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# Efforts on membrane properties and enzymes by adding divalent cations and sodium carboxymethyl cellulose

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ABSTRACT

In this paper, the addition of sodium carboxymethyl cellulose (NACMC) and cobalt ions to alginate gel significantly improved the inner and outer surface properties of membranes and the activity of the enzymes. The results showed the optimization was sodium alginate (SA) and NACMC at ratio 1:1, and the concentration of $CoCl_2$ at 0.05 mol/L. The combined SA-NACMC gel bead apparently had a more porous and higher mechanical strength than that of the SA gel bead by scanning electron microscopy (SEM). The microcapsule surface roughness was measured by atomic force microscopy (AFM), the roughness was 155 ± 15.82 mm. Otherwise, the Brunauer–Emmett–Teller (BET) analysis showed that with the addition of NACMC the surface area and most of pore size of the microcapsules was 76.471 $m^2/g$, and distributed in 3–25 nm, respectively. In 28d, immobilized enzyme had a higher degradation rate, and the atrazine residue of the immobilized enzyme with 5% content was 21.79%.



## 1. Introduction

Atrazine is toxic to animals, aquatic plants, and even human beings (Kotrikla, Gatidou, & Lekkas, 1999; Ralebitso, Senior, & van Verseveld, 2002; Villanueva, Durand, Coutte, Chevrier, & Cordier, 2005). It is clear that treatment of contaminated soil and water is of great practical significance for environmental protection. Although the multiple methods are available for removing atrazine from contaminated soil and water, based on efficiency, bioremediation has showed its important role in complete degrading atrazine to ammonia and $CO_2$ (Struthers, Jayachandran, & Moorman, 1998).

Enzymes play an important part in the bioremediation: the breakdown of organic and inorganic pollutants (Walsh, 2001). While the value and applications of enzymes are limited by their instability and non-reusability, enzyme immobilization is an effective way to overcome these limitations to some extent. Although several papers have studied the TrzN, AtzB, and AtzC to degrade atrazine (Boundy-Mills, de Souza, Mandelbaum, Wackett, & Sadowsky, 1997; Mulbry, Zhu, Nour, & Topp, 2002; Sebai, Devers-Lamran, Changey, Rouard, & Martin-Laurent, 2011), there was less far research on the three enzymes under immobilized conditions.

Sodium alginate (SA) is a naturally occurring biopolymer extracted primarily from brown algae. It has been used in the biotechnology industry as a thickening agent, a gelling agent or a colloidal stabilizer. Alginate also has a unique capacity to be sued as a matrix for the entrapment and/or delivery of a variety of molecules or particles (Rousseau, Cerf, Picton, Argillier, & Muller, 2004). In the presence of divalent cations, alginate shows gelling properties. Addition of calcium ions (divalent cations) induces a cooperative effect between G-blocks until a 3D network is formed according to the well-known “egg-box” model. Clark and Ross-Murphy (1987) had studied the influence of the divalent cations on the strength of the gel.

With its pronounced visco-elastic and structure-forming properties, the ether sodium carboxymethyl cellulose (NACMC) is employed as a flow enhancer, a stabilizer, and also as an agent for binding, suspending and thickening. As starch, NACMC is also low-cost and environmentally friendly (He, Zhao, Liu, & Roberts, 2007).

Chitosan, a natural biodegradable polymer, which is derived from chitin by deacetylation (Huang, Yang, Zheng, & Wang, 2012), is a low acetyl substituted form of chitin named (1-4)-2-amino-2-deoxy-(D-glucose) is a natural positively charged polysaccharide

*Abbreviations:* NACMC, sodium carboxymethyl cellulose; SA, sodium alginate; PEC, polyelectrolyte complexes.

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**Table 1**
Orthogonal design table of microcapsule immobilizing atrazine degrading enzyme.

| Levels | A<br>NACMC:SA (g:g) | B<br>Chitosan concentration (%) | C<br>The $Co^{2+}$ concentration (M) | D<br>Chitosan solution (pH) |
|---|---|---|---|---|
| 1 | 0:3 | 0.3% | 0.05 | 5.0 |
| 2 | 1:1 | 0.5% | 0.07 | 5.5 |
| 3 | 2:1 | 0.7% | 0.09 | 6.0 |

having a pKa ≈ 6.3–7.0 (Rinaudo & Domard, 1989) with its unique properties such as excellent adsorbability, biodegradability, nontoxicity, anti-bacterial effect and biocompatibility (Suyatma, Copinet, Legin-Copinet, Fricoteaux, & Coma, 2011). It is widely used in agriculture (Chmielewski et al., 2007), food (Aider, 2010). Also, Chitosan can be used in environmental pollution control, for example, it could be grafted in Multiwalled Carbon Nanotube for heavy metals removal from aqueous solutions (Shao, Hu, & Wang, 2010).

