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Isolation, Identification and Optimization Conditions of Antagonistic Strain *Bacillus subtilis* LZ2-70 Against *Verticillium dahliae*

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The strains having antagonistic activity against *Verticillium dahliae* Kleb were isolated and screened from the soil of cotton fields from different regions. A strain named LZ2-70 with a rather higher antagonistic activity was obtained. The morphology characteristics, physiological and biochemical properties and 16S rRNA gene sequence of this strain were further studied. The strain LZ2-70 was finally identified as a type of *Bacillus subtilis*. The antagonistic substance was antifungal protein. Through single factor experiment and orthogonal experiment, the optimal shaking flask fermentation condition of strain LZ2-70 was determined as follows: medium composed of 5% amidulin, 1% peptone, 0.03% $MnCl_2$, 0.01% KCl, initial pH 8.0 and 10% inoculation volume, media volume 30/250 (mL/mL), fermentation temperature 37°C, rotating speed 200 r/min, fermentation time 96 h. A distinguished elevation of the antagonistic activity was observed at about 41.1%.

Key words: Antagonistic bacterium, antifungal protein, *Bacillus subtilis*, fermentation, isolation and identification, *Verticillium dahliae*.

Cotton Verticillium wilt is a common disease caused by the fungus *Verticillium dahliae* Kleb, and it is one of the most harmful diseases of cotton (Bell, 1992). It is a widespread fungal disease with a significant negative effect on the world's yield of cotton **(**Fradin and Thomma, 2006*)*. Different methods were used to prevent or control cotton Verticillium wilt disease, such as selection of disease-resistant varieties, seed treatment, strengthening field management, chemical control, biological control and so on (Lin *et al*., 2013; Tjamos *et al*., 2000a). Generally, biological control, as a promising control method, does not adversely affect other beneficial microorganisms in the agroecosystems (Tjamos *et al*., 2000b). At the same time, with the properties of high biocide activity and low dosage, it is easy to be decomposed by edaphon, avoiding accumulation in nature (Tjamos, 2000). When the antagonistic strain is used for biological control, it should be easy to implement in large-scale industrial production with microbial agents having high activity, strong stability, and long shelf life (Berg and Ballin, 1994). Therefore, this experiment is committed for screening spore-producing antagonistic bacteria that exhibit easy control of the liquid fermentation of bacteria and spore resistance to heat, pressure and chemical agent. These make the commercialization of the microbial agents more realistic.

Those biocontrol agents often produce a variety of antifungal substances such as antibiotics, peptides, bacteriocins, and protein substances which have high activity to specific pathogens (Tamehiro *et al*., 2002). The discovery of peptide and protein substances provides a new

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biological resource and the antifungal genes for disease resistance genes engineering. Making use of genetic engineering means crop varieties can be modified to show exogenous resistance, opening up new avenues for breeding for disease resistance (Chet *et al*., 1993). The key for achieving this goal is the effective separation of the gene. Medium composition and culture conditions are important factors in the formation of antagonistic substances.

Using *Verticillium dahliae* as test microorganism, the strain ZL2-70 with a relatively high rivalry activity was obtained after preliminary and secondary screening. The morphological, physiologic and biochemical characteristics and the 16S rRNA gene sequence of this strain were further studied. Through its identification, more details of its physiologic and biochemical characteristics were obtained which laid a good foundation for the studies on the mechanism of bacterial antagonism. This study also optimized the fermentation medium and culture conditions in the production of antifungal proteins. The benefit of this study is two folded, on one hand, it will help to fully exploit the benefits of microbial resources for the biological control of plant diseases; on the other hand, to lay a theoretical genetic foundation for molecular cloning of the antifungal protein gene and construction of transgenic engineering bacteria.

MATERIALS AND METHODS

Strains and medium

The tested pathogen was *V. dahliae* V-190 preserved in the bio-pharmaceutical laboratory of College of Life Science, Agricultural University of Hebei, China.

NA medium, NB medium and potato medium (PDA) were prepared in this study according to the "Microbiology Experiment" (Shen *et al*., 1999), while the media for identifying physiologic or biochemical characteristics were prepared according to the "Manual of System Determinative Common Bacteriology" (Dong and Cai, 2001). The fermentation medium for the secondary screening contained 3.0% of corn meal, 3.0% of beef extract, 0.05% K_2HPO_4, and 0.05% $CaCl_2$ with the initial pH of 7.0.

Seed medium contained 1.0% peptone, 1.0% glucose, 0.2% $NaH_2PO_4 \cdot 2H_2O$, 0.4% $Na_2HPO_4 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$ with the initial pH of 7.0~7.2.

Basic fermentation medium contained 1.0% carbon source, 1.0% nitrogen source, 0.2% $NaH_2PO_4 \cdot 2H_2O$, 4.0% $Na_2HPO_4 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2$, with the initial pH of 7.0-7.2.

Soil samples

Soil samples at the depth of 10–15 cm were collected by shovel from diseased cotton fields, and then stored in plastic bags after air drying, with sampling time and locations recorded. A total of 20 soil samples were screened, including Hebei, Jilin, Hubei, Hunan and other areas.

