ORIGINAL RESEARCH

# Preparation and biosorption evaluation of *Bacillus subtilis*/alginate–chitosan microcapsule

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**Abstract:** The aim of this study was to assess the effect of alginate–chitosan microcapsule on viability characteristics of *Bacillus subtilis* and the ability of *B. subtilis*/alginate–chitosan microcapsule to remove uranium ion from aqueous solution. The effects of particle size, chitosan molecular weight and inoculum density on viability characteristics were studied using alginate–chitosan microcapsule-immobilized *B. subtilis* experiments. In addition, the effects of pH, immobilized spherule dosage, temperature, initial uranium ion concentration and contact time on removal of uranium ion were studied using batch adsorption experiments. The results showed that alginate–chitosan microcapsule significantly improved the viability characteristics of *B. subtilis* and that *B. subtilis*/alginate–chitosan microcapsule strongly promoted uranium ion absorption. Moreover, the optimum values of pH was 6; immobilized spherule dosage was 3.5; temperature was 20°C; initial uranium ion concentration was 150 mg/L; contact time was 3 h of uranium ion absorption and the maximum adsorption capacity of uranium ion was 376.64 mg/g.

**Keywords:** alginate–chitosan microcapsule, *Bacillus subtilis*, viability characteristics, uranium ion, adsorption

## Introduction

Pollutions caused due to heavy metals has become a serious environmental problem over the past few decades. Final industrial wastewater contains numerous heavy metal ions, which are extremely dangerous even at low concentrations due to their toxicity.[1] For example, cadmium concentrations in the range of 0.1–100 mg $\times$ dm$^{-3}$ are typical in wastewater from several industries, and an intake of excessively large doses by humans could lead to serious kidney failure.[2,3] Uranium is an essential radioactive element and a nuclear fuel, but at a higher level it is toxic to plants, animals and humans.[4] Uranium consumption in high doses could produce serious toxicological concerns such as cancer, kidney and liver injuries.[4] Uranium is widely used in important nuke industrial applications; hence, its removal from wastewater is important for environmental protection and human health.

Biosorption course has been discovered to be better than other technologies because of its high efficiency, convenient operation, low cost, regeneration of biosorbents and recovery of metals, compared to the traditional effluent treatments.[5,6] It was evident from a literature survey of >100 recent papers that low-cost adsorbents had demonstrated outstanding removal capabilities for various pollutants.[7] Biosorption utilizes all kinds of microorganisms, including yeast, bacteria, algae, fungi and protozoa, which could

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be discovered everywhere.[8] The mechanisms for removal of heavy metals include adsorption, uptake, reduction, methylation and oxidation.[9] Living, dead and immobilized bacteria could be used in the process. Immobilized bacteria are usually easier to handle, require less complex separation systems, allow a high biomass density to be maintained and provide a greater opportunity for reuse and recovery.[10]

A previous study showed that *Bacillus subtilis* possessed high physiological activity in an industry waste treatment.[6] The structure of *B. subtilis* cell wall is well known and consists primarily of peptidoglycan and teichoic acid.[8] Peptidoglycan is a polymer of acetylmuramic and acetylglucosamine acids that display mainly carboxylic and hydroxyl functional groups.[8] Teichoic acid is a polymer of copyranosyl glycerol phosphate that comprises mainly phosphate and hydroxyl groups.[11,12]

In this study, we reported alginate–chitosan as a *B. subtilis* microcapsule. The *B. subtilis*/alginate–chitosan microcapsule was composed of *B. subtilis*, sodium alginate, chitosan and calcium chloride. Therefore, in sterile conditions, *B. subtilis* was mixed with sodium alginate solution, and then the mixed solution was dropped into calcium chloride solution to immobilize using microcapsule preparation instrument. *B. subtilis*-loaded calcium alginate gel beads were obtained after immobilizing, and *B. subtilis*-loaded calcium alginate gel beads were mixed with chitosan solution to obtain the *B. subtilis*/alginate–chitosan microcapsule. The microcapsule system had good mechanical strength, flexibility and biocompatibility between *B. subtilis* and microcapsule. In addition, internal three-dimensional network structure of the microcapsule provided a sufficient space for *B. subtilis* growth and good encapsulating stability. Biosorption studies were conducted by the administration of *B. subtilis*/alginate–chitosan microcapsule to uranium-polluted wastewater, and the significant biosorption ability was evaluated in order to remove uranium ion from aqueous solution using free *B. subtilis* as the control.

