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Characterization of metal removal of immobilized *Bacillus* strain CR-7 biomass from aqueous solutions

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ABSTRACT

Bacillus strain CR-7 of multiple metal and antibiotic resistances was isolated. Its metal adsorption under different pretreatments and immobilizations from aqueous solution was characterized. Pretreatment with NaOH (0.1 mol L^{-1}) significantly improved Cu^{2+} adsorption capacity of the bacterial biomass. Sodium alginate (2%) was the ideal immobilization matrix. The immobilized and pretreated biomass had an obvious "orderliness", following the order of $Cu^{2+} > Zn^{2+}$ in the solution containing these two metals, and following the order of $Pb^{2+} > Al^{3+} > Cr^{6+} > Cu^{2+} > Fe^{3+} > Zn^{2+} = Ni^{2+} > Cd^{2+} = Co^{2+} > Mn^{2+}$ in the solution containing these 10 metals. ΔH° and ΔS° of Cu^{2+} adsorption were +7.68 J/mol and +16.628 J/mol K, respectively. The infrared peak of –N–H shifted greatly after Cu^{2+} adsorption. After adsorption treatment, some molecular groups disappeared in un-immobilized biomass but were still present in the immobilized biomass. Cu^{2+} adsorption fit both Langmuir and Freundlich isotherm models. It was concluded (1) that the Cu^{2+} adsorption process was endothermic, (2) that –N–H is a most important Cu^{2+}-binding group, (3) that immobilization prevents loss or damage of the Cu^{2+}-binding molecular groups, and (4) that Cu^{2+} adsorption of pretreated and immobilized biomass is homogeneous.

1. Introduction

Growing amount of heavy metal-polluted wastewater is rooted in the aggressive industrialization and urbanization [1]. If no further treatment, the heavy metals in the wastewater are likely eventually absorbed by and accumulated in living organisms, and threaten health of the living organisms. Even if some trace elements such as copper are essential to growth and development of the living organisms, they have toxic effects on the living organisms at high concentrations [2]. The polluted wastewater has therefore received much concern [3]. Methods on treatment of heavy metal-polluted wastewater can be divided into (1) physical and/or chemical reactions such as chemical precipitation, ion exchange, filtration, and (2) adsorption either by biomaterials such as microbial and plant derived biomasses [4–6] or by non-biomaterials such as fly ash, carbon slurry, red mud, kaolinite, baggage fly ash, bentonite, electric furnace slag, and montmorillonite [7–10]. Physical and/or chemical reactions are not fast for removal processes but easy to commercialization and application. However, physical and/or chemical reactions-based methods have unacceptable defects such as high costs, high energy consumption, secondary pollution, and/or production of a large amount of toxic chemical sludge which is difficult to treat [5,11,12]. Relatively, adsorption methods have an advantage over physical and/or chemical methods because of low cost and low energy, and particularly due to no the secondary pollution [4,13]. However, if no other auxiliary measures, the microbial biomass-based treatment methods are hardly applied because of several major defects such as solid–liquid separation problems, possible biomass swelling, inability to regenerate/reuse, use in the continuous mode, and development of high pressure drop in the column mode [14,15]. To overcome these defects, immobilization techniques for microbial biomasses have been developed [14,16].

Most researches on immobilization techniques-based metal removal from the solutions that have been conducted were based on the use of granulized microbial adsorbents packed in columns [16]. This approach seems to be inapplicable to metal removal by free cells because the resulting bed is easily plugged by free cells

Abbreviations: AAS, atomic absorption spectrometry; CDC, Cu^{2+} adsorption capacity; CRE, Cu^{2+} removal efficiency; ΔH°, enthalpy change; ΔS°, entropy change; EDAX, energy dispersive x-ray analysis; IAS, infrared absorption spectra; IR, Infrared; PB, NaOH (0.1 mol L^{-1})-pretreated biomass; PVA, polyvinyl alcohol; rpm, revolutions per min; SEM, scanning electron microscope; UUB, un-pretreated and un-immobilized biomass.

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[16]. Therefore, microbial immobilization techniques still needs further study. Toward this goal, choice of the immobilization matrices is a key step. By now, numbers of immobilization matrices have been developed and used, such as sodium or calcium alginate, polysulfone, polyacrylamide, polyurethane and silica [14]. Even so, immobilization techniques specific to bacterial species are required not only because of bacterial species diversity [17] but also owing to difference in the nature of immobilization matrices.

Although *Bacillus* biomass has been used for removal of heavy metal from aqueous solution [14,16] researches on heavy metal removal by immobilized Bacillus biomass from the solutions were very limited. Immobilization matrices used in *Bacillus* biomass included Diaion SP-850 resin [18], silica gel [21], Amberlite XAD-4 [22], and calcium alginate [23]. However, little is known about characteristics of heavy metal removal by *Bacillus* biomass from aqueous solution with mixed metals, and effects of immobilization on metal-binding molecular groups of the biomass. In this study, *Bacillus* strain CR-7 was chosen as an adsorbent because of its resistance to multiple metals. The aim of this study was to characterize metal adsorption of the bacterial biomass immobilized with sodium alginate, gelatin, and polyvinyl alcohol (PVA).

2. Materials and methods

2.1. Isolation of bacteria

The soil sample from disposal sites of the tailings of a copper mine in Guangxi of China was used to isolate heavy metal-resistant bacteria. Briefly, the soil suspension prepared with sterile water was plated onto plates of Luria-Bertani's (LB) agar medium containing different concentrations of $CuSO_4{\cdot}5H_2O$. The plates were placed for incubation of bacteria for 24 h at 37 °C.

2.2. Analysis of metal and antibiotic resistance profiles of bacteria

One millilitre of overnight bacterial culture with an OD_{600} value of 0.2 was transferred onto 10 mL LB medium containing different concentrations of metal salts and antibiotics, and cultured for 24 h at 37 °C by rotation at 180 rpm. One hundred microlitres of the resulting bacterial culture were plated onto plates of LB agar medium without metal salts and antibiotics, and then placed for 24 h at 37 °C. The resistance was reported as maximum inhibitory concentration (MIC) that could inhibit growth of bacteria.

2.3. DNA manipulation and, amplification, cloning and sequencing analysis of bacterial 16S rDNA sequence

DNA manipulation referred to conventional methods in the literature [24]. Bacterial 16S rDNA was amplified from the bacterial genomic DNA as the method in the literature [25] by polymerase chain reaction with primers (forward 5′-TAGGGTTACC-TTGTTACGACTT-3′, and backward 5′-AGAGTTGATCATGGCTCAG-3′). The amplified DNA was cloned into the pUCm-T vector (Sangon, Shanghai, China) and then sequenced.

2.4. Treatment of bacteria

Two millilitres of overnight bacterial culture with an OD_{600} value of 0.5 were added to 200 mL LB medium, and cultured at 37 °C by rotation for 24 h at 180 rpm. The resulting bacterial culture was collected by centrifugation for 10 min at 4500 rpm. The collected bacteria were washed twice with sterile water until pH of the filtrate reached 7, and collected again by centrifugation.

