Enhanced removal of cadmium from wastewater with coupled biochar and *Bacillus subtilis*

Jing Ding, Weiguang Chen, Zilan Zhang, Fan Qin, Jing Jiang, Anfei He and G. Daniel Sheng

ABSTRACT

Shortcomings of individual biochar or microbial technologies often exist in heavy metal removal from wastewater and may be circumvented by coupled use of biochar and microorganisms. In this study, *Bacillus subtilis* and each of three biochars of different origins (corn stalk, peanut shell, and pine wood) were coupled forming composite systems to treat a cadmium (Cd, 50 mg/L) wastewater formulated with $CdCl_2$ in batch tests. Biochar in composite system enhanced the activity and Cd adsorption of *B. subtilis*. Compared with single systems with Cd removal up to 33%, the composite system with corn stalk biochar showed up to 62% Cd removal, which was greater than the sum of respective single *B. subtilis* and biochar systems. Further analysis showed that the removal of Cd by the corn stalk composite system could be considered to consist of three successive stages, that is, the biochar-dominant adsorption stage, the *B. subtilis*-dominant adsorption stage, and the final biofilm formation stage. The final stage may have provided the composite system with the ability to achieve prolonged steady removal of Cd. The biochar-microorganism composite system shows a promising application for heavy metal wastewater treatment.

Key words | *Bacillus subtilis*, biochar, cadmium removal, composite system, wastewater

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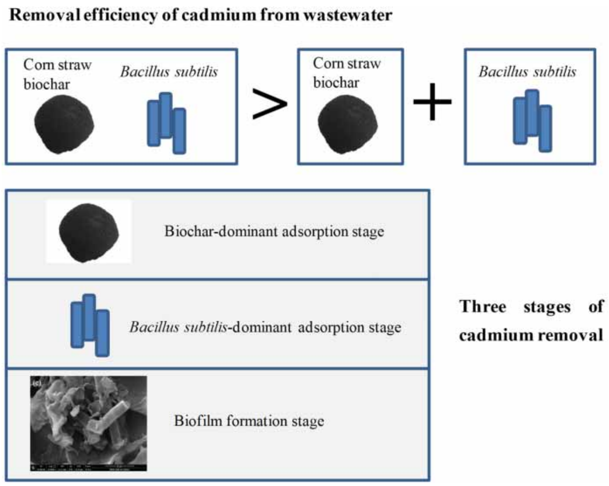
HIGHLIGHTS

- Biochar enhanced the activity and cadmium adsorption of *Bacillus subtilis*.
- Corn stalk composite system had higher cadmium removal efficiency than peanut shell and pine wood composite systems.
- Cadmiun removal in corn stalk composite system was higher than the sum of the single biochar and Bacillus subtilis systems.
- Cadmium removal in corn stalk composite system could be divided into three stages.

doi: 10.2166/wst.2021.138

GRAPHICAL ABSTRACT



INTRODUCTION

Heavy metals can destroy the community structure of the benthos in natural ecosystems and thereby seriously endanger ecosystems. Organisms can accumulate heavy metals, thus posing risks to the health of other members in the food chain including humans (Tan *et al.* 2017). Among heavy metals, cadmium (Cd) has the highest potential ecological risk. Cd exposure can cause a number of illnesses including kidney disease, early atherosclerosis, hypertension, and cardiovascular diseases (Nogawa *et al.* 2004). Cd can accumulate in rice crops, which resulted in the development of known itai-itai disease in local Japanese rice consumers during World War II. Cd in the environment comes from the use of various chemicals, such as pesticides, chemical fertilizers, and biological raw materials (e.g., Nogawa *et al.* 2004). Another source of Cd is from wastewater treatment plant (WWTP). The US Environmental Protection Agency (EPA) has established the maximum contaminant level (MCL) of 0.005 mg/L for Cd drinking water (http://www.epa.gov/safewater/pdfs/factsheets/ioc/cadmium.pdf). The World Health Organization (WHO) established a guideline of 0.003 mg/L for lifetime consumption (http://www.who.int/water_sanitation_health/dwq/chemicals/cadmium.pdf). As current WWTP is not capable of effectively removing Cd from wastewater, there is a need to develop a long-term and steady Cd removal method.

Given the degree of Cd pollution in water environment, removal technologies have been developed including chemical precipitation (Ku & Jung 2001), membrane filtration (Zhou *et al.* 2019), and ion exchange (Chitpong & Husson 2017). Adsorption has been one of the most cost-effective method for removing Cd (Demirbas 2008). Biochar arising from pyrolysis of biomass, in particular, is a low-cost heavy metal adsorbent rich in carbon. The porous structure and surface functional groups can interact physically and chemically with Cd, thereby promoting the removal of the metal from water (Wang *et al.* 2019). On the other hand, microbial adsorption technology has been a major focus of research thanks to its advantages of low operating costs, high removal efficiency, and no secondary pollution (Blanco *et al.* 1999). However, both biochar and microorganisms have shortcomings associated with treating Cd-containing wastewater. For example, biochar has a poor fixation effect on Cd, and desorption may occur during long-term wastewater treatment, resulting in secondary pollution (Ahmad *et al.* 2014). The environmental conditions and toxicity of the metal can affect microbial activity and result in an unsatisfactory treatment effect (Moore *et al.* 2005).

