Preparation of 4A zeolite modified enteromorpha biochar and its application in bacillus thuringiensis microspheres

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The *Enteromorpha prolifera* biochar and 4A zeolite-modified biochar based on different pyrolysis temperatures were prepared. The biochar of good biocompatibility with Bt was selected by studying the effects of different biochar on the survival rate and salt tolerance of *Bacillus thuringiensis* (Bt). The biochar microspheres loaded with Bt were prepared with sodium alginate, pectin, and chitosan. The effects of the addition of biochar on the particle size and ball formation rate of the microspheres were investigated. The structure of the microspheres was characterized by SEM and FTIR, and the SR of the microspheres under different pH conditions were evaluated. The results showed that 4A zeolite-modified biochar prepared at 500 ℃ (GH500) had good compatibility with Bt, and could improve the salt tolerance of Bt. The prepared microspheres have a uniform shape and particle size, and their swelling characteristics were pH-responsive, the microspheres containing biochar exhibited good swelling properties under alkaline conditions.

(Received September 21, 2023; Accepted January 4, 2024)

Keywords: *Enteromorpha prolifera*, Modified biochar, *Bacillus thuringiensis*, Microspheres, Swelling characteristics

1. Introduction

Bacillus thuringiensis (Bt) is a Gram-positive bacterium, which can produce spores and parasporal crystals [1]. Parasporal crystal is a kind of protein with insecticidal activity, also known as δ-endotoxin, which contains insecticial crystal protein (ICPs) with specific toxicity to pests and is harmless to people, animals, and the environment. The application of such biopesticides conforms to the concept of green ecological prevention and control [2-4]. However, microorganisms are easy to lose their activity in adverse natural environments, especially in saline-alkali soil, the prevention and control effect is easily lost or significantly reduced. Therefore, improving the ability of microbial agents to resist adverse environments is an urgent problem to be solved in biological control [5].

Biochar refers to the solid product of bio-organic materials (biomass) after thermal cracking in hypoxia and hypoxia environments [6]. Biochar is widely used in the fields of thermal power generation, gas and water purification, soil remediation, and saline-alkali land improvement [7-8]. Studies have shown that biochar can improve soil permeability, change pH and salinity in saline-alkali land, provide nutrients and a living environment for microorganisms, enhance the viability of biocontrol bacteria in the soil environment, and promote crop growth [9]. It is a natural biomass material with great application prospects [10-11]. *Enteromorpha prolifera* is a common macroalga in Qingdao, Shandong Province, with the characteristics of wide distribution, rapid growth, and mass reproduction, which leads to the perennial flooding of *enteromorpha prolifera*

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https://doi.org/10.15251/DJNB.2024.191.53

and obvious damage to the marine ecosystem, how to deal with enteromorpha prolifera is very important [12-14]. It is an important way to utilize *enteromorpha prolifera* resources by preparing *enteromorpha prolifera* into biochar and using its good adsorption and biocompatibility to immobilize microorganisms to achieve soil remediation and improvement [15]. As a stable adsorption material, 4A zeolite has excellent ion exchange performance and adsorption performance, and the stability of biochar can be improved by constructing a composite material with biochar [16].

Both sodium alginate and pectin are natural polymer materials and polysaccharides, which have good biocompatibility and can cross-link with calcium chloride to form a more excellent composite membrane by synergistic effect [17]. Chitosan is a deacetylated derivative of chitin, which is composed of glucosamine and N-N-acetylglucosamine units. Chitosan carried high-density positive charges can electrostatically interact with the sodium alginate carried negative charges which deposit a thin film on the polysaccharide-calcium ion gel system and improve the stabilization of gel matrix to control release [18-21].

In this study, *enteromorpha prolifera* biochar and 4A zeolite-modified *enteromorpha prolifera* biochar were prepared at different pyrolysis temperatures. the compatibility of biochar with *Bacillus thuringiensis* and its influence on its salt tolerance were further studied to determine the suitable biochar. Biochar microspheres loaded with *Bacillus thuringiensis* were prepared with sodium alginate, pectin, and chitosan. The structure of the microspheres was characterized by scanning electron microscopy, infrared spectroscopy, and thermogravimetric analysis, and their swelling characteristics at different pH conditions were evaluated. The research will provide a reference for the application of *enteromorpha prolifera* biochar in biological agents.