## Materials and methods
### Strains and materials
*B. subtilis* was provided by College of Life Science and Engineering, Southwest University of Science and Technology. Alginate, chitosan, $U_3O_8$ and other reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

### *B. subtilis* cultivation
Cultivation of *B. subtilis* was carried out in 250 mL conical flasks with 100 mL culture medium on a rotary shaker at 200 rpm at a constant temperature of 37°C. The culture medium contained 3 g/L beef extract, 10 g/L peptone, 20 g/L glucose, 5 g/L NaCl and 0.005 g/L $MnSO_4$. The pH of the medium was adjusted to 7.0.

## Preparation of *B. subtilis*/alginate–chitosan microcapsule
A total of 15 mL of *B. subtilis* suspension of logarithmic phase was centrifuged for 10 min at 6,000 rpm. After centrifugation, *B. subtilis* was mixed with sodium alginate (5 mL, 15 g/L), and the concentration of the thallus was adjusted to $2.0 \times 10^6$/mL in miscible liquids. Then, $CaCl_2$ was added to the solution (15 g/L) and immobilized for 20 min to obtain *B. subtilis*/calcium alginate gel beads using microcapsule preparation instrument. Chitosan solutions were chosen (4 g/L) to mix with *B. subtilis*/calcium alginate gel beads, and *B. subtilis*/alginate–chitosan microcapsule was obtained after 20 min.

## *B. subtilis*/alginate–chitosan microcapsule cultivation
Cultivation of *B. subtilis*/alginate–chitosan microcapsule was carried out in 250 mL conical flasks with 100 mL culture medium on a rotary shaker at 200 rpm and a constant temperature of 37°C.

## Morphologic observation of *B. subtilis*/alginate–chitosan microcapsule
The microcapsule sample was collected at regular intervals during *B. subtilis*/alginate–chitosan microcapsule cultivation, and the viability characteristics of *B. subtilis* were observed in microcapsules using an inverted microscope.

## Measurement of cell biomass
The cultivation solution was collected in the free sample of cell culture at regular intervals, and the optical density was measured using a spectrophotometer at a wavelength of 650 nm to calculate the cell biomass. The microcapsule was opened after collecting the sample to release *B. subtilis*, and the optical density was measured using a spectrophotometer at a wavelength of 650 nm to calculate the cell biomass. The methods had been described previously.[13]

## Configuration of the uranium solution and standard curve measurement
$U_3O_8$ (1.1790 g) was taken in a 1,000 mL volumetric flask to prepare 1 g/L uranium solution. The concentration of uranium ion in the experiment was measured by atomic absorption spectrophotometry. Standard stock solution of uranium ion was collected to prepare 0.02, 0.04, 0.06, 0.08 and 0.1 mg/L uranium solution using distilled water, and

then, the absorbency was measured and the standard curve was drawn.

## Characterization

The overall shape and surface characteristics of particles were observed using a scanning electron microscope (SEM; Quanta 200FEG; FEI Company, USA).

## Adsorption experiment

A certain amount of immobilized *B. subtilis* adsorbent was taken in a 100 mL metallic solution at appropriate pH, oscillated at 100 rpm and centrifuged for 15 min at 10,000 rpm, then the supernatant was collected and the residual heavy metal ion concentration was measured.

$$\text{Adsorption capacity}: q_t = \left(C_0 - C_t\right) V / M$$

$$\text{Removal efficiency}: Y = \left(C_0 - C_t\right) \times 100 / C_i$$

where $q_t$ is the adsorption capacity of heavy metal ion under "t" time that the heavy metal ion was absorbed by per gram of immobilized adsorbent and was a index (mg/g) of measuring the adsorption capacity; $C_0$ is the initial concentration (mg/L) of heavy metal ion, $C_t$ is the concentration (mg/L) of heavy metal ion from solution after reactions, M is the absorbent dosage (g), V is the solution volume (L) and Y is the removal efficiency (%) of heavy metal ion. The methods had been described previously.[8]

## pH experiment

The effect of adsorption process was observed under different pH values (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 5.5, 6.0, 6.5 and 6.8) by reacting for 24 h at 37°C and 100 rpm in an EZ thermoshake. In this process, the dosing quantity of immobilized pellets was 3.5% (w/v), and the initial concentration of heavy metal ion was 100 mg/L.

## Adsorbent additive amount experiment

The effect of adsorption process was observed under different dosing quantities of immobilized pellets (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5% [w/v]) by reacting for 24 h at 37°C and 100 rpm in an EZ thermoshake. In this process, the pH of the solution was 6.0, and the initial concentration of heavy metal ion was 100 mg/L.

## Temperature experiment

The effect of adsorption process was observed under different temperatures (10, 15, 20, 25, 30, 35, 40, 45 and 50°C) by reacting for 24 h at 100 rpm in an EZ thermoshake. In this process, the pH of solution was 6.0, the dosing quantity of

immobilized pellets was 3.5% (w/v) and the initial concentration of heavy metal ion was 100 mg/L.

