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Short Report

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Isolation and Identification of Antarctic Microorganisms with Activity against *Pseudomonas aeruginosa* and Characterization of Their Antibacterial Mechanism

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Abstract

Pseudomonas aeruginosa (PA) is a highly pathogenic and multidrug-resistant bacterium and one of the common pathogens responsible for nosocomial infections. Therefore, safe and effective preventive and therapeutic approaches were urgently needed. In this study, a strain named TJ31 was isolated from sediment samples collected from Antarctic Lake Tuanjie, demonstrated potent antibacterial activity against PA and was subsequently identified as *Bacillus velezensis*. To maximize the antibacterial activity of TJ31, single-factor experiments and response surface methodology were used to determine the optimal medium and fermentation conditions for this strain. Subsequently, a series of experiments were conducted to investigate the antibacterial mechanism of the TJ31 crude extract. The alkaline phosphatase (AKP) assay and protein leakage assay demonstrated that the crude extract effectively disrupted the cellular integrity of PA, a finding further corroborated by scanning electron microscopy (SEM) analysis. TJ31 crude extract inhibited respiratory chain dehydrogenases in PA and reduced the membrane potential of PA cells, and impaired cellular metabolic activity. Furthermore, the crude extract downregulated the expression of virulence-associated genes, effectively inhibited twitching motility, weakened the colonization ability of PA, disrupted biofilm formation, reduced the production of pyocyanin by PA, and lowered its pathogenicity.

Keywords: PA; Antarctic microorganism; *Bacillus velezensis*; antagonistic mechanism

1. Introduction

Infectious diseases are among the top three global causes of mortality, while antibiotic misuse has promoted widespread drug resistance in pathogenic bacteria, intensifying their threat to public health[1]. *Pseudomonas aeruginosa* (PA), a highly contagious bacterium, was first isolated from wound exudate by Gessard (1882)[2]. As a Gram-negative bacterium, PA exhibits high environmental adaptability, potent infectivity, significant virulence, and a high propensity for nosocomial transmission [3,4]. Additionally, PA exhibits intrinsic antibiotic resistance and can develop enhanced

drug resistance through the acquisition of resistance genes via mutation, resulting in continuously rising rates of both clinical isolation and antimicrobial resistance [5,6]. Among all Pseudomonas species, *Pseudomonas aeruginosa* is the most important species affecting humans and is responsible for serious debilitating and life-threatening infections[7].Consequently, developing novel therapeutic approaches against PA represents an urgent global priority.

Polar regions are characterized by unique geographical conditions featuring extreme aridity, prolonged freezing temperatures, and high-intensity UV radiation. To survive these extremes, polar microorganisms have evolved specialized physiological adaptations and often biosynthesized unique bioactive metabolites [8,9].Kim M K et al. isolated *Ochrolechia sp*. from Arctic lichens, with subsequent antibacterial assays revealing inhibition zones of 8–12 mm diameter against six common clinical pathogenic bacteria, including PA[10]. Florent Magot et al. isolated 77 microbial strains from a depth of 2454 m in the Arctic Ocean seabed, with most strains exhibiting certain antibacterial activity against MRSA and *Enterococcus faecium*[11]. Zhang et al. also found that the *Chrysogenum* CCTCCM 2020019 strain isolated from Antarctic penguin feces has broad antibacterial activity[12].

Given this context, Antarctic microorganisms are hypothesized to synthesize unique antimicrobial compounds that differ structurally and functionally from conventional antibiotics, presenting significant potential for the discovery and development of innovative therapeutic agents against PA. This study aimed to isolate a bacterium from Antarctica and evaluate the inhibitory activity and mechanism of action of *Bacillus velezensis* TJ31 against PA. We believe that this research will provide a theoretical basis for inhibiting PA.

2 Material and Methods

2. 1 Isolation and purification of Antarctic microorganisms

Sediments for single colony isolation were collected from Antarctic Lake Tuan Jie. Strain isolation used the dilution spread plate method[13]. 0.5 g of sediment from Antarctic Lake Tuan Jie was added to a test tube with 4. 5 mL of physiological saline. The mixture was mixed thoroughly and allowed to stand for 2 hours. Subsequently, 0. 5 mL of the supernatant was transferred to another tube with 4. 5 mL of physiological saline. Serial dilutions generated seven different concentration gradients. For each gradient, 100μL of diluted suspensions (ranging from $10^{-6}$to 10^{-2}) were spread onto plate media. Inoculated petri dishes were incubated at 12°C and 37°C for 5–6 days in constant-temperature incubators. Bacterial colonies with distinct morphologies were selected for purification by the streak plate method, which was repeated 2–3 times. Finally, purified single colonies were preserved using the glycerol preservation method for further study.

2. 2 Screening of PA-resistant strain

A double-layer agar well diffusion method was used for preliminary screening of Antarctic strains with antagonistic activity against PA. The lower layer was LA medium. After mixing 100μL of PA culture (at log phase, 1×10^{8}CFU/mL) with LA medium, the mixture was poured as the upper layer. A 12-mm puncher was used to create wells through the upper medium, and 150μL of 24-hour Antarctic microbial fermentation

broth was added to each well. Plates were co-cultured at 37°C for 12 hours to observe the formation of inhibition zones.

Strains with initial PA-inhibitory activity were re-screened using the agar diffusion method[14]. Briefly, 100μL of the inhibitory strain was inoculated into an LB medium and cultured at 37°C for 24 hours. The fermentation broth was centrifuged at 8000 rpm for 10 minutes at 4°C, and the supernatant was concentrated via rotary evaporation until completely dried. The concentrate was filtered through a 0. 22 μm sterile microfiltration membrane to obtain crude fermentation broth[15]. Next, 100μL of PA culture (log phase, 1×10^8CFU/mL) was mixed with 40 mL of LA medium, poured into plates, and allowed to solidify. Wells (12 mm) were punched into the medium, and 300μL of TJ31 crude extract was added to each well. Plates were incubated at 37°C for 12 hours, and the diameter of inhibition zones was measured using a vernier caliper.

2. 3 Inhibition Spectrum of the Antagonistic Strains

Eight typical pathogenic bacteria were chosen to conduct the tests in the present research (Table 1). All bacterial strains are preserved at the School of Life Sciences, Dalian Minzu University, China.The ability to inhibit bacterial pathogens was tested according to the method described in Section 2. 2.

Table 1 PAthogens used for inhibition spectrum research

PAthogen	Type of culture medium
Staphylococcus aureus	LB Broth
Escherichia coli	LB Broth
Vibrio Parahaemolyticus	LB Broth
Candida albicans	LB Broth
Enterococcus faecium	LB Broth
Salmonella typhimurium	LB Broth
Klebsiella Pneumoniae	Nutrient Broth
Acinetobacter baumannii	Brain Heart Infusion

2. 4. Identification of PA-resistant strain

The 16S rDNA sequence was amplified using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The cycling parameters were as follows: (1) initial denaturation at 94 °C for 5 min; (2) 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min; and (3) final extension at 72 °C for 10 min[16].The PCR products were sent to Majorbio Technology Pharmaceutical Co., Ltd. (Shanghai, China) for sequencing. The obtained sequences were submitted to the NCBI database to determine the putative identity of the strain, and a polygene phylogenetic tree was constructed in MEGA 11.0.

2.5 Screening for Optimal Ingredients in Fermentation Medium

To evaluate the effects of different types and concentrations of carbon sources, nitrogen sources, and inorganic salts on the antibacterial activity of TJ31 against PA, the composition of LB medium was modified. Control groups consisted of LB medium formulations specifically lacking either carbon sources, nitrogen sources, or inorganic salts. The tested carbon sources included starch, lactose, sucrose, mannitol, and glucose.

The tested nitrogen sources included ammonium sulfate, tryptone, peptone, potassium nitrate, and beef extract. The tested inorganic salts included NaCl, $MgSO_4$, K_2HPO_4, $CaCl_2$, KCl, and KH_2PO_4. Subsequently, for each selected component type (carbon source, nitrogen source, inorganic salt), the effect of varying its concentration on the antibacterial activity was assessed using the agar well diffusion assay described in Section 2.2. Details of the experimental design for component screening are provided in Table 2.