2. Experimental

2.1. Materials

Bacillus thuringiensis (strain B-Y7-1) was provided by the College of Botany, Qingdao Agricultural University. Chitosan (deacetylation degree ≥95%), Shanghai McLean Biochemical Technology Co., Ltd., Sodium Alginate, Tianjin Bodi Chemical Co., Ltd., 4A zeolite, AR, Taiyuan Fakaj Chemical Co., Ltd.. Other reagents chemical reagents were analytical grade and could be commercially available. *Enteromorpha prolifera* was collected from Qingdao, Shandong Province.

2.2. Methods

2.2.1. Preparation of enteromorpha biochar and modified biochar

(1) Preparation of biochar: the washed *enteromorpha prolifera* was dried in an oven at 80℃ for 24 h, crush it with a high-speed pulverizer and seal the dried *enteromorpha prolifera* for later use after passing through a 100 mesh sieve. An appropriate amount of enteromorpha granules was put into a pyrolysis reactor and placed in a muffle furnace, heated to 500℃, 600℃ and 700℃ at a rate of 10 ℃/min, and then maintained for 2h. The prepared biochar products were stored in sealed bag, and were labeled as H500, H600, and H700 respectively according to the temperature.

(2) Preparation of zeolite-modified biochar: 4 g 4A zeolite and 20 g *enteromorpha prolifera* were put in 1000 mL distilled water to fully stir for 3h, then filtered to obtain a mixture of zeolite and biochar. According to the method of 2.2.1 to prepare modified biochar with pyrolysis temperature of 500℃, 600℃ and 700℃. The modified enteromorpha biochar was labeled as GH500, GH600, and GH700 respectively.

2.2.3. Characterization of biochar performance

With the method of reference [22], the basic physical and chemical indexes such as pH, yield, and ash were determined. Fourier transform infrared spectrometer (FTIR) was used to analyze the functional groups on the surface of biochar, and a scanning electron microscope (SEM) was used to observe the morphology and structure of biochar.

2.2.4. Biocompatibility between biochar and Bt

The mixed LB culture solutions were prepared by maintaining the mass concentration of biochar at 0.1%, 0.5%, 1%, and 1.5%. LB culture solutions were poured into a sterile Petri dish after sterilization. When the LB culture medium cooled and solidified, covered 100 uL of Bt

bacterial suspension with appropriate concentration on flat plate, cultured in an incubator at 30℃ for 24 h, and counted the number of colonies. Each treatment was repeated in 3 groups.

2.2.5. Effect of biochar on salt tolerance of Bt

(1) Salt tolerance of Bt: Firstly, the culture media with a salt content of 1%, 3%, and 5% (based on the mass and volume concentration of sodium chloride) were prepared, inoculated with Bt bacteria (streaking method), and inverted cultured in an incubator at 30℃ for 24 hours to observe the growth of Bt.

(2) Effect of biochar on the salt tolerance of Bt: the salt-containing medium was added different kinds of biochar with the mass-volume ratio of 0.1%, 0.5%, 1.0%, and 1.5%, inoculated Bt bacteria (streaking method), and cultured in an incubator at 30℃ for 24 hours to observe the growth of Bt.

2.2.6. Preparation of Bt microspheres

By measuring the compatibility of different kinds of biochars with Bt and their effects on their salt tolerance, the optimum kinds of biochars were determined, and the gel microspheres loaded with Bt were further prepared. The specific preparation method was as follows:

(1) Sodium alginate/pectin microspheres: 2% sodium alginate solution and 2% pectin solution were mixed with the volume ratio of 4:1, then added 10 mL of Bt suspension (bacterial content: 1.0×10^8cfu/ml), stirred at a constant speed for 30 min to ensure uniform dispersion of Bt, and slowly dropped the mixed solution into 100 mL of 4% (w/v) calcium chloride solution with a syringe. The initial microspheres were filtered and washed with sterile water three times. The microspheres were placed in 2% (w/v) chitosan solution (chitosan solution was prepared with 1% acetic acid solution), stirred at low speed for 2 hours to solidify the microspheres, then filtered and separated the microspheres, washed with sterile water three times. Finally, the microspheres were dried in an oven at 60℃ for 4 hours to obtain Bt composite microspheres, labeled as SA/CJ -MC, and sealed at 4℃.

