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Antimicrobial mechanism and biocontrol effect of *Bacillus cereus* XZ30-2 on *Aspergillus niger*

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RESEARCH ARTICLE

Abstract

Aspergillus niger is a major mold-causing spoilage in cereals, fruits and vegetables. Controlling of mold in stored grains is essential for safety of food. Currently, application of microorganisms to control *A. niger* is a safer and more effective method. In this study, strain XZ30-2 against *A. niger* was isolated and identified as *Bacillus cereus* according to morphological and biochemical characteristics as well as 16 Svedberg ribosomal ribonucleic acid (*16S rRNA*) gene sequence analysis. The investigation of action mechanism showed XZ30-2 culture filtrate caused the mycelia inflated or contract, increasing the membrane permeability, leading to the intracellular leakage and nucleic acids release, disrupting the proton pump, decreasing the ergosterol content, inducing the membrane lipid peroxidation and reactive oxygen species (ROS) accumulation in *A. niger*. Moreover, *B. cereus* XZ30-2 culture filtrate could produce hydrolases and lipopeptides, including iturin, surfactin and fengycin. This work also evaluated the control effect of XZ30-2 on *A. niger* in wheat grains, and indicated that 40 µL/g of culture filtrate significantly controlled the infection of *A. niger*. Therefore, *B. cereus* XZ30-2 can be developed as a biological agent for controlling *A. niger* in stored grains.

Keywords: *Aspergillus niger*; *Bacillus cereus* XZ30-2; culture filtrate; antimicrobial mechanism; biocontrol effect

Introduction

Wheat (*Triticum aestivum* L.) is the most widespread global crop; however, up to half of the global harvested grain is lost to poor storage, wastage, and mycotoxin contamination (Mesterházy *et al.*, 2020). During storage, *Aspergillus niger* causes mold in wheat with high water content (Gherbawy *et al.*, 2021; Mannaa and Kim, 2017). Furthermore, *A. niger* produces a lot of mycotoxins and other secondary metabolites, which have been known to cause various diseases and ailments in humans, such as nephrotoxic, immunosuppressive, teratogenic and carcinogenic disorders (Frisvad *et al.*, 2018; Gil-Serna *et al.*, 2019). In addition to health fallouts, *A. niger* infection also causes substantial economic losses. Stored grains and food products infected with mold may become unsuitable for consumption, resulting in economic difficulties and losses for farmers, food producers and distributors (Wilson *et al.*, 2002).

In the conventional agricultural production, the control of *A. niger* mainly depends on physical and chemical methods (Agriopoulou *et al.*, 2020). Physical measures required to prevent the growth of *A. niger* include controlling temperature and water activity of stored grains (Fleurat-Lessard, 2017). Nevertheless, there are deficiencies in the practical application of physical measures. For example, incidental fungal spoilage in stored grains also occurs due to water condensation because of 'cold wall contact' effect and moisture transfer resulting from temperature differences in bulk grains. The chemical method mainly inhibits propagation of *Aspergillus* by fumigation

(Thorpe, 2008). Common chemical reagents used are ozone gas, phosphine, propionic acid and plant essential oils (Rutenberg *et al.*, 2018). However, these chemical agents have persistent toxicity when used to control fungal spoilage in stored grains. Hence, a safer and more effective method is needed to prevent mold propagation in stored grains.

At present, biological control presents a promising approach for combating *A. niger* infestation, which is more reliable ecologically and economically sustainable. Researchers mainly use microorganisms or their metabolites to reduce the impact of other harmful organisms to prevent and control mold in stored grains (de Andrade Santiago *et al.*, 2018; Santra and Banerjee, 2020). *Kluyveromyces thermotolerans* (Ponsone *et al.*, 2011) and some antagonistic bacteria (*Paraburkholderia fungorum*, *Bacillus subtilis,* and Lactobacillus) were found to inhibit *A. niger* during grain storage (Rahayu *et al.*, 2021; Yassein and Elamary, 2021). Antagonistic yeast *Cyberlindnera jadinii* volatiles were reported to inhibit the growth of *A. niger* and had a significant biocontrol ability against the pathogen (Alkuwari *et al.*, 2022). Meanwhile, most bioactive metabolites, such as alkaloids, lipopeptides and lactones could also control *A. niger* (Li *et al.*, 2020, 2022; Wang *et al.*, 2017).

Recently, *B. cereus*is has been reported to play an important role in controlling pathogens and molds. For instance, *B. cereus* B25 inhibited the growth of the phytopathogen *Fusarium verticillioides* P03 (Báez-Astorga *et al.*, 2022). *B. cereus* F-BC26 promoted pepper growth and protected it against *Xanthomonas euvesicatoria* (Hernandez-Huerta *et al.,* 2023). Also, *B. cereus* can eliminate zearalenone (a nonsteroidal estrogenic mycotoxin) in mice and regulate gut microbiota (Wang *et al.*, 2018). *B. cereus* secretes various secondary metabolites against bacterial and fungal growths, including lipopeptide antibiotics synthesized by nonribosomal peptide synthetase (NRPS). Some studies on the antifungal mechanisms of biocontrol bacteria have shown their effects on fungal cells, such as cell membrane disorder, membrane potential imbalance, and effect on physiological metabolism (Ju *et al.*, 2023; Molina-Hernández *et al.*, 2022; Niu *et al.*, 2022).