Table 2 Screening for Optimal Ingredients in Fermentation Medium

Optimization Factor	Experimental Operation	Tested Substances	Concentration (g/L)	Control Group
Carbon Source	Type Screening	Starch, Lactose, Sucrose, Mannitol, Glucose	5	LB without Yeast Extract
	Concentration Optimization	Best carbon source	2, 4, 6, 8, 10	—
Nitrogen Source	Type Screening	Ammonium sulfate, Tryptone, Peptone, Potassium nitrate, Beef extract	10	LB without Tryptone
	Concentration Optimization	Best nitrogen source	6, 8, 10, 12, 14	—
Inorganic Salts	Type Screening	NaCl, $MgSO_4$, K_2HPO_4, $CaCl_2$, KCl, KH_2PO_4	5	LB without NaCl
	Concentration Optimization	Best inorganic salt	2, 4, 6, 8, 10	—

2.6 Single-Factor of Fermentation Parameters on the Antibacterial Activity of TJ31

An effective composition of culture medium would lead to the execution of a single factor experiment in order to determine the influence of changing the fermentation conditions on the inhibitory behavior of TJ31. The initial conditions were defined as such: quantity of inoculation of 100 μL/mL volume of 100 mL bottle, duration of fermentation of 24 h and temperature of 37℃. In order to learn the influences of individual factors it was altered, and all others were kept constant. The test conditions were: amount of inoculation: 80 μL/mL、100 μL/mL、120 μL/mL、140 μL/mL、160 μL/mL, volume of bottling: 50 mL, 75 mL, 100 mL, 125 mL and 150 mL;; temperature: 33 ℃, 35 ℃, 37 ℃, 39 ℃ and 41 ℃; time: 12 h, 24 h, 36 h, 48 h, 60 h. The antibacterial activity was measured using the experimental process given

in section 2.2

2.7 Response Surface Optimization Test

The four most critical factors that will be optimized based on the findings of the single-factor test are bottling volume (A), inoculation amount (B), temperature (C) and time (D). Response surface optimization was planned with Box Behnken method which was done with the Design Expert 13 software as per table 3.

Table 3 Factors and levels of Box–Behnken tests.

Levels	Factors			
	A Bottling volume (mL)	B inoculation amount	C Temperature (℃)	D Time(h)
-2	50	80	33	12
-1	75	100	35	24
0	100	120	37	36
1	125	140	39	48
2	150	160	41	60

2. 8 Preparation and Separation of Antibacterial Extracts

The TJ31 strain was inoculated into 100 mL LB medium at a 0. 13%(v/v) inoculation rate and incubated in a shaker (180 rpm) at 37℃ for 41 h. After incubation, the bacterial suspension was centrifuged at 8, 000 rpm for 10 minutes at 4℃and the supernatant was collected as a sterile filtrate. This aseptic filtrate was then extracted three times with ethyl acetate, and the extracts were concentrated using a rotary evaporator (R-100, Buqi, Switzerland).

2. 9 Determination of Minimum Inhibitory Concentration (MIC) of TJ31 against PA

The broth microdilution technique of the American Clinical Laboratory Standards Institute (CLSI) [17] was used to determine the MIC of crude extract against PA. In particular, 100ul LB medium was introduced in each of the wells (96-wells plate) and 100 ul crude extract (200mg /ml) was introduced in the first well. Two-fold serial dilutions were applied to produce a concentration range of 50 mg/ml to 0.024 mg/mL. Then 100uL of active PA (1x108 CFU/mL) were added to the wells with different concentrations of crude extract and 12 experimental groups with three replicate each group were prepared. Positive controls were PA grown in LB medium without addition of crude extract and negative controls were the medium with crude extract of different concentrations without PA. The sample was incubated in a constant temperature incubator at 37 degrees Celsius over 24 hours. MIC was defined as the smallest volume of crude extract, wherein no bacterial growth was found either pre-incubation or post-incubation through gentle shaking of the plate.

2. 10 Effects of Crude Extract on AKP Content in Culture Medium

The crude extract was included in LB broth in the concentrations of 1/2 MIC, 1 MIC, and 2 MIC until inoculation. Three replicate samples were made on every concentration. The control (0 MIC) was grown in pure LB broth, without crude extract supplementation. After shaking test tubes containing suspensions, they were incubated at 37 ℃ (180 rpm) in a incubator with 8 h. Supernatants were serated after incubation by centrifugation of the samples at 7000 rpm at 4deg C during 10 min. It was measured

by AKP Assay Kit (Jian Cheng Bioengineering Institute, Jiangsu, China) and measured at the absorbance of 520 nm.

2. 11 Effects of Crude Extract on the protein leakage of PA

The cultures were cultivated until the late-logarithmic stage according to the protocol provided in BCA Protein Assay Kit. The bacterial suspensions were placed in sterile tubes and serially exposed to the crude extract (0 MIC, 0.5 MIC, 1 MIC and 2 MIC). Upon incubation, cells were harvested by centrifugation (8,000 rpm, 10 min, 4 °C) and then washed twice with PBS and finally resuspended in PBS buffer. Cell lysis was performed by sonication at low temperatures to avoid protein denaturation. Working BCA reagent (50:1 v/v A: B solution) was newly prered and applied to the lysates which were incubated at 37 °C during 30 min. The absorbance was read with a microplate reader (H1, Berten Instruments Limited, USA) at 562 nm.

2. 12 Effects of Crude Extract on Cell Morphology of PA

The effects of cell morphology were measured by scanning electron microscopy (SEM)[18] The short process is: was grown in LB medium and the crude extract was added to LB medium to obtain final concentrations of 1/2 MIC, 1 MIC and 2 MIC respectively. The control was the sample with 0 MIC. It was then shaken (180 rpm) at 37℃ and incubated further for 4 hours. The centrifugation of the bacterial suspension was done at 8000xg to separate bacteria cells at 10 mins at 4 oC. Bacterial cells were washed with PBS three times before being fixed with 2.5% glutaraldehyde (stored overnight at 4 oC in a fridge). The next step included the dehydration with 25, 50, 75 and 100 percent concentration of ethanol during 10 min each. The samples were afterwards placed on silicon wafers. The conductive coating was put onto the cells which were viewed with SEM (S-4800, Hitachi, Jan)

.2. 13 Effects of the Crude Extract on respiratory chain dehydrogenases of PA

The damage to the PA respiratory chain was assessed by quantifying respiratory chain dehydrogenases activity[8]. Briefly, 1 mL of PA bacterial suspension (1×10^8 CFU/mL) was placed into a test tube, followed by sequential addition of 2 mL of 0. 05 mol/L Tris-HCl buffer (pH 8. 6), 2 mL of 0.1 mol/L glucose solution, and 2 mL of 1 mg/mL TTC solution. After thorough mixing, TJ31 crude extract was added to achieve final concentrations of 0, 1/2 MIC, 1 MIC, and 2 MIC. A sterile water blank was included. Tubes were incubated at 37 ℃for 5 hours, after which 2 drops of concentrated sulfuric acid were added to terminate reactions. Samples were extracted with n-butanol to PArtition products into the supernatant. Following phase separation, the upper layer was collected and centrifuged at 4000 rpm for 10 minutes. Absorbance was measured at 490 nm using n-butanol as a reference.

2. 14 Effects of Crude Extract on the Membrane Potential of PA

The method used to assess the membrane potential was Rhodamine 123 fluorescence staining. Final concentrations of 1/2 MIC, 1MIC, and 2 MIC of crude extract on were achieved by growing them in logarithmic phase, 0 MIC was used as the positive control and sterile water as the negative control. After three hours of inoculation in a shaker (180 rpm) at 37℃, the cultures were centrifuged at 7000 rpm at 4℃ (10 minutes) and cells were collected. The cells were washed with 0. 01 mol/L phosphate buffered saline (PBS, pH 7. 2). A bacterial solution containing Rhodamine

123, which had been prepared as a 1mg/ml solution in 0. 01 mol/L PBS buffer, had a final concentration of 2 g/ml. It was incubated under dark condition at 30 min and then washed and resuspended in 0. 01 mol/L PBS. Mean fluorescence intensity (MFI) was determined with a microplate reader (H1, Berten Instruments Limited, USA) and measured at excitation wavelength of 480 nm and emission wavelength of 530 nm and slit width of 10 nm. Results are presented as MFI.

2. 15 Effects of the crude extract on pyocyanin production of PA

PA cultures were grown to logarithmic phase before supplementation with TJ31 crude extract at final concentrations corresponding to 2 MIC, MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, 1/16 MIC, and control group (0 MIC). Following 24 h incubation at 37℃, pyocyanin was extracted using chloroform. The chloroform phase was transferred to fresh tubes and re-extracted with 2 mL of 0.2 M HCl. The acid aqueous layer was collected, centrifuged (10,000 rpm, 5 min), and the resultant supernatant was analyzed spectrophotometrically at 520 nm. Absorbance at 520 nm showed positive correlation with pyocyanin concentration.

2. 16 Effects of the Crude Extract on the twitching motility of PA

The twitching motility of PA was quantified using the methodology described by Rashid to assess the antibacterial activity of the TJ31 crude extract[19]. LB agar plates (1% agar) served as blank controls, while experimental groups comprised LB medium supplemented with crude extract concentrations of control group (0 MIC), 1/2 MIC, MIC, and 2 MIC. Overnight-grown bacterial colonies were stab-inoculated onto the plate surface with a sterile toothpick, ensuring penetration to the base of the Petri dish. Post-inoculation, plates were incubated at 37℃ for 24 hours. Twitching motility was evaluated by measuring the radial expansion of the diffusion zone at the agar-plate interface.

