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Effects of solution conditions on the physicochemical properties of stratification components of extracellular polymeric substances in anaerobic digested sludge

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Abstract

The composition and effects of solution conditions on the physicochemical properties of the stratification components of extracellular polymeric substances (EPS) in anaerobic digested sludge were determined. The total EPS in anaerobic digested sludge were extracted by the cation exchange resin method. Another EPS extraction method, the centrifugation and sonication technique was employed to stratify the EPS into three fractions: slime, loosely bound (LB)-EPS, and tightly bound (TB)-EPS from the outside to the inside of the anaerobic digested sludge. Proteins and polysaccharides were dispersed uniformly across the different EPS fractions, and humic-like substances were mainly partitioned in the slime, with TB-EPS second. Protein was the major constituent of the LB-EPS and TB-EPS, and the corresponding ratios ranged from 54.0% to 65.6%. The hydrophobic part in the EPS chemical components was primarily comprised of protein and DNA, while the hydrophilic part was mainly composed of polysaccharide. In the slime, the hydrophobic values of several EPS chemical components (protein, polysaccharide, humic-like substances and DNA) were all below 50%. The protein/polysaccharide ratio had a significant influence on the Zeta potentials and isoelectric point values of the EPS: the greater the protein/polysaccharide ratio of the EPS was, the greater the Zeta potential and the higher the isoelectric point value were. All Zeta potentials of the EPS showed a decreasing trend with increasing pH. The corresponding isoelectric point values (pH) were 2.8 for total EPS, 2.2 for slime, 2.7 for LB-EPS, and 2.6 for TB-EPS. As the ionic strength increased, the Zeta potentials sharply increased and then gradually became constant without charge reversal. In addition, as the temperature increased (< 40°C), the apparent viscosity of the EPS decreased monotonically and then gradually became stable between 40 and 60°C.

Key words: anaerobic digested sludge; extracellular polymeric substances; stratification components; physicochemical properties; solution conditions

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Introduction

In biological wastewater treatment systems, extracellular polymeric substances (EPS) are high-molecular weight compounds originating from bacterial secretion, cell lysis and hydrolysis, leakage of exocellular constituents, and adsorbed organic matter from the surrounding wastewater (Urbain et al., 1993; Frølund et al., 1996; Wingender et al., 1999). In recent years, the stratification components of EPS have generated great research interest. **Figure 1** presents the evolution of the understanding of the stratification components of the EPS. Nielsen et al. (1997) subdivided EPS into bound and soluble fractions. According to Rosenberger and Krume (2003), EPS can be divided into extractable EPS and soluble EPS (also called slime polymers). Lansky (2003) thought that EPS could be differentiated into slime and sheath by centrifugation. Ramesh et al. (2006) and Li and Yang (2007) observed that bound EPS exhibit a dynamic double-layered structure, composed of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). After EPS are extracted, the residual cells are recognized as the pellet (Yu et al., 2008a). Hence, on the basis of the aforementioned findings, Yu et al. (2008b) further separated EPS into slime, LB-EPS, and TB-EPS. The EPS fractions and the pellet together comprise the multi-fractioned structure of the sludge from the outer surfaces to the cores of the granules.

Over the past few decades, numerous studies have indicated that EPS have a great influence on the bioflocculation, settling, and dewatering of sludge (Jia et al., 1996;