Bacillus spp. plays an important role in inhibiting fungal growth by changing the cell structure and disrupting energy, transportation, and cell membrane permeability in fungi (Zhang *et al.*, 2022a). Further research in this field could contribute to the development of effective biocontrol strategies for management of *A. niger*. Compared to other microorganisms, little is known about the antifungal potential of *B. cereus* against food spoilage fungi. Therefore, in this study, antagonistic bacteria *B. cereus* XZ30-2 were screened and identified against *A. niger*. The action mechanism of strain XZ30-2 was analyzed and its biocontrol efficacy on *A. niger* in wheat grains was evaluated to develop a new biological agent for controlling the pathogen.

Materials and Methods

Materials

In all, 48 of soil samples from Tibet, China were provided by Dr. Yu Shi of the Henan University on 30 August 2017 for isolating biocontrol bacteria. Antagonistic bacteria *B. cereus* XZ30-2 and *A. niger* were stored at -20°C in the laboratory of the School of Biological Engineering, Henan University of Technology. The wheat seeds of Zhengmai 366 were obtained from Henan Academy of Agricultural Sciences. Potato dextrose agar (PDA) medium (20-g glucose, 200-g potato, 15-g agar, and 1-L sterile water; pH 7) was used to culture *A. niger*. Luria-Bertani (LB) medium (5-g yeast extract, 10-g peptone, 10-g NaCl, and 1-L sterile water; pH 7) was used to culture bacteria.

Isolation and screening of antagonistic bacteria

Bacteria were isolated and screened according to the method described by Zhai *et al.* (2021) with modification. In brief, 10-g soil samples were added into a series of flasks and gradually diluted. The diluted suspensions were coated onto the LB medium and incubated at 35°C for 48 h. Antagonistic ability of all isolates against *A. niger* were tested using the plate confrontation method. The screened bacteria were inoculated onto liquid LB medium and cultured in a shaker at 180 rpm and 35°C for 48 h. Then the culture filtrate of bacteria was centrifuged at 10,000 rpm and prepared by filtering through a 0.22-μm filter. The inhibitory activity of culture filtrate against *A. niger* was tested using the mycelial growth rate method (Yi *et al.*, 2022). PDA inoculated with *A. niger* without culture filtrate treatment was treated as a control. Following formula was used for calculating the inhibition rate:

$$\text{Inhibition rate (\%)} = [(C - T)/C] \times 100 \qquad (1)$$

Where C represents the mycelial radial diameter of *A. niger* in the control group, and T is the mycelial radial diameter of *A. niger* in the treatment group. Antagonistic bacteria with the strongest inhibitory effect were selected for further study.

Identification of antagonistic strain

Antagonistic bacteria XZ30-2 were cultured onto LB medium at 37°C for 24 h to observe colony characteristics, including morphology, color and growth properties. Physiological and biochemical tests were carried out according to Berger's Manual of Systematic Bacteriology (Vos *et al.*, 2009).

Molecular identification of strain XZ30-2 was performed by 16 Svedberg ribosomal ribonucleic acid (*16S rRNA*) gene sequence analysis. The genome DNA of antagonistic strain was extracted by the cetyltrimethylammonium bromide (CTAB) method (Yan *et al.*, 2020). The *16S rRNA* was amplified using the universal primers F (5'-AGAGTTTGATCATGGCTCAG-3') and R (5'-ACGGTTACCTTGTTACGACTT-3'). The conditions for polymerase chain reaction (PCR) were as follows: predenaturation at 94°C for 5 min, then 32 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. PCR amplification products were verified by 1.2% agarose gel electrophoresis and sent to Sangon Biotech Co. Ltd. (Shanghai, China) for sequencing. Sequences were searched and compared by the Basic Local Alignment Search (BLAST) tool in GenBank (Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov). Sequence alignment and phylogenetic tree construction were performed through the MEGA 7.0 program using the neighbor-joining method (Saitou and Nei, 1987).

Action analysis of XZ30-2 culture filtrate on *A. niger* cells

Inhibition of XZ30-2 culture filtrate on mycelial morphology of A. niger

The effects of culture filtrate on mycelial morphology were observed using an optical microscope based on the method described by Tso *et al.* (2021) with minor modifications. Briefly, *A. niger* was grown on a PDA plate containing XZ30-2 culture filtrate (200-μL/mL) at 28°C for 2 days. *A. niger* cultured on PDA plate not containing culture filtrate was taken as a control. Then the mycelia of *A. niger* were collected and observed.

Detection for cell membrane permeability of A. niger treated with culture filtrate

Cell membrane permeability reflects the degree of cell membrane injury. A fluorescent probe propidium iodide (PI) was used to detect cell membrane permeability. *A. niger* mycelia were treated with 200-μL/mL culture filtrate for 48 h. Then the mycelia were collected and incubated in 2-μmol/L prodidium iodide (PI) staining solution for 20 min and rinsed with phosphate buffer solution (PBS), followed by visualization with fluorescence microscope. Mycelia without any culture filtrate treatment were set as a control.