Isolatation strains from soil

Isolation and purification of bacteria from soil samples were conducted by the following procedures. 1.0 g soil sample was grounded and added to tubes filled with 9.0 mL sterile distilled water, then shaken for 20 min in a water bath at 80°C. Next, the 1.0 mL sample solution was diluted into 1×10^{-6} by ten times gradient dilution, and then 0.1 mL diluent was coated on the plate of Beef extract peptone agar medium medium in two gradient concentrations of 1×10^{-4} and 1×10^{-5}, respectively, cultured and inverted for 24 h at 30°C. Single colonies in different shape, size and color were transferred respectively into the slant of Beef extract peptone agar medium medium. Then, the strains numbered and cultured for 24 h at 30°C were finally stored in refrigerator at 4°C for future use, and the purification of each strain was conducted and tested by Streak Plate Method.

Screening antagonistic strains against *Verticillium dahliae*

To activate the strains, they were transferred into slant of Beef extract peptone agar medium and PDA medium, respectively. The cotton wilt pathogen *V. dahliae* V-190 and the isolated bacteria from the 4°C fridge were kept at room temperature for 1 h. The isolated bacteria were cultured at 30°C for 48 h and the cotton wilt pathogen was stored at 25°C for 7–10 days for future use.

Preliminary screening for the antagonistic bacteria against *V. dahliae* V-190 was conducted as follow: 5.0 mL sterile distilled water was added into the slant of the pathogen cultured for 10 days. Spores were scraped gently with the inoculation

loop and shaken up to obtain the spore suspension, which was added into the PDA medium, cooled to 50°C after melting and rapid shaking. The spore suspension was then poured into plates and prepared for future use after solidification. Some bacterial lawn were selected from the slant of the isolated bacteria with inoculation loop and inoculated on the PDA medium plate to culture for 24–48 h at 30°C. Finally, antagonistic strains with the inhibition zone were selected for future use.

Secondary screening for the antagonistic bacteria against *V. dahliae* V-190 was done by inoculating the antagonistic bacteria obtained from the preliminary screening into the 250 mL Erlenmeyer flask with 50 mL activated fermentation medium, which was shaken in rotary vibrator for 48 h at 30°C at 180 r/min. Sterile membrane filter (0.22 µm) was used to remove the thalli. 5.0 mL sterile distilled water was added into the slant of the pathogen cultured for 10 days, the spores were scraped gently with inoculation loop and shaken up to obtain the spore suspension. Then, the spore suspension was added into the PDA medium and cooled to 50°C after melting and rapid shaking. Finally, the spore suspension was poured on the plate and prepared for future use after solidification.

Regular pores were perforated with the puncher after the medium was cooled. 70 µL antagonistic bacteria broth fermented already for 48 h without the thalli was added into each pore and cultivated for 3 days at 30°C after standing for 30 min. Finally, the diameter of inhibition zone was measured and the strains with large and clear inhibition zone were selected.

Effect of ZL2-70 against *Verticillium dahliae* in pot

The sterilized field soil was put into the nutrition pots. And healthy cotton seedling was transplanted into each nutrition pot, cultured and managed normally. The spores of *V. dahliae* V-190 cultivated for 7 days were washed with 0.1% Tween 80. The concentration of spores was adjusted to 10^4/mL. The spore suspension was evenly applied on the cotton leaves using a writing brush. After 48 h, the fermented liquid of ZL2-70 (at about 10^6/mL) was diluted to 5 times volume. These cottons were treated in three groups with the diluted fermentation liquor as Group 1, Group 2 treated with the 50% carbendazol wettable powder that had been diluted to 500 times as medicine control, and Group 3 treated with the water as a blank control. There were 20 cotton seedlings in each group, with 3 replicates. In 7 d, the cotton seedlings were removed to investigate the incidence of cotton wilt disease (Table 1) and calculate the disease index and the control efficacy by the flowing equations:

$I = (N \times R)/(M \times T)$, where I is disease index, N is the number of the diseased plant in a certain disease level per plant, R is the numeric value of the corresponding disease level, M is the highest numeric value of the investigated cotton and T is the total number of cotton investigated per plant.

Control efficacy can be calculated with the following equation (Zhang, 2001):

Control efficacy (%) = [(CK Disease index – Pt Disease index) / CK Disease index] × 100.

Effect of ZL2-70 against *Verticillium dahliae* by field experiment

The field with cotton planted for 2 years was selected at Agricultural University of Hebei. The spore suspension of *V. dahliae* V-190 was sprayed on young seedling of these cottons. 60 h later, these cottons were treated twice in a row with the fermented liquid of ZL2-70 (10^6/mL) diluted to 5 times volume as Group 1, treated with the 50% carbendazol wettable powder diluted to 500 times as Group 2 (a medicine control), and treated with the water as Group 3 (a blank control). There were 20 m^2 in each group, with 3 replicates. The incidence of cotton wilt disease was investigated. The disease index and the control efficacy were calculated.