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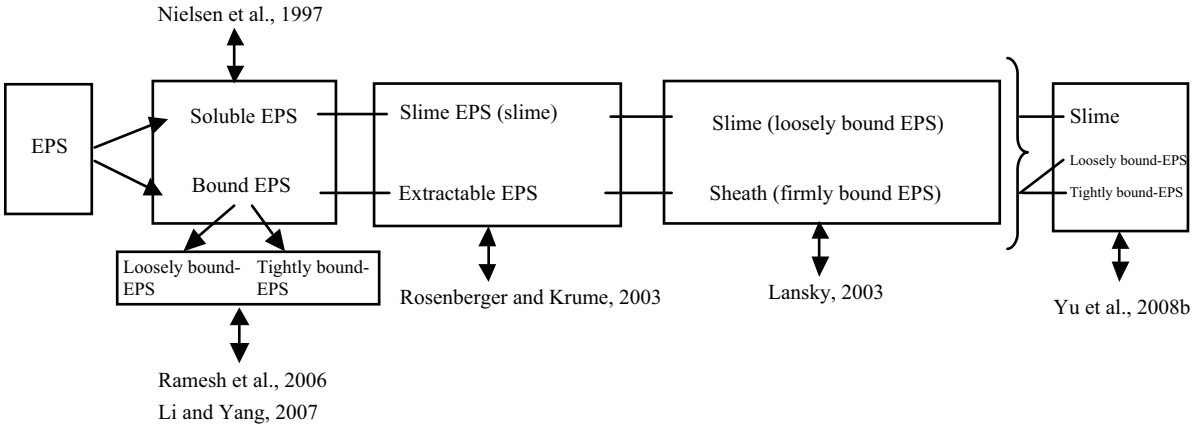

Fig. 1 Evolution of the understanding of the stratification components of EPS.

Higgins and Novak, 1997; Houghton et al., 2001; Cetin and Erdincler, 2004). Furthermore, considerable research has been conducted on the effects of operating conditions on components, contents, and physicochemical properties of EPS in batch reactors for anaerobic digestion (Yu et al., 2008a; Ramesh et al., 2006; Nielsen et al., 1996; Houghton and Stephenson, 2002). However, few studies have focused on the variations in physicochemical properties of EPS with solution conditions. Liao et al. (2002) found that the major source of surface charge of the sludge came from EPS. Extremes of pH (acidity or alkalinity) can make the proteins of EPS lose their natural shapes (Watson et al., 1987). The different functional groups of EPS were found to remain protonated at pH above the isoelectric point after being in the deprotonated state at pH below the isoelectric point (Braissant et al., 2007), which in turn led to the decrease of the surface charge (Zheng et al., 2007) and Zeta potentials (Wang et al., 2012) of EPS with increasing pH. Accordingly, the Zeta potentials of the sludge became more negative (Liu et al., 2010) and other sludge characteristics changed as well (Zheng et al., 2007). Wang et al. (2012) found that the EPS from *Bacillus megaterium* TF10 presented a denser and compact structure at lower pH, and released chains at higher pH. The highest flocculation efficiency was achieved near the isoelectric point. They also recognized that complex and subtle intra/inter-actions among the EPS functional groups, such as hydrophobicity, intermolecular hydrogen bonds and coulombic forces, were responsible for the pH dependence of flocculation. Their work gives insight into the observation that microbial flocculation is dependent heavily on pH in solution. In addition, ionic strength can greatly affect the Zeta potentials (Liu et al., 2010) and the stability of sludge (Zita and Hermansson, 1994). Hence, solution conditions are the main contributors to changes in the physicochemical properties of EPS and the sludge characteristics. Nevertheless, the information regarding the effects of solution conditions on the physicochemical properties of EPS is still insufficient. Therefore, this study attempts to investigate the effects of solution conditions such as pH, ionic strength, and temperature on the physicochemical properties of different EPS in anaerobic digested sludge. Variations in zeta potentials, conductivity, and apparent viscosity of these EPS were identified. The results are expected to provide preliminary data on the effects of EPS on the physicochemical properties of sludge.

1 Materials and methods

1.1 Sludge samples

The anaerobic digested sludge samples were collected from a municipal wastewater treatment plant in Beijing, China. The collected samples were transported to the laboratory within 2 hr after sampling and subsequently filtered through a 1.2-mm sieve. Filtered samples were then stored at 4°C. **Table 1** shows the characteristics of two sludge samples collected at two different times. As indicated in **Table 1**, the total suspended solid (TSS) or volatile suspended solid (VSS) contents of sample #1 were about twice those of sample #2; the chemical oxygen demand (COD), Zeta potential, and apparent viscosity of sample #1 were also higher than those of sample #2. Other characteristics, such as pH, conductivity, and soluble COD (SCOD), were lower in sample #1 than in sample #2. All experiments were carried out within a week after sampling.

