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Effects of carbohydrate sources on biosorption properties of the novel exopolysaccharides produced by *Arthrobacter* ps-5

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ABSTRACT

The crude exopolysaccharides (EPSs) were obtained from *Arthrobacter* ps-5 fermentation using various carbohydrate sources followed by centrifugation, ethanol precipitation, and the isolated EPSs were further deproteinized and lyophilized. Carbohydrates from various sources resulted in different yield of EPSs from the fermentation and different molecular weight of EPSs. A maximum yield of 0.27 mg/g was achieved by using the culture medium supplemented with sucrose. The EPS produced by glucose-supplemented medium had the maximum content of acidic polysaccharides, subsequently presented the highest biosorption capacity for Cu^{2+} and Pb^{2+} at 257.9 mg/g and 331.8 mg/g, respectively. The ratio of acidic to neutral polysaccharides presented in EPSs was a key factor to explicate the biosorption mechanism, the higher the ratio, the stronger the biosorption capacity.

1. Introduction

As a result of rapid industrialization heavy metals have been used widely, such as in the industrial processes of electroplating, manufacturing, mining, and automotive. Consequently, heavy metals have been released to the environment. As hazardous pollutants, they are difficult to remove from the environment due to the fact that they are resistant to degradation through chemical, biological and photolytic processes. For instance, copper (Cu) and lead (Pb) are applied in some metabolic processes of microorganisms in the environment, nevertheless, acute exposure to large amounts of Cu^{2+} and Pb^{2+} causes health problems such as anemia, damage to liver and kidneys, brain damage and ultimately death (Davis, Ramirez, Mucci, & Larsen, 2004; Davis, Volesky, & Vieira, 2000). As a result, removal of these pollutants from industrial effluents has become a high priority task that is reflected by more stringent environmental regulations enacted in many countries. This creates the demand of efficient and cost-effective treatments in handling large volumes of heavy metal-bearing waste water from mining industries (Davis, Volesky, & Vieira, 2000).

Much of the conventional methods have been employed to remove heavy metals, such as precipitation, oxidation/reduction, ionic exchange, adsorption, filtration, electrochemical processes, membrane separations and evaporation, among which adsorption is highly effective and economical. In addition, biosorption could be an even more economical option, since it utilizes various microorganisms naturally available, including bacteria, fungi, yeast, and algae. These biosorbents possess metal sequestering properties and can effectively decrease the concentration of heavy metal ions in dilute solutions that contain the pollutants at trace levels, leading to aggregation or precipitation of the metals. Therefore biosorption can be an ideal option for treatment of large volumes of waste water which contains heavy metals at relatively low concentrations (Veglio & Beolchini, 1997; Volesky & Holan, 1995).

Biopolymers naturally generated by microorganisms are biodegradable and are degradation intermediates, which are harmless to human being and the environment. They can be used as biosorbents in heavy metals treatment and performed well at the common conditions. Among biopolymers exopolysaccharides (EPSs) are sugar polymers that are produced by mainly bacteria and cyanobacteria, which have been proved to be effective in aggregation and binding of heavy metals (Freire-Nordi, Henriques Vieira, & Nascimento, 2005). Adsorption of heavy metals by EPSs is considered non-metabolic and energy independent as it is resulted from interaction between metal cations and negative charge of acidic functional groups of EPSs (Francois et al., 2012; Kim, Kim, Kim, & Oh, 1996). During the bacterial growth, EPSs are released and consequently bind the metal ions in the environment. The structures of the EPSs are typically found with long-chain polysaccharides containing branched and sugars or sugar derivatives

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such as glucose, fructose, mannose and galactose as distinct units.

Carbohydrates are the major sources of cellular carbon and energy in all living organisms. Composition of carbohydrates from various sources exhibits significant effects on the yield, monosaccharide composition and molecular mass of EPSs during microorganism fermentation (Cerning et al., 1994; Tallon, Bressollier, & Urdaci, 2003; Torino, Hébert, Mozzi, & Valdez, 2005), and subsequently impacts on other physical and chemical properties of EPSs (Fukuda et al., 2010; Kaplan, Christian, & Arod, 1987). However, the mechanisms of how source carbohydrates may alter monosaccharide composition of EPSs that are generated from strains cultivation remain unclear. In this paper, we aimed to evaluate the effects of different carbohydrate sources used in culture media on the yield, chemical structure and biosorption capacity of the EPSs produced by *Arthrobacter* ps-5. Possible determinants leading to the biosorption of Cu^{2+} and Pb^{2+} in the EPSs will be discussed.