(2) Sodium alginate/pectin/biochar microspheres: Weighed 0.3 g of biochar and 10 mL of bacterial liquid, put it on a shaking table (180 rpm, 30℃) to shake and absorb for 30 min, a certain amount of mixed liquid of sodium alginate and pectin were poured into the above suspension of bacteria and biochar, fully stirred to obtain mix suspension, and prepared microspheres according to step 2.6.1, marked as SA/CJ/BC-MC.

2.2.7. Effect of biochar on granulation rate and particle size of microspheres

Refer to 2.6 for the preparation of microspheres, and calculate the granulation rate and average particle size according to the following formula and method.

Granulation rate % = (mass of microspheres after drying/mass of solid matter in solution) ×100%

Average particle size: Select 10 complete microspheres and arrange them neatly, measure the particle size of microspheres with a ruler and calculate the average value.

2.2.8. Structural characterization of Bt microspheres

The appearance and structure of the microspheres were observed by scanning electron microscope (SEM). The functional groups of different Bt microspheres were analyzed by Fourier transform infrared spectrometer. The thermal stability of Bt microspheres in the range of 30~700℃ was measured by the synchronous thermal analyzer.

2.2.9. Swelling properties of microspheres

The swelling properties of microspheres were investigated by setting different pH values. Experimental reference [23] method: Weighed a certain mass of microspheres and put them in PBS buffer solutions with different pH (set to 5, 7, and 9). Take out the microspheres every 30 minutes, absorb the surface moisture with filter paper, weigh them quickly, and calculated the swelling rate according to the following formula:

Swelling rate% = [(mass of microspheres after swelling-mass of microspheres before swelling)/mass of microspheres before swelling] ×100%

3. Results and discussion

3.1. Physical and chemical properties of biochar

Table 1 shows the yield, ash content, and pH value of different biochars. It can be seen from Table 1 that the yield of modified enteromorpha biochar generally decreased with the increase of temperature, and the yield of modified enteromorpha biochar remained at a relatively stable level of 27%~28%, while the yield of unmodified biochar decreased significantly with the increase of temperature, from 29.46% at 500℃ to 20.44% at 700℃, this may be due to the thermal stability of 4A zeolite in the modified biochar, thereby maintaining a constant weight of the modified enteromorpha biochar. The ash content of biochar increases with the increase of preparation temperature, as the higher the preparation temperature, the more residual inorganic substances in biochar, resulting in the ash content also increaseing [24]. However, under the same preparation temperature, the ash content of modified biochar is lower than that of unmodified biochar, which may be because volatile substances gradually escape from the surface and inner part of biomass organs under high-temperature pyrolysis [25], while the adsorption of zeolite on the surface of enteromorpha caused a relatively small amount of volatile substances to escape, which leads to more combustible burning products during re-burning, resulting in lower ash content. All biochars are alkaline, and the addition of 4A zeolite is not obvious influence on the pH value. The pH has a lettle increase with the increase of temperature, because the content of basic functional groups in biochar increases with the increase of temperature, and the alkaline substances of biochar will be produced at higher temperatures [26-27].

Table 1 Physical and chemical properties of biochar.

Physicochemical property / Biochar	Yield (%)	Ash (%)	pH
H500	29.46	41.86	10.1
H600	26.54	54.01	10.3
H700	20.44	55.98	10.4
GH500	28.11	39.90	10.0
GH600	27.36	53.60	10.1
GH700	27.36	55.30	10.3

Fig. 1 is the FTIR of 4A zeolite and enteromorpha biochar, the peak at 3444 cm^{-1} is the characteristic peak of phenolic hydroxyl and alcoholic hydroxyl. With the increase of pyrolysis temperature, the intensity of the - OH peak of biochar decreases and redshifts, mainly due to the separation of a large number of water molecules and the break of the bound of - OH to - H [6].

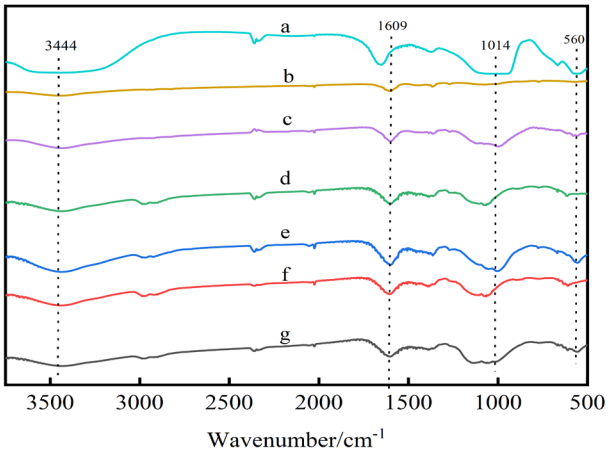

Fig. 1. Infrared characterization of Enteromorpha biochar; a: 4A zeolite, b: H500, c: GH500, d: H600, e: GH600, f: H700, g: GH700.