For pretreatment, the collected bacterial biomass was added in NaOH, HCl or HNO_3 solution as a ratio 1 (g):100 (mL) of the bacterial culture to reagent, and incubated for 30 min at 35 °C by rotation at 150 rpm. The control treatment was conducted in sterile water in parallel with procedures of the pretreatment. All procedures for washing and collecting bacterial culture were performed as indicated above. The treated bacteria were collected and dried for 24 h at 80 °C. Dried bacteria were ground into powder and sieved through a 100-mesh sieve for further use.

2.5. Immobilization of bacteria

Powdered bacterial biomass was immobilized in sodium alginate, gelatin, and PVA. Immobilization procedures followed the methods in the literature [26]. Briefly, matrix solutions were sterilized as conventional moist heat sterilization method. Powdered bacterial biomass was mixed with the matrix solution as a ratio 1 (g):100 (mL). The mix was slowly dripped through a syringe with a needle 12 into 4% (w/v) $CaCl_2$ solution, generating immobilization beads. After laid aside for 24 h in $CaCl_2$ solution, the resulting immobilization beads were rinsed for further use with sterile water until pH of the washing effluent was up to 7.

The strength of immobilization beads was assayed as the method in the literature [26]. In brief, 50 beads were added to 100 mL water solution and subjected to rotation for 24 h at 220 rpm at 50 °C to observe breakage of the beads.

For assay of mass transfer resistance, immobilization beads were mixed with methylene blue dye as a ratio 5 (g):100 (mL) and laid aside for 24 h at room temperature. After that, the beads were fully rinsed with sterile water, and cut from the middle with a shaving blade to observe the internal coloring of the beads.

2.6. Batch experiment of metal adsorption

The metal adsorption was conducted as the rotation method in 100 mL solution containing metal (s). The conditions for adsorption were detailed in the figure legends. Quantitative analysis of metals in samples was conducted by atomic absorption spectrometry (AAS) on a Hitachi Z-8000 atomic absorption spectrophotometer (Tokyo, Japan) equipped with a graphite tube atomizer following standard procedures.

Energy dispersive x-ray analysis (EDAX) was performed for identification of metals using the Hitachi 3400-N scanning electron microscope (SEM) with the EDAX Genesis program and a SUTW-SAPPHIRE detector. Preparation of the samples, i.e. adsorbents, for SEM analysis referred to the procedures in the literature [27] but with some modifications. The samples were fixed in 0.1 mol L^{-1} phosphate buffer (pH 7.3) containing 2.5% glutaraldehyde for 30 min at room temperature, and then washed three times with the phosphate buffer followed by further dehydration in gradient concentrations of ethanol for 10 min each. The dehydrated samples were vacuum-dried. The dried samples were ground into powder, and then sputter-coated for scanning with gold. For parameters for instrument's operation, detector's inclination was 35°, spectra was registered from 0 to 20 keV with a resolution of 130.87 eV, and pulse reading time was 100 s.

The infrared absorption spectra (IAS) of the samples were recorded within the range of 4000–400 cm^{-1} with a resolution of 6 cm^{-1} on a Fourier transform infrared spectrometer (Nicolet 5700) with a DTGS detector. The samples were dried at 70 °C till constant weight and ground into powder. Each powdered sample (2 mg) was mixed with KBr (200 mg). The sample–KBr mix was pressed the mixture into a pellet, which was analyzed by the Fourier transform infrared spectrometer. The average over 64 scans was collected for each measurement.

Construction of adsorption isotherms referred to the methods described in the literature [19,28]. The thermodynamic parameters on the adsorption, enthalpy change (ΔH°) and entropy change (ΔS°), were assessed as the equations described in the literature [10].

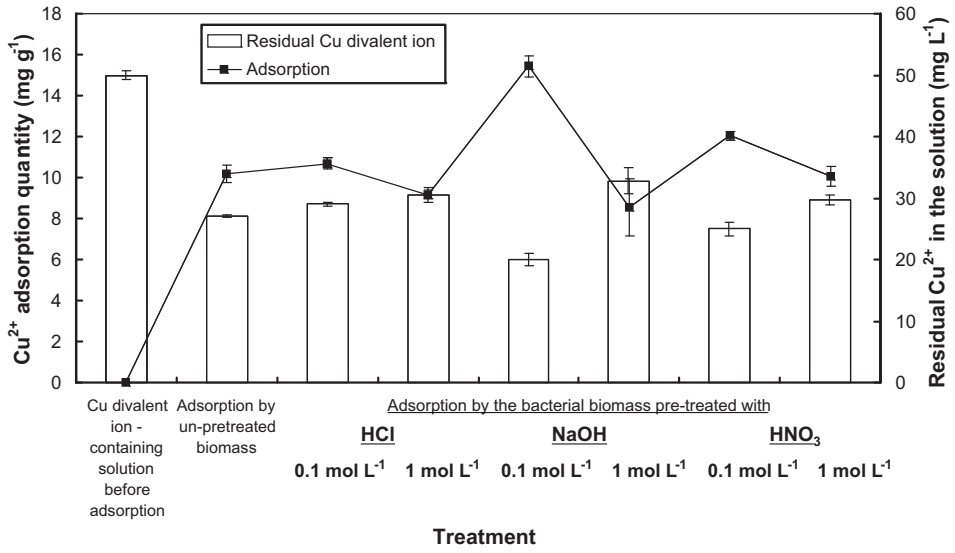

Fig. 1. The effect of pretreatment of *Bacillus* strain CR-7 biomass on Cu^{2+} adsorption and removal. Adsorption was conducted at 35 °C for 30 min under rotation at 150 rpm in 100 mL solution with an initial pH 7 and containing Cu^{2+} (50 mg L^{-1}) and powdered bacterial biomass (10 g L^{-1}). Cu^{2+} was quantified by AAS. Each datum was presented with the mean ± standard deviation (SD) from three batches of experiments. X axle indicates different types of the biomasses, pretreated and un-pretreated. AAS, atomic absorption spectrometry.

3. Results and discussion

3.1. Cu^{2+}-resistant bacteria

Although metal resistance of bacteria is not absolutely associated with their capacity of metal adsorption, the resistance is a marker for selection of target bacteria. As indicated in the literature [14,16], even in belonging to the same genus, bacterial biomass displayed great difference in metal adsorption capacity. As for *Bacillus* biomass, Cu^{2+} adsorption capacity is relatively lower [14,16].

Mine tailings are the materials remaining after extraction and beneficiation of ores [29]. We firstly conducted isolation of Cu^{2+}-resistant bacteria form the soil in disposal sites of the copper mine tailings, where copper content in the soil was analyzed by ASS to be 0.98 ± 0.02 mg kg^{-1}. As a result, a Cu^{2+}-resistant bacterial strain, numbered CR-7, was obtained on the plate of LB agar medium containing 7 mmol Cu^{2+} L^{-1}.

