Characterization of Extracellular Polymeric Substances (EPS) Extracted from both Sludge and Pure Bacterial Strains Isolated from Wastewater Sludge for Sludge Dewatering

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Abstract: At present, the nature and characteristics of extracellular polymeric substances (EPS) produced by activated sludge microorganisms, which play an important role in sludge settling and dewatering are not clear. In order to understand the chemistry of biopolymers, sludge EPS were extracted from fresh activated sludge. Six pure EPS producing bacterial strains were isolated and were grown in synthetic medium. The individual EPS produced by these strains as well as those isolated (extracted) from activated sludge were characterized in terms of proteins and carbohydrate content and zeta potential. The effect of sludge EPS as well as those from pure isolated bacterial strains was studied on sludge settling characteristics (sludge volume index). Better sludge settling characteristics were observed with individual EPS than the EPS extracted from activated sludge. Zeta potential characterizations of EPS revealed that all individual EPS produced by pure culture strains were anionic in nature but their charge varied to a large extent. The reason for sludge EPS poorly performed to settle sludge than EPS of pure strains were discussed.

Keywords: Wastewater Sludge, Bioflocculation, Biocoagulation, Microorganisms, and EPS

INTRODUCTION
Sludge dewatering, is one of the most important steps in wastewater treatment and for sludge recycle. Better dewaterability of sludge leads to economical disposal and reuse, such as production of polyhydroxyalkanoates (PHA or bioplastics), bricks for construction, and use as a raw material for growth of industrial microorganisms (Tyagi et al., 2002; Yezza et al., 2004). Microorganisms of particular interest are those producing enzymes, bioflocculants and biopesticides.

Sludge is found to be negatively charged (approximately -30mV), and hence cationic synthetic polymers are generally employed to neutralise the sludge charge, which facilitates the sludge settling. These polymers are expensive, and pollute environment and safety precautions must be followed during their handling (Changa et al., 2002). To overcome these existing problems an alternative and suitable way is using biocoagulants/bioflocculants produced by bacterial strains derived from activated sludge.

During the past five decades researchers have been venturing to bioflocculate the sludge using microorganisms. Microorganisms are capable of growing in diverse environments and producing secondary metabolites during their growth. Microorganisms growing in sludge produce secondary metabolites mainly consisting of carbohydrates (Extra Cellular Polysaccharides, alginate and chitosan), proteins (lectins), lipids (fatty acids) and DNA & RNA. It has been well documented that microbial EPS play an important role in bioflocculation process by interacting with the sludge solids. The microbial EPS may be non-ionic or may contain cationic, anionic and/or both charges (Garnier et al., 2005).

Sludge settling ability of EPS mainly depends on the concentration and characteristics of EPS. The concentration of EPS produced by sludge is affected by sludge characteristics, EPS biochemical characteristics, operational parameters in a wastewater treatment plant as well as type of microbial community dominating in that treatment plant. Generally, there is an optimal concentration of EPS for sludge settling (Urbain et al., 1993). EPS concentration below or above the optimal level is detrimental to sludge settling. Sludge is a complex and diverse microbial community. Different microorganism produces different types of EPS with different characteristics and concentration. In order to study the mechanism of EPS for sludge settling and to analyze
their properties, it is necessary to isolate the EPS producing microbial strains from sludge. Therefore, the objective of this study was to isolate EPS producing bacterial strains from activated sludge and to characterize and compare the physical and biochemical properties of these EPS.

**MATERIALS AND METHODS**

**Sludge Sample Collection and isolation of EPS producing strains**

Wastewater sludge samples were collected from Communauté Urbaine du Québec (CUQ, Québec). Six EPS producing bacterial strains were isolated on Plate Count Agar (PCA) using serial dilution techniques. Plates were incubated at 25°C for 72 hrs. The isolated bacterial strains were named from B1 to B6 (Bala et al., 2006).

