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Research Article

Characterization and Molecular Identification of Extracellular Polymeric Substance (EPS) Producing Bacteria from Activated Sludge

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Abstract

The aim of this study was identification and characterization of highly efficient Extracellular Polymeric Substance (EPS) producing bacteria in activated sludge. Among 74 isolated bacteria, 20 EPS-producing bacteria were obtained from wastewater treatment plant of Bojnourd. EPS extraction was performed using absolute ethanol after glucose-enriched culture. Dry weight, concentration, and the ratio of carbohydrate to protein for the EPS of each isolate was then determined. Molecular identification of four bacteria with the highest EPS production was carried out using 16S rRNA gene amplification. The results showed that these bacteria are belonging to the genus *Bacillus*, *Pseudomonas* and *Klebsiella*. EPS productions were studied in different conditions (carbon and nitrogen sources, different levels of glucose and yeast extract and temperature) for the genus *Bacillus* and *Pseudomonas*.The production of EPS was observed highest while in the presence of 2.5 to 3% glucose and 0.5% yeast extract at temperature of 30 to 37°C.

***Keywords*:** EPS-producing bacteria, Sludge, 16S rRNA gene

Introduction

Many bacteria are able to excrete extracellular polymeric substances (EPS) outside of their cell walls (Vu et al., 2009). EPS exists in two forms, either attached to the cell (capsule) or as extracellular secretion (slime) (Hirst et al., 2003). EPS produced by microorganisms have attracted the attention of many researchers due to their versatile applications and various advantages. They play an important role in biotechnology such as textiles, pharmaceuticals, food, oil recovery and wastewater treatment processes (Maugeri et al., 2002; Zhang et al., 2003; Parikh et al., 2006; Yuksekdag et al., 2008; Patel et al., 2010). Nichols et al., (2005) reported that the extreme environmental conditions affect the bacterial behavior and consequently the production of EPS. It seems that EPS protect bacteria from environmental stresses. Studies have shown that some environmental conditions such as pH, temperature (Pengfu et al., 2001; Singh et al., 2011) and nutrient source (Mata et al., 2006; Pawar et al., 2013) can influence the EPSs production. EPSs are composed of carbohydrates, proteins, lipids, nucleic acids, glycoproteins and phospholipids (Lazarova et al., 1995; Czaczyk and Myszka, 2007). In recent years, EPS producing bacteria were isolated and identified from different habitats such as saline water (Llamas et al., 2010; Mata et al., 2006), soil (Pawar et al., 2013; Razack et al., 2013), food (Gamar- Nourani et al., 1998) and petroleum contaminated soil (crude petroleum oil) (Zaki et al., 2011). Identification of these bacteria using culture-dependent and molecular-based techniques indicates that they belong to different taxonomic groups (Vu et al., 2009). Identification of EPS producing bacteria in activated sludge helps not only to have better insight into the physico-chemical properties of sludge, but allows predicting its related processes such as sludge settling and dewatering. A fail flocculation of bacteria may occur due to insufficient EPS in activated sludge composition. EPS constituents are different depending on the type of microorganisms, microbial biofilms age and conditions of EPS-formation. These conditions include the amount of oxygen, nitrogen content, cell density, and environmental parameters (Vu et al., 2009).

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Activated sludge is a complex consortia of microorganisms formed in aeration conditions and responsible to remove the organic material from wastewater. In general, bacterial extracellular polymeric substances improve the formation of bioflocs in activated sludge and contribute to its structural, surface charge and settling properties (Houghton et al., 2001). Therefore, EPS produced by microorganisms affect sludge characteristics and consequently the efficiency of flocculation processes in wastewater treatment plants (More et al., 2014). Sheng et al., (2010) described that EPS secreted by bacteria play an important role in sludge bioflocculation because all active sites of EPS are free and able to form floc with biomass. In this study, the objectives were: (i) characterization and identification of EPS producing bacterial strains from municipal wastewater sludge and (ii) evaluation of different conditions in EPS production.