Detection for intracellular leakage of A. niger treated with culture filtrate

The effects of culture filtrate on intracellular leakage of *A. niger* were determined using the intracellular conductivity detection method with minor modifications (He *et al.*, 2021). After culturing for 5 days at 28°C, *A. niger* spores (10^6 colony forming unit [CFU]/mL) were inoculated with 100-mL potato dextrose broth (PDB) and cultured in a shaker at 110 rpm and 28°C for 48 h. Then XZ30-2 culture filtrate was added to the mold culture fluid to make the final concentration of 200-μL/mL. The mold culture fluid without XZ30-2 culture filtrate was set as a control. After shaking for an additional 24 h, the mycelia of *A. niger* were collected on double gauze and rinsed twice with sterile water; 0.5-g dried mycelia were suspended in 20-mL sterile water for 0, 5, 10, 20, 40, 80, 100, 120, 140, 180, 200, and 220 min at 25°C. Then the electric conductivity was measured using a conductivity meter to assess intracellular leakage through cell membranes. The relative conductivity of mycelia was calculated using the following formula:

$$\text{Relative conductivity (\%)} = (\text{Conductivity}/\text{Final conductivity}) \times 100 \quad (2)$$

Determination of release of nucleic acids in A. niger

The release of cellular components was performed by the method described by Ke *et al.* (2022). The culture of the mold was prepared described previously. After centrifugation for 25-min at 4,000-rpm to obtain mold supernatant; absorbance (OD_{260}) of the supernatant was measured using UV spectrophotometer.

Assay for the extracellular pH of A. niger treated with culture filtrate

Proton pump of the cell membrane can transfer intracellular protons to extracellular environment. Activities of *A. niger* proton pump were detected by monitoring the glucose-induced acidification of external medium by determining pH according to the method described by Tao *et al.* (2014) with slight modifications. *A. niger* was cultured and mycelia were prepared by the method described previously; 1.0-g mycelia was treated with 40-mL KCl (50-mmol/L) for 18 h at 4°C; Then XZ30-2 culture filtrate was added to mycelia culture to make the final concentration of 200-μL/mL. Mycelia without treatment of culture filtrate were set as a control. The mixture was incubated at 25°C for 10 min and treated with 20-mL 10% glucose solution to make medium acidification for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.0, 3.5, and 4.0 h. The value of extracellular pH was obtained by using pH meter.

Determination of ergosterol content in A. niger

Ergosterol is found in fungal cell membranes, and the growth of fungus is evaluated based on the content of its ergosterol. Ergosterol content in the plasma membrane of *A. niger* was determined by the method described by Prakash *et al.* (2014). In brief, *A. niger* mycelia were treated with 200-μL/mL culture filtrate for 48 h. Mycelia without culture filtrate treatment were set as a control.

After treatment, the mycelia were harvested and washed twice with sterile water. The wet weight of the mycelia was recorded. Then, 5 mL of 25% alcoholic potassium hydroxide solution (25-g KOH and 35-mL sterile distilled water, diluted to 100-mL with anhydrous ethanol) was added to each sample. The mixture was vortexed for 2 min and incubated at 85 ± 2°C in a water bath for 5 h. Subsequently, a mixture of 2-mL sterile distilled water and 5-mL n-hexane was added to extract sterols. After 1 h, the n-hexane layer was analyzed using a scanning spectrophotometric method between 230 nm and 300 nm. The amount of ergosterol was determined using the formula provided by Tian *et al.* (2012).

Determination of lipid peroxidation of A. niger

Malondialdehyde (MDA) concentration was used as an indicator of lipid peroxidation level. In brief, mycelia of *A. niger* were treated with 200-µL/mL culture filtrate for 48 h. Mycelia without culture filtrate treatment were set as a control. After cultivation, mycelia were collected and rinsed twice with sterile water and its wet weight was determined. Then the MDA content was measured using a lipid peroxidation (MDA) assay kit (Solarbio, Beijing, China).

Detection of reactive oxygen species (ROS) accumulation in A. niger

Reactive oxygen species are primarily produced in the mitochondria by the oxidative respiratory chain. Oxidative stress might cause damage to plasma membrane damage and enhance membrane permeability. The accumulation of ROS was monitored by following the method described by Yi *et al.* (2022). Mycelia of *A. niger* were treated with 200-µL/mL culture filtrate for 48 h. The mycelia were stained with 10-µM DCFH-DA solution (Beyotime, Shanghai, China). After incubation in the dark at 28°C for 30 min, the samples were observed and photographed using a fluorescence microscope.

Detection of antimicrobial substances in strain XZ30-2

The antimicrobial substances produced by XZ30-2, including protease and cellulase, were determined by using skimmed milk and carboxymethyl cellulose (CMC) according to the protocol described by Ben Khedher *et al.* (2021). Amylase was determined by the starch agar plate method (Nxumalo *et al.*, 2020). Surfactin was determined by the oil spreading assay (Rani *et al.*, 2020).

PCR detection of lipopeptides in strain XZ30-2

The genome DNA of strain XZ30-2 was extracted by the CTAB method (Yan *et al.*, 2020). Ten pairs of primers of lipopeptide genes (*sfp*, *srfAA*, *srfAD*, *fenA*, *fenB*, *fenD*, *ituA*, *ituB*, *ituC* and *ituD*) were used for PCR amplification of lipopeptide synthesis-related genes (Table 1). Sequencing of PCR-amplified products was performed at Sangon Biotech Co. Ltd. (Shanghai, China), and the sequences were compared with the NCBI database (Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov) to analyze sequence homology with lipopeptide genes.

Assay of XZ30-2 culture filtrate for controlling *A. niger* infection

The control effect of strain XZ30-2 was evaluated by the method described by Duan *et al.* (2023). In this test, sterilized 120-g wheat seeds were divided into three groups, including the control group, in different tubes. XZ30-2 culture filtrate, 20 and 40-µL/g, was added into tubes. In the control group, culture filtrate was replaced by LB medium. Next, 100-µL of *A. niger* spores suspension (10^7-CFU/mL) was added into each tube containing wheat seeds and shaken well; the tubes were sealed and stored at 28°C. After 2 weeks, the number of fungal colonies in wheat seeds was calculated.