Biochar has been suggested to be a robust carrier for fixing microorganisms (Warnock *et al.* 2007), as it provides not only a favorable living environment for microorganisms but also carbon source, energy, and mineral nutrition for their growth. Microorganisms can also help biochar complete the long-term fixation of heavy metals (Chen *et al.* 2019). To date, few studies have carefully examined the treatment of heavy metal wastewater by biochar coupled with microorganisms, especially on the heterogeneity effect in biochar characteristics in heavy metal removal (Ahmad *et al.* 2014). The microbial communities associated with different biochars in the environment are often distinctive, and thus differential in their metabolic potential (Lehmann *et al.* 2011). Evaluation of the performance of biochar coupled with microorganisms in the treatment of heavy metal wastewater is in great need for improving the application potential of this technology.

Biochars made from, for example, corn stalk (CSB), peanut shell (PSB), and pine wood (PWB) usually have high performance in the treatment of heavy metal wastewater (Ahmad *et al.* 2014). *Bacillus subtilis* is widely used as a model strain in heavy metal treatment because of its strong reproductive capacity, lack of pathogenicity, ubiquity in the environment, and strong environmental adaptability (Moore *et al.* 2005). Our objective was to evaluate the performance of biochar coupled with *B. subtilis* in removing Cd from wastewater and its underlying mechanism. To do so, each of above-mentioned three representative biochars coupled with *B. subtilis* was tested for the treatment of a Cd-containing wastewater formulated by dissolving CdCl in deionized water. Composite systems (i.e., coupling) were compared to those of respective single systems in the treatment efficiency and characteristics. Coupling biochar with *B. subtilis* to treat wastewater has not been previously tested. The results of this study can provide a good reference for future studies in the treatment of heavy metal wastewater.

MATERIALS AND METHODS

Bacterial culture and biochar preparation

The *B. subtilis* used in this study was purchased from China General Microbiological Culture Collection Center (CGMCC No. 1.4255). *B. subtilis* was inoculated into lysogeny broth (LB) medium at a 7% ratio and activated on a rotary mixer for 2 h at 30 °C and 170 rpm. The activated *B. subtilis* was then transferred to fresh LB medium to cultivate for 12 h when it was in the logarithmic growth stage.

The raw materials of biochar were corn stalk, peanut shell, and pine wood. They were pulverized using an electric saw and a grinder, collected in a casserole, then wrapped with tin foil, and finally placed in a muffle furnace. The materials were pyrolyzed at 500 °C for 3 h and then removed after natural cooling for 6 h. The obtained biochars were ground in a mortar, sieved, and stored in sealed bags.

Biochar adsorption experiment

The adsorption determinations were conducted in 250-mL Erlenmeyer flasks. The total volume of the adsorption system was 100 mL. According to our pretest of the biochar adsorptivity, biochar was added with a concentration of 1 $g{\cdot}L^{-1}$. Tests were in triplicate. Four tests were performed. (1) Effect of pH. The initial concentration of Cd was 50 $mg{\cdot}L^{-1}$. The initial pH of the solution was adjusted using 1 mol/l HCl and NaOH solutions to 4.0, 5.0, 6.0, 7.0, and 8.0. The effect of pH on the adsorption of Cd by biochar was tested after 24 h of reaction at 30 °C. (2) Kinetics of adsorption. The initial concentration of Cd was 50 $mg{\cdot}L^{-1}$. This test was performed at a constant pH by adjusting pH, in this study, to 7.0. The adsorption kinetics of Cd on biochar was obtained by fitting the experimental data to the quasi-first-order and quasi-second-order kinetic models. (3) Adsorption isotherms. The initial concentrations of Cd were 10, 20, 40, 50, 80, and 100 $mg{\cdot}L^{-1}$. The pH of the reaction solution was adjusted to 7.0. After adsorption at 30 °C for 24 h, samples were taken to determine the Cd content in the solution. The experimental data were fitted to the Langmuir and Freundlich models. (4) Adsorption in single and composite systems. Three groups of adsorption were conducted, i.e., *B. subtilis* + Cd medium (LB +50 $mg{\cdot}L^{-1}$ Cd), biochar (CSB, PSB, PWB) + Cd medium (LB +50 $mg{\cdot}L^{-1}$ Cd), *B. subtilis* + biochar (CSB, PSB, PWB) + Cd medium (LB +50 $mg{\cdot}L^{-1}$ Cd). The pH was adjusted to 7.0, and the total experimental duration was 150 h. Our pretests showed that the Cd adsorption in all systems reached equilibrium within 120 h.

Monitoring the bacterial growth

The growth of *B. subtilis* was monitored in the following three systems in triplicate: i. *B. subtilis* + LB medium; ii. *B. subtilis* + Cd medium (LB +50 $mg{\cdot}L^{-1}$ Cd); and iii. *B. subtilis* + biochar (CSB, PSB, PWB) + Cd medium (LB +50 $mg{\cdot}L^{-1}$ Cd). *B. subtilis* at the logarithmic growth stage was inoculated into the system at a dose of 7% (V/V), and biochar was added to the system iii with a concentration of 1 $g{\cdot}L^{-1}$. The total solution volume was 100 mL. The pH was adjusted to 7.0. All the systems were cultured at 30 °C and 170 rpm.

The growth status of *B. subtilis* in the systems was recorded by regularly measuring the OD_{600} value using a spectrophotometer at 600 nm (Thermo Fisher Scientific, USA). In system iii, biomass was separated from biochar by high-speed centrifugation at ×4,302 g (Thermo Fisher Scientific, USA) before determining the OD_{600} value. The growth curve of *B. subtilis* was plotted versus culturing time.

