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Influences of Environmental Factors on Bacterial Extracellular Polymeric Substances Production in the Process of Artificial Injection

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ABSTRACT

Bioclogging of natural porous media occurs frequently under a wide range of conditions. It may influence the performance of permeable reactive barrier and constructed wetland. It is also one of the factors that determine the effect of artificial groundwater recharge and in situ bioremediation process. In this study, a series of percolation column experiments were conducted to simulate bioclogging process in porous media. The predominant bacteria in porous media which induced clogging were identified to be Methylobacterium, Janthinobacterium, Yersinia, Staphylococcus and Acidovorax, most of which had been shown to effectively produce viscous extracellular polymeric substances (EPS). The column in which EPS production was maximized also coincided with the largest reduction in saturated hydraulic conductivity of porous media. In addition, carbon concentration was the most significant factor to affect polysaccharide, protein and EPS secretion, followed by phosphorus concentration and temperature. The coupled effect of carbon and phosphorus concentration was also very important to stimulate polysaccharide and EPS production.

1. INTRODUCTION

Microorganisms play a crucial role in the degradation of organic contaminants in subsurface environments. This biodegradation process involves microbes that utilize organic compounds as their growth substrates to keep cells growing, which leads to the removal of contaminants. Besides these metabolism activities (Cajthaml et al., 2009; Stasinakis, 2012; Yang et al., 2013a; Yang et al., 2013b), microbes also become significant for their carrier function in solute transport in subsurface environments, which directly affects the spreading and decay of contaminant plumes in the subsurface. In the presence of high nutrient loading, biomass accumulation can cause drastic reduction in saturated hydraulic conductivity of porous media due to clogged pores, known as bioclogging (Baveye et al., 1998; Vandevivere and Baveye, 1992a,b; Vandevivere et al., 1995). Bioclogging is widely found in permeable reactive barrier (Liang et al., 2000), aquifer storage and recovery (Pavelic et al., 2007), microbial enhanced oil recovery (Cai et al., 2013; Jiménez et al., 2012), drip irrigation (Puig-Bargues et al., 2005), bioremediation of organic contaminants in subsurface environments (Calderer et al., 2014), and landfill leachate collection systems (Beaven et al., 2013; Singhal and Islam, 2008). Bioclogging could trigger deleterious consequences such as the reduction of artificial recharge efficiency and increasing operating cost; therefore, its mechanisms are worthy of great value.

Bioclogging in porous media due to bacterial cells was first observed by McCalla (1951). Following his work, researchers found that the microbial exopolymers contributed more to bioclogging than cells (Mitchell and Nevo, 1964; Vandevivere and Baveye, 1992b). Exopolymers, also known as extracellular polymeric substances (EPS) (Ras et al., 2011), were slimy or gummy materials excreted by microorganisms. Vandevivere and Baveye (1992b) performed sand column experiments, with both EPS producing and non-producing bacteria strains, and identified the EPS as main contributor to bioclogging.

As EPS are considered to be one of the major factors in bioclogging process (Vandevivere and Baveye, 1992b), exploring the impacts of various environmental factors on EPS production would pave the way for bioclogging prediction and control. Numerous environmental factors have been documented to influence EPS contents. Some researchers studied the influences of aerobic and anaerobic conditions on EPS yields (Nielsen and Frølund, 1996). Miqueleto et al. (2010) discussed the effects of different carbon sources and C/N radio on EPS production in an anaerobic sequencing batch biofilm reactor. Fang et al. (2002) studied the influence of Zn on the EPS production by sulfate-reducing bacteria. A similar work done by Yang et al. (2013) revealed the coupled effect between heavy metals and biofilm EPS in porous media. For most of these studies, the researchers focused on a single factor once at a time without considering the effects of the interactions among different factors. Single-factor experiments were generally time and energy consuming. In this work, we handle this issue by applying the Response Surface Methodology (RSM), which is an effective statistical technique to solve multivariate scenarios. RSM has been widely used in many fields, such as the manufacturing of biochemical and biotechnological products (Aybastıer and Demir, 2010; Chatterjee et al., 2012).

In our study, based on sand and inoculum sampled from a natural aquifer, we conducted molecular biology identification, saturated hydraulic conductivity measurement and EPS production experiments. The specific objectives of this work were to: (1) isolate and identify the dominant bacteria in porous media using polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), and analyze their characteristics; (2) build correlation between microbial EPS yield and relative saturated hydraulic conductivities of porous media and (3) investigate the influences of key environmental factors and their combined effect on the yield of microbial polysaccharide, protein and EPS, by applying both the single-factor experiment method and RSM analysis. This work will contribute to the precise prediction and efficient control of bioclogging in porous media in the future.