Statistical analysis

All tests are performed in triplicate. The data were expressed as the mean ± standard error (SE), and analyzed statistically by independent-sampled test of variance (ANOVA) using SPSS 20.0 (SPSS Co. Ltd., Chicago, IL, USA). Significant differences between mean values at $p < 0.05$ were determined by multiple comparisons using Duncan's multiple range test (DMRT).

Results and Discussion

Isolation and identification of antagonistic strain

Of the 30 bacteria strains isolated, six strains had antifungal effect on *A. niger* (Figure 1A). Among them, strain XZ30-2 showed higher antagonistic effects for *A. niger*. When strain XZ30-2 was cultured on LB plate, Gram-positive bacteria presented big, rod-shaped, milky white, opaque irregular edges. In addition, XZ30-2 was positive for nitrate reduction, glucose reaction, citrate, indole, amylolysis, and catalase test, whereas it was negative for methyl red test. Meanwhile, the *16S rRNA* gene sequence of XZ30-2 (GenBank accession No.: MZ646135) was arranged in a phylogenetic tree (Figure 1B). Results of morphological and biochemical characteristics and molecular identification discovered strain XZ30-2 as *Bacillus cereus*, showing 100% resemblance to *B. cereus* ATCC 14579.

Table 1. Ten pairs of primers of lipopeptide synthesised genes.

Genes	Primers	Sequences (5' → 3')	Amplified size / bp
sfp	*Sfp-F*	ATGAAGATTTACGGAATTTA	675
	Sfp-R	TTATAAAAG CTC TTC GTACG	
srfAA	*srfAA-F*	GCCCGTGAGCCGAATGGATAAG	1,600
	SrfAA-R	CCGTTTCAGGGACACAAGCTCCG	
srfAD	*srfAD-F*	CCGTTCGCAGGAGGCTATTCC	1,300
	SrfAD-R	CGCCCATCCTGCTGAAAAAGCG	
fenA	*fenA-F*	GCTGTCCGTTCTGCTTTTTC	1,000
	fenA-R	GTCGGTGCATGAAATGTACG	
fenB	*fenB-F*	CTATAGTTTGTTGACGGCTC	1,600
	fenB-R	CAGCACTGGTTCTTGTCGCA	
fenD	*fenD-F*	TTTGGCAGCAGGAGAAGTTT	1,600
	fenD-R	GACAGTGCTGCCTGAAA	
ituA	*ituA-F*	ATGTATACCAGTCAATTCC	1,047
	ituA-R	GATCCGAAGCTGACAATAG	
ituB	*ituB-F*	CAACGGTATCGAAGCA	449
	ituB-R	CGTCTCGGGTATCATTT	
ituC	*ituC-F*	GGCTGCAGATGCTTTAT	423
	ituC-R	TCGCAGATAATCGCAGTGAG	
ituD	*ituD-F*	ATGAACAATCTTGCCTTTTTA	1,203
	ituD-R	TTATTTTAAAATCCGCAATT	

Effects of *B. cereus* XZ30-2 culture filtrate on *A. niger*

Changes of mycelial morphology of A. niger

Effects of XZ30-2 culture filtrate on mycelial morphology of *A. niger*, observed using optical microscope (Figure 2A), showed that mycelia were either inflated or compacted, compared to the control, particularly at mycelial tips. It showed that culture filtrate could remarkably affect the mycelial morphology of *A. niger.*

Cell membrane permeability of A. niger

The growth of *A. niger* was inhibited due to cell membrane damage (Ju *et al.*, 2020). In damaged cells, PI dye entered the nucleus through cell membrane. After PI staining, red fluorescence was obviously observed in the mycelia of *A. niger* treated with culture filtrate (Figure 2B). The result indicated that the membrane permeability of mycelia was increased by XZ30-2 culture filtrate. It was consistent with the inhibitory effects of cinnamaldehyde vapor on the cell membranes of *A. niger* HY2 (Niu *et al.*, 2022).

Intracellular leakage of A. niger

In case fungal cell membrane is damaged, intracellular electrolyte solution flows out and increases the conductivity of extracellular solution. Hence, changes in the electric conductivity of extracellular solution are important indicators reflecting intracellular leakage (Yan *et al.*, 2023). In this study, electric conductivity increased after exposure to culture filtrate (Figure 3A), which indicated that XZ30-2 culture filtrate resulted in the intracellular leakage of *A. niger*.

Release of nucleic acids of A. niger

Release of nucleic acids after culture filtrate treatment was measured by spectroscopy at 260 nm. As shown in Figure 3B, compared to OD_{260} (0.123) in the control group, the absorbance value of DNA treated with culture filtrate was 0.211. The results indicated that culture filtrate showed a significant effect on the intracellular leakage of *A. niger*. The absence of nucleic acids in cells could disrupt the normal activities of cells and even cause cell death (Niu *et al.*, 2022).

Extracellular pH of A. niger

As shown in Figure 4A, the extracellular pH declined slowly after treatment with culture filtrate (200-μL/mL) at different periods, compared to that of the control group. Decrease in extracellular pH reflects disruption of the proton pump of *A. niger* (Tian *et al.,* 2012). The results showed that XZ30-2 culture filtrate could disrupt the proton pump of *A. niger*.

