Carbohydrate Polymers 101 (2014) 50–56

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Biosorption of Cu^{2+}, Pb^{2+} and Cr^{6+} by a novel exopolysaccharide from *Arthrobacter* ps-5

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ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 26 August 2013
Accepted 10 September 2013
Available online 14 September 2013

Keywords:
Arthrobacter
Exopolysaccharide
Biosorption
Metal ions

ABSTRACT

The biosorption behaviors and mechanisms for Cu^{2+}, Pb^{2+} and Cr^{6+} by a novel exopolysaccharide (EPS) from *Arthrobacter* ps-5 have been studied in the paper. The influences of EPS concentration, solution pH and ionic strength on adsorption property were investigated. The EPS showed strong biosorption capability, up to 169.15 mg/g of Cu^{2+}, 216.09 mg/g of Pb^{2+} and 84.47 mg/g of Cr^{6+}, respectively. With the additional concentration of Ca^{2+} and K^{+} increased, the biosorption ability on Cu^{2+}, Pb^{2+} and Cr^{6+} decreased significantly and followed the order of $K^{+} < Ca^{2+}$. Infrared spectrometry analysis demonstrated that the groups of O—H, C=O, C—O—C and C=O—C of the EPS involved in metal biosorption process were the main functional groups for binding metal ions.

1. Introduction

Heavy metals in the environment are difficult to remove by chemical or biological means. Today, many heavy metals constitute a global environmental hazard (Mejáre & Bülow, 2001). Heavy metals usually can be toxic, carcinogenic or mutagenic even at trace levels. It has been established that all metals or elements with metallic characteristics can form compounds that are toxic to human (Goyer, 1991).

Copper (Cu), lead (Pb) and chromium (Cr) are applied in some metabolic processes, such as electroplating, manufacturing, mining, automotive and so on (Ogunbileje et al., 2013). Nevertheless, acute exposure to Cu^{2+}, Pb^{2+} and Cr^{6+} causes health problems such as anemia, diseases of liver and kidneys, brain damage and ultimately death (Raungsomboon, Chidthaisong, Bunnag, Inthorn, & Harvey, 2008; Zhou, Wang, Shen, Hou, & Zhang, 2009). As a result, removal of these toxins from industrial effluents has become an important priority that is reflected in a tightening and enforcement of environmental regulations. Large volumes of industrial heavy metal-bearing waste waters require efficient and high-value treatment (Davis, Volesky, &Vieira, 2000).

So far, there have been a number of studies considering the possibility of removal and recovery heavy metals from diluted solutions (Saeed, Akhter, & Iqbal, 2005; Wu, Kuo, & Lo, 2009). The traditional methods for removing include precipitation, oxidation/reduction, ionic exchange, filtration, electrochemical processes, membrane separations and evaporation. These methods have several disadvantages like high cost, incomplete removal, low selectivity and high energy consumption, and they generate toxic slurries which are difficult to eliminate. These methods are unsuitable, especially when the concentration of metals is lower than 100 mg/L (Cushnie, 1985; Paknikar, Palnitkar, & Puranik, 1993). Ion exchange, membrane technologies and activated carbon adsorption process are extremely expensive, especially when treating a large amount of wastewater containing heavy metals at low concentration, so they cannot be used at large scale. Alternative process is biosorption, which utilizes various natural materials of biological origin, including bacteria, fungi, yeast, algae and so on. These biosorbents possess metal sequestering properties and can decrease the concentration of heavy metal ions in solution from ppm to ppb level. They can sequester effectively and quickly dissolved metal ions out of dilute complex solutions. Therefore biosorption is an ideal candidate for treatment of large volume and low concentration complex wastewater (Veglio & Beolchini, 1997; Volesky & Holan, 1995).

Most of bacteria and cyanobacteria produce extracellular polysaccharides (EPSs), which play a key role not only in aggregation but also in binding of heavy metals (Freire-Nordi, Henriques Vieira, & Nascimento, 2005; Salehizadeh & Shojaosadati, 2003). Most of microorganisms from soil or wastewater sludge could produce EPSs (Li, Zhou, Zhang, Wang, & Zhu, 2008), and the adsorption of heavy metals by EPSs is considered non-metabolic, energy independent and can be caused by interaction between metal cations and negative charge of acidic functional groups of EPSs (Kim, Kim,

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http://dx.doi.org/10.1016/j.carbpol.2013.09.021

Kim, & Oh, 1996). EPSs are long-chain polysaccharides containing branched, repeating units of sugars or sugar derivatives such as glucose, fructose, mannose and galactose, which are secreted into their surrounding environment during the bacterial growth (Ismail & Nampoothiri, 2010). EPSs also contain different functional groups such as carboxyl, sulphate, carbonyl, amino, and amido (Kurane & Matsuyama, 1994).

In this paper, biosorption characteristics of EPSs for Cu^{2+}, Pb^{2+} and Cr^{6+} in different conditions which were generally encountered in wastewater treatment process were determined. The mechanisms and potential functional groups were also investigated.

