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Biochemical diversity of the bacterial strains and their biopolymer producing capabilities in wastewater sludge

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HIGHLIGHTS

- Phenotype study of sludge isolates and flocculation activity of EPS from sludge.
- *Bacillus*, *Serratia* and *Yersinia* spp. had varied carbon source utilization spectrum.
- Sludge was first time used as a medium for EPS production.
- EPS from these strains exhibited distinct flocculation behavior.

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ABSTRACT

The biochemical characterization of 13 extracellular polymeric substances (EPS) producing bacterial strains were carried out by BIOLOG. The bacterial strains were cultured in sterilized sludge for EPS production. Flocculation and dewatering capabilities of produced EPS (broth, crude slime and capsular) were examined using kaolin suspension combined with calcium (150 mg of Ca^{2+}/L of kaolin suspension). BIOLOG revealed that there were 9 *Bacillus*, 2 *Serratia* and 2 *Yersinia* species. Most of these bacterial strains had the capability to utilize wide spectrum of carbon and nitrogen sources. EPS concentration of more than 1 g/L was produced by most of the bacterial strains. Concentration of EPS produced by different *Bacillus* strains was higher than that of *Serratia* and *Yersinia*. Broth EPS revealed flocculation activity more than 75% for *Bacillus* sp.7, *Bacillus* sp.4 and *Bacillus* sp.6, respectively. Flocculation activity higher than 75% was attained using very low concentrations of broth EPS (1.12–2.70 mg EPS/g SS).

1. Introduction

Chemical flocculants commonly used in wastewater sludge treatment are inorganic such as alum, ferric chloride, lime, and polyaluminum chloride (PAC), and organic synthetic polymers such as polyacrylamide (PAM) and polyethylene amine (Salehizadeh and Shojaosadati, 2001, 2002). There are environmental and health concerns about neurotoxicity and less degradability of organic synthetic polymer flocculants and also, carcinogenic nature of their degraded monomers such as acrylamide (Subramanian et al., 2010; Zhang et al., 2010). Therefore, environmentally safe, biodegradable and sustainable bioflocculants such as chitosan, sodium alginate and microbial flocculants (e.g. extracellular polymeric substances (EPS), filamentous fungi (FF)) have been studied for their potential applications in wastewater sludge flocculation to replace chemical polymers completely or partially (Salehizadeh and Shojaosadati, 2001; More et al., 2010; Subramanian et al., 2010; Zhang et al., 2010).

EPS are the biopolymers secreted by microorganisms outside or on the cell wall, which are the structural and functional integrity of microbial aggregates such as biofilms, flocs and sludge (Wingender et al., 1999). Polysaccharides and proteins are the major compo-

Abbreviations: B-EPS, broth EPS; BIOLOG, BIOLOG bacterial identification system; Ca-Kaolin-EPS, Calcium (Ca^{2+}), kaolin suspension in water and EPS; C-EPS, capsular EPS; CFU, colony forming unit; CST, capillary suction time; EPS, extracellular polymeric substances; GN/GP, gram's negative/gram's positive; INRS-ETE, Institut national de la recherche scientifique, Centre Eau, Terre et Environnement; Kaolin control, Kaolin suspension in deionized water; NTU, Nephelometric Turbidity Units; P (AM-DMC), poly(acrylamide[2-methacryloyloxy)ethyl] trimethylammonium chloride; PAC, polyaluminium chloride; PAM, polyacrylamide; rpm, revolutions per minute; S-EPS, slime EPS; sp., one species; spp., two or more species; TS, total solids; TSA, tryptic soy agar; TSS, total suspended solids; VS, volatile solids; VSS, volatile suspended solids.

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nents of EPS, whereas lipids, nucleic acids and other biopolymers as minor components (Higgins and Novak, 1997a,b; Wingender et al., 1999). Viscous matrixes of EPS play an important role in the sludge flocculation because of their adsorption capability, biodegradability, hydrophobicity/hydrophilicity and the presence of charged functional groups (Urbain et al., 1993; Higgins and Novak, 1997a). EPS occur in the sludge naturally but sludge seldom settles as its own and hence it requires addition of flocculating agents (Flemming and Wingender, 2001; Houghton et al., 2001; Tian, 2008). The concentration and type of EPS present in the sludge are considered as the important parameters for sludge settling and dewatering (Houghton et al., 2001). Recent studies have reported that microbial flocculants/polymers i.e. EPS produced from various microorganisms were applied as a flocculant material which could be used alone, or in combination with multivalent cations, or in combination with conventional polymers to improve the sludge settling and dewatering characteristics (Subramanian et al., 2010; Zhang et al., 2010). Microbial flocculant, TJ-F1 has been reported to improve the sludge dewaterability when used alone as well as in combination with conventional poly(acrylamide((2-methacryloyloxy)ethyl) trimethylammonium chloride) (P(AM-DMC)) and $CaCl_2$ (Zhang et al., 2010). The previous study conducted in our lab (Subramanian et al., 2010) revealed that bacterial strains isolated from wastewater sludge were capable of producing viscous EPS in synthetic mineral medium having high flocculating ability.