**Identification of bacterial strains**

Bacterial strains were identified based on 16S rDNA sequencing. Isolated genomic DNA from individual isolated bacterial strains was subjected to PCR amplification of 16S rDNA using universal primers (Weisburg et al., 1991). Amplified products were gel purified using Qiagen gel extraction kit and purified products were sequenced (Bala et al., 2007b). Obtained 16S rDNA gene sequences were blasted into the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for similarity search.

**EPS Production**

To produce EPS, microbial strains were grown on tryptic soy broth (TSB) and peptone water media. Isolated strains were grown individually as pure cultures and consortium (0.1% V/V secondary sludge used as inoculum) as well. Bacterial strains were inoculated in the liquid broth from the PCA slants, and TSB broth medium was incubated in an orbital shaker at 250 rpm for 5 days at 25°C. At the end of 3 days, the broth became highly viscous (data not shown). After incubation, culture broths of individual bacterial strains and bacterial consortium containing EPS were collected and stored at 4°C for further studies. After EPS production, the culture medium was centrifuged at 8,000g for 10 minutes to obtain EPS in supernatant (slime) and in bacterial pellet (capsular EPS and microorganisms). The harvested crude EPS was then stored at 4°C for characterization and further studies.

**Sludge Volume Index (SVI)**

Several researchers have studied the bioflocculation process using extracellular polymeric substances produced by microorganisms (Higgins and Novak, 1997a, b; Sobek and Higgins, 2002). Bioflocculants are generally secreted outside the cell and named as exocellular polymeric substances (EPS). Two types EPS are mainly found to play an important role in sludge settling such as slime and capsular EPS. Slime types of EPS are generally washed out from cell during centrifugation/harvesting and the capsular EPS are stable and attached on the cell wall of microorganisms during this process. These EPS play an important role in flocculation, settling and dewatering of the wastewater sludge. EPS helps in formation of bioflocs in the activated sludge and contributes to its structural, surface charge and settling properties of bioflocs. Many studies were carried out using only one type of EPS from individual microorganisms for sludge settling. Hence we studied the sludge settling efficiency of extracted slime EPS (supernatant after centrifugation), capsular EPS (pellet after centrifugation) and bacterial broth (combination of both slime and capsular EPS obtained without centrifugation).

Sludge settling characteristics were measured in terms of sludge volume index (SVI) to investigate the performance of EPS produced by isolated microorganisms; EPS in supernatant (slime EPS), bacterial pellet (Capsular EPS) and bacterial broth (Slime, capsular EPS and bacterial strains) were studied (Bala et al., 2006; 2007a). SVI was measured to determine the sludge settling efficiency of the produced EPS. The SVI of the sludge with the addition of supernatant EPS, bacterial pellet or bacterial broth (with and without addition of cations (Ca++)) was measured. Based on the previous study, an optimal concentration of Ca++ ions 100 (mg/l) was used along with bacterial broth. To measure SVI, fresh sludge sample collected from municipal wastewater treatment plant (CUQ, Quebec) was first mixed thoroughly. The well mixed sludge was transferred into five beakers (1L in each beaker). Followed by the addition of 1% EPS (v/v) (test 1), 2% EPS (v/v) (test 2),
3% EPS (v/v) (test 3) and 4% EPS (v/v) (test 4). The fifth beaker served as control (no addition of EPS). After addition of EPS, the samples were mixed in two different stages, at 117 rpm for 5 min and followed by 10 rpm for 10 min. First stage of mixing enables the biopolymer to mix and contact with sludge solids, second stage of mixing enables to form flocs before sludge settling. Each mixed sludge sample was then transferred into 1L graduated measuring cylinder for SVI measurement. Sludge settling efficiency in each cylinder was monitored at an interval of 5, 10, 20 and 30 min (Bala et al., 2006).

**EPS Extraction**

EPS extraction from pure cultures strains and consortium. EPS extraction was carried out using modified protocol from Smitinont et al., (1999). Bacterial culture broth was centrifuged at 6000 rpm for 20 min at 4°C to remove bacterial cells. The supernatant was precipitated with 2.2 volume of absolute chilled ethanol by incubating the mixture at -20°C for 1 hr. Precipitated EPS was collected by centrifugation at 6000 rpm for 20 min at 4°C. Supernatant was decanted and pellet containing EPS was dried at room temperature in laminar hood for 6 hrs. Dry weight of the extracted EPS (biopolymer) was measured as mentioned below.