2. 17 Effects of Crude Extract on PA Biofilm Formation

Crude extract ability to inhibit biofilm formation of was measured using semi-quantitative crystal violet staining technique[20]. Crude extract was introduced in a 24-well plate with , with three replicates at every concentration to get the final concentration of 1/2 MIC, 1 MIC and 2 MIC. Control group (0 MIC) was cultured in the same volume of LB in another 24 well plates. They were afterwards incubated at 37 ℃ after 48 hours incubation time and rinsed with 0.01 mol/L PBS and stained with 1ml methanol. Then, 500ml of 0. 1 percent crystal violet solution was added to all wells containing completely dry biofilms. Due to 30 minutes of dark staining, the excess crystal violet was eliminated with three washes with PBS. They were turned upside down and dried in an oven at 37° C and then 500uL of 33 percent glacial acetic acid was added. They were allowed to shake on the shaker to dissolve the crystal violet. Lastly, 50uL of such solution acquired was put in an empty 96-well plate and measured at 590nm. The calculation of the biofilm inhibition rate was done by using equation[21](1).

$$\text{Inhibition rate (\%)} = OD_{\text{positive control}} - OD_{\text{assay}} / OD_{\text{positive control}} \quad (1)$$

2. 18 Impact of the Crude Extract on Virulence Gene Expression in PA

Virulence genes *algA*, *rhlI*, and *pslA* represent critical determinants of drug resistance and pathogenicity in PA, directly participating in its quorum sensing (QS)

systems[22,23]. PA cultures were propagated to the late logarithmic phase and treated with 1 MIC of the antagonistic strain crude extract (dissolved in methanol) for the experimental group, while an equal volume of methanol served as the negative control. Relative expression levels of QS-associated virulence genes (*algA*, *rhlI*, *pslA*) were quantified in triplicate experiments.

3. Results

3. 1 Isolation and purification of Antarctic microorganisms

A total of 64 Antarctic microbial strains were isolated from sediment samples collected in the Antarctic Lake Tuan Jie. Images of some strains were shown in Fig.1 All isolates were cryopreserved in glycerol stocks.

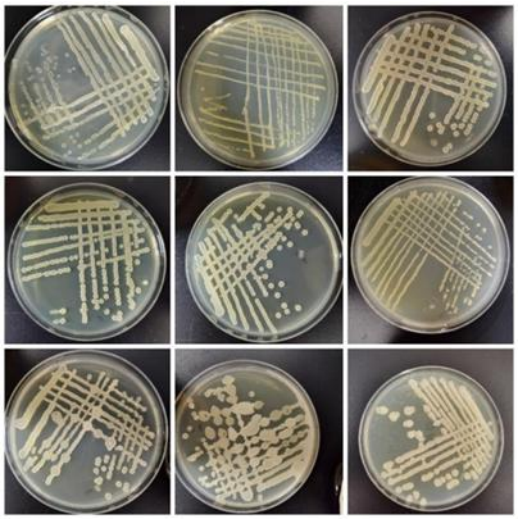


Fig. 1 Partial strain maps obtained through isolation and purification.

3. 2 Screening of PA-resistant strain

Initial screening identified eight strains exhibiting significant anti-PA activity (Fig. 2). Confirmatory screening results are presented in Figure 3 and Table 4. Strain TJ31 demonstrated superior antibacterial efficacy, produced an inhibition zone of 22.3 ± 0.7 mm.

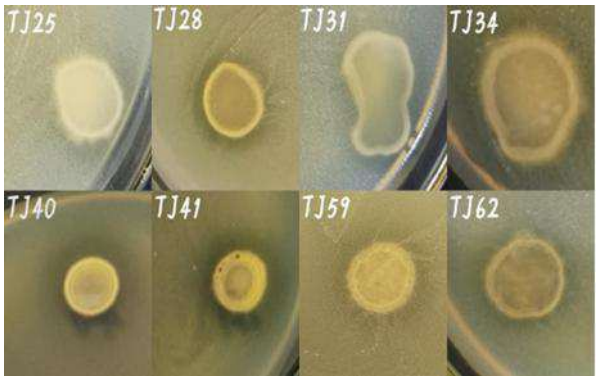


Fig. 2 Preliminary screening results of PA-resistant Antarctic samples

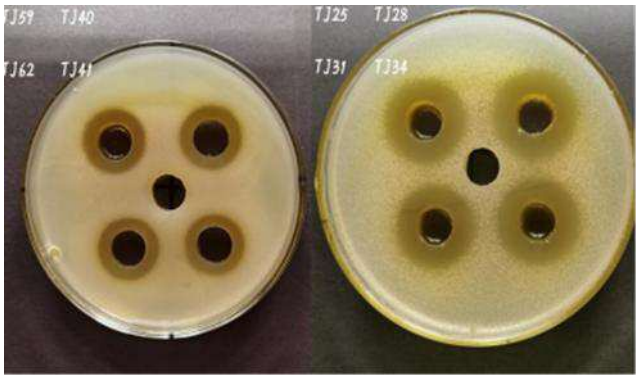


Fig. 3 Rescreening Results

Table 4 Screening results of Antarctic microbial strains against PA.

Strain Number	Inhibition Zone Diameter(mm)	Strain Number	Inhibition Zone Diameter(mm)
TJ25	21. 5±0. 8	TJ40	20. 0±0. 1
TJ28	22. 2±0. 2	TJ41	19. 4±0. 2
TJ31	22. 3±0. 7	TJ59	19. 0±0. 1
TJ34	21. 5±0. 3	TJ62	20. 0±0. 1

3. 3 Identification of a PA-Resistant Strain

The sequence was analyzed in NCBI, and the phylogenetic tree as shown in Fig. 4 indicated that strain TJ31 clustered together with *Bacillus velezensis*, showing the closest genetic relationship. The strain was preliminarily identified as *Bacillus velezensis*.

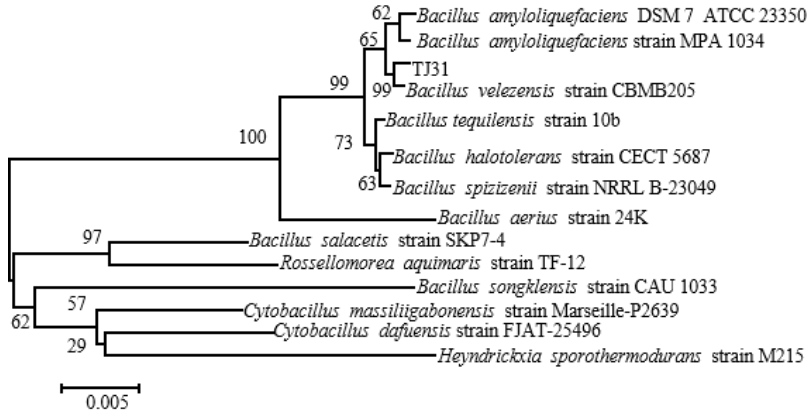


Fig. 4 Phylogenetic tree of strain TJ31.

3. 4 Inhibition Spectrum of the Antagonistic strain

As shown in Table 5, strain TJ31 exhibited varying degrees of inhibition against eight common pathogenic bacteria, indicating a broad antibacterial spectrum. Specifically, strong inhibition was observed against *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*, with inhibition zone diameters of 27.4 ± 0.4 mm, 28.1 ± 1.0 mm, and 27.6 ± 0.3 mm, respectively. In contrast, weaker inhibition was observed against *Acinetobacter baumannii*, *Enterococcus faecium*, and *Candida albicans*, The inhibition zone diameters were 17. 8±0. 7 mm, 18. 1±0. 1 mm and 18. 1±0. 9 mm, respectively.

Table 5 Inhibition spectrum of TJ31.

PAthogen	Inhibition Zone Diameter(mm)	PAthogen	Inhibition Zone Diameter(mm)
Escherichia coli	27. 4±0. 4	*Salmonella typhimurium*	27. 6±0. 3
Staphylococcus aureus	20. 7±0. 1	*Acinetobacter baumannii*	17. 8±0. 7
Candida albicans	18. 1±0. 9	*Enterococcus faecium*	18. 1±0. 1
Vibrio Parahaemolyticus	20. 0±1. 4	*Klebsiella Pneumoniae*	28. 1±1. 0

3.5 Results of Fermentation Medium Components through Systematic Screening

The growth performance of TJ31 varied across different culture media, resulting in differences in the antibacterial activities of its crude extracts against PA. The results demonstrated that variations in medium composition significantly affected the inhibition zone diameter of the TJ31 fermentation solution. Notably, the fermentation medium containing Glucose (Fig. 5A), beef extract (Fig. 5B), and K_2HPO_4 (Fig. 5C) yielded significantly larger inhibition zones against PA compared to other formulations (p<0.05). When the concentrations were 6 g/L (Fig. 5D), 10 g/L (Fig. 5E), and 10 g/L (Fig. 5F), strain TJ31 exhibited maximal antimicrobial activity with zone diameters of 23.08±0.67 mm, 22.87±1.11 mm, and 24.09 ±0.39 mm.