Materials and Methods

Site Sampling

An initial sample was taken from activated sludge in municipal wastewater treatment plant of Bojnourd. 50 ml of activated sludge was added in to 450 ml TSB (tryptic soy broth) medium, then incubated at 30ºC for 30 min at 150 rpm. After serial dilution, 100 µl of each dilution were cultured on TSB containing agar. In order to have maximum isolation, the culture plates were incubated at 22°C and 30°C for 24-48 h. The EPS-producing bacteria were selected based on their viscous and mucoid characteristics that enable colonies to have a string formation. The seed culture was produced in 20 ml TSB medium. 200 µl of standard bacterial suspension (1.5×10^8 CFU/ml) was added to the culture medium and incubated at 150 rpm and 30°C for 48 h. After getting a logarithmic growth phase, the isolates were inoculated in glucose medium (25 g/l glucose, 0.2 g/l $MgSO_4$-$7H_2O$, 2 g/l K_2HPO_4, 1 g/l KH_2PO_4, 1 g/l NH_4Cl and 0.05 g/l yeast extract). The initial pH of media was adjusted to 7.0. Glucose and $MgSO_4$ were sterilized separately and mixed aseptically with other ingredients before inoculation. In order to achieve sufficient viscosity, all samples were incubated in a shaker at 150 rpm and 30°C for 72 h (Subramanian et al., 2010).

EPS extraction

The glucose medium at the end of 72 h, became highly viscous. In order to extract the EPS, the culture medium was centrifuged at the speed of 6000 *g* at 4°C for 10 min to remove bacterial cells. The supernatant phase mixed with 2.2 volumes of absolute ethanol and then incubated at -20°C for 2 h. To collect the precipitated EPS, the mixture was centrifuged at 6000 *g* and 4°C for 15 min. The supernatant was removed and the pellet containing slime EPS was dried at room temperature in a laminar hood for 6 h (Subramanian et al., 2010). The dry weight of the extracted EPS was measured (APHA, 2005).

EPS Analysis

The total amount of protein and carbohydrate in EPS was quantified using the Bradford method (Bradford, 1976) and phenol-sulfuric acid method (Dubois et al., 1956) respectively. A standard curve of bovine serum albumin (BSA) was applied to calculate the amount of protein in each sample.

Bacterial Growth

EPS are referred to secondary metabolite produced in the late logarithmic phase or at the early stationary phase of growth. To evaluate the EPS production rate, 20 ml TSB medium was prepared in a 100 ml flask. Approximately 1.5×10^8 CFU/ml was added into the medium. The growth rate of bacteria was determined by the measurement of optical density of bacterial suspensions at 600 nm using spectrophotometer.

Effect of Different Carbon and Nitrogen Sources

To determine the best source of carbon for the production of EPS, various carbon sources such as glucose, fructose, sucrose, lactose and non-carbon sources were investigated by adding 1% (w/v) of each one to the production medium. 10% of each mentioned carbon sources were added to the culture medium. After inoculation, the samples were incubated at 30°C and 150 rpm for 24 h. After sufficient time, the EPS of desired strains were extracted. To determine the effect of glucose level in EPS production, glucose was used at 1, 1.5, 2, 2.5 and 3% (w/v) concentrations. Then, the effect of nitrogen sources (peptone, urea, ammonium nitrate, yeast extract and nitrogen-free medium) was evaluated by adding 0.1% (w/v) of each one to the production medium. To examine the effect of yeast extract level as the best nitrogen source, yeast extract was used at 0.005, 0.1, 0.3, 0.5, 0.7 and 0.9 % (w/v) concentration. To evaluate the effect of temperature, flasks containing inoculated production media were incubated at 20, 30, and 37°C. Culture conditions and extraction is similar to the previous step.

Bioflocculation Activity

Flocculation activities influenced by different extracted EPS, were calculated and measured using a modified method (Yun and Park 2003). A mixture of 5 g/l powdered activated carbon suspension with 0.1 ml extracted EPS in the presence of $CaCl_2$ (100 mg/l, as coagulant) was stirred with rapid mixing at 230 rpm for 2 min. A suspension without any bioflocculant addition, was used as a control under similar conditions. The suspension was then allowed to flocculate for 10 min and the supernatant of settled activated carbon suspension was withdrawn to measure the absorbance using UV spectrophotometer at 550 nm. The flocculation activity was calculated according to the following equation:

$$Flocculation\ activity\ (\%) = \left[\frac{A-B}{A}\right] \times 100$$

Where A and B are the supernatant optical density of the control and sample respectively, at 550 nm.

Biochemical and Molecular Identification of Bacteria

In order to identify the selected strains, gram staining, capsule staining, motility test, catalase activity, citrate utilization, H_2S production, indole production, and sugar fermentation (glucose, lactose, and mannitol) were performed. DNA genome analysis was conducted using the FastDNA® SPIN Kit. Genomic DNA of selected bacteria with high EPS production was extracted from fresh culture according to the manufacturer's instructions. The amplification of 16S rRNA gene was performed using universal primers (27F and 1492R). PCR was performed using 1.5 mM $MgCl_2$, 30 mM KCl, 10 mM Tris-HCl, 2.5 mM of each dNTP, 5–10 pmol of each primer, and 1U of *Taq* polymerase. PCR was carried out as follows: initial denaturation at 95°C for 2 min, 30 denaturation cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and final elongation at 72°C for 7 min. The sequencing was carried out at Macrogen, South Korea.