Determination of Cd concentration

Cd concentration in solution was determined by atomic absorption spectrometry (PinAAcle900 T, Platinum Elmer, USA). Cd standard solution (1,000 $\mu g \cdot mL^{-1}$) was purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Samples were filtered by a 0.45-μm cellulose filter membrane and diluted with 1% nitric acid to prevent the hydrolysis of Cd. The Cd adsorption and the Cd removal rate were calculated as follows (Al-Ananzeh 2021),

$$q_e = \frac{(C_0 - C) \times V}{M} \quad (1)$$

$$R(\%) = \frac{(C_0 - C)}{C_0} \times 100 \quad (2)$$

where q_e is the equilibrium adsorption ($mg \cdot g^{-1}$); V is the volume of solution (L); M is the amount of biochar added (g); R is the removal rate of Cd; C_0 is the initial Cd concentration in the solution ($mg \cdot L^{-1}$); and C is the Cd concentration in the solution after adsorption ($mg \cdot L^{-1}$).

Characterization of biochars

Pure and Cd-adsorbed biochar materials were washed three times and dried for electron microscopy. According to the method in the previous studies (Tao *et al.* 2018; Wang *et al.* 2018), the *B. subtilis*-Cd adsorbed biochar was placed into glutaraldehyde (2.5%) to fix at 4 °C for 24 h. Glutaraldehyde was then removed by centrifugation for 5 min (12,000 rpm) and washed three times with phosphoric acid buffer (pH = 7). Next, the samples were sequentially dehydrated with 30, 50, 70, 90, and 100% ethanol solution, and further treated by vacuum freeze-drying. Finally, the samples were sprayed with gold for scanning electron microscopy (SEM) using a field emission scanning electron microscope S-4800 (Hitachi High-Technologies Corp., Honshu, Japan).

After filtering, the Cd-adsorbed and *B. subtilis*-Cd adsorbed biochar materials were washed three times with distilled water and dried at 60 °C. The samples were ground in a mortar, followed by the addition of 200 mg of spectral pure KBr, mixing, pressing, and analysis by Fourier transform infrared spectroscopy (FTIR) using a Nicolet 6700 FTIR spectrometer (Nicolet Instrument Corp., USA). All of the biochar materials were sampled from respective systems at 120 h.

RESULTS AND DISCUSSION

Cd adsorption characteristics of three biochars

The Cd adsorption by three biochars at different pH values (4, 5, 6, 7, and 8) was compared. The Cd adsorption was highest at pH 7, and all the subsequent experiments were therefore conducted at this pH (Figure S1, Supplementary Material). Heavy metal ions such as Cd with positive charges are considered to be adsorbed by biochars via electrostatic forces between the metal ion and the oxygen-containing acidic functional groups on the biochar surfaces (Demirbas 2008). The degree of dissociation of these groups increases with increasing pH, resulting in enhanced metal adsorption by biochar, as indicated by the Cd adsorption here. Comparatively lower Cd adsorption at pH 8.0 than at pH 7.0 may have resulted from the speciation of Cd at the former pH with more negatively charged soluble Cd complexes (Figure S1). The Cd adsorption by the three biochars also differed, probably due to the difference mainly in their surface acidity.

Adsorption kinetics can describe the adsorption rate at the solid-liquid interface. The kinetic curves showed that corn stalk biochar adsorbed Cd rapidly from 0 to 12 h, and thereafter increased slowly until reaching equilibrium (Figure 1(a)). Peanut shell biochar adsorbed Cd rapidly from 0 to 1 h, and the adsorption at the 23th hour did not significantly increase compared with that at the first hour. By comparison, pine wood biochar had a poor adsorption ability for Cd, and its adsorption approached equilibrium at the 12th hour. Both the quasi-first-order and quasi-second-order kinetic models can satisfactorily simulate the adsorption by corn stalk and peanut shell biochar (Table 1). The q_e value (35.54) of the quasi-first-order kinetics of corn stalk biochar differed from the actual value by 6.72%, and the q_e value (38.07) of the quasi-second-order kinetics differed from the actual value by 0.07%. Similarly, the q_e value (35.54) of the quasi-first-order kinetics of peanut shell biochar differed by 1.33% from the actual value, and the q_e value (38.07) of the quasi-second-order kinetics differed by 0.06% from the actual value. The R^2 showed that the quasi-second-order kinetics ($R^2 = 0.947$) better simulated the adsorption process of corn stalk and peanut shell biochars than the quasi-first-order kinetics ($R^2 = 0.874$). While Cd adsorption process often involves multiple mechanisms, chemical reaction (the formation of chemical bonds between Cd ion and the adsorbent functional groups) appeared to be the predominant mechanism here. The adsorption rate of corn stalk biochar was lowest compared with peanut shell and pine wood biochars, but its