In this survey, the polyelectrolyte complexes (PEC) by SA/Chitosan/NACMC/Cobalt ions ($Co^{2+}$) was done with the purified enzyme that have atrazine degrading gene trzN, atzB and atzC. The composite was characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM), and Brunauer–Emmett–Teller (BET), and then the application of the microcapsules for the atrazine contaminated soil remediation was also examined.

## 2. Materials and methods

### 2.1. Materials

Chitosan (degree of deacetylation ≥90%), NACMC and SA were purchased from solarbio Biochemical Co., Ltd. (Beijing, China), Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and Sanpu Chemical Co., Ltd. (Shanghai, China), respectively. Other reagents and solvents were analytical grade and purchased from Beijing Chemical Reagent Company.

### 2.2. Preparation of microcapsules

The purified enzyme was obtained from the Environmental Science Laboratory, Northeast Agricultural University, Heilongjiang, China, and it can express the specific protein of recombinant strains for atrazine degrading gene trzN, atzB and atzC (Zhang et al., 2011). In the aqueous solution (SA/NACMC), the mass fraction of SA/NACMC was 2% and the mass ratios of them were 1:1, 1:2 and 3:0, respectively, with the purified enzyme (the dosage of it was 1/10). The above-mentioned solution was inhaled using syringe then through a 0.35 mm micro-sieve and injected uniformly into 0.75% Chitosan solution. The soluble salts such as calcium chloride ($CaCl_2$), cobalt chloride ($CoCl_2$), zinc chloride ($ZnCl_2$) were added to the aqueous solutions under concentrations ranging from 0 to 0.1 mol/L at the interval of 0.03 mol/L prior to encapsulation. After a 40 min residence time, the microcapsules were separated from the precipitation bath and rinsed several times with distilled water. The microcapsules were then stored in a 0.9% (w/v) NaCl solution. The determination of microcapsule properties was carried out after microcapsule swelling first and then being stabilized. The obtained microcapsules have a relatively narrow size distribution, with diameter of 0.4 mm (a coefficient of variation of 15%). The experimental procedures were conducted at room temperature.

### 2.3. Optimization of the preparation process for microcapsules

The preparation for microcapsules were optimized by orthogonal experiments ($L(9)3^4$) using encapsulation efficiency (EE) as the target index. The ratio of NACMC:SA, Chitosan concentration, $Co^{2+}$ concentration and Chitosan solution (pH) with three levels were chosen in the orthogonal experiments (Table 1).

EE, one of the important parameters during the process, was calculated as follows, which can be defined as:

$$EE(\%) = \frac{M_m}{M_t} \times 100\%$$

where $M_t$ refers to total added pure enzyme mass and $M_m$ refers to the pure enzyme mass in the microcapsules.

### 2.4. Microcapsule characterization

#### 2.4.1. Mechanical strength

Mechanical strength was determined by electronic balance, namely the microcapsules being situated on a holder on the top of the balance, permanently stressed until bursting and the final reading was the burst point. Each batch test included 50 microcapsules.

#### 2.4.2. SEM

The microstructure of various microcapsules was observed by SEM (Hitachi S-3400N, Hitachi Company, Japan). The specimens were fractured in pretreatment, and then fixed on stubs with sputter coated with gold before observation.

#### 2.4.3. AFM

The microcapsules in aqueous dispersion were filtered through a regenerated cellulose polymeric filter membrane and then air-dried. AFM images were obtained using a Nanoscope III Multimode Atomic Force Microscope (Digital Instruments Inc.) in air, using the contact mode. A triangular $Si_3N_4$NP probe (Veeco Instruments, CA), with a nominal cantilever spring constant of 0.12 N/m and nominal frequency of 20 kHz, was used. The tip height was 2.5–3.5 μm, with a nominal radius of 20 nm and a side angle of 35°. On each sample, five different areas of dimension 20 μm × 20 μm, were scanned at a rate of 0.5 Hz. Average values were determined from this data for the roughness and height of each sample.