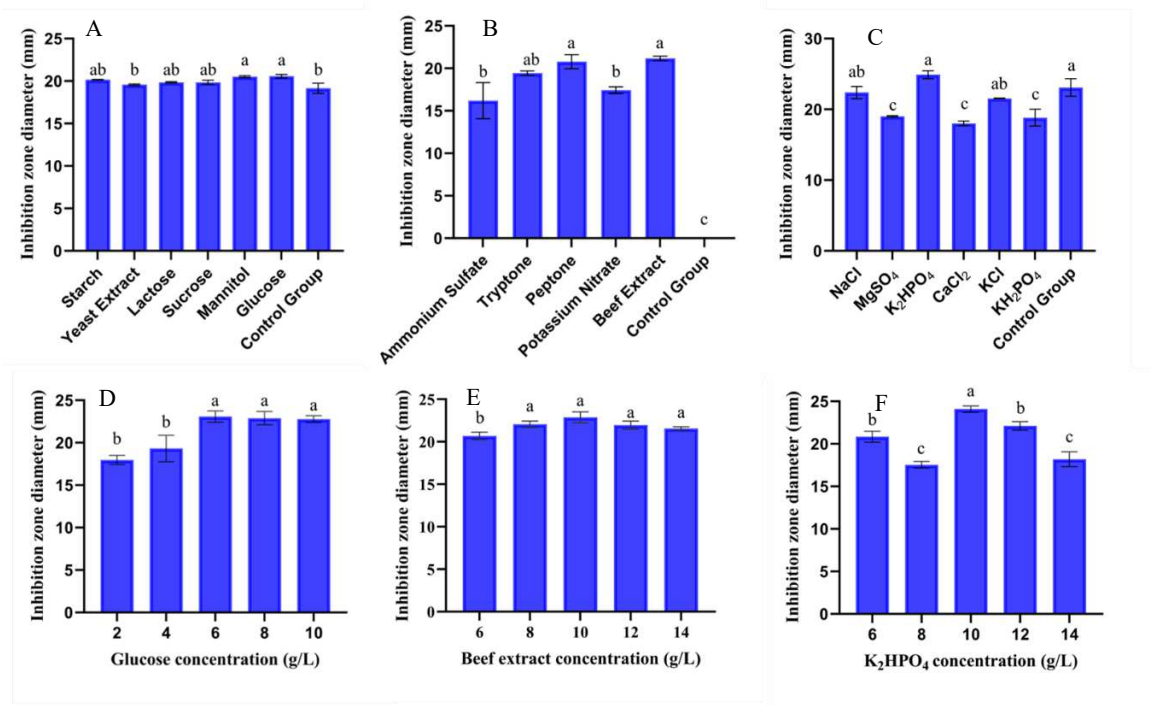


Fig.5 Effect of different carbon sources (A), nitrogen source (B), and inorganic salt (C) and different glucose concentration(D) beef extract concentration(E) K_2HPO_4 concentration(F) on the inhibition zone diameter of TJ31. Vertical bars represent standard error, and different letters indicate significant differences between groups (n=3, p<0.05).

3.6 Results of Single-Factor Experimental Analysis

The antibacterial efficacy of PA fermentation products demonstrated significant dependence on cultivation parameters, exhibiting optimal bioactivity under specific fermentation conditions. Maximum inhibition zone diameters were achieved at 150 mL bottling volume (26.30 ± 0.45 mm, p < 0.05) (Fig. 6A), inoculation amount 100 μL/mL

(25.01 ± 0.45 mm, $p < 0.05$) (Fig. 6B), 37 °C incubation temperature (27.42 ± 0.71 mm, $p < 0.05$) (Fig. 6C), and 48-hour fermentation time (25.01 ± 0.40 mm, $p < 0.05$) (Fig. 6D). These findings collectively demonstrated the critical importance of precise parameter control for maximizing the bioactive potential of TJ31.

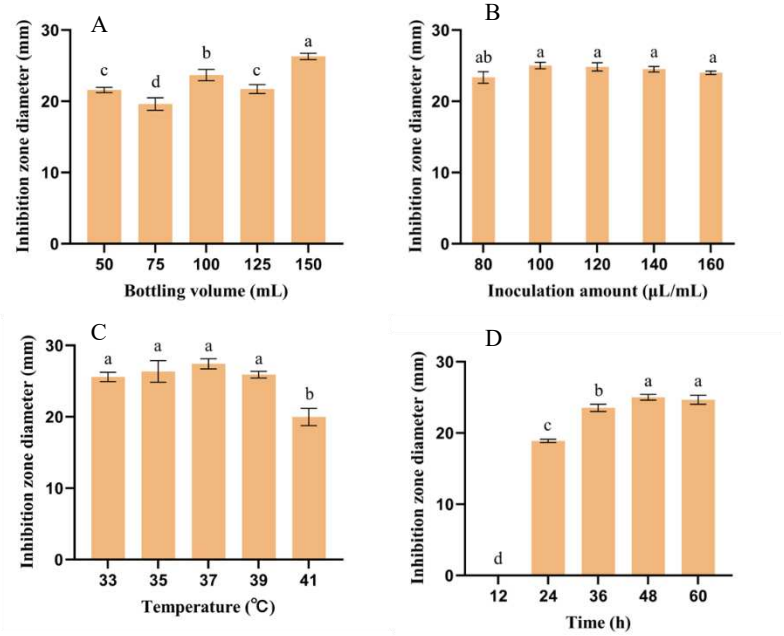


Fig.6 Effect of inoculation amount (A), bottling volume(B),incubation temperature (C) and time (D) on the inhibition zone diameter of TJ31. Vertical bars represent standard error, and different letters indicate significant differences between groups (n = 3, p < 0.05).

3.7 Optimization Outcomes from Response Surface Methodology Experiments

Based on the Box-Behnken design principle, the Design-Expert 13 was used to perform response surface optimization analysis on the cultivation conditions. Using the inhibition zone diameter (Y) as the response value, a quadratic polynomial regression model was established to describe its relationship with bottling volume (A), inoculation amount (B), temperature (C), time (D):

$$Y=21.95+1.57A+0.60B-0.13C-0.04D+0.62AB+0.3AC-0.26AD-0.08BC-0.02BD+0.30CD- 2.21A^2-1.97B^2-0.91C^2-0.33D^2$$

Table 6 Response surface experimental results.

No.	Time	Temperature	Inoculation amount	Bottling volume	Diameter of the inhibition zone(mm)
1	-1	0	-1	0	18.07
2	0	-1	0	-1	19.62
3	-1	0	0	1	17.79
4	-1	0	1	0	17.27
5	0	0	0	0	21.83
6	1	0	0	1	20.57

7	0	1	-1	0	19.61
8	0	-1	-1	0	18.12
9	0	1	0	1	19.79
10	0	1	1	0	19.27
11	0	1	0	-1	20.27
12	0	0	0	0	21.46
13	1	-1	0	0	18.36
14	0	0	0	0	22.25
15	0	0	0	0	22.06
16	0	0	1	-1	20.37
17	0	0	1	1	21.05
18	0	0	-1	1	20.86
19	-1	0	0	-1	17.15
20	0	0	0	0	22.13
21	1	0	-1	0	19.93
22	0	-1	0	1	19.22
23	1	0	0	-1	20.95
24	1	0	1	0	20.33
25	-1	-1	0	0	15.91
26	0	0	-1	-1	21.39
27	0	-1	1	0	18.09
28	1	1	0	0	21.27
29	-1	1	0	0	16.33

According to Table 6, the P-value of model is less than 0.01, indicating extremely high significance. This demonstrated the successful construction of the model and its ability to reflect changes in the response value. The lack-of-fit term's P-value is 0.1379, exceeding 0.05, indicating it is not significant. This suggested a good fit for the regression equation. The coefficient of determination R^2 is 0.9668, and the R^2adj is 0.9336. This indicated that the model's predictions are close to the actual values, and the predicted values can be used as a reference. The order of influence of the various factors on the inhibition zone diameter is A > B > C > D.

Table 7 Analysis of variance for regression models.

Source of Variation	SS	DF	MS	F-value	P-value	*
Model	265.02	14	18.93	29.13	< 0.0001	**
A	63.16	1	63.16	97.19	< 0.0001	**
B	5.17	1	5.71	8.79	0.0102	
C	0.2133	1	0.2133	0.3283	0.5758	

D	0.066	1	0.066	0.1016	0.7547	
AB	2.5	1	2.5	3.84	0.0702	
AC	0.2652	1	0.2652	0.4081	0.5332	
AD	0.8649	1	0.8649	1.33	0.268	
BC	0.0169	1	0.0169	0.026	0.8742	
BD	0.0009	1	0.0009	0.0014	0.9708	
CD	2.58	1	2.58	3.96	0.0664	
A2	113.64	1	113.64	174.86	< 0.0001	**
B2	102.07	1	102.07	157.06	< 0.0001	**
C2	36.03	1	36.03	55.44	< 0.0001	**
D2	18.59	1	18.59	28.61	0.0001	
Residual	9.1	14	0.6499			
Lack-of-fit	8.08	10	0.8082	3.18	0.1379	
Pure Error	1.02	4	0.254			
Total	274.12	28				

R=0.9668 Radj=0.9336

Using Design-Expert 13 software, three-dimensional response surface analysis plots were generated, and the results were shown in Fig. 7. As can be seen from the figure, all graphs had an opening facing downward and exhibited a trend of first increasing and then decreasing. Based on the steepness of the surfaces, relatively steeper contours indicated significant interaction effects between the two corresponding conditions, while flatter contours indicated less significant interaction effects.