2. Materials and methods

2.1. Microorganism and chemicals

Arthrobacter ps-5 was screened from the rhizosphere soil of *ginkgo biloba* trees and stored in our laboratory. The strain was cultivated at 28 °C for 2 d on basic medium consisting of (per liter) 5.0 g glucose, 3.0 g beef extract, 10.0 g peptone, 5.0 g sodium chloride, 20.0 g agar and then maintained at 4 °C. Seed culture was carried out at 28 °C on a rotary shaker incubator at 150 rpm for 2 d in 250 mL conical flask with 50 mL liquid medium consisting of (per liter) 10.0 g glucose, 3.0 g beef extract, 10.0 g peptone and 5.0 g sodium chloride at initial pH 6.0–7.0.

DEAE–cellulose was purchased from Shanghai Sheng Gong Biological Engineering Technology Service Company, Shanghai, China. All other chemicals used were all analytical grades and purchased from Tianjin Kermel Chemical Reagent Company, Tianjin, China.

2.2. Production, isolation and purification of the EPS

2.2.1. Liquid fermentation of Arthrobacter ps-5

The culture medium fermented from *Arthrobacter* ps-5 was used to harvest EPSs. Liquid medium for *Arthrobacter* ps-5 was 20.0 g sucrose, 25.35 g yeast extract, 3.0 g beef extract, 1.98 g K_2HPO_4, 0.5 g $CaCl_2$ and 0.5 g $MnSO_4$ per liter. *Arthrobacter* ps-5 was propagated in 250 mL conical flasks with 50 mL of liquid medium and shaken at 160 rpm for 48 h on a rotary shaker. The fermentation temperature, initial pH value, inoculation proportion were 28 °C, 7.0 and 4.0% (v/v), respectively according to our previous report (Ye, Liu, Wang, Wang, & Zhang, 2012).

2.2.2. Isolation and purification of the EPS

After cultivation as described above, the culture medium was separated by centrifugation (4000 rpm, 20 min). The supernatant was collected and mixed with 3 volumes of 95% ethanol (v/v), and then was left overnight at 4 °C for polysaccharide precipitation.

The precipitate in the centrifuging tube was rinsed thoroughly with water, deproteinized with Sevag reagent (chloroform:*n*-butanol at 5:1, v/v) for 3 times to remove the residual protein. The EPS in supernatant was precipitated again by ethanol and left overnight. The resulting precipitate was re-dissolved in distilled water and dialyzed (MWCO 7000) using running tab water for 48 h and distilled water for another 48 h (Ye et al., 2012). The dialyzed solution was concentrated and lyophilized. The lyophilized EPS named the crude EPS was used for further EPS fractionation.

The yield of EPS was determined by an anthrone colorimetric method, through sulphuric acid hydrolysis of EPS in the presence of anthrone agent at 100 °C. The absorbance of the sample solution was measured at 620 nm and calibrated using glucose as a standard (Leung, Shuna, Ho, & Wu, 2009; Priester et al., 2006).

The EPS fractionation was carried out in a DEAE-52 anion-exchange chromatography column (2.6 cm × 50 cm), with successive elution using distilled water and NaCl solutions in a linear gradient of 0–0.5 M, at a flow rate of 0.5 mL/min. Fraction volume was set at 5 mL per each tube. The main fraction was further purified through a Sephadex G-100 column (2.6 cm × 50 cm) and the column was eluted with 0.05 M NaCl solution at a flow rate of 0.4 mL/min. The carbohydrate content was then quantified using the anthrone colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The main fraction was collected, dialyzed, concentrated and lyophilized, resulting in purified EPS which was used for subsequent analyses.

2.3. Analysis of the EPS physical properties

2.3.1. Purity of the EPS

The UV absorption spectrum was recorded using a Lambda 35 spectrophotometer (Perkin Elmer, U.S.) between 190 and 500 nm, in order to examine the existence of proteins and nucleic acids (Wang, Cui, & Xu, 2007).

2.3.2. Molecular weight

The molecular weight of EPS was measured using high liquid chromatography (HPLC) with a TSK-Gel G3000SWXL column (7.5 nm × 300 nm, Waters-2690, USA). The column was eluted with 0.05 M NaCl solution at a flow rate of 0.4 mL/min. The eluent was monitored with a refractive index detector (RID) (Lin et al., 2009). Dextrans with different molecular weights were used as references for determination of the molecular weight of the EPS.

2.3.3. Monosaccharide composition of EPS

The monomer composition of the EPS was analyzed by thin layer chromatography (TLC) using silica gel coated aluminum plates. The purified EPS (2 mg) was hydrolyzed in 250 μL of 2 M trifluoroacetic acid (TFA) at 100 °C for 5 h. Excess TFA was removed by rotary evaporation, and the hydrolysate was washed thoroughly with water and lyophilized. The lyophilized powder was dissolved in 100 μL of water, and 5 μL aliquot was used for thin-layer chromatography (TLC). Development was done twice on a silica gel TLC plate (20 cm × 20 cm) using a developing solvent of *n*-butanol:ethanol:water (2:1:1, v/v). Carbohydrates were visualized by heating the TLC plate after spraying with 5% (v/v) sulfuric acid in ethanol. Glucose, galactose, and mannose were used as standard monosaccharides (Fukuda et al., 2010).