The 16S rDNA sequence of bacterial strain CR-7 has a highest homology (99%) with that (GenBank accession no. EU915719.1) of *Bacillus* sp. ZSA, which has been released in GenBank database under accession no. GU564472. This bacterial isolate was therefore named *Bacillus* strain CR-7. *Bacillus* strain CR-7 showed resistances to multiple metals, including Hg^{2+} ($HgCl_2$, 0.001 mmol L^{-1}), Cd^{2+} ($CdCl_2{\cdot}2H_2O$, 0.01 mmol L^{-1}), Zn^{2+} ($ZnSO_4{\cdot}7H_2O$, 0.3 mmol L^{-1}), Al^{3+} ($Al_2(SO_4)_3{\cdot}18H_2O$, 0.5 mmol L^{-1}), Co^{2+} ($CoCl_2{\cdot}6H_2O$, 0.5 mmol L^{-1}), Fe^{3+} ($FeCl_3{\cdot}5H_2O$, 2 mmol L^{-1}), Ni^{2+} ($NiCl_2{\cdot}6H_2O$, 3 mmol L^{-1}), Cr^{6+} ($K_2Cr_2O_7$, 4 mmol L^{-1}), Pb^{2+} ($Pb(CH_3COO)_2{\cdot}3H_2O$, 4 mmol L^{-1}), Cr^{3+} ($CrCl_3{\cdot}6H_2O$, 8 mmol L^{-1}), and Mn^{2+} ($MnCl_2{\cdot}4H_2O$,17 mmol L^{-1}). The metal resistance of this bacterial isolate is much higher than that of reference bacteria *Escherichia coli* [30]. *Bacillus* bacteria of resistance to multiple heavy metals were not reported before. The multiple metal resistance likely reflects its adaption to the soil environment in disposal sites of the copper mine tailings.

It has been suggested that there is a co-selection of antibiotic and metal resistances in prokaryotes [30–32]. Therefore, resistance of *Bacillus* strain CR-7 to antibiotics was assayed. As a result, *Bacillus* strain CR-7 showed a wider spectrum of antibiotic resistance, including kanamycin (300 μg mL^{-1}), ampicillin (100 μg mL^{-1}), spectinomycin (50 μg mL^{-1}), streptomycin (5 μg mL^{-1}), chloromycetin (3 μg mL^{-1}), rifampicin (2 μg mL^{-1}), cefotaxime (1 μg mL^{-1}) and gentamycin (1 μg mL^{-1}). Co-existence of metal and antibiotic resistances results from heavy metal resistance that may contribute to generation of antibiotic resistance genes by increasing the selective pressure of the environment [30]. Another main reason for this is that antibiotic and metal resistance systems in prokaryotes share structural and functional characteristics [31].

To our knowledge, this is the first study that characterizes metal and antibiotic resistance profiles of *Bacillus* bacteria.

3.2. Cu^{2+} adsorption of the pretreated bacterial biomass

Usually, the density of metal-binding sites or groups on a biomass is very low. Therefore, most of the bioadsorbents require pretreatment in the metal adsorption [14]. It is reported that metal adsorption of bacterial biomass can be significantly enhanced by appropriate pretreatments [14,16]. However, effect of pretreatment on metal adsorption of bacterial biomass varies greatly with biomass species, pretreatment reagents, and metal species. In application of bacterial biomass to metal adsorption, dead biomass is easily controlled than living cells because of no environmental safety risks. In view of this, batch experiments of Cu^{2+} adsorption were done with powdered biaomass of *Bacillus* strain CR-7. We conducted pre-experiments on effects of pretreatments with different concentrations (0.1, 0.3, 0.5, 0.7, 0.9 and 1 mol L^{-1}) of NaOH, HCl and HNO_3 on Cu^{2+} adsorption capacity (CDC) of the bacterial biomass before this study. The CDC of the biomass was decreased with increasing alkaline and acid concentrations in pretreatments (data not shown). Therefore, two concentrations of NaOH (HCl and HNO_3), 0.1 mol L^{-1} and 0.9 mol L^{-1}, were chosen in the following pretreatment experiments.

In comparison with control (un-pretreatment), pretreatment with 0.1 mol L^{-1} NaOH made CDC increased by 50%, and pretreatments with 1 mol L^{-1} NaOH led to an decrease in CDC by 16.1%; pretreatment with 0.1 mol L^{-1} HNO_3 enhanced CDC by 17.9%. However, pretreatments with 0.1 mol L^{-1} HCl, 1 mol L^{-1} HCl and 1 mol L^{-1} HNO_3 made no significant effects on CDC.

The acid pretreatment is considered unfavorable for metal adsorption by biomass because acid can make the biomass protonated, on the contrary, alkaline pretreatment can improve the

Table 1
Characteristics of immobilization beads.

Immobilization	Preparation	Shape	Breakage percentage of beads	Mass transfer resistance of beads
Sodium alginate (2%) + PB (1%)	Easy	Sphere of uniform size (about 0.5 cm in diameter)	0	Lowest
Sodium alginate (2%) + gelatin (1%) + PB (1%)	Easy	Sphere of uniform size (about 0.5 cm in diameter)	0	Lowest
Sodium alginate (2%) + PVA) (7%) + PB (1%)	Difficult	Irregular	12%	High
Sodium alginate (2%) + gelatin (1%) + PVA (7%) + PB (1%)	General	Near sphere	2%	General

PB, NaOH (0.1 mol L^{-1})-pretreated biomass; and PVA, polyvinyl alcohol.

metal adsorption capacity because alkaline can increase the overall negative charge on the surface of cells, and make the relevant functional groups de-protonated [14].

However, pretreatments with high concentrations of alkalines made decrease in CDC (Fig. 1) likely because high concentrations of alkalines not only do a severe damage to cell wall structures and consequently lead to the loss of more contents in the biomass but impair also the metal-binding molecular groups on biomass surfaces.

3.3. Characteristics of immobilized bacterial biomass

Bacillus strain CR-7 biomass was immobilized in matrices of sodium alginate, gelatin and PVA. Characteristics of the resulting immobilization beads were summarized in Table 1. Different immobilization approaches resulted in different effects on CDCs of the biomass. Biomass pretreated with NaOH (0.01 mol L^{-1}) and immobilized with 2% single sodium alginate displayed the highest CDC (18.5 mg g^{-1}) and the highest Cu^{2+} removal efficiency (CRE) (76%) in comparison with other biomasses (Fig. 2).