The peak at 1609 cm^{-1} attributed to the C = C stretching vibration and asymmetric -COOH stretching vibration corresponding to the aromatic C - C bond, which contains nitrogen or oxygen for biochar's rich aromatic ring structure [27-29].

The peak at 560 cm^{-1} in the spectra of 4A zeolite is O-Si(Al)-O, which also exists in modified biochar, indicating that zeolite is successfully loaded on biochar. The stretching vibration of the C-O-C glycosidic bond is at 1100 cm^{-1} [30], while the peak intensity of modified biochar will be weakened and shifted to the right, indicating that a more stable organic complex may be formed in biochar after zeolite modification [31]. The modified biochar with 4A zeolite not only has the same absorption peak as unmodified biochar, but also significantly enhances biochar absorption intensity, showing that the addition of 4A zeolite can enrich the functional groups of biochar.

3.2. SEM morphology of biochar

Fig. 2 shows the SEM morphology of H500 and GH500. It can be seen that the surface of enteromorpha biochar presents a porous lotus-like structure, and the pore arrangement is relatively regular [32], which can improve the adsorption capacity of enteromorpha biochar and the efficiency of carrying bacteria. On the other hand, 4A zeolite particles can be observed in the SEM image of the modified enteromorpha biochar, indicating that 4A zeolite is successfully loaded on enteromorpha biochar. 4A zeolite particles with special pore structure protrude on the surface of biochar which is beneficial to improve adsorption capacity.

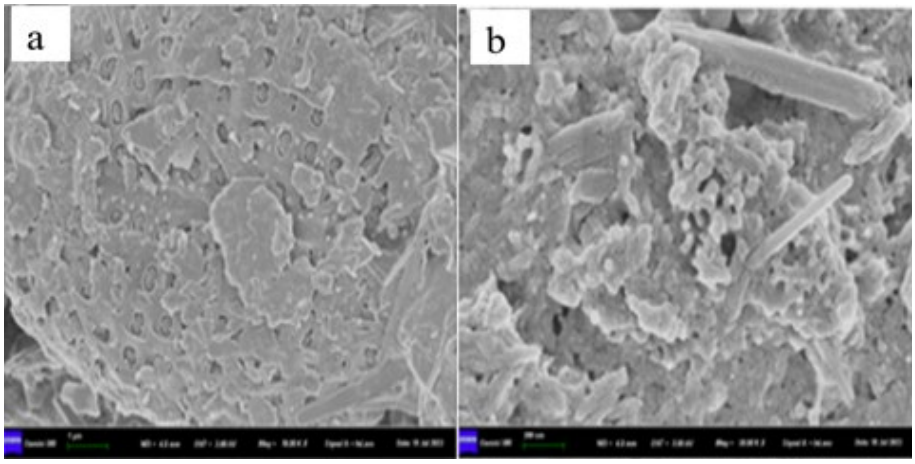

Fig. 2. Scanning electron microscopy images of biochar (a:H500; b:GH500).

3.3. Compatibility of biochar with Bt

To evaluate the compatibility of biochar with Bt, the effects of different biochars on the growth of Bt was determined (Fig 3). It can be seen from fig.3 that the compatibility of modified enteromorpha biochars with Bt were better than unmodified biochar, and the growth of Bt was the best when the amount of GH500 was 0.5%, and the number of CFU is significantly higher than other treatment groups. However, biochars prepared at high temperatures had higher inhibition rate on the growth of Bt, because *enteromorpha prolifera* contains rich polysaccharides that could produce tar during pyrolysis at high temperatures, and tar would gradually generate polycyclic aromatic hydrocarbons such as naphthalene and anthracene, those chemicals were not conducive to the survival of bacteria. From the analysis of the additional amount of biochar and the growth of Bt, with the increase of the additional amount, some biochar showed the trend of first promoting and then inhibiting, but the overall trend was not obvious, mainly because of the difference of components in prepared biochars at different temperatures, which leads to different effects on Bt. However, biochar prepared at high temperature, such as *enteromorpha prolifera* biochar prepared at 700℃, showed higher inhibition with the increase of dosage, indicating that metal ions in biochar would precipitate at high temperatures leading to its inhibition of Bt[35].