## Initial concentration of heavy metal ion experiment

The effect of adsorption process was observed under different initial concentrations of heavy metal ion (1, 5, 10, 20, 30, 50, 80, 100 and 150 mg/L) by reacting for 24 h at 37°C and 100 rpm in an EZ thermoshake. In this process, the pH of the solution was 6.0 and the dosing quantity of immobilized pellets was 3.5% (w/v).

## Time experiment

The effect of adsorption process was observed under different initial concentrations of heavy metal ion (1, 50 and 100 mg/L) at 5 min, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h and 24 h. In this process, the pH of the solution was 6.0, the dosing quantity of immobilized pellets was 3.5% (w/v) and the reacted temperature was 37°C.

## Desorption experiment

The desorption studies were performed using 3.5% (w/v) immobilized pellets with 100 mL of 100 mg/L uranium solution. After adsorption experiments (24 h), the adsorbed metal ions were eluted with 0.01 mol/L $Na_2CO_3$. The eluted biosorbent was then washed thoroughly with distilled water until the pH of the elute solution reached 7.0 and placed into metal solution for the subsequent adsorption–desorption cycle.

$$\text{Desorption efficiency}: R\left(\%\right) = C_e \times 100 \left(C_o - C_{eo}\right)$$

where $C_e$ is the concentration of the metal ion in the solution after desorption. $C_o$ and $C_{eo}$ are the concentrations of the heavy metal ion in the solution before and after adsorption.

# Results

## Growth state of *B. subtilis* in microcapsules

Very few *B. subtilis* were found in *B. subtilis*-loaded microcapsules, which were transparent gel microspheres. After cultivation for 8 h, *B. subtilis* grew and formed mycelial morphology in microcapsules, which further increased and the density of *B. subtilis* enlarged in the microcapsules after cultivation for 24 h (Figure 1A). The primary culture of microcapsules was transferred to fresh culture medium, where *B. subtilis* adapted to the new environment to achieve growth stability, and the density of *B. subtilis* further increased (Figure 1B).

# Effect of chitosan molecular weight on the growth characteristics of microencapsulated *B. subtilis*

The chitosans of 20,000 u, 50,000 u and 100,000 u molecular weight were chosen as microcapsule membrane. It was observed that *B. subtilis* reached the maximum quantity after 10 h in the sample of 100,000 u molecular weight chitosan, after 15 h in the sample of 50,000 u molecular weight chitosan and after 35 h in the sample of 20,000 u molecular weight chitosan. The maximum quantity of *B. subtilis* was similar in all 3 samples (Figure 2).

# Effect of microcapsule particle size on the growth characteristics of microencapsulated *B. subtilis*

The microcapsule carriers of 100 and 1,000 μm particle size were chosen. It was observed that the culture growth of *B. subtilis* in the microcapsule carrier with a particle size of 100 μm was better than that in the microcapsule carrier with a particle size of 1,000 μm during primary culture and batch culture (Figure 3A and B).

# Effect of inoculative density on the growth characteristics of microencapsulated *B. subtilis*

*B. subtilis* of $2.0 \times 10^6$, $2.6 \times 10^6$ and $5.2 \times 10^6$/mL inoculative density was chosen. It was observed that excess inoculative density led to decline of microcapsule mechanical strength, and the microcapsule ruptured. In addition, too low inoculative density caused the growth rate of *B. subtilis* to reach
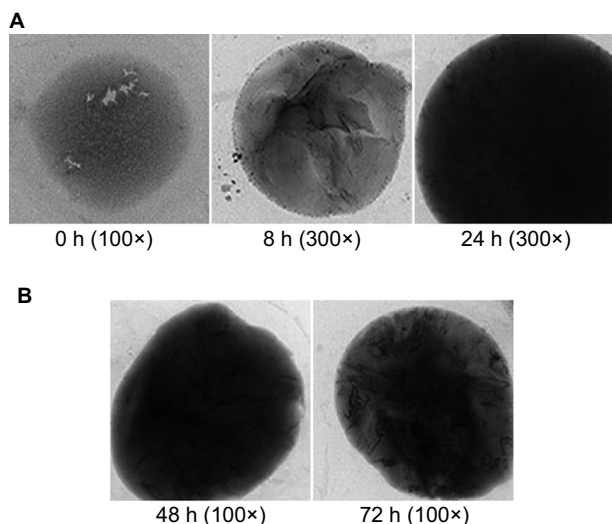


**Figure 2** Effect of chitosan molecular weight on the growth characteristics of *Bacillus subtilis*/alginate–chitosan microcapsule.
**Abbreviation:** lgc, logarithmic expression of cell number.