By now, different matrices have been used for immobilization of microbial biomass for metal removal from the solution. Compared with alginate, PVA is a better matrix for immobilization of fungal biomass for gold adsorption [33,34] especially for passive immobilization of fungal spores because this matrix has extensive surface area, depressions and cavities [35]. In comparison with alginate, polyacrylamide, polysulfone and polyurethane, polyurethane is more suitable for immobilization of biomass of *Pseudomonas aeruginosa* strain CSU for uranium adsorption [36]; Alginate is a better matrix for immobilization of *Rhizopus arrhizus* biomass for chromium VI adsorption [37]; polyacrylamide gel is an ideal matrix for immobilization of *P. aeruginosa* biomass for adsorption of lanthanide such as La, Eu and Yb [38]. These results strongly suggest that selection of the immobilization matrices should be based on both biomass types and metal species. For bacterial biomass, this is at least because there are great differences between cells in the chemical composition of the cell wall [16]. For instance, 18 strains

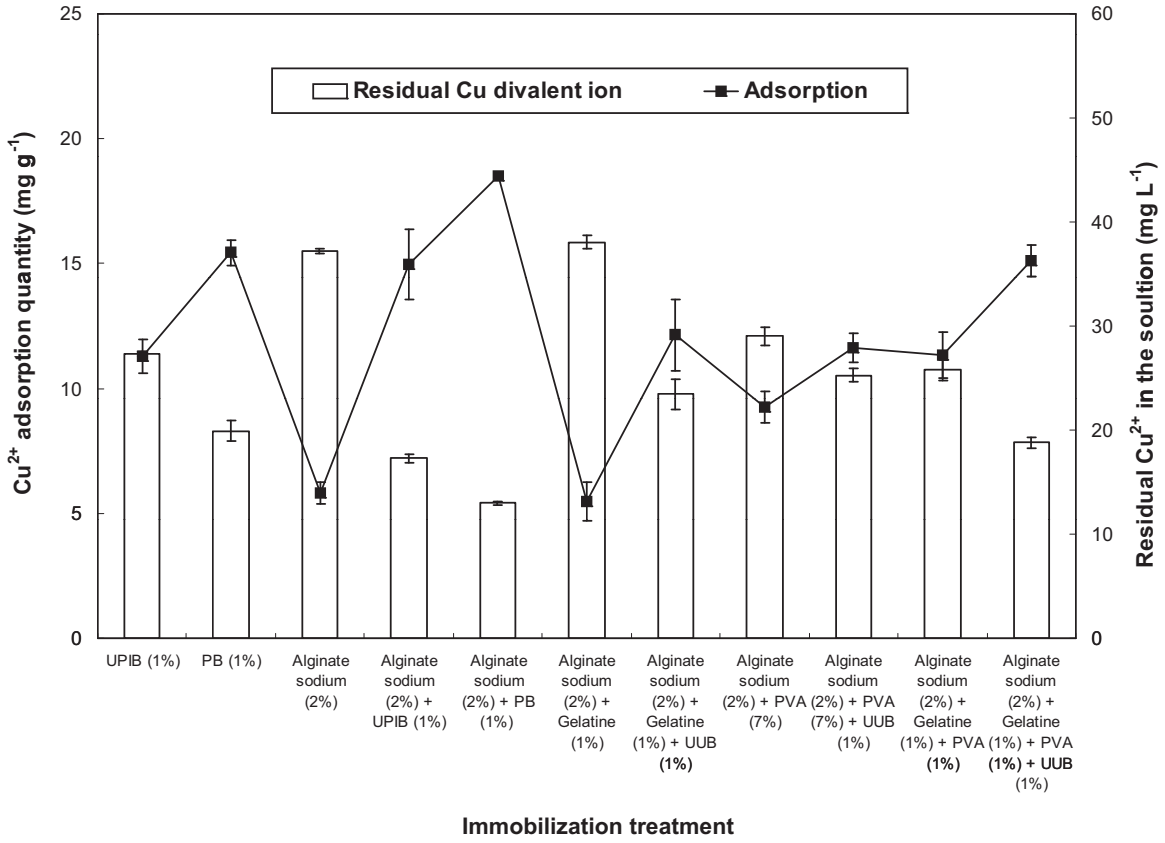

Fig. 2. Cu^{2+} adsorption and removal by immobilized biomass of *Bacillus* strain CR-7. Adsorption was conducted at 35 °C for 1 h under rotation at 150 rpm in 100 mL solution with an initial pH of 7 and containing Cu^{2+} (50 mg L^{-1}) and adsorbents (0.5 g L^{-1}). Cu^{2+} was quantified by AAS. Each datum was presented with the mean ± SD from three batches of experiments. *X* axis indicates different types of the biomasses: pretreated and un-pretreated, as well as immobilized and un-immobilized. AAS, atomic absorption spectrometry; PB, NaOH (0.1 mol L^{-1})-pretreated biomass; PVA, polyvinyl alcohol; and UUB, un-pretreated and un-immobilized biomass.