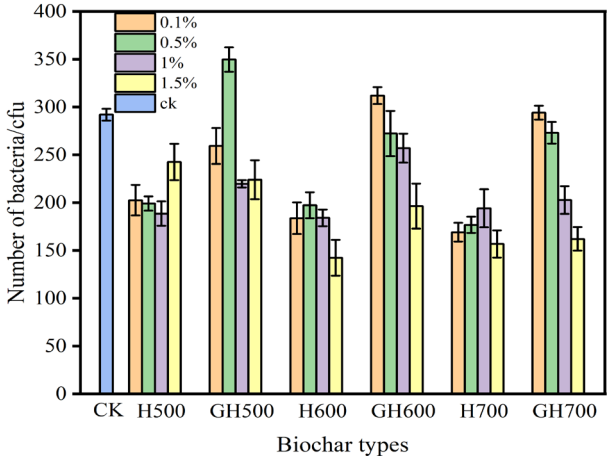

Fig. 3. Compatibility between moss biochar and Bacillus thuringiensis.

3.4. Effect of biochar on salt tolerance of Bt

The biggest problem of bacteria in practical application is environmental stress. The high salt content in saline-alkali land makes it difficult for microorganisms to survive and proliferate, thus affecting the application of biological agents in saline-alkali land [36]. As can be seen from Fig. 4 a, with the increase of salt content, the growth of Bt decreased, and it was inhibited under 3% salt content, it could not grow when the salt content was increased to 5%, so this experiment chooses 3% salt content for subsequent experiments. Fig. 4 b shows the effect of different biochars on the salt tolerance of Bt. It can be seen that Bt could grow normally on a 3% saline medium by adding 0.1% biochar, the growth of Bt is inhibited to some extent with the increase of biochar addition. When the addition of H500 and GH500 increased to 1.5%, Bt could still grow normally. However, when biochar is prepared at high temperatures, the nutrients are easily digested which is not conducive to the growth of Bt. On the whole, the modified biochar prepared at low temperatures was more suitable for Bt and could effectively improve the salt tolerance of bacteria, which has reference significance for the use of microbial pesticides in saline-alkali land.

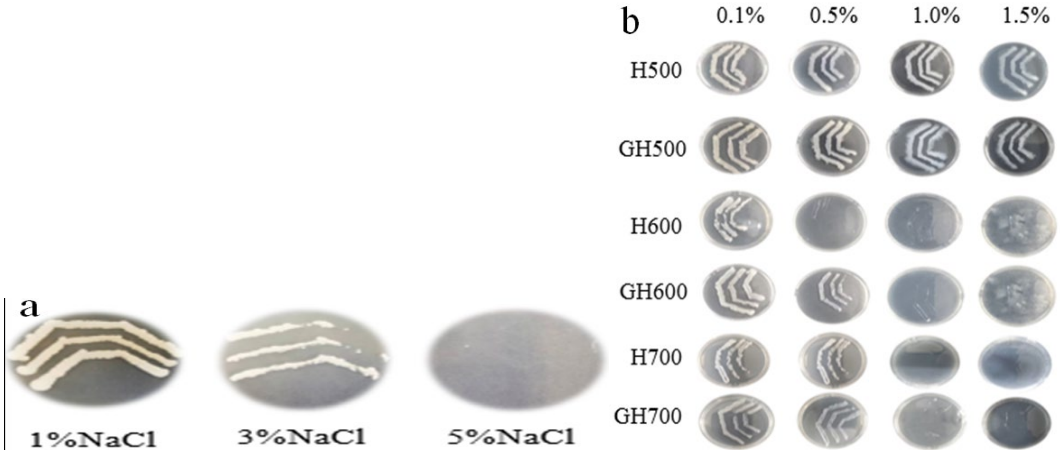

Fig. 4. The effect of different biochar on the salt tolerance of Bt(a: Salt resistance of Bt; b: Effect of biochar on salt resistance of Bt).