2. MATERIALS AND METHODS

2.1 Sample collection

Sand samples were taken from a natural aquifer near Dagu river (N36.38024, E120.12089°) in Jiaozhou city, Shandong province of China. The sand samples were sieved according to the Specification of Soil Test (Ministry of Water Resources, 1999) and the

average particle size were measured to be 0.539 mm. Before repacked in the columns, the sand samples were soaked in HCl solution (0.25 M) for 24 h, followed by NaOH solution (0.25 M) for 24 h. Then they were washed by Milli-Q water and incinerated at 550 ℃ for 2 h to remove any organic matter on the sand surface.

2.2 Inoculum

A mixture of microbial consortium and water collected from a recharge well near Dagu river was used as inoculum. After the water sample was taken from the site, it was inoculated and fully aerated for 2 h. Then, the sample was settled for 30 min to form a uniform suspension before introduced into the sand columns.

2.3 Synthetic nutrient solution

After the water sample was taken from the field, the aqueous profile of the sampled groundwater was measured in the lab. This included concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+}, NH_4^+, Cl^- and SO_4^{2-}. In order to stimulate bacteria growth and EPS production, synthetic nutrient solution according to the water sample profile was introduced, which contained glucose (variable), 5 g/L NaCl, 1.91 mg/L NH_4Cl, K_2HPO_4 (variable), 45 mg/L $MgSO_4 \cdot 7H_2O$, 20 mg/L $CaCl_2 \cdot 2H_2O$, and 1 mL of a trace elements solution, containing 2 mg/L $FeSO_4 \cdot 7H_2O$, 0.4 mg/L $CuSO_4 \cdot 5H_2O$, 0.04 mg/L $MnSO_4 \cdot 6H_2O$, 0.04 mg/L $Na_2MoO_4 \cdot 2H_2O$, 2 mg/L H_3BO_3 and pH 7.0–7.2. Before column experiments, the nutrient medium was autoclaved at 121 ℃ for 15 min.

2.4 Operation of the column

The sand column experiments were conducted in columns (22 cm in length and with an inside diameter of 5 cm) constructed from cast acrylic tubing. The inlet and outlet of the columns were covered by 80-mesh stainless steel screens. In addition, a 2-cm-thick glass bead (diameter 1 cm) layer was placed on the top of the inlet screen to make an even influent passing through the porous media surface. Piezometers were installed at the column inlet and outlet to monitor the hydraulic head difference gradient. Before packing, the columns and connections were sterilized by ultraviolet carefully. The columns were packed with wet sand in an increment of 2 cm to avoid air entrapment and homogenize every layer. The packed columns had an average porosity of 0.385.

The columns were saturated slowly with 12 pore volumes of sterilized Milli-Q water and then 8 pore volumes of the suspended bacteria prepared in Section 2.2. Peristaltic pumps (BT100-2J,Longerpump, Baoding, China) were used to inject the sterilized nutrient medium into the columns in an upward-flow mode. The pumping rate at inlet was kept at 5 mL/min. After 9 days' incubation, the columns were dismantled for bacterial strains identification and EPS quantification.

2.5 Saturated hydraulic conductivity evaluation

A constant flow rate test was used to measure the saturated hydraulic conductivity K_s [L/T], according to Darcy's law:

$$Q = \mathrm{A} \cdot K_s \frac{\Delta H}{L} \qquad (1)$$

where Q is the flow rate [L^3/T], A is the cross sectional area of the column (19.625 cm^2 in this work), DH is the hydraulic head difference between the column inlet and outlet, and L is the distance between the two points where DH is measured (18 cm in this work).

The calculated K_s value was normalized by K_{s0}, the initial saturated hydraulic conductivity of the sand column. The ratio K_s/K_{s0} was used to describe the degree of clogging in different columns.

2.6 Microbial community analysis

2.6.1 Genomic DNA extraction

After a 9-days' incubation, the column was dismantled and sand samples of 2 g each were transferred into a sterile plastic tube containing 10 mL aseptic phosphate buffered (PB, 10 mM, pH 7.4) solution and 1 g of sterile glass beads. The genomic DNA was extracted using a bead beating protocol by means of the commercial Power Soil™ isolation kit (MO-BIO Laboratories, Inc., Carlsbad, CA, USA), following manufacturer's instructions.