AFM roughness and height data were obtained using the Nanoscope program (Nanoscope 5.30 r3sr3, Veeco Instruments, CA). The average roughness ($R_a$) for the image was defined as the arithmetic average of the absolute values of the surface height deviations measured from the mean plane, rather than the frequency or spacing of the features. The height comparisons were performed using the maximum roughness ($R_{max}$), defined as the maximum vertical distance between the highest and lowest data points in the image.

#### 2.4.4. BET

BET theory is a popular method of interpreting nitrogen adsorption isotherms for determining the specific surface area.

Probe gas $N_2$ used as adsorbate has remained universally preeminent at 77 K and sub-atmospheric pressure and can be used for routine quality control and research of new materials. If it is applied over a wide range of relative pressures ($p/p_0$), $N_2$ adsorption isotherms will provide some information on size distributions in the micro-, meso-, and macro-porosity range (approximately 0.5–200 nm). The classical pore size model based on the Kelvin

**Table 2**
The Young's modulus of different metal ions.

| Metal ions | Mechanical strength (MPa) |
|---|---|
| $Ca^{2+}$ | 5.78 ± 0.417 |
| $Co^{2+}$ | 6.97 ± 0.123 |
| $Zn^{2+}$ | 6.35 ± 0.107 |

Values are means ± standard deviation ($n$ = 50).

equation that was developed by Barret, Joyner and Halenda (BJH) corrected for multilayer adsorption, was widely used for calculations of the PSD at the range of meso pore and part of the macro pore. The Surface area determined by the BET method.

### 2.5. The application of the microcapsules for the atrazine contaminated soil remediation

The atrazine contaminated soil remediation experiments were performed in order to test whether immobilized enzymes had a higher degradation rate than the free enzymes on the removal of atrazine in soil. The soils with a concentration of 20 mg/kg atrazine were incubated (at a temperature of 27 °C and 40% water holding capacity) for 28 days. The soils were treated as follows: (i) original collected soil with atrazine as the blank control (CK); (ii) the soil containing atrazine and 1‰ (w/v) free enzymes; (iii) the soil containing atrazine and 5‰ (w/v) free enzymes; (iv) the soil containing atrazine and 1‰ (w/v) immobilized enzymes; (v) the soil containing atrazine and 5‰ (w/v) immobilized enzymes. All experiments were carried out in four replicates. 50 g soil was sampled from each treatment mentioned above at 0, 7, 14, 21 and 28 days to measure the residual atrazine concentration in the soil.

The above soil samples (10 g) were extracted with methanol aqueous solutions (9:1), concentrating the methanol, and then extracted with chloroform. After concentrating to a defined volume, GC-14C gas chromatographic and FID detector were used to determine the residual atrazine in the soil (Zhang et al., 2012).

### 2.6. Statistical analysis

Analysis of ANOVA using LSD (least significant difference) test was performed to assess the significance of differences ($P$ < 0.05) in SPSS 19.0 software (SPAA, Inc.). Origin 8.0 was used to find the linear regression and correlation between various parameters.

## 3. Results and discussion

### 3.1. Effect of different divalent metal cations on the microcapsule and the optimal ratio of the microcapsule material

Generally, PEC formation was strongly influenced by the presence of the salts due to the charge screen effect (Bartkowiak, 2002). From Table 2, the strengths of different divalent $Co^{2+}$, $Zn^{2+}$, $Ca^{2+}$ were 6.97, 6.35, 5.78 MPa, respectively. An interesting phenomenon, observed during capsule formation, was that the mechanical strength of these capsules was contrary to the radius of the ions (Zhang, Guang, Ji, & Yao, 2006). The order of radius of the ions was $Ca^{2+}$ (1.12Å) > $Zn^{2+}$ (0.745Å) > $Co^{2+}$(0.735Å) (Shannon & Prewitt, 1969). Improvement of the mechanical strength could extend the applications of the capsules (Bartkowiak, 2002).

Table 3 shows the effect of different $Co^{2+}$ concentrations on embedding rate. When the $Co^{2+}$ concentration was 0.05 mol/L, 0.075 mol/L and 0.1 mol/L, the embedding rate reached 59.34%, 74.67% and 78.31%, respectively. The microcapsule was regular and easy to ball. Orthogonal experiments were carried out to obtain the optimal ratio of microcapsule materials. When the optimization was SA:NACMC at ratio 1:1, and at $CoCl_2$ concentration of 0.05 mol/L, the orthogonal experiment (Table 4) showed best embedding effect.

