Structure and Stability Analysis of Antibacterial Substances Produced by Selenium Enriched *Bacillus Cereus*

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Abstract

Microorganisms can produce many antibiotics against bacteria and fungi, which have been used as potential choice of new antibiotics. In this paper, *Bacillus cereus*, was used to study the properties of antibacterial substance in its fermentation supernatant. The results showed that the source of antibacterial activity of *Bacillus cereus* was mainly the antibacterial substance produced by acid precipitation method, and further characterization speculates that the antibacterial substance may be lipopeptide substance. Then the antibacterial spectrum of the antibacterial substance was investigated, which showed that the antibacterial substance only had good inhibitory effect on Gram-positive bacteria and fungi, and selenium enrichment could significantly enhance the antibacterial activity of lipopeptide antibacterial substance produced by *Bacillus cereus*, and the inhibition mode of antibacterial substance to indicator bacteria was determined. The effects of different treatment methods on the stability of antibacterial substance were studied and the results showed that the antibacterial substance were stable to heat, ultrasonic and ultraviolet treatment, and their antibacterial activity would not be greatly affected. However, they were sensitive to pepsin. The optimum pH range of antibacterial activity was 3-5. This study may contribute to reuse the fermentation supernatant often discarded in the previous fermentation process. At the same time, the lipopeptide antibacterial substance extracted from the fermentation broth of selenium enriched *Bacillus cereus* can be used in the development of antibiotics and biopesticides, and open up a new way for the control of plant diseases.

1. Introduction

Since the discovery of penicillin in 1928, antibiotics have been considered as the only effective way to inhibit microorganisms. But the researchers still have not given up the search for new and more effective antibiotics (Guy and Mallampalli, 2008). At present, although the research of antibiotics has made a great breakthrough, the use of antibiotics has been close to saturation. Due to the shortcomings of some antibiotics and the inappropriate use of antibiotics by manufacturers, microbial resistance to antibiotics has been enhanced, thus limiting the use of antibiotics (Blomberg, 2008). Based on the above facts, while reducing microbial resistance to antibiotics, the demand for new antibiotics is also increasing, and natural antibiotics are the potential choice of new antibiotics. Microorganisms can produce many natural antibacterial substance to inhibit bacteria and fungi, and are also an important source of antibiotics for various diseases (Thakur et al., 2007). Therefore, the production of natural antibiotics by microorganisms is of great significance.

Members of bacillus are facultative anaerobic or oxygen aerobic Gram-positive bacteria that can produce a variety of lipopeptide antibiotics (Liang et al., 2017), regulate the intestinal microecological balance, promote intestinal development and nutrient absorption, and is one of the widely used strains (Du et al., 2017). At present, it has been known that *Bacillus subtilis*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus thuringiensis*, *Bacillus circulans* and *Bacillus brevis* can produce a variety of antimicrobial substance including lipopeptides, peptides and bacteriocins (Gordon et al., 1983). *Bacillus cereus* is a facultative anaerobic Gram-positive bacillus, which widely exists in soil

and other environments, it can secrete a variety of secondary metabolites against bacterial and fungal, including lipopeptide antibiotics synthesized by non ribosomal peptide synthetase (NRPS). Antimicrobial substance produced by microorganisms have the advantages of high safety, less side effects and less drug resistance compared with chemical synthetic antibiotics (Fengying Li et al., 2016a).

Previous studies have shown that inorganic selenium can be converted into organic selenium to produce selenium enriched strains by using microbial strains (Wang and Fang, 2011). Many strains have the effect of inhibiting intestinal harmful bacteria, and many studies have shown that this effect can be significantly improved after selenium enrichment. On the one hand, selenium can reduce the microbial activity, on the other hand, it can improve the antibacterial properties of the fermentation products. Generally speaking, the antibacterial activity of selenium enriched strains is affected by the corresponding strain carriers. At present, the research on selenium enriched strains is more about lactic acid bacteria and yeast, but less about *Bacillus cereus*. Lipopeptide antibacterial substance is one of the main antibacterial substance produced by *Bacillus*, which has been considered to be an important reason for its ability to inhibit plant diseases as a biological pesticide (Hsieh et al., 2008). It is a cyclic peptide composed of fatty acids and peptide chains, and has good preventive effect on *Aspergillus flavus* (Moyne et al., 2001), scab (Xiang et al., 2015), Fusarium wilt (Xiang et al., 2016) and many plant diseases. Zhang and others optimized the culture medium and fermentation conditions of a *Bacillus subtilis* strain producing lipopeptide antibacterial substance, and successfully increased the antibacterial potency of the antibacterial substance by 3.51 times, and achieved the effect of high yield, low cost and short fermentation time (Zhiyan Zhang et al., 2019). Jiang et al. studied the lipopeptide antibacterial substance produced by rapeseed endophytic bacterium and the results showed that the extracellular secretory substance contained peptide bond and had ring structure, then the antibacterial substance was identified as surfactin of cycle lipopeptide antibiotics by high performance liquid chromatography and mass spectrometry (Jiang et al., 2010).