3.5. Appearance and morphology of microspheres

By measuring the effects of different biochar on the survival rate and salt tolerance of Bt, suitable biochar was selected. Based on this, the biochar microspheres loading Bt were prepared using sodium alginate, pectin, and chitosan as natural carriers. Figs. 5 a and b show the undried

morphology of SA/CJ-MC and SA/CJ/BC-MC, it can be seen from the figures that the microspheres without biochar were transparent and had a relatively regular spherical shape, while the microspheres with biochar were dark in color, but their appearance was still relatively regular. Figs. 5 c, d, e, and f are SEM surface and cross-sectional views of two kinds of microspheres. It can be seen that the surface wrinkles of SA /BC-MC are more obvious after adding biochar, which enhances the roughness of the surface of microspheres. The cross-section of SA /BC-MC was rougher than SA-MC, indicating the presence of biochar. Moreover, the existence of biochar improves the internal structure of microspheres and is more conducive to the fixation and adsorption of Bt.

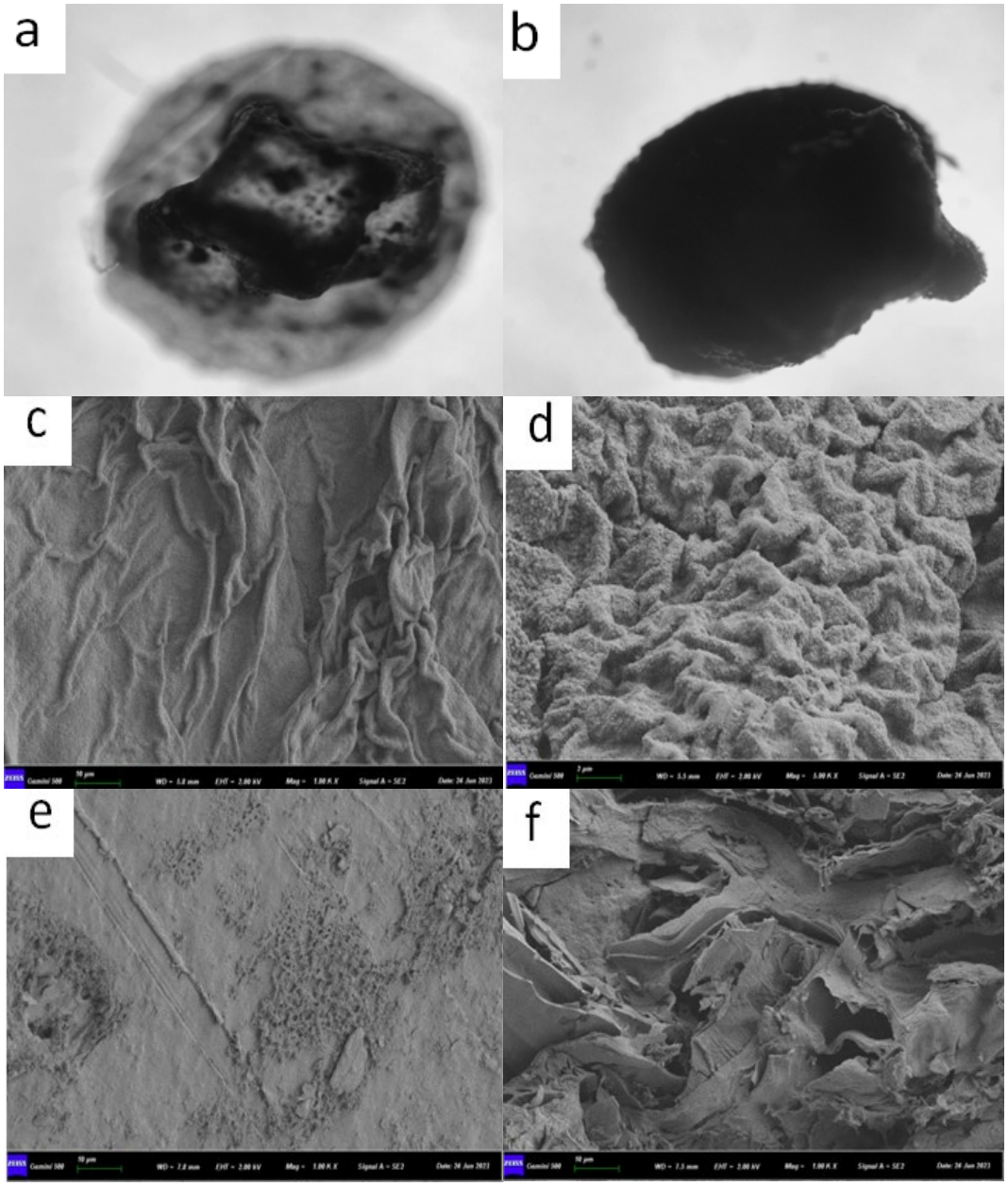

Fig. 5. Appearance and cross-sectional structure of microspheres (a,c,e: SA/CJ-MC ; b,d,f: SA/CJ/BC-MC).