EPS extraction from fresh sludge. Sludge EPS was extracted as mentioned in Urbain et al., (1993). Collected secondary sludge was concentrated to total solids 37.4 g/l, suspended solids 30.35 g/l and dissolved solids 7.05 g/l. Concentrated 100 ml sludge sample (in duplicate) was sonicated thrice using sonicator (Ultrasonic processor, CV33, Cole Parmer, USA) at 50W for 15s with a time interval of 10s. The sonicated sludge samples were well mixed with equal volume of Milli-Q water and then the mixture was allowed to stand for 45 min at 4°C. Well mixed samples were centrifuged at 14000g for 20 min at 4°C. Then collected supernatant containing sludge EPS was precipitated and extracted with ethanol by following similar EPS extraction procedure as used for pure bacterial strains and consortium. The dry of weight of the extracted polymer was measured as described below.

**EPS characterization**

Dry weight of the extracted EPS was measured by drying at 105°C to a constant weight (APHA, 1995). Characterization of charge (zeta potential $[\zeta]$) of the produced EPS was determined using Zetaphoremeter (Zetaphoremeter IV, Zetacom pact 28000, CAD Instrumentation, France) with the application of Smoluckowski equation. Surface charge of the fresh sludge EPS was also measured using zetaphoremeter. The total carbohydrates (TC) content of extracted EPS was determined by the phenol-sulfuric acid method (Dubois et al., 1956). The total protein (TP) content of the extracted EPS was investigated by the Bradford (1976) method with bovine serum albumin as standard.

**RESULTS AND DISCUSSION**

**Isolation and identification of Bacterial Strains**

Six bacterial strains were isolated from the municipal wastewater sludge. These microorganisms were selected based on their slimy colony formation on the growth medium and string forming ability by touching with inoculating loop (Bala et al., 2006; 2007a). All these 6 bacterial strains (named as B1 to B6) were screened to study their potential in EPS production and were identified based on 16S rDNA sequencing (Table-1).

**Sludge Settling**

SVI studies involving the EPS in supernatant (slime), bacterial pellet (capsular EPS) and bacterial broth (slime as well as capsular EPS and bacteria) with and without addition of Ca++ aimed to investigate the effectiveness of different forms of EPS on bacterial mediated co-aggregation of the sludge. The results showed that at different EPS concentration, their sludge settling efficiency or bioflocculating activity decreased in the following order: bacterial supernatant EPS$>$bacterial pellet$>$ bacterial broth with calcium ions$>$ bacterial broth without calcium. EPS in supernatant showed the best sludge settling efficiency compared to those in bacterial pellet and bacterial broth. The possible reason is that supernatant EPS active sites were fully available to form bioflocs with sludge solids.

Another important point was that addition of Ca++ to the sludge in conjunction with bacterial broth increased the sludge settling performance. This phenomenon is probably due to the reduction of negative surface charge...
of the sludge particles or compression of electrical double layers surrounding the sludge particles by Ca++, which can reduce the repulsive force hindering the agglomeration between the sludge particles and the EPS. In addition, divalent cations such as Ca++ possessing two positively charged groups on their structure can act as a bridge to link two negatively charged molecules, and this may be either in between the anionic charged EPS and EPS or in between anionic charged EPS and sludge solids and/or microorganisms. At certain extent, Ca++ forms a three-dimensional bioflocs by combining with EPS (externally added and/or naturally existing in sludge), microorganisms and sludge solids, thereby resulting in enhancing sludge settling.

With regard to the EPS in supernatant (slime), at 4% (V/V) of biopolymer concentration, B1 & B2 showed relatively good results in sludge settling compared to other bacterial strains (B3 to B6). Similar phenomenon can generally be observed at different biopolymer concentrations (1 to 3% v/v). This result implies that B1 & B2 strains may be able to provide comparatively long polymeric chain than the others so that its EPS may possess more active sites to bind with the sludge particles, microorganisms and bridging agents (cations) (Flemming and Wingender, 2001; Korstgens et al., 2001; Higgins and Novak, 1997a, b).