The purpose of this study was to investigate the properties of antibacterial substance in the fermentation supernatant of *Bacillus cereus*. The antibacterial substance of protein and lipopeptide were extracted from the fermentation supernatant of *Bacillus cereus* by ammonium sulfate precipitation method and acid precipitation method respectively, and their antibacterial activities were compared to determine the main source of antibacterial activity of *Bacillus cereus*. The antibacterial substance was preliminarily analyzed by biuret method and ninhydrin method, and further analyzed by Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy and matrix assisted laser desorption time of flight mass spectrometry. And then the action mode of antibacterial substance on indicator bacteria was studied, and the effects of different treatment methods on the stability of antibacterial substance were compared.

2. Material And Methods

Strain material

The experimental *Bacillus cereus* strain were isolated from soil that was taken from Enshi (China) and obtained by Se-Run Health Tech Development Co., Ltd in Enshi (China). The strain was collected at -80 °C and inoculated into Luria-Bertani (LB) agar medium plates, activated at 37 °C for 6 h, and then single colonies were selected from it and inoculated into liquid LB medium. The strain seed liquid was obtained by culture at 150rpm on a shaking incubator HZ-150L(Ruihua Instrument & Equipment, China) with a rotational radius of 20 mm(same rotational radius below) for 12 h, and the concentration is 3×10^7 cfu/mL.

Chemicals and reagents

Analytical grade sodium selenite, analytical grade hydrochloric acid, sodium chloride, copper sulphate, ammonium sulphate, methanol, and ninhydrin were purchased from Sinopharm Chemical Reagent Co.,Ltd (Shanghai, China). Guaranteed potassium bromide was purchased from Aladdin Biochemical Technology Co.,Ltd(Shanghai, China). Tryptone was purchased from OXOID (Hants, UK). Yeast Extract was purchased from Aoboxing Biotechnology (Beijing, China). Agar was purchased from Biofroxx (Germany). Pepsin was purchased from Solarbio (Beijing, China).

Isolation and characterization of antimicrobial substance from *Bacillus cereus* supernatant

Identification of the source of bacteriostasis in the supernatant of *Bacillus cereus*

The antibacterial activity of antibacterial substance solution produced by *Bacillus cereus* was determined by Oxford cup method (Zhengyu Fan et al., 2018). The strain seed liquid was inoculated into 2L liquid LB medium with 7%(v/v) inoculation amount, and selenium enriched group and non selenium enriched group were set up. The selenium enriched group was cultivated at 33°C and 170 rpm for 4 h, and then added with 10 mg/L sodium selenite solution to make the concentration of sodium selenite reach 150µg/mL, and then continue to cultivate for 24 h. The non selenium enriched group was cultivated at 33°C and 170 rpm for 24 h. Then the bacterium solution was centrifuged at 4000 rpm for 25 min to collect the supernatant, and then sterilized by 0.22µm membrane. Finally, the supernatant was concentrated 10 times by rotary evaporator to obtain the supernatant containing antibacterial substance of *Bacillus cereus*, and then the antibacterial substance were prepared by ammonium sulfate precipitation and acid precipitation respectively. The detailed methods are as follows (Hongxiao Li et al., 2016b):

1. Extraction of antimicrobial substance from *Bacillus cereus* by ammonium sulfate precipitation

62.6g ammonium sulfate was ground into powder, and then added into 200mL supernatant containing antibacterial substance at room temperature while stirring, so that the ammonium sulfate saturation in the solution was 50%, then the mixed solution was placed at room temperature for 6h to precipitate the antibacterial substance. After that, the solution was centrifuged at 4 °C and 6000 rpm for 30 min. The precipitates were freeze-dried and stored for use.