The molar ratio of the hydrolysate was analyzed using HPLC (Waters-2690, USA) equipped with RID and a Sugar-pak-1 column (Waters-2690, USA). Ultrapure water was used as the solvent at a flow rate of 1.0 mL/min. 25 μL of sample or glucose, galactose each as a standard was injected. The molar ratio of glucose and galactose was calculated from the ratio of time and areas of the peak (Jin, Wang, & Xu, 2010).

2.3.4. Infrared analysis

The infrared (IR) spectrum of EPS was determined using a Fourier transform infrared (FTIR) spectrophotometer (Spectrum One-B, Perkin Elmer, U.S.) for detection of various functional groups. The purified EPS was ground with KBr powder and pressed into pellets for FTIR measurement in the frequency range of 4000–400 cm^{-1} (Zhbankov, Adnanov, & Marchewka, 1997).

2.4. Metal adsorption

Synthetic stock solution of Cu^{2+}, Pb^{2+} and Cr^{6+} (100 mg/L) was prepared by dissolving calculated quantity of copper sulphate and lead nitrate and potassium dichromate (AR Grade) in double distilled water and working standards were obtained by further dilutions.

The adsorption experiments were carried out by adding 2.0 mL crude EPS (The purity of EPS was 82.3%, w/v) solution (10 mg/L) in a

250 mL flask containing 50 mL of metal solution (10 mg/L) and then shaking on a shaker. Each of the sets was prepared in triplicates. The control was one sample without heavy metal ions. The flasks were operated at 200 rpm.

Samples were centrifuged (10,000 rpm) and solution was filtered through a 0.45 μm cellulose acetate membrane. Then residual Cu^{2+}, Pb^{2+} and Cr^{6+} in the supernatant were determined using an atomic absorption spectrophotometer (models 180-80, Hitachi, Japan).

To investigate the effect of the initial concentration of EPSs on the biosorption performance for metals, the concentration of EPSs was prepared in the range of 0.2–1.5 g/L.

To investigate the effect of pH on the adsorption of EPSs (1.0 g/L) for heavy metals, the solution pH was adjusted with adding a small amount of dilute HCl (0.1 mol/L) or NaOH (0.1 mol/L) solution using a pH meter in the pH range 3.0–7.0.

The effects of salt concentration on biosorption amounts for Cu^{2+}, Pb^{2+} and Cr^{6+} were analyzed over the KCl and $CaCl_2$ concentration range from 0 to 4.0 mmol/L.

The biosorption capacity, Q (mg/g), can be determined by the following equation:

$$Q = \frac{V(C_0 - C_e)}{W}$$

C_0 and C_e are the initial and end concentrations of metals in the sample solutions (mg/L), respectively, V is the volume of sample solutions (L) and W is the mass of sorbent (g).

2.5. Experimental statistics

Three replicates were measured for each sample during the above-mentioned assays. All experimental data were presented as mean values ± standard deviations.

3. Results

3.1. Purification and characterization of EPSs

3.1.1. Purification of EPSs

In the present study, the crude EPS was prepared from the fermentation broth of *Arthrobacter* ps-5. The EPS solution (10 mg/L) were firstly separated through an anion-exchange chromatography of DEAE-52 with distilled water and a gradient elution of NaCl in the range of 0–0.3 M.

The EPS was separated to two independent elution fractions EPS-1 and EPS-2 (Fig. 1(A)). Since DEAE is an anion exchanger, positive charged groups and neutral groups in the samples are removed with eluate, whereas negative ions in the samples can exchange with balance ions and combine to the chromatography column. Sub-fraction EPS-1 was a neutral polysaccharide as it was eluted with water, and sub-fraction EPS-2 should be an acidic polysaccharide as it was eluted with NaCl solution (Chang, 1987). EPS-1 was the major component by the peak area in anion-exchange chromatography. The ratio of EPS-2 and EPS-1 was 0.22. To prepare conveniently and improve the content, EPS-1 fractions was collected, concentrated and further purified through a Sephadex G-100 column eluted with 0.05 M NaCl solution. There was one sub-fraction EPS-1-1 (Fig. 1(B)).

3.1.2. Physical properties of the EPS

The purified EPS was light yellow in color and in a form of odorless powder. It was soluble in water but insoluble in ethanol and other organic solvents, such as ether and acetone, which conferred with general properties of polysaccharides. The EPS solution was a clear, homogeneous liquid with a color of light yellow and no precipitation occurred after centrifugation. An ultraviolet scan spectrum analysis of the EPS was shown in Fig. 2, indicating an absence of proteins and nucleic acids due to the negative response – no absorption peaks at 260 or 280 nm.