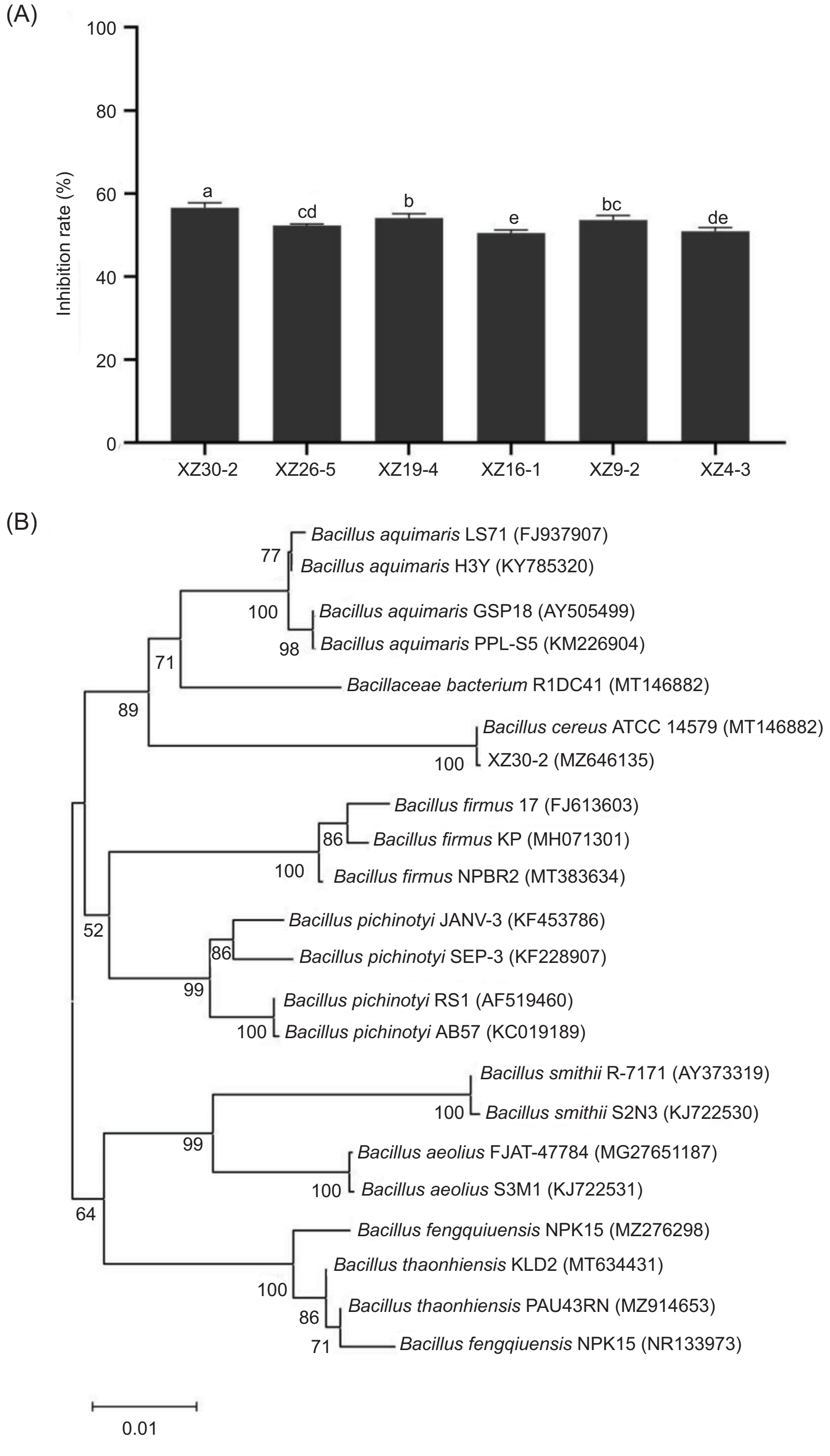

Figure 1. Screening and identification of bacteria against *A. niger*. (A) Inhibition proportions of six strains against *A. niger*. (B) Phylogenetic tree obtained from *16S rRNA* gene sequences of strain XZ30-2 and other closely related bacteria. Numbers at branch points are bootstrap values of parsimony-based analysis. Different lowercase letters in each column represent extremely significant differences at $p < 0.05$.

Ergosterol content of A. niger

Changes in ergosterol content are presented in Figure 4B. Compared to the control, treatment with culture filtrate at 200-μL/mL resulted in a 26.90% decrease in the ergosterol content of *A. niger*. Fungal ergosterol is a classical drug target because it is important for plasma membrane structure and functioning as well as localization of plasma membrane proteins (Georgopapadakou and