**Table 3**
The effect of different $Co^{2+}$ concentration on embedding rate.

| $Co^{2+}$ concentration (mol/L) | Preparation and the difficulty of the formation | Embedding rate (%) ($n$ = 4) |
|---|---|---|
| 0.02 | Scattered into shape, shape outline is not obvious | 19.23 |
| 0.04 | The fractionation tends to be regular and is easy to ball | 31.61 |
| 0.05 | The fractionation is regular and easy to ball | 59.34 |
| 0.075 | The fractionation is regular and easy to ball | 74.67 |
| 0.1 | The fractionation is regular and easy to ball | 78.31 |

When $Co^{2+}$ was grafted into the combined SA-NACMC cellulose gel bead by OLYMPUS BX51, it formed microcapsules with better particle size and also replaced the $Ca^{2+}$ in the traditional alginate gel. The addition of $Co^{2+}$ had a similar effect on microcapsules with NACMC, and it was used as stabilizer and also as agents for binding, thickening and suspension. Usually, $Co^{2+}$ is a kind of heavy metal ions that has been researched in the environmental pollution cleanup by many researchers (Liu, Chen, Hu, Wu, & Wang, 2011). However, many researchers had also observed that $Co^{2+}$ exhibited the promotion of enzyme activity. $Ni^{2+}$ and $Co^{2+}$ had been found to be the most efficient replacements for $Zn^{2+}$ in the proteins (Jackman, Raetz, & Fierke, 1999). Both $Ni^{2+}$ and $Co^{2+}$ restored the activity level of AtzC (Shapir, Osborne, Johnson, Sadowsky, & Wackett, 2002). $Co^{2+}$ was the only divalent cation tested that stimulated hydrolase activity (threefold) (Bouquard, Ouazzani, Prome, Michel-Briand, & Plesiat, 1997).

### 3.2. Effect of NACMC concentration on inner and outer surface properties of membranes

When the $Co^{2+}$ concentration was 0.05 mol/L, to determine the effects of the application of the NACMC on the topography of the PEC membranes, SEM and AFM imaging were used.

Compared with microcapsules prepared with the combined SA-NACMC gel bead and SA gel bead, the inner structure of microcapsules were observed to have a special structure of multilayer films by SEM. Fig. 1 showed the morphology of the gel beads with the immobilized enzyme by SEM. The combined SA-NACMC gel

**Table 4**
Orthogonal design results of microcapsule immobilizing atrazine degrading enzyme.

| No. | Factors | | | | EE (%) |
|---|---|---|---|---|---|
| | A | B | C | D | |
| 1 | 1 | 1 | 1 | 1 | 46.21 |
| 2 | 1 | 2 | 2 | 2 | 59.52 |
| 3 | 1 | 3 | 3 | 3 | 43.42 |
| 4 | 2 | 1 | 2 | 3 | 47.64 |
| 5 | 2 | 2 | 3 | 1 | 58.10 |
| 6 | 2 | 3 | 1 | 2 | 74.92 |
| 7 | 3 | 1 | 3 | 2 | 67.27 |
| 8 | 3 | 2 | 1 | 3 | 55.68 |
| 9 | 3 | 3 | 2 | 1 | 47.86 |
| T1 | 49.33 | 53.33 | 58.33 | 50.33 | |
| T2 | 59.67 | 57.33 | 51 | 66.67 | |
| T3 | 56.33 | 54.67 | 56 | 48.33 | |
| R | 10.34 | 4.00 | 7.33 | 18.34 | |

T1, total EE of each factor in its first level; T2, total EE of each factor in its second level; T3, total EE of each factor in its third level; $R$, range EE of each factor in its each level.