2. Extraction of antimicrobial substance from *Bacillus cereus* by acid precipitation

The supernatant containing antibacterial substance was adjusted to pH 2.0 with 6 mol/L HCl and precipitated overnight at 4 °C, and then centrifuged at 4 °C for 30 min at 6000 rpm. After discarding the supernatant, methanol was added into the precipitate and stirring for 2 h, then the solution was centrifuged at 4 °C and 6000 rpm for 20 min, and the methanol solution at the upper layer was collected. Adding 1mol/L NaOH to adjust the pH to 7.0, then rotating evaporation at 45 °C to remove methanol, freeze-drying the remaining aqueous solution to obtain antibacterial material powder for use.

The antibacterial activity of the two antibacterial substance solution was compared with the initial supernatant to explore and separate the antibacterial substance which play a major role in the supernatant.

Preliminary analysis of antimicrobial substance produced by *Bacillus cereus*

The antibacterial substance was preliminarily inferred by biuret method and ninhydrin method, and the detailed methods are as follows (Chen, 2008):

1. Biuret reaction of antibacterial substance

The antibacterial substance solution and control solution were added to the two test tubes respectively. Add 10 drops of 10% sodium hydroxide and 2 drops of 1% copper sulfate into each test tube, and observe the color change of the solution after full mixing.

2. Ninhydrin reaction of antibacterial substance

Two tubes were added with antibacterial solution, another tube was added with control solution, and then ninhydrin reagent was added to each tube and the color change of solution was observed. Then put a tube of antibacterial solution into a boiling water bath and observe the color change of the solution again.

Analysis of antimicrobial substance produced by *Bacillus cereus*

Infrared spectrum analysis of antibacterial substance

Appropriate amount of antibacterial substance samples were mixed with potassium bromide powder evenly at room temperature, and then the mixed powder was extruded into flakes through a tablet press. The sample pieces were put into an infrared spectrometer Nicolet NEXUS670(Thermo Nicolet Corporation, US) for infrared spectrum scanning, with the scanning range of 4000-400cm⁻¹.

Analysis of antibacterial substance by time of flight mass spectrometry

The molecular weight of lipopeptide antimicrobial substance produced by *Bacillus* is generally in the range of 900 – 2000 (Youzhou Liu et al., 2017). The sample plate was dripped with 1 µL methanol solution of antibacterial substance and covered with 1 µL α-Cyano-4-hydroxycinnamic acid matrix working solution. After the plate was naturally dried, time of flight mass spectrometry AB SCIEX MALDI-TOF/TOF 5800(AB Sciex Pte. Ltd., US) was used for high-resolution positive ion detection under 337 nm excitation light source.

NMR analysis of antimicrobial substance

20 mg antibacterial substance sample was accurately weighed and dissolved in deuterated methanol, and determined by nuclear magnetic resonance BRUKER AVANCE 400(Bruker Corporation, Germany) with 16 scanning times.

Determination of antibacterial spectrum

Staphylococcus aureus, *Micrococcus gambiae*, *Escherichia coli*, *Salmonella*, yeast and *Aspergillus niger* were inoculated into LB liquid medium respectively until the colony total was approximately the same, and then 100 µL bacterial liquid was spread on LB solid medium, and Oxford cup was placed on the medium, the antibacterial substance solution obtained from selenium enriched and non selenium enriched culture was added into the Oxford cup respectively. Then the diameter of inhibition zone was determined after 24 h incubation at 37 °C to obtain the antibacterial spectrum of *Bacillus cereus*.

Study on the antibacterial action mode of antibacterial substance produced by *Bacillus cereus*

A small agar block was cut from the bacteriostatic area of the plate with inhibition zone, and put it into 10 mL liquid LB medium. Then the agar block was broken into pieces by Vortex oscillator(IKA, Germany) and was cultivated at 37°C and 150 rpm for 24 h. The initial and final OD_{600nm} of bacterial liquid were determined, and the viable bacteria were counted (Hao et al., 2006).

Add 10 mL LB liquid medium into two tubes respectively, and add bacteriostatic substance solution into one tube and control solution into the other tube. After that, the activated indicator bacteria solution was inoculated into it and cultured at 37 °C for 3 h, then the supernatant was discarded and the precipitates of two groups of bacteria were collected after centrifugation. The precipitates of two groups of bacteria were collected and washed repeatedly with sterile water before freeze-drying. The two groups of bacteria powder were placed under scanning electron microscope for microscopic morphology observation.

