  were varied from 1-30  $\text{mg l}^{-1}$  and 0-100 mM, respectively. The pH of the activated carbon suspensions was adjusted using HCl and NaOH in the pH range of 1-11. The temperature of activated carbon suspension was changed in water bath in the range of 10-100 °C. The effects of various metal cations (monovalent: NaCl, KCl; divalent:  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{CdCl}_2 \cdot \text{H}_2\text{O}$ ;

and trivalent:  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in the range of 0-100 mM concentration) on flocculation of activated carbon suspension were also studied.

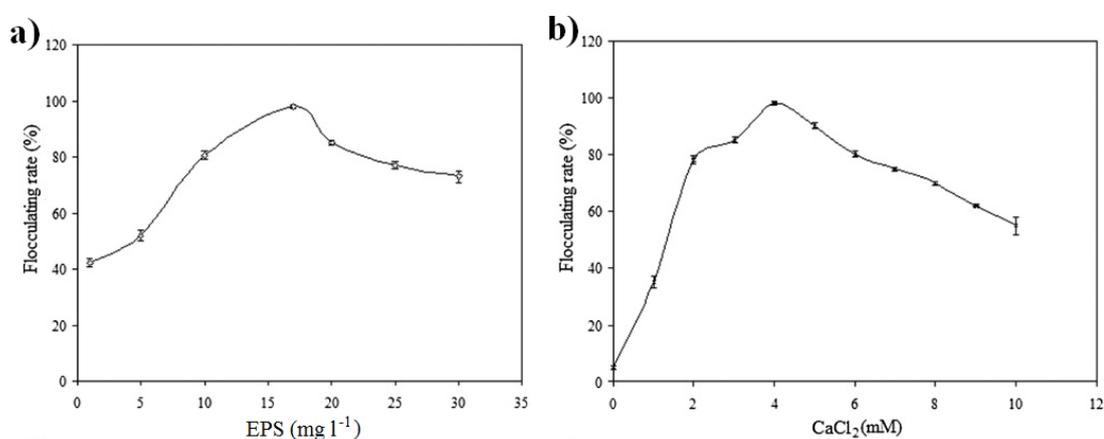
### 4.3. Results

#### 4.3.1. Emulsifying activity measurement

The EPS showed characteristic emulsifying activity with toluene (66.6%), n-hexadecane (65%), olive oil (63.3%) and kerosene (50%).

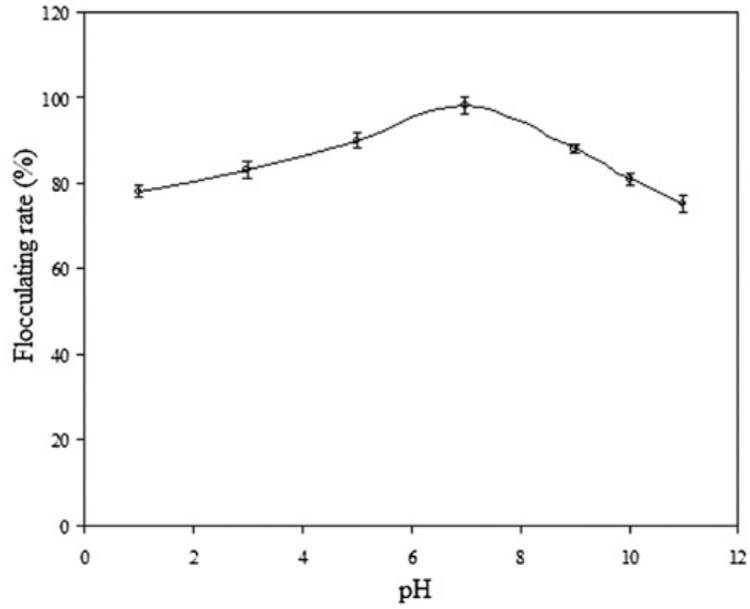
#### 4.3.2. Effect of dosage, pH, temperature and metal ions on the flocculating rate

Effect of higher or lower dosage of both  $\text{CaCl}_2$  and EPS on flocculating rate was studied in order to determine optimal dosage. The flocculating rate of 98% was achieved with EPS dosage of  $17 \text{ mg l}^{-1}$  and 4 mM  $\text{CaCl}_2$  at pH 7. Higher or lower dosage of EPS and  $\text{CaCl}_2$  caused poor flocculation (Fig. 4.2a & b).