Phylogenetic Analysis and Comparison of Sequences

The partial 16S rRNA sequences of the isolates were compared with the NCBI and Ez-taxon databases. The GenBank accessions of the sequences are KR185341 to KR185344. Multiple sequence alignments were performed using online tools SINA (http://www.arb-silva.de/aligner/). The phylogenetic dendrograms were constructed by the Maximum likelihood method, and phylogenetic tree was evaluated by performing bootstrap analysis of 1000 data sets using MEGA (Tamura et al., 2011).

Statistical analysis

One-way analysis of variance and Tukey HSD test were done by Tukey's test with the R software (The R Development Core Team, 2007).

Results and Discusion

Screening and Isolation of EPS Producing Bacteria

A number of EPS producing bacteria have been isolated and characterized in last few years and protocols have been presented in literature (Subramanian et al., 2010; Zaki et al., 2011; Pawar et al., 2013). In this study, we aimed to isolate EPS producing bacteria from the slimy colony formation on the growth medium. Out of total seventy four isolated strains from mucoid colonies, 42 (56%) were gram-positive and the remaining 32 (44%) were gram-negative having morphology ratio as 45 rods and 29 cocci shaped strains. Among these 74 isolates, 20 strains having the highest appearance of mucoid colonies were selected and the EPS of each isolate extracted using ethanol. The EPS dry weight and EPS concentration varies from 0.06 to 0.46 g and 2.2 to 15.7 g/l, respectively (Table 1). The EPS concentration from bacterial strains of municipal wastewater sludge was reported as 4-35 g/l by Subramanian et al., in 2010. Right after, Razack et al., (2013) also showed that EPS concentration from *Bacillus subtilis* varied from 3.5 to 5.5 g/l. It was also discussed that variation in EPS concentration depends upon the environmental parameters and extraction method (Metzger et al., 2009; Orr et al., 2009). Isolate BS2 in gram-positive bacteria and BS8, BS9 and BS20 in gram-negative bacteria produce the highest EPS concentration, and have selected for future studies.

Chemical Composition of EPS

Total carbohydrate and total protein was extracted and evaluated from EPS using phenol-sulfuric acid and Bradford method (Table 1). The carbohydrate/protein (TC/TP) ratio measured in all extracted EPS samples varied from 1.42 to 15. This result is similar to the previous investigations which exhibit that carbohydrate was usually dominated in EPS composition. Subramanian et al., (2010) and Zaki et al., (2011) showed that this ratio vary between 0.8 to 7.74 and 2.3 to 5.7, respectively.

Identification of EPS Producing Bacteria

In this work, four isolates were selected for further analysis and biochemical tests (Table 2).

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Table 1. Partial characterization of extracted EPS from twenty bacterial strains.

	Morphology	Gram reaction	Dry weight	EPS concentration	TC (%)	TP (%)	TC/TP ratio	Other components
BS1	rods	Positive	0.15 ± 0.01	4.83 ± 0.02	4.76	1.86	2.56	93.38
BS2	rods	Positive	0.26 ± 0.09	15.79 ± 0.04	3.04	1.11	2.75	95.86
BS3	rods	Negative	0.21 ± 0.09	7 ± 0.03	6.71	2.14	3.13	91.14
BS4	coccus	Negative	0.06 ± 0.03	4.82 ± 0.06	9	2.37	3.8	88.63
BS5	rods	Negative	0.18 ± 0.04	6.11 ± 0.03	6.05	1.64	3.7	92.31
BS6	rods	Positive	0.29 ± 0.01	9.5 ± 0.05	5.89	0.84	7.01	93.26
BS7	rods	Negative	0.29 ± 0.01	9.89 ± 0.04	4.55	0.51	8.9	94.94
BS8	rods	Negative	0.46 ± 0.08	15.33 ± 0.07	3.72	0.26	14.25	96.02
BS9	rods	Negative	0.44 ± 0.04	14.67 ± 0.08	3.89	0.55	7.13	95.57
BS10	coccus	Positive	0.3 ± 0.03	9.78 ± 0.03	6.14	0.41	15	93.45
BS11	rods	Negative	0.06 ± 0.01	2.22 ± 0.02	18.45	3.6	5.13	77.95
BS12	rods	Positive	0.3 ± 0.04	10.67 ± 0.01	6.94	0.84	8.22	92.22
BS13	rods	Negative	0.3 ± 0.05	10.01 ± 0.01	4	1.01	3.97	95.02
BS14	rods	Positive	0.2 ± 0.08	7.01 ± 0.02	5.14	0.57	9.01	94.29
BS15	rods	Positive	0.26 ± 0.07	8.5 ± 0.02	3.88	1.29	3	94.82
BS16	rods	Negative	0.12 ± 0.05	3.89 ± 0.01	7.97	2.31	3.4	89.71
BS17	rods	Negative	0.07 ± 0.04	2.22 ± 0.02	18.9	3.6	5.25	77.5
BS18	rods	Negative	0.24 ± 0.01	7.83 ± 0.01	5.36	1.79	2.99	92.85
BS19	rods	Positive	0.14 ± 0.04	4.67 ± 0.02	7.07	3	2.36	89.93
BS20	rods	Negative	0.34 ± 0.06	11.17 ± 0.01	1.52	1.07	1.42	97.4