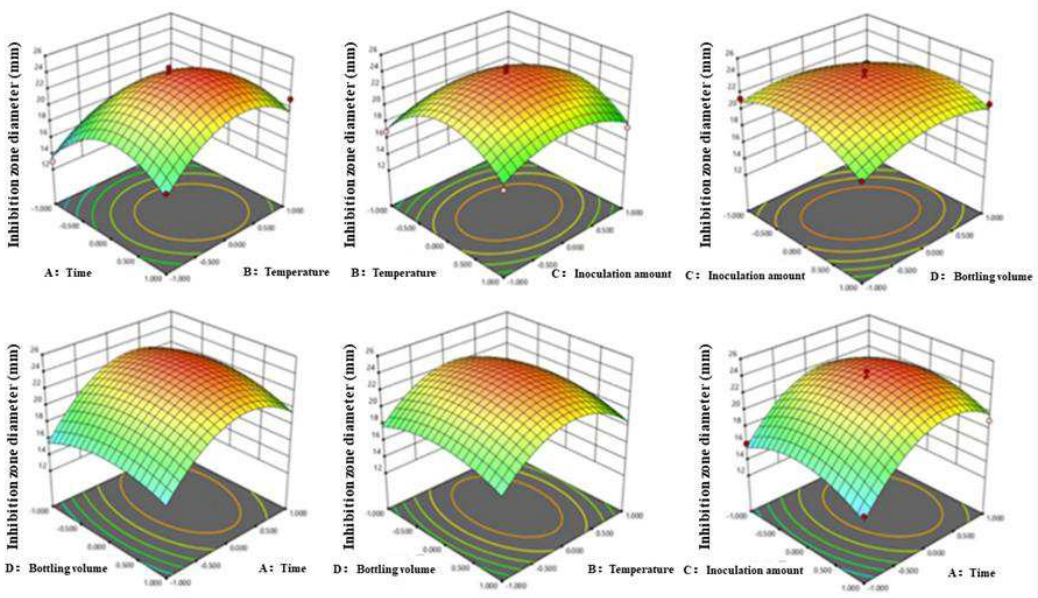


Fig.7 Response surface and contour map of each factor.

Quadratic regression fitting via the software revealed the optimal cultivation conditions for TJ31 as follows: cultivation time of 41.095 h, cultivation temperature of 36.526 °C, inoculum size of 129.835 μL, and medium volume of 133.854 mL. The

predicted inhibition zone diameter was 24.562 mm. Considering the feasibility of practical operation, the optimal conditions were revised to a cultivation time of 41 h, temperature of 37 °C, inoculation amount of 130 μL/mL, and bottling volume of 134 mL. Experimental results under these revised conditions showed an inhibition zone diameter of 25.9 mm. This value showed a small relative error compared to the predicted theoretical value, indicating good agreement between the regression model predictions and experimental results, and confirming the reliability of the findings.

3. 8 Minimum Inhibitory Concentration (MIC) of TJ31 against PA

As the concentration of the crude extract decreased, the optical density at OD_{590} increased, indicating an elevation in the turbidity of the culture medium in the 96-well plate. At a crude extract concentration of 1.563 μg/mL, the culture in the well plate remained clear and inhibited 80% of the PA growth (Table 8). Therefore, the MIC of the crude extract against PA was determined to be 1.563 μg/mL.

Table 8 Inhibition of PA by crude extracts of different concentrations.

Concentration of Crude Extract(μg/mL)	OD_{590}	Concentration of Crude Extract(μg/mL)	OD_{590}
50. 00	1.719	1. 56	0.134
25. 00	1.435	0. 78	0.278
12. 5	0.204	Negative control	0.046
6. 25	0.154	Positive control	0.870

3. 9 Results of Crude Extract on AKP Content in Culture Medium

AKP is localized in the periplasmic space between the inner membrane and cell wall of PA. Disruption of the cell wall resulted in significant release of AKP into the culture medium, and the detected AKP activity was proportional to the extent of cell wall damage[16]. The experimental data revealed a concentration-dependent effect of the crude extract on PA membrane integrity (Fig. 8). Furthermore, AKP activity was observed to increase progressively over time in the lower concentration groups.

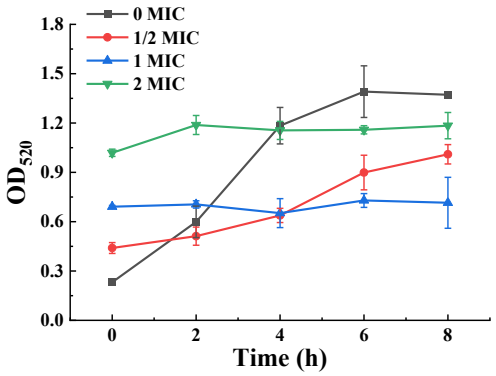


Fig. 8 Effect of TJ31 on AKP leakage in PA.

3. 10 Results of Crude Extract on the intracellular protein leakage of PA

Intracellular proteins, localized beneath the inner membrane, were found to exhibit minimal extracellular release under standard cultivation conditions. The level of detected intracellular protein was found to correlate linearly with the OD_{562}. As shown in Fig. 9, an increase in the concentration of TJ31 crude extract was associated with a progressive decrease in OD_{562} values. This reduction was interpreted that substantial

protein leakage resulting from damage to PA cells. Notably, at 2 MIC, protein in the precipitate was nearly undetectable. These findings collectively demonstrated that the crude extract caused significant damage to the inner membranes of PA cells.

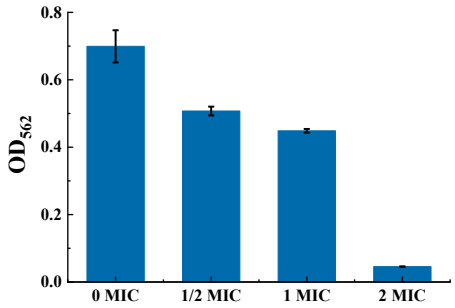


Fig. 9 TJ31 crude extract induced protein leakage in PA. Vertical bars represent standard error (n = 3, p <0.05).

3. 11 Results of Crude Extract on Cell Morphology of PA

Scanning electron microscopy (SEM) images of PA cells are presented in Figure 10 A-C. Untreated control cells (Fig.10A) exhibited intact morphology, uniform distribution, and smooth surfaces. Following 4 hours of treatment with 1/2 MIC crude extract (Fig.10B-4h), subtle surface damage and minor morphological alterations were observed. Extended treatment for 8 hours (Fig. 10B-8h) induced PArtial structural collapse, cellular deformation, and occasional membrane rupture. Increasing the crude extract concentration to MIC induced significant changes in cellular appearance. Within 4 hours (Fig.10C-4h), cells exhibited breakage, deformation, shrinkage, and aggregation. By 8 hours (Fig. 10C-8h), complete cellular rupture was observed. Collectively, these findings demonstrated that the crude extract disrupted the cellular membranes of PA, leading to morphological damage and ultimately resulting in cell death.

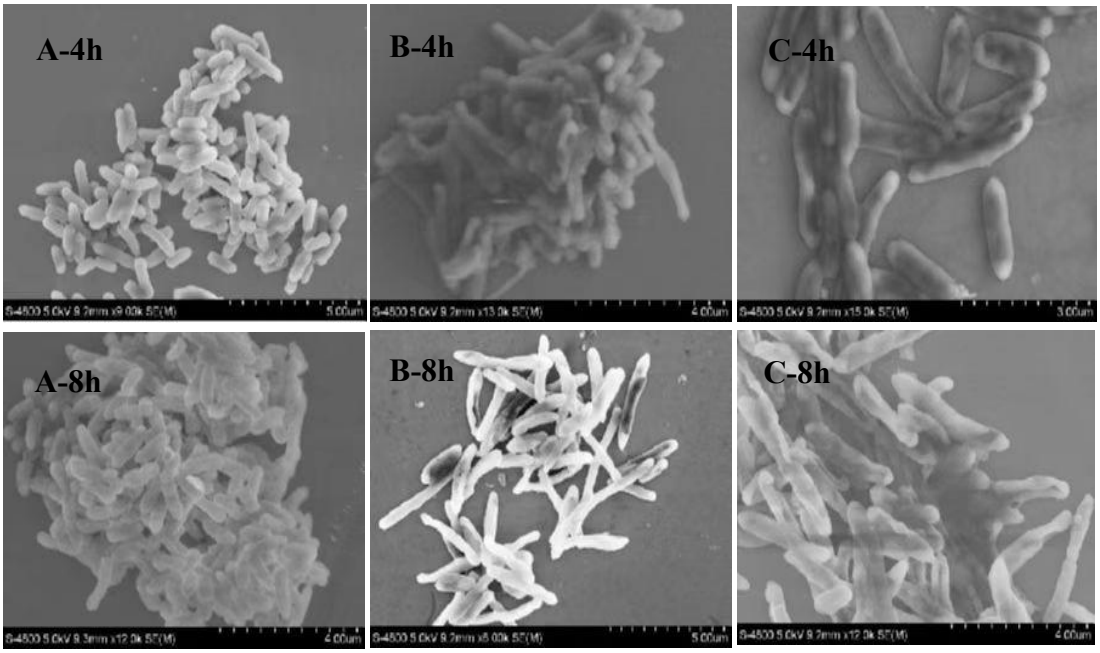


Fig. 10 The morphology of PA treated with methanol(A), 1/2 MIC(B)and F(C)was observed under electron microscopy.

