PREPARATION AND BIOASSAY OF BACILLUS THURINGIENSIS MICROCAPSULES BY COMPLEX COACERVATION

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In this study, the gelatin-gum arabic microcapsules loaded *Bacillus thuringiensis* (*Bt*) were prepared by complex coacervation. Based on single factor experiment and orthogonal test, the preparation process parameters such as encapsulating temperature and pH, the concentration of getatin and gum Arabic, and rotational speed were investigated and optimized. The obtained optimum parameters of preparation were as follows: the encapsulating temperature and pH were 40℃ and 3.9, the concentration of getatin and gum arabic were 10 $g^{-1}L^{-1}$, the volume ratio of core (10^{10} cfu/L *Bt* suspension) to wall (10 $g^{-1}L^{-1}$ gelatin and Arabia gum solution) was 1:2, and stirring speed was 350 rpm. With the optimized preparation process, the size and span value of obtained *Bt* microcapsule were about 12.7 μm and 1.03, and encapsulation efficiency was up to 87%. The experiment of biological activity to grub showed that the *Bt* microcapsule had better persistent effect compared with the control, when the number of spores was 109$cfu^{-1}ml^{-1}$, the mortality of grub were 75.65% and 86.09% on the 7th and the 14th day.

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1. Introduction

Microencapsulation by coacervation is the phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media[1]. Generally, the coacervation process is divided into two types: simple coacervation and complex coacervation, which is determined by the number of polymeric ingredients [2]. Simple coacervation is the result of the interaction of a dissolved polymer with a low-molecular substance. Complex coacervation is achieved by the interaction of two oppositely charged macromolecular polymers [3]. The word ‘complex coacervation’ was first proposed by Bungenberg de Jong and Kruyt for the system of gum arabic-gelatin in order to distinguish it from the simple coacervation of a single polymer[4]. The first step in complex coacervation method is the preparation of the emulsion via dispersion of core material (usually oil) into an aqueous polymer solution. Then the deposition of shell material onto the core particles is occurred by adding the second aqueous polymer solution followed by the addition of salt or by changing the pH, temperature, or by dilution of the medium. Finally, the microcapsules are stabilized by addition of a crosslinking agent, desolvation or heat treatment [5,6] .

Bacillus thuringiensis (*Bt*) is a spore-forming Gram-positive rod-shaped bacteria that is the world's most studied and widely used microorganism insecticide for human and livestock safety without destroying the ecological balance [7-9]. It can form insecticidal activity in the process of spore forming parasporal crystal, which is composed of one or more insecticidal crystal proteins

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(ICPs) or delta-endotoxins with highly specific insecticidal activity crystal protein composition [10]. These proteins have specific activity on the larval stage of certain pests [11]. Once absorbed by insects, these crystals dissolve in the midgut and then activated by midgut proteases and binds to specific recipient of the insect cell membranes resulting in cell division and insect death [12,13]. Li Changyou isolated and screened *Bt* strains (B-Y7-1) with insecticidal activity to grub pests [14]. With high insecticidal activity and good environmental compatibility, B-Y7-1 shows a broad application prospect in the prevention of underground pest grub. However, spores and toxins are easily inactivated by solar radiation, which limits the use of conventional *Bt* as a pesticideunder field conditions [15,16]. As of now, a lot of formulations have been developed with addition of various screens, but some of the UV screens have been shown to detrimental to the environment [17-19]. To protects the spores of *Bt* from ultraviolet radiation, Ana produced microcapsules encapsulating *Bt* by spray drying with starch granules as wall material [20]. Khorramvatan et al. prepared *Bt* microcapsule formulations by emulsion technology using starch [21], gelatin and sodium alginate to protect crystalline proteins from UV radiation.