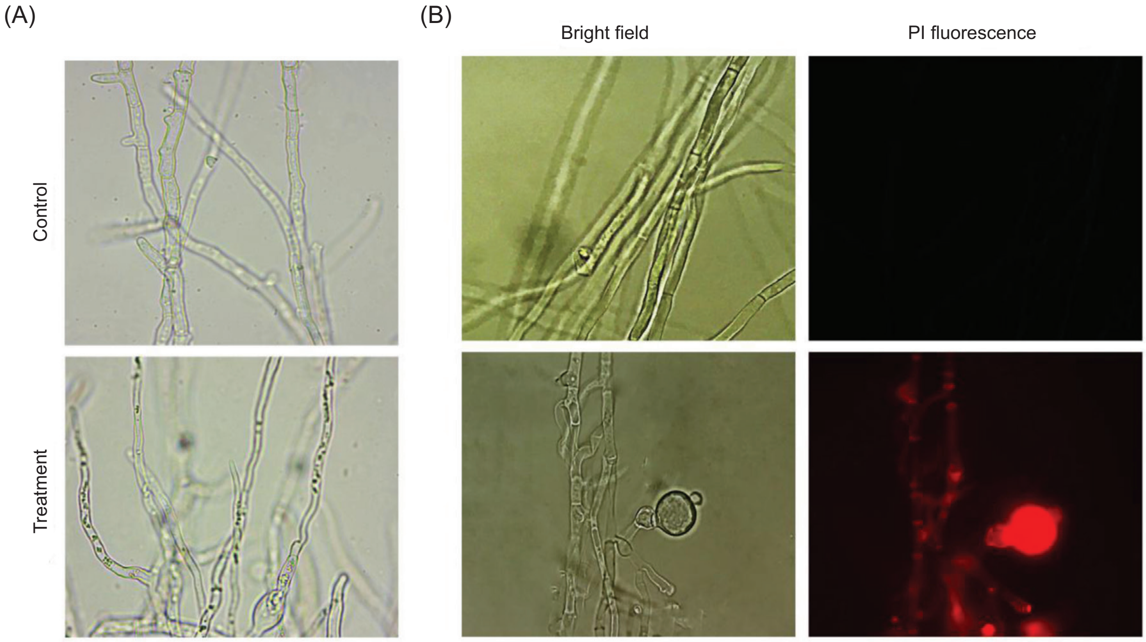

Figure 2. Effects of XZ30-2 culture filtrate on the mycelia of *A. niger*. (A) Effects on the morphology of *A. niger* mycelia. (B) Effects on the serosal membrane injury of *A. niger*. Red fluorescence represents PI-stained mycelia. Micrographs were taken using a fluorescence inverted microscope with 40× magnification.

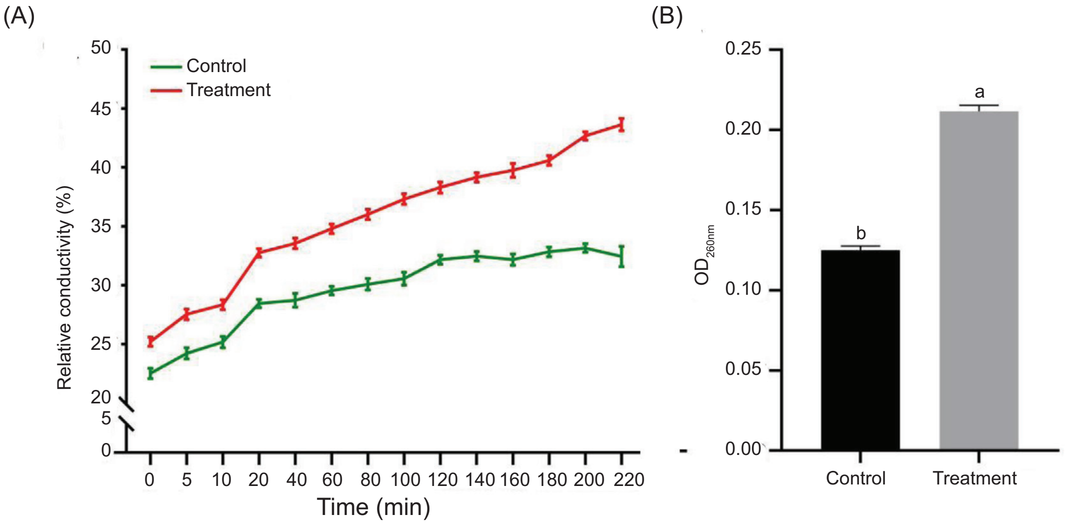

Figure 3. Effects of XZ30-2 culture filtrate on the (A) relative conductivity and (B) OD_{260} of *A. niger* mycelia. Each value is presented as the average of three replicates. Different lowercase letters in each column represent extremely significant differences at $p < 0.05$.

Tkacz, 1995). Therefore, XZ30-2 culture filtrate could decrease the biosynthesis of ergosterol, thereby affecting the growth of *A. niger*.

Lipid peroxidation of A. niger

The MDA level reflects the extent of lipid peroxidation (Ju *et al.*, 2020). As shown in Figure 4C, compared to the control, treatment with culture filtrate at 200-μL/mL resulted in an increase in the MDA content of *A. niger* by 0.339. This result showed that culture filtrate treatment induced membrane lipid peroxidation of *A niger.*

Reactive oxygen species accumulation of A. niger

Reactive oxygen species plays a crucial regulatory role in normal cellular metabolism and under stress conditions. However, excessive ROS levels can lead to oxidation