2. Materials and methods

2.1. Cultivation of Arthrobacter ps-5 and reagents

Arthrobacter ps-5 was screened from the rhizosphere soil of *ginkgo biloba* trees and maintained in our laboratory, which can produce EPSs during the fermentation. The strain was cultivated 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 as described in our previous report (Ye, Zhang, Yang, Wang, & Xiao, 2014).

All reagents used were analytical grade. Synthetic stock solution of Cu^{2+} and Pb^{2+} (100 mg/L) was prepared by dissolving calculated quantity of copper sulphate and lead nitrate (AR Grade) in double distilled water.

2.2. Isolation and purification of the EPS

The culture medium prepared as mentioned above was used to ferment *Arthrobacter* ps-5 and for harvesting EPSs. *Arthrobacter* ps-5 was propagated in 250 mL conical flasks with 50 mL of liquid medium, using a rotary shaker (HZQ-X100A) when operated at 160 rpm for 48 h. Sucrose, glucose or starch were added in the culture medium as different carbohydrate sources with a final concentration of 2% (w/v). The fermentation temperature, initial pH, inoculation proportion were held at 28 °C, 7.0 and 4.0% (v/v), respectively, based on our previous study results (Ye et al., 2014). According to the culture medium supplemented with different carbohydrates, such as sucrose, glucose and starch, these EPSs obtained were named EPS_{Suc}, EPS_{Glu} and EPS_{Sta} respectively.

Crude EPS and its purified fractions were prepared as described in our previous report (Ye et al., 2014), using DEAE-52 anion-exchange chromatography column and Sephadex G-100 column. Then acidic or neutral polysaccharides were obtained (Chang, 1987).

2.3. Composition analysis of the EPSs

The yield of EPSs was determined by an anthrone colorimetric method (Li, Zhou, Zhang, Wang, & Zhu, 2008), through sulphuric acid hydrolysis of EPSs in the presence of anthrone agent at 100 °C. The reaction solution had a blue-green color. The absorbance of the sample solution was measured at 620 nm and calibrated using glucose as a standard reference (Leung, Shuna, Ho, & Wu, 2009). The carbohydrate content was then quantified using the anthrone colorimetric method as described above.

2.4. UV analysis of the EPSs

The UV absorption spectrum (190–500 nm) was recorded using a Lambda 35 spectrophotometer (Perkin Elmer, USA) to examine the existence of proteins and nucleic acids. The detailed protocols were reported previously (Wang, Cui, & Xu, 2007).

2.5. Characterization of the EPSs

2.5.1. Estimation of molecular mass of the EPSs

The molecular mass distribution of EPSs was estimated using high liquid chromatography (HPLC). The purified EPS was dissolved in water (1 mg/mL) and this solution was loaded onto 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, Wang, Chang, Inbaraj, & Chen, 2009). A series of dextrans with known molecular weights were used as the standard (AOAC 980.13).

2.5.2. Monosaccharide components of EPS

Determination of monosaccharide components was followed as per the methods of Xu, Zhu, Jin, and Yu, 2010 and Fukuda et al. (2010) with some modifications. 5 mg of the purified EPS was dissolved in 1 mL of 12 M hydrochloric acid (HCl) and the EPS solution was hydrolyzed in 250 μL by autoclaving at 100 °C for 5 h. Excess HCl was neutralized by NaOH solution, and the hydrolysate was washed thoroughly with deionized water and then lyophilized. The lyophilized powder was dissolved in 100 μL of deionized water, and 5 μL aliquot was used for TLC. A mixed solvent system (n-butanol, ethanol, water) was used at a ratio of 2:1:1 (v/v) for separation of carbohydrates. Carbohydrates were visualized on the TLC plate (20 cm × 20 cm) by heating (60 °C) after spraying with sulfuric acid (5%, v/v). The commercial sugars, including glucose, galactose and mannose were used as standards for identification of single sugar composition present in the sample. The molar fraction of the hydrolysate was analyzed using an HPLC (Waters-2690, USA) equipped with RID and a Sugar-pak-1 column (Waters, USA). The molar ratio of glucose versus galactose in each sample was calculated from the peak areas. Detailed procedures are reported previously (Ye et al., 2014).

