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Efficient removal of heavy metals by synergistic actions of microorganisms and waste molasses

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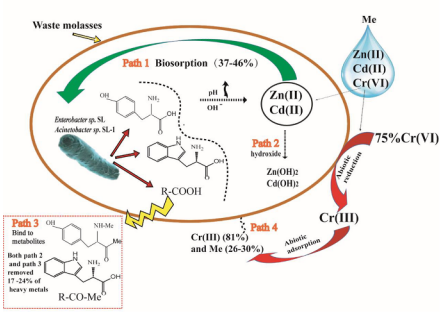
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, two bacteria strains (*Enterobacter* sp. SL and *Acinetobacter* sp. SL-1) and waste molasses (carbon source) were used to remove Zn(II), Cd(II), Cr(VI), and Cr(Total) in the liquid solution (87 mg·L). The results showed the removal efficiencies of Cr(Total) and Cr(VI) could reach over 98.00% after reaction, and the removal efficiencies of Zn(II) and Cd(II) were all about 90.00% by the synergistic actions of microorganisms and waste molasses. In this process, waste molasses provides nutrients for microorganisms and has the characteristics and capability of Cr, Zn, and Cd. Microorganisms mainly use biological adsorption (36.95% and 45.69%) and metabolism (24.37% and 17.05% by producing humic-acid and fulvic-acid like substances) to remove Zn(II) and Cd (II), while waste molasses could to remove Cr(Total) (81.24%) and Cr(VI) (75.90%). This study has potential application value for the treatment of wastewater containing high concentrations of heavy metals.

1. Introduction

Heavy metal-containing wastewaters are produced in many industries, and their direct discharge has caused serious impacts on human health and environment (Zhu et al., 2018). The popular methods for treatment of heavy metal-containing wastewater include the ion exchange method (Dai et al., 2015), electrochemical method (Kobya et al., 2017), photocatalysis (Luo et al., 2017), and biological method (Wen et al., 2018). Among them, biological method has been widely studied due to the low energy consumption, small secondary

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pollution, and environmental friendliness. (Zhou et al., 2013).

However, applying survivable microbes for biological treatments in toxic environments has become the main challenge (Pradhan et al., 2019) because heavy metal-containing wastewater not only has complex heavy metal compositions but also may contain other toxic substances such as cyanide (a phenolic organic matter) (Wen et al., 2018). In addition, the characteristics of heavy metal-containing wastewater may vary moderately; especially the change of pH can significantly inhibit the bacterial activities (Alexandrino et al., 2014). Moreover, biological treatments of heavy metals normally have been used for low concentration ranges (10–50 $mg{\cdot}L^{-1}$) of heavy metals (Guo et al., 2017), and some bacteria can only work with specific single heavy metal [e.g., Zn(II) or Cr(VI)] in the wastewater (Peng et al., 2019; Singh et al., 2011; Wang et al., 2018). Due to the high concentration of heavy metals on microbial stress, it is extremely difficult for microorganisms to remove many kinds of heavy metals simultaneously (Dhal et al., 2013; Guo et al., 2017).

Therefore, previous studies have used chemical pretreatment (Mejias Carpio et al., 2016) or the addition of substances such as iron ore (Guo et al., 2017) to reduce heavy metal content or toxicity. Although these methods can improve the activity of microorganisms, they also bring trouble to the subsequent treatment of the final effluent. Alexandrino et al. (2014) and Yan et al. (2018) applied a high concentration of sodium lactate ($7.0\ g{\cdot}L^{-1}$) as a carbon source to stimulate bacteria growth and heavy metal removal. However, these expensive carbon sources burden wastewater treatment (Li et al., 2019; Wang et al., 2018). In addition, it has been reported that microorganisms need specific nutrient composition to be able to function, and the complex wastewater system (containing heavy metals) will inhibit its activity (Mejias Carpio et al., 2016; Peng et al., 2019; Pradhan et al., 2019). Therefore, biotreatments are usually limited in industrial application due to the multi heavy metals contained wastewater. So, it is important that to develop an economic biotreatment method with multi heavy metals tolerance performance. Moreover, it is critical to differentiate the contributions of biotic (e.g., via microbial metabolisms) and abiotic (the chemical properties of the carbon source) reactions for heavy metals removal in complex wastewater system.

To fill the knowledge gap, this study was conducted to evaluate the feasibility of using *Enterobacter* sp. SL and *Acinetobacter* sp. SL-1 with waste molasses (sugar industry by-product) as the carbon source (an free carbon source) for efficiently simultaneous removal of heavy metals Zn(II), Cd(II), Cr(Total), and Cr(VI) in synthetic wastewater by combined biotic and abiotic reactions and how to elucidate the associated mechanisms for better understanding. This study mainly describes how the molasses and microbes work synergistically for efficient removal if heavy metals as well as the associated mechanisms. This study provides a new and inexpensive method for removing heavy metals; the elucidated mechanism may allow for better control of the systems for treatment of industrial wastewater contaminated with high concentrations of heavy metals.

2. Materials and methods

2.1. Waste molasses and chemicals

The waste molasses used in this experiment was taken from Qinzhou Lianfeng Sugar Co., Ltd., Guangxi, China. Its main components include fructose (15.00%), sucrose (33.00%), reducing substances (8.00%), glucose (9.00%), Colloid (6.42%), and others (20.00%). Waste molasses (store at 4 °C) was autoclaved (sterilized at 121 °C for 20 mins) before being used for preparation of the medium (see below).

The heavy metal-containing wastewater was synthesized by adding $ZnCl_2$, $CdSO_4$, and K_2CrO_7, and then sterilized by UV for 30 min. All the chemicals used in this research were of analytical grade and purchased from Sinopharm, China.