Table 2. The biochemical characteristics of EPS producing bacteria isolated from activated sludge at wastewater treatment plant of Bojnourd

	BS2 (*Bacillus muralis*)	BS8 (*Pseudomonas fragi*)	BS9 (*Klebsiella variicola*)	BS20 (*Pseudomonas hunanensis*)
Bacterial morphology	rod	rod	rod	rod
Gram reaction	Positive	Negative	Negative	Negative
Indole production	+	+	+	+
H_2S production	-	-	-	-
MR	-	+	+	+
VP	+	-	-	-
Citrate utilization	-	+	+	+
Sugar fermentation				
Glucose	+	+	+	+
Lactose	+	-	+	+
Mannitol	-	-	-	-
Sucrose	+	-	-	+
DNase	-	+	+	+

These isolates were subjected to 16S rRNA gene identification. The sequencing results obtained showed that these isolates are belonging to two main groups: Gamma-proteobacteria and Firmicutes (Figure 1).

The results of the analysis revealed that BS2, BS8-BS20 and BS9 were characteristic of *Bacillus*, *Pseudomonas* and *Klebsiella*, respectively.

BS2 isolate showed a 98.65% resemblance with *Bacillus muralis* (AJ628748). BS8 showed a 97.92% similarity to *Pseudomonas fragi* (AF094733). The most similarity (99.21%) was found between BS9 and *Klebsiella variicola* (AJ783916).

BS20 showed a 97.67% similarity to *Pseudomonas hunanensis* (JX545210). These bacteria from activated sludge were found capable of producing EPS (Subramanian et al.,, 2010). The EPS producing bacteria have been reported by taxonomically diverse bacteria (Sutherland 2001). The bacteria from the orders and families *Lactobacillales*, *Leuconostocaceae* and *Streptococcaceae* (*Firmicutes*) and *Burkholderiales*, *Pseudomonadales* and *Xanthomonadales* (*Proteobacteria*) are producers of polysaccharides that are employed in a range of different commercial applications (Rehm, 2009; Naessens et al., 2005).

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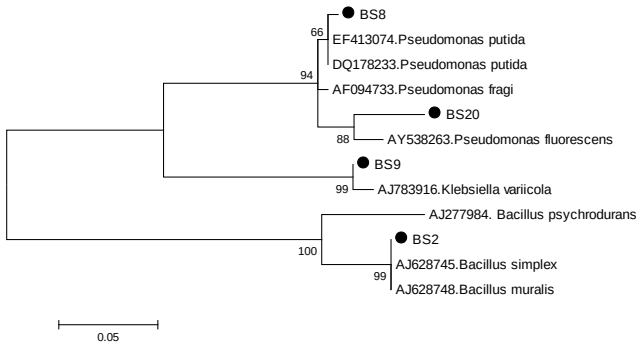

Figure 1: The phylogenetic tree of isolated strains. Clustering was performed using the maximum likelihood method with 1,000 bootstraps.