2.6.2 PCR-DGGE

The extracted genomic DNA was used as the template for PCR. Amplifications were performed using the universal primers BA101F (5'-TGGCGGACGGGTGAGTAA-3', Invitrogen, Shanghai) and BA534R (5'-ATTACCGCGGCTGCTGG-3', Invitrogen, Shanghai). A 40 bp GC-clamp was linked to the forward primer BA534R. PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA) and the products were analyzed by DGGE using a Bio-Rad™ universal mutation detection (Bio-Rad Laboratories, USA), according to the procedure described by EI Sheikha (2010). The gel was electrophoresed at 150 V for 9 h. After that, the gel was stained by silver staining and gel images were photographed.

2.6.3 Sequencing of DGGE bands and homology search

DNA sequencing was performed using a DNA sequenator (ABI377 DNA Analyzer, Applied biosystems, USA). The obtained 16S rDNA sequence were submitted to the Genbank online database (http://www.ncbi.nlm.nih.gov/BLAST) for homology search.

2.7 Extraction and quantification of polysaccharide and protein in EPS

In this study, polysaccharide and protein were quantified and the sum of them was calculated to be EPS yield. EPS was extracted following the formaldehyde-NaOH method depicted by Liu and Fang (2002) with slight modifications. The polysaccharide content (expressed as μg /g sand) was determined according to the phenol–sulfuric acid method with a glucose standard (Frølund et al., 1996). The protein yield (expressed as μg/g sand) was assessed by the Bradford assay (Sharma and Babitch, 1980) with albumin

trom bovine serum (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) as standard. All biological samples were analyzed using 2800 UV/VIS Spectrophotometer (UNICO, USA).

3. RESULTS AND DISCUSSION

3.1 Identification of predominant bacteria

The identification of predominant bacterial strains in porous media was assayed by 16S rDNA PCR-DGGE using universal primer followed by sequencing of the DGGE bands.

The DGGE result is shown in Fig. 1. X1 and X2 were duplicate samples, revealing a good repeatability. Eleven bands were considered to be major bacterial strains in our experiment (Fig. 1) and all of them were sequenced. The sequenced data were submitted to the Genbank online system and the result is shown in Table 1. It was found that, except for band 2, 5 and 10, the rest of the eight strains had more than 98% homology to the known bacteria in GenBank. They belonged to Methylobacterium, Janthinobacterium, Yersinia, Staphylococcus, and Acidovorax. In addition, band 9 also displayed 99% homology with an unknown and uncultured bacterium in the GenBank database.

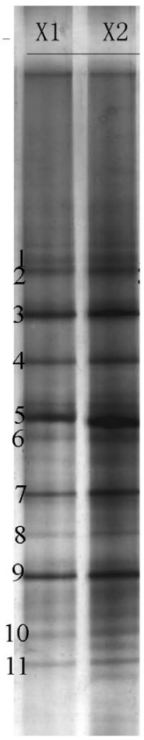

Figure 1: DGGE 16S rDNA profile of bacterial strains in porous media.

The Methylobacterium, Janthinobacterium, Staphylococcus, and Acidovorax were regarded as preponderant strains in porous media, and have been shown to effectively produce viscous EPS in previous studies (Nakamura et al., 1976). The conclusion obtained in this study was consistent with Vandevivere and Baveye (1992b) who pointed out that the growth of slime producing bacteria in porous media played a very important role in the bioclogging.

3.2 The correlation between microbial EPS yield and porous mediapermeability

There are several potential causes that induce bioclogging, (1) accumulation of cell bodies. Many earlier experiments indicated that bacterial cells could dramatically reduce the K_s of the pore space, particularly when they were strategically located at grain contacts or at pore necks (Vandevivere and Baveye, 1992a,b,c). (2) Production of bacterial extracellular polymers. Characklis (1971) concluded that exopolymers could affect K_s either by increasing the viscosity of the fluid or by decreasing the volume and size of fluid-conducting pores. In addition, the presence of exopolymers may decrease the incidence of predation of protozoans (DeLeo et al., 1997; Mattison et al., 2002). (3) Entrapment of gaseous end-products. Gas bubbles in porous media, if sufficiently large to become trapped in the network of pores, causes a reduction of K_s because the bubbles decrease the size of the water-conducting pores, in the same manner as solid particles would (Orlob and Radhakrishna, 1958). In this work, a series of 17 column experiments were conducted. The data in Fig. 2 depicted that the column in which EPS production was maximized also coincided with the largest reduction in saturated hydraulic conductivity of porous media. Besides that, we also investigated the effects of environmental factors that lead to the EPS production.