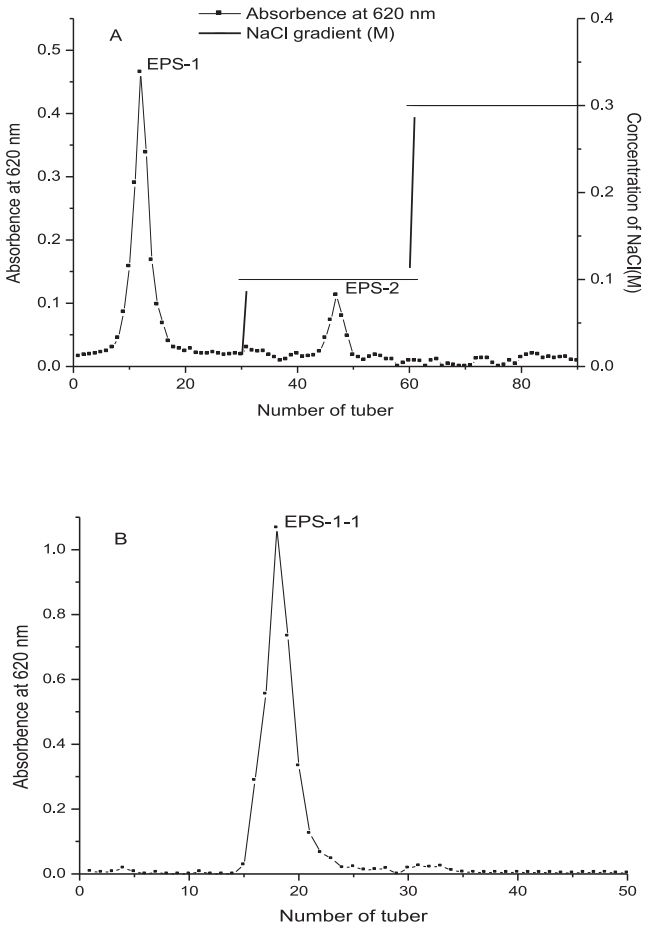

Fig. 1. Purification of EPS by DEAE-52 anion-exchange chromatography (A) and elution curve of fractions (EPS-1) on Sephadex G-100 column (EPS-1-1) (B).

3.1.3. Molecular of EPS

The molecular weight of EPS-1-1 was measured by HPLC with a size exclusion column (Fig. 3(A)). Based on a calibration curve obtained using dextrans as references at with various molecular

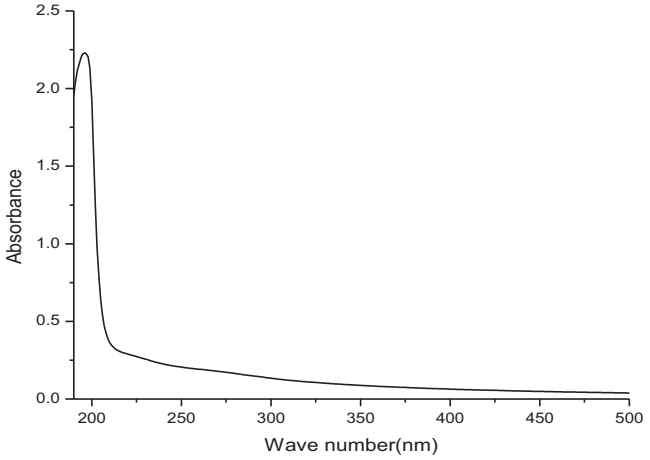

Fig. 2. The ultraviolet scan spectrum of EPS.