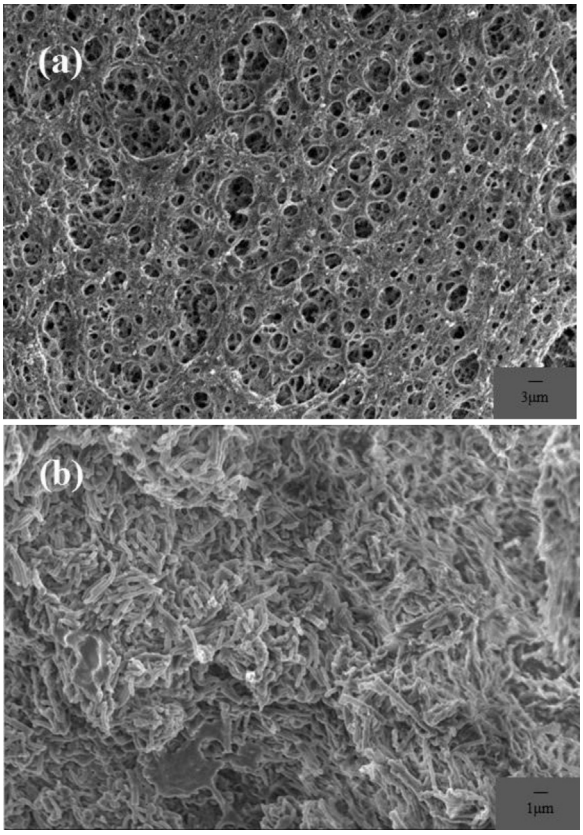


**Fig. 1.** Scanning electron microscopy photographs of the microcapsules. (The conditions of microcapsule preparation SA:NACMC = 1:1(a) and SA:NACMC = 3:0 (b) after 30 min reaction time, Chitosan as reaction medium, then poured into 0.05M$CoCl_2$.)

bead (NACMC:SA = 1:1) (Fig. 1a) was apparently more porous compared with that of the SA gel bead (Fig. 1b). With a large number of pores to ensure the effective transfer of degraded organic pollutants, the enzyme could be restricted in the microcapsules. Similar observation has also been reported by Thi, Van, and Van (2013) and Kim, Park, Gu, and Kim (2012).

Fig. 2 showed the effect of NACMC addition on the AFM imaging. As represented in Table 5, there was an obvious difference in surface roughness parameters due to the different weight ratios of NACMC to SA. It indicated that the addition of appropriate NACMC could increase the surface roughness of microcapsules, while excess NACMC was likely to cover the formative rough membrane because of its sticky nature. The optimum ratio of 1:1 was selected for enzyme in the combined SA-NACMC gel bead.

### 3.3. *Effect of NACMC concentration on surface area and pore size of microcapsule*

When the cobalt ion concentration was 0.05 mol/L, the combined SA-NACMC gel beads and the SA gel beads were analyzed by BET method using nitrogen adsorption–desorption isotherms, according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature (Sing et al., 1985). Fig. 3a showed

**Table 5**
The roughness of different membrane.

| NACMC:SA (g:g) | Roughness (nm) |
|---|---|
| 0:3 | 137 ± 24.79 |
| 2:1 | 123 ± 22.38 |
| 1:1 | 155 ± 15.82 |

Values are means ± standard deviation ($n$ = 4).

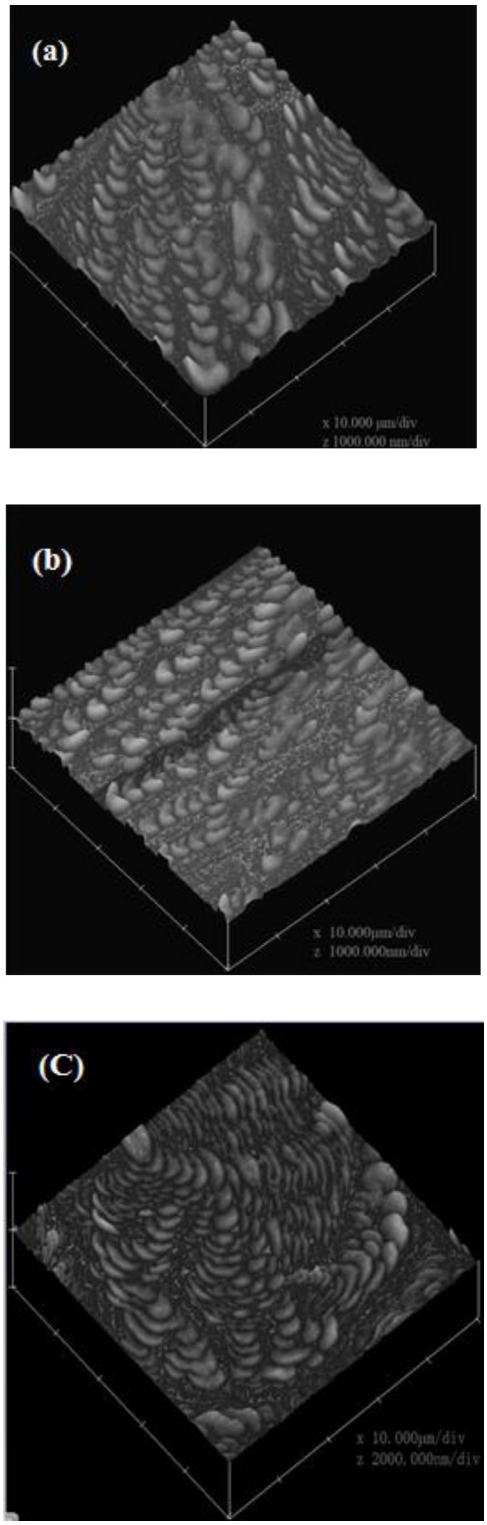


**Fig. 2.** The effect of NACMC addition on the atomic force microscopy imaging (the conditions of microcapsule preparation SA:NACMC = 1:1(a) and SA:NACMC = 3:0(b) and SA:NACMC = 1:2(C) after 30 min reaction time, Chitosan as reaction medium, then poured into 0.05M$CoCl_2$.).