2.2. Bacterial screening and enrichment

Strains of *Enterobacter* sp. SL and *Acinetobacter* sp. SL-1 were obtained by this research group (Sun et al., 2019), and preserved in the China Center for Type Culture Collection (CCTCC). After separation, 16rSDNA showed that SL may belong to *Enterobacter* sp., and the bacterial deposit number is CCTCC M 2018892. SL-1 may belong to *Acinetobacter* sp., and the bacterial deposit number is CCTCC M 2019063 (http://www.cctcc.org/). SL and SL-1 were mixed in a 1:1 ratio and cultured to 1×10^8 (cfu) mL^{-1} for using (pure bacteria were cultured in the same way as mixed bacteria). Gram staining results showed that the two mixed bacteria had similar concentrations.

The medium components used in the bacterial culture stage and heavy metal removal experiments are as follows: $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ $0.5\ g{\cdot}L^{-1}$, Na_2SO_4 $0.5\ g{\cdot}L^{-1}$, NH_4Cl $1.0\ g{\cdot}L^{-1}$, $MgSO_4{\cdot}7H_2O$ $2.0\ g{\cdot}L^{-1}$, K_2HPO_4 $0.5\ g{\cdot}L^{-1}$, NaCl $2.0\ g{\cdot}L^{-1}$, yeast extract $1.0\ g{\cdot}L^{-1}$, and waste molasses of $1.5\ g{\cdot}L^{-1}$ (as the carbon source). The pH of the medium was adjusted to 7.0 with 0.5 M NaOH and 0.5 M H_2SO_4. Then, a certain amount of the medium (depending on different tests, see below; via an autoclave at 121 °C for 20 mins) was transferred into an anaerobic bottle (GL45, Shanghai shupei Experimental Equipment Co., Ltd., China). The mixed bacteria (SL and SL-1) were inoculated into the anaerobic bottle at 10% ratio (v/v) (depending on different tests, see below) and then fushed with N_2 for 10 mins. Finally, the anaerobic bottle was placed in the biochemical incubator (SHP-250, Shanghai Senxin laboratory instrument co., Ltd., China) and incubated for 2 days.

2.3. Experimental design for heavy metal removal

2.3.1. Heavy metal removal experiments

In order to explore the effects and mechanisms of heavy metal removal, six groups of experiments were conducted as shown in Table 1.

Table 1
Test conditions.

Test Groups	WM	Zn(II)	Cd(II)	Cr(VI)	Bacterial & Inoculum (v/v)	Test Purpose
	$g{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$		
WM + BF	1.5	87	87	87	SL and SL-1 (10%)	General performance
WM	1.5	87	87	87	Sterile water (10%)	Control
BF	0	87	87	87	SL and SL-1 (10%)	Control
WM + Ia	1.5	87	87	87	Inactivated SL and SL-1 (10%)	Biosorption
WM + SL	1.5	87	87	87	SL (10%)	Effect of pure bacteria
WM + SL-1	1.5	87	87	87	SL-1 (10%)	Effect of pure bacteria

Note: 1) All tests had an initial pH of 7.0 with 3 replicates (n = 3); 2) waste molasses was weighed and added; 3) the culture temperature was 35 °C. WM: waste molasses; BF: bacterial fluid; and Ia: inactivation (i.e., The biomass was centrifuged dried at 60 °C for 5 d and used in heavy metal removal experiments, this method referred to the study of Chojnacka et al. (Chojnacka, 2007)); The dosage of inactivated SL and SL-1 was added by drying in accordance with 10% volume of bacterial solution in other experiments; and 4) the deviation of heavy metal concentration in each experimental group was ± $0.5\ mg{\cdot}L^{-1}$.

In Group WM + BF, 135 mL cultural medium (see Section 2.2) was poured into to a 250 mL-anaerobic bottle, which then was inoculated with 10% (V/V) cultured mixed bacterial fluid (BF, 15 mL), added with waste molasses (WM) of 1.5 g·L^{-1} and Zn(II), Cd(II), and Cr(VI) (each with 87 mg·L^{-1}) (WM + BF, Table 1). In Group BF as a control, no WM was added into the medium, and the other conditions were the same as Group WM + BF. Group WM was also a control, which had sterile water [ultra-pure water (0.085 μS cm^{-1}, 25 °C) at 121 °C for 20 min] instead of the bacteria fluid with the other conditions being the same as WM + BF. Finally, each anaerobic bottle was flushed with N_2 for 10 mins and then placed in a biochemical incubator under 35 °C (SHP-250, Shanghai Senxin laboratory instrument co., Ltd., China). Samples (5 mL) were taken every 24 h for heavy metal, pH, and ORP measurements; after sampling, the bottle was flushed with N_2 for another 10 mins to maintain the anaerobic environment.

2.3.2. Effect of initial pH

The activity of bacteria varies under acidic or alkaline conditions; therefore improper pH environments inhibit the heavy metals removal efficiencies. In order to explore the influence of pH on heavy metal removal, the initial pH of the medium in the test bottles of Group WM + BF was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with 0.5 mol·L^{-1} NaOH and 0.5 mol·L^{-1} H_2SO_4, and the performance of the systems were evaluated with the same methods as tests in Group WM + BF shown in Table 1.

2.3.3. Biosorption and pure bacteria experiment

To explore the contribution of bacterial sorption on heavy metals, the mixed bacteria of SL and SL-1 were inactivated (the biomass was centrifuged and dried at 60 °C for 5 d and used in heavy metal removal experiments, this method referred to the study of Chojnacka et al. (Chojnacka, 2007), other conditions are shown in Table 1) to study the adsorption efficiencies of microbial rich metals with the experimental conditions shown in Table 1 (Group WM + Ia). In order to explore the differences between pure bacteria and mixed bacteria in heavy metal removal and the effects of two strains of bacteria on heavy metals removal, pure SL and pure SL-1 bacteria (Table 1) were used to remove Zn(II), Cd(II), and Cr(VI). The sampling procedures for all six groups of tests were the same.