2.6. Quantification of metal adsorption

In a 250 mL flask, 50 mL of 10 mg/L metal solution was mixed with 2.0 mL crude EPS (the purity of EPS was 82.3%, w/v) solution that has a concentration of 10 mg/L). The mixing was operated at 25 °C and 200 rpm in the dark for 4 h. After centrifugation at 10,000 rpm for 10 min, the concentration of Cu^{2+} and Pb^{2+} was determined by atomic absorption spectrophotometer (models 180-80, Hitachi, Japan).

All tests were replicated and a blank sample, replacing EPS fraction with deionized water was used to calibrate the AAS. All experimental data were processed using Excel and presented as mean values ±standard deviations.

The metal uptake, Q (mg/g), was then calculated by the following equation:

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

where 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 (crude EPS) added in the metal solutions (g).

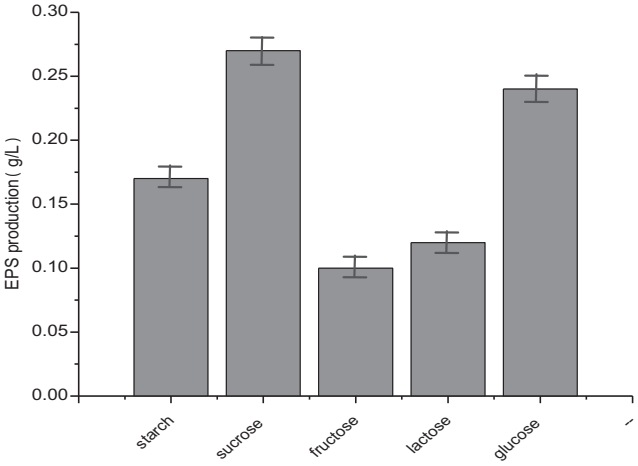

Fig. 1. Effects of carbohydrate sources on the EPS production.

2.7. Fourier transform infrared spectroscopy analysis

The infrared (IR) spectra of EPS before and after metal adsorption tests were determined using a Fourier transform infrared (FTIR) spectrophotometer (Spectrum One-B, Perkin Elmer, USA) equipped with detectors for differentiating various functional groups. The samples were freeze-dried, mixed with dry KBr powder and pressed into pellets. The spectrum was recorded between 400 and 4000 cm^{-1} (Kanmani et al., 2011).

3. Results

3.1. Effects of different carbon sources on production of EPSs

Production of EPSs was carried out through fermentation of *Arthrobacter* ps-5 using a culture medium supplemented with the selected carbohydrates, i.e., sucrose, glucose, starch, fructose or lactose, at a pre-determined concentration level of 2% (w/v). After 48 h cultivation, the maximum EPSs production was achieved at 0.27 g/L in the culture medium supplemented with sucrose. EPSs production was 0.24 g/L in the culture medium supplemented with glucose (Fig. 1). Next, the EPSs produced from the Carbohydrate-enriched culture media were separated and purified for further studies of their effects on monosaccharide composition and biosorption capacity of EPSs.

3.2. Characteristics of crude and purified EPSs

3.2.1. Isolation and purification of EPSs

The EPS_{Suc} was separated into two independent elution peaks after DEAE anion exchanger, i.e., sub-fractions S_u-1 and S_u-2 (Fig. 2S_u-A). Sub-fraction S_u-1 was a neutral polysaccharide as it was eluted out with water, while sub-fraction S_u-2 was more likely acidic as it was eluted with NaCl solution. This is in agreement with other literature indications (Chang, 1987). S_u-1 was the major component, shown by its greater peak area in anion-exchange chromatography (Fig. 2S_u-A). The ratio of S_u-2 and S_u-1 was 0.22. S_u-1 was collected and further purified through a Sephadex G-100 column and then renamed as S_u-1-1 for purified sample (Fig. 2S_u-B).

The EPS_{Glu} was separated into two independent elution fractions, named as sub-fractions G_l-1 and G_l-2 (Fig. 2G-A). G_l-1 was a neutral polysaccharide, and G_l-2 was an acidic polysaccharide, whereas the ratio of G_l-2 and G_l-1 was 1.82. G_l-1 and G_l-2 fractions were collected respectively and further fractioned through a Sephadex G-100 column eluted with 0.05 M NaCl solution, resulting in two purified samples of G_l-1-1 and G_l-2-1 (Fig. 2G-B).

