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Characterization and bioactive component analysis of filamentous bacterium XJ-16

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ABSTRACT

Actinomycetes, which can produce a variety of bioactive compounds in the metabolic process, is one of the important sources of novel drugs, enzymes, anti-tumor drugs and enzyme inhibitors. It has been the focus of researchers to find and develop *Actinomycetes* with special characters. Strain XJ-16 is a blue alkali-resistant filamentous bacterium with high antimicrobial activity isolated from saline-alkali land of Xinjiang. Based on the classification, the enzyme production, metabolite antibacterial activity, and antibacterial substance isolation of XJ-16 were explored. which showed that XJ-16 belongs to the blue group of *Streptomyces* sp, and it can secrete cellulase, lipase, urease, protease, catalase and oxidase during metabolism. In addition, the bacteriostatic substance secreted by the strain XJ-16 showed inhibitory effects against both Gram-positive and Gram-negative bacteria, as well as the yeast *Candida albicans*. Then it was found that the bacteriostasis produced by XJ-16 has strong tolerance to acid, weak tolerance to alkali, and easy to be inactivated. After tested by HPLC, the retention time of antimicrobial substance was 13.261 min. This study provides new research ideas and theoretical support for searching for new antibacterial compounds and further developing the blue alkaline *Actinomycete* XJ-16.

1. Introduction

Among the natural antibiotics of microbial origin in clinical use, 67% are derived from *Actinomycetes*,[1] with penicillin and cefotaxime being notable exceptions.[2] At the same time, the natural secondary metabolites produced by *Actinomycetes* include alkaloids, polyketides, terpenes, peptides, glycosides, ketones, flavonoids and phenols, which have a wide range of applications in medical, agricultural, food-related, chemical, environmental protection and other fields.[3,4] In recent years, the overuse of broad-spectrum antibiotics has led to the emergence of multi-drug resistant microorganisms (MDRM),[5] which greatly increases the difficulty of clinical treatment and requires the development of new antibiotics. *Actinomycetes* play an important role as one of the most important sources of antibiotics.[6] Although there are extremely rich resources of *Actinomycetes* in nature, due to factors such as isolation technology, culture methods, and source of strains, the redundancy of isolated *Actinomycetes* continues to increase.[7] However, a new promising approach is the isolation of novel *Actinomycetes* from extreme environments.

The concept of extremophiles was first proposed in 1974 to describe the newly discovered group of microorganisms living in environments that were previously considered inhospitable to life.[8] Among *Actinomycetes* there are alkaliphilic, halophilic, acidophilic, thermophilic, and psychrophilic species. After a long period of natural screening, these *Actinomycetes* have evolved new genes, proteins, and physiological mechanisms not found in ordinary microorganisms, and it stands to reason that novel compounds and secondary metabolites with special functions can be found in extremophiles.[9,10]

At the end of the 20th century, *Actinomycetes* were also discovered in highly alkaline environments.[11] Compared with ordinary *Actinomycetes*, their secondary metabolites are relatively new and their enzymes are also highly stable at high pH. Specific examples include the xylanase

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produced by alkaliphilic *Actinomycetes*, which has high enzymatic activity and thermal stability.[12] When it was used in the paper industry, the quality of paper could be improved.[13,14] In addition, alkaline enzymes produced by alkaliphilic *Actinomycetes* could also be used in sewage treatment, food, textiles, and other areas.[15] Therefore, the discovery of alkaliphilic *Actinomycetes* in extreme environments plays an important role in finding new bacterial resources and synthesizing new lead compounds.