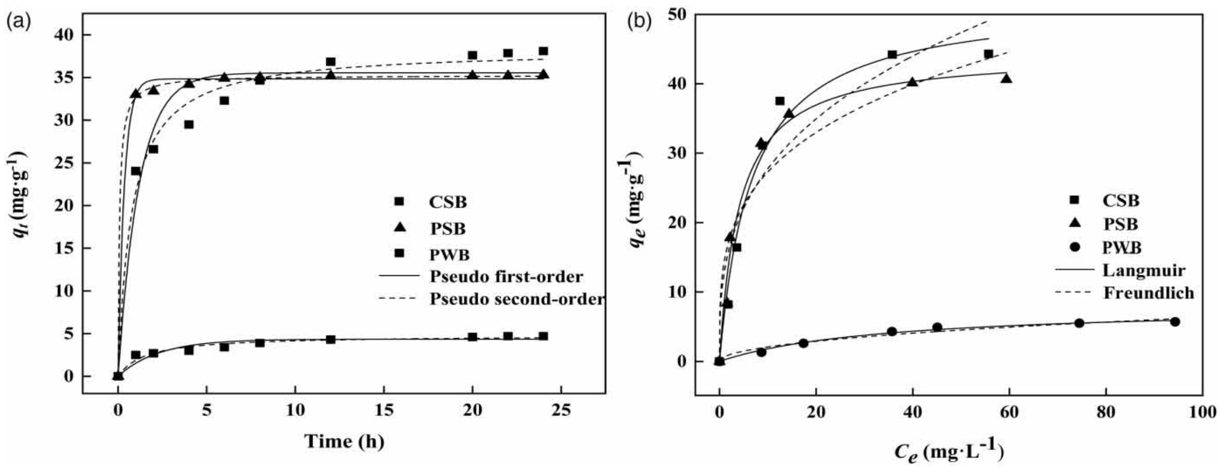


Figure 1 | Model fitting of Cd adsorption at 30 °C and solution pH of 7.0 on three biochars. (a) Adsorption kinetics curves; (b) adsorption isotherms. CSB: corn stalk biochar; PSB: peanut shell biochar; PWB: pine wood biochar.

Table 1 | Kinetics of Cd adsorption on biochars and fitting parameters of adsorption isotherms

	Pseudo-first-order			**Pseudo-second-order**		
Sorbent	q_1 (mg·g^{-1})	k_1 (h^{-1})	R^2	q_2 (mg·g^{-1})	k_2 (g·(mg·min)$^{-1}$)	R^2
CSB	35.54	0.84	0.934	38.07	0.03	0.977
PSB	34.83	2.87	0.997	35.28	0.35	0.999
PWB	4.35	0.44	0.874	4.80	0.14	0.947
	Langmuir			**Freundlich**		
Sorbent	Q_m (mg·g^{-1})	K_L (mg·g^{-1})	R^2	1/n	K_F	R^2
CSB	52.1	0.15	0.981	0.33	12.93	0.892
PSB	44.44	0.25	0.981	0.27	14.54	0.895
PWB	8.07	0.03	0.987	0.48	0.69	0.951

Note: The temperature of adsorption was 30 °C. CSB: corn stalk biochar; PSB: peanut shell biochar; PWB: pine wood biochar.

adsorption capacity was the highest. This pattern may have stemmed from the fact that corn stalk biochar had a higher pore volume, and the adsorption of Cd in the pore required a longer period of equilibration. The total adsorption capacity was also higher. Peanut shell and pine wood biochars had smaller pore volumes and reached adsorption equilibrium more quickly with a relatively limited adsorption capacity.

The Langmuir and Freundlich models were used to fit the adsorption data of three biochars (Figure 1(b), Table 1). The correlation coefficients from the Langmuir model were higher than the respective ones from the Freundlich model, indicating that the adsorption of Cd by the biochars was mainly in monolayer configuration. Based on the comparison of 1/n and K_F values between the biochars, the adsorption capacity of peanut shell biochar was better than that of corn stalk biochar. The adsorption performance of pine wood biochar was the lowest. Ma et al. (2016) also showed that the adsorption characteristics of corn stalk biochar for Cd ions in aqueous solution were more consistent with pseudo-second-order kinetics and the Langmuir model. Although the removal efficiency of peanut shell biochar was slightly lower than that of corn stalk biochar, its adsorption equilibrium time was comparatively shorter. Use of peanut shell biochar could apparently shorten the treatment process of Cd-containing wastewater.

Growth activity of *B. subtilis* in composite systems

After inoculation into LB medium, *B. subtilis* rapidly entered the logarithmic phase and later the stable phase at approximately the 20th hour. The OD value reached 2.085 (Figure 2). With the constant consumption of substrate,

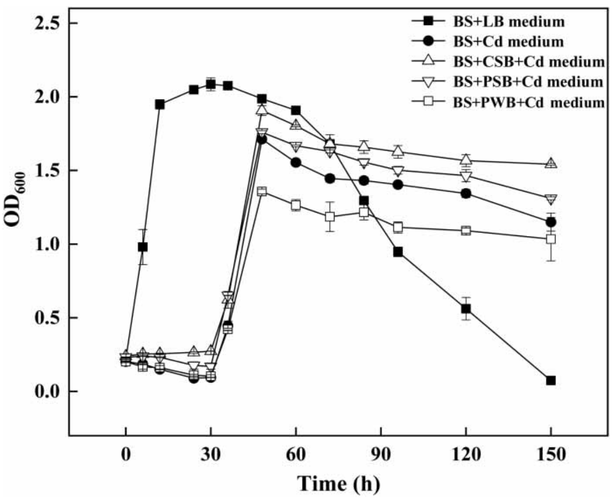


Figure 2 | Growth of *B. subtilis* in different systems at 30 °C. BS: *B. subtilis*; CSB: corn stalk biochar; PSB: peanut shell biochar; PWB: pine wood biochar.

B. subtilis entered the decay period after 60 h. In Cd medium, the growth activity of *B. subtilis* was inhibited within 0 ~ 30 hours, grew logarithmically during the following 18 hours, and entered the stable phase at the 48th hour, with maximum OD value of 1.714. Compared with pure culture medium, there was a longer lag period in the growth of *B. subtilis* in Cd medium, reflecting the inhibitory effect of Cd stress. In this case, *B. subtilis* needed to adjust its growth and metabolism to adapt to Cd stress. The stable period of *B. subtilis* was longer in the Cd-containing environment, which may stem from the fact that the bacteria can activate the active factors (such as *CzrA* and *CadA* genes) when the bacteria adapt to Cd stress. *CadA* is the main factor responsible for Cd resistance in *B. subtilis*, and the *CzrA* gene can not only regulate the *CadA* gene but also play an active role in the *B. subtilis* physiological metabolism, which stabilizes the biomass of *B. subtilis* for long periods (Moore *et al.* 2005).