2.3.4. Bacterial extracellular polymeric substances (EPS) extraction

The bacterial Extracellular Polymeric Substances (EPS) (WM + BF group and mixed bacteria liquid in Section 2.2) were extracted refer to heat extraction method (Ma et al., 2018; Gu et al., 2018). To remove the supernatant, samples (approximately 50 mL) were taken from the microbial culture stage (168 h) and Group WM + BF (168 h), and then centrifuged at 10,000 rpm (10,777 *g*, 4 °C) for 15 min. After discarding the solution, the remaining precipitation were re-suspended by using 0.90% NaCl (aq.), followed by a heat treatment (in a water bath at 80 °C) for 20 min and then centrifuged again under the same operating conditions. Finally, the re-suspended precipitation of the samples after the second centrifugation were filtrated through a 0.45 μm membrane filter (Shanghai Funi Biotechnology Co., Ltd., China), and then the EPS in the filtrate was retained in a 10 mL centrifuge tuber (RF1185, Shanghai Hengfei biotechnology co., Ltd., China) and then used for analyzing the EPS with an Excitation-Emission-Matrix (EEM) spectrometer immediately.

2.4. Heavy metal removal kinetics

The removal efficiency was calculated as Eq. (1)

$$R(\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (1)$$

where R (%) represents the removal efficiency; C_0 and C_t (mg·L^{-1}) were initial concentrations of heavy metals and that in the solution at time t (h), respectively. In order to describe the characteristics and behaviors of heavy metals removal, the pseudo-first-order model and the pseudo-second-order model were used for dynamics fitting. The pseudo-first-order model can be represented by Eq. (2):

$$\frac{dR_t}{dt} = K_1'(R_e - R_t) \quad (2)$$

Take the boundary conditions and sort out Eq. (3):

$$\ln(R_e - R_t) = \ln R_e - K_1' t \quad (3)$$

The pseudo-second-order model is represented with Eq. (4):

$$\frac{dR_t}{dt} = K_2'(R_e - R_t)^2 \quad (4)$$

After rearranging, it becomes Eq. (5):

$$\frac{t}{R_t} = \frac{1}{K'_2 R_e^2} + \frac{t}{R_e} \quad (5)$$

where K_1' (h^{-1}) and K_2' (mL·mg^{-1}·h^{-1}) represent rate constants for first-order kinetics and second-order kinetics, respectively (Jia et al., 2017). R_e (%) represents the removal efficiency at equilibrium; and R_t (%) was the removal efficiency of heavy metals at time t (h).

2.5. Analytical methods

The sample was passed through a 0.45 μm filter, and the concentrations of Zn(II), Cd(II), and Cr(Total) were measured by an atomic absorption spectrometer (iCE 3500, Thermo Fisher Scientific). The determination method of hexavalent chromium is diphenylcarbazide dip spectrophotometry as per the reference (Sun et al., 2019). ORP and pH were determined by a portable multi-parameter water quality analyzer (MTC101, Hach Company, USA). The WM + BF and the WM groups were reacted for 168 h, and the precipitates (after freeze-drying by a freeze dryer, FD-2A-80, Shanghai Jipu Electronic Technology Co., Ltd., China) were determined by X-ray photoelectron spectroscopy (XPS, Thermo Scientific, USA). The morphology of the precipitated material was observed by field emission scanning electron microscope-energy dispersive spectrometer (FESEM-EDS, SU8010, Hitachi, Ltd., company, Japan). Fourier infrared spectroscopy (FT-IR, Tracer-100, Shimadzu Co., Ltd.) was used to study the changes of functional group type and structure of the precipitated materials.

The EPS extracted form Groups WM + BF and SL and SL-1 were measured by Excitation-Emission-Matrix spectra (EEM, F-4600, Hitachi High-Tech Co., Japan). Excitation and emission wavelengths ranged from 200–600 nm with a scan gap of 5 nm and a scan speed of 30,000 nm·min^{-1}.

3. Results and discussion

3.1. Removal efficiencies of heavy metals

The removal efficiencies of heavy metals in Groups WM + BF, WM, and BF were shown in Fig. 1. When the microorganisms and waste molasses coexisted (WM + BF), the removal efficiencies of Zn(II), Cd(II), Cr(Total), and Cr(VI) at 168 h were 91.86%, 89.39%, 99.61%, and 98.48%, respectively. The removal efficiencies of Zn(II) and Cd(II) by WM was only about 30.00%, but the removal efficiency of Cr(Total) and Cr(VI) were as high as 81.24% and 75.32%. In contrast, the removal of Zn(II), Cd(II), Cr(Total), and Cr(VI) by microorganisms (BF) were 76.19%, 59.06%, 29.84% and 50.36%, respectively. The removal efficiencies of heavy metals were the highest, with the action of microorganisms and waste molasses. Without energy substance (WM), the