2. Materials and methods

2.1. Biological identification of the strain

The alkali-tolerant filamentous bacterium XJ-16 isolated from Xinjiang saline-alkali soil was grown in Gauze's Medium No.1 at 28 °C for 10 d. During this period, the growth characteristics of the strain were observed and recorded. Then, 100 μL of fermentation broth was spread on the culture medium, and a cover glass was inserted into medium at a 45-degree angle, followed by incubation at 28 °C. On the 3rd, 7th, and 14th day, the hyphae characteristics were observed under a microscope (1000 ×) (Shanghai Yongke Optical Instrument Co., Ltd). The partial sequence of the 16SrDNA gene was then used for phylogenetic analysis. The genomic DNA was extracted using the Ezup column bacterial genomic DNA extraction kit from Sangon Biotech Co., Ltd. and the PCR product was amplified using the 16SrDNA universal primers 27F (5′-GTTTGATCCTGGCTCAG-3′) and 1492R(5′-GGTTACCTTGTTACGACTT-3′) with the genomic DNA as template. After purification, the amplified DNA was sent to Sangon Biotech Co., Ltd for sequencing. Then, the sequencing results were analyzed by performing a BLAST search against the NCBI database, and compared with other strains. Finally, MEGA7.0 software was used to construct a phylogenetic tree to determine the genus of the strain.[16]

2.2. Enzyme activity of the strain

Strain XJ-16 was streaked and grown in Gauze's Medium No.1 at 28 °C for 14 d to obtain a single colony. Them, sterile toothpicks were used to pick an appropriate amount of single colonies into different media for the detection of catalase, amylase, cellulase, chitinase, protease, urease, lipase and oxidase activity, as described before.[17]

2.3. Antimicrobial inhibition spectrum

The activated XJ-16 strain was grown in Gauze's liquid medium No. 1 at 28 °C and 180 rpm for 7 d. The fermentation broth was centrifuged at 8000 rpm for 10 min, and then the supernatant was passed through a 0.22 μm pore-size filter membrane to obtain a sterile culture supernatant as described before.[18]

In order to determine the inhibitory effect of strain XJ-16, pathogenic bacteria were grown in LB liquid medium at 37 °C and 220 rpm for 20 h. Then, 200 μL of bacterial solution was added to cooled down but still liquid LB agar and poured into plates. When the medium solidified, a sterile punch with a diameter of 6 mm was used to inject 50 μL of the sterile-filtered culture supernatant of XJ-16. The plates were incubated at 37 °C for 4 d, after which the size of the inhibition zone was measured.

In addition, pathogenic fungi such as *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Pseudomonas rotundus* were cultured in PDA liquid medium at 28 °C and 180 rpm for 4 d.[19] Then, 100 μL of the culture was spread on agar plates, and 50 μL of sterile fermentation broth was injected. After incubation at 28 °C for 4 d, the inhibitory effect of the XJ-16 strain on pathogenic fungi was detected.

2.4. Physicochemical properties of the fermentation broth

The strain XJ-16 was grown in Gauze's Medium No.1 at 28 °C and 180 rpm for 7 d, and the seed solution was divided into 250 mL Erlenmeyer flasks. Subsequently, the inoculation amount and culture conditions were varied to determine the inhibitory effect of the fermentation broth on *Bacillus subtilis* with different temperatures, pH values and carbon sources. Finally, the measured data was analyzed using Origin 8.6.

2.5. Crude extraction of active antibacterial substances

The n-butanol, butyl acetate, ethyl acetate, dichloromethane, benzene, and petroleum ether were selected as extractants with different polarities, and were each mixed with the culture supernatant in equal proportions. After culture at 28 °C and 200 rpm for 24 h, the organic phase and the aqueous phase were separated, and the most suitable extractant for the antibacterial substance of strain XJ-16 was selected.

The XJ-16 strain was grown in 10 L of Gauze's liquid medium No. 1 for 10 d. In order to obtain the supernatant, the fermentation broth was filtered with absorbent cotton, and the filtrate was centrifuged at 6000 rpm for 20 min. The obtained supernatant was mixed with the optimal extractant in a ratio of 3:1 and then extracted. After the extract was concentrated on a rotary evaporator (Shanghai Yarong Biochemical Instrument Factory), 50 mL of extractant was added to re-dissolve it, and the antibacterial activity of the extract was finally determined.

To evaluate the acid-base properties of the antibacterial active substances, citric acid-disodium hydrogen phosphate buffers spanning pH 2.2–8, and glycine-sodium hydroxide buffers spanning pH 8–9 were used. Chromatographic paper was soaked with the different buffers, and placed in 50% methanol, 50% ethanol and 50% acetone. The indicator bacteria were spread on LB plates, and incubated with the dried treated chromatography paper at 37 °C for 20 h. After 20 h, the Rf value was calculated, and then the type of bacteriostatic substance was judged according to the position of the trace.