Table 1 Characteristics of the anaerobic digested sludge samples

Sample	pH	Conductivity (mS/cm)	TSS (g/L)	VSS (g/L)	VSS/TSS (%)	COD (mg/L)	SCOD (mg/L)	Zeta potential (mV)	Apparent viscosity (mPa·s)
#1	6.90 ± 0.01	2.19 ± 0.03	23.32 ± 0.31	14.27 ± 0.18	61.20 ± 0.70	30124.0 ± 4.3	98.9 ± 0.6	–13.8 ± 0.6	1.74 ± 0.01
#2	7.12 ± 0.02	4.75 ± 0.01	12.00 ± 0.18	6.55 ± 0.03	54.60 ± 1.05	8964.0 ± 8.5	210.4 ± 0.7	–17.2 ± 0.7	1.25 ± 0.01

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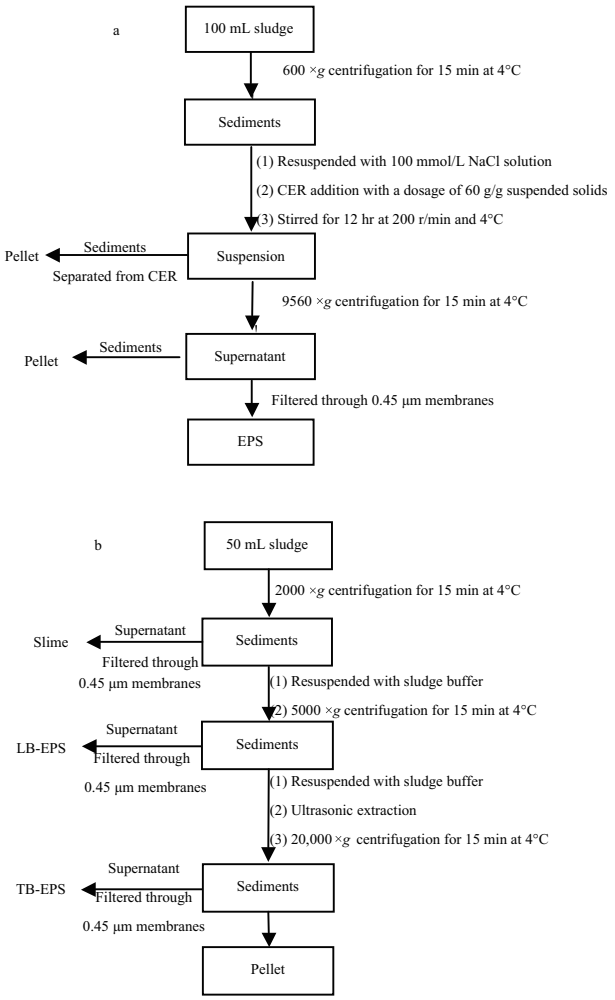

Fig. 2 EPS extraction protocols: (a) total EPS; (b) different EPS fractions. CER: cation exchange resin.

1.2 EPS extraction

Figure 2 displays the EPS extraction protocols for sludge. As indicated in **Fig. 2a**, the total EPS were extracted using the cation exchange resin technique according to the procedure of Ni et al. (2009). The total EPS were dissolved in 100 mmol/L NaCl solution. In **Fig. 2b**, the stratified EPS of sludge samples were extracted by centrifugation and sonication (Yu et al., 2007) in turn. The slime included the organic matter obtained from the supernatant after low-speed centrifugation. The LB-EPS and TB-EPS of the sludge samples were dissolved in a pH 7 buffer solution consisting of Na_3PO_4, NaH_2PO_4, NaCl, and KCl (molar ration 2:4:9:1). The conductivities of the buffers were adjusted with distilled water to match those of the sludge samples listed in **Table 1**.