Stability analysis of antibacterial substance produced by *Bacillus cereus*

1 mL of antibacterial substance solution produced by *Bacillus cereus* cultured in selenium enriched and non selenium enriched medium were respectively put into test tube, then treated at 40 °C, 60 °C, 80 °C and 100 °C for 30 min for thermal stability analysis, and 20, 30, 40, and 50 min with ultrasonic (100 W) treatment for ultrasonic stability, the ultraviolet stability was analyzed by exposure the antibacterial substance solution to ultraviolet lamp (40 W) for 20, 30, 40 and 50 min, and the pH value of antibacterial substance solution was adjusted to 3, 5, 7, 9, and 11 with 0.1mol/L NaOH or 0.1mol/L HCl, then the pH value was returned to the neutral after 24 h of treatment at 37°C for acidic and alkaline stability analysis. The stability of antibacterial substance solution under the action of pepsin was analyzed by adding 1 mg/mL pepsin to the supernatant at 37 °C and pH 7.0 for 6 h.

100 µL of indicator bacteria liquid was spread on LB solid medium, and aseptic Oxford cup was placed in medium. The antibacterial substance solution produced by *Bacillus cereus* cultured in selenium enriched and non selenium enriched medium were added to Oxford cup respectively, and the control solution was set. The change of antibacterial activity was determined by measuring the diameter of inhibition zone.

Statistical analysis

All trials were carried out in triplicate and all the data were reported as means $\pm$ SD (standard deviation). The statistics significance was evaluated using Student's t-test and $P < 0.05$ or 0.01 was taken as significant.

3. Results And Discussion

Identification of the source of bacteriostasis in the supernatant of *Bacillus cereus*

As shown in Figure 1, compared with the control of the supernatant containing antibacterial substance of *Bacillus cereus*, the antibacterial substance obtained by acid precipitation method have almost the same antibacterial activity, while the antibacterial substance obtained by ammonium sulfate precipitation method do not show obvious antibacterial effect. Therefore, it is inferred that the antibacterial activity of

Bacillus cereus mainly comes from the lipopeptide antibacterial substance obtained by acid precipitation method. The antibacterial substance obtained by this method were analyzed in subsequent experiments.

Preliminary analysis of antimicrobial substance produced by *Bacillus cereus*

1. Biuret reaction of antibacterial substance

It can be seen from Figure 2(a,b) that biuret reaction of antibacterial substance produced by *Bacillus cereus* showed positive of purple phenomenon. This indicates that antibacterial substance produced by *Bacillus cereus* contains peptide bond, which may belong to protein or polypeptide.

2. Ninhydrin reaction of antibacterial substance

It can be seen from Figure 2(c,d,e) that there was no color change when ninhydrin was added to the aqueous solution of antibacterial substance produced by *Bacillus cereus*, so it is inferred that the antibacterial substance does not contain free α -amino group. After boiling in boiling water bath for a few minutes, the solution in the tube appeared positive reaction and turned purple, which indicated that heating promoted the partial decomposition of antibacterial substance and produced free α -amino group.

In conclusion, the structure of antibacterial substance produced by *Bacillus cereus* has peptide bond, and does not contain free α -amino group. Therefore, it is preliminarily inferred that the antibacterial substance belongs to the cyclic peptide. Cheng et al. (2009) isolated and analyzed the antimicrobial peptide produced by the endophytic bacteria PCE45 of *Paris polyphylla* var. *chinensis*. The results showed that the ninhydrin reaction test was negative, indicating that the substance had no free N-terminal. After acid hydrolysis, the ninhydrin reaction was positive again, so it was speculated that the substance might be a cyclic peptide. Lanyan Fan et al. (2013) carried out color reaction test on czk1 antibacterial substance produced by Endophytic *Bacillus subtilis* from *Hevea brasiliensis*. The results showed that the antibacterial substance solution had obvious color change after biuret reaction for 10 min. There was no obvious reaction after mixing with ninhydrin, and the color became darker after boiling water heating, indicating that the substance may belong to cyclic peptide.