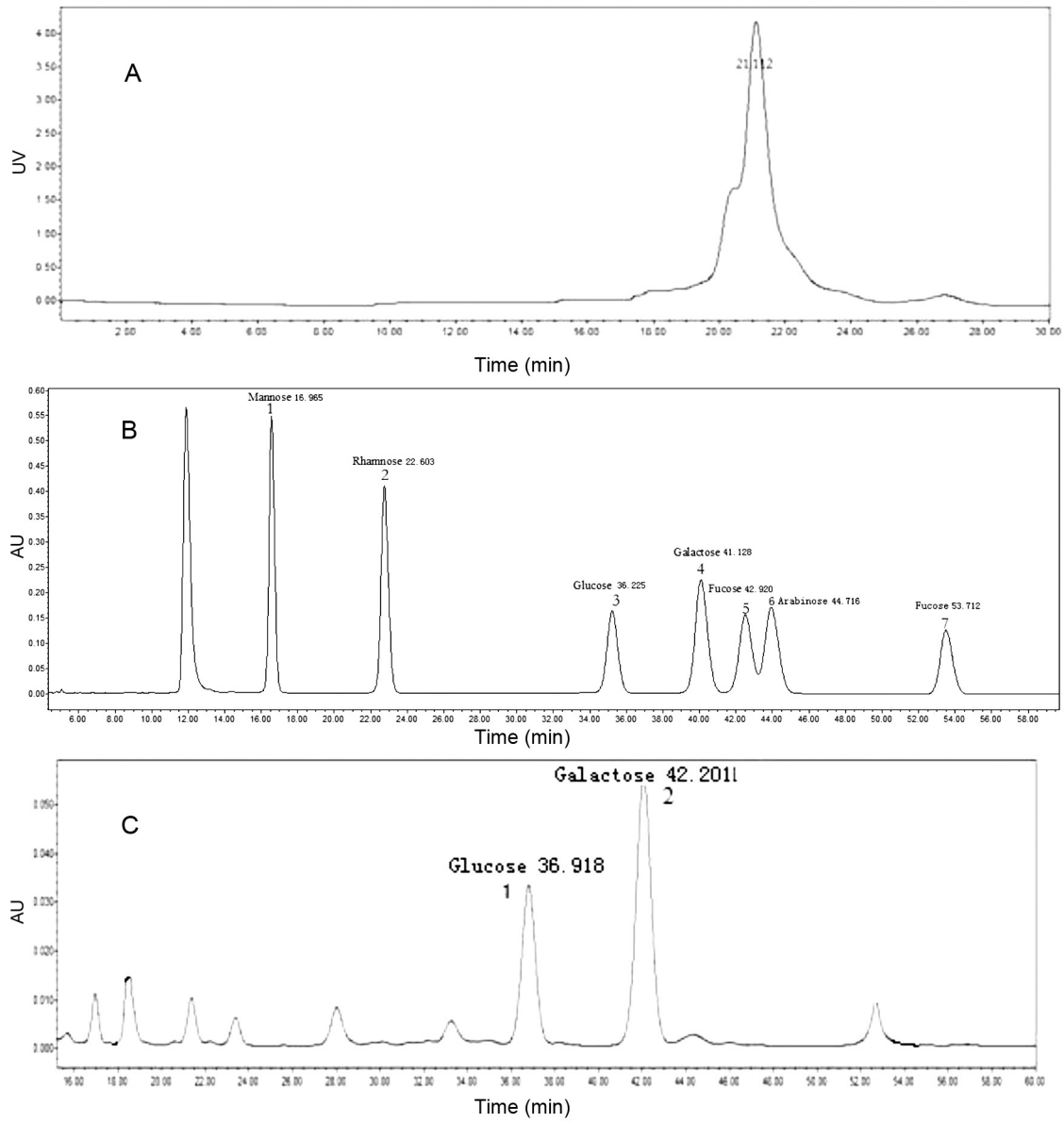

Fig. 3. HPLC chromatograms of EPS-1-1 about molecular weight distribution (A), PMP derivatives of monosaccharide standard samples (B) and PMP derivative of hydrolysate of fraction EPS (C).

weight cut-offs, the average molecular weight of EPS-1-1 was estimated to be 5.81×10^5 Da.

3.1.4. Monosaccharide composition of EPS

Only glucose and galactose were detected in the EPS-1-1 by TLC. The molar ratio of glucose to galactose was 2.81 and HPLC chromatograms were presented in Fig. 3(B) and (C).

3.2. Metal adsorption of EPS

3.2.1. Effect of initial EPS concentration

The influence of EPS concentration in equilibrium adsorption was shown in Fig. 4(A). Results indicated that with the dosage of the EPS increasing from 0.4 to 1.5 g/L, the biosorption amounts for all heavy metal ions increased significantly, which increased from 81.05 to 139.75 mg/g for Cu^{2+}, from 79.05 to 163.5 mg/g for Pb^{2+} and from 31.57 to 70.49 mg/g for Cr^{6+}, respectively. It could be attributed to more available binding sites for metal ions biosorption presented by the increased EPS at low concentration. Although adsorption sites increased at excessive EPS, but the bound metal ions may be unstable and the metal ions surrounded by unit mass of EPS decreased (Zhou et al., 2009).

3.2.2. Effect of pH on metals adsorption

The effect of solution pH on metals adsorption was shown in Fig. 4(B). The biosorption capacity of the EPS for the metals increased as pH increased, and then it decreased at high pH. The maximum uptakes were 169.15 mg/g for Cu^{2+}, 216.09 mg/g for Pb^{2+} and 84.47 mg/g for Cr^{6+} at pH 5.0, 5.5 and 6.0, respectively.

3.2.3. Effect of ionic strength on metals adsorption

Salts such as KCl and $CaCl_2$ with different concentrations (1.0–4.0 mmol/L) were added in the solution to test influence of ionic strength on the biosorption capability of the EPS on Cu^{2+}, Pb^{2+} and Cr^{6+}, respectively. The results were shown in Fig. 5. In addition, different types of salts also presented different effects on the metals uptake under the same concentration. Salt ions with positive charge can decrease the biosorption capacity of EPS for heavy