Effects of Various Parameters on EPS Production

The strains of BS2 (*Bacillus*) and BS20 (*Pseudomonas*) grew in various carbon source. The amount of EPS produced varied while in the presence of different nutrients. The maximum EPS production occured in the presence of glucose as carbon source (Figure 2). These results are similar to the investigation carried out by Mata et al (2006) and Petry et al (2000), in contrast to the results obtained by Pawar et al., 2013. The effect of glucose percentage on the EPS production is shown in Figure 2. The results indicate that the optimum production of EPS for both strains was at 2.5% and 3% glucose. Investigation of various nitrogen sources showed that the maximum EPS production occured in a medium containing yeast extract. A maximum of EPS was also observed when concentration of 0.5% yeast extract was used as nitrogen source. The results suggest that EPS production in the presence of yeast extract increases in comparison with other sources of nitrogen. Wang et al., (2006) have shown that yeast extract was the most effective nitrogen source for the production of bacterial EPS, which might be due to the presence of higher levels of free amino acids, short peptides and growth factors in the yeast extract. The optimum EPS production for two isolates was detected at 30 and 37 °C in mesophilic temperature (Figure 2). The numerous studies have shown that environmental conditions such as pH, temperature and oxygen concentration and nutritional factors such as carbon and nitrogen sources (Cerning, 1990; Looijesteijn, 1999; Vaningelgem et al., 2004) are important factors in the synthesis of extracellular polymeric substances (Conti et al., 1999; Duta et al., 2006).

The results of bioflocculation activity test for four strains is shown in Figure 3. As it can be seen, the most activity for flocculation is belonging to the BS8 and BS9. This behaviour may be related to the chemical composition of these strains, where the TC/TP ratio is higher comparing to two other strains. In fact, carbohydrates play an important role in flocculation of activated sludge, Due to their ability to form bridges between negatively charged groups and divalent cations in the sludge (Higgins and Novak, 1997).

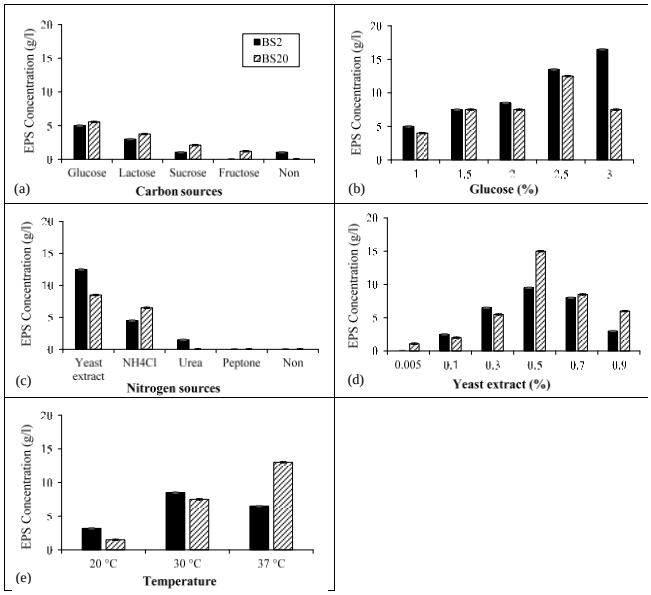

Figure 2. The effect of different variables on EPS production of BS2 (*Bacillus* sp.) and BS20 (*Pseudomonas* sp.).

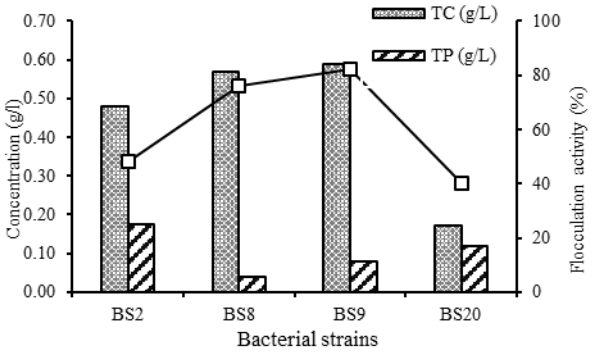

Figure 3. Bioflocculation activity of strains BS2, BS8, BS9 and BS20 in culture medium.

Conclusion

Among 20 species of EPS producing bacteria, four strains were found to produce the highest EPS. Molecular identification showed these bacteria belong to the genus *Bacillus*, *Pseudomonas* and *Klebsiella*. The EPS dry weight and EPS concentration for all bacteria vary between 0.06 and 0.73 g and 72.67-139.34 g/l respectively. EPS concentration in different bacteria and even in a strain are different with respect to the composition of the culture medium and culture conditions such as pH and temperature. Assessment of

environmental conditions showed that EPS production increases at 2.5 to 3% glucose, 0.5% yeast extract and mesophilic temperature. The effect of other environmental factors such as pH, oxygen concentration, incubation time, metal ions, surfactants and NaCl concentration on EPS production have been considered as the effective parameters in EPS production which can be investigated in future studies.

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