3.6. Effect of biochar on spheronization rate and particle size of microspheres

The granulation rate and particle size are the basic properties of microspheres. The influence of adding biochar on the spheronization rate and particle size of microspheres is shown

in Fig. 6. It can be seen that after adding biochar, the granulation rate of microspheres increased and the particle size decreased, which shows that adding biochar could reduce the loss of materials and improve the spheronization rate of microspheres.

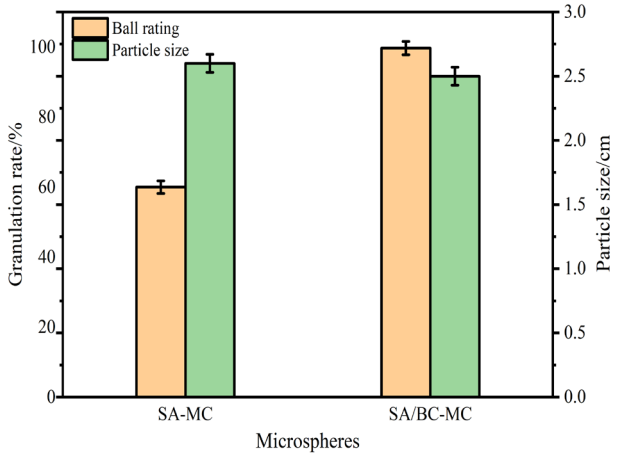

Fig. 6 .Granulation rate and particle size of different microspheres.

3.7. Infrared and thermogravimetric analysis of different microspheres

Fig. 7 is an infrared and thermogravimetric spectrum of the prepared microspheres. Fig.7a shows the infrared spectra of different microspheres. According to the literature analysis [37], the absorption peak at 3444 cm^{-1} is -OH, the absorption peak at 1739 cm^{-1} is -C=O, and the peak at 1622 $cm^{-1}$is -COOH, which shows that microspheres and biochar are rich in oxygen-containing functional groups. The peak at 560 cm^{-1} is the O-Si (Al) - O of 4A zeolite, indicating the presence of modified biochar in SA/CJ/BC-MC. The peak at 1025cm^{-1} is -C-O in polysaccharides, which verifies the polysaccharide characteristics of sodium alginate microspheres. The peak at 1609 cm^{-1} is -C=C, indicating biochar exists in SA/BC-MC. The existence of aromatic carbon can provide π –electron to shift the peak of microspheres to the right at 1622 cm^{-1}, indicating the carboxyl group carried by polysaccharide chelates with Ca^{2+} to generate more stable substances [38-39].

According to the thermogravimetric analysis of microspheres in Fig. 7 b, the degradation of microspheres and bacteria can be divided into two stages. In the first stage, the mass loss rate of bacteria was 15%, the mass loss rate of SA-MC was 13%, and the mass loss rate of SA /BC-MC was 25%. This stage was mainly the evaporation of water, and SA /BC-MC lost more water than other samples. The material was mainly degraded within the range of 250℃~700℃, and the mass loss rate of bacteria, SA-MC and SA /BC-MC was 65%, 54%, and 30%, respectively. This showed that the thermal stability of microspheres was better than that of bacteria, and the thermal stability of microspheres with biochar was significantly improved.