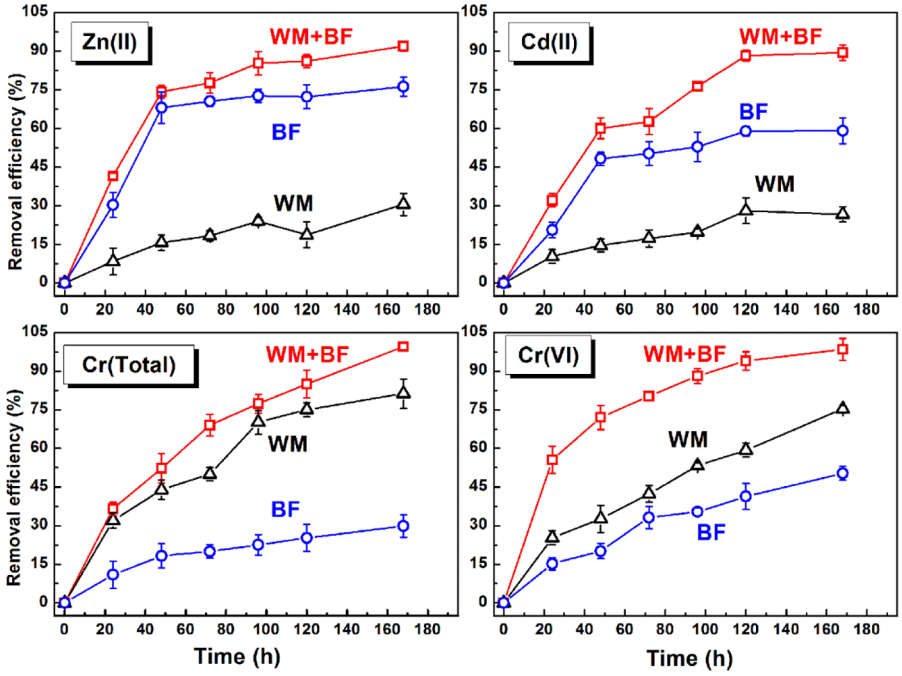

Fig. 1. The removal efficiency of heavy metals by bacteria and waste molasses. The test conditions of Group WM + BF, WM, and BF are shown in Table 1.

activity of microorganisms will be inhibited; at the same time, waste molasses can absorb some heavy metals (FT-IR Analysis, it mainly depends on the action of carboxyl group and amino group), which could reduce the toxicity of heavy metals. This is why Group WM + BF has better effects on heavy metals removal when compared with WM and BF.

In Fig. 1, the removal efficiencies of BF(single) + WM(single) > BF + WM for Zn(II), BF(single) + WM(single) = BF + WM for Cd(II), BF(single) + WM(single) > BF + WM for Cr(Total), BF(single) + WM (single) < BF + WM for Cr(VI) before 60 h and BF(single) + WM (single) > BF + WM for Cr(VI) after 60 h. In the 60 h, Cr(VI) is reduced to Cr(III) during the glycolysis of sugars in waste molasses by microorganisms in BF + WM, and the productions of the glycolysis are also reducing sugars, which could continuously reduce the Cr(VI) and the reduction process is accelerated due to the increasing amount of total electron donors.

Mixed bacteria may also have more intense metabolic activity by using waste molasses as carbon source. The reductive metabolites could also help reduce Cr(VI) to Cr(III). However, the generated reducing environment has limited effects on Cd(II) and Zn(II) due to the removal mechanisms of them are not rely on redox reactions. Instead, the adsorption of waste molasses is the major mechanism of Cd(II) and Zn(II) removal. Thus, the amount of waste molasses could affect the overall removal efficiencies of both Cd and Zn. WM are consumed by the bacteria in WM + BF, therefore lead to a smaller removal efficiencies of Cd(II) and Zn(II) in BF(single) + WM(single). After 60 h, the gradually reduced bacteria metabolic activities result in a decreasing of Cr(VI) reduction so that BF(single) + WM(single) has a higher Cr(VI) removal than BF + WM after 60 h.

Guo et al. (2017) used sulfate-reducing bacteria to remove heavy metals [Cr(VI) and Zn(II)] with 4.0 $g{\cdot}L^{-1}$ sodium lactate being used energy substance. The reaction time was 250 h, and their experiment also required zero-valent iron to participate in the removal of heavy metals, which increased the processing cost. The carbon source used in this study was a sugar by-product, which not only saves processing costs but also has a shorter reaction time (e.g., 168 h).

3.2. Effect of initial pH on removal efficiency of heavy metals

Fig. 2 shows the effect of initial pH on heavy metals removal. The removal efficiencies of Zn(II) and Cd(II) under alkaline conditions (pH = 8.0–10.0) were higher than that under acidic conditions, and it took only 48 h to reach equilibrium. The removal efficiencies of Zn(II) at 168 h were 2.89% (pH = 3.0), 26.42% (pH = 4.0), 52.72% (pH = 5.0), 51.89% (pH = 6.0), 89.54% (pH = 7.0). The removal efficiencies of Cd(II) at 168 h were 11.58% (pH = 3.0), 21.94% (pH = 4.0), 40.32% (pH = 5.0), 44.37% (pH = 6.0), 89.39% (pH = 7.0). However, the removal efficiencies of Cr(Total) and Cr(VI) under acidic conditions were faster than that at alkaline conditions (Fig. 2). The Cr(VI) removal efficiencies at 24 h were 98.61% (pH = 3.0), 93.23% (pH = 4.0), 68.71% (pH = 5.0), 66.96% (pH = 6.0), and 48.54% (pH = 7.0) respectively.

pH has significant effects on removal efficiency. For Zn(II), pH 8.0 was better than pH 7.0 before 90 h. For Cd(II), pH 8.0–10.0 were better than pH 7.0. For Cr(Total) and Cr(VI), pH 3.0–6.0 were better than pH 7.0. In general, the removal of Cd and Zn was inhibited at low pH. Especially at pH = 3, the removal efficiency of Cr(VI) reached equilibrium after 24 h. This is due to the fact that hexavalent chromium ions exist mainly as $Cr_2O_7^{2-}$ in the solution when the pH is lower than 4.25 (Gola et al., 2016). The reduction process of hexavalent chromium under the action of bacteria and waste molasses ($C_xH_yO_z$) is shown in Eq. (6). So in the acidic case, it's more favorable for the reduction of Cr (VI). The removal of hexavalent chromium was limited, when the pH was low than 2.0, this is because the activity of microorganisms were inhibited.