Morphological identification of ZL2-70

The observation of colonial morphology: some thalli were selected from the slant of the isolated bacteria with inoculation loop, the thalli were put into a tube with 10.0 mL sterile distilled water, and then the tube was shaken. 1 mL was taken into another tube with 9 mL sterile distilled water; it was diluted into a variety of concentrations of bacterial suspension. 0.1 mL of the diluents with two gradient concentrations of 10^{-4}, 10^{-5} were coated on the plate of NA medium, respectively, and cultured invertly for 24 h at 30°C. Some thalli of the isolated bacteria were selected from the slant for gram stain or spore staining, and the staining method was described in the "Manual of System Determinative Common Bacteriology"

(Dong and Cai, 2001; Shen *et al*., 1999). The morphological characteristics of thalli and spores were observed by microscope.

Physiologic and biochemical identification

According to the "Manual of System Determinative Common Bacteriology" (Dong and Cai, 2001), the antagonistic bacteria were identified based on many physiologic biochemical tests (Table 2) such as nitrate reduction, indole test, hydrogen sulfide, starch hydrolysis, and citrate test.

Extraction of the genomic DNA and PCR reaction

The genomic DNA of the antagonistic bacteria was extracted (Sambong *et al*., 1995; Rainey *et al*., 1996). The quality of DNA was detected by 1% agarose electrophoresis. The concentration of the DNA was measured by the BIO-RAD Quantity One software. Primers are the universal primer (Lane, 1991), forward primer is 27F: 52 -AGAGTTTGATCCTG GCTCAG-32 and reverse primer is 1495R: 52 - CTACGGCTACCTTGTTACGA-32 . They are respectively located at 8–27 bp and 1495–1514 bp of 16S rRNA gene (the *Escherichia coli* as a standard). The PCR reaction system was as follows: DNA (70 ng/µL) template 2 µL; dNTP Mixture (2.5 mmol/L) 3.5 µL; 27F (20 µmol/L) 1.0 µL; 1495R (20 µmol/L) 1.0 µL; 10 × ExTaq Buffer (Mg^{2+} pluse) 5 µL; ExTaq DNA Polymerase 0.5 µL; and ddH_2O 50 µL. The PCR amplification conditions were as follows: denature at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min and at 72°C for 3 min, with the final extension at 72°C for 5 min. After purification, the PCR products were sent for sequencing (Sagon Company).

16S rRNA gene sequence analysis and phylogenetic tree construction

Resemblance analysis of the 16S rRNA gene sequence was done through the GenBank database using the BLAST method. Multiple alignments were carried out among the sequences with high resemblance in the Clustal X (1.8) program. Finally, a multiple alignment array was established, with gaps instead of lines, and a phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987).

Extraction and characterization of antagonistic substances produced by LZ2-70

Antagonistic bacteria LZ2-70 strain was inoculated into the 250 mL erlenmeyer flask with 50 mL NB fermentation medium, which was shaken in rotary vibrator for 48 h at 30°C at 180 r/min. Fermentation broth was centrifuged at 4°C at 8000 r/min for 5min, supernatant was treated as follows (Dong and Cai, 2001; Lane, 1991): 1) sterile membrane filter (0.22 µm) was used to remove the thalli; 2) non-thalli supernatant was treated at 100! water bath for 30 min; 3) non-thalli supernatant was mixed with the same volume of chloroform, static stratified after shaking for 60 min, taking the upper phase liquid. 4) the supernatant fluid was added with ammonium sulfate to 80% saturation, 4! static overnight, centrifuged for 10 min at 4! at 8000 r/min to collect the precipitation, precipitation was dissolved using 0.1 mol/L pH 7.5 phosphate buffer solution, then dialysised using the same buffer (8000 D) for 2 days, 1% $BaCl_2$ was used to test dialysis effect, freeze-drying after hemodialysis completely, dissolved with Tris-HCl buffer solution, made into 3g/L antifungal protein solution, finally sterile membrane filter (0.22 µm) was used to remove the solid impurities. 200 µL of the above 5 solutions was used to determine antifungal activity with tube plate method (Lane, 1991).

Optimization conditions for LZ2-70

Inclined plane strain LZ2-70 was inoculated into 250 mL erlenmeyer flask with 50 mL seed medium, which was shaken in rotary vibrator for 16 h at 30°C at 180 r/min. Fermentation media was prepared according to the experimental needs into 250 mL erlenmeyer flask (50 mL each flask). Carbon, nitrogen sources and inorganic salt fermentation conditions were studied by single factor experiment method. 4% liquid strain was inoculated into fermentation medium, then shaken in rotary vibrator for 48 h at 30°C at 200 r/min. Fermentation broth was filtrated with sterile membrane filter (pore size of membrane filter is 0.22 µm) in order to remove the thalli, the antifungal activity of the supernatant fluid was determined. The antifungal activity of fermentation protein can be characterized by the size of the bacteriostatic circle.