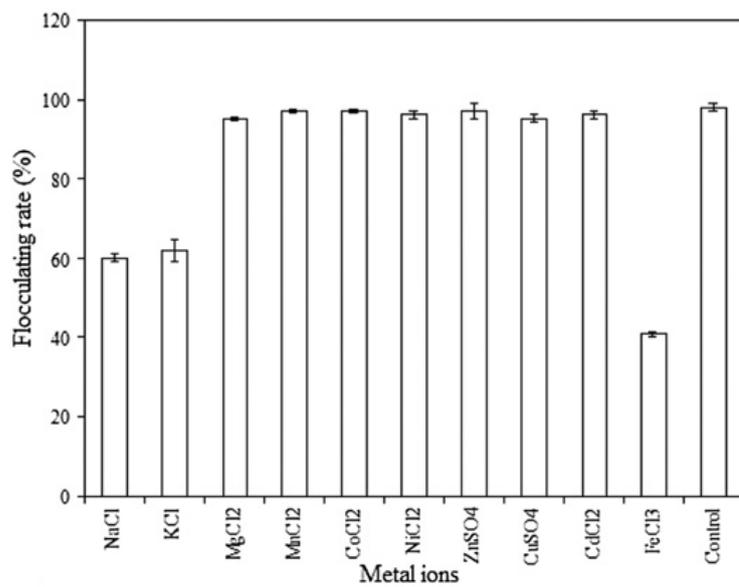
**Fig. 4.2.** a) Effect of EPS dosage on flocculating rate. b) Effect of  $\text{CaCl}_2$  concentration or dosage on flocculating rate.

In the pH range of 3-10, flocculating rates were above 80% showing maximum (98%) at pH 7 in presence of 4 mM  $\text{CaCl}_2$  (Fig. 4.3).



**Fig. 4.3.** Effect of pH on flocculating rate in presence of 4 mM CaCl<sub>2</sub>.

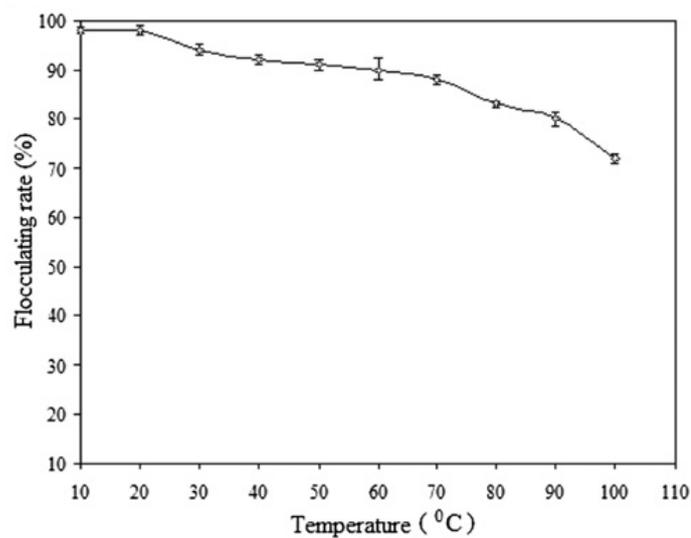
Effects of various cations other than Ca<sup>2+</sup> on the flocculating rate of the EPS were studied (Fig. 4.4) and compared with the flocculating rate in presence of CaCl<sub>2</sub> (control).



**Fig. 4.4.** Effect of various metal cations on flocculating rate

The optimal concentration of  $Mg^{2+}$  or  $Mn^{2+}$  or  $Co^{2+}$  or  $Ni^{2+}$  or  $Zn^{2+}$  or  $Cu^{2+}$  or  $Cd^{2+}$  was found to be 4 mM (same as that  $Ca^{2+}$ ) whereas, the optimal concentration of  $Fe^{3+}$  was 1 mM and for monovalent cations,  $Na^+$  and  $K^+$ , the optimum dosage was 10 mM. The choice of using  $CaCl_2$  over  $FeCl_3$  in flocculation studies was due to the ease of handling the  $Ca^{2+}$  induced large and compact floc in contrast to the gelatinous floc produced by  $Fe^{3+}$ .