that the adsorption isotherms were apparently classified as the types IV and the hysteresis loop belonged to the type H4, typical slit-shaped mesopores, whose sizes and shapes were also non-uniform. It could be seen that the adsorption capacity increased gently at the low pressure stage, by this time $N_2$ molecules were adsorbed on the inner surface of mesoporous from monolayer to

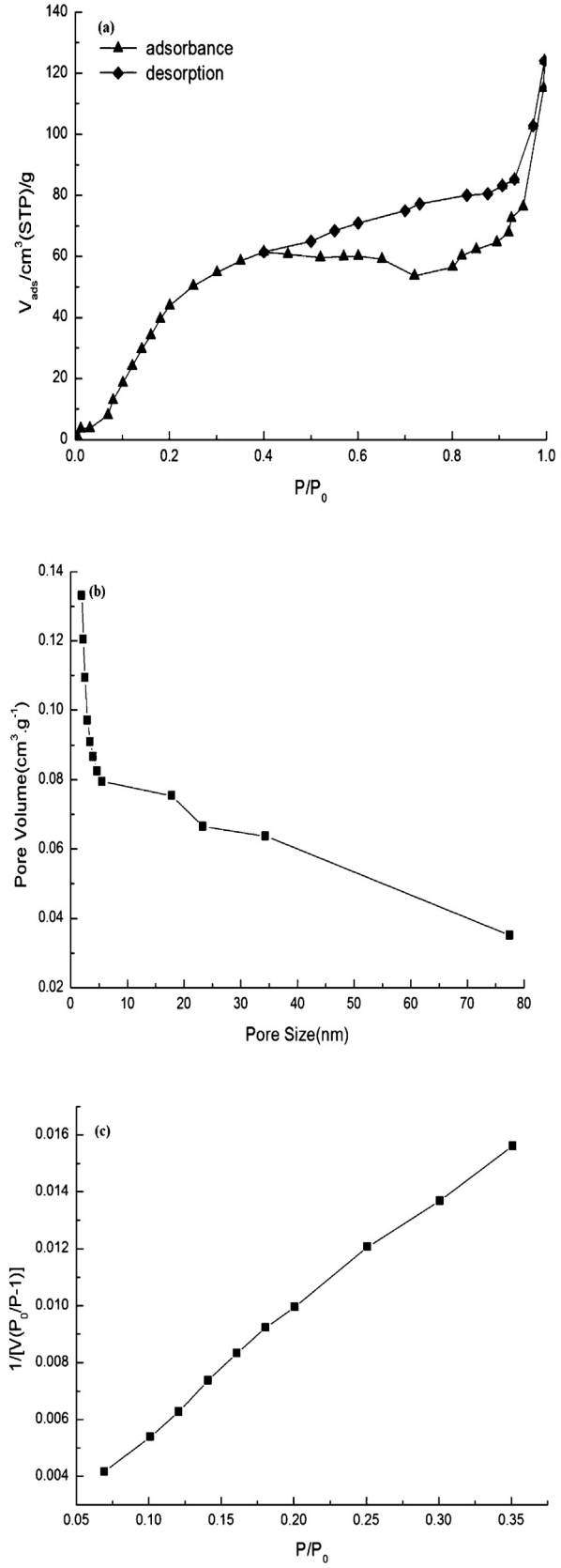


**Fig. 3.** With NACMC absorption–desorption isotherm linear (a), cumulative pore (b), BET(c). (The conditions of microcapsule preparation SA:NACMC = 1:1, after 30 min reaction time, Chitosan as reaction medium, then poured into 0.05M$CoCl_2$.)