The microcapsules by complex coacervation can be divided into mononuclear microcapsules and multinuclear microcapsules according to their internal structure [22]. Mononuclear microcapsules are formed when one core particle is encapsulated by coacervates and multinuclear microcapsules are formed through aggregation among many mononuclear microcapsules. In order to prevented mononuclear microcapsules accumulating into multinuclear microcapsules, it is necessary to use special kind of wall materials [23], add the shock-preventing agent, modify the process parameters in the coacervation or change the hardening treatment process in the process of microcapsulation [3]. Microcapsules by complex coacervation have controlled release ability based on mechanical stress, temperature or sustained release. The release characteristics largely depend on the morphology, size and loading. Therefore, it is necessary to know how to control these properties in terms of the preparation conditions. In the complex coacervation stage, the positively charged gelatin molecules interact with negatively charged acacia molecules by adjusting pH to form water-insoluble complex aggregates, which are deposited on the surface of the emulsion droplets to form microcapsules wall [24].

The aim of this study was to develop a *Bt* microcapsules employing gelatin and gum arabic as wall materials by complex coacervation. The effect factors such as ration of core and wall, rotational speed, temperature and pH on the *Bt* microcapsules encapsulation efficiency (EE) and span value (SV) were examined, and the optimized conditions of preparation were obtained. Besides, the insecticidal activity of Bt microcapsule against grub were demonstrated to prove the persistence of biological activity. This work provides a promising approach of preparation of *Bt* microcapsules with low cost and simple preparation process to improve the persistence of microbial pesticide *Bt*.

2. Experimental

2.1. Materials

Gelatin (Tianjin basf chemical co., LTD.), gum arabic (Tianjin Bodi Alagh Khan chemical co., ltd) were used as natural polymers. Tween80 (Shanghai try chemical reagent co., ltd) as an emulsifier. All other reagents were local products of analytical grade.

2.2. Bacterial strains preparation

Bacillus thuringiensis strains (*Bt* serovar B-Y7-1) were given by the research center of invertebrate cell in Shandong. The *Bt* freeze-dry powder was activated in fresh sterilized Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) overnight at 30℃and was diluted (1%) and cultured in a rotary shaker (120 rpm) at 30℃ in LB medium for approximately 48 h. All *Bt* cultures were collected by centrifugation at 8,000 rpm for 15 min without washing. The cultured were stored in deionized water at 4℃before use [25].

2.3. Preparation of microcapsules

The preparation of the *Bt* microspsules were carried out according to a procedure reported by LiShan [26]. The *Bt* strains was added to a certain amount of distilled water, adding a certain amount of 20 % tween 80, placed in a magnetic stirrer for 30min, the stirring speed at low speed 300 rpm to produce a *Bt* suspension (*Bt* concentration was 10^7 cfu/mL). After the bacteria solution was evenly dispersed and then added a proper amount of gelatin solution, the temperature slowly raised to 40 ℃ and dropped 10% acetic acid solution for the pH to 4. The system was stirred for 20 min, the same amount of gum arabic solution was added to the emulsion at 400 rpm of the rotation speed. Continue to stir for 30min, the system was arranged in the ice bath, the temperature dropped to 5 to 10 ℃, adding a certain amount of curing agent glutaraldehyde (25%) solution, stirring under low temperature 20 min, and then remove the microcapsule solution for centrifugation. The microcapsule under the tube were precipitated and washed, finally obtained and dried at room temperature. The dried substance was *Bt* microcapsule powder, preserved at the temperature of 4℃. In order to optimize the optimal conditions of gelatin-Arabia gum microcapsules, single factor experiments and orthogonal experiments were carried out in succession, and the measurements of the encapsulation efficiency and particle size span value are essential.

2.4. Characterization of microcapsules

The morphology and internal structure of the microcapsule were observed through optical microscope and scanning electron microscopy (SEM) (JEOL 7500F, Japan). For optical observation, the microcapsules were dispersed in distilled water and dropped on a clean glass slide. For SEM, the dried samples were sputtered with a thin layer of gold before observed.

The particle size and size distribution of the *Bt* microcapsules were measured with a particle size analyzer (Bettersize Instrument Ltd., Bettersize2000) by dynamic light scattering (DLS) technique [27]. The microcapsules were appropriately diluted in distilled water before measurement. The average diameter was reported as the volumetric mean diameter, and the particle size distribution was evaluated by span value (SV), as defined as the following expression (1).

$$SV = \frac{D90 - D10}{D50} \tag{1}$$

where D_N% (N = 10, 50, and 90) is the volume percentage of microcapsules with diameters less than a certain value equal to N%. The smaller the SV is the narrower the particle size distribution of the microcapsules.