Table 1
The monosaccharide composition of EPSs supplemented with various carbon sources.

EPS	Molar ratio (glucose/galactose)	ρ
EPS_{Suc}	2.81	0.22
EPS_{Glu}	2.75	1.82
EPS_{Sta}	2.78	1.08

ρ, mean the ratio of acidic polysaccharide/neutral polysaccharide.

The EPS_{Sta} was also separated into two independent elution fractions S_t-1 and S_t-2 (Fig. 2S_t-A). Similarly, S_t-1 was a neutral polysaccharide and S_t-2 was an acidic polysaccharide. The ratio of S_t-2 and S_t-1 was 1.08. S_t-1 and S_t-2 fractions were collected and further purified respectively, resulting in two purified samples of S_t-1-1 and S_t-2-1 for further tests (Fig. 2S_t-B).

The ratio of acidic polysaccharide/neutral polysaccharide in all crude EPSs was shown in Table 1.

3.2.2. Physical properties of the EPS

The purified EPSs were 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 aqueous solutions prepared by all purified EPSs were clear, homogeneous liquids with a color of light yellow and no precipitation occurred after centrifugation. An ultraviolet scan spectrum analysis of the EPSs was shown in Fig. 3, indicating the absence of proteins and nucleic acids due to a negative response – no absorption peaks at 260 or 280 nm. So the EPSs were thought to be pure.

3.2.3. Estimation of EPSs molecular mass

The molecular mass of sub-fractions, S_u-1, G_l-1, G_l-2, S_t-1 and S_t-2 was measured by HPLC with a size exclusion column. Based on a calibration curve obtained using dextrans as references at various molecular weight cut-off points, the average molecular weights of S_u-1, G_l-1, G_l-2, S_t-1 and S_t-2 were estimated to be 8.83×10^5, 3.48×10^5, 1.21×10^6, 1.01×10^5 and 7.36×10^5 Da, respectively. Molecular mass distribution followed by the order of $EPS_{Glu} < EPS_{sta} < EPS_{suc}$.

3.2.4. Monosaccharide composition of EPSs

Only glucose and galactose were detected in the three purified EPSs. The molar ratio of glucose to galactose ranged from 2.7 to 2.8 depending on sub-fractions (Table 1). The results showed that the monosaccharide composition of EPS_{Suc}, EPS_{Glu} and EPS_{Sta} was very similar, which suggests there is little effect of carbon sources on EPSs composition.

3.3. Metal adsorption of EPSs

As shown in Fig. 4, the metal biosorption capacity of EPS_{Suc}, EPS_{Glu} and EPS_{Sta} varied. The EPS_{Glu} had the highest biosorption amounts of 257.9 mg/g for Cu^{2+} and 331.8 mg/g for Pb^{2+}, respectively. The biosorption capacity of EPS_{Sta} and EPS_{Suc} was slightly lower than that of EPS_{Glu}, which may be related to the slight variation of structures in these EPSs.

3.4. FTIR analysis results

The FTIR analyses of the three EPSs and the metal-bearing EPSs were shown in Fig. 5. The strong bands in the region 3420–3468 cm^{-1} were related to the stretch vibration of O—H or hydrogen bond existing in all polymers (Kanmani et al., 2011). The strong absorption peaks at 2932–2934 cm^{-1} can be assigned