1.3 EPS and sludge characterization

Proteins and humic-like substances were determined using the modified Lowry method (Frølund et al., 1995), with bovine serum albumin (Beijing Aoboxing Biotechnology Co., Ltd., China) and humic acid (Sigma, America) as standards, respectively. Polysaccharides were measured using the anthrone method (Gaudy, 1962), with glucose as the standard. DNA was determined using the diphenylamine citric method (Sun et al., 1999) with 2-deoxy-D-ribose (Beijing Ruibo Bitechnology Co., Ltd., China) as the standard. The sum of the amounts of total proteins, humic-like substances, polysaccharides, and DNA was considered representative of the total amount of EPS.

COD and SCOD analyses were conducted using a COD expedited testing apparatus (HATO CTL-12, Huatong Environmental Protecting Instruments Co., Ltd., Chengde, China). The pH was obtained using a pH meter (PB-10, Sartorius Stedim Biotech Co., Ltd., Beijing, China). Conductivity was determined using a conductivity meter (EC215, Beijing Kanggaote Science and Technology Co., Ltd., China).

The Zeta potential was measured with a Malvern instrument, using a Zetasizer Nano Z system (Malvern Co., UK). The apparent viscosity of the sludge mixture and the EPS was measured using a rotating torque cylinder (ULA#0). The apparent viscosity was measured at a shear rate of 60/sec to keep samples in suspension for 5 min; the temperature was maintained at about 20°C. Sludge samples were diluted 50 times with distilled water to measure the Zeta potential and apparent viscosity. The hydrophobicity of EPS was evaluated according to the method reported by Jorand et al. (1998) and expressed as the mass proportion of the hydrophobic part to the corresponding total components (proteins, polysaccharides, humic-like substances, DNA) in the extracted EPS.

Other sludge parameters, including TSS and VSS, were analyzed following standard methods (APHA et al., 1998). All chemical analyses were carried out in duplicate using chemicals of analytical grade.

1.4 Effect of solution conditions on physicochemical properties of EPS

The pH or ionic strength was adjusted to investigate the effects on the Zeta potentials, conductivity, and apparent viscosity of EPS solutions. The pH of EPS solutions was varied from 1 to 12 by adding either HCl or NaOH, and NaCl was added to the EPS solutions to control their ionic strength. Temperature was varied from about 20 to 60°C to study the effect of temperature on the apparent viscosity of EPS solutions.

2 Results and discussion

2.1 Contents of chemical components in different EPS fractions

Table 2 illustrates the different chemical components in EPS from the two sludge samples. It was observed that the DNA accounted for about 7% and 5% of the total EPS in the samples. Liao et al. (2001) demonstrated that DNA measurement can be used to determine whether or not bac-

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terial cells are ruptured during the EPS extraction process and further showed that such cells are not destroyed when the proportion of DNA in the extracted EPS ranges from 2% to 15% of the total EPS. Therefore, the aforementioned DNA ratios of about 7% and 5% indicate that the cation exchange resin technique did not lead to cell rupture during the total EPS extraction. The ratios of proteins to polysaccharide for the two sludge samples were 3.7 and 2.7. Moreover, the weight ratios of the total EPS to the TSS of these two sludge samples were approximately 9.3% and 13.9%, similar to a report by Karapanagiotis et al. (1989).