Structure analysis of antibacterial substance produced by *Bacillus cereus*

Infrared spectrum analysis of antibacterial substance

As shown in Figure 3, the characteristic absorption peak at 3220.71 cm^{-1} is caused by the stretching vibration of amide bond, indicating the existence of peptide bond, and the characteristic absorption at 1733.63 cm^{-1} is caused by the contraction vibration of lactone carbonyl, it shows that there is a structure

of C=O of ester carbonyl, and 1630.75-1659.10 cm^{-1} is the stretching vibration of free amino group and ketone group, indicating the existence of N-H group and C=O group. The above characteristic absorption indicates that the antibacterial substance has peptide chain. The absorption at 1402.41 cm^{-1} and 1453.24 cm^{-1} is the stretching vibration of C-H bond in aliphatic carbon chain, and the stretching vibration of aromatic =C-H group at 3055.17 cm^{-1} . The above characteristic absorption indicates that the antibacterial substance contains fatty acid molecules. 1093.19 cm^{-1} is the stretching vibration of C-O-C bond. Based on the above results, it is inferred that the antibacterial substance produced by *Bacillus cereus* is a kind of lipopeptide with ring structure. Similar results were found in the spectral properties of lipopeptide surfactants produced by *Alkalihalobacillus clausii* (Hazra et al., 2014), *Bacillus pumilus* (Morikawa et al., 1992) and *Bacillus licheniformis* (Yakimov et al., 1995).

Analysis of antibacterial substance by time of flight mass spectrometry

As shown in Figure 4(A), there are ion peaks at m/z 1008.502, m/z 1022.536 and m/z 1036.588, and the molecular weight difference is 14Da, which was exactly the length of a fatty acid chain ($-\text{CH}_2$). It was speculated that these ion peaks are the molecular weight of a group of surfactin which are homologous to each other (C13-C15). As shown in Figure 4(B), there are ion peaks at m/z 1021.533, m/z 1035.533 and m/z 1049.577, with a molecular weight difference of 14Da. It was speculated that these ion peaks are the molecular weights of a group of bacillomycin L (C14-C16) which are homologous to each other. The above results were consistent with the reports in the literature (Luo et al., 2015).

NMR analysis of antimicrobial substance

As shown in Figure 5, multiple overlapping methylene ($-(\text{CH}_2)_n-$) signal characteristic resonances were observed in the range of 1.45-1.0 ppm, indicating the existence of long-chain fatty acid structure in the molecule. The resonance signal between 5.2 and 4.4 ppm indicates the presence of fatty acid functional groups and C-H bonds in peptide. The characteristic signal in the range of 8.0-7.2ppm indicates the existence of N-H characteristic resonance in peptide chain. At the same time, the figure also shows the presence of -C-OH structure(δ 2.17ppm), and the signal at the chemical shift of 3.37ppm was the solvent peak produced by deuterated methanol. Based on the above results, it was speculated that there is lipopeptide structure in the antibacterial substance (Sharafi et al., 2014). Andreia et al. studied the biosurfactant produced by a *Bacillus subtilis* strain Isfm-05 by ^1H NMR. The results clearly showed that the substance had long fatty acid chain, peptide main chain and aliphatic carbon hydrogen bond, indicating that the surfactant was lipopeptide (Faria et al., 2011). Liu et al. also reported the same results. They confirmed and characterized c15-surfactant-o-methyl ester produced by *Bacillus subtilis* HSO 121, a lipopeptide producing strain (Xiangyang Liu et al., 2009).

Determination of antibacterial spectrum

After the indicator bacteria were cultured for 24 hours, a clear antibacterial circle appeared around the Oxford cup on the plates of *Staphylococcus aureus*, *Micrococcus luteus* and *Aspergillus niger*, but there was no significant change on the plates of *Salmonella*, *Escherichia coli* and yeast. The measured diameter of inhibition zone was shown in Table 1. It can be seen that the antibacterial substance produced by *Bacillus cereus* has inhibitory effect on Gram-positive bacteria and some fungi, but has no inhibitory effect on Gram-negative bacteria, and selenium enrichment can significantly enhance its antibacterial activity. Oscáriz et al. studied the bacteriocin cerein 7 produced by a *Bacillus cereus* in 1999 and 2000, and the results showed that this antibacterial substance had broad-spectrum antibacterial activity against Gram-positive bacteria, but had no inhibitory effect on Gram-negative bacteria (Oscáriz et al., 1999; Oscáriz and Pisabarro, 2010). In 2009, Lappe and others found that cerein 8A, an antibacterial substance produced by *Bacillus cereus*, could inhibit Gram-negative bacteria including *Salmonella* (Lappe et al., 2009). It can be seen that the range of antibacterial spectrum of the strains in this study is different from that of other similar studies, which may be due to the great differences in DNA level and physicochemical properties of different *Bacillus cereus*, resulting in the differences in physicochemical properties, antibacterial mechanism and chemical structure of antibacterial substance produced by the strains (Lina Zhang, 2012). At the same time, due to the obvious inhibitory effect of the antibacterial substance on *Staphylococcus aureus*, *Staphylococcus aureus* was selected as the indicator bacteria in the follow-up test.