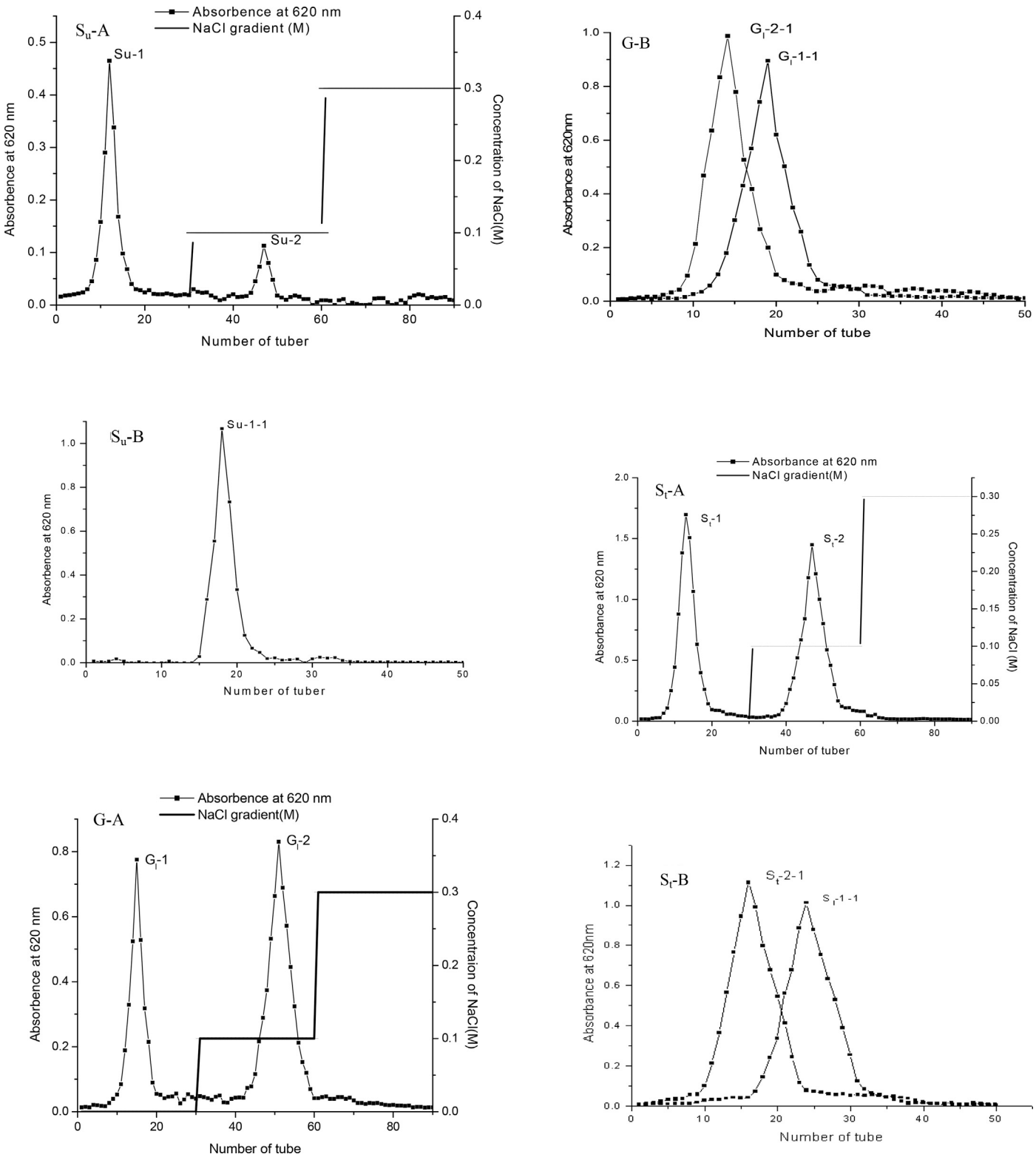

Fig. 2. Purification of EPSs by DEAE-52 anion-exchange chromatography and elution curve of fractions through a Sephadex G-100 column. S_u-A: EPS_{Suc} by DEAE-52 anion-exchange chromatography; S_u-B: S_u-1 through a Sephadex G-100 column; G-A: EPS_{Glu} by DEAE-52 anion-exchange chromatography; G-B: G_l-1 and G_l-2 through Sephadex G-100 column; S_t-A: EPS_{Sta} by DEAE-52 anion-exchange chromatography; S_t-B: S_t-1 and S_t-2 through Sephadex G-100 column.

to associate with the stretching vibration of C—H in the sugar ring, and the strong absorption peaks were observed around 1653–1655 cm^{-1}, representing the stretching vibration of C=O and carboxyl group (Pradhan, Singh, & Rai, 2007). The 1000–1125 cm^{-1} range is characteristic of uronic acid, O–acetyl ester linkage bond (Morillo Perez et al., 2008). The FTIR spectra revealed the presence of many functional groups in EPSs, such as hydroxyl, carboxyl and glycosidic bonds.