In the different EPS fractions extracted from these two sludge samples, proteins and polysaccharides were dispersed evenly. humic-like substance were mainly found in the slime fraction, and secondly in the TB-EPS fraction. The corresponding weight ratios of humic-like substances to slime were 68.3% and 66.4% for both sludge samples, respectively; while such ratios of humic-like substances to TB-EPS were 21.5% and 18.6%. Compared with other fractions, the LB-EPS fraction had the lowest concentration of proteins, polysaccharides, humic-like substances, and DNA. The humic-like substances contents were higher in the slime than in other components. For both the LB-EPS and TB-EPS, protein was the predominant component, at quantities of 65.6% to 54.3%, followed by polysaccharide at 15.9% to 21.6%, and humic-like substances accounted for a small proportion of EPS.

The total EPS content was not equal to the sum of the slime, LB-EPS, and TB-EPS contents. Such a discrepancy may probably be attributed to differences in the EPS extraction methods employed. As listed in **Table 2**, protein, polysaccharide, and humic-like substances were the major constituents of EPS.

2.2 Hydrophobicity of EPS

The presence of hydrophilic and hydrophobic groups in the EPS molecules indicates that the EPS are amphiphilic. The hydrophobicity of the total EPS and EPS fractions in sample #1 is shown in **Fig. 3**. For the total EPS, the hydrophobicity values of protein and DNA fractions were 73% and 55%, respectively. In the different EPS fractions, the hydrophobicity values were all below 50% for the slime, while a hydrophobic part of approximately 71% or 1% was observed in the corresponding protein or polysaccharide component of the TB-EPS, respectively. Variations in the results obtained may be due to the different distributions of chemical components across the three EPS fractions.

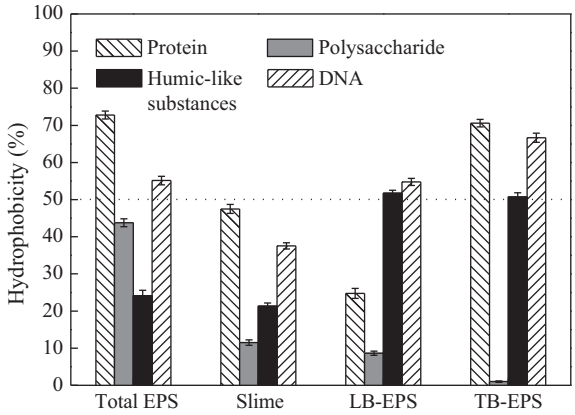

Fig. 3 Hydrophobicity of EPS.

As indicated in **Fig. 3**, compared with other components of the same EPS fractions except the LB-EPS, protein showed the highest hydrophobicity, whereas polysaccharide presented the lowest hydrophobicity compared to the other chemical components of all tested EPS. These results suggest that the hydrophobic chemical components in EPS were primarily composed of protein and DNA, and that the hydrophilic ones mainly consisted of polysaccharide, which is more or less in agreement with a report by Jorand et al. (1998). Their previous study revealed that the hydrophobic part accounted for about 7% in the corresponding EPS component and mainly consisted of protein, whereas the hydrophilic part mainly consisted of polysaccharide. Dignac et al. (1998) found that approximately 24% of the amino acids in EPS were hydrophobic. However, some researchers have found that the alkyl groups in polysaccharide have some hydrophobicity (Singh et al., 1987). Long et al. (2008) indicated that the TB-EPS had a higher hydrophobicity than the LB-EPS. Variations among reports could be partly due to differences in the sludge types and EPS extraction methods applied.

2.3 Effect of solution conditions on physicochemical properties of EPS

2.3.1 Effect of pH and ionic strength on Zeta potentials of EPS

Figure 4a demonstrates the effects of pH on the Zeta potentials of EPS in sample #1. The EPS extracted from the original sludge were negatively charged under neutral conditions, and the corresponding Zeta potentials were –19.6 mV for total EPS, –15.1 mV for slime, –22.8 mV for LB-EPS, and –20.1 mV for TB-EPS.