$$C_xH_yO_z + Cr_2O_7^{2-} + H^+ \xrightarrow{\text{Bacteria}} Cr^{3+} + H_2O + CO_2 \quad (6)$$

Previous studies have shown that the removal of Zn(II) and Cd(II) by microorganisms was mainly dependent on biosorption (Cai et al., 2017). Hydrogen ions ionize microorganisms and their metabolites under acidic conditions are thus positively charged. Therefore, the removal efficiencies of Zn(II) and Cd(II) were relatively low, while the removal efficiencies of hexavalent chromium and total chromium were relatively high. It has been shown in Fig. 1 that the main role of

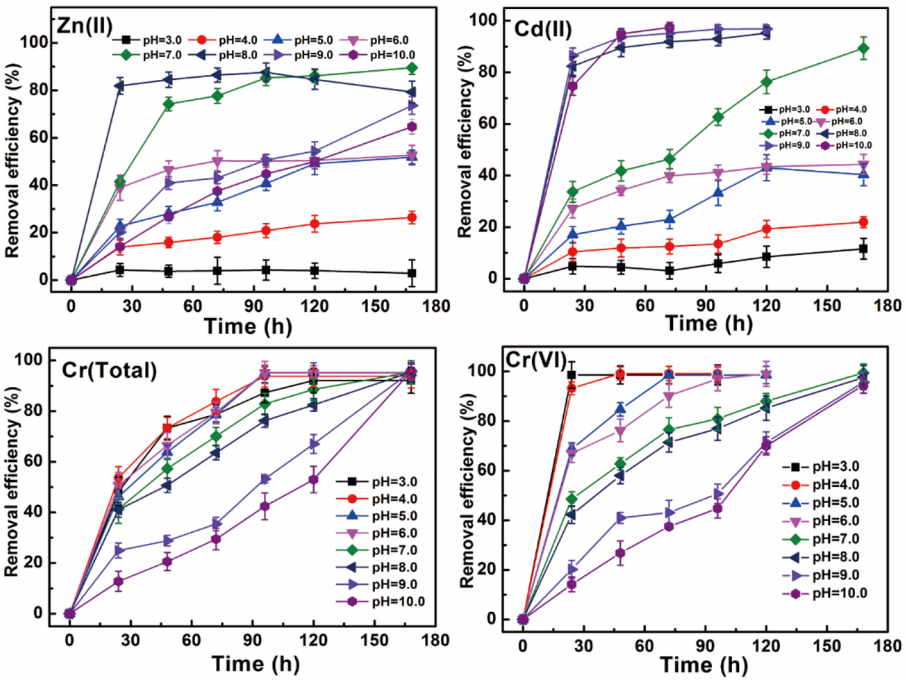

Fig. 2. Removal efficiency of heavy metals at different pH. The test conditions were the same as Group WM + BF in Table 1 except for different initial pH.

removing Zn(II) and Cd(II) were microorganisms. Therefore, the removal efficiencies of Zn(II) and Cd(II) under acidic conditions was relatively low, but increased with pH (pH between 3.0 and 6.0 in Fig. 2). In addition, the optimum pH of the microorganism was neutral (Ramrakhiani et al., 2016), and pH lower or higher than the neutral one would inhibit the activity of microorganisms and the formation of EPS (Fig. 2). However, Cd(II) and Zn(II) were also precipitated [$Cd(OH)_2$ and $Zn(OH)_2$] as a hydroxide under the alkaline conditions. Since Zn $(OH)_2$ is amphoteric hydroxide, it is easy to dissolve when the pH is greater than 9.0, resulting in $Zn(OH)_4^{2-}$ (Gao et al., 2014). Microbial activity was also inhibited at high pH, which resulted in low removal rate of Zn (II).

The removal of Cr(Total) and Cr(VI) mainly depends on the action of waste molasses (Fig. 1), while the macromolecules in waste molasses were hydrolyzed under acidic conditions to further produce reducing substances (Dhal et al., 2013). Under the combined action of bacteria and molasses Cr(VI) was reduced to Cr(III).

3.3. Mechanism of heavy metals removal

3.3.1. Proportion of biotic/abiotic removal of heavy metals and EEM

In addition to secreting EPS, bacteria can also remove some heavy metals through their own metabolism. The removal efficiencies of Zn (II) by microorganisms after inactivation treatment (WM + Ia, 67.49%) was close to that in the WM + SL (64.72%) and WM + SL-1 (66.71%) systems (Fig. 3a). The removal efficiency of Cd(II) in the WM + SL system (90.32%) was almost the same as that in the WM + BF system (89.39%). However, the removal of Cr(VI) was mainly through waste molasses reduction. In general, mixed bacteria had the best removal effects on heavy metals with the action of waste molasses.