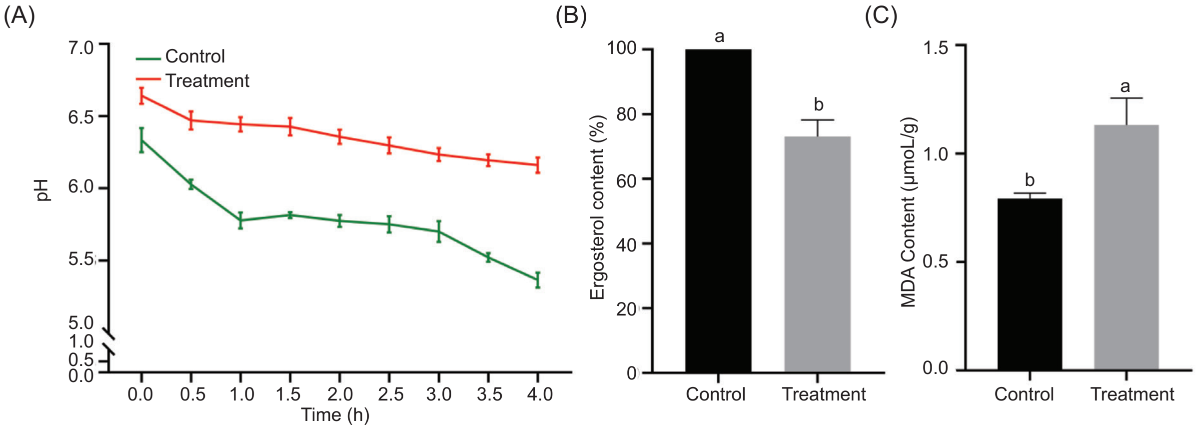

Figure 4. Effects of XZ30-2 culture filtrate on (A) pH, (B) ergosterol content, and (C) MDA content of the mycelia of *A. niger.* C indicates the control group and T indicates the mycelia treated with culture filtrate. Different lowercase letters on the bars indicate significant difference at *p* < 0.05.

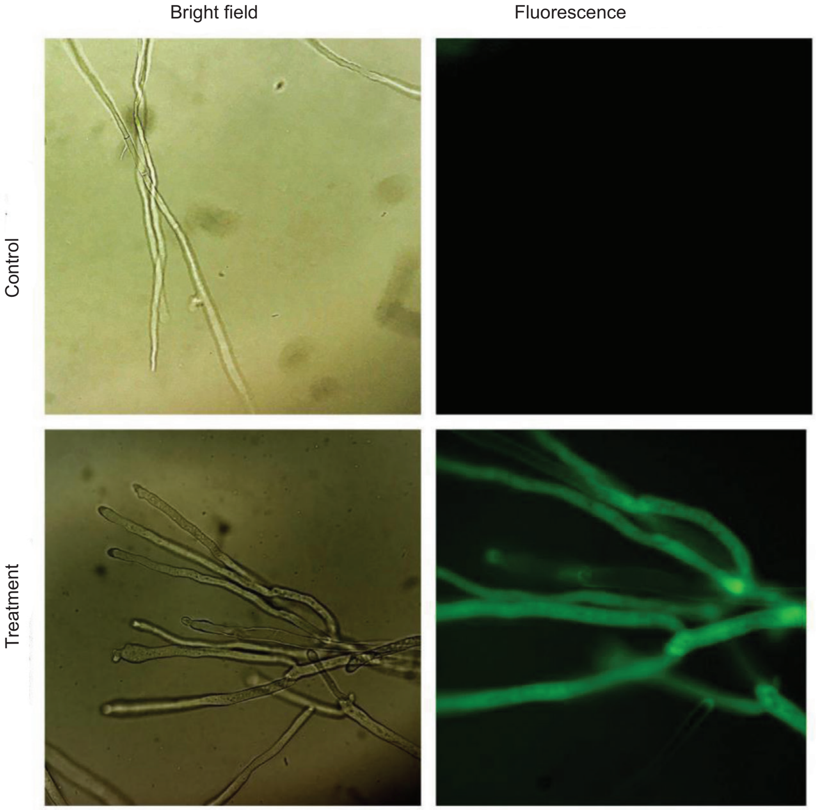

Figure 5. Effects of XZ30-2 culture filtrate on ROS. C indicates the control group, and T indicates the treatment group. The samples were visualized/photographed using microscope at 40× magnification.

stress and cellular damage (Ju *et al.*, 2020). As shown in Figure 5, treatment with culture filtrate (200 μL/mL) significantly increased the level of green fluorescence, compared to the control group. Therefore, culture filtrate resulted in a significant accumulation of ROS in *A. niger* and could lead to cell damage.

Active substances produced by *B. cereus* XZ30-2

Antimicrobial action through the production of hydrolase is probably an important mechanism of biocontrol bacteria to inhibit pathogens (van Schie *et al.*, 2021). Hydrolase of *B. cereus* XZ30-2 were detected and exhibited transparent circles on identification media (Figures 6A–6C), which indicated that XZ30-2 could produce protease, cellulase and amylase. Meanwhile, the diameter of the oil drainage circle was 7.35 cm if 20-μL culture filtrate was added to the media, indicating the presence of surfactin in the culture filtrate (Figure 6D). Similarly, antimicrobial substances produced by *B. cereus* had an inhibitory effect and could be taken as antifungal agents (Durval *et al.*, 2021; Ming *et al.*, 2022).

Lipopeptides produced from *B. cereus* XZ30-2

Among the useful metabolites produced by *Bacillus* spp., the main antimicrobial substances are lipopeptides (Hu *et al.*, 2023; Zhang *et al.*, 2022b). After the PCR amplification of 10 known primer pairs, five fragments of *ituA*, *ituC*, *ituD*, *srfAD* and *fenB* genes were analyzed according to sequence comparison with the lipopeptide genes of NCBI database (Table 2). It indicated that *B. cereus*

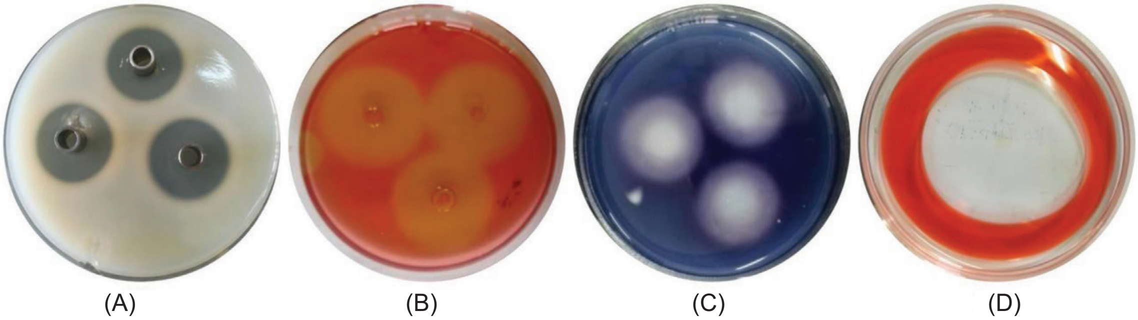

Figure 6. **Active substances of *B. cereus* XZ30-2. (A) Protease, (B) cellulase, (C) amylase and (D) surfactin.**