As seen in **Fig. 4a**, the Zeta potentials of EPS solutions

Table 2 Contents of different EPS fractions

	Sample #1				Sample #2			
	Total EPS	Slime	LB-EPS	TB-EPS	Total EPS	Slime	LB-EPS	TB-EPS
Protein (mg/g VSS)	91.31 ± 2.06	16.99 ± 1.73	16.65 ± 1.05	20.42 ± 1.11	126.08 ± 4.78	34.19 ± 2.34	33.56 ± 1.85	45.42 ±2.15
Polysaccharide (mg/g VSS)	24.71 ± 0.12	13.84 ± 0.19	4.04 ± 0.11	8.14 ± 0.21	46.81 ± 2.78	20.41 ± 0.61	17.18 ± 0.36	26.04 ± 0.24
Humic-like substances (mg/g VSS)	24.69 ± 2.40	19.42 ± 1.11	2.90 ± 0.21	6.11 ± 0.34	67.89 ± 3.54	36.78 ± 1.09	8.26 ± 0.36	10.32 ± 0.11
DNA (mg/g VSS)	11.10 ± 1.03	2.61 ± 0.00	1.79 ± 0.23	2.94 ± 0.04	13.63 ± 2.35	5.30 ± 0.35	1.55 ± 0.12	2.36 ± 0.12

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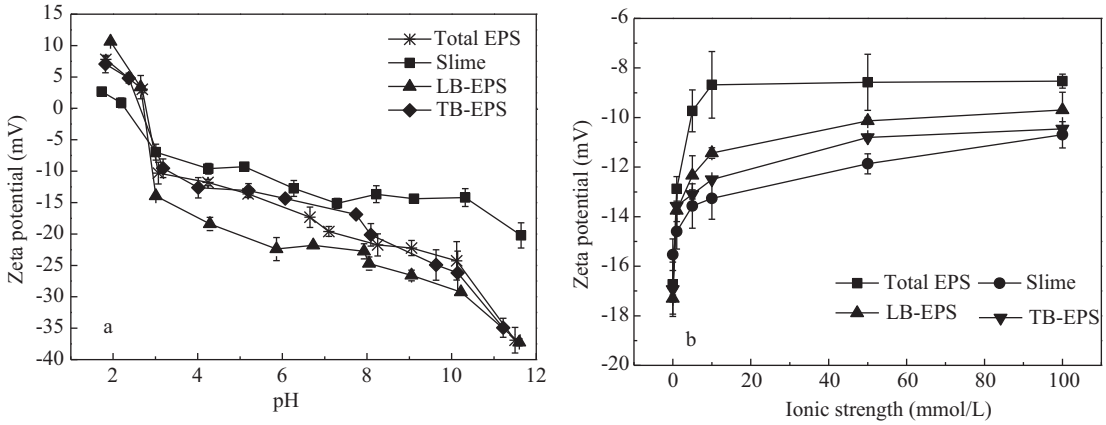

Fig. 4 Effect of pH (a) and ionic strength (b) on Zeta potentials of EPS.

decreased with increasing pH, and the corresponding isoelectric point values were in the range of acidic pH values; the corresponding order was $pH_{total\ EPS}$ (2.8) > $pH_{LB\text{-}EPS}$ (2.7) > $pH_{TB\text{-}EPS}$ (2.6) > pH_{slime} (2.2), consistent with the order of protein/polysaccharide ratios of EPS in the original sludge: total EPS (3.7) > LB-EPS (2.5) > TB-EPS (2.1) > slime (1.2). EPS carried increasingly positive charges less than 11 mV as pH decreased below the isoelectric point. In contrast, the Zeta potential values were negative and the maximum absolute value approached 37 mV at pH values above the isoelectric point. Tenney and Stumm (1965) reported that the isoelectric point (pH) of bacteria ranged from 2 to 4. The isoelectric point (pH) values obtained in this study were within this reported range. However, in contrast to our findings, Liu et al. (2010) observed that the isoelectric point (pH) values of the LB-EPS and TB-EPS contained in anaerobic sludge were 2.02 and 2.00, respectively.