balance in the subsequent culture. The appropriate initial inoculative density was $2.6 \times 10^6$/mL (Figure 4).
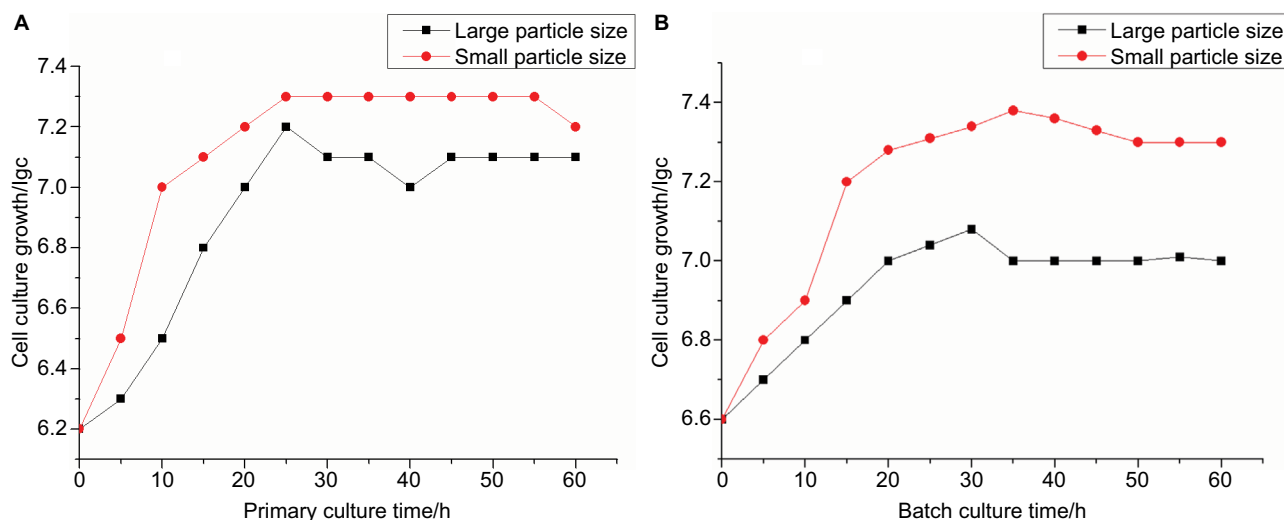
## Standard curve measurement

As shown in Figure 5, the abscissa is uranium concentration and the ordinate is absorbency. It was observed that the uranium concentration showed a linear relation with the absorbency at 0.02–0.1 mg/L.

## SEM analysis

The surface areas of *B. subtilis*/alginate–chitosan microcapsule before and after uranium biosorption were observed by SEM. The SEM images are shown in Figure 6. Many tiny interspace structures distributed on the surface of the biosorbent could be clearly observed in Figure 6A, which thus provided the maximum surface area for the biosorption of uranium. Figure 6B reveals that the surface of the biosorbent became rough and had more protrusions.

## Effect of initial pH on uranium adsorption

Figure 7 presents the effect of initial pH on the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule, with pH from 1.5 to 6.8. The removal efficiencies of uranium onto both adsorbents had the same trend, which increased with increasing pH. When the initial pH was adjusted to 1.5, a little uranium was removed by *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule. The removal efficiency of uranium increased sharply at the pH range of 1.5–5 while it increased slightly at pH >5. When the initial pH was 6, the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule



**Figure 1** The morphology of *Bacillus subtilis*/alginate–chitosan microcapsule in primary culture (**A**) and in batch culture (**B**).

**Figure 3** Effect of microcapsule particle size on the growth characteristics of *Bacillus subtilis*/alginate–chitosan microcapsule in primary culture (**A**) and in batch culture (**B**).
**Abbreviation:** lgc, logarithmic expression of cell number.
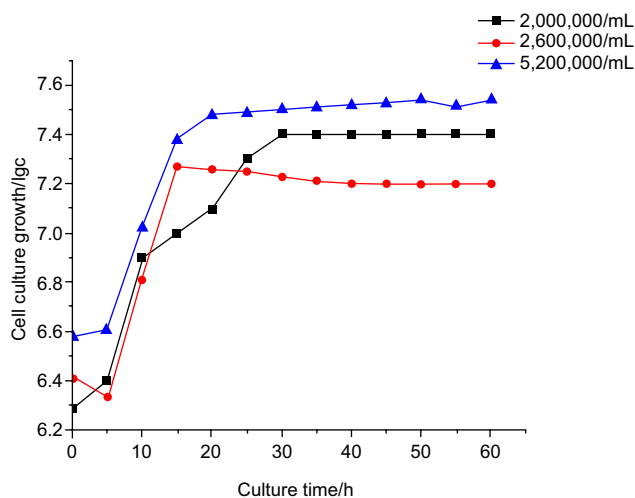


**Figure 4** Effect of inoculative density on the growth characteristics of *Bacillus subtilis*/alginate–chitosan microcapsule.
**Abbreviation:** lgc, logarithmic expression of cell number.

and alginate–chitosan microcapsule reached the maximum. When the initial pH was >6.5, the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule significantly decreased, and the removal efficiency of uranium on *B. subtilis*/alginate–chitosan microcapsule was higher than that on alginate–chitosan microcapsule at all pH ranges.