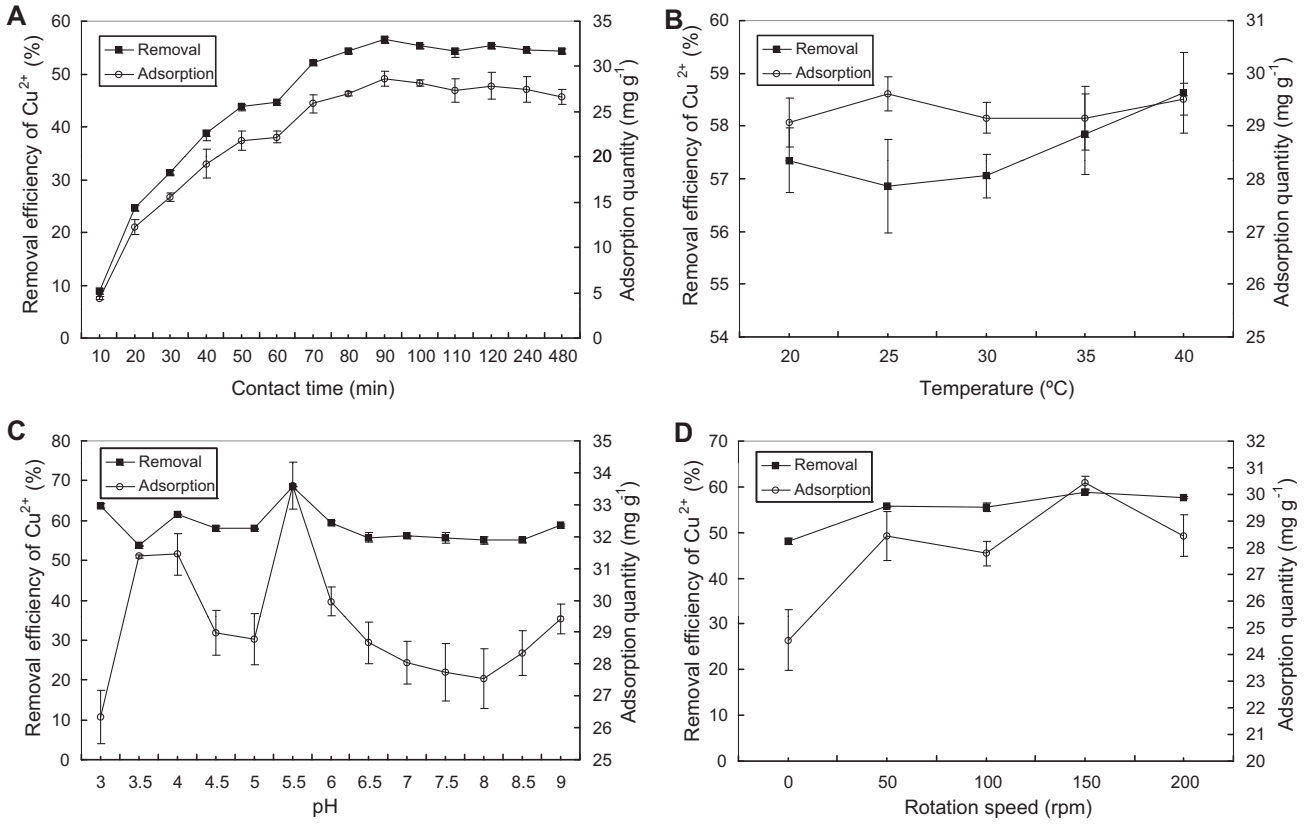

Fig. 3. Cu^{2+} adsorption and removal by alginate (2%)-immobilized PB of *Bacillus* strain CR-7 under different conditions. A: Contact time effect. Adsorption was conducted at 35 °C under rotation for different time at 150 rpm in 100 mL solution with an initial pH of 7 and containing Cu^{2+} (50 mg L^{-1}) and alginate (2%)-immobilized PB (0.5 g L^{-1}). B: Temperature effect. Adsorption was conducted for 90 min at different temperatures following other conditions as described above. C: Initial pH effect. Adsorption was conducted for 90 min at 35 °C following other conditions as described above in the solution with different initial pH. D: Rotation speed effect. Adsorption was conducted for 90 min at 35 °C under different rotation speeds following other conditions as described above in the solution with an initial pH of 5.5. Cu^{2+} was quantified by AAS. Each datum was presented with the mean ± SD from three batches of experiments. AAS, atomic absorption spectrometry; and PB, NaOH (0.1 mol L^{-1})-pretreated biomass.

of *Pseudomonas* bacteria showed a significant difference in the fluoride adsorption abilities [39]; *P. aeruginosa* PU21 biomass has a strong ability to adsorb heavy metals such as Pb^{2+} because there are very strong negatively charged SH groups on the cell wall [40]; The great differences in metal adsorption between *Bacillus* biomasses were also observed [16] maybe due to differences in some functional molecules such as extracellular polymer [19] and S-layer proteins [20].

3.4. Characterization of CDC and CRE of pretreated and immobilized bacterial biomass

CDC and CRE of NaOH (0.1 mol L^{-1})-pretreated and sodium alginate (2%)-immobilized biomass of *Bacillus* strain CR-7 were further studied under different conditions. As a result, CDC and CRE slowly increased with contact time and were highest 90 min after contact (Fig. 3A). Obviously, CDC and CRE maintained a steady state over 90 min (Fig. 3A), suggesting that Cu^{2+} adsorption equilibrium occurred at the time point of 90 min after contact.

CDC and CRE slightly, but not significantly, increased with increasing temperature from 20 to 40 °C (Fig. 3B). ΔH° and ΔS° of Cu^{2+} adsorption were estimated to be +7.68 J/mol and +16.628 J/mol K, respectively, suggesting that the Cu^{2+} adsorption process was endothermic. The ΔH° value implies formation of Cu^{2+} complexation with anionic oxygen ligands, and the ΔS° value is indicative of inner sphere complexation by multiple ligands [41]. This partially explains why CDC and CRE slightly increased with the temperature. Additionally, high temperatures can overcome mass transfer resistance of the immobilization matrices and therefore increase matter exchange chances [42].

CDC was greatly affected by initial pH of the solution. It was weakest at pH 3, significantly enhanced with increasing pH from 3.5 to 4, decreased again with further increasing pH from 4.5 to 5, reached a highest level (33.3 mg Cu^{2+} g^{-1}) at pH 5.5, and sharply declined again over pH 5.5 (Fig. 3C). Such change in Cu^{2+} adsorption with initial pH of the solution was associated in part with pH effects on immobilization matrix. For example, the carboxylic parts of alginate can attract H^{+} under the acid conditions, thereby decreasing H^{+} concentration around the active parts of immobilized cells [43]. Unlike dramatic decline in CDC, CRE stayed at a relatively stable high-level state under initial pH of >5.5. This is likely because high pH can cause the formation of metal hydroxide and other metal–ligand complexes which can significantly reduce the amount of metal ions [14]. On the other hand, microprecipitation results from formation of metal hydroxide under high pH, and thereby indirectly leads to decrease in metal content in the solution [44].

Mass transfer resistance is a factor that affects metal adsorption of immobilized biomass [14,16]. On the whole, CDC increased with rotation speed and was maximum (30.4 mg Cu^{2+} g^{-1}) at 150 rpm, but CRE slightly, not significantly, increased (Fig. 3D). This indicates that mass transfer is not an absolute factor limiting Cu^{2+} adsorption of immobilized biomass of *Bacillus* strain CR-7. A higher rotation speed, 200 rpm, could not cause cracks or ruptures of the adsorbent beads. A similar result was that increase of rotation speed to more than 150 rpm caused no considerable change in production of 11α-hydroxyprogesterone by immobilized *Aspergillus terreus* biomass [45].

Increasing the amount of the adsorbent beads from 0.5 to 1.5 g L^{-1} led to a sharp decline in CDC, and caused a slight decrease in CRE (Fig. 4). Such decreases in CDC and CRE with used amount of the adsorbent beads were probably due to increase in external mass transfer resistance.

3.5. Identification of metal(s) absorbed by immobilized bacterial biomass

By EDAX, NaOH (1 mol L^{-1})-pretreated biomass (PB) (Fig. 5A), sodium alginate (Fig. 5B), and sodium alginate (2%)-immobilized PB (Fig. 5C) only had some constituent elements such as C, N, O, Na or Fe before metal adsorption. After adsorption in Cu^{2+}-containing solution, PB (Fig. 5D), sodium alginate (Fig. 5E) and sodium alginate (2%)-immobilized PB (Fig. 5F) all contained Cu^{2+}, but Cu^{2+} content in the immobilized PB was highest (Fig. 5F). Adsorption of the immobilized PB in the solution containing mixed metals was further characterized. As a result, metal adsorption showed an obvious "orderliness", following the order of $Cu^{2+} > Zn^{2+}$ in the solution containing these two metals (Fig. 5G), and following the order of $Pb^{2+} > Al^{3+} > Cr^{6+} > Cu^{2+} > Fe^{3+} > Zn^{2+} = Ni^{2+} > Cd^{2+} = Co^{2+} > Mn^{2+}$ in the solution containing these 10 metals (Fig. 5H). The orderliness of metal adsorption differed slightly from that in the literature