2.6. Separation and purification of antibacterial active substances

In order to further identify the antibacterial active substance, thin-layer chromatography was carried out as described before.[20] The GF254 silica gel plate was chosen as the solid phase, while dichloromethane and methanol were mixed in different proportions as the mobile phase. The Rf value was calculated after color development using CAM developer. Additionally, the candidate band on the silica gel was scraped off, and dissolved in dichloromethane for evaluation of antibacterial activity.

According to the results of TLC, silica gel column chromatography was used, and several ratios of the best developing agent were selected for elution. Eluates of the same composition were combined for rotary evaporation, after which the antibacterial activity was determined. In order to determine the absorption spectrum of the antibacterial active substance, a multifunctional microplate reader (Hangzhou Allsheng Instruments CO., Ltd) was used to scan several concentrated solutions with good antibacterial effects in the full wavelength range.

Finally, semi-preparative high performance liquid chromatography was performed on a Shim-pack PREP-ODS(H)KIT column (250 × 20 mm) (Shanghai Yi Sai Scientific Instrument Co., Ltd) to analyze the components obtained by silica gel column chromatography.[21] The injection volume was set to 2 mL, the flow rate was 5 mL/min, and the detection wavelength was 280 nm. The mobile phase was methanol: water in gradient elution, and the antibacterial ability of the eluate fractions was determined. Then, the fraction with antibacterial activity was prepared in large quantities under the same conditions, and the purity of the collected material was checked by HPLC on a Kromasil 100-5-C 18 column (4.6 × 250 mm) (Shanghai Mailing Technology Co., Ltd).[22] The injection volume was set to 20 μL, the flow rate was 1 mL/min, the detection wavelength was 280 nm, and the mobile phase was methanol: water (80:20). Finally, the collected single peak fraction was used to detect the antibacterial activity.

2.7. Effect of NaCl and pH on the antibacterial substance

The Gauze's Medium No.1 was chosen as the basal medium, and was divided into 250 mL erlenmeyer flasks. The pH and sodium chloride concentration of each bottle were adjusted using hydrochloric acid, NaOH and NaCl. Subsequently, 1 mL of seed culture was added to each flask for cultivation. In this experiment, *B. subtilis* was chosen as the indicator strain, and the inhibition zone diffusion method was used to determine the antibacterial activity. Finally, Origin8.6 software was used to determine the effects of pH and NaCl on the antibacterial active substance.

3. Results and discussion

3.1. Strain identification

Colonies of the strain XJ-16 grew rapidly on Gauze's Medium No.1, and after 24 h, white aerial hyphae were formed (Fig. 1a). When the strain was cultured for 48 h, it started producing a blue pigment, and after 72 h, the colonies became purple-blue. Similarly, the culture turned purple-blue when the strain was cultured in shake-flasks for 7 d. In addition, the aerial hyphae became velvety pink after 168 h of cultivation, and the white spore filaments were also distributed radially. At this time, the surface of the colony was rough and dense (Fig. 1c).

The morphology of the basal hyphae, aerial hyphae and spores of the strain was assessed using the glass insert method. After 3 d of cultivation, the intrabasal hyphae were branched, while the aerial hyphae exhibited 1–2 spirals. In addition, when the basal hyphae and spores were cultivated for 7 d, the basal hyphae developed a diaphragm, and the basal filaments were not broken. At this moment the spores were round, with spore chains but no sporangia (Fig. 1f).

Subsequently, part of the 16SrDNA gene of XJ-16 was cloned and sequenced. Based on the sequencing results, a phylogenetic tree was constructed (Fig. 2). Strain XJ-16 exhibited 99% sequence similarity with *Streptomyces gulbargensis* DAS 131, so it could be preliminarily concluded that XJ-16 belonged to the genus *Streptomyces*. Then, according to the description of the intrabasal hyphae, aerial hyphae and pigment in the "*Streptomyces* Identification Manual", the strain XJ-16 could finally be classified as belonging to the blue cyaneus group in the genus *Streptomyces*.