## Effect of dosing quantity of immobilized pellets on uranium adsorption

The effect of dosing quantity of initial immobilized pellets on the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan

microcapsule was assessed by varying the dosing quantity of immobilized pellets from 1.0 to 5 (w/v). The removal efficiencies of uranium onto both adsorbents had the same trend, which increased with increasing dosing quantity of immobilized pellets. When the initial dosing quantity of immobilized pellets was adjusted to 1.0 (w/v), a little uranium was removed by *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule. The removal efficiency of uranium increased sharply at the dosing quantity range of immobilized pellets of 1.0–3 (w/v), whereas it increased slightly when the dosing quantity of immobilized pellets was >3 (w/v). When the initial dosing quantity of immobilized pellets was 3.5 (w/v), the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule reached the maximum. When the initial dosing quantity of immobilized pellets was >4 (w/v), the removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule significantly decreased. The removal efficiency of uranium onto *B. subtilis*/alginate–chitosan microcapsule was higher than that onto alginate–chitosan microcapsule at all dosing quantity ranges of immobilized pellets (Figure 8).

## Effect of temperature on uranium adsorption

The effects of temperature on adsorption capacity of *B. subtilis*/alginate–chitosan microcapsule and alginate–chitosan microcapsule for uranium are shown in Figure 9. It could be observed from the figure that the adsorption capacity of *B. subtilis*/alginate–chitosan microcapsule for uranium
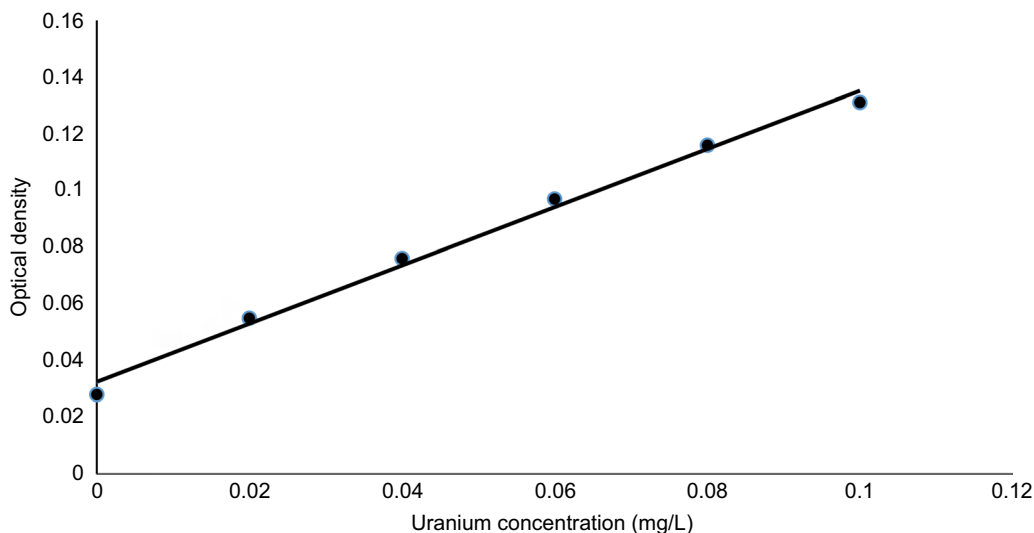
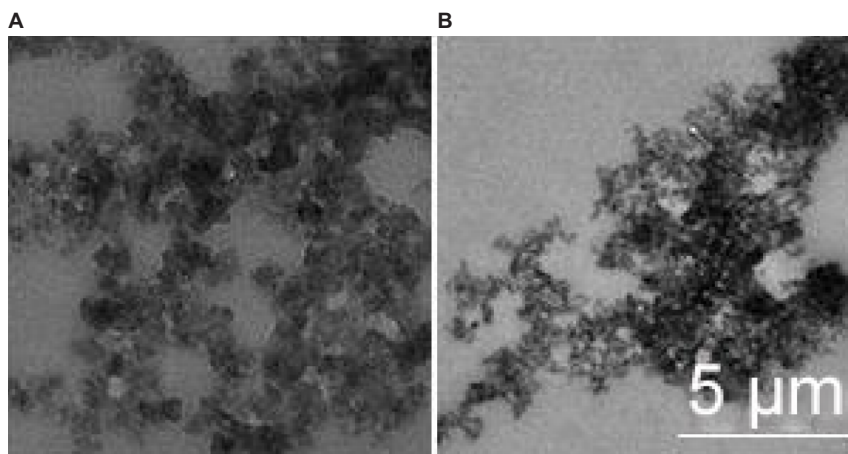**Figure 5** The standard curve of uranium ions.