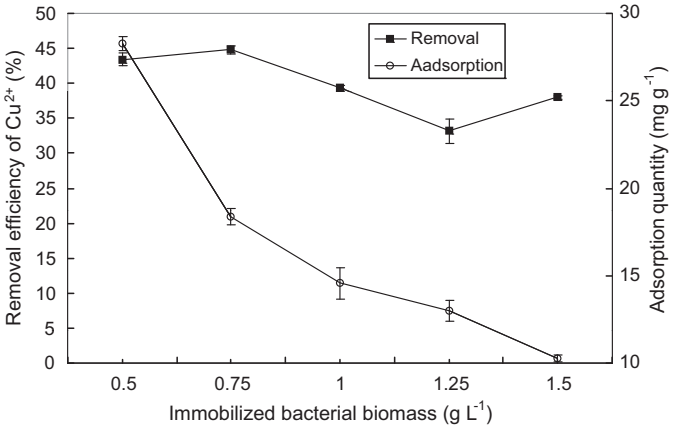

Fig. 4. Change in Cu^{2+} adsorption and removal with dose of alginate (2%)-immobilized PB of *Bacillus* strain CR-7. Adsorption was conducted at 35 °C by rotation for 90 min at 150 rpm in 100 mL solution with an initial pH of 5.5 and containing Cu^{2+} (50 mg L^{-1}) and different doses of alginate (2%)-immobilized PB. Cu^{2+} was assayed by AAS. Each datum was presented with the mean ± SD from three batches of experiments. AAS, atomic absorption spectrometry; and PB, NaOH (0.1 mol L^{-1})-pretreated biomass.

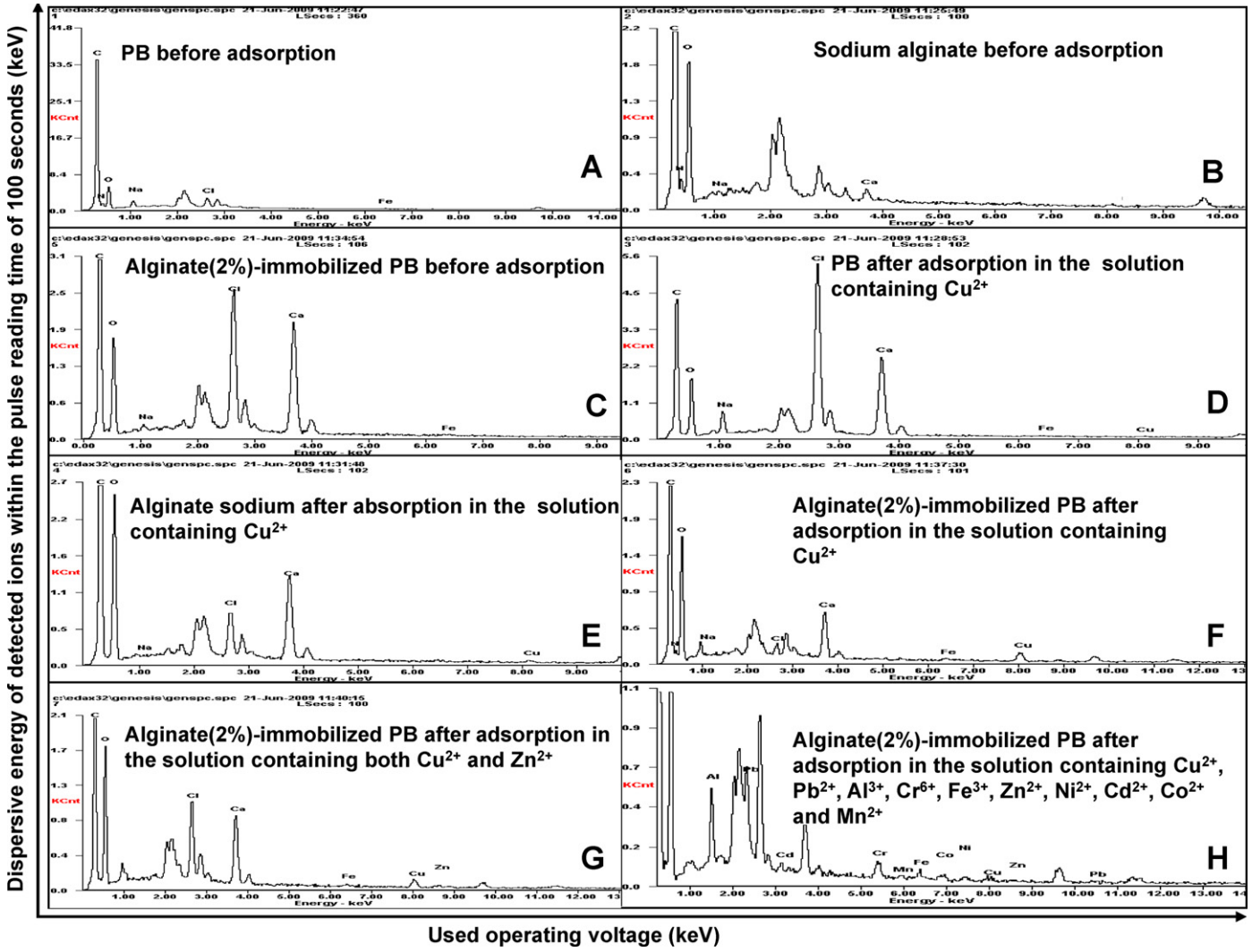

Fig. 5. Identification of metal ions absorbed by the adsorbents. Adsorption was conducted at 35 °C by rotation for 90 min at 150 rpm in 100 mL solution with an initial pH of 5.5 and containing Cu^{2+} (50 mg L^{-1}), metal (s) and adsorbents. The concentration of each metal and dose of each adsorbent were 50 mg L^{-1} and 0.5 g L^{-1}, respectively. Metals were identified by EDAX. PB, NaOH (0.01 mmol L^{-1})-pretreated biomass; and EDAX, energy dispersive x-ray analysis.