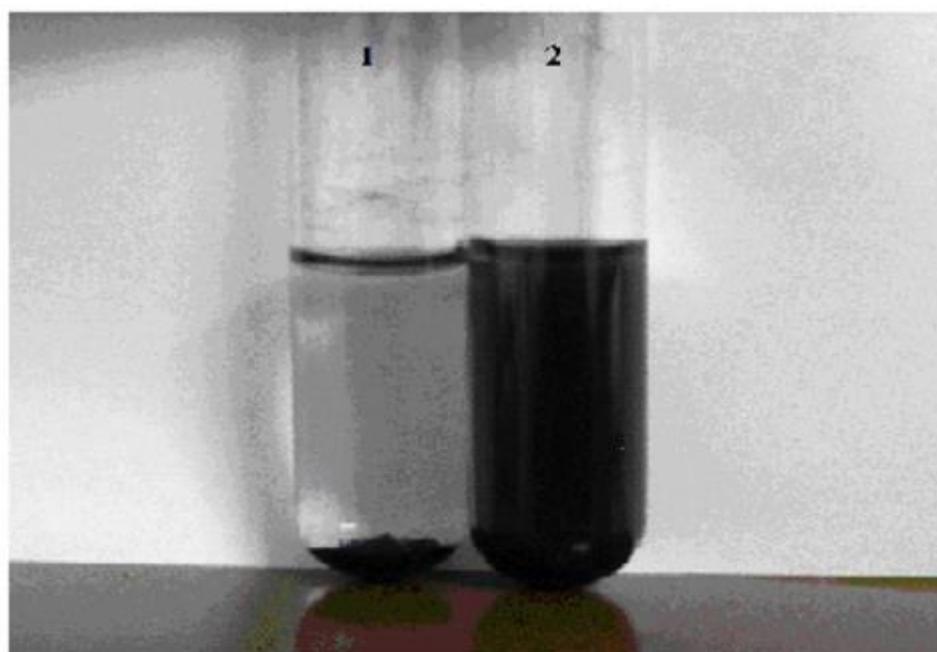
Effect of temperature on flocculating rate was also studied. The flocculating rate remained above 90% in the temperature range of 10-50 °C. Maximum flocculation of 98% was observed in both 10 °C and 20 °C (Fig. 4.5).



**Fig. 4.5.** Effect of temperature on flocculating rate

#### 4.4. Discussion

Emulsifying activities of the EPS obtained from *K. pneumoniae* PB12 against hydrophobic compounds like n-hexadecane, toluene and olive oil were well comparable with emulsification index for the EPS produced by gram-negative bacteria *Pseudomonas oleovorans* (Freitas et al., 2009). Presently there is growing demand for biodegradable and renewable flocculants instead of chemical flocculants. In this study, flocculating property of EPS obtained from *K. Pneumoniae* PB12 was studied. The flocculating activity was determined by activated carbon assay. Flocculation of activated carbon was more than 98% with 17 mg l<sup>-1</sup> of EPS dosage (Fig. 4.2a & Fig. 4.6).



**Fig. 4.6.** Photograph showing flocculation of activated carbon in 5 min; sample 1: with EPS; sample 2: without EPS.

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). An EPS produced by a micro-alga, *Gyrodinium impudicum* KG03 required 7mM CaCl<sub>2</sub> for maximum flocculation (61%) of activated carbon (Yim et al., 2007). Results showed that at lower EPS dosage ineffective bridging caused poor flocculation; on the other side, over addition of negatively charged EPS caused incomplete dispersion of excess EPS leading to poor stability (Suh et al., 1997). 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). In alkaline condition, the flocculating rate decreases, suggesting that increase in OH<sup>-</sup> concentration causes increment of negative charge density on activated carbon particles and consequently the neutralizing effect of CaCl<sub>2</sub> gets inhibited resulting in dispersion of suspended particles. In the absence of Ca<sup>2+</sup> ion no effective flocculation was observed at pH 7 which dictates the requirements of CaCl<sub>2</sub> for effective flocculation by forming Ca<sup>2+</sup> 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 flocculating rates determined with divalent cations Mn<sup>2+</sup>, Co<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, and Ni<sup>2+</sup> were better than other tested cations. The flocculating rate in presence of Fe<sup>3+</sup> was 53% lesser than that of Ca<sup>2+</sup> induced flocculation. This may be because of its effect on changing the surface charge of activated carbon particle and coverage the EPS adsorb-sites. The competition of the positively charged particles and less adsorb sites reduce the flocculation efficiency

(Gong et al., 2008; Zheng et al., 2008). Such reduction in flocculation was also noted when excess of  $\text{CaCl}_2$  was added; may be because of the similar phenomenon (Fig. 4.1B). Monovalent cations like  $\text{K}^+$  and  $\text{Na}^+$  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 PB12 EPS 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.

The optimal dosages for flocculation of activated carbon suspension were 17 mg l<sup>-1</sup> EPS and 4 mM  $\text{CaCl}_2$ . EPS showed flocculating rate of above 80% over a wide range of pH (pH 3-10) whereas, more than 90% rate was noted in the temperature range (10-50 °C) tested in presence of  $\text{CaCl}_2$ . Moreover, EPS showed characteristic emulsifying activity with toluene (66.6%), n-hexadecane (65%), olive oil (63.3%) and kerosene (50%). Results showed that EPS was found suitable to be applied in alkaline, neutral,

and acidic conditions. Both the emulsifying and flocculating properties of the EPS were comparable with those of other commercially available flocculants and emulsifying agents. Thus, it is anticipated that EPS obtained from *K. pneumoniae* PB12 would be an attractive candidate for future use in environmental bioremediation processes like enhanced oil recovery.