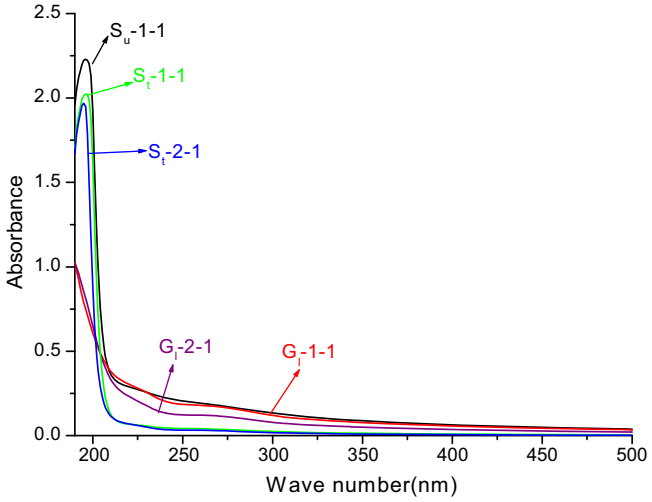

Fig. 3. The ultraviolet scan spectrum of EPSs.

Compared to the purified EPSs, the dominant peaks and assignments of the metal-bearing EPSs shifted obviously. For instance, $EPS_{Suc} + Cu^{2+}$ and $EPS_{Suc} + Pb^{2+}$, O—H shifted from 3399.72 cm^{-1} to 3420.99 cm^{-1} and 3421.92 cm^{-1}, respectively, C=O shifted from 1640.02 cm^{-1} to 1636.47 cm^{-1} and 1636.84 cm^{-1}, C—O shifted from 1418.17 cm^{-1} to 1384.74 cm^{-1} and 1384.78 cm^{-1}, C—O—C shifted from 1129.51 cm^{-1} to 1127.82 cm^{-1} and 1127.70 cm^{-1}, respectively. The intensity of absorbance peaks also changed.

4. Discussion

Carbohydrates are essential nutrients during synthesis of microbial exopolysaccharides in all microorganisms. Differences in carbohydrate composition from various sources shows obvious effects on the yield and molecular weight of the EPSs obtained from *Arthrobacter* ps-5 fermentation. Although only glucose and galactose were detected in all hydrolyzed solutions of the EPSs, the molar ratio of glucose to galactose was different in three purified EPSs that were harvested from the culture media supplemented by three different carbon sources. In addition, the ratio of acidic versus neutral polysaccharide varied significantly in three EPSs. This may be one of the reasons to explain the differences of the three EPSs in the adsorption capacity of heavy metals.

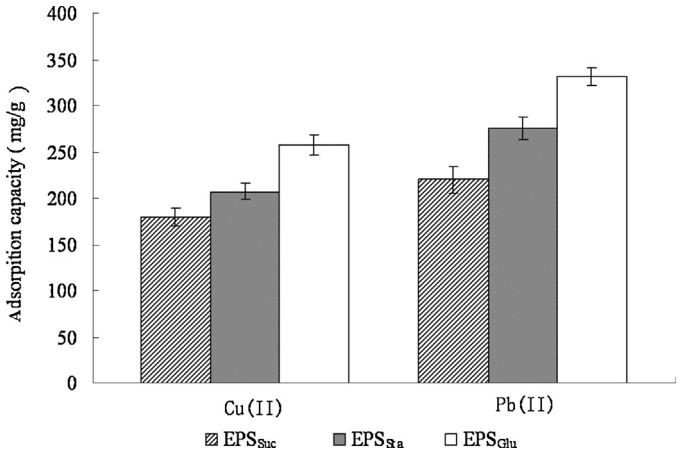

Fig. 4. The metal biosorption capacity of EPS_{Suc}, EPS_{Glu} and EPS_{Sta} for Cu (II) and Pb (II).

As reported in the literature many EPSs have the adsorption capacity for heavy metals. For example, Zhou et al. reported that the optimum biosorption capacity was observed for Cu^{2+} at 48.0 mg/g from the EPS obtained from deep-sea mesophilic bacterium SM-A87 (Zhou, Wang, Shen, Hou, & Zhang, 2009). But in our study as shown in Fig. 4, the biosorption activities for Cu^{2+} were 179.3 mg/g, 257.9 mg/g and 207.5 mg/g for EPS_{Suc}, EPS_{Glu} and EPS_{Sta}, respectively, and the biosorption activities for Pb^{2+} were 219.9 mg/g, 331.8 mg/g and 275.5 mg/g for EPS_{Suc}, EPS_{Glu} and EPS_{Sta}, respectively. Thus, we believed the EPSs obtained from *Arthrobacter* ps-5 fermentation presented strong biosorption capacity in general for both Cu^{2+} and Pb^{2+}.