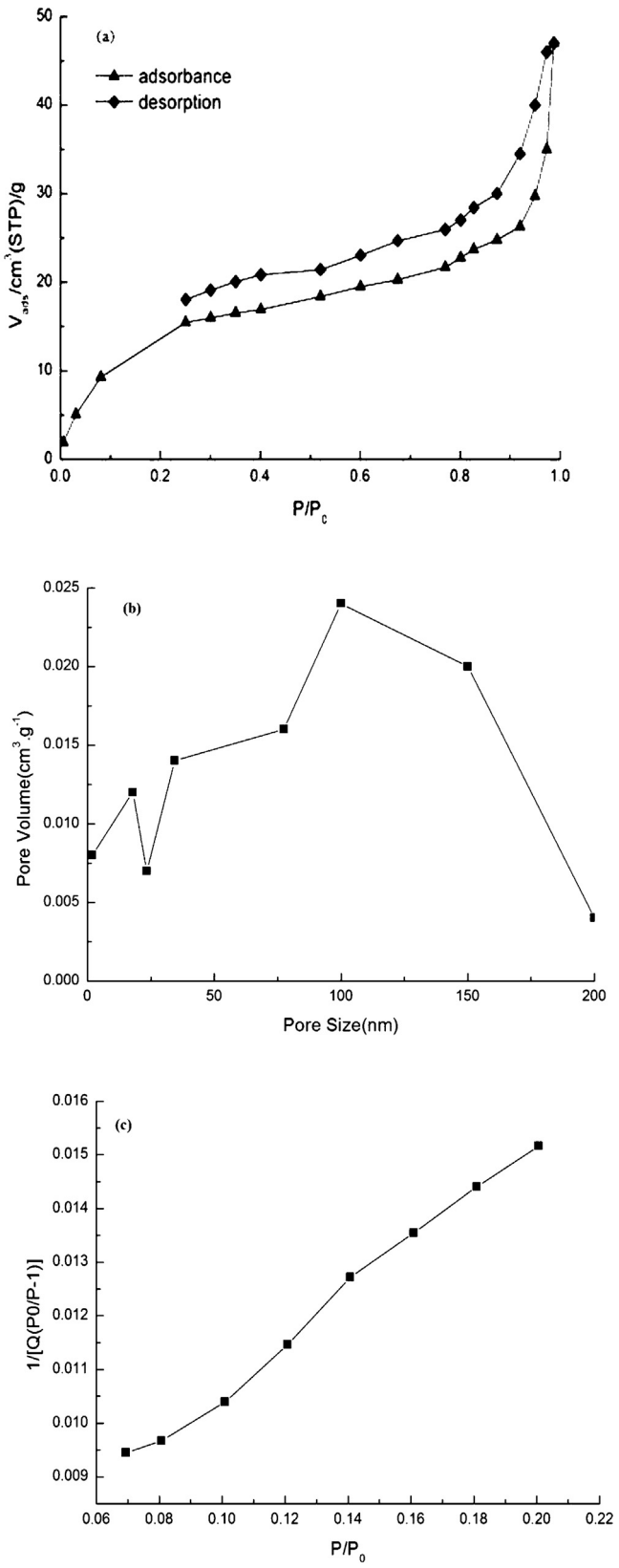


**Fig. 4.** Without NACMC absorption–desorption isotherm linear (a), cumulative pore (b), and BET (c). (The conditions of microcapsule preparation SA:NACMC = 3:0 after 30 min reaction time, Chitosan as reaction medium, then poured into 0.05M$CoCl_2$.)

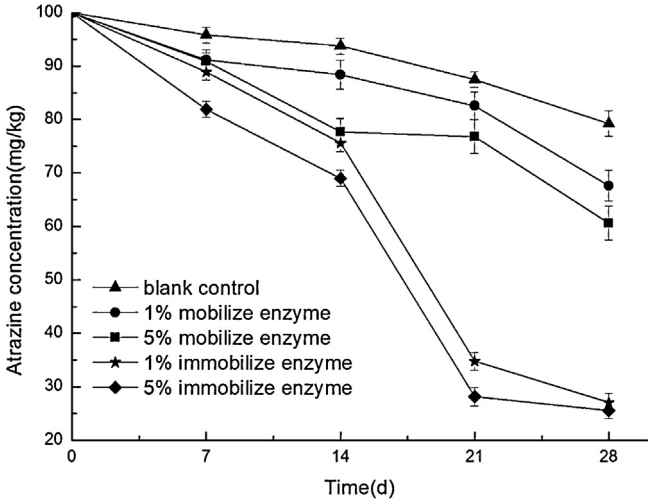


**Fig. 5.** The residual concentration of atrazine after different treatment. Error bars are ±standard deviation ($n = 4$).