RESULTS

Preliminary screening for the antagonistic bacteria

Total 891 strains were isolated from soil samples, Most of the strains morphological

characteristics were gray, a small number of yellow, opaque colonies, ranging in size, flat, dry surface, folds, showed a crater-like, radial or irregular colonies neat edge. 83 strains with larger diameter of the inhibition zone and stronger antifungal activity than the other strains were obtained through preliminary screening (figure 1A).

Secondary screening for the antagonistic bacteria

20 strains with better antagonistic activity than the other strains were obtained from the secondary screening (figure 1B). The diameter of inhibition zone for 4 strains were more than 16 mm, the growth of cotton wilt pathogen was effectively inhibited. The diameter of the inhibition zone for strain ZL2-70 reached 19 mm, strain ZL2-70 had the highest antagonistic effect on *V. dahliae* V-190 among the strains investigated.

Control effect of ZL2-70

The result of basin culture trials (table 2) showed that, compared with the blank control, the treatment with ZL2-70 significantly reduced the disease index, with 76.37% control efficacy, indicating a higher certain preventive effect on cotton wilt disease than that of 50% carbendazol wettable powder diluted to 500 times. The result of the plot trials (table 2) showed that ZL2-70 had a highly visible preventive effect on cotton wilt disease, with 79.07% control efficacy higher than the 50% carbendazol wettable powder diluted to 500 times.

From the growth condition of cotton, plants treated by ZL2-70 grew more vigorously. The strain ZL2-70 would secrete certain materials prompting plant growth. The mechanism needs further research

Colonial and thallus morphology of the strain ZL2-70

The single colony of the antagonistic strain LZ2-70 on NA medium plate was circular, white and opaque with regular neat edges, and thick and moist uneven and wrinkly surface. Furthermore, there were Crater-like protuberances in the middle (figure 2A). Cells of strain LZ2-70 were rod-shaped under the microscope after staining at about 2.0-2.5 µm length and 0.7-0.9 µm width while the gram stain showed positive. The thallus formed ellipsoidal and swelled spores, and then budded from the middle, length 1.2 ¼m, width 0.7 µm (figure 2B). The test of motility and metachrome showed positive results while the acid-fast stain showed negative results.

Physiologic and biochemical identification of LZ2-70

Physiological and biochemical identification of the strain LZ2-70 was shown in Table 3. Strain LZ2-70 was identified as *Bacillus*

Table 1. Cotton wilt disease classification standard

Disease level	Degree	Numeric value
1	No symptoms	0
2	Leaves with small brown spots, l-3	1
3	Leaves with brown spots, 4-8	2
4	Leaves with brown spots,9 or more	3

Table 2. The control effect of ZL2-70 against *V. dahliae*

Treatment	Treatment pattern	*P* = 0.05	Total number	Diseased plant	Disease index	Control efficacy (%)
Basin culture trials						
ZL2-70	spray	a	60	13	16.85	76.37
Carbendazol wettable powder	spray	a	60	15	17.82	75.00
Water	spray	b	60	55	71.30	-
Plot trials						
ZL2-70	spray	a	120	9	7.50	79.07
Carbendazol wettable powder	spray	a	120	14	28.13	78.51
Water	spray	b	120	43	35.83	-

Note: "-" means no control effect.

(Dong and Cai, 2001; Buchanan and Gibbons, 1984).

16S rRNA gene sequence analysis of strain ZL2-70

The sequence length of 16S rRNA gene of the strain LZ2-70 is 1469 bp. The resemblance analysis was carried out between this sequence and other bacterial 16S rRNA gene sequences using the BLAST program. Strain LZ2-70 and the other nine strains together formed a cladogram according to resemblance. Based on the results shown in figure 3, the LZ2-70 strain was preliminarily identified as *Bacillus*. Furthermore, the similarity between the LZ2-70 strain and the strain AJ276351 of *B. subtilis* was 99.78%. According to its morphological characteristics, physiologic and biochemical characteristics and the 16S rRNA gene sequences analysis, the strain LZ2-70 was identified as *B. subtilis*. The 16S rRNA gene sequences of strain LZ2-70 has been submitted to GenBank, accession number EU336956.

Characterization of antagonistic substance produced by LZ2-70

According to Cylinder-Plate Method antagonistic test, the ammonium sulfate precipitate of strain LZ2-70 fermentation broth was found to exhibit antifungal effect on pathogenic bacteria, but no antifungal activity was found in the chloroform extracts of the fermentation broth filtrate. The proceeds of ammonium sulfate precipitate can withstand 100°C, 30 min heat treatment. From all above, it can be confirmed that the antifungal substances in the strains of *B. subtilis* LZ2-70 fermentation broth are proteins.

Effect of carbon sources on yield of antimicrobial protein produced by LZ2-70

Different carbon sources at 1% concentration were added to fermentation medium, then fermentation to produce antifungal protein test was implemented. All results were shown in figure 4. In fact, the highest activity of antifungal protein in the fermentation broth will be appeared when soluble starch as carbon source. Each treatment of the following tests was repeated three times, the value of the inhibition zone diameter expressed as: mean ± standard deviations.