2.5. Determination of encapsulation efficiency of *Bt* microcapsules

A certain volume of bacterial suspension was diluted to a certain volume, and the number of bacteria (B_0) was measured by using the cell count plate under a optical microscope [28, 29]. The same volume of bacterial suspension was used to make the same volume of microcapsule solution (B_1), and the number of bacteria was measured, and the encapsulation efficiency (EE) as defined as the following expression (2).

$$EE(\%) = 1 - \frac{B_1}{B_0} \tag{2}$$

2.6. Insecticidal activity of *Bt* microcapsules on grub

Determination of the insecticidal activity against grub: The LC_{50} of the *Bt* broth, *Bt* suspension(SC) and *Bt* suspensions capsule(CS) were measured separately [30]. According to the results of the pre-test, *Bt* broth, *Bt* SC and *Bt* CS were diluted to different concentrations at a concentration of 3 times in a certain concentration range. The soil is sterilized and packed into a moisturizing box. Each moisturizing box is divided into 100 g soil and 5g soaked with different concentration of bacteria solution. The remaining solution is poured into the soil and stirred evenly.

The worms were pre treated for 5 h, 30 worms for each treatment, 3 times in parallel, and distilled water was used as control group. Fresh potato blocks were replaced every 2 days to check the number of larval survival after 7 d and 14 d respectively. The mortality rate (R1) and adjusted mortality rate (R2) were calculated as the following expression [31].

$$R1(\%) = \frac{N1}{N0} \times 100 \tag{3}$$

$$R2(\%) = \frac{R1 - R0}{1 - R0} \times 100 \tag{4}$$

N1: the number of dead insects, N0: the total number of insects, R0: the mortality rate of the control group.

3. Results and discussion

3.1. Process optimization

In order to obtain better conditions for *Bt* microcapsule preparation, the effects of pH value, temperature, ratio of core to wall, and stirring speed on the span value and encapsulation efficiency of microcapsules were investigated. The effects of various factors on the embedding rate and span value of microcapsules are shown in Fig. 1.

Fig. 1(a) illustrates the effects of the solution pH on the encapsulation efficiency and particle size of the microcapsules. Generally speaking, Arabia gum molecules are negatively charged, except for the solution of pH <3.0, the isoelectric point of gelatin is less than 4.9 [32]. To ensure the occurrence of complex coacervation, gelatin molecules must be positively charged, so it is necessary to select suitable pH regions for experiments. The results show that with the increase of pH, the embedding rate increases first and then decreases, but the span value has been reduced. Fig. 1(b) shows the influence of core material and wall material concentration on encapsulation efficiency and particle size span of microcapsules. When the core wall ratio is less than 1:2, the particle size distribution is uniform and the span value is small. With the increase of the ration of core and wall, the viscosity of microcapsules becomes more serious, and the uneven particle size makes the SV value increase significantly. Temperature has a great influence on the embedding rate and SV of *Bt* microcapsules as shown in Fig.1(c). When the temperature is low, both the embedding rate and the SV are suitable, while the temperature is too high, the gelatin hydrolysis of gelatin makes the encapsulation rate of microcapsules drop suddenly, so the temperature range of 30-50℃ is selected for further screening. Seen from the Fig. 1(d), it can be seen that the encapsulation rate of microcapsules is higher at low rotational speed, and the maximum encapsulation rate is 82.86% at 300rpm. With the increase of rotational speed, the entrapment efficiency and particle size span of microcapsules gradually decreased. Because the excessive rotation speed destroys the capsule wall formed by the complex coacervation of gelatin and gum arabic, the embedding rate is reduced. Most of the particles in the solution are unincorporated cells or capsules with smaller particle sizes, making the SV smaller.