**Figure 6** SEM images of *Bacillus subtilis*/alginate–chitosan microcapsule before (**A**) and after (**B**) biosorption of uranium ions.
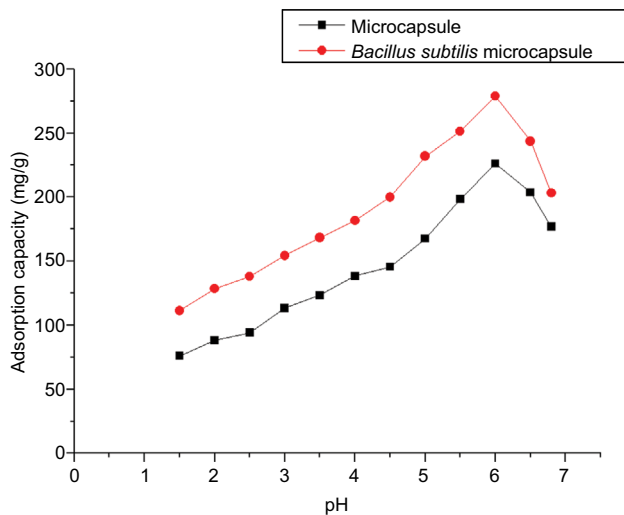**Abbreviation:** SEM, scanning electron microscope.



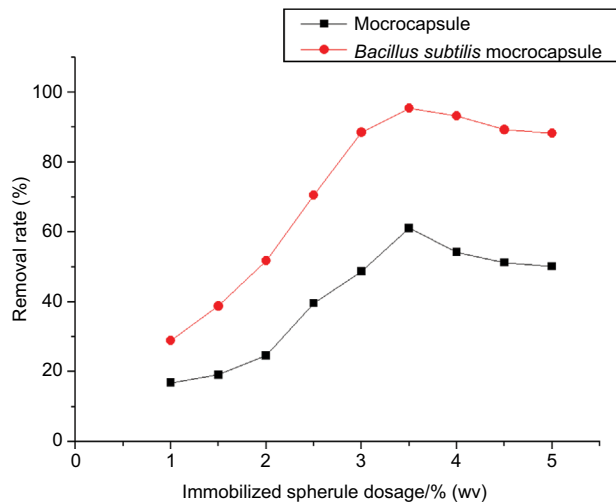**Figure 7** Effect of initial pH on uranium ion adsorption by *Bacillus subtilis*/alginate–chitosan microcapsule.



**Figure 8** Effect of dosing quantity of immobilized pellets on uranium adsorption by *Bacillus subtilis*/alginate–chitosan microcapsule.

increased with the increase in temperature from 10 to 20°C. The adsorption capacity reached a maximum at 20°C and then decreased with the increase in temperature to 50°C. This small change at 10−20°C led to a great change in adsorption capacity. These results indicated that *B. subtilis* was very sensitive to temperature. The adsorption capacity of alginate–chitosan microcapsule increased with increasing temperature, which indicated the endothermic nature of the adsorption process.

## Effect of initial uranium concentration on uranium adsorption

The removal efficiency and adsorption capacity at different uranium concentrations are presented in Figure 10. At a low uranium concentration of 1 mg/L, the removal efficiency of *B. subtilis*/alginate–chitosan microcapsule reached a maximum of 98.7% and then reduced with the increase in initial uranium concentration. The high biosorption efficiency (close to 99%) at low concentrations indicated the potential application of *B. subtilis*/alginate–chitosan microcapsule for removal of a trace amount of uranium from wastewater. The removal efficiency of uranium dropped sharply with an increase in initial uranium concentration from 5 to 150 mg/L. The adsorption capacity of *B. subtilis*/alginate–chitosan microcapsule increased with increasing initial uranium concentration and reached 376.64 mg/g at the initial uranium concentration of 150 mg/L.