3. 12 Results of the Crude Extract on respiratory chain dehydrogenases of PA

Progressive decrease in OD_{490} was observed with increasing concentrations of the antagonistic bacterial crude extract (Fig. 11). Given the established positive correlation between OD_{490} and respiratory chain dehydrogenase activity, the observed decrease was indicative of inhibited dehydrogenase function in PA. Furthermore, as oxidative metabolism is primarily mediated by dehydrogenase-catalyzed reactions in the respiratory chain, the crude extract was demonstrated to inhibit the electron transport chain of PA [18].

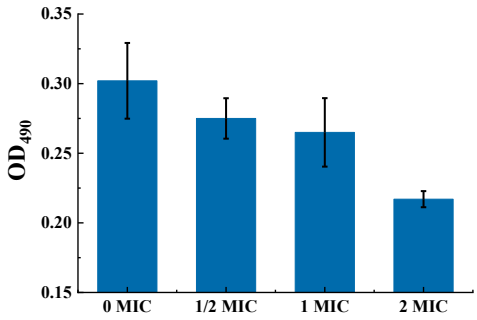


Fig. 11 Respiratory chain dehydrogenase activity in PA. Vertical bars represent standard error (n = 3, $p < 0.05$).

3. 13 Results of Crude Extract on the Membrane Potential of PA

Significant depolarization effects of the crude extract against PA were demonstrated by membrane potential measurements (Fig. 12). A mean fluorescence intensity of 1021 AU was observed in the control group. In contrast, significantly lower intensities were recorded for the treatment groups: 771 AU at 1/2 MIC, 669 AU at MIC, and 661 AU at 2 MIC. These intensities represented concentration-dependent decreases of 30.3%, 34.5%, and 34.2% compared to control, respectively. Collectively, these findings demonstrated that treatment with the crude extract induced depolarization of the membrane potential in PA. This depolarization disrupted cellular homeostasis, ultimately resulting in bactericidal effects due to compromised metabolic processes.

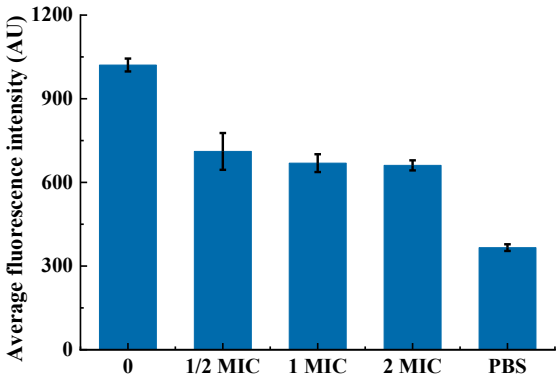


Fig. 12 Membrane potential alterations in PA reflected by fluorescence intensity changes. Vertical bars represent standard error (n = 3, p <0.05).

3. 14 Results of the Crude Extract on the twitching motility of PA

Pilus-dependent twitching motility in PA was mediated by the quorum sensing (QS) system, facilitating early bacterial adhesion, pathogenic biofilm formation, persistent infection, and enhanced drug resistance[24]. In the control group, distinct, broad staining patterns were observed (Fig. 13). These patterns, corresponding to PA twitching motility tracks, featured uniformly diffuse circular halos, indicating robust motility. In contrast, significantly reduced staining areas were observed on plates treated with 1/2 MIC crude extract. Treatment with MIC crude extract confined the staining to the puncture site. Collectively, these results demonstrated that PA twitching motility was potently inhibited by the crude extract.

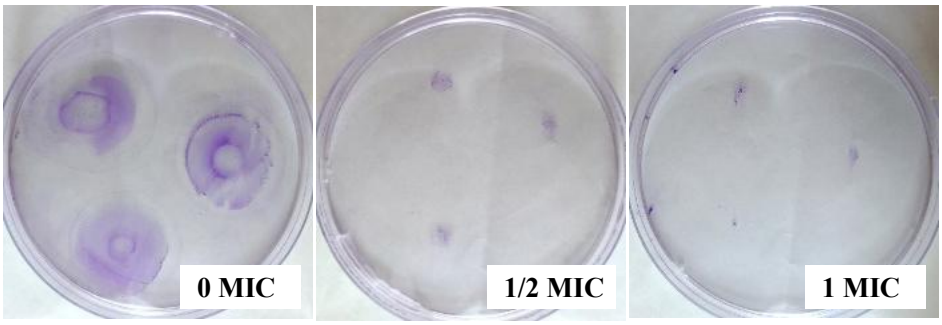


Fig. 13 Crystal violet staining quantification of PA twitching motility.

3. 15 Results of Crude Extract on PA Biofilm Formation

Biofilm is a structured aggregate of bacterial cells encased within a self-produced polymeric matrix. Biofilms formed by PA are found to confer significant resistance to antimicrobial agents, rendering the associated infections more difficult to eradicate. In this study, biofilms were stained using crystal violet. The bound crystal violet was subsequently dissolved in glacial acetic acid for quantification. A concentration-dependent increase in the inhibition rate of PA biofilm formation was observed with increasing concentrations of crude extract (Table 9). Biofilm formation was inhibited by the crude extract at a concentration of 1/2 MIC. Furthermore, the maximum inhibition rate (86.6%) was achieved at the 2 MIC concentration.

Table 9 Inhibition of PA biofilm formation by crude extract.

Concentration relative to MIC	OD_{590}	Inhibition rate (%)
0(Control)	1. 408±0. 110	-
1/2 MIC	1. 116±0. 057	21. 8%
1 MIC	0. 290±0. 104	79. 4%
2 MIC	0. 189±0. 041	86. 6%

3. 16 Results of Crude Extract on the Pyocyanin production of PA

As shown in the Fig. 14, increasing concentrations of the crude extract resulted in a progressive decrease in pyocyanin production and a concomitant suppression of PA viability, relative to normal growth conditions. Pyocyanin production was markedly suppressed at 1/2 MIC, MIC, and 2 MIC concentrations of the crude extract, confirming its potent inhibitory effect on this virulence factor.

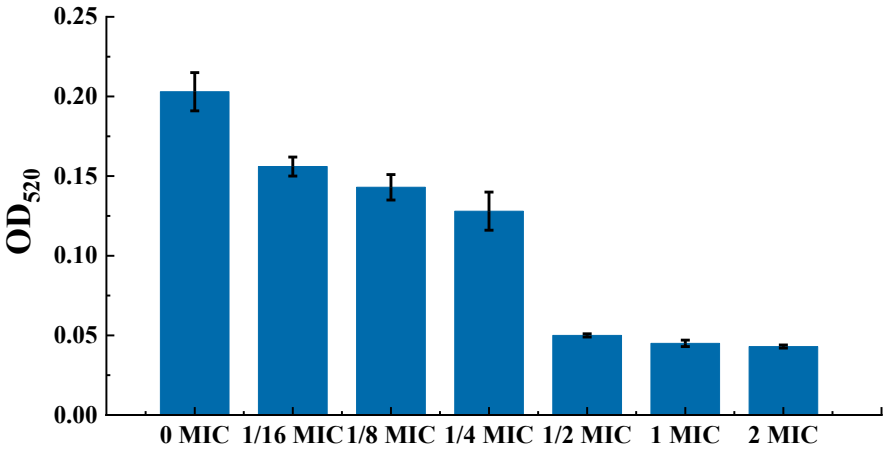


Fig. 14 Results of Crude Extracts on Pyocyanin Production in PA. Vertical bars represent standard error (n = 3, p <0.05).

3. 17 Impact of the Crude Extract on Virulence Gene Expression in PA

After exposure to the crude extract, the expression levels of *algA*, *rhlI*, and *pslA* in PA were significantly downregulated (Fig.15). The gene *rhlI*, encoding a signal molecule synthase, is a critical component of the quorum sensing system, while *algA* and *pslA* are involved in the biosynthesis of virulence-associated extracellular polysaccharides[25]. Notably, the transcription of all three genes was significantly suppressed by the crude extract.