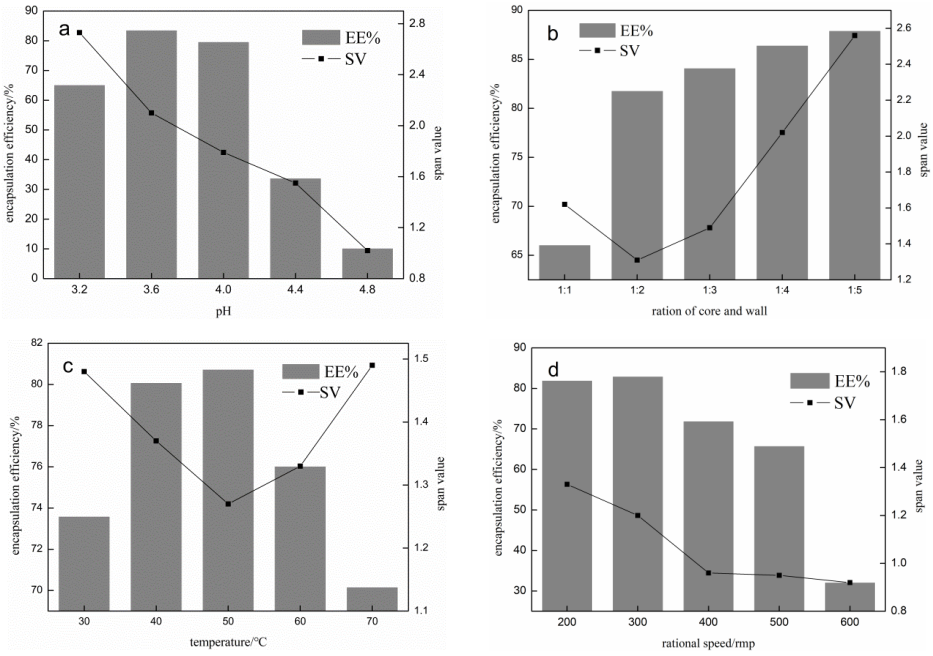

Fig. 1. Effects of variation of factors on encapsulation efficiency and particle span value of encapsulated Bt microcapsules (a: pH; b: volume ration of core and wall(10^{10} cfu/L Bt suspension:10 $g^{-1}L^{-1}$ gelatin and Arabia gum solution); c: temperature; d: rational speed)

The good levels of four factors concluded from single factor experiments are as follows: rotational speed is 300~400rpm, the volume ratio of core and wall is 1:1.5~1:2.5, pH value 3.5~3.9 and temperature is 35~45℃. To further investigate the effects of factors such as rotational speed, core wall ratio, pH, and temperature on the characteristic of microcapsules, talking encapsulation efficiency (EE) and span value (SV) as respond value, orthogonal experiment was designed with four factors and three levels, as shown in Table 1 and Table 2.

Table 1. Factors and levels of orthogonal experiment of the Bt Microcapsules.

Levels	Factor			
	A/Rotational speed (rpm)	B/pH	C/ volume ratio of core wall	D/temperature (℃)
1	300	3.5	1.0：1.5	35
2	350	3.7	1.0：2.0	40
3	400	3.9	1.0：2.5	45

Note: 'core' is 10^{10} cfu/L *Bt* suspension and 'wall' is 10 $g^{-1}L^{-1}$ gelatin and Arabia gum solution.

Table 2. The result of orthogonal experiment of the $L_9(3^4)$.

Experiment	Factor								
	A	B	C	D	EE (%)	D10	D50	D90	SV
1	1	1	1	1	77.86	7.96	15.54	31.3	1.50
2	1	2	2	2	83.05	9.11	18.16	30.24	1.16
3	1	3	3	3	81.24	9.73	19.36	37.52	1.44
4	2	1	2	3	85.78	10.12	20.88	31.06	1.00
5	2	2	3	1	82.9	8.16	17.29	29.97	1.26
6	2	3	1	2	85.21	10.59	21.36	32.7	1.04
7	3	1	3	2	80.25	8.66	13.5	28.66	1.48
8	3	2	1	3	85.31	9.85	17.32	34.11	1.40
9	3	3	2	1	85.48	7.69	14.89	29.13	1.44