After the biochar and *B. subtilis* were added to the Cd medium, the biomass of *B. subtilis* at the lag period in the composite system was slightly higher than that in single bacteria system with Cd medium, and the biomass of corn stalk biochar composite system was the highest. This finding indicated that all the three biochars could alleviate Cd toxicity for *B. subtilis* to a certain extent, and corn stalk biochar had the greatest ability. During the stable period, the OD values of *B. subtilis* in corn stalk, peanut shell, and pine wood biochar composite system were 1.907, 1.760, and 1.357, respectively. The biomass in corn stalk and peanut shell biochar composite systems was higher than that in single *B. subtilis* system, whereas the biomass in pine wood biochar composite system was lower than that in single *B. subtilis* system. This finding indicated that the addition of corn stalk biochar and peanut shell biochar played a stimulative role in the growth of *B. subtilis*. The biomass of *B. subtilis* remained stable for long periods in composite systems of corn stalk and peanut shell biochar, which might be attributed to the fact that biochar provides nutrition for the growth of *B. subtilis* and creates a favorable living environment, hence facilitating the maintenance of a high level of bacterial biomass for a long period. Similar findings were obtained with cotton stalk biochar, which was found to be an efficient inoculation carrier of *B. subtilis*, and the concentration of bacteria was higher in the biochar system compared with the no-biochar system (Tao *et al.* 2018). Pine wood biochar appeared to lack such stimulative nutrition. Therefore, corn stalk biochar is suggested to be more widely used for the treatment of Cd-contaminated wastewater in the future. Corn stalk biochar cannot only be combined with some pure cultured microorganisms but also be mixed with activated sludge to facilitate the removal of heavy metals.

Cd removal efficiencies in composite systems

At the end of the bacterial growth experiment, the Cd removal rates in three composite systems (biochar and *B. subtilis*) were higher than those in single systems (Figure 3). The removal efficiency in corn stalk biochar-*B. subtilis* composite system was the highest (62%), greater than the sum of single *B. subtilis* (26%) and corn stalk

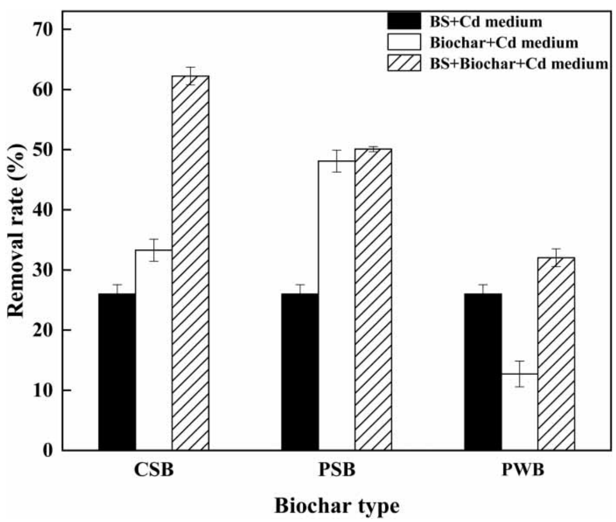


Figure 3 | Removal efficiency of Cd in different systems. BS: *B. subtilis*; CSB: corn stalk biochar; PSB: peanut shell biochar; PWB: pine wood biochar.

biochar (33.3%) systems. The removal effect in pine wood biochar-*B. subtilis* composite system was the lowest (32%).

The Cd removal efficiency in composite systems was as follows: corn stalk biochar composite system > peanut shell biochar composite system > pine wood biochar composite system. However, the treatment efficiency in composite systems cannot be simply attributed to the adsorption properties of biochar, as microorganisms also played an important role in treatment efficacy. In the medium containing Cd, peanut shell biochar had the highest removal rate of Cd in biochar only systems, but became lower in corresponding composite system than in corn stalk composite system. This may stem from the fact that the adsorption of Cd by corn stalk biochar was greatly enhanced by other components in the culture medium. Because of the strong adsorption of other elements in the solution, the growth and fixation of *B. subtilis* on the surface of corn stalk biochar were also enhanced. In the end, the composite system had a strong synergistic removal effect on Cd. Therefore, the selection of biochar in a composite system not only requires consideration of its adsorption capacity of heavy metals but also its influence on microbial growth and fixation. It was also pointed out that the adsorption properties of different biochars for Cd affected the Cd treatment efficiency in their respective composite systems (Wang *et al.* 2018).

Removal of Cd from corn stalk biochar-*B. subtilis* system

Based on the Cd removal efficiency in single biochar systems, the growth of *B. subtilis* in composite systems, and the Cd removal efficiency in composite systems, corn stalk biochar-*B. subtilis* composite system was further studied as it showed the greatest treatment efficiency. The Cd removal rate in the composite system was much higher than those in single *B. subtilis* and biochar systems (Figure 4) that, when stable, the Cd removal rate in the composite system was 139.4% higher than that in single *B. subtilis* system and 86.9% higher than that in single biochar system. According to the difference between the composite system and the single system, the adsorption and removal of Cd by the composite system can be roughly divided into three stages: biochar adsorption-dominated stage, *B. subtilis* adsorption-dominated stage, and biofilm formation stage.