Many studies have reported that the molecular mass of EPSs is related to their physical-chemical properties, such as chelating properties (Kaplan, Christian, & Arod, 1987) and viscosity (Fukuda et al., 2010). Molecular mass distribution followed the order of $EPS_{Glu} < EPS_{sta} < EPS_{suc}$ in our experiment, which indicates the bacteria may have a better utilization of glucose (simple sugar) during the fermentation compared to other di- or polysaccharides. Such variation in molecular structure can be used to explain the difference in their biosorption capacity, which is not only related to the metal ion size (Ye, Yin, Peng, & Jia, 2001), but also related to the size of sorbents.

The existence of various functional groups is one of the key information required to explicate the mechanism of metal binding activity of EPSs. Peaks appearing in the FTIR transmission spectra of EPSs were assigned to various groups and can be associated with different binding sites of heavy metal ions. When comparing between the purified and metal-bearing EPSs, it can be observed that there is a shift in wave lengths of dominant peaks. This shift is corresponding to the metal binding process that takes place on the surface of biosorbent (Pavasant et al., 2006; Zhou et al., 2009). The O—H stretching group and the C=O stretching group are involved in metal biosorption. Once metals are bound to these functional groups, the changes in wavelengths occur, consequently resulting in the shift of wavelengths of other groups such as C—H group, C=O group. All these structural changes as revealed by FTIR spectra proved that a metal binding process occurred on the surface of EPSs. In the study, Cu^{2+} and Pb^{2+} might result in changes in the absorption frequencies of the various functional groups present in the EPSs. This is in good agreement with other literature indications (Pal & Paul, 2008).

Acidic polysaccharides are likely to contain more uronic acids with negative charges. Such acidic polysaccharides are the important components of EPSs that affect metal removal, and thus, indicate the potential of EPSs to bind with heavy metals including Cu^{2+} and Pb^{2+}. Based on the results in our study, we can conclude that the source carbohydrates had impact on metal adsorption capacity of various EPSs obtained through *Arthrobacter* ps-5 fermentation. One of the reasons is due to the difference in the ratio of acidic versus neutral polysaccharide in various EPSs samples. The highest ratio was shown in EPS_{Glu} (1.82), followed by the ratio of EPS_{Sta} (1.08), and the lowest ratio was in EPS_{Suc} (0.22) (Table 1). There are more carboxylic groups (—COOH, O—H, C=O) in acidic polysaccharides that can be protonated when the pH of solution reaches its pK_a value, consequently the deprotonated COO— prompts the binding of metal cations (Fig. 1). This mechanism would be then responsible for metal biosorption capacity in various EPS samples (Ruangsomboon, Chidthaisong, Bunnag, Inthorn, & Narumon, 2007). Therefore, in this investigation, the EPS_{Glu} produced by *Arthrobacter* ps-5 using glucose supplemented culture medium presented a higher metal cation adsorption due to its abundant acidic functional groups.