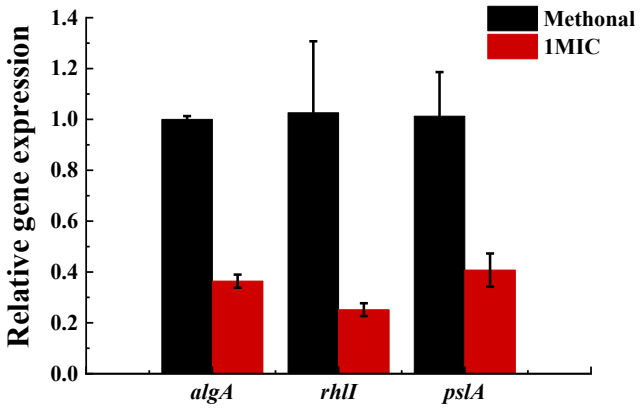


Fig. 15 Effect of sample on gene expression of *algA*, *rhlI* and *pslA*. Vertical bars represent standard error (n = 3, p <0.05).

4. Discussion

Pseudomonas aeruginosa (PA) is a major nosocomial threat, designated by the

WHO as a Priority 1 Critical pathogen because of its combination of intrinsic virulence and increasing multidrug resistance. PA exhibits extensive drug resistance, mediated in part by its efflux pump systems and quorum sensing, which effectively prevent drug accumulation. It is highly pathogenic and significantly contributes to morbidity, being found in over 90% of chronic wound infections[26].

Additionally, PA easily acquires drug-resistant genes through mutation, and the abuse of antibiotics has further exacerbated this resistance problem, leading to its current resistance to most traditional antibiotics. Carbapenem-resistant strains of *Pseudomonas aeruginosa* (CRPA) exhibit extremely high mortality rates in ICUs and have developed resistance to last-resort antibiotics such as polymyxins. WHO emphasizes that PA epitomizes the post-antibiotic era crisis, demanding urgent R&D for novel anti-pseudomonal agents targeting its resistance machinery[27].

Due to the unique living environments of polar microorganisms, their metabolites differ significantly from those of ordinary microorganisms, and they may help reduce the likelihood of inducing drug resistance. It has become one of the research hotspots in international microbial studies[28,29,30]. Notably, Yuan et al. isolated 13 bacterial strains from Antarctic krill that exhibited antibacterial activity against *Staphylococcus aureus*, and 6 strains showed activity against[31].

While numerous studies have confirmed the efficacy of polar microbial metabolites against pathogenic bacteria, significant gaps persist in strain characterization, compound optimization development.

In this study, we isolated multiple strains from sediment samples in Antarctic Lake Tuan Jie, among which several exhibited significant antibacterial activity against PA. Among these strains, TJ31 showed particularly potent antibacterial effects and was preliminarily identified as *Bacillus velezensis* through 16S rDNA sequencing.

Optimal culture medium composition and fermentation parameters are critical determinants of microbial metabolite production. This study identified the crude extract of TJ31 as exhibiting maximal antibacterial activity. Through systematic optimization, the ideal medium formulation was established as containing Glucose, beef extract, and K_2HPO_4 in aqueous solution. Among evaluated fermentation parameters, cultivation temperature exerted the most pronounced effect on antibacterial production, followed sequentially by time, inoculum size, and bottling volume. The optimized conditions yielded 25.9 mm inhibition zone against PA. Improved by 4.1mm compared to that before optimization.

The cell membrane, as an important protective structure, serves as a permeability barrier for small ions and ensures the stability of intracellular components such as nucleic acids, enzymes, and proteins[32]. Alkaline phosphatase (AKP) is mainly present intracellularly, with minimal content in the fermentation broth. A decrease in cell membrane integrity triggers irreversible apoptosis, initiating cell death. Previous studies have shown that bacterial cells undergo changes in membrane permeability under the influence of antimicrobials, leading to the release of intracellular substances[33]. In this study, the addition of different concentrations of the crude extract resulted in increased AKP and protein levels in the PA fermentation broth, consistent with findings in existing literature. Scanning electron microscopy (SEM)

analysis further confirmed that the crude extract disrupted the cell membrane of PA. Therefore, it was speculated that the crude extract of TJ31 inhibited the growth of PA by damaging the integrity of its cell membrane.

Quorum sensing (QS) is a cell-to-cell communication mechanism employed by bacteria to coordinate collective behaviors in response to population density. Through QS, bacteria regulate diverse biological processes, including the secretion of virulence factors, biofilm formation, and the development of resistance to antimicrobial agents. Biofilms are primarily composed of microbial EPS. These polymers serve as structural scaffolds to encapsulate bacteria on surfaces and protect them from environmental stresses. Microbial biofilms adhere firmly to biological and abiotic surfaces, forming a protective barrier that enhances microbial survival under extreme conditions. This protection covers environmental challenges, the effects of antibiotics and antifungals, as well as defense of the host immune system responses during infection[32] Microorganisms within biofilms exhibit stronger drug resistance than their planktonic counterparts. In this study, crystal violet assay showed that the inhibitory effect on biofilm formation became more pronounced with the increase in crude extract concentration. Notably, the twitching motility ability was also inhibited, significantly affecting the colonization capacity.

Pyocyanin is a toxin produced by PA. With the increase in crude extract concentration, pyocyanin production decreased in a gradient manner. The crude extract of TJ31 significantly inhibited the ability of PA to produce pyocyanin, thereby reducing PA toxicity. *algA* and *pslA* are associated with the production of exopolysaccharides, cyclic di-GMP, and biofilm polysaccharides, while *rhlI* is one of the key genes in the quorum sensing system. The crude extract of TJ31 showed significant inhibitory effects on the expression levels of these genes.

This study isolated an Antarctic-derived microorganism exhibiting potent antibacterial activity against PA, optimized its culture medium and fermentation conditions, and investigated the antimicrobial mechanisms of its crude extract, including effects on the PA QS system. These findings provided new perspectives for developing anti-PA therapeutics.

5. Conclusion

In this study, a strain isolated with potent antibacterial activity against PA from sediment samples in Antarctic Lake Tuan Jie. This strain exhibits broad-spectrum antimicrobial properties and was identified as *Bacillus velezensis* TJ31.

Through culture optimization, this study identified that a medium containing as the Glucose carbon source, beef extract as the nitrogen source, and K_2HPO_4 as the inorganic salt yielded the most significant growth inhibition of PA. Fermentation parameter optimization revealed that antibacterial activity was most influenced by cultivation temperature, followed by time inoculum size and bottling volume. The optimal fermentation conditions were established as: 41 h, temperature of 37 ° C, inoculation amount of 130 μL/mL, and bottling volume of 134 mL. Under these optimized conditions, the inhibition zone diameter reached 25.9 mm, representing a 4.1 mm increase compared to the index according to baseline conditions.

Antibacterial mechanism studies were subsequently performed. The minimum

inhibitory concentration (MIC) against PA was determined to be 1.563 mg/mL for the crude extract. Scanning electron microscopy (SEM)observations revealed that the crude extract could disrupt the integrity of PA cells. Results from protein leakage assays and alkaline phosphatase experiments further confirmed that the crude extract damaged both the cell membrane and cell wall of PA. Additionally, membrane potential assays and respiratory chain dehydrogenase experiments confirmed that the crude extract impaired PA metabolism, leading to bacterial death. Furthermore, the crude extract interferes with virulence of PA by suppressing extracellular pyocyanin secretio, inhibiting biofilm formation, and downregulating pathogenic genes expression.

This indicated that it possessed substantial development potential as a novel antimicrobial agent against PA infections. This investigation advanced fundamental knowledge in Antarctic microbiota while demonstrating their therapeutic utility against multidrug-resistant pathogens. Collectively, these findings will lay a theoretical foundation for future drug development.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Author Contributions

J.H. participated the writing, reviewing and editing.L. X. and M.L. participated the investigations, data collection, and writing original drafts. Y.H. and W.M. participated the methodologies and investigations. D.G. and W.M. participated the formal analyses and investigations. H.C. offered strain resources and technical help. X.H. participated the supervision and reviewing drafts. L.J. and C.Q. participated the conceptualizations, supervision, offering resources, project administration, writing, reviewing editing.

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References

[1] Micek, Scott T., Richard G. Wunderink, Marin H. Kollef, Catherine Chen, Jordi Rello, Jean Chastre, Massimo Antonelli et al. "An international multicenter retrospective study of *Pseudomonas aer*ug*inosa nosocomial* pneumonia: impact of multidrug resistance." Critical Care 19, no. 1 (2015): 219.

[2] Fujitani, Shigeki, Hsin-Yun Sun, L. Yu Victor, and Jeremy A. Weingarten.

"Pneumonia due to *Pseudomonas aer*ug*inosa*: Part I: epidemiology, clinical diagnosis, and source." Chest 139, no. 4 (2011): 909-919.