The biochar adsorption-dominant stage occurred in 0–24 h. The difference of removal rate between composite system and *B. subtilis* system was greater than that between composite system and biochar system (Figure 4). This observation stems from the fact that *B. subtilis* had not adapted to

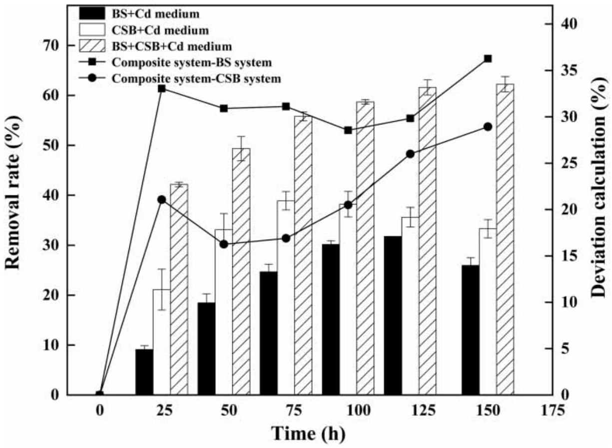


Figure 4 | The removal of Cd in different systems and the difference in removal rate between different systems. The incubation temperature was 30 °C. BS: *B. subtilis*; CSB: corn stalk biochar.

the Cd toxicity in the solution at this stage, greatly inhibiting its metabolic activity that biochar played a major role in the rapid adsorption of Cd in the system. At this stage, the removal of Cd in the composite system is mainly the non-metabolism dependent adsorption, thus limiting the removal effect. It was obtained, however, that the removal rate in the composite system (42.2%) was greater than the sum of the rates in single systems (9.1% and 21.1%). There appeared to be a synergistic effect existing in the composite system. The presence of biochar in the composite system alleviated the stress pressure of Cd on *B. subtilis* via adsorption, improving the bacterial activity and the absorption of Cd. The facilitated Cd absorption by *B. subtilis* reduced the amount of Cd on the biochar and thus enhanced additional Cd adsorption by biochar. There was a similar observation that after bioleaching the heavy metal-adsorbed biochar with *Thiobacillus ferrooxidans*, microorganisms could not only remove heavy metals from the biochar but also improve the functional groups and pore structure of the biochar (Wang *et al.* 2013).

In the 24–120 h stage, the absorption rate difference between the composite system and the *B. subtilis* system began to approach that between the composite system and the biochar system; thus, *B. subtilis* was suspected to play a dominant role in the adsorption stage. Consistent with this speculation, the optical density (OD) value of *B. subtilis* in the system at this stage suggested that *B. subtilis* rapidly proliferated and reached the stable stage, and could adsorb Cd by dual metabolism-dependent and non-metabolism-dependent mechanisms (Blanco *et al.* 1999). That is, Cd absorbed by extracellular functional groups began to be gradually transferred into living cells because of bacterial metabolic activities (Tahir 2019), and the vacant adsorption sites became available for Cd binding again. Within 24–48 h, however, both of the two plots showed a temporary decline. This may stem from the proliferation of *B. subtilis*, which caused some sites of biochar to be occupied by bacteria. Consequently, the total removal rate in the composite system was less than the sum of the removal rates in the single systems. After 48 h, corn stalk biochar was nearly in a dynamic equilibrium state of adsorption saturation. At this time, *B. subtilis* was in a stable period and could further adsorb Cd.

The last 120–150 h period can be regarded as the biofilm formation stage. The removal rate of Cd in single system showed a decreasing trend, whereas in composite system it remained stable (Figure 4). This pattern stems from the release of Cd ions by *B. subtilis* with the onset of the decay period, causing secondary Cd pollution. At the same time, in the long-term treatment process, the Cd adsorbed on the surface of biochar in a single system does not form a stable complex, with a certain risk of desorption. Frankel and colleagues pointed out that it takes six days for a robust biofilm to form and that the biofilm can be used as a repository for the adsorption of metals by biochar (Frankel *et al.* 2016). In addition, some studies have shown that organic acids secreted by microorganisms form a weak acidic microenvironment on the surface of biochar, promoting the release of phosphorus. Heavy metals can thus form stable phosphorus compounds and be immobilized on the surface of biochar for a prolonged time (Chen *et al.* 2019). In the composite system, the formation of biofilm on corn stalk biochar may thus be the main factor stimulating the long-term immobilization of Cd. In addition, the formation of biofilm can maintain the biomass of bacteria and decrease the rate of bacteria entering the decay period, which is important for the long-term immobilization of Cd in the composite system.

Morphology and chemical composition of biochar before and after adsorption

SEM revealed that all the biochars had rich pore structure (Figure 5(a), Figures S2 and S3). Among them, corn stalk biochar had smaller mesopore diameters, with a rough pore surface. The mesopore diameters of pine wood biochar were relatively large, with a smooth surface. The coarse and rich pore structure enhanced the adsorption of Cd by biochar, as was in the case of corn stalk biochar. After adsorption, the pore surface of corn stalk biochar was filled with more materials (Figure 5(b)), while there was no obvious filling on the surfaces of peanut shell and pine wood biochars (Figures S2 and S3). In corn stalk composite system, *B. subtilis* could adhere to the surface of the biochar and grow in the pores, forming complexes (Figure 5(d) and 5(e)). The bacterial morphology in the biochar pore was relatively full, which indicated that the pore structure of corn stalk biochar could help *B. subtilis* effectively avoid Cd toxicity and promote its growth. The *B. subtilis* on the surface of biochar showed clear shrinkage and adhesion, which primarily stemmed from the high stress of Cd on the surface of biochar. As a result, the cells shrank, and the bacteria secreted more extracellular polymers, further facilitating the adhesion between bacteria (Dogan *et al.* 2011). The surface of corn stalk biochar became wrapped by *B. subtilis*, reflective of the biofilm formation.