## Effect of contact time on uranium adsorption

The effects of contact time for *B. subtilis*/alginate–chitosan microcapsule on the adsorption capacity at different initial concentrations (1, 50 and 100 mg/L) are shown in Figure 11.
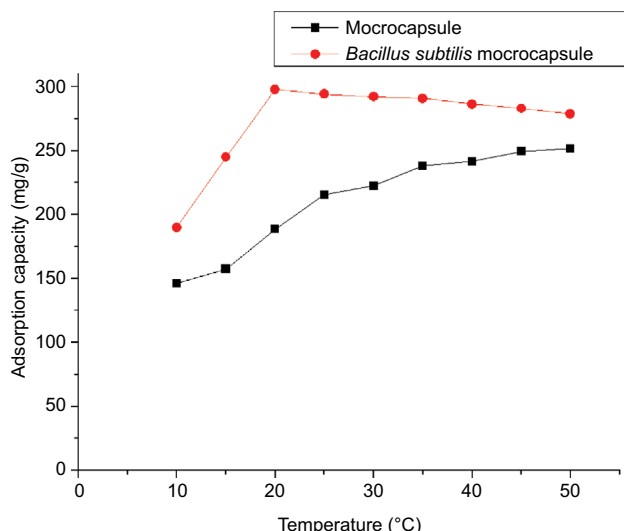
**Figure 9** Effect of temperature on uranium adsorption by *Bacillus subtilis*/alginate–chitosan microcapsule.
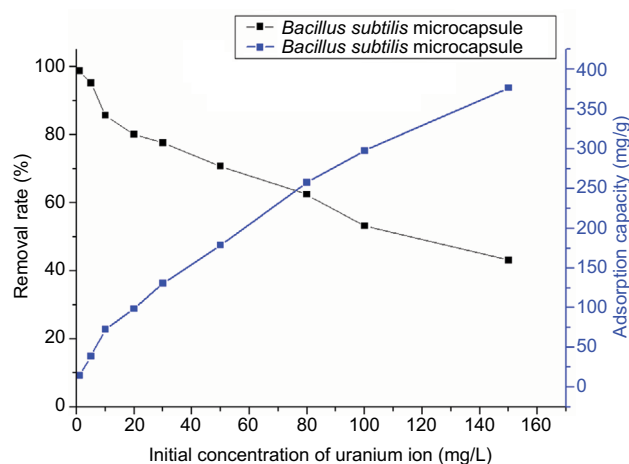
**Figure 10** Effect of initial uranium concentration on uranium adsorption by *Bacillus subtilis*/alginate–chitosan microcapsule.
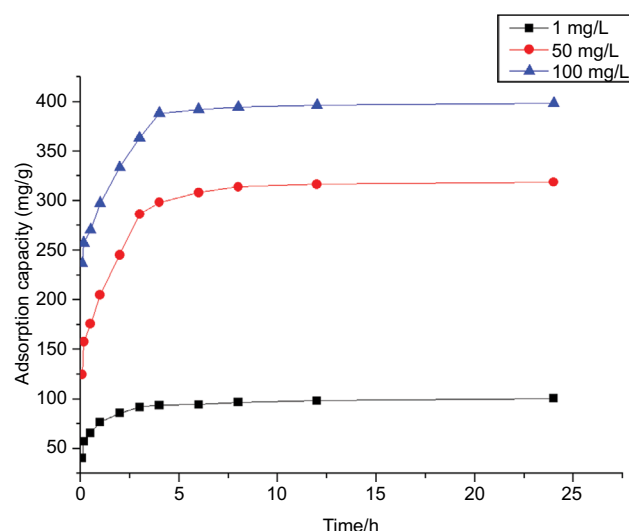
**Figure 11** Effect of contact time on uranium adsorption by *Bacillus subtilis*/alginate–chitosan microcapsule.

**Table 1** Desorption ratio of uranium ions adsorbed by *Bacillus subtilis*/alginate–chitosan microcapsule

| Cycle | Adsorbed capacity (mg/g) | Desorption ratio (%) |
|---|---|---|
| 1 | 94.8 | 97.8 |
| 2 | 91.2 | 95.3 |
| 3 | 88.5 | 90.2 |
| 4 | 85.6 | 84.6 |
| 5 | 82.1 | 79.4 |

It was observed that a rapid uptake occurs within the first 3 h and then followed by a slow increase until the equilibrium state was attained after 8 h at all the initial uranium concentrations. After this equilibrium period, the amount of uranium adsorbed did not change significantly with time.

## Effect of desorption study

Table 1 lists the adsorption capacity and desorption ratio of uranium on the *B. subtilis*/alginate–chitosan microcapsule in

5 adsorption–desorption cycles. The adsorption capacity and desorption ratio of *B. subtilis*/alginate–chitosan microcapsule decreased by only 5.2 mg/g and 2.2% after the first cycle, which could still be maintained at 82.1 mg/g and 79.4%, respectively, at the 5th cycle.