multilayer. In contrast, in Fig. 4a, there was not a closed hysteresis loop, which illustrated variation ranges of shape and size of pores being large, namely the pore size of this type microcapsule distributed unevenly. When the specific area of ordered mesoporous material was calculated by BET, the relative pressure ($P/P_0$) between 0.05 and 0.35 was more appropriate. When $P/P_0$ was between 0.05 and 0.35 (Figs. 3c and 4c), the graphics had a better linear relationship, the specific surface areas of the combined SA-NACMC gel beads and the alginate gel beads were 76.471 $m^2/g$ (Fig. 3a) and 43.34 $m^2/g$ (Fig. 4a), respectively. The addition of NACMC make specific surface area of the microcapsule increased about 1.7 times. The increase in specific surface area of the gel beads would improve the mass transfer during the enzymatic reaction (Thi et al., 2013). Carboxymethyl cellulose (CMC) is grafted on Multiwalled Carbon Nanotubes (MWCNT) by using plasma techniques, the CMC grafted MWCNT (MWCNT-g-CMC) have larger specific surface area, which is easily dispersed in solution and has very high sorption capacity in the removal of $UO_2^{2+}$ from aqueous solution (Shao, Jiang, Wang, Li, & Meng, 2009). The specific pore sizes of the combined SA-NACMC gel beads and the SA gel beads distributed in 3–25 nm (Fig. 3b) and 120–130 nm (Fig. 4b), respectively.

### 3.4. The remediation of atrazine polluted soil

The effect of free enzyme and immobilized enzyme on soil atrazine removal efficiency in 28 days was shown in Fig. 5. In 14d, atrazine residue of the treatments was below the atrazine residue in the blank control. In 28d, atrazine residues of the immobilized enzyme treatment groups were less than 30% (atrazine residue of the immobilized enzyme treatment with 1% content was 27.06%, while atrazine residue of the immobilized enzyme with 5% content was 21.79%); by contrast, atrazine residue of the control group was up to 79.22%, and atrazine residues of the free enzyme treatment groups were above 60%.

The atrazine residues of degrading enzyme groups with different forms were significantly lower ($p < 0.05$) than the control group. At the 28d after treatment application, immobilized enzyme had a higher degradation rate; atrazine residues of free enzyme treatment groups were both above 60%. The reason might be the free enzyme had existed in the soil environment for a long time, the surrounding microorganisms or the nature of soil environment led to the change of enzyme properties, leading to the deactivation, and thus no longer able to degrade atrazine. On the other hand, the activity of immobilized enzyme still remained high, such that the degradation efficiency of atrazine was up to 70%. This was due to a decrease in protein loss during the gel bead formation in the enzyme immobilization procedure and an increase in specific surface area of the gel beads (Thi et al., 2013). The similar degradation effect had also been obtained by some researchers using immobilization (Siripattanakul, Wirojanagud, McEvoy, Casey, & Khan, 2009a; Siripattanakul, Wirojanagud, McEvoy, Casey, & Khan, 2009b) and other technology methods (Bianchi, Pirola, Ragaini, & Selli, 2006; Ghosh & Philip, 2004; Granados-Oliveros, Paez-Mozo, Ortega, Ferronato, & Chovelon, 2009; Singh, Suri, & Cameotra, 2004) for atrazine degradation. Thus, immobilized enzyme can be applicable to the remediation of atrazine contaminated soil.

## 4. Conclusions

Influences of metal ions and NACMC on the formation of microcapsules were investigated in detail. The optimization of the formation of microcapsules was SA and NACMC at ratio 1:1, and $CoCl_2$ concentration at 0.05 mol/L. The microcapsules had good characteristics and had higher degrading rate in soil remediation on the removal of atrazine (atrazine residue of the immobilized enzyme treatment with 1% content and 5% content was 27.06% and 21.79%, respectively). $Co^{2+}$ can replace the $Ca^{2+}$ ions in the traditional alginate gel and have better particle size. Meanwhile, $Co^{2+}$ exhibited the promotion of enzyme activity. The properties of microcapsule were observed by SEM, AFM and BET. When the $Co^{2+}$ concentration was 0.05 mol/L, the analyses of SEM and AFM demonstrated that compared with the SA gel beads, the combined SA-NACMC gel beads apparently had rougher surface, more porosity, multilayer internal structure and higher mechanical strength. BET analysis demonstrated that the microcapsules had larger specific surface area and appropriate pore size. It was beneficial for the embedment of enzyme.

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