Moreover, the Zeta potential values showed significant decreases for the total EPS and EPS fractions as pH values increased from 2 to 3 or from 10 to 12, but they changed only slightly at other pH values. When the pH was over 3.0, the LB-EPS showed the lowest Zeta potential values whereas the slime presented the highest.

The effects of ionic strength on the Zeta potentials of EPS in sample #2 were determined and are presented in **Fig. 4b**, where the horizontal ordinate is expressed as the concentration of NaCl which was added in the EPS solutions for ionic strength adjustment. The Zeta potentials of EPS in the raw sludge were –16.7 mV for total EPS, –15.5 mV for slime, –17.3 mV for LB-EPS, and –16.9 mV for TB-EPS. The Zeta potentials became less negative with increasing ionic strength. A sharp increase was observed when the ionic strength was increased to 10 mmol/L. Afterwards, as more and more NaCl was added to the system, the Zeta potentials of the total EPS began to produce fewer responses and changed minimally; for the EPS fractions, the rate of increase also slowed down. These findings are consistent with the compression of the electrical double layer of Na^+, that is, by increasing the ionic strength of EPS samples, the Zeta potentials of the system first increase and then remain stable without charge reversal after exceeding a certain concentration.

The order of Zeta potentials of all EPS samples of the same ionic strength was total EPS > LB-EPS > TB-EPS > slime, and the order of the corresponding protein/polysaccharide ratios was total EPS (2.7) > LB-EPS (2.0) > TB-EPS (1.8) > slime (1.7), which was consistent with the former. These findings indicate that the greater the protein/polysaccharide ratio of EPS is, the

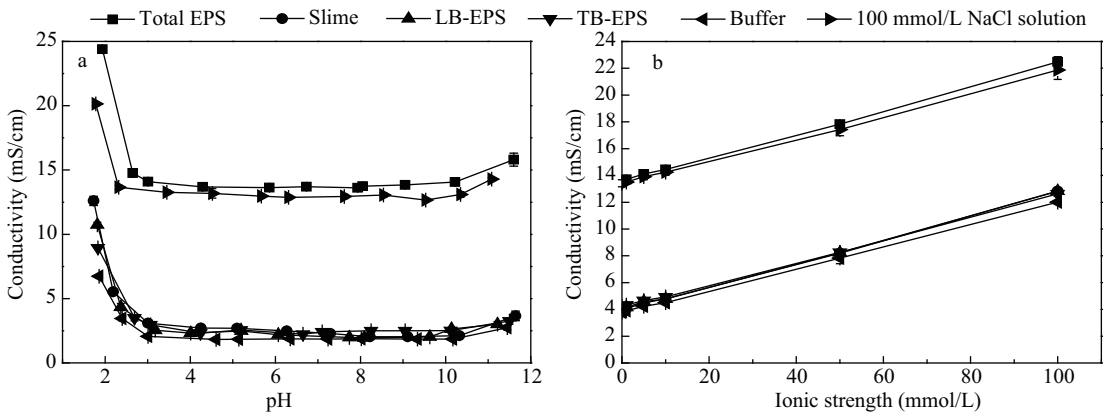

Fig. 5 Effect of pH (a) and ionic strength (b) on the conductivity of EPS.

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greater the Zeta potential would be.

2.3.2 Effect of pH and ionic strength on conductivity of EPS

Variations in the conductivity of EPS solutions in sample #1 with varying pH are given in **Fig. 5a**. The total EPS containing conducting substances was dissolved in 100 mmol/L NaCl solution, and the conductivity of the total EPS was greater than that of the 100 mmol/L NaCl solution. As pH increased from 1 to 3, the conductivity of the total EPS fell drastically, which may be attributed to the sharp decrease in H^+ concentration. The conductivity remained almost unchanged with further increases in pH. In the pH range from 10 to 12, the increase in conductivity observed is likely due to the increase in OH^- concentration. Conductivities of the EPS fractions showed a trend similar to that observed in the total EPS. In addition, the EPS fractions showed slightly higher conductivity than the buffer solution.