3.2. Enzyme activity of strain XJ-16

Strain XJ-16, did not produce amylase and chitinase under the tested culture conditions, but was positive for catalase, oxidase, urease, protease, cellulase and lipase. Among them, the diameters of cellulase, protease and lipase were larger than the others (Table 1), which indicated that strain XJ-16 had a better ability to produce these three types of enzymes, making it a potential development strain for high yield production of lipase, cellulase and protease in the future.

3.3. Determination of the antibacterial spectrum of the fermentation product of strain XJ-16

The inhibitory effect of strain XJ-16 on pathogenic bacteria was assessed based on the diameter of the inhibition zone, as shown in Fig. 3. Strain XJ-16 had no inhibitory effect on *K. pneumoniae* S, and its inhibitory effect on *E. faecalis* TCC29212 and *S. aureus* ATCC6538 was relatively weak. By contrast, XJ-16 had a strong inhibitory effect on *E. coli* ATCC8091, *E. cloacae* ATCC45031, and *K. pneumoniae* R.

In addition, strain XJ-16 inhibited the growth of the yeast *M. albicans* ATCC10231, but it had little inhibitory effect on the growth of *F. oxysporum*, *B. cinerea*, *R. cerealis*, *B. berengeriana*, and *F. graminearum* (Fig. 3).

On the basis of these two experimental results, it could be concluded that the bacteriostatic substance secreted by the strain XJ-16 had an inhibitory effect on Gram-positive and Gram-negative bacteria, as well as the yeast *Candida albicans*.

3.4. Physicochemical properties of the fermentation broth

The influence of the inoculation amount on the antibacterial activity of the fermentation broth of strain XJ-16 is shown in Fig. 4. It was found that with the increase of inoculation amount, the size of the inhibition zone did not continue to expand, and reached the maximum when the inoculation amount was 1 mL. After analyzing the reasons for this trend, it was found that when different volumes of seed liquid were added to the basic medium, the obtained strain fermentation liquid had different

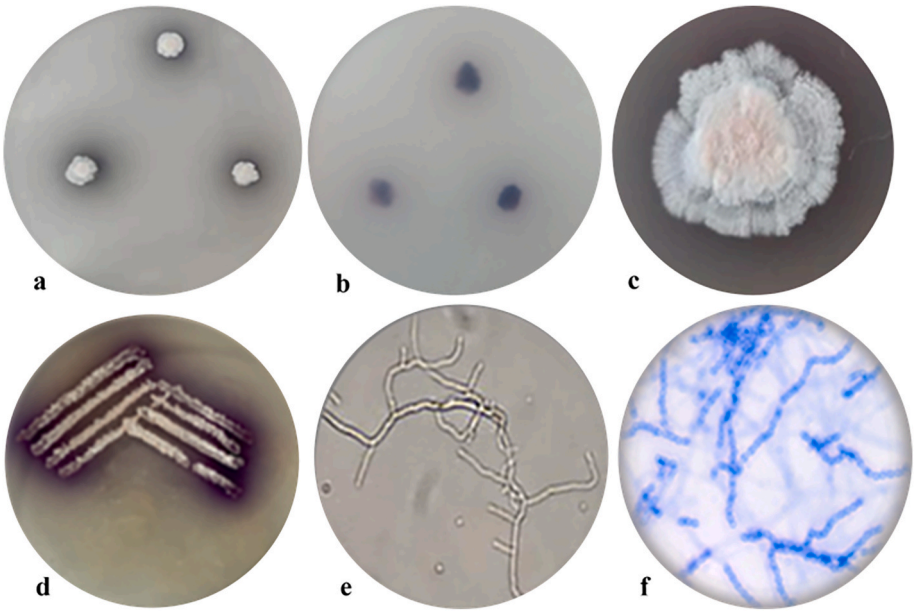

Fig. 1. Observation of the colony morphology and pigment color of strain XJ-16 (a. Colony frontal morphology; b. Dorsal morphology of colony; c. Colony morphology when secreting red metabolites; d. Mycelial morphology of secreting blue-purple metabolites; e. Mycelial morphology; f. Mycelial morphology when secreting blue-purple metabolites).