Alexandrino et al. (2014) studied the protective effects of microorganisms of different species (Alexandrino et al., 2014). One of the

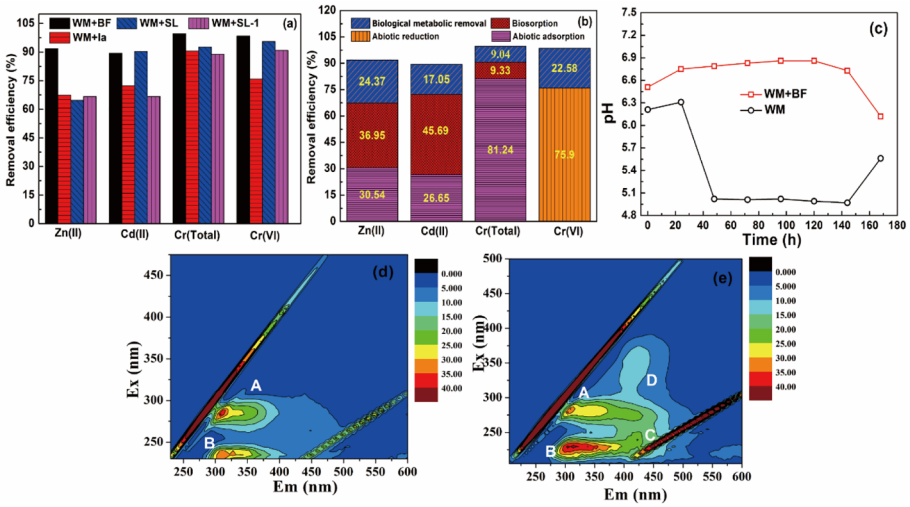

Fig. 3. Removal efficiency of heavy metals by different strains (the test conditions of WM + BF, WM + SL, WM + SL-1, and WM + Ia are shown in Table 1) (a); contribution of heavy metal removal by different mechanisms (b); changes in pH in Groups WM + BF and WM (c); EEM analysis of precipitates in mixed strains cultured for 168 h without heavy metal (d) and in Group WM + BF after 168 h reaction (e).

microorganisms reduced the concentration of a certain heavy metals to protect another microorganism. The removal efficiencies of Cr(Total) in the inactivated microorganisms (WM + Ia), WM + SL, and WM + SL-1 systems were 90.54%, 92.76%, and 88.83%, respectively. This also verifies that the Cr(Total) removal by microorganisms is primarily via biosorption. The removal of Cr(VI) in the SL and SL-1 systems all showed better removal efficiencies than inactivated microorganisms (WM + Ia).

The ratios of the action of waste molasses and microorganisms to Zn (II), Cd(II), Cr(Total), and Cr(VI) are shown in Fig. 3b. Abiotic adsorption and reduction refer to the action of waste molasses. In general, microorganisms mainly remove Zn(II) and Cd(II), and waste molasses mainly removes Cr(Total) and Cr(VI). The removal of Zn(II) and Cd(II) depends on the metabolism of microorganisms (24.37% and 17.05%), biosorption (36.95% and 45.69%) and adsorption of waste molasses (30.54% and 26.65%). The removal of Cr(Total) depends on the metabolism of microorganisms (9.04%), biosorption (9.33%) and waste molasses adsorption (81.24%). The reduction of Cr(VI) was accomplished by bioreduction (22.58%) and molasses reduction (75.90%).

Previous studies by our research group were shown that *Enterobacter* sp. SL reduced Cr(VI) of 100 $mg{\cdot}L^{-1}$ within 42 h with waste molasses as carbon source (Sun et al., 2019). However, the co-existence of the three different heavy metals with high concentrations in the testing systems used in this study created more complex environments, and thus led to a certain level of inhibitory effects on the activities of microorganisms. In other words, the reduction rates of Cr(VI) by microbial processes were mitigated and reduced, but the overall reduction efficiency was still reached over 99.00%. It can also be seen in Fig. 3b that waste molasses plays a major role in the removal of Cr(Total) and Cr(VI), which also reduces the load of microbial treatment, and thus provides a certain degree of protection for the microorganisms in the system. Moreover, the added waste molasses concentration in this study was 1.5 $g{\cdot}L^{-1}$, and the non-biological reducing agent was reduced as compared to the waste molasses concentration of 2.5 $g{\cdot}L^{-1}$ used in the experiments of Sun et al. (2019). Therefore, Cr(VI) reduction in the study of Sun et al. (2019) had a faster rate (finished in 42 h).

Fig. 3c shows that in the presence of heavy metals, the pH of the system with microorganisms (Group WM + BF) increases, while the pH of the WM system decreases from 6.3 to 5.1 after 24 h. The decrease of pH in the WM system may be due to chromate ions in normal hydrolysis will produce hydrogen ions (Eq. (7)), making the solution acidic. Then the pH rises again as the reducing substance ($C_kH_lO_i$) in the molasses reacts with hexavalent chromium, consuming some of the hydrogen ions (Eq. (8)).

The pH rised a bit in Group WM + BF systems might be due to the self-protection of microorganisms by producing sorptive substances to reduce the toxic effects of heavy metals. This because the heavy metal removal behavior by *Enterobacter* sp. SL and *Acinetobacter* sp. SL-1 strains were different. Heavy metals may be removed by complexation or direct adsorption. The increase of pH may be due to the large amount of ornithine and arginine secreted by SL during the bacteria self-protection process and the toxicity of heavy metals to the bacteria can be reduced (Vrancken et al., 2009). However, after 144 h of reaction, due to the toxicity of heavy metals and the consumption of waste molasses, the microbial activity decreased and the alkali production ability decreased. Thereby the pH of the system was lowered.

$$Cr_2O_7^{2-} + H_2O \rightleftharpoons 2HCrO_4^- \rightleftharpoons 2CrO_4^{2-} + 2H^+ \quad (7)$$

$$2CrO_4^{2-} + 2H^+ + C_kH_lO_i \rightarrow Cr^{3+} + H_2O + CO_2 + C_qH_wO_r \quad (8)$$

The results of the EEM can reflect changes in the species (Fig. 3d), content and composition of EPS (Dhal et al., 2013). In the study, EPS was mainly composed of tryptophan/tyrosine protein (A) and tryptophan/tyrosine (B) in the absence of heavy metals (Osburn et al., 2012). After adding heavy metals, in addition to the two peaks A and B, two new fluorescent peaks C and D appear (Fig. 3e), which represent the polycarboxylic acid, humic acid, and the fulvic acid peaks (Gao et al., 2017). It shows that the metabolism and products of bacteria changed under the influence of heavy metals, and the fluorescence intensity of tryptophan/tyrosine (B) was increased. The peak B was blue-shifted along the Ex axis as a whole, which was related to the change of macromolecular structures caused by the elimination of specific functional groups such as carbonyl, hydroxyl, and amino groups (Li et al., 2019).