3.3 Influence of environmental factors on EPS production

3.3.1 Single-factor experiment

Given the conditions for microbial activity, nutrient levels and external conditions are considered to have potential effects on microorganism growth and EPS secretion. Therefore, we looked into the influences of carbon, nitrogen, phosphorus, salt concentration, and temperature on the EPS production. Previous studies in our lab showed that EPS production reached maximum

when carbon, nitrogen, phosphorus, salt concentration, and temperature were 50 mg/L, 5 mg/L, 1 mg/L, 5 g/L and 30 ℃ respectively. In addition, carbon, phosphorus concentration, and temperature were the main factors influencing EPS production (Xia et al., 2014). To further reveal their relative importance, carbon, phosphorus and temperature were chosen in this study for the following RSM experiments, with nitrogen and salt were controlled at constant concentrations of 5 mg/L and 5 g/L respectively.

3.3.2 Response surface methodology (RSM)

To determine the coupled effect of carbon, phosphorus, and temperature on EPS production, a three-factor three-level Box–Behnken experimental design was carried out. The series of experiments and data obtained for polysaccharide (Y1), protein (Y2) and EPS yields (Y3) are summarized in Table 2. The letter A, B and C represent carbon, phosphorus, and temperature. They are given in terms of coded (actual) value in Table 2.

The complete design involved 17 experiments, including 12 factorial and 5 replications at the central points. The 5 replications were run for the purpose of experimental error. All the experiments were conducted in triplicate and the obtained data were analyzed using the software Design Expert (version 7.0.0). Our statistical analyses were performed in the following three steps, (1) first the regression model fitting, followed by (2) the analysis of variance (ANOVA) and finally (3) plotting the response surface.

Table 1: Comparison of genomic sequences in dominant DGGE bands by sequencing and BLAST analysis.

DGGE band no.	Accession number	Bacterial identification results	The identity with sequences in the GenBank databases
1	KF680873	Methylobacteriumpersicinum	385/385(100%)
2	KF680874	Bacteriovoraxstolpii	422/449(94%)
3	KF680875	Janthinobacteriumlividum	426/431(99%)
4	KF680876	Yersiniaaldovae	421/426(99%)
5	KF680877	Microbulbiferagarilyticus	268/292(92%)
6	KF680878	Staphylococcuscapitis	436/440(99%)
7	KF680880	Acidovoraxdefluvii	422/432(98%)
8	KF680882	Yersiniaaldovae	425/431(99%)
9	KF680884	Aquabacteriumcommune	426/432(99%)
10	KF680887	Aeromonassharmana	421/434(97%)
11	KF680888	Janthinobacteriumlividum	429/432(99%)

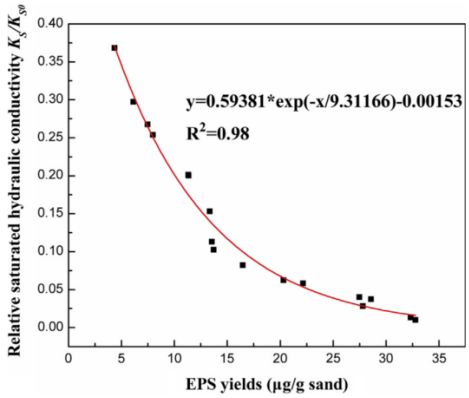

Figure 2: Relationship between EPS content and relative saturated hydraulic conductivity of porous media after a 9-days' incubation.

3.3.3 Influence factors on polysaccharide content

The predicted model of polysaccharide content can be depictedas follow:

Table 2: Box–Behnken design and experimental results.

Run	Factors			Responsevalues		
	A Carbon(mg/L)	BPhosphorus(mg/L)	CTemperature(℃)	Y1Polysaccharide(μ g/g sand)	Y2Protein(μ g /gsand)	Y3EPS(μ g /gsand)
1	+1(75)	-1(0.5)	0(30)	18.12±1.26	4.05±0.34	22.17±1.56
2	-1(25)	0(1.0)	+1(45)	4.82±1.00	2.65±0.24	7.47±1.21
3	0(50)	0(1.0)	0(30)	23.90±1.31	4.67±0.22	28.57±1.53

4	+1(75)	0(1.0)	+1(45)	16.31±0.66	3.99±0.13	20.30±0.75
5	0(50)	+1(1.5)	-1(15)	6.69±0.12	4.66±0.22	11.35±0.11
6	0(50)	-1(0.5)	-1(15)	9.69±0.86	3.85±0.10	13.54±0.84
7	0(50)	0(1.0)	0(30)	27.53±0.54	4.81±0.17	32.34±0.54
8	0(50)	0(1.0)	0(30)	28.11±1.59	4.66±0.22	32.77±1.75
9	0(50)	-1(0.5)	+1(45)	12.56±0.40	3.91±0.05	16.47±0.45
10	+1(75)	0(1.0)	-1(15)	10.04±1.21	3.67±0.11	13.71±1.16
11	0(50)	0(1.0)	0(30)	23.20±0.51	4.27±0.10	27.47±0.59
12	0(50)	+1(1.5)	+1(45)	7.22±0.33	4.10±0.19	11.32±0.52
13	-1(25)	+1(1.5)	0(30)	5.63±0.27	2.34±0.08	7.97±0.30
14	-1(25)	-1(0.5)	0(30)	2.22±0.21	2.12±0.18	4.34±0.38
15	+1(75)	+1(1.5)	0(30)	9.80±0.05	3.54±0.11	13.34±0.14
16	-1(25)	0(1.0)	-1(15)	4.05±0.46	2.08±0.10	6.13±0.52
17	0(50)	0(1.0)	0(30)	23.46±0.24	4.34±0.07	27.80±0.27