The experimental results and analysis are shown in Table 3 and Table 4. Table 3 is based on microcapsules embedding rate as an inspection index, the results show that the factors affecting the microcapsule embedding rate in order of speed, core wall ratio, pH and temperature. The optimum is A2B3C2D3, that is the rotation speed 350 rpm, ratio of core and wall 1:2, pH 3.9, temperature 45℃. Tab.4 shows the particle size span of the microcapsules as an index. The results show that the factors affecting the particle size span of the microcapsules are the rotational speed, the ratio of the core to the wall, the temperature and the pH. The optimum is A2B2C2D2, that is the rotation speed 350 rpm, ratio of core and wall 1:2, pH 3.7, temperature 40℃. Two kinds of preparation conditions were verified respectively. The optical photomicrograph of *Bt* microcapsules from two processes are shown in Fig. 2.

Table 3. Range analysis of microencapsulation efficiency.

K_1	242.14	243.89	248.38	246.24
K_2	253.89	251.26	254.31	248.51
K_3	251.04	251.93	244.38	252.33
Range	11.73	8.04	9.93	6.09
Effect order	A>C>B>D			
Optimum	A2B3C2D3			

Note: K_i means the sum value of the experimental results corresponding to the level of i (i =1,2,3) under each factor.

Table 4. Range analysis of Bt microcapsule span value.

K_1	4.10	3.98	3.94	4.20
K_2	3.30	3.82	3.60	3.68
K_3	4.32	3.92	4.18	3.84
Range	1.02	0.16	0.58	0.53
Effect order	A>C>D>B			
Optimum	A2B2C2D2			

Note: K_i means the sum value of the experimental results corresponding to the level of i (i =1,2,3) under each factor.

Fig. 2(a) show the appearance of *Bt* microcapsules for conditions: rotational speed is 350 rpm, the ration of core and wall is 1:2, pH 3.9 and temperature 45℃. While Fig. 2(b) is prepared at a pH of 3.7 and a temperature of 40℃. In Fig. 2(a), the microcapsules were spherical, and the size of the particles was uniform and almost no adhesion. Compared with a, microcapsules in Fig. 2(b) adhesion was serious and the particle size distribution was uneven, which was not conducive to the feeding of insects and reduced the effect of microcapsules. And when the value of pH is too low, it is not conducive to the survival of *Bacillus thuringiensis*. Therefore, the optimal conditions are: rotational speed is 350 rpm, the ration of core and wall is 1:2, pH 3.9 and temperature are 45℃. The encapsulation efficiency (EE) and span value (SV) are 87.0% and 1.03, respectively.

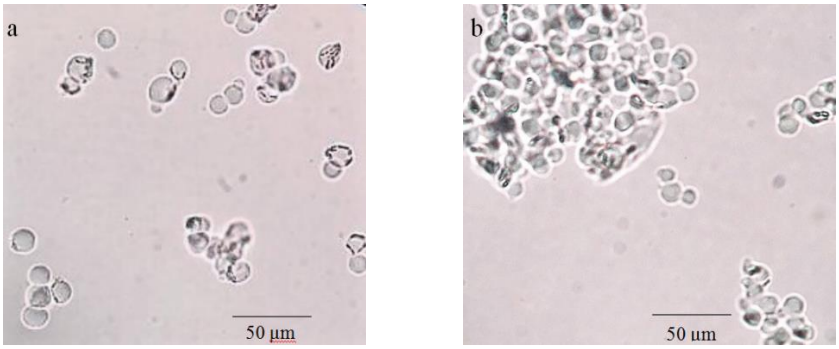

Fig. 2. Optical photomicrograph of the morphology of Bt microcapsules.

3.2. Morphology of the microcapsules

The microcapsules of *Bt* presented irregular spherical forms of a wide range of diameters, which is about 12.7 μm. Their surface appeared to be smooth and without porous or external cracking. Spherical shape deformations could have been produced as result of the loss of water content during the dried process (Fig.3). Gao et al. [28] used the same microencapsulation method and reported similar microcapsule morphology to that observed in their work, while using capsaicin as active componenta and a mixture of gelatin and carboxymethyl cellulose as wall materials.