Effect of nitrogen sources on yield of antimicrobial protein produced by LZ2-70

On the basis of 1% soluble starch as carbon source, different nitrogen sources at 1% concentration were added to fermentation medium, then fermentation to produce antifungal protein

Table 3. Results of physiologic and biochemical identification of the strain LZ2-70

Item	Result
Culture in broth medium (pH 5.7)	+
Anaerobic growth	-
Catalase test	+
Cellulose decomposition	-
Tyrosine hydrolysis	-
Anti-lysozyme	+
Starch hydrolysis	+
Nitrite reduction	+
NNitrate reduction	-
Citrate utilization	+
Gelatin liquefaction	+
Methyl red	-
Litmus	A
Fluorochrome	+
Tryptophan deaminase	-
V.P determination	+
pH of V.P determination	6.12
Casein hydrolyzate	+
Indole	+
Phenylalanine deaminase	-
Lecithinase	-
Malonic acid utilization	-
Salt tolerance	
2%	+
5%	+
7%	+
10%	+
12%	-
Indole	-
Catalase	+
Carbon sources utilization	
Glucose	+
Mannitol	+
Lactose	-
Growth temperature	
5°C	-
10°C	+
20°C	+
30°C	+
40°C	+
50°C	-
Tartrate utilization	
Denitrification test	
Urease	-
Lipase test	+

Note: "+" means positive while "-" means negative

Table 4. Designation and result analysis of medium orthogonal experiment

Number	A Soluble starch	B Peptone	C $MnCl_2$	D KCl	Antifungal circle diameter /mm
1	0.5	0.5	0.01	0.01	12.25 ± 0.35
2	0.5	1.0	0.03	0.02	19.75 ± 0.35
3	0.5	2.0	0.05	0.05	15.75 ± 0.35
4	0.5	3.0	0.07	0.10	14.50 ± 1.12
5	1.0	0.5	0.03	0.05	15.00 ± 0.00
6	1.0	1.0	0.01	0.10	14.75 ± 1.77
7	1.0	2.0	0.07	0.01	14.25 ± 0.35
8	1.0	3.0	0.05	0.02	15.25 ± 1.06
9	2.0	0.5	0.05	0.10	16.25 ± 0.35
10	2.0	1.0	0.07	0.05	14.25 ± 1.06
11	2.0	2.0	0.01	0.02	13.25 ± 1.77
12	2.0	3.0	0.03	0.01	16.00 ± 2.12
13	5.0	0.5	0.07	0.02	16.25 ± 1.77
14	5.0	1.0	0.05	0.01	16.50 ± 1.41
15	5.0	2.0	0.03	0.10	18.25 ± 1.06
16	5.0	3.0	0.01	0.05	16.75 ± 0.35
K1	15.56	14.94	14.25	14.75	
K2	14.81	16.31	17.25	16.13	
K3	14.94	15.38	15.94	15.44	
K4	16.94	15.63	14.81	15.94	
R	2.13	1.37	3.00	1.38	
Optimal combination		A4B2C2D2			

K stands for average value, R stands for Standard Deviation

Table 5. Designation and result analysis of culture condition orthogonal experiment

Number	A Fermentation time/h	B Liquid volume of shaking bottle /mL	C Inoculation volume/%	D pH	Antifungal circle diameter /mm
1	24	30	2	6.0	17.25 ± 1.77
2	24	50	4	7.0	17.00 ± 0.71
3	24	750	8	8.0	17.25 ± 2.48
4	24	100	10	9.0	16.75 ± 1.06
5	48	30	4	8.0	21.25 ± 1.06
6	48	50	2	9.0	17.75 ± 0.35
7	48	75	10	6.0	17.75 ± 1.77
8	48	100	8	7.0	19.25 ± 0.35
9	72	30	8	9.0	19.75 ± 1.06
10	72	50	10	8.0	22.25 ± 1.77
11	72	75	2	7.0	20.50 ± 1.41
12	72	100	4	6.0	19.00 ± 1.41
13	96	30	10	7.0	23.25 ± 1.06
14	96	50	8	6.0	21.00 ± 1.41
15	96	75	4	9.0	20.75 ± 2.48
16	96	100	2	8.0	19.00 ± 1.41
K1	17.06	20.38	18.63	18.75	
K2	19.00	19.50	19.50	19.93	
K3	20.38	19.06	19.31	19.94	
K4	21.00	18.50	20.00	18.75	
R	3.94	1.88	1.37	1.19	