Based on the elemental analysis on the surface of biochar (Figure 5(a) and Figure S3), corn stalk biochar had slightly higher carbon content on the surface than the pine wood and corn stalk biochars. It contained a small amount of mineral elements. They may play a role in promoting the adsorption of Cd by biochar. The elements adsorbed on the surface of corn stalk biochar were mainly Cd and nutrients in the medium (Figure 5(b)). The adsorbed nutrient elements were much less on the surface of peanut shell biochar and almost absent on the surface of pine wood biochar (Figures S2 and S3). It can thus be seen that the corn stalk biochar had high adsorption capacity for nutrients in the culture medium, which affected its adsorption of Cd. This observation also explains why the adsorption efficiency of corn stalk biochar for Cd in pure water was slightly higher than that of peanut shell biochar but lower than that of peanut shell biochar in Cd medium. The high adsorption effect of corn stalk biochar on nutrient elements greatly promotes the growth of *B. subtilis* on its surface, thereby enhancing the Cd removal of the *B. subtilis*-corn stalk biochar composite system. After adding *B. subtilis*, the elements on the surface of corn stalk biochar were further changed (Figure 5(c)), that N and Na disappeared, Si increased significantly, and P appeared. The disappearance of N and Na stemmed from the fact that the nutrients adsorbed by corn stalk biochar were utilized by *B. subtilis* attached to the surface. Some studies have pointed out that mineral elements in biochar could transform water-soluble heavy metals into stable solid states through precipitation (Cao & Harris 2010). The increase in *B. subtilis* on the surface of corn stalk biochar might stimulate the release of mineral components in the biochar, which led to a significant increase in Si element and the appearance of P, strengthening the fixation of Cd. The P element also existed

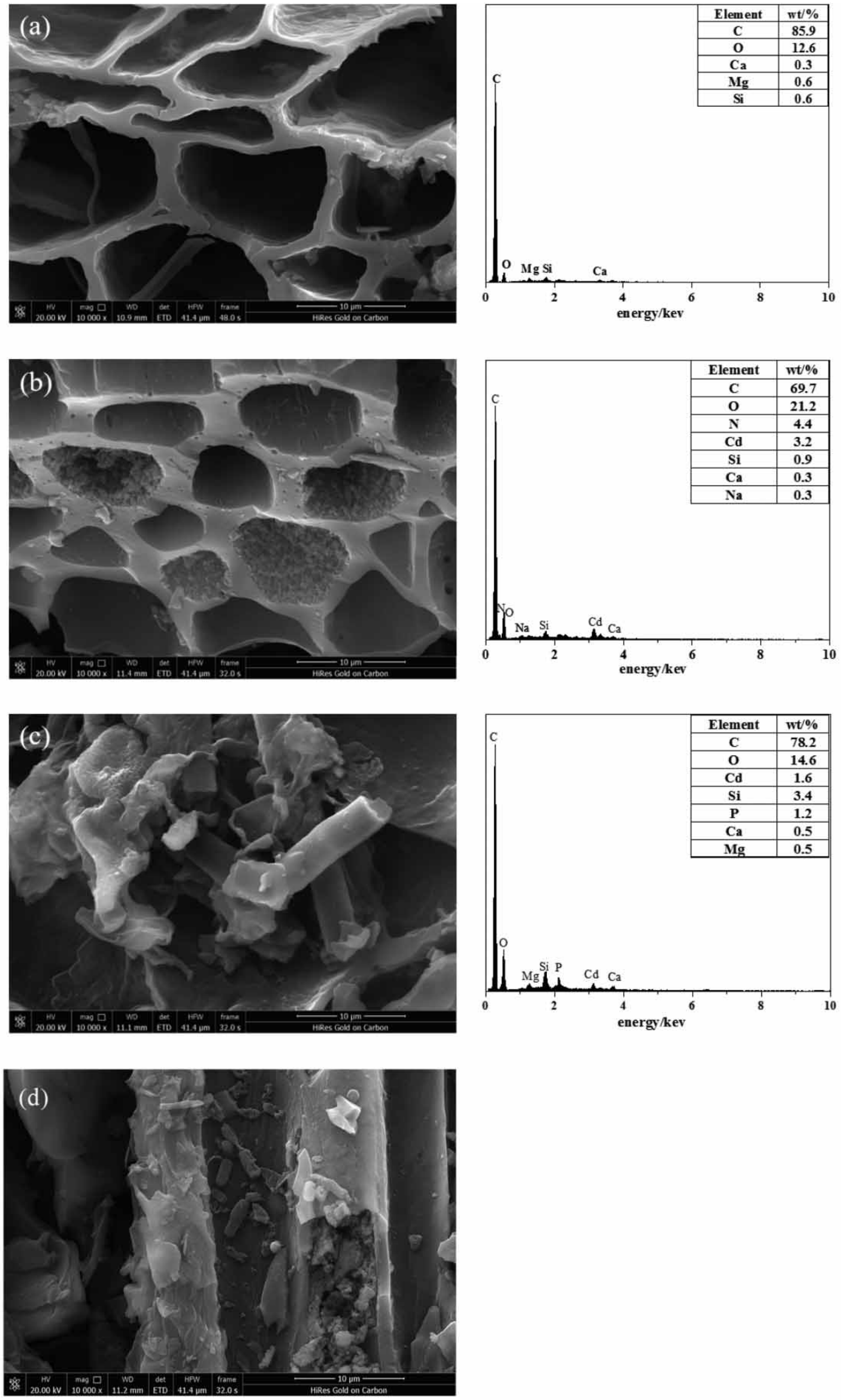


Figure 5 | SEM and EDS images of corn stalk biochar in different systems. (a) Corn stalk biochar without Cd adsorption; (b) corn stalk biochar with adsorbed Cd; (c) corn stalk biochar with adsorbed *B. subtilis* and Cd; (d) the surface of corn stalk biochar after adsorption.