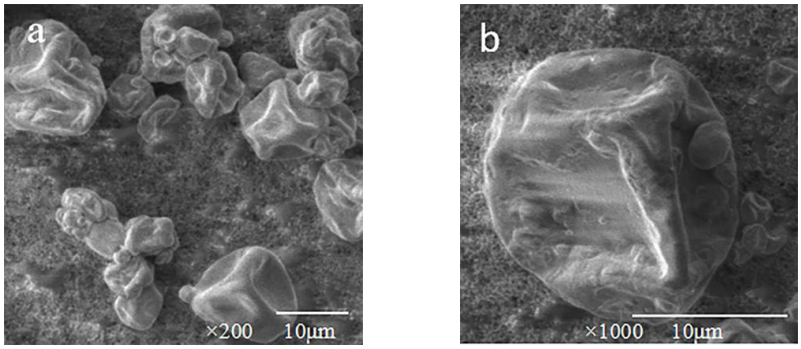

Fig. 3. SEM image of Bt microcapsules.

3.3. Insecticidal activity of *Bt* microcapsules on grub

The insecticidal activity of *Bt* broth, *Bt* SC and *Bt* microcapsules (*Bt* CS) were shown in Fig. 4. It could be seen from Fig.4 that the three *Bt* preparations have the same tendency for grub. With the concentration of the preparation increases, the adjusted mortality of the test larvae also increases. Whether from 7th day of control effect or 14th day of control effect, *Bt* CS has the best insecticidal effect, followed by the insecticidal effect of *Bt* broth, and the effect of *Bt* SC insecticide is the worst. When the number of spores was $10^9 cfu^{-1} ml^{-1}$, the adjusted mortality of *Bt* CS was 75.65% and 86.09% on the 7th and the 14th day, *Bt* broth was 67.96% and 77.03%, while *Bt* SC was only 58.10% and 66.66%, respectively. Compared with the other two formulations, *Bt.* microcapsule has obvious advantages in control pest, the wall material could be bioactive components of slow release and effectively protect the persistence of core material.

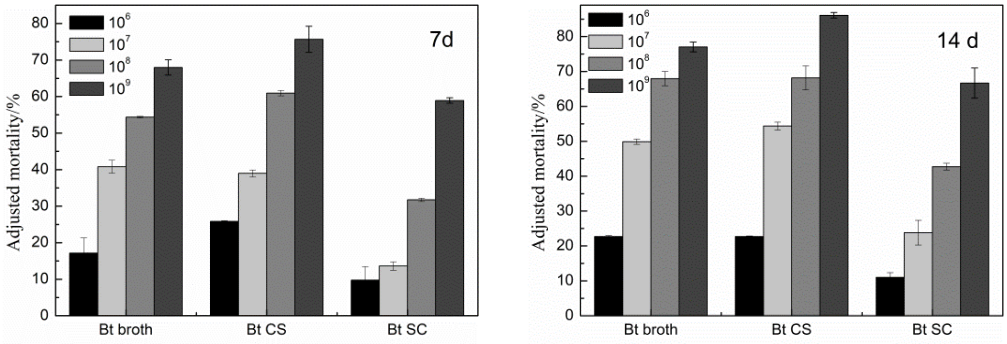

Fig. 4. Insecticidal activity of Bt on grub Error bars represent standard deviation from the mean (n = 3).

4. Conclusions

With gelatin and gum arabic as wall materials and *Bacillus thuringiensis* as core material, microcapsules were prepared by coacervation. The effect of various processing parameters, including the wall and core ration, pH value, stirring speed and temperature on the morphology, particle size distribution and microencapsulation efficiency was investigated. The optimal conditions for preparing spherical microcapsules were as follows: ratio of core and wall 2:1, pH 3.9, temperature 40℃ and stirring speed 350 rpm. The microencapsulation efficiency and span value of microcapsules was 87% and 1.03. The results of indoor bioassay showed that the *Bt* microcapsule suspension had the best insecticidal effect, followed by the insecticide effect of the *Bt* liquid, and the suspension insecticide had the worst effect, but the differences among the three were not significant.

Acknowledgments

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