Urbain et al. (1993) described that, though activated sludge possess significant quantity of natural EPS, the optimal level of EPS is about 40 mg/l, lower or higher EPS concentration is detrimental to sludge settling and dewatering. However, in this study we observed that even addition of different quantity of slime EPS produced by B1 and B2 strains, did not show any detrimental effect on SVI (Bala et al., 2006). The reason could be due to the fact that EPS used were produced from pure culture strains, they may possess different chemical composition and zeta potential value (charge) compared with the EPS produced by natural microbial community in sludge. Furthermore, the optimal and detrimental concentration of EPS produced by pure culture strains can be measured by performing SVI and capillary suction time (CST) at varying concentrations of EPS. This may provide the optimum value of EPS required for higher sludge settling.

EPS concentration, EPS charge and EPS hydrophobicity, are three important factors that govern the bioflocculation. Some authors reported that sludge requires an optimal EPS concentration (Urbain et al., 1993) whereas others reported that sludge requires high EPS concentration (Mikkelsen and Keiding, 2002) for better sludge settling. The EPS charge reduction is not directly related to floc formation, the floc formation could be due to polymer entanglement (Eriksson et al., 1992). The EPS hydrophobicity is good for floc formation but not for sludge settling. In fact, the sludge settling and its relation to EPS properties are frequently studied but remain debatable, and most often the results are controversial. However, all these studies were carried out on EPS extracted from the fresh sludge.

**EPS Characteristics**

**EPS concentration and charge**

Slime EPS concentration produced by pure bacterial strains varied from 2.1 to 3.2 g/l (Figure 1). This variation was due to the fact that the individual bacterial strains have different metabolic activity. Slime EPS extracted from consortium which contain all microbial strains (contained in sludge) showed 2.4 (g/l) and EPS extracted directly from sludge showed concentration of 2 (g/l) (Fig. 1). Isolated pure bacterial strains produced maximum of 3.2 g/l but when they grew in consortium or presence in sludge they failed to produce maximum quantity of EPS.
Figure -1. Extracted slime EPS concentration from pure bacterial strains (B1 to B6), consortium and sludge. Zeta potential study of extracted slime EPS revealed that microbial polymers were anionic in nature. EPS produced by pure bacterial strains showed higher negative charge than that of microbial consortium and sludge EPS (Fig. 2). Though the EPS from pure bacterial strains showed higher negative charge but they had better ability for sludge settling than sludge EPS. And also the concentration of extracted EPS is not directly related to the EPS charge. The results from this study agreed with the statement which reported by Mikkelsen and Keiding (2002), the zeta potential value is not directly related to sludge settling. EPS produced by pure bacterial strains were different in terms of EPS concentration and charge (Fig. 2) than the EPS obtained from consortium and fresh sludge. Hence pure bacterial strains showed better sludge settling than those with consortium and natural sludge EPS.

Based on earlier SVI study results (Bala et al., 2006) we found that bacterial slime EPS showed better settling than the slime EPS produced by the bacterial consortium and from the extracted sludge EPS (data not shown). We observed that slime EPS produced by pure bacterial strains were effective in sludge settling than microbial consortium.

Figure-2. Zeta potential value of extracted EPS from pure bacterial strains (B1 to B6), consortium and sludge.
**EPS bio-chemical characteristics**

The total carbohydrates (TC) concentration in EPS is higher than total protein (TP) concentration in all cases except the bacterial consortium (Fig. 3). Thus, it is obvious that carbohydrates fraction should play a dominant role than proteins fraction in sludge settling. As reported in literature, polysaccharides were suggested to play a major role in flocculation due to their capacity to form bridges between their negatively charged groups and divalent cations available in sludge (Flemming and Wingender, 2001; Korstgens et al., 2001; Higgins and Novak, 1997a, b). However, in many studies, proteins were found to be the main component for sludge floc formation (Liao et al., 2001; Fang and Jia, 1996; Urbain et al., 1993). The protein contribution to flocs binding strength is explained by hydrophobic interactions and polyvalent cation bridging, both enhancing the stability of the biopolymer network (Jorand et al., 1998).