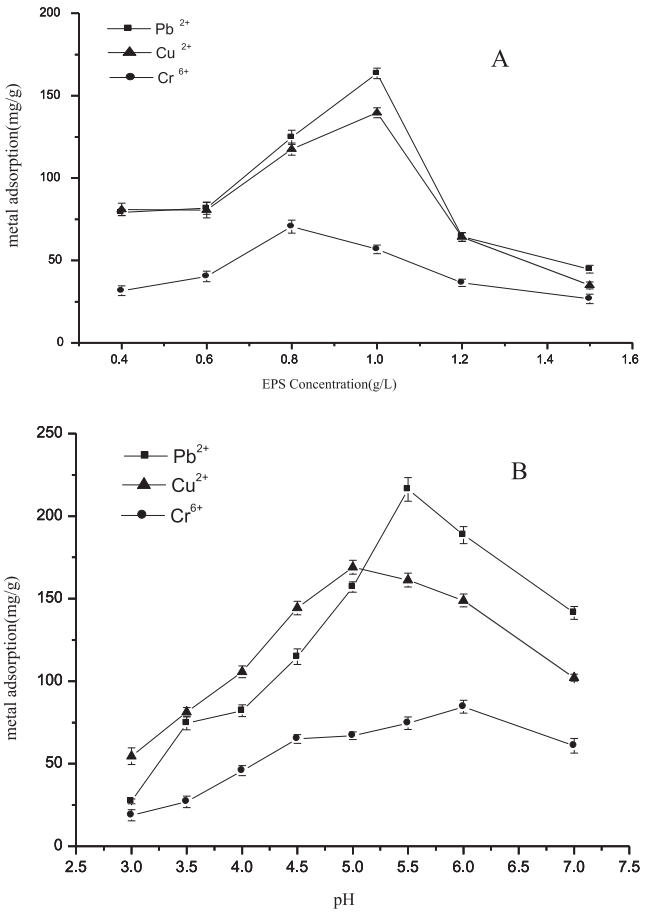

Fig. 4. Influence of EPS concentration (A) and pH (B) on adsorption of heavy metal ions.

metal ions by competing the adsorption site. At the same salt concentration, Ca^{2+} can adsorb more electronegative adsorption site than K^+. So, its effect on electrical double layer in metal biosorption is much stronger than K^+ (Wang, Zhou, Shen, Hou, & Zhang, 2009). As a result, addition of salts decreased biosorption amount of Cu^{2+}, Pb^{2+} and Cr^{6+} followed the order of $K^+ < Ca^{2+}$.

3.2.4. Infrared analysis

The IR analysis of the EPS was shown in Fig. 6. The peaks assignments of the EPS were as follows: 3400–3200 cm^{-1} was related to the stretch vibration of O—H or hydrogen bond existing in all polymers. The signals at 3000–2800 cm^{-1} were related to the stretch vibration of C—H bond, and the signals at 1850–1600 cm^{-1} were due to the stretch vibration of C=O bond. The signals at 1400–1370 cm^{-1} were attributed to the vibration of C—O bond and the signals at 900–1150 cm^{-1} for C—O—C bond (Sun, Mao, & Chen, 2009). There were shifts in wave numbers of peaks at 3399.72 cm^{-1}, 3399.72 cm^{-1}, 2936.10 cm^{-1}, 1640.02 cm^{-1}, 1418.17 cm^{-1} and 1063.64 cm^{-1} after loading Cu^{2+}, Pb^{2+} and Cr^{6+} (Table 1).

Table 1
Wave numbers (cm^{-1}) of dominant peaks obtained from FTIR transmission spectra of EPS + Cu^{2+}, EPS + Pb^{2+}, EPS + Cr^{6+}.

Sample	Functional groups				
	O—H	CH_2	C=O	C—O	C—O—C
EPS	3399.72	2936.10	1640.02	1418.17	1063.64
EPS + Cu^{2+}	3420.99	2941.17	1636.47	1384.74	1060.13
EPS + Pb^{2+}	3421.92	2943.01	1636.84	1384.78	1060.02
EPS + Cr^{6+}	3419.23	2927.34	1635.18	1381.56	1131.28