$$Y1 = -89.88 + 1.63A + 75.01B + 2.16C - 0.23AB + 3.67\times10^{-3}AC - 0.078BC - 0.013A^2 - 32.13B^2 - 0.036C^2 \quad (2)$$

where Y1 is polysaccharide yield (μ g/g sand), A is carbon concentration (mg/L), B is phosphorus concentration (mg/L), and C is temperature (℃).

The result of ANOVA for model (2) is described in Table 3. The model was highly significant with $p < 0.0001$ while the lack of fit was not significant relative to the pure error ($p > 0.05$). This indicated the model was sufficient to explain the actual relationship between polysaccharide yield and crucial parameters. Based on the results of ANOVA, carbon was found to be the most important factor that influenced polysaccharide content ($p < 0.01$), followed by phosphorus. The temperature was considered to be insignificant ($p > 0.05$). The combination of carbon and phosphorus was considered to be very significant to affect the polysaccharide concentration ($p < 0.05$), while the combined effect of carbon/temperature, phosphorus/temperature were not significant ($p > 0.05$).

When the temperature (C) was fixed at 30 ℃, the combined effect of various carbon (A) and phosphorus (B) on the polysaccharide yield is illustrated in Fig. 3(a). With increasing amount of carbon concentration, the polysaccharide first increased rapidly and then decreased. It meant there was an optimum carbon concentration to stimulate microbial polysaccharide secretion. It also suggested that polysaccharide yield could be enhanced in the presence of suitable carbon concentration. If carbon concentrations were higher than the optimum value, microbial cellular growth and exopolysaccharide secretion would be inhibited. Our results are consistent with Celik et al. (2008), who showed that the production of exopolysaccharide was stimulated in the PAP medium with 3% xylose (a carbon source), while higher concentrations of xylose suppressed polysaccharide production. Fig. 3(a) also indicated that polysaccharide yield was enhanced along with the increasing phosphorus concentration. When it was greater than a specific optimum value, polysaccharide started to decrease. Krey et al. (2013) showed that phosphorus was of great importance regarding microbial cellular membrane formation. However, higher phosphorus concentrations could also inhibit the production of microorganism metabolites. Table 3 showed that the coupled effect of carbon and phosphorus was very significant to polysaccharide production ($p < 0.05$). It had been reported that carbon and phosphorus had synergistic effect on microbe growth. More precisely, microorganisms could utilize phosphates as carbon source to maintain cell growth and metabolic activity when carbon source was limited (Spohn and Kuzyakov, 2013).

Table 3: ANOVA for polysaccharide yield.

Source	Sumofsquares	Degreeoffreedom	Meansquare	F-value	P-value
Model	1194.24	9	132.69	34.96	<0.0001
A-COD_{Cr}	176.25	1	176.25	46.43	0.0003
B-Phosphorus	21.95	1	21.95	5.78	0.0472
C-Temperature	13.62	1	13.62	3.59	0.1000
AB	34.40	1	34.40	9.06	0.0197
AC	7.56	1	7.56	1.99	0.2010
BC	1.37	1	1.37	0.36	0.5671
A2	287.71	1	287.71	75.79	<0.0001

B2	271.58	1	271.58	71.54	<0.0001
C2	280.96	1	280.96	74.02	<0.0001
Residual	26.57	7	3.80	–	–
Lackoffit	3.97	3	1.32	0.23	0.8688
Pureerror	22.61	4	5.65	–	–
Cortotal	1220.81	16	–	–	–