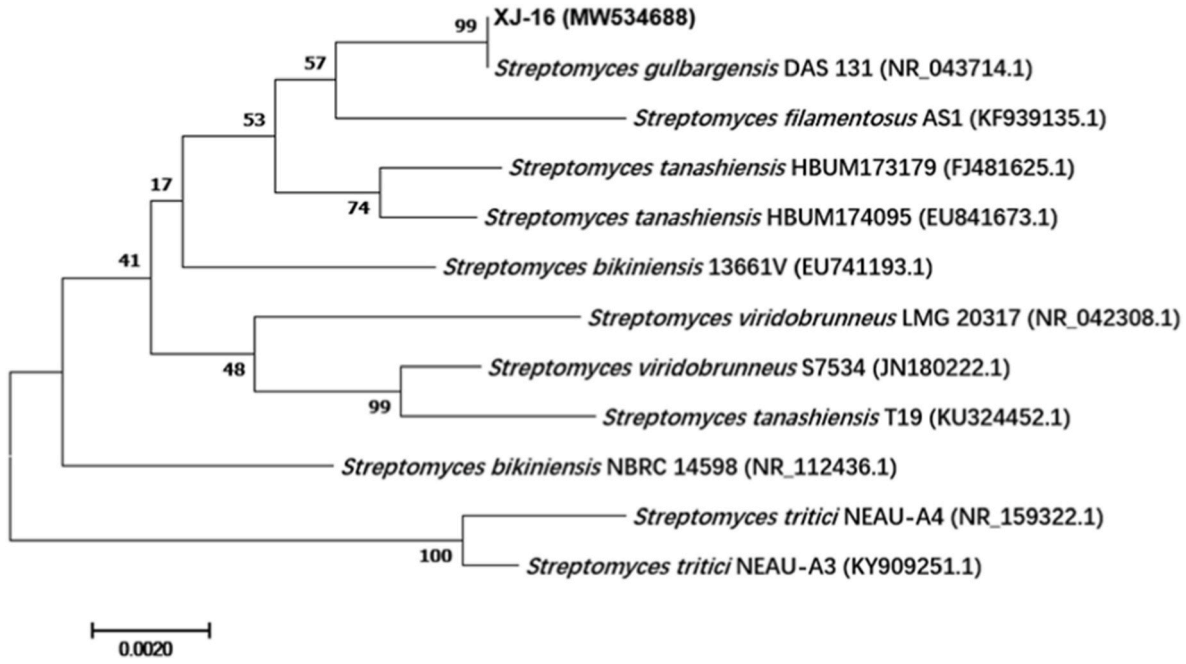

Fig. 2. Phylogenetic tree of strain XJ-16 16SrDNA.

Table 1
Test results of enzyme activity of strain XJ-16 (n = 3).

Enzyme types	Positive	Negative	Zone of Inhibition (mm)
Catalase	+	0	–
Oxidase	+	0	–
Urease	+	0	–
Protease	+	0	12 (±0.2)
Cellulase	+	0	17 (±2)
Lipase	+	0	23 (±1)
Amylase	0	-	0
Chitinase	0	-	0

antibacterial activities against the indicator strains. With the increase in the amount of seed culture, the lag phase was prolonged, which may have affected the synthesis of secondary metabolites.

The optimal inoculum size was then used to analyze the inhibitory effect of the fermentation broth on the indicator strains at different culture temperatures. As shown in Fig. 5, and the diameter of the inhibition zone did not exhibit a simple linear relationship with the temperature. When the culture temperature was increased to 30 °C, the diameter of the inhibition zone was the largest, demonstrating that the antibacterial activity of the fermentation broth was the highest. With the increase of temperature, the inhibition zone gradually became smaller, which indicated that the thermal stability of the fermentation broth of strain XJ-16 was poor.

When the inoculation amount was 1 mL and the culture temperature was adjusted to 30 °C, the pH of the basic medium was altered to detect the influence of pH changes on the inhibitory effect of the secondary metabolites of strain XJ-16 on the indicator strains. As shown in Fig. 6, the diameter of the bacteriostatic zone was the largest when the pH was adjusted to 5, indicating that the fermentation broth had the strongest bacteriostatic activity at this pH. In addition, it was also found that the fermentation broth of the strain maintained a certain antibacterial activity against the indicator strains under both acidic and alkaline conditions, which demonstrated that the antibacterial active substance was not sensitive to pH.