Optimal combinationA3B1C4D3

K stands for average value, R stands for Standard Deviation

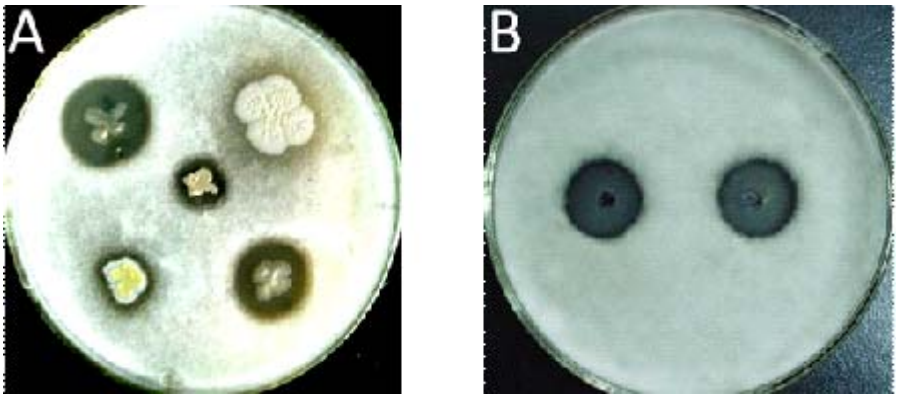
test was implemented. All fermentation results were shown in figure 5. The ability of strain LZ2-70 to use organic nitrogen is generally better than the use of inorganic nitrogen and the highest activity of antifungal protein in the fermentation broth was detected when peptone acted as the nitrogen source.

Effect of different inorganic ions on yield of antimicrobial protein produced by LZ2-70

In order to investigate the influence of inorganic ions on the production of antifungal proteins, different types of salts at 0.02% concentration were added to fermentation medium when fermentation tests were conducted. The results shown in figure 6 indicated that antifungal proteins showed higher activity in the presence of Mn^{2+}, K^{+}, while Zn^{2+} and Cu^{2+} have a strong inhibitory effect on the production of antifungal proteins. So we choose Mn^{2+}, K^{+} as the best inorganic salt components in the fermentation medium.

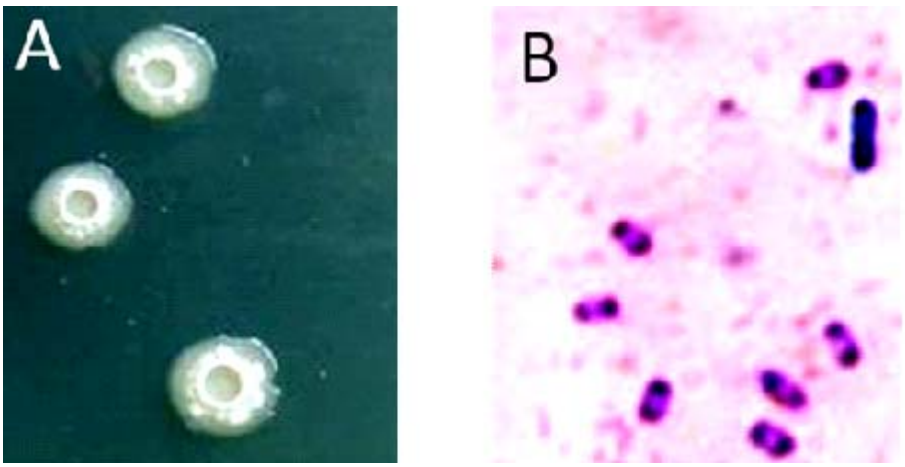
Orthogonal test to optimize the medium composition

On the basis of all these single-factor test results, the soluble starch, peptone, inorganic salts $MnCl_2$and KCl were selected as four factors to design four-level orthogonal test (Table 6). It can be inferred that $MnCl_2$ has the main impact, soluble starch being the second, based on visual analysis of the range R of $MnCl_2$> R of soluble starch >R of peptone> R of KCl in orthogonal experiment results. As seen in table 4, antifungal proteins with highest



A: Preliminary screening B: Secondary screening

Fig. 1. Antagonistic effect of isolated bacteria strains against the pathogen



A: Colony morphology on NA medium B: Morphological shapes of thallus and spore

Fig. 2. Colonial and thallus morphology of the strain ZL2-70

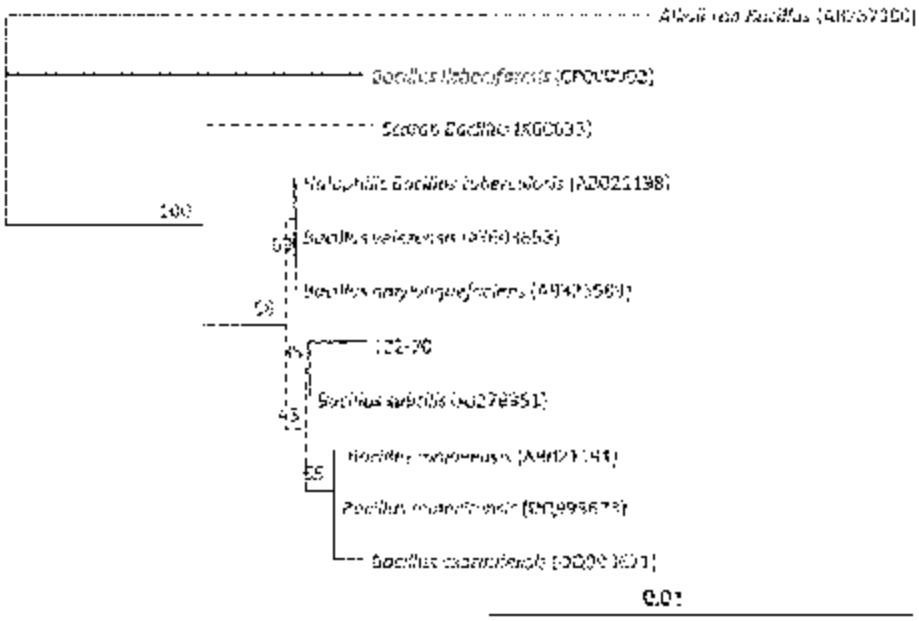


Fig. 3. Phylogenetic tree of the 16S rRNA gene sequence of LZ2-70 strain and related strains