The conductivity of the total EPS in sample #2 and 100 mmol/L NaCl solution was found to increase linearly with increasing ionic strength (**Fig. 5b**). The variation pattern of the conductivity of the EPS fractions was similar to that achieved in the total EPS. The similarity of conductivity values between total EPS and its solvent-NaCl solution or EPS fractions and their solvent-buffer solutions indicates that addition of extra solvents mainly contributes to the conductivity of the corresponding solutions.

2.3.3 Effects of pH, ionic strength, and temperature on apparent viscosity

The apparent viscosity of EPS solutions in sample #1 showed no significant change around values of approximately 1.20 mPa·s under varying pH and ionic strength.

The apparent viscosity of EPS in sample #1 was greatly influenced by temperature changes. These results are plotted in **Fig**. **6**. The apparent viscosity of EPS declined monotonically as the temperature increased from 20 to 40°C and then reached near-constant levels at temperatures ranging from 40 to 60°C. It has been reported that at temperatures of 50 to 70°C and 70 to 95°C, DNA and protein started to rupture, respectively (Häner et al., 1994). However, in this study the DNA accounted for little and the operation temperature ranged from 20 to 60°C, so the protein of EPS was not denatured and the temperature reached in this work did not severely affect the components of EPS. Generally, as the temperature increased, molecular and molecular chain movements occurred, leading to the decreased twisting of chains, increased intermolecular distances, and reductions in apparent viscosity. Although the thermal motion was intensified, the structure and distances of the molecules remained stable and the apparent viscosity of the system gradually tended to level off when the system was heated to higher temperatures. The total EPS had the highest apparent viscosity, followed by the slime, LB-EPS, and TB-EPS at temperatures ranging from 20 to 40°C. Variations in the apparent viscosity of the EPS solutions are attributed to many factors, such as the concentration of macromolecular substances, molecular structure, and temperature (Hoffman, 1992).

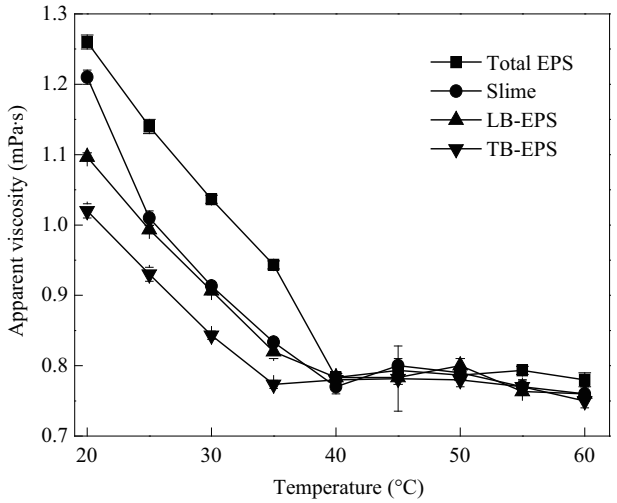

Fig. 6 Effect of temperature on the apparent viscosity of EPS.

3 Conclusions

Protein and polysaccharide were distributed uniformly in the three EPS fractions. Humic-like substances were mainly dispersed in the slime fraction, followed by the TB-EPS, and found least in the LB-EPS fraction.

All Zeta potentials of EPS decreased with increasing pH. The effects of ionic strength on Zeta potentials of the EPS are consistent with the compression of the electrical double layer. The greater the protein/polysaccharide ratio of EPS, the greater the Zeta potential and the higher the isoelectric point value were. The addition of H^+/OH^- and extra solvents may mainly be responsible for the variations in the conductivity of EPS with pH and ionic strength, respectively.

Temperatures ranging from 20 to 40°C had a significant influence on the apparent viscosity of EPS, and the ranking order of the apparent viscosity of EPS was total EPS > slime > LB-EPS > TB-EPS.

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