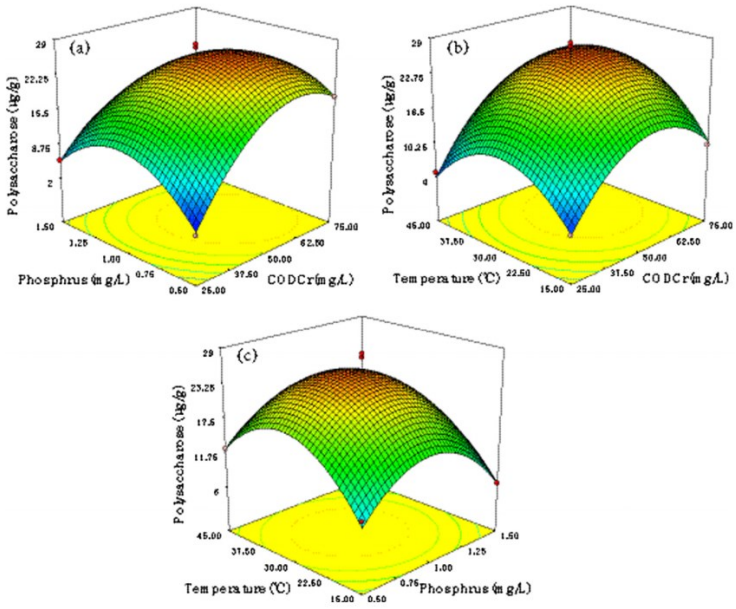

Figure 3: Response surface plots showing the effects of (a) carbon and phosphorus concentration, (b) carbon concentration and temperature and (c) phosphorus concentrationand temperature on polysaccharide content.

Fig. 3(b) depicted the response surface plot of variable carbon concentration (A) and temperature (C) on polysaccharide yield. For every carbon concentration, there was an optimum temperature to suit for polysaccharide secretion. This may due to the fact that all microorganisms in porous media were mesophiles, and further increase in temperature would lead to the growth inhibition (Sun et al., 2013). This optimum temperature effect was also observed in several previous studies. Faria et al. (2010) pointed out that the optimum temperature for microbial growth and xanthan gum production was 30 ℃. Both lower or higher temperatures led to the reduction of the xanthan production. Arockiasamy and Banik (2008) found that maximum gellan gum produced by Sphingomonas paucimobilis was reached at 30–31 ℃. The influence of phosphorus (B) and temperature (C) on polysaccharide production was illustrated in Fig. 3(c). It showed a similar plot as for the carbon/phosphorus (Fig. 3(a)) and carbon/temperature plot (Fig. 3(b)).

3.3.3.1 Influence factors on protein content. The predicted model of protein content is shown in Eq. (3)

$$Y2 = -5.66 + 0.26A + 3.55B + 0.077C - 0.015AB - 1.67\times10^{-4}AC - 0.021BC - 2.06\times10^{-3}A^2 - 1.01B^2 - 7.44\times10^{-4}C^2 \quad (3)$$

where Y2 is protein yield (μ g/g sand), A is carbon concentration (mg/L), B is phosphorus concentration (mg/L) and C is temperature (℃).

Table 4 revealed the analysis of protein yield for the regression equation. The p-value revealed the relative significance of the investigated factor. The low-values were highly significant, while the lack of fit was not significant ($p > 0.05$). Table 4 showed that carbon content was the most relevant variable ($p < 0.01$), while phosphorus and temperature were of little significance to the microbial protein secretion. As for the combined effects, they were not significant to protein production (Table 4).

The joint action of carbon (A) and phosphorus (B) on protein production shown in Fig. 4(a) revealed that protein concentration increased with the addition of carbon. Same as in the polysaccharide case, this trend reversed as it was beyond an optimum value. This phenomenon indicated that proper carbon source could promote microorganisms metabolic activity to secrete extracellular protein, while the excessive carbon repressed protein production. Geisseler and Horwath (2008) were concerned that excessive carbon could favor protease activity which led to the degradation of extracellular protein. Fig. 4(a) also demonstrated that the relationship between phosphorus and protein yield was not obvious. Similar as our results, Sun et al. (2014) also concluded that phosphorus concentration had less significant effect on protein synthesis when compared with nitrogen.

Table 4: ANOVA for protein yield.

Source	Sum of squares	Degree offreedom	Mean square	F-value	P-value
Model	12.57	9	1.40	14.57	0.0009
A-COD_{Cr}	4.59	1	4.59	47.88	0.0002
B-Phosphorus	0.063	1	0.063	0.66	0.4442
C-Temperature	0.019	1	0.019	0.20	0.6695
AB	0.13	1	0.13	1.39	0.2770
AC	0.016	1	0.016	0.16	0.6985
BC	0.096	1	0.096	1.00	0.3501
A2	6.95	1	6.95	72.52	<0.0001
B2	0.27	1	0.27	2.80	0.1382
C2	0.12	1	0.12	1.23	0.3037
Residual	0.67	7	0.096	–	–
Lackoffit	0.45	3	0.15	2.80	0.1728
Pureerror	0.22	4	0.054	–	–
Cortotal	13.24	16	–	–	–