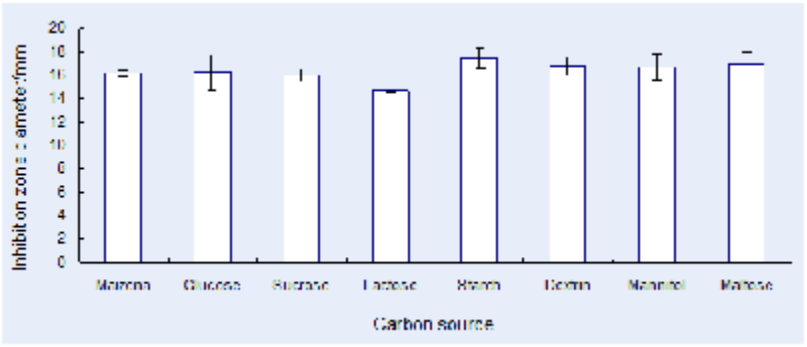


Fig. 4. Effect of different carbon sources on antifungal protein production of LZ2-70

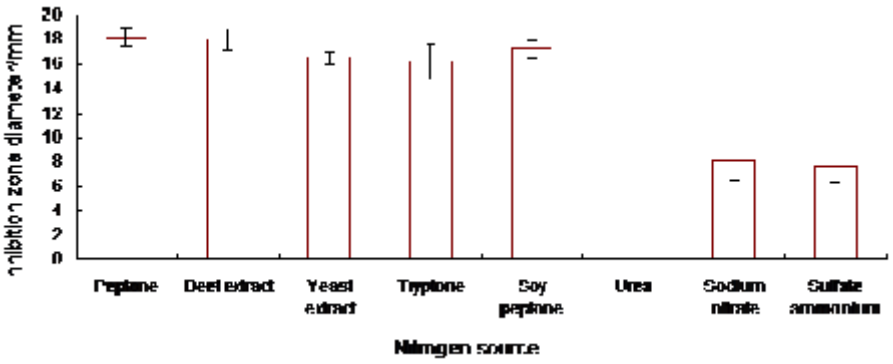


Fig. 5. Effect of different nitrogen sources on antifungal protein production of LZ2-70

activity were found in the 2nd test combination and the best combination A4B2C2D2 is obtained by the analysis of mean K. Because of no such combination in the orthogonal table, the re-test between combination A4B2C2D2 and 2nd is verified. The inhibition zone diameter of combination A4B2C2D2 is 22.4 mm, while the No. 2 combination is 20.0 mm. Therefore the optimal medium composition of strain LZ2-70 was determined: 5% of the soluble starch, peptone 1%, 0.03% of $MnCl_2$ and KCl (0.02%).

Effect of fermentation conditions on yield of antimicrobial protein produced by LZ2-70

Four factors and four levels of orthogonal test to optimize the fermentation time, inoculum size, initial pH, liquid volume. The experiment results are shown in Table 5.

From table 5, it can be seen that the antimicrobial protein with the highest activity is in the 13th test combination and the best combination is A4B1C4D3 by the analysis of mean K. The results of re-test validation between the combination A4B1C4D3 and 13th test combination showed that the inhibition zone diameter of A4B1C4D3 is 23.5 mm while 13 the combination (A4B1C4D2) diameter is 22.5 mm. Therefore, the optimization fermentation conditions for the fermentation time of 96 h, bottling capacity of 250 mL flask 30 mL, inoculum size 10%, initial pH value of 8.0.

Effect of rotation speed and incubation temperature on yield of antimicrobial protein produced by strain LZ2-70

Under the conditions of initial pH 8.0, 250 mL flask of 30 mL, 10% of the inoculum size at 30°C, the different fermentation tests of strain LZ2-70 were implemented with the speed 150, 180, 200, 220 r/min for 96 h, respectively. According to antagonist activity measurements of protein in the fermentation broth, it is confirmed that the rotation speed 200 r/min is the best speed rotation speed (figure 7).