3.3.2. XPS analysis of precipitation

As shown in Fig. 4, Zn and Cd after the reaction were mainly present in the form of sulfide metal (ZnS and CdS) and oxide (ZnO and CdO) (refer to NIST X-ray Photoelectron Spectroscopy Database). The oxide was mainly formed by the formation of a hydroxide precipitate, which was then washed and dried to be converted into an oxide. The action of microorganisms reduces SO_4^{2-} to S^{2-}, which in turn forms a sulfide metal precipitate (Singh et al., 2011). The main forms of Cr were CrOOH, Cr_2O_3 and $Cr(OH)_3$ (Fig. 4d). Srinath et al.'s results showed that a large amount of reductase and reducing substances existed on the cell membrane (Srinath et al., 2002). Therefore, a part of Cr(VI) will be reduced on the biofilm and converted into $Cr(OH)_3$ (a precipitate) to protect the microorganisms (Srinath et al., 2002). In addition, in the presence of various heavy metals, microorganisms produce alkaline substances in order to protect the cells, which also causes partial formation of chromium hydroxide.

3.4. Heavy metal removal kinetics model

The kinetic fitting results show that the removal of Zn, Cd, and Cr (Total) batter correlated with the pseudo-second-order kinetics model [$R^2_{(Zn\ and\ Cd)} > 0.98$, $R^2_{(Cr(Total)} > 0.94$, Fig. 5a–c]. Thus, the removal of Zn, Cd, and Cr(Total) may be controlled by chemical reactions, and the removal process involves the valence or covalence between heavy metals and microorganisms or waste molasses (Jia et al., 2017). The removal of Zn(II), Cd(II) and Cr(Total) could involve complex reactions (Kenawy et al., 2018). However, Cr(VI) accords with the pseudo-first-order kinetics model [$R^2_{Cr(VI)} > 0.96$, Fig. 5d]. The removal of Cr(VI) was dominated by reduction (Nakajima and Baba, 2004). The possible mechanism of heavy metal removal by bacteria and waste molasses is shown in Fig. 6.

When bacteria and waste molasses were present in wastewater containing heavy metals [Zn(II), Cd(II), and Cr(VI)], Zn(II), Cd(II), and Cr(Total) were removed in four paths: 1) biosorption of *Enterobacter* sp. SL and *Acinetobacter* sp. SL-1; 2) the action of microorganisms will increase the pH of the system (produces basic amino acids such as ornithine and arginine), so that heavy metals can be removed in the form of hydroxide; 3) microorganisms can produce tryptophan/tyrosine, polycarboxylic acid-like humic acids and fulvic acid-like substances, which react with heavy metalsto form new compounds; and 4) abiotic adsorption of waste molasses (mainly dependent on carboxyl and amino groups). The total chromium was removed mainly by route 4 (81%). Cr (VI) was mainly removed by abiotic reduction and biological reduction, and abiotic reduction played a dominant role (75%). It was noted that in the study of this process, the BF group had a slight error in the results because the medium contained a small amount of nutrients.

3.5. Implications

This research has a value for potential applications to treatment of wastewater contaminated with high concentrations of heavy metals, such as Zn smelting wastewater (Feng et al., 2019; Tang et al., 2019; Xu et al., 2017), electroplating wastewater (Chen et al., 2020), and heavy metals in smelting fume (Berger et al., 2018; Maweja et al., 2009), etc. Microbial species used in this study can tolerate heavy metals and reduce the concentration of heavy metals at the same time, while the activity of common microorganisms may be inhibited by heavy metals.

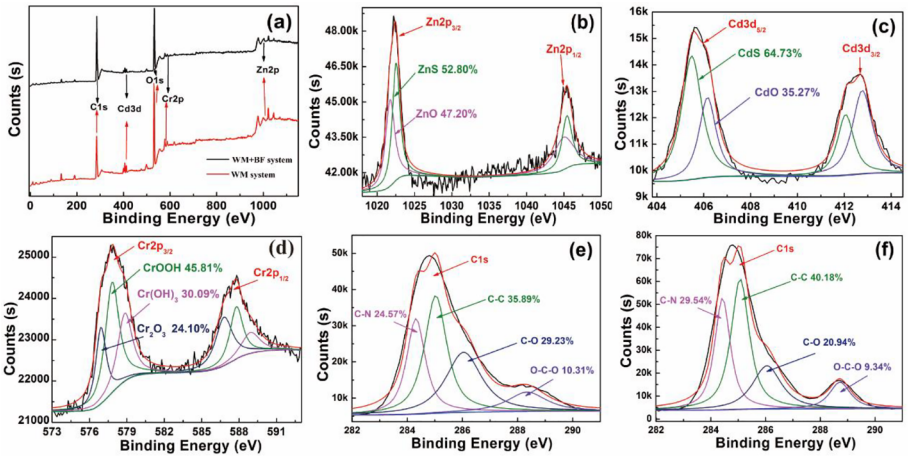

Fig. 4. XPS survey spectra (a); Zn 2p (b), Cd 3d (c), Cr 2p (d) core level spectra of precipitation of Group WM + BF; C 1 s core level spectra of precipitation of WM group (e) and WM + BF (f).