The flocculating activity and production cost of bioflocculants are the two major limiting factors with regard to their applications. The economical and efficient production of the biopolymers can be achieved by cheaper production medium such as wastewater sludge. Wastewater sludge is cheap and readily available source of carbon, nitrogen and other nutrients. Microorganisms can utilize carbon, nitrogen, phosphorus and micronutrients available in the municipal and industrial wastewater sludge (Drouin et al., 2008). As per our knowledge, there is no study reported so far where wastewater sludge has been used as a raw material for EPS production by microorganisms. The EPS synthesis by microbial cells depends upon availability of the carbon and nitrogen in the culture medium and environmental conditions. The organisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH, which are the critical factors for maximum EPS production (Wingender et al., 1999). To understand the EPS producing capabilities of pure microbial cultures in wastewater sludge, it is essential to have knowledge of their metabolic, biochemical properties, and susceptibility to certain chemicals and antimicrobial agents. In this context, BIOLOG plate technique is a rapid and convenient tool for studying and comparing physiological profiles of the bacterial strains. Analysis of physiological profiles allows estimation of similarity between bacterial strains. BIOLOG systems investigate the metabolic potential of the bacterial strains which are capable of metabolically active and growing in the certain conditions (Konopka et al., 1998). Therefore, the objective of this work was to study metabolic and biochemical properties of each individual strains isolated from sludge, their EPS producing capabilities using sludge as raw material and to determine flocculation activity of the EPS.

2. Method

2.1. Identification and biochemical characterization

Thirteen EPS producing bacterial strains isolated from the wastewater sludge and identified by 16S rDNA sequence were used in this study (Subramanian et al., 2010). BIOLOG system (BIOLOG Inc., Hayward, USA) was used for the identification and the biochemical characterization of bacterial strains. Identified strains were grown on the tryptic soy agar plates and stored at 4 °C and sub-cultured fortnightly.

BIOLOG system was used to investigate biochemical diversity or metabolic potential of the bacterial strains which is based on their ability to oxidize different carbon and nitrogen sources (Konopka et al., 1998). All the steps were performed according to the manufacturer's instructions. BIOLOG plates were inoculated with 100 μL of the bacterial suspensions and incubated at 25 °C for 24 h, respectively to allow the utilization of carbon and nitrogen sources. Utilization reactions are indicated when a purple colour forms in the wells and no reactions remain colourless. Growth of the bacterial strains in different pH, carbon sources, amino acids/proteins, carboxylic acids, esters and fatty acids and presence of antibiotic, reducing power, Grams staining characteristics were studied (Becker et al., 2009). BIOLOG system enables to understand what stimulates or inhibits growth of the strains.

2.2. Sludge as a growth medium

Secondary wastewater sludge (without chemical polymers) was collected from biofiltration unit (biolite filter media) of Communauté Urbaine du Québec (Municipal wastewater sludge treatment plant, CUQ, Québec, Canada). The sludge was first settled by gravity for 1 h and the concentrated sludge was collected by discarding the supernatant. Characteristics of the sludge, such as pH, total suspended solids (TSS), and volatile suspended solids (VSS), total carbon, total nitrogen, and total phosphorous were determined using Standard Methods (APHA, 2005). The sludge used was at pH 6.5, TSS of 10 g/L and VSS of 7.4 g/L. Viscosity was measured by viscometer (DV-II + PRO, Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA), at constant higher shear rate of 30 s^{-1} and room temperature. Zeta potential (ζ) was measured using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France). The sludge was stored at 4 °C for further use.

2.3. EPS production and harvesting

Sludge sterilization was done by autoclaving at 121 °C for 15 min. After cooling to ambient temperature, pH of sterilized sludge was adjusted to 7. All the bacterial strains were grown in sterilized sludge by incubating at 250 rpm, 25 °C for 24 h and used as inoculum. Standard plate count method was used to study growth of the bacterial strain by colony forming unit (CFU) on the TSA plates and was reported as CFU/mL. Inoculum (3×10^6 CFU) was used to inoculate in 100 mL of sterilized sludge in 500 mL Erlenmeyer flasks and then incubated at 250 rpm, 25 °C for 72 h, respectively. EPS produced were measured at the end of the cultivation. After incubation (72 h), broth samples were centrifuged at 6000*g* for 15 min at 4 °C to obtain supernatant (containing slime EPS; termed as crude S-EPS) and pellets (containing capsular EPS (C-EPS) with bacterial cells along with residual sludge material and termed as crude C-EPS) (Subramanian et al., 2010; Wingender et al., 1999).

S-EPS in the supernatant was precipitated with 2.2 volume of absolute chilled ethanol by incubating the mixture at −20 °C for 1 h. The precipitates (containing pure S-EPS) were collected by centrifugation at 6000*g* for 15 min at 4 °C. Precipitates of S-EPS were dried at room temperature in a laminar hood for 6 h and dry weight of the precipitates was measured and denoted as S-EPS (APHA, 2005). To determine C-EPS, the pellet (crude C-EPS) was re-suspended in deionized water. The re-suspended pellet was first heated at 60 °C in water bath for 30 min to release C-EPS followed by centrifugation at 6000*g* for 15 min at 4 °C (Wingender et al., 1999; Li and Yang, 2007). The supernatant (containing C-EPS) was used to precipitate C-EPS using same procedure as