After calculating the percentage of TC and TP, we found that EPS produced by pure culture strains mainly consist of other components such as lipids, DNA and RNA (Table 1). In sludge EPS the major portion could be lipids, mineral phase, organic acids, DNA, RNA and others (undefined compounds). This conclusion was drawn based on literature (Garnier et al., 2005), all biological compounds mainly comprise of carbohydrates, proteins and lipids. After determining the percentage of TC and TP from the original dry weight of the extracted EPS, the remaining subtracted value was considered as other components. Calculated TC/TP ratio showed pure bacterial strains possess maximum TC/TP ratio than the consortium and sludge EPS (Table-1). But in this study we found that carbohydrates and other (lipids, DNA, RNA) components found to play a major role in sludge settling. This showed that bacterial strains isolated from municipal wastewater sludge produced more quantity of carbohydrates and other components. Further, role of these compounds in floc formation and sludge settling needs to be studied.

For the sake of understanding and achieving the bioflocculation in future, the characterized individual EPS and their major components could be purified in terms of carbohydrates, proteins and lipids. Then purified components shall be studied for sludge settling and then we could understand the reason why individual pure bacterial strains showed better results than consortium and sludge EPS. Also molecular characterization of extracted biopolymers in terms of their molecular weight, chemical nature and active groups needs to be undertaken. This may give a new path to attain and improve the bioflocculation using the purified polymers.

![Figure-3](image-url)  
**Figure-3.** Extracted slime EPS total carbohydrates (TC) and total protein (TP) concentration from pure bacterial strains (B1 to B6), consortium and sludge.
Table 1. Extracted slime EPS concentration, TC (%), TP (%) and TC/TP concentration from pure bacterial strains (B1 to B6 and their identification results), consortium and sludge.

<table>
<thead>
<tr>
<th>EPS source</th>
<th>TC/TP ratio</th>
<th>TC (%)</th>
<th>TP (%)</th>
<th>Other components (%)</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>7.740</td>
<td>5.739</td>
<td>0.741</td>
<td>93.520</td>
<td>Pseudomonas sps</td>
</tr>
<tr>
<td>B2</td>
<td>1.890</td>
<td>5.267</td>
<td>2.787</td>
<td>91.947</td>
<td>Pseudomonas fluorescens</td>
</tr>
<tr>
<td>B3</td>
<td>3.380</td>
<td>7.000</td>
<td>2.071</td>
<td>90.929</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>B4</td>
<td>1.468</td>
<td>6.000</td>
<td>4.086</td>
<td>89.914</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>B5</td>
<td>3.520</td>
<td>4.476</td>
<td>1.271</td>
<td>94.252</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>B6</td>
<td>2.255</td>
<td>4.438</td>
<td>1.967</td>
<td>93.595</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>consortium</td>
<td>0.802</td>
<td>4.625</td>
<td>5.765</td>
<td>89.610</td>
<td>-</td>
</tr>
<tr>
<td>Sludge EPS</td>
<td>1.601</td>
<td>11.400</td>
<td>7.120</td>
<td>81.480</td>
<td>-</td>
</tr>
</tbody>
</table>

CONCLUSIONS

The EPS produced by six isolated strains and activated sludge were characterized in terms of proteins and carbohydrate ratio and zeta potential. The effect of sludge EPS as well as those from pure isolated bacterial strains was studied on sludge settling characteristics (sludge volume index). Extracted EPS contains major portions of other components (lipids, DNA, RNA) than total carbohydrates and total proteins. EPS concentration and charge are not directly related to the sludge settling. Better sludge settling characteristics were observed with individual EPS than the EPS extracted from activated sludge. Zeta potential characterizations of EPS revealed that all individual EPS produced by pure culture strains were anionic in nature but their charge varied to a large extent.

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