## Discussion

Uranium is an environmentally important element because of its toxicity and to a lesser extent because of its radioactivity.[14] With the rapid development of uranium mining industry, a large amount of wastewater containing uranium has been discharged into the environment. In addition, malfunctions of nuclear reactors and leaks of cooling water have also endangered the environment.[15,16] Therefore, it is important to remove uranium from wastewater from uranium mining, reactor operations, fuel reprocessing and other anthropogenic activities.[17] Conventional methods for removing uranium (VI) from wastewaters include chemical precipitation, coagulation and ion-exchange process. However, these conventional methods are expensive and ineffective, particularly at low metal concentrations.[18,19]

Many studies have shown that different groups of microorganisms, such as algae, bacteria, yeasts and fungi, could be used as natural adsorbents for uranium (VI) from aqueous solutions, and, among these, fungi and algae showed the greatest potential due to their high adsorption capacity and low cost.[20–22] However, the physical and mechanical characteristics of the microbial biomass, such as the small particle size, difficult separation of solids and liquids and poor mechanical strength, had impeded their commercial application as a biosorbent.[23,24] In order to overcome these difficulties, some researchers developed immobilization methods for biomass, and the immobilized biomass showed advantages over the free biomass in that it had higher mechanical strength and could more easily be separated from the aqueous solutions and the adsorbed metal ions could more easily be recovered.[25,26]

In our study, we report the synthesis of alginate–chitosan microcapsule with immobilized *B. subtilis* and showed that alginate–chitosan microcapsule had good mechanical strength, elasticity and biocompatibility with *B. subtilis*. Its internal three-dimensional network structure provided ample space for *B. subtilis* growth and good encapsulating stability. And *B. subtilis* was mycelial growth and did not leak in the culture process. In this study, microcapsule of small particle size had a high mass transfer efficiency, large specific surface area, easy to be the characteristics of a stable suspension and was advantageous to the pouch *B. subtilis* growth metabolism. Chitosan molecular weight

had a significant effect on the microcapsule membrane performance and the microcapsule cell growth. It was observed that 100,000 u molecular weight chitosan could be used to prepare thin microcapsule membrane, which had good mechanical strength and mass transfer performance. Although *B. subtilis* inoculation density showed a positive correlation with the increase in growth of microcapsule *B. subtilis*, excess inoculation density led to microcapsule rupture. Therefore, in our study, it is demonstrated that $2.1 \times 10^6$/mL inoculation quantity was advisable.

On the other hand, in the biosorption evaluation of uranium, it was found that *B. subtilis*/alginate–chitosan microcapsule significantly improved biosorption of uranium. In the SEM analysis, many adsorbent particles were clearly observed on the surface of the biosorbent, which was attributed to the reactions occurring on the surface of the biosorbent that afterward changed the structure of alginate–chitosan microcapsule. Assessment of the effect of pH, dosing quantity of immobilized pellets, temperature, initial uranium concentration, contact time and desorption study on uranium adsorption showed that uranium ions could be effectively adsorbed by *B. subtilis* immobilized into alginate–chitosan microcapsule compared with alginate–chitosan microcapsule and that *B. subtilis*/alginate–chitosan microcapsule has great potential as a low-cost heavy metal biosorbent.

Although the removal of radionuclide, uranium, had been reported earlier, to our knowledge, this study is the first comprehensive report on its accumulation by alginate–chitosan microcapsule, which shows the feasibility of possible application of biosorbents in consecutive biosorption cycles. These results showed that immobilization methods could further promote the biosorption of uranium and increase the biomass of *B. subtilis*. This result was consistent with the results of previous studies;[25,27,28] therefore, the immobilized *B. subtilis* alginate–chitosan microcapsule has great potential for removing uranium (VI) from aqueous solutions.

## Conclusion

The following points can be concluded from this study: 1) *B. subtilis* was effectively encapsulated by alginate–chitosan; 2) Uranium ions could be effectively adsorbed by *B. subtilis*/alginate–chitosan microcapsule; 3) The pH, immobilized spherule dosage, temperature, initial uranium ion concentration and contact time highly affect the uranium biosorption; 4) Our results showed that the maximum adsorption capacity of uranium ion was 376.64 mg/g; and 5) The desorption rate was maintained at 79.4% after 5 cycles. This suggested that the *B. subtilis*/alginate–chitosan microcapsule has great potential as a low-cost uranium ion biosorbent.

# Acknowledgment

# Author contributions

Ke Tong designed, carried out the experiment and wrote the manuscript. The author also revised the manuscript, gave useful suggestions on the early version and read and approved the final manuscript.

# Disclosure

The author reports no conflicts of interest in this work.

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