Fig. 4(b) described the response surface plot of carbon and temperature on protein concentration. The protein yield was slowly increased with increasing temperature and then decreased as temperature continued to increase for given a fixed carbon concentration. Li et al. (2008) reported that, for mesophilic microorganisms, increased metabolism rate with the increasing culture temperature from 15 to 35 ℃, while higher temperatures caused oxidative damage at cellular level and subsequent reduction in microbial metabolism ability. The combined effect of carbon and temperature on protein concentration was of little importance on protein production ($p > 0.05$ in Table 4).

The relationship between variables (phosphorus and temperature) and protein yield was investigated by response surface plot and shown in Fig. 4(c). It can be seen clearly that there was a slowly rising trend of protein concentration in response to the increase of phosphorus concentration and temperature. This meant that high phosphorus and temperature could favor the growth and metabolism activity, but the presence of phosphorus and temperature, as well as their combined effect, was not significant to the protein yield (Table 4).

3.3.3.2 Influence factors on EPS yield. EPS predicted model is shown in Eq. (4)

$$Y3 = -95.54 + 1.89A + 78.56B + 2.24C - 0.25AB + 3.5\times10^{-3}AC - 0.099BC - 0.015A^2 - 33.14B^2 - 0.037C^2 \quad (4)$$

where Y3 stands for EPS (μ g /g sand), A is carbon concentration (mg/L), B is phosphorus concentration (mg/L) and C is temperature (℃).

Table 5 described the analysis result of EPS production for the quadratic polynomial model. The model p-value was less than 0.0001, which indicated that it was highly significant and sufficient to describe the relationship between the EPS production and environmental factors. Furthermore, the "lack of fit F-value" of 0.24 implied the lack of fit was not significant relative to the pure error. According to the ANOVA, carbon was proved to be the most important factor to influence EPS production with p-value < 0.01, followed by phosphorus while temperature was not very significant. The coupled effect of carbon and phosphorus was also significant ($p < 0.05$), while the combined effect of phosphorus/temperature, carbon/temperature were not significant (Table 5).

The response surface plots of carbon, phosphorus, and temperature on EPS yield are shown in Fig. 5 which showed a similar trend as Fig. 3. With the increase of carbon concentration, EPS production was increasing rapidly and then stated to decrease slowly. As an easily biodegradable substrate, glucose can be directly used in microbial cells growth. Miqueleto et al. (2010) reported that activated monosaccharides such as glucose were continuously adsorbed to a lipid carrier to form glycogen in microbial cells. With microbial metabolism proceeding, the glycogen passed through the cell membrane to the outside of cells and formed the EPS matrix.

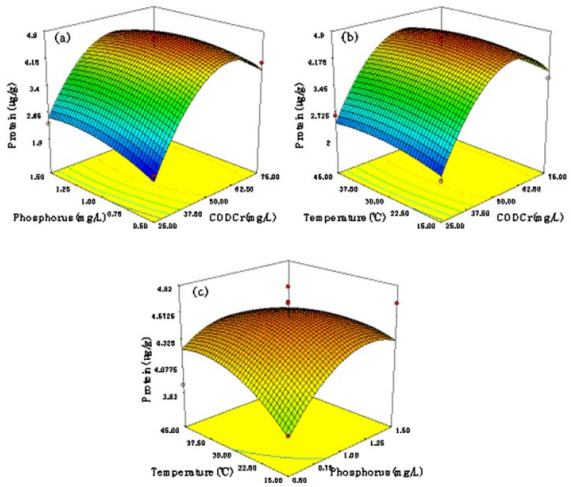

Figure 4: Response surface plots showing the effects of (a) carbon and phosphorus concentration, (b) carbon concentration and temperature and (c) phosphorus concentrationand temperature on protein content.

Table 5: ANOVA for EPS production.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	1398.46	9	155.38	35.18	<0.0001
A-COD_{Cr}	237.73	1	237.73	53.82	0.0002
B-Phosphorus	19.66	1	19.66	4.45	0.0428
C-Temperature	14.66	1	14.66	3.32	0.1113
AB	38.81	1	38.81	8.79	0.0210
AC	6.89	1	6.89	1.56	0.2518
BC	2.19	1	2.19	0.50	0.5041
A^2	384.11	1	384.11	86.95	<0.0001
B^2	288.93	1	299.93	65.41	<0.0001
C^2	292.60	1	292.60	66.24	<0.0001
Residual	30.92	7	4.42	–	–
Lack of fit	4.71	3	1.57	0.24	0.8650
Pure error	26.21	4	6.55	–	–
Cor total	1429.38	16	–	–	–