On the basis of the above experimental results, the carbon source in the basic medium was changed to test the inhibitory effect of the fermentation broth on the indicator bacteria. As shown in Fig. 7, when the carbon source was corn meal, the maximum value of the inhibition zone diameter was 21 mm (±1). However, there was no inhibitory effect when the carbon source was replaced with maltose. In addition, after the diameters of inhibition zone of fermentation broths obtained with corn flour and soluble starch were compared, it was found that the diameter of inhibition zone was larger when soluble starch was used as the carbon source, and the antibacterial activity of the fermentation broth was also stronger. In previous studies, starch was found to be easier to hydrolyze into maltose and glucose under acidic conditions,[23] which could be fully absorbed and used by strain XJ-16. Therefore the use of soluble starch as the carbon source in the acidic environment was more conducive to improving the antibacterial activity of the strain.

3.5. Preliminary isolation and identification of antibacterial active substances

The antibacterial activity of the substance obtained by extracting the fermentation supernatant with different extractants is shown in Fig. 8. The ability of the extractants to concentrate the antibacterial active substances into the organic phase was in the order n-butanol, ethyl acetate, butyl acetate, dichloromethane, benzene, and petroleum ether. The inhibitory effects of the remaining aqueous phase on the indicator trains were in the order petroleum ether, benzene, n-butanol, butyl acetate, dichloromethane and ethyl acetate. After ethyl acetate extraction, the aqueous phase had the least antibacterial active substances, and the organic phase had a better antibacterial effect. Therefore, ethyl acetate was selected as the extractant for subsequent experiments. Furthermore, when extracting the fermentation supernatant with different extractants, it was also found that the polarity of antibacterial substance produced by strain XJ-16 was very low, since they could not be extracted with high-polarity organic solvents.[24]

Subsequently, the crude extract obtained with ethyl acetate was subjected to pH paper chromatography tests with 50% methanol, 50% ethanol and 50% acetone as the mobile phase. As shown in Fig. S1, when the chromatography papers were developed at pH 2.2, 3, and 4, the zone of inhibition appeared, and the Rf values were between 0.8 and 0.85, 0.73–0.85, and 0.72–0.82, respectively. However, when 50% acetone was used as the mobile phase and the pH was 2.2, two zones of inhibition of different sizes appeared. The Rf values at this time were 0.32 and