Table 1. Inhibition spectrum of *Bacillus cereus*.

Indicator bacteria		Diameter of bacteriostatic zone/mm	
		Selenium enrichment	Non Selenium enrichment
Gram positive bacteria	<i>Staphylococcus aureus</i>	15.1±0.2	9.0±0.2
	<i>Micrococcus luteus</i>	11.2±0.6	8.7±0.4
Gram negative bacteria	<i>Escherichia coli</i>	-	-
	<i>Salmonella</i>	-	-
Fungus	<i>Saccharomycetes</i>	-	-
	<i>Aspergillus niger</i>	13.2±0.4	8.7±0.2

Morphological effects of antibacterial substance produced by *Bacillus cereus* on indicator bacteria

As shown in Table 2, the number of living bacteria in the medium increased significantly after the agar block in the inhibition zone was cultured at 37 °C for 24h, indicating that the indicator bacteria in the inhibition zone were not completely killed by the antibacterial substance, and its inhibition mode was to temporarily inhibit the growth activity of the indicator bacteria, and the indicator bacteria could continue to grow when the conditions were appropriate (Lina Zhang, 2012).

The effect of antibacterial substance on *Staphylococcus aureus* was observed by scanning electron microscope. The results are shown in Figure 6. In the blank control group, *Staphylococcus aureus* showed the characteristics of full shape, smooth and complete surface, clear outline, and obvious spherical shape. However, after treated with antibacterial substance produced by *Bacillus cereus* for 3 hours, the surface of *Staphylococcus aureus* in the experimental group became rough, and a large number of cells showed folds and depressions with abnormal cell morphology. This indicates that the antibacterial substance can effectively destroy the surface structure of the cells and make their morphology abnormal. Cao et al. (2019) studied the antibacterial mechanism of *Bacillus subtilis* fermentation extract on *E.coli* by scanning electron microscope. The results showed that the surface of *E.coli* cells became rough and wrinkled after the effect of the extract, and a large number of abnormal morphological bacteria appeared, which confirmed the destructive effect of antibacterial substance produced by *Bacillus subtilis* on the surface structure of indicator cells.

Table 2. The OD_{600nm} and bacterial accounts antibacterial circle area.

	0h	24h
OD _{600nm}	0.122	1.7045
Number of live bacteria /cfu	1.6×10 ³	2.3×10 ⁹

Stability analysis of antibacterial substance produced by *Bacillus cereus*

As shown in Figure 7, selenium enrichment can significantly enhance the antibacterial activity of antibacterial substance produced by *Bacillus cereus*. It seems that selenium promotes the production of soluble metabolites related to antibacterial activity. When selenium replaces sulfur in disulfide bond of antibacterial peptide, its antibacterial activity may be improved. Of course, the increase of biomass may also be the reason for the enhancement of antibacterial activity. Kheradmand et al. (2014) also found a similar phenomenon in the experiment, the antibacterial activity of *Lactobacillus plantarum* and *Lactobacillus johnsonii* against *Candida albicans* was increased after selenium enrichment.

In addition, after different levels of temperature, ultrasound and UV treatment, the antibacterial activity of antibacterial substance decreased slightly, which may be due to the partial inactivation of antibacterial substance caused by high temperature, high power ultrasonic and ultraviolet treatment. But in general,

the antibacterial substance produced by *Bacillus cereus* had good stability to temperature, ultrasound and UV treatment ($p < 0.05$). At the same time, the antibacterial substance have antibacterial activity in the range of pH 3-11, but the optimal pH range for its antibacterial activity is pH 5-7. The pH value above or below this range will lead to a significant decrease in antibacterial activity. P A et al. (2010) isolated a substance with antibacterial activity from *Bacillus cereus* ATCC 14579 in 2004. It has inhibitory effect on clinically related *Staphylococcus aureus* and *Micrococcus luteus*, and has high antibacterial activity in the range of pH 2.0-9.0. And after pepsin treatment, the antibacterial activity of the antibacterial substance solution decreased significantly, which confirmed that the antibacterial substance was protein or polypeptide again.