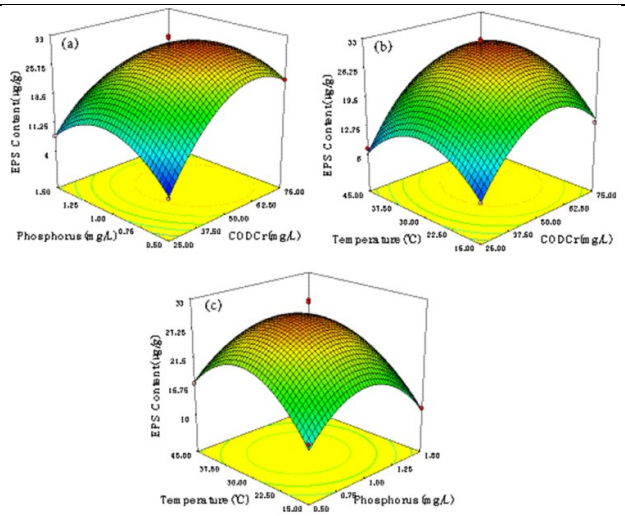

Figure 5: Response surface plots showing the effects of (a) carbon and phosphorus concentration, (b) carbon concentration and temperature and (c) phosphorus concentrationand temperature on EPS yield.

However, when there was an excess carbon concentration, other essential nutrients, such as nitrogen and phosphorus, could limit cell growth and metabolism which led to the reduction in EPS yield. At the same time, the energy from excess carbon source was used to synthesize EPS, so the yield of EPS reduced slowly as shown in Fig. 5(a). The effect of phosphorus on EPS production shown in Fig. 5(a) demonstrated that phosphorus concentration had little effect on EPS yield while the combined effect of carbon and phosphorus was significant to EPS production ($p < 0.05$ in Table 5).

The combined effect of carbon/temperature, phosphorus/temperature on EPS production were shown in Fig. 5(b) and (c) respectively. With the increase of nutrient solution, microbes grew rapidly and started to produce polysaccharide and protein to the outer layer. Under the optimal condition, microbial EPS production reached a peak. Any further increase of carbon, phosphorus and temperature led to the excessive growth of microorganisms and consumed a lot of oxygen in the porous media. This process produced large amount of bacteria in the outer layer. Meanwhile, the surviving bacteria in deeper layer became even more active to produce polysaccharide and protein. This explained why the EPS yield decreased slowly at higher carbon, phosphorus and temperature. Zhou et al. (2011) obtained a similar result.

In this study, polysaccharide yield was more abundant than protein content. This indicated the polysaccharide was the main component of EPS. Our result agreed with Dean et al. (2008). They concluded cells had a preferential ability to synthesize polysaccharide rather than protein as energy, in response to the changing external growth condition. Consequently, the trend in Fig. 5 was consistent with trend in Fig. 3.

3.3.3.3 Validation of EPS production predictive model. The optimal environmental condition to stimulate EPS production was described by RSM, which revealed that carbon was at a concentration of 58 mg/L, phosphorus at a concentration of 0.92 mg/L and temperature of 31.7℃. Under this optimum condition, a maximum EPS yield of 30.86 μg/g sand was achieved according to Eq. (4). The validation experiment was carried out using this optimal condition. The EPS yield value measured in this experiment was 31.02 μg/g sand, which was in good agreement with the one predicted by the model (30.86 μg/g sand). This close match verifies that our model, is useful to depict the effects of carbon, phosphorus and temperature on EPS production.

4. CONCLUSIONS

In this study, a series of column experiments were conducted to identify the bacteria that was contributing to the bioclogging process. We found the predominant bacteria in porous media belonged to Methylobacterium, Janthinobacterium, Yersinia, Staphylococcus, and Acidovorax, most of which had been shown to effectively produce viscous EPS. The column in which EPS production was maximized also coincided with the largest reduction in saturated hydraulic conductivity of porous media. According to RSM analysis, carbon concentration was the most significant factor to the production of polysaccharide, protein and EPS. It was followed by phosphorus concentration and temperature. The combined effect of carbon and phosphorus concentration was also very significant to stimulate polysaccharide and EPS secretion. Polysaccharide component was found to be more abundant than protein in this work. It indicated that polysaccharide was the main component of EPS. Further studies are needed to focus on the contributions of different EPS components, including polysaccharide and protein, on the bioclogging process. The predicted models obtained by fitting the experimental data are adequate and reliable to depict the effects of carbon, phosphorus and temperature on EPS production.

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