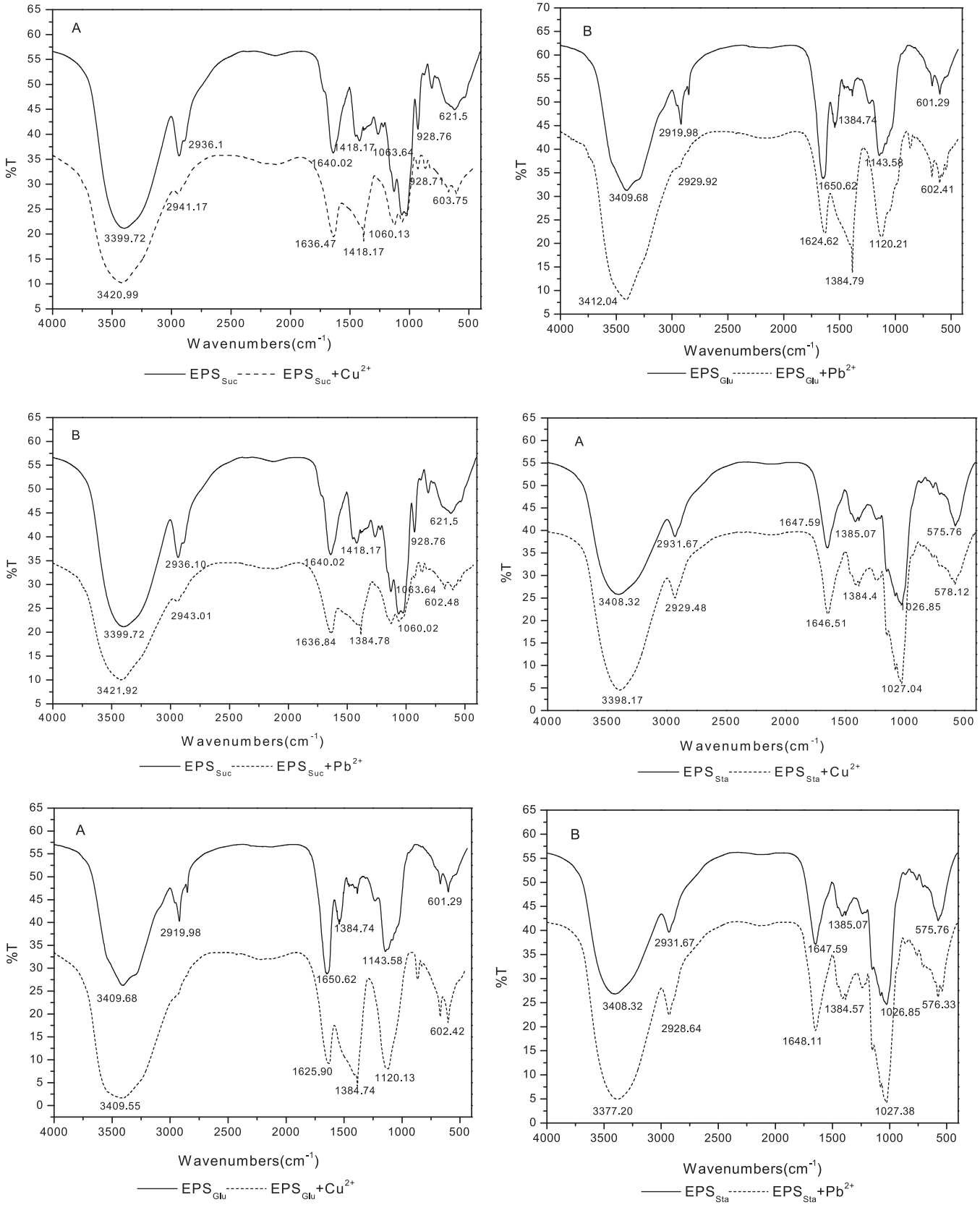

Fig. 5. IR spectra of EPS_{Suc}, EPS_{Glu} and EPS_{Sta}.

5. Conclusions

We have demonstrated, for the first time, *Arthrobacter* ps-5 from the rhizosphere soil of *ginkgo biloba* trees could produce useful EPSs with desirable heavy metal adsorption capacity when using various sugar-supplemented culture media. The source carbohydrates had significant impact on EPS production yields, their composition of monomers and functional groups, as well as biosorption activities.

Our results indicated that the EPS productions using the culture medium supplemented with sucrose resulted in a maximum yield at 0.27 g/L. The EPS produced with supplementation of glucose (EPS_{Glu}) had the maximum content of acidic polysaccharides, subsequently presented the highest biosorption capacity for Cu^{2+} and Pb^{2+} at 257.9 mg/g and 331.8 mg/g, respectively. The ratio of acidic versus neutral polysaccharides presented in the EPSs was a key factor to explicate the biosorption mechanism, the higher the ratio, the stronger the biosorption capacity, which was in good agreement with other literature findings. Interactions between metal ions and various functional groups in EPSs were confirmed by FTIR, showing with the shifts in wavelength readings from various functional groups.

Since it is a promising way using EPS as a biosorbent for heavy metal removal, it is important to study different fermentation factors such as carbon sources in culture media so to understand their effects on fermentation process and to obtain EPSs with desirable chemical and physical properties. Future research will investigate effects of nitrogen sources and mineral salts on chemical and physical properties of the EPSs.

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