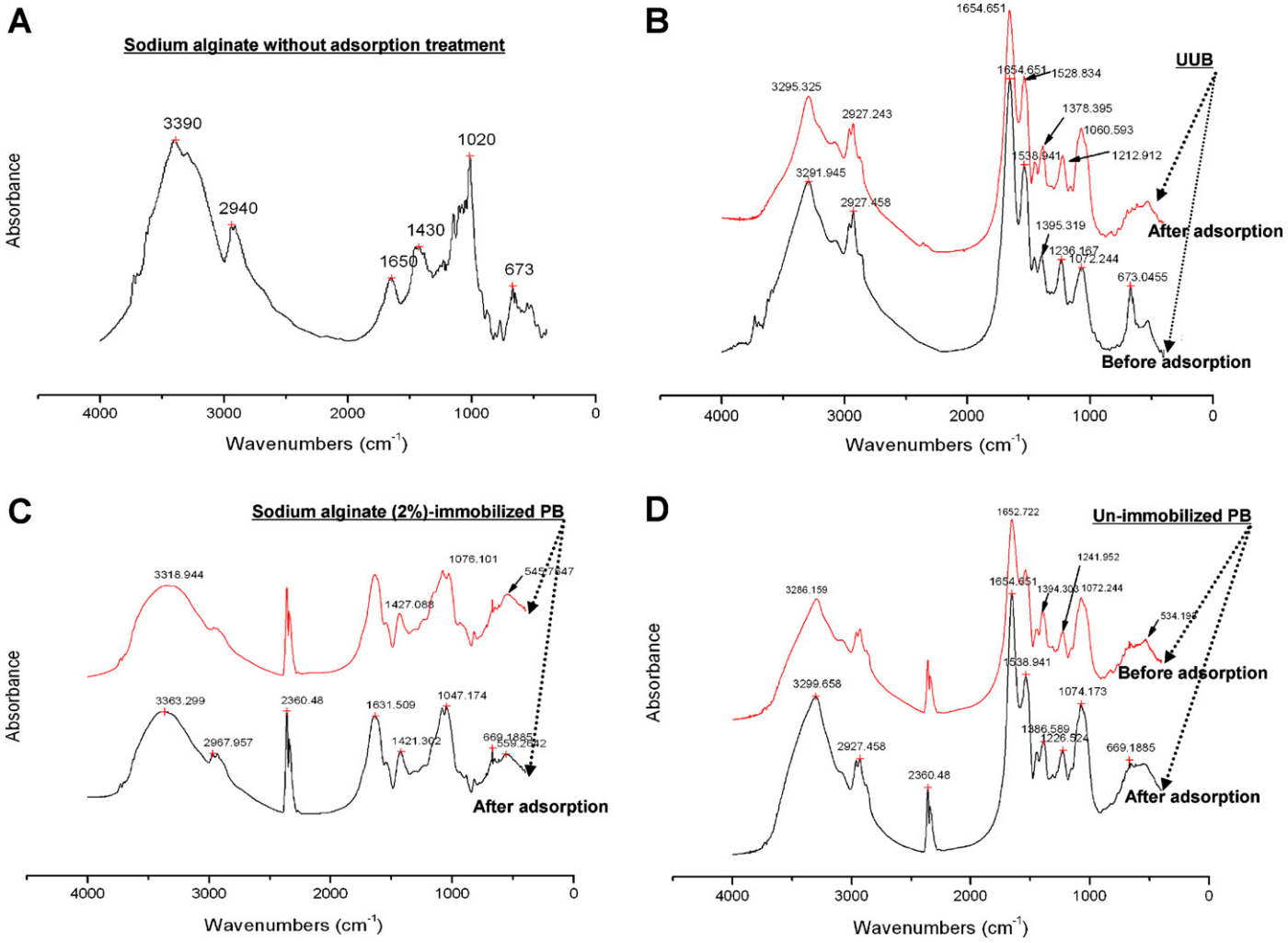

Fig. 6. IASs of adsorbents before and after adsorption of Cu^{2+}. Adsorption was conducted at 35 °C by rotation for 90 min at 150 rpm in 100 mL solution with an initial pH of 5.5 and containing Cu^{2+} (0.5 g L^{-1}) and adsorbent (50 mg L^{-1}). IAS, infrared absorption spectra; PB, NaOH (0.01 mmol L^{-1})-pretreated biomass; and UUB, un-pretreated and un-immobilized biomass.

[16] likely due to differences in experimental conditions [42]. Additionally, orderliness of metal adsorption results not from the differences between metals in entropy [10], but also from the number and density of metal-binding ligands in the specific biomass. For example, softer ions such as Cd^{2+} are expected to bind to nitrogen and sulfur donor atoms of the ligands on the algal cell wall, whereas borderline ions such as Cu^{2+} and Pb^{2+} would bind to any of the ligands [46].

3.6. Cu^{2+}-binding molecular groups

Some metal-binding groups can be determined by IAS analysis [16,47]. Usually, peak stretching in infrared (IR) absorbance implies that corresponding molecular groups are functional [21]. IASs of sodium alginate, UUB, sodium alginate (2%)-immobilized PB and un-immobilized PB before and after Cu^{2+} adsorption were therefore compared (Fig. 6). Consequently, IAS of sodium alginate (Fig. 6A) differed greatly form that of UUB (Fig. 6B), sodium alginate (2%)-immobilized PB (Fig. 6C) and un-immobilized PB (Fig. 6D). Change in some important IR peaks and corresponding molecular groups were summarized in Table 2. These molecular groups fallen into the category of metal-binding groups previously reported in the literature [16,47]. As shown in Fig. 6, IR peaks of most molecular groups were wider in sodium alginate (2%)-immobilized PB (Fig. 6C) than in UUB (Fig. 6B) and un-immobilized PB (Fig. 6D), agreeing with an opinion that immobilization increased the thermal stability of the cells [43]. –N–H was likely a group key to Cu^{2+} binding because its IR peak in sodium alginate (2%)-immobilized PB shifted more from 3318.944 before adsorption to 3363.299 cm^{-1} after adsorption (Table 2). Some molecular groups disappeared in un-immobilized biomass after adsorption. For example, after adsorption treatment, –OH at 3730 cm^{-1} as well as γOH at 673.045 cm^{-1} disappeared in UUB (Fig. 6B) and appeared in the sodium alginate (2%)-immobilized PB (Fig. 6C). These results also strongly indicate that immobilization prevents loss or damage of some molecular groups.

3.7. Isotherm models

The kinetics of the adsorption is usually explained with the equilibrium adsorption isotherms such as Langmuir and Freundlich isotherm models. The Langmuir isotherm equation is represented by $q_e = q_{max}K_LC_e/(1 + K_LC_e)$ [48]. In this study, q_e was the equilibrium Cu^{2+} concentration (mg g^{-1}) on the adsorbent, C_e was the equilibrium Cu^{2+} concentration (mg L^{-1}) in the solution, q_{max} was the monolayer adsorption capacity (mg g^{-1}) of the adsorbent, and K_L was the Langmuir adsorption constant (l mg L^{-1}). A Cu^{2+} adsorption experiment was conducted with sodium alginate (2%)-immobilized biomass of *Bacillus* strain CR-7 at 35 °C. The resulting data were shown in Fig. 7A, and turned into a linear model (Fig. 7B).

Table 2
Stretching changes of IR absorbance peaks of important molecular groups of the adsorbents before and after Cu^{2+} adsorption.