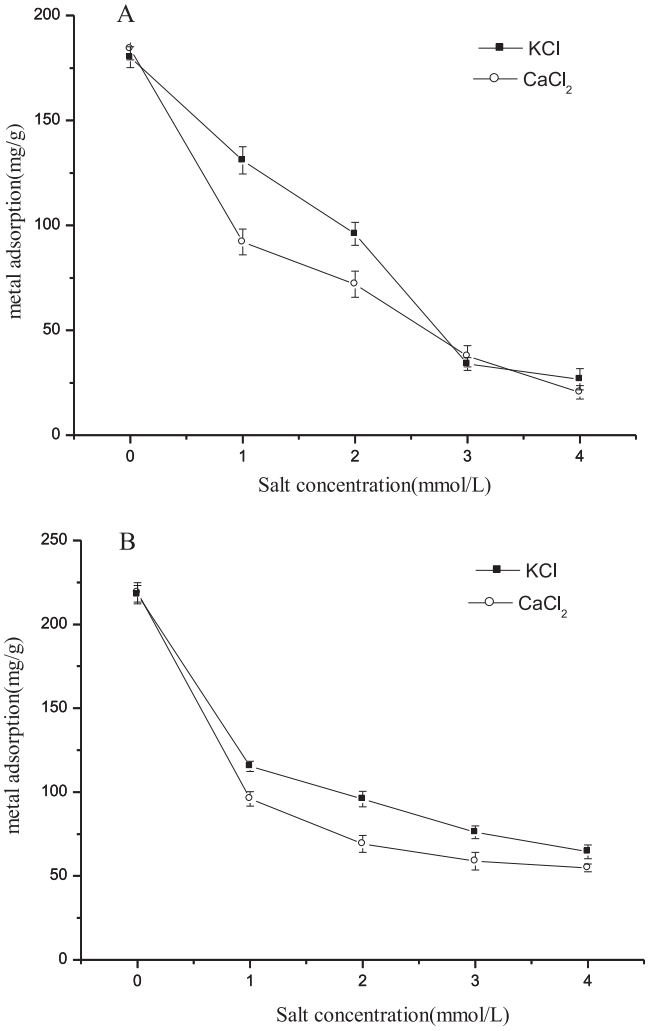

Fig. 5. Effect of ionic strength on Cu^{2+} (A), Pb^{2+} (B) and Cr^{6+} (C) adsorption by EPS.

4. Discussion

The biosorption capacity of EPS is selective. It depends on structure and functional groups of adsorbent and state, size and bond energy of metal ions. However, binding sites for metal ions biosorption increased and metal ions surrounded by unit mass EPS decreased when EPS concentration was too high (Ye, Yin, Peng, & Jia, 2001). But metal ions may be unable combined and could be divorced from EPS. The biosorption capacities of EPS for three kinds of metal ions followed the order of $Pb^{2+} > Cu^{2+} > Cr^{6+}$. The main reason for this phenomenon could result from atomic mass, molecular size and deformability of metal ions (Ye et al., 2001).

The solution pH, which affects the biosorption performance of biosorbent, is an important controlling parameter in the process (Han et al., 2006). The effect of pH on the metal biosorption can be achieved through the competition between H_3O^+ ions and metal ions for the biosorptive sites. At low pH, little biosorption occurs, as the large amount of H_3O^+ exists in solution and the competition biosorption takes place (Cruz, Costa, Henriques, & Luna, 2004). Moreover, the protonation state of ligands on the surface will endorse reaction with metal ions (Krishnani, Meng, Christodoulatos, & Buddu, 2008). As pH increased, these negatively charged functional groups would be exposed in cations to accumulate in the double layer surrounding the polyelectrolyte (Lamelas, Benedetti, Wilkinson, & Slaveykova, 2006). Hence the biosorption