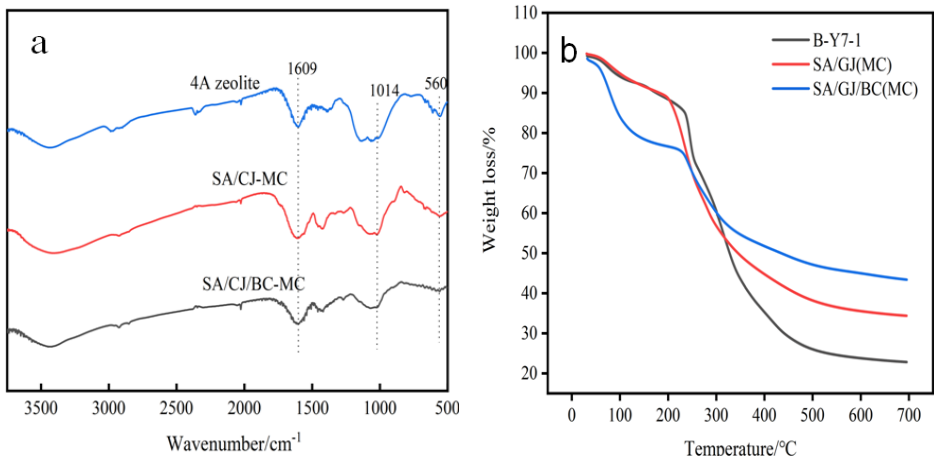

Fig. 7. Infrared spectrum and thermogravimetric analysis of different microspheres.

3.8. Swelling properties of different microspheres

Fig. 8 shows the swelling rate of SA/CJ-MC and SA /CJ/BC-MC at different pH (5, 7, 9). The swelling rate of SA/CJ-MC and SA /CJ/BC-MC were faster at pH 9, and the swelling rate of both microspheres was the highest at 180 min, but the swelling rate was relatively low at pH 5 and 7. The main components of two microspheres are polysaccharide substances such as sodium alginate and pectin, which can form alkaline sensitive gel with calcium ions causing the microspheres to swell and break easily under alkaline conditions. SA /BC-MC reached the swelling peak first due to the alkaline properties of biochar at a pH of 9. Overall, microspheres have the characteristics of pH response.

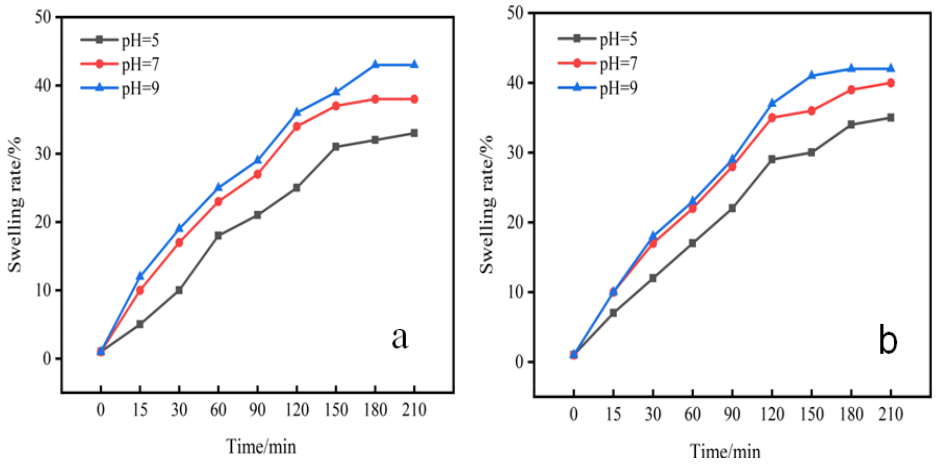

Fig. 8. Swelling properties of different microspheres
(a: swelling ratio of SA -MC; B: swelling ratio of SA/BC-MC).

4. Conclusion

In this study, enteromorpha biochar modified by 4A zeolite was prepared at different temperatures. By measuring the influence of biochars on the survival rate and salt tolerance of *Bacillus thuringiensis*, it was determined that the biochar with good compatibility with Bt was GH500 biochar, and the optimal addition mass ratio was 0.5%. On this basis, sodium alginate/pectin/biochar microspheres loaded with Bt were further prepared. It was found that the addition of modified biochar improved the granulation rate of microspheres, the prepared microspheres had relatively small particle size and regular morphology, and the internal porosity of microspheres increased, which was beneficial to the fixation and proliferation of Bt. Moreover, the microspheres have good thermal stability and swelling performance, and the swelling performance shows pH responsiveness, the swelling rate was accelerated under alkaline conditions.

Acknowledgments

This work was supported by the Innovation Improvement Project of Scientific and Technological Minor Enterprises of Shandong Province (2022TSGC1311, 2021TSGC1249), the Key R&D Plan of Shandong Province (2022CXGC020709), Ningxia Province Natural Science Foundation (2022AAC03638), and Qingdao Science and Technology Demonstration Project for Agriculture Inovation (22-3-7-xdny-6-nsh).

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