Adsorbent	Before adsorption		After adsorption	
	IR peak (cm^{-1})	Assigned molecular group	Stretching or status of IR peak (cm^{-1})	Assigned molecular group
UUB	3730	–OH from –COOH	Disappeared	
	3291.945	–N–H from $–NH_3$	3295.325	
	1395.319	$–CH_2$	1378.395	
	1236.167	Amide	1212.912	
	1072.244	–C–OH from polysaccharide	1060.593	
	673.045	γOH from phenol	Disappeared	
Sodium alginate (2%)-immobilized PB	3318.944	–N–H from–NH_3	3363.299	
	No peak		2967.957	γC–H from $–CH_3$
	1427.088	$–CH_2$	1421.302‘	
	1076.101	–C–OH from polysaccharide	1047.174	
	545.764	γOH from phenol	559.264	
Un-immobilized PB	3299.658	–N–H from $–NH_3$	3286.159	
	No peak		2927.458	γC–H from CH_3
	1652.722	–C═O from amide I	1654.651	
	1394.303	–C–H from amide III	1386.589	

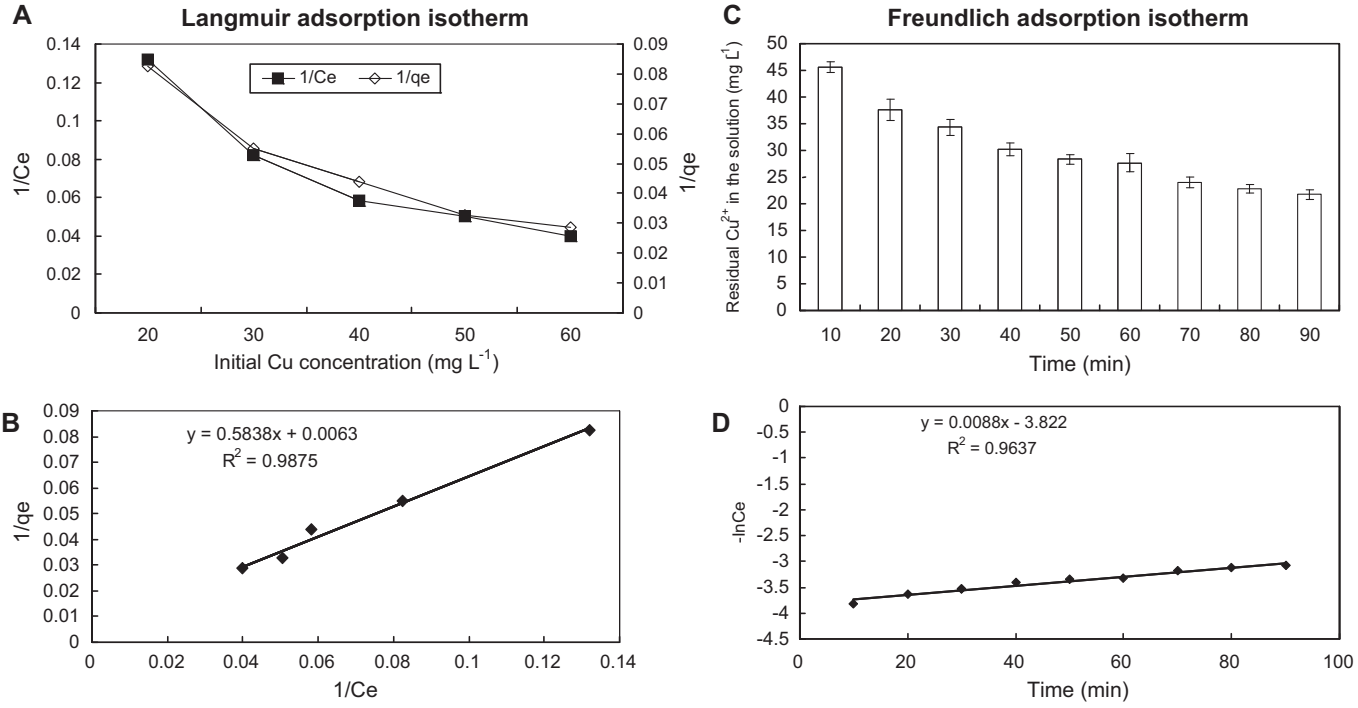

Fig. 7. Kinetics of Cu^{2+} adsorption of sodium alginate (2%)-immobilized PB of *Bacillus* strain CR-7. For construction of Langmuir adsorption isotherm, adsorption was conducted at 35 °C by rotation for 90 min at 150 rpm in 100 mL solution with an initial pH of 5.5 and containing alginate (2%)-immobilized PB (0.5 g L^{-1}) and different concentrations of Cu^{2+}. For construction of Freundlich adsorption isotherm, adsorption was conducted at 35 °C by rotation for different times at 150 rpm in 100 mL solution with an initial pH of 5.5 and containing Cu^{2+} (0.5 g L^{-1}) and sodium alginate (2%)-immobilized PB (0.5 g L^{-1}). PB, NaOH (0.01 mmol L^{-1})-pretreated biomass.

A high correlation coefficient (Fig. 7B), $R^2 = 0.9875$, indicated that Cu^{2+} adsorption fit the Langmuir isotherm model very well, suggesting that homogeneous adsorption within the adsorbent and further meaning that once a Cu^{2+} occupy a site, nor can adsorption take place at that site any further [19].

Another experiment of Cu^{2+} adsorption with time course was conducted. The obtain data (Fig. 7C) were linearized as Freundlich isotherm model as the described method in the literature [19] (Fig. 7D). The correlation coefficient, $R^2 = 0.9637$, indicated that Cu^{2+} adsorption also followed the Freundlich isotherm model. In fact, the Freundlich isotherm model has the same meaning as the Langmuir isotherm model, and it assumes a heterogeneous energetic distribution of the active binding sites on the biomass as well as interactions between the adsorbed molecules [49].

Two isotherm models also in part explain why metal adsorption of biomass of *Bacillus* strain CR-7 varied with the conditions.

4. Conclusions

Bacillus strain CR-7 isolated is of multiple metal and antibiotic resistances. Pretreatment with 0.1 mol L^{-1} NaOH significantly improved CDC of *Bacillus* strain CR-7 biomass. Pretreatment with a high concentration (1 mol L^{-1}) of NaOH led to decrease in CDC. When compared to gelatin and PV, sodium alginate (2%) was the ideal matrix for immobilization of the bacterial biomass. The alginate (2%)-immobilized and NaOH (0.1 mmol L^{-1})-pretreated biomass had an obvious “orderliness” of metal adsorption, following the order of $Cu^{2+} > Zn^{2+}$ in the solution containing these two metals, and following the order of $Pb^{2+} > Al^{3+} > Cr^{6+} > Cu^{2+} > Fe^{3+} > Zn^{2+} = Ni^{2+} > Cd^{2+} = Co^{2+} > Mn^{2+}$ in the solution containing these 10 metals. ΔH° and ΔS° of Cu^{2+} adsorption were +7.68 J/mol and +16.628 J/mol K, respectively. The IR peak of –N–H shifted greatly after Cu^{2+} adsorption. After

adsorption treatment, some molecular groups such as –OH and γOH disappeared in un-immobilized biomass but were still present in the immobilized biomass. Cu^{2+} adsorption by pretreated and immobilized biomass fit the both Langmuir and Freundlich isotherm models. It could be concluded (1) that the Cu^{2+} adsorption process was endothermic, (2) that –N–H is a most important Cu^{2+}-binding group, (3) that immobilization prevents loss or damage of the Cu^{2+}-binding molecular groups, and (4) that Cu^{2+} adsorption of pretreated and immobilized biomass is homogeneous.

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