On the basis of the fermentation

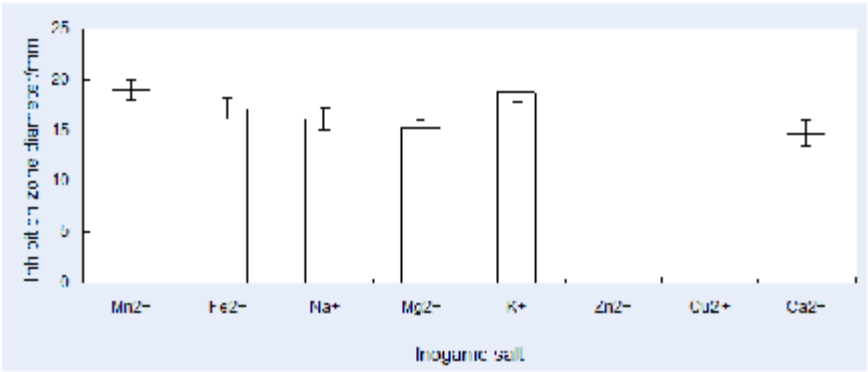
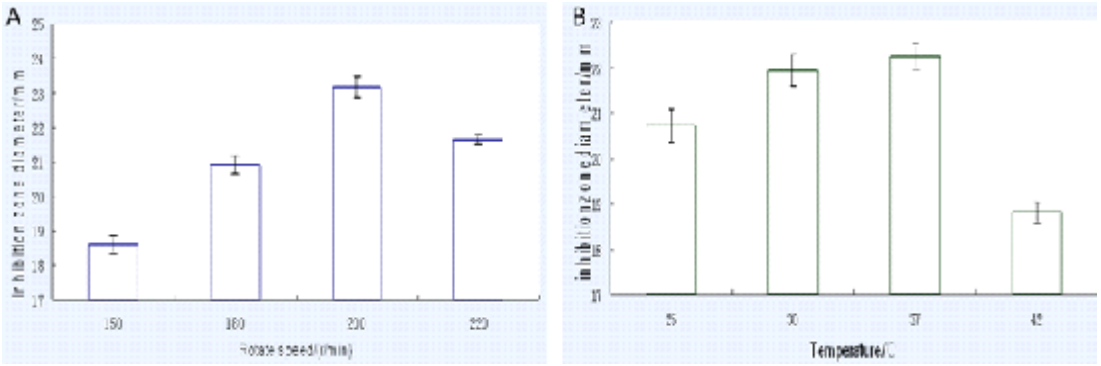


Fig. 6. Effect of different inorganic salts on antifungal protein production of LZ2-70



A: Rotate speed B: culture temperature

Fig. 7. Effect of different rotate speed and culture temperature on antifungal protein activity of LZ2-70

conditions of initial pH 8.0, 250 mL flask of 30 mL, 10% of the inoculum size, fermentation temperature at 30°C and speed of 200 r/min, the different fermentation tests of strain LZ2-70 were implemented at 25°C, 30°C, 37°C and 42°C for 96 h, respectively.

According to antagonist activity measurements of protein in the fermentation broth, it is shown that the highest activity of antagonistic protein in the fermentation broth will appear at 37°C. So the optimum fermentation temperature is determined to be 37°C.

DISCUSSION

In order to reduce the harmful effects imposed on the ecological environment and human health by chemical fungicide and pesticides, researchers around the world began to seek for safe and more effective alternative products. The application of biological control microbes and their secondary metabolites, antibacterial activity of plant material, phytoalexin, antifungal protein and the efficient, broad-spectrum, lower toxic, less-persistent chemicals, as widely concerned hot issues, are especially worth investigating (Chen and Huang, 2003). *Bacillus* is the predominant microorganism in the microecology of soil and plants because it can produce endogenous spores, with very strong biotic potential for industrial production, disease resistance and stress tolerance. Furthermore, it is a nonpathogenic bacterium widely existing in the nature, harmless to humans and animals without polluting the environment. Many natural isolated strains with excellent traits have been successfully applied to the biological control of plant diseases.

It had been confirmed that many fungi or bacteria could produce peptide or protein material that are biologically active (Sivapalan, 1993). Bacillus is a successful microbial population in soil and plant micro ecology, it can produce endogenous spores, rapid micropropagation and industrial production is easy to realize. In addition, they have a strong resilience and antibacterial action against diseases. They are non-pathogenic bacteria widely existing in nature, harmless to human and livestock, and do not pollute the environment. Therefore, since 1945, Johnson reported *B. subtilis* could produce antifungal material, for nearly half a century, researchers from various countries have showed great interest and attention to the bacteria due to its potential to become a new biological control factor. Many excellent natural strains have been successfully isolated and used for the biocontrol of plant diseases (Zhang, 2001). They are the dominant microbial populations in soil and plant micro-ecological, so is relatively easy for the colonies to take hold in soil, which is a very important factor in the production of in antagonistic probiotics.

In this study, an antagonistic bacterium named LZ2-70 was identified as *B. sublitis* with a strong antifungal activity. It has a high application value in plant disease for biological control. Therefore, further research should be carried out, such as the research on bacteriostatic mechanism, using genetic engineering methods to build more efficient biological control strains. Overall, it demonstrated that LZ2-70 is a successful biocide strain for controlling cotton wilt disease, a wide-spread disease around the world. Further investigation would concentrate on the practice of large-scale industrial production, such as the cost and fermentation conditions, etc.

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