4. Conclusion

In this study, the antibacterial substance were extracted from the fermentation broth of *Bacillus cereus* by ammonium sulfate precipitation method and acid precipitation method respectively, and the inhibition zone test proved that the antibacterial activity of *Bacillus cereus* mainly came from the substance extracted by the latter method. The results of biuret reaction and ninhydrin reaction showed that the antibacterial substance belonged to protein or peptide. Furthermore, the results of FTIR, NMR and MALDI-TOF-MS indicated that the antibacterial substance might be lipopeptide. The results of antibacterial spectrum test showed that *Bacillus cereus* had good inhibitory effect on Gram-positive bacteria and fungi, but no obvious inhibitory effect on Gram-negative bacteria, and selenium enrichment could significantly enhance the antibacterial activity of lipopeptide antibacterial substance produced by *Bacillus cereus*. And the mechanism of the antibacterial substance on the indicator bacteria is inhibition rather than killing, so when the growth conditions are suitable, the inhibited indicator bacteria can grow again, and the antibacterial substance can effectively destroy the surface structure of the cell, making its morphology appear abnormal changes. The results of stability test showed that the antibacterial substance produced by *Bacillus cereus* were stable to heat treatment, ultrasonic time treatment and ultraviolet treatment. In the range of pH 3-11, the antibacterial substance has antibacterial activity, but the optimum pH range is 3-5. The antimicrobial substance produced by *Bacillus cereus* was sensitive to pepsin, which confirmed that the antimicrobial substance was peptide substance again. The antibacterial substance showed good stability, which provided theoretical foundation for their structural identifications and future applications. This study may contribute to provide a theoretical basis and idea for the extraction and reuse of metabolites such as antibacterial substance in the fermentation broth often discarded in the previous fermentation process. Subsequently, the antibacterial substance can be studied by means of genetic engineering, and try to construct and express it in other organisms. At the same time, this study confirmed that selenium enrichment can improve the antibacterial activity of the strain, which is conducive to the further utilization of selenium enriched *Bacillus cereus*. However, the mechanism of selenium enrichment enhancing antibacterial activity needs further study.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

Availability of data and material

Samples of the strain are not available from the authors.

Code availability

Not applicable.

Authors' contributions

Conceptualization: Zhenzhou zhu; Methodology: Shanxiu Ming, Xujun Chen and Shuyi Li; Formal analysis and investigation: Shanxiu Ming, Xujun Chen; Data curation: Shanxiu Ming; Writing-original draft preparation: Xujun Chen, Na Zhang; Writing-review and editing: Shanxiu Ming, Xujun Chen and Zhenzhou zhu; Funding acquisition: Shuiyuan Cheng; Resources: Zhenzhou zhu; Supervision: Shuyi Li and Zhenzhou zhu; Project administration: Shuiyuan Cheng.

Research involving Human Participants and/or Animals

Not applicable.

Informed consent

Not applicable.

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Figures

Figure 1

Comparison of antibacterial activity of different antimicrobial substance produced by *Bacillus cereus* A, antibacterial substance obtained by acid precipitation method. B, antibacterial substance obtained by ammonium sulfate precipitation method. C, the supernatant containing antibacterial substance. D, the blank control

Figure 2

Biuret reaction(a,b) and ninhydrin reaction(c,d,e) of antibacterial substance, in which a and c are blank control of pure water, b is biuret reaction of antibacterial substance, d is solution of antibacterial substance added with ninhydrin, and e is the antibacterial substance solution boiled after adding ninhydrin.

Figure 3

The IR spectra of antimicrobial substance from *Bacillus cereus*.

Figure 4

MALDI-TOF-MS of antimicrobial substance produced by *Bacillus cereus*.

Figure 5

¹H NMR spectra of antibacterial substance produced by *Bacillus cereus*.

Figure 6

Effect of antimicrobial substance produced by *Bacillus cereus* on cell morphology and structure of *Staphylococcus aureus*.

Figure 7

Stability analysis of antibacterial substance produced by *Bacillus cereus* against temperature(A), ultrasonic time(B), UV(C), acid-base(D) and pepsin(E).