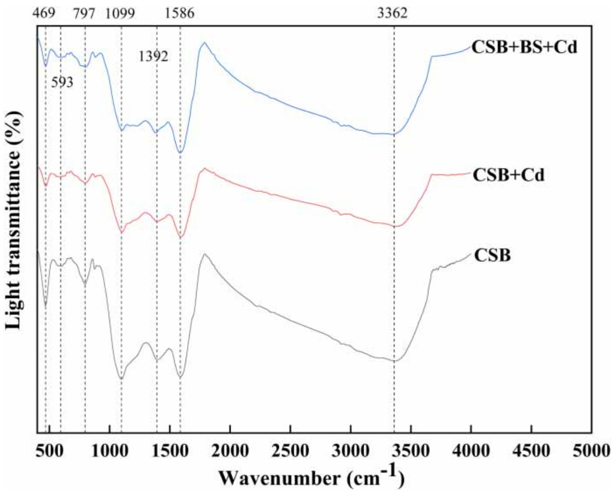


Figure 6 | FTIR spectra of corn stalk biochar before and after Cd adsorption. CSB: corn stalk biochar, BS: *B. subtilis*.

on the surface of peanut shell biochar, but the amount was comparatively lower than that on corn stalk biochar. Only a few *B. subtilis* were observed in the pores of pine wood biochar, and there was no significant change of elements composition on the biochar surface before and after absorption (Figures S2 and S3). Therefore, the formation of biofilm on the surface of corn stalk biochar may be an excellent platform for long-term immobilization of Cd.

As shown in Figure 6, the main FTIR characteristic absorption peaks of corn stalk biochar appear at the wavenumbers of 3,362, 1,586, 1,392, 1,099, 797, 593, and 469 cm^{-1}. The three peaks at the highest wavenumbers (i.e., 3,362, 1,586, and 1,392 cm^{-1}) represent, respectively, the extension of –OH (Du *et al.* 2016), the stretching vibration of -COOH, -OH and lactone group C-O, C = C, as well as the stretching vibration of the C = O bond (Wu *et al.* 2012). The other three peaks at 1,099, 797, and 469 cm^{-1} correspond to the vibration absorption of Si-O-Si (Yang *et al.* 2008). The remaining peak at 593 cm^{-1} is the stretching vibration of C-H (Kołodyńska *et al.* 2012). Clearly, carboxyl and hydroxyl groups exist in the structure of biochar, and thus effectively bind Cd. The peak intensities at 3,362, 1,586, 1,099, and 797 cm^{-1} of corn stalk biochar in the Cd medium shifted, suggesting that the functional groups of -COOH, -OH, C-O, and C = C on the surface of biochar reacted with Cd and nutrient elements in solution or via an electrophilic substitution with aromatic compounds on the surface. The peak intensities at 3,362, 1,586, and 1,392 cm^{-1} weakened following the adsorption of Cd in the composite system, which indicated that Cd and nutrient elements in the medium exchanged with the functional groups and formed a stable structure on the surface. A new peak at 881 cm^{-1} appeared, which was considered related to the functional groups of 1,2,4-trisubstituted benzene, indicating that *B. subtilis* may have occupied the adsorption sites on the surface. In addition, Figure S4 showed that corn stalk biochar had more obvious characteristic peaks than peanut shell and pine wood biochars, especially following adsorption. This indicated that the intensity and amplitude of most peaks of pine wood and peanut shell biochars did not significantly change compared with corn stalk biochar. Therefore, the interactions of pine wood/peanut shell biochars with Cd and/or *B. subtilis* were not clearly existent that the removal efficiency for Cd was poor. An abundance of functional groups of corn stalk biochar appeared to play an important role in Cd removal (via, i.e., electrophilic substitution, surface complexation, and ion exchange).

CONCLUSIONS

This study compared the Cd removal from wastewater by three biochars (corn stalk, peanut shell, and pine wood) with or without *B. subtilis* present in the wastewater. In composite systems, biochar alleviated the toxicity of Cd to *B. subtilis* and thus improved the bacterial activity and biomass. Its rough surface and rich pore structure provided favorable conditions for the growth and fixation of *B. subtilis*. Among three biochar composite systems, the corn stalk system presented the highest Cd removal efficiency, being greater than the sum of the respective single biochar and bacteria systems. No apparent Cd desorption was observed in long-term treatment process. In corn stalk biochar-*B. subtilis* composite system, the Cd removal process was considered to consist of three successive stages, i.e., the biochar-dominated stage, the *B. subtilis*-dominant stage, and the biofilm formation stage. The last stage played an important role in long-term immobilization of heavy metals. In summary, the biochar-microbial composite systems appear to provide a promising technology for heavy metal wastewater treatment, as they may achieve prolonged and steady removal efficiency of heavy metals in wastewater.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support from the Six Talent Peaks Project of Jiangsu Province (TD-JNHB-003), the Innovative and Entrepreneurial Team of Jiangsu Province (2018–2017), National Natural Science Foundation of China (41807413), Jiangsu Provincial Natural Science Foundation of China (BK20180967), the Innovative and Entrepreneurial Doctor Program of Jiangsu Province, and the Postgraduate Practice Innovation Program of Jiangsu Province (SJCX19_0832).

DISCLOSURE STATEMENT

No competing financial interests exist.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 21 January 2021; accepted in revised form 28 March 2021. Available online 7 April 2021