[3] Shi, Qingyi, Chen Huang, Tingting Xiao, Zhenzhu Wu, and Yonghong Xiao. "A retrospective analysis of *Pseudomonas aeruginosa* bloodstream infections: prevalence, risk factors, and outcome in carbapenem-susceptible and-non-susceptible infections." Antimicrobial Resistance & Infection Control 8, no. 1 (2019): 68.

[4] Zilberberg, Marya D., Brian H. Nathanson, Kate Sulham, Weihong Fan, and Andrew F. Shorr. "A novel algorithm to analyze epidemiology and outcomes of carbapenem resistance among patients with hospital-acquired and ventilator-associated pneumonia: a retrospective cohort study." Chest 155, no. 6 (2019): 1119-1130.

[5] Liu, Xue, Jiaxin Cai, Haiming Chen, Qiuping Zhong, Yaqi Hou, Weijun Chen, and Wenxue Chen. "Antibacterial activity and mechanism of linalool against *Pseudomonas aeruginosa*." Microbial Pathogenesis 141 (2020): 103980.

[6] Holbrook, Selina YL, and Sylvie Garneau-Tsodikova. "Evaluation of aminoglycoside and carbapenem resistance in a collection of drug-resistant *Pseudomonas aeruginosa* clinical isolates." Microbial drug resistance 24, no. 7 (2018): 1020-1030.

[7] Araos, Rafael, and Erika D'Agata. "*Pseudomonas aeruginosa* and other *Pseudomonas* species." (2019).

[8] Rizzo, Carmen, and Angelina Lo Giudice. "Life from a snowflake: diversity and adaptation of cold-loving bacteria among ice crystals." Crystals 12, no. 3 (2022): 312.

[9] Efimenko, T. A., O. V. Efremenkova, E. V. Demkina, M. A. Petrova, I. G. Sumarukova, B. F. Vasilyeva, and G. I. El'-Registan. "Bacteria isolated from Antarctic permafrost are efficient antibiotic producers." Microbiology 87, no. 5 (2018): 692-698.

[10] Kim, Mi-Kyeong, Tae-Jin Oh, and Hyun Park. "Antibacterial and antioxidant capacity of polar microorganisms isolated from Arctic lichen *Ochrolechiasp.*" (2014).

[11] Magot, Florent, Gwendoline Van Soen, Larissa Buedenbender, Fengjie Li, Thomas Soltwedel, Laura Grauso, Alfonso Mangoni, Martina Blümel, and Deniz Tasdemir. "Bioactivity and metabolome mining of deep-sea sediment-derived microorganisms reveal new hybrid PKS-NRPS macrolactone from *Aspergillus versicolor* PS108-62." Marine Drugs 21, no. 2 (2023): 95.

[12] Ren, Zilin, et al. "Study on the Secondary Metabolites of the Antarctic Intertidal Fungus Pseudeurotium sp. HDN17-894." Chinese Journal of Marine Drugs 43, no. 2 (2024): 67-72. https://doi.org/10.13400/j.cnki.cjmd.2024.02.010

[13] Schön, Thomas, Jim Werngren, Diana Machado, Emanuele Borroni, Maria Wijkander, Gerard Lina, Johan Mouton et al. "Multicentre testing of the EUCAST broth microdilution reference method for MIC determination on *Mycobacterium tuberculosis*." Clinical Microbiology and Infection 27, no. 2 (2021): 288-e1.

[14] Shu, Qin, Yongwu Niu, Wenjun Zhao, and Qihe Chen. "Antibacterial activity and mannosylerythritol lipids against vegetative cells and spores of *Bacillus cereus*." Food Control 106 (2019): 106711.
[15] Santos, Suikinai Nobre, Faustos Klabund Ferraris, Ana Olivia de Souza, Maria das Graças Henriques, and Itamar Soares Melo. "Endophytic fungi from Combretum leprosum with potential anticancer and antifungal activity." Symbiosis 58, no. 1 (2012): 109-117.
[16] Tang, Xiaodi, Shenghai Chang, Wen Qiao, Qinghua Luo, Yuejia Chen, Zhiying Jia, James Coleman et al. "Structural insights into outer membrane asymmetry maintenance in Gram-negative bacteria by MlaFEDB." Nature Structural & Molecular Biology 28, no. 1 (2021): 81-91.
[17] Wayne, P. A. "Clinical and laboratory standards institute: performance standards for antimicrobial susceptibility testing: informational supplement, M100." Clinical and Laboratory Standards Institute (CLSI) (2018).
[18] Durán, Nelson, Marcela Durán, Marcelo Bispo De Jesus, Amedea B. Seabra, Wagner J. Fávaro, and Gerson Nakazato. "Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity." Nanomedicine: nanotechnology, biology and medicine 12, no. 3 (2016): 789-799.
[19] Xu, Can, Jinglei Li, Liuqing Yang, Fang Shi, Liu Yang, and Ming Ye. "Antibacterial activity and a membrane damage mechanism of Lachnum YM30 melanin against *Vibrio Parahaemolyticus* and *Staphylococcus aureus*." Food Control 73 (2017): 1445-1451.
[20] Essar, D. W., L. Eberly, A. Hadero, and I. P. Crawford. "Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications." Journal of bacteriology 172, no. 2 (1990): 884-900.
[21] Zhao, Kun, Boo Shan Tseng, Bernard Beckerman, Fan Jin, Maxsim L. Gibiansky, Joe J. Harrison, Erik Luijten, Matthew R. Parsek, and Gerard CL Wong. "Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms." Nature 497, no. 7449 (2013): 388-391.
[22] Leid, Jeff G., Carey J. Willson, Mark E. Shirtliff, Daniel J. Hassett, Matthew R. Parsek, and Alyssa K. Jeffers. "The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing." The journal of immunology 175, no. 11 (2005): 7512-7518.
[23] Richter, Anika K., Emmanuel Frossard, and Ivano Brunner. "Polyphenols in the woody roots of Norway spruce and European beech reduce TTC." Tree physiology 27, no. 1 (2007): 155-160.
[24] Popat, Roman, Shanika A. Crusz, Marco Messina, Paul Williams, Stuart A. West, and Stephen P. Diggle. "Quorum-sensing and cheating in bacterial biofilms." Proceedings of the Royal Society B: Biological Sciences 279, no. 1748 (2012): 4765-4771.
[25] Overhage, Jörg, Mirle Schemionek, Jeremy S. Webb, and Bernd HA Rehm. "Expression of the psl operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation." Applied and environmental

microbiology 71, no. 8 (2005): 4407-4413.
[26] JP, BOWMAN. "Diversity and association of psychrophilic bacteria in Antarctic Sea Ice." Appl. Environ. Microbiol. 63 (1997): 3-068.
[27] World Health Organization. WHO bacterial priority pathogens list, 2024: bacterial pathogens of public health importance, to guide research, development, and strategies to prevent and control antimicrobial resistance. World Health Organization, 2024.
[28] Kayani, Hajra, Muhammad Asif Rasheed, Wadi B. Alonazi, Farrukh Jamil, Annam Hussain, ChangHui Yan, Raza Ahmed, and Muhammad Ibrahim. "Identification and genome-wide analysis provide insights into the genetic diversity and biotechnological potentials of novel cold-adapted *Acinetobacter* strain." Extremophiles 27, no. 2 (2023): 14.
[29] Silva, Tiago R., Alysson WF Duarte, Michel RZ Passarini, Ana Lucia TG Ruiz, Caio Haddad Franco, Carolina Borsoi Moraes, Itamar Soares de Melo, Rodney A. Rodrigues, Fabiana Fantinatti-Garboggini, and Valéria Maia Oliveira. "Bacteria from Antarctic environments: diversity and detection of antimicrobial, antiproliferative, and antiparasitic activities." Polar Biology 41, no. 7 (2018): 1505-1519.
[30] Núñez-Montero, Kattia, and Leticia Barrientos. "Advances in Antarctic research for antimicrobial discovery: a comprehensive narrative review of bacteria from Antarctic environments as potential sources of novel antibiotic compounds against human pathogens and microorganisms of industrial importance." Antibiotics 7, no. 4 (2018): 90.
[31] Jeong, Pyeong-Hwa, Yong-Suk Kim, and Dong-Hwa Shin. "Changes of physicochemical characteristics of Schizandra chinensis during postharvest ripening at various temperatures." Korean Journal of Food Science and Technology 38, no. 4 (2006).
[32] Chen, Chris Zhisheng, and Stuart L. Cooper. "Interactions between dendrimer biocides and bacterial membranes." Biomaterials 23, no. 16 (2002): 3359-3368.
[33] Xing, Ke, Xi Guang Chen, Ming Kong, Cheng Sheng Liu, Dong Su Cha, and Hyun Jin PArk. "Effect of oleoyl-chitosan nanoPArticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of and *Staphylococcus aureus*." Carbohydrate Polymers 76, no. 1 (2009): 17-22.