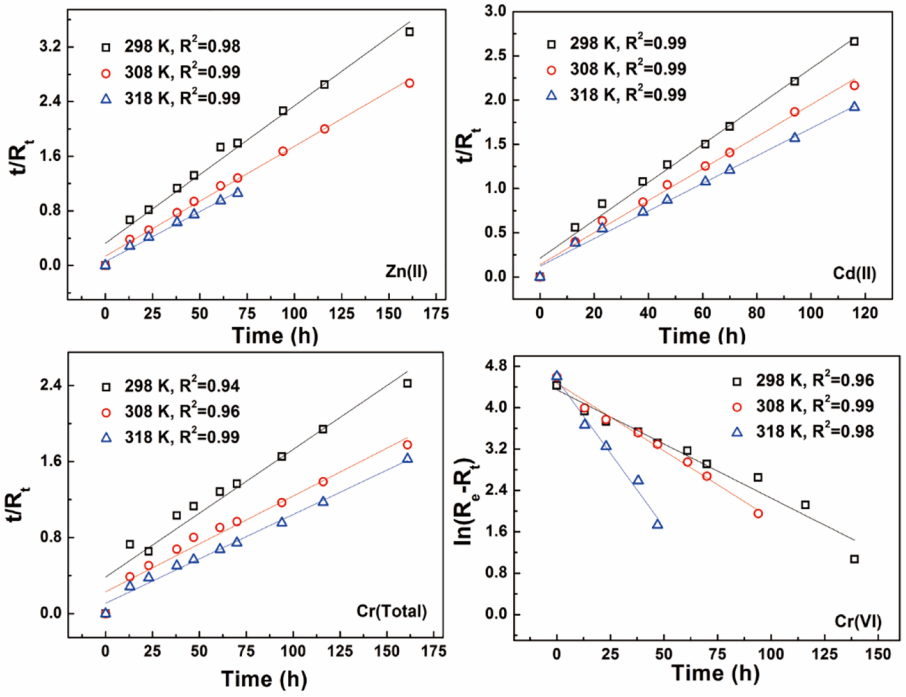

Fig. 5. Curve fitting for determination of kinetic parameters based on pseudo-first-order and pseudo-second-order models.

For weak acidic wastewater, *Enterobacter* sp. SL and *Acinetobacter* sp. SL-1 can also increase the pH of the system and thus, accelerate the precipitation of heavy metals. Waste molasses is a free carbon source and can save the cost for wastewater treatment. This has great economic advantages for small and medium-sized enterprises in wastewater treatment. Using waste molasses (a coproduct in sugar industry) as a carbon source of bacteria would achieve the goal of waste reuse. However, the residual concentrations of Zn(II) and Cd(II) after biological treatment were about 8 mg·L^{-1}, and further treatment is required.

4. Conclusion

Under anaerobic conditions with the synergistic actions of microorganisms and waste molasses the removal efficiencies of Zn(II), Cd(II), Cr(Total), and Cr(VI) (the initial concentrations were 87 mg·L^{-1}) were 91.86%, 89.39%, 99.61%, and 98.48% within 168 h, when the pH was 7.0, the concentration of molasses was 1.5 g·L^{-1}, and the temperature of 35 °C. Moreover, the removal pathways of the three heavy metals and the proportion of each pathways were described, which has potential value for industrial application of this technology.

CRediT authorship contribution statement

Yan Sun: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft. **Jirong Lan:** Validation, Writing - review & editing. **Yaguang Du:** Writing - review & editing. **Zhuang Li:** Investigation. **Xi Liao:** Investigation. **Dongyun Du:** Supervision, Funding acquisition, Writing - review & editing. **Hengpeng Ye:** Resources, Funding acquisition. **Tian C. Zhang:** Writing - review & editing. **Shaohua Chen:** Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

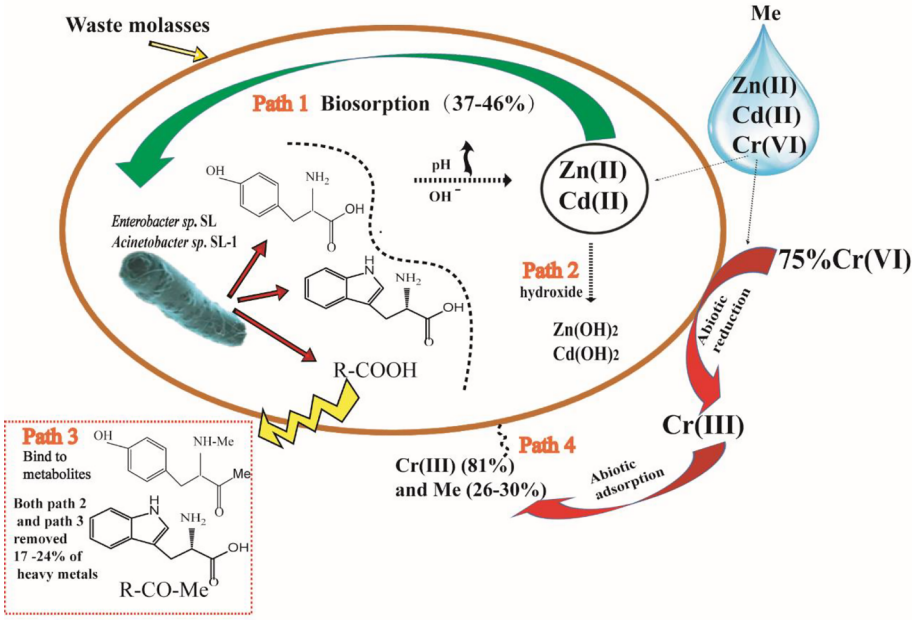

Fig. 6. Schematic diagram of the proposed mechanism for removal of heavy metals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.122797.

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