Table 2. **PCR amplification of fragments from *B. cereus* XZ30-2.**

Genes	Product size(bp)	Probable genes	Identity (%)
srfAD	1300	*srfAD* of *B. subtilis*	98.56%
fenB	1600	*fenB* of *B. amyloliquefaciens*	97.22%
ituA	1047	*ituA* of *B.velezensis*	99.26%
ituC	423	*ituC* of *B.velezensis*	97.11%
ituD	1203	*ituD* of *B.amyloliquefaciens*	97.82%

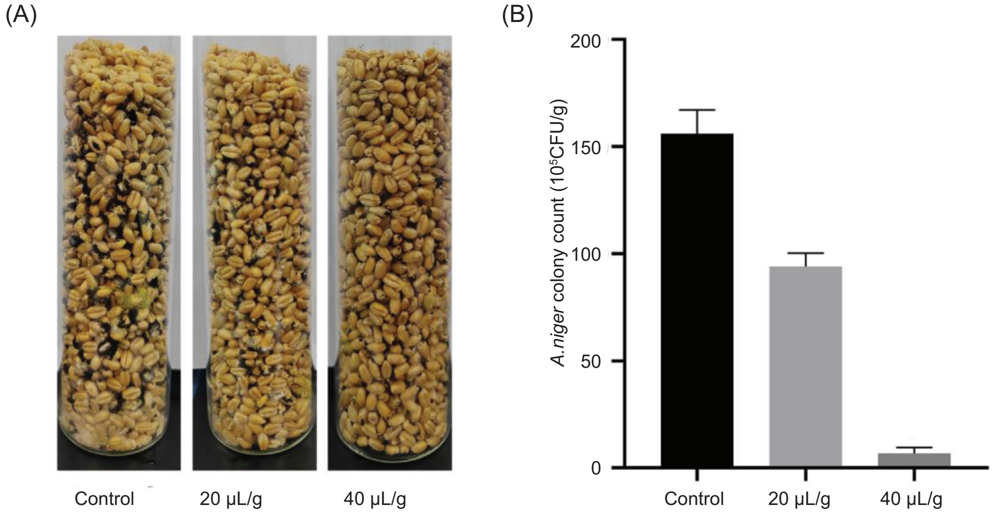

Figure 7. **Control effects of XZ30-2 culture filtrate on *A. niger* in stored wheat grains. (A) Mold infection on wheat in different treatment groups. (B) *A. niger* colony count after 2 weeks when exposed to different concentrations of culture filtrate. Different lowercase letters on the bars indicate significant difference at *P* < 0.05.**

XZ30-2 could produce lipopeptides, the main antimicrobial substances, including iturin, surfactin, and fengycin (Ayangbenro and Babalola, 2020; Li *et al.*, 2021).

Control effects of XZ30-2 culture filtrate on *A. niger* infection in wheat grains

The control effects of XZ30-2 on wheat grains are shown in Figure 7. The *A. niger* infection in wheat grains was obviously visible with different moldy levels. Colony counting of *A. niger* in wheat samples treated with different concentrations of culture filtrate showed the following results: colony count in the control group was 1.68 CFU×10^8/g; colony count in the treatment group with 20-μL/g of culture filtrate was 9.4 CFU×10^7/g; colony count in the treatment group with 40 μL/g of culture filtrate was 6.69 CFU×10^5/g. The results showed that *B. cereus* XZ30-2 effectively controlled *A. niger* and reduced moldiness in stored wheat grains. Comparatively, the control effect of XZ30-2 culture filtrate was similar to or better than other candidate biocontrol agents reported previously (Oztopuz *et al.*, 2018; Ul Hassan *et al.*, 2019).

Conclusions

According to morphological, biochemical and molecular analysis, antagnostic strain XZ30-2 of *B. cereus* was identified to combat *A. niger*. *B. cereus* XZ30-2 controlled *A. niger* by inhibiting mycelia development and increasing membrane permeability, thus leading to intracellular leakage and nucleic acid release, disrupting proton pump, decreasing the ergosterol content, inducing membrane lipid peroxidation and ROS accumulation, and producing hydrolases and lipopeptides, including iturin, surfactin, and fengycin. The culture filtrate of strain XZ30-2 effectively controlled the infection of *A. niger* and reduced moldiness in stored grains. In summary, the present study demonstrated the controlled potential of *B. cereus* XZ30-2 and provided a new microbial resource for controlling *A. niger*.

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Conflicts of interest

The authors stated that they had no conflict of interest to declare.

Author Contributions

The manuscript was written and revised by Yanjie Yi and Zhipeng Hou. Experiment design and execution were performed by Zhipeng Hou, Qian Yang, Heng Lu, Yang Liu, Yuyanqiao Zhang, and Yuan Chen. Data collection and analysis were performed by Zhipeng Hou, Liuqing Cui, Yang Liu, and Ruifang Li. All authors read and approved the published version of the manuscript.

Data Availability

All original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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