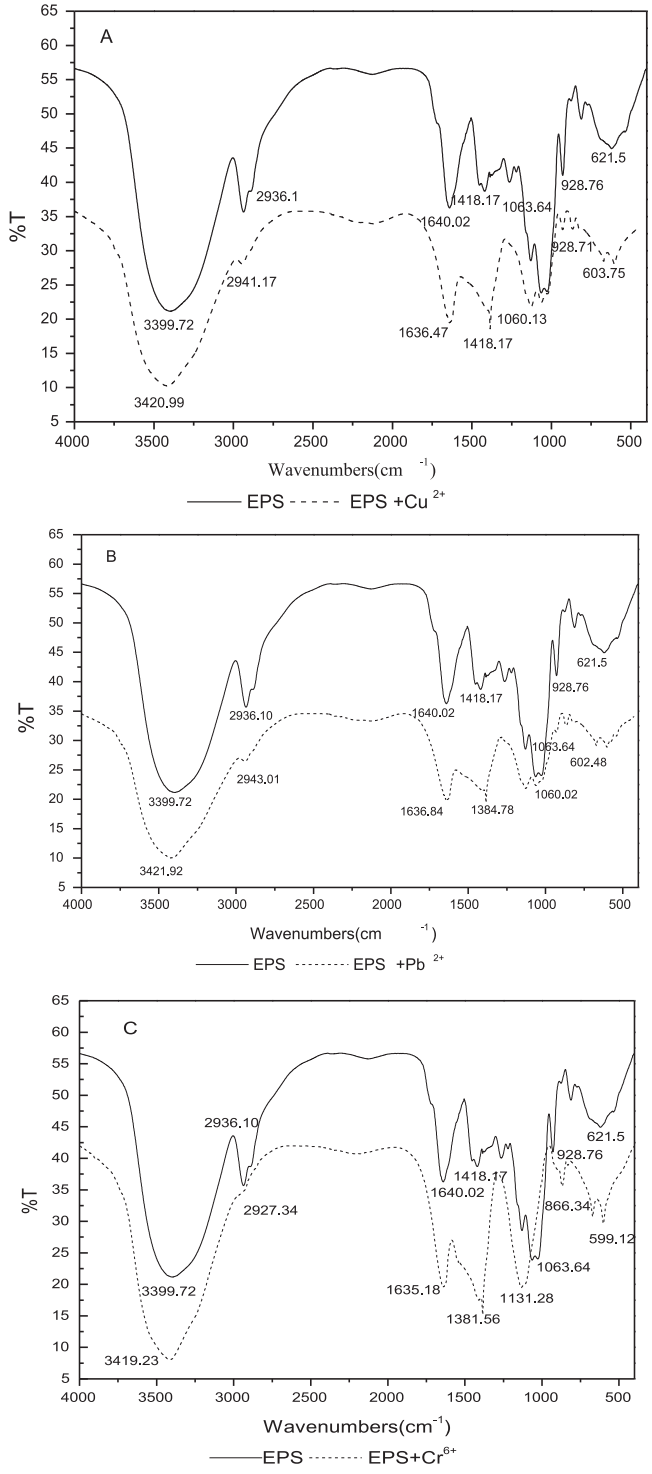

Fig. 6. FTIR spectra of EPS and EPS loaded with Cu^{2+}, Pb^{2+} or Cr^{6+}.

on the EPS surface was increased, which was corresponding to the maximum biosorption capacity obtained at pH 5.0 for Cu^{2+}, 5.5 for Pb^{2+} and 6.0 for Cr^{6+}, respectively. At high pH, the precipitation of metals occurs by the formation of metal hydroxides, so it is impossible for the biosorption (Veglio & Beolchini, 1997).

Ionic strength, which is a general factor of the solution, can affect the affinity between adsorbate and biosorbent (Kiran, Kaushik, & Kaushik, 2007). Furthermore, the salts ions added in solution may compete with Cu^{2+}, Pb^{2+} and Cr^{6+} for electrostatic binding onto EPS, so the biosorption capacity for heavy metals decreased (Ye et al., 2001). With ionic strength increased, the biosorption capacity of EPS for the metals decreased significantly, which presented the ionic strength-dependent of metal ions biosorption and the adverse effects of high ionic strength. With the salt concentration increased, both the property of electrical double layer and the activity coefficient were changed, which limited the metals to transfer from solution onto EPS surface (Krishnani et al., 2008).

The functional group is one of the keys to analyze the mechanism of metal binding onto the EPS. Peaks appearing the FTIR transmission spectra of the EPS were related to various groups and bonds in accordance with respective wave numbers (cm^{-1}). The FTIR spectra revealed the presence of many functional groups such as hydroxyl, carboxyl and glycosidic bond on the EPS surface. In comparing between the EPS and the metal-laden EPS, it can be observed that there were some shifts in wave number of dominant peaks associated with the metal-laden EPS. These shifts in wave number were corresponding to the metal binding process taking place on the surface of biosorbent (Pavasant et al., 2006). The O—H stretching group and the C=O stretching group were involved with metal biosorption. The wave numbers of the other groups such as C—H group, C—O—C group and C—O—O group were also changed (Fig. 6 and Table 1). This phenomenon showed that there existed a metal binding process on the surface of EPS. This is because O of the polysaccharide structure complexes with Cu^{2+}, Pb^{2+} and Cr^{6+} in adsorption process to reduce electron cloud density of functional groups containing oxygen and to change their vibration frequency and vibration intensity (Pal & Paul, 2008; Sun, Zhou, & Zhang, 2006). The wave numbers of several functional groups by Cu^{2+} and Pb^{2+}-laden EPS were close than those by Cr^{6+}-laden EPS, which may be related to the number of electric charges of metal ions. The IR analysis results in this study demonstrated that O—H, C=O, C—O—C and C=O—C played an important role in the binding Cu^{2+}, Pb^{2+} and Cr^{6+} by the EPS.

5. Conclusions

In this study, an exopolysaccharide was extracted and purified from *Arthrobacter* ps-5, through a series of processing such as liquid fermentation, ethanol precipitation, sevage purification, ion exchange fractionation, size exclusion, dialysis and freeze drying. There were two polysaccharide sub-fractions after separation. Sub-fraction EPS-1 was a neutral polysaccharide and sub-fraction EPS-2 was an acidic polysaccharide and the ratio of EPS-2 and EPS-1 was 0.22. The purified EPS-1 was comprised of glucose and galactose, and the molar ratio of glucose to galactose was 2.81. The EPS biosorption capacity on heavy metals was tested and the results indicated that the biosorption capacity on Cu^{2+}, Pb^{2+} and Cr^{2+} was obviously affected by EPS concentration, pH and ionic strength. The optimum biosorption capacity on Cu^{2+}, Pb^{2+} and Cr^{6+} was 169.15 mg/g, 216.09 mg/g and 84.47 mg/g, respectively. The biosorption capacity on Cu^{2+}, Pb^{2+} and Cr^{6+} decreased significantly with the addition of Ca^{2+} and K^{+} (in the order of $K^{+} < Ca^{2+}$). Interactions between metal ions and functional groups of the EPS were determined by FTIR. The results demonstrated that the groups of O—H, C=O, C—O—C and C=O—C of the EPS involved in metal biosorption process were the main functional groups for binding metal ions. The overall results suggest that the EPS obtained from *Arthrobacter* ps-5 has potentials as a novel biosorbent in waste water treatment and the future work will be focused on the removal of trace metal ions.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (31370554).

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