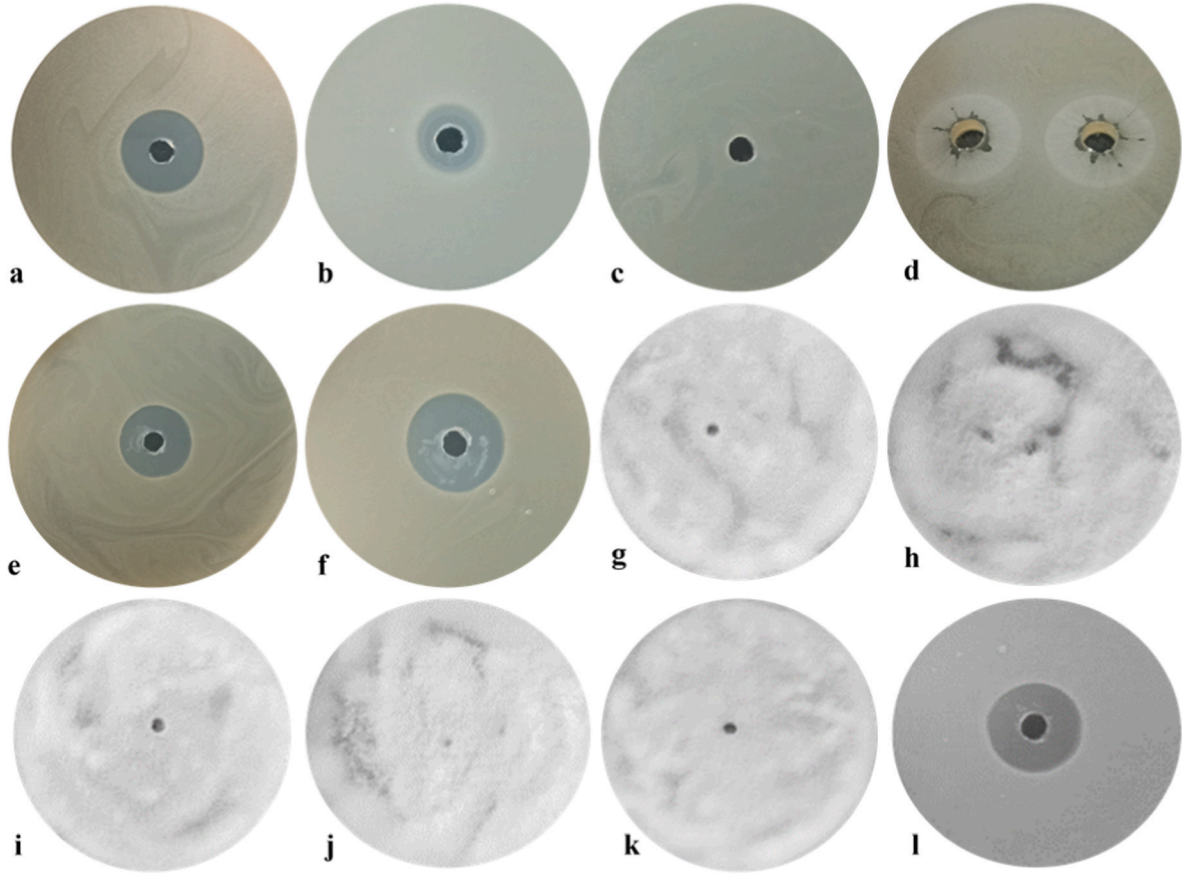

Fig. 3. The inhibitory effect of strain XJ-16 on pathogenic bacteria (a. *E. coli*; b. *S. aureus*; c. *K. pneumoniae S*; d. *E. faecalis*; e. *E. cloacae*; f. *K. pneumoniae R*; g. *Botrytis cinerea*; h. *Cucumber Fusarium wilt*; i. *Apple Ringworm*; j. *Rhizoctonia solani*; k. *Fusarium head blight*; l. *Candida albicans*).

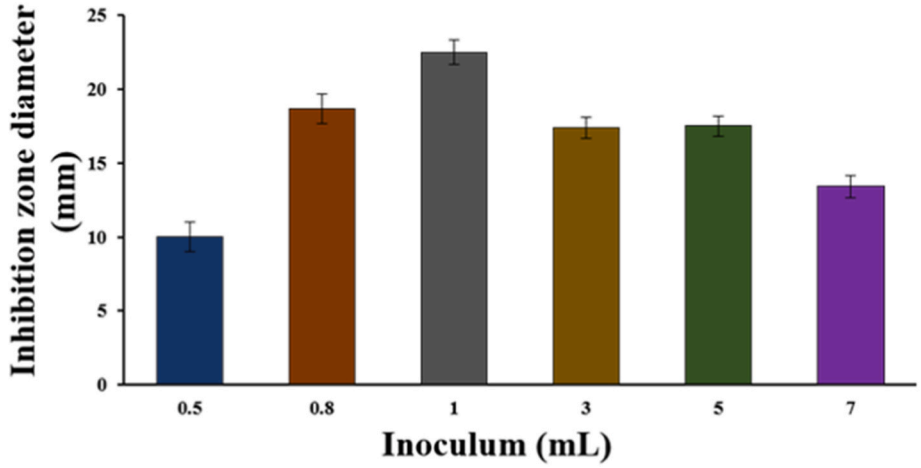

Fig. 4. The effect of strain XJ-16 inoculum on the antibacterial activity of fermentation broth.

0.93, respectively, and the zone of inhibition of the band with an Rf value of 0.32 was greater. These results showed that there were multiple antibacterial active substances in the XJ-16 fermentation broth. In addition, it was also found that under acidic conditions, the active substance existed in the form of ions, which had a strong solubility and inhibitory effect on the indicator strains. By contrast, the substance was not ionized under alkaline conditions, so that its solubility was reduced, and the original inhibitory effect was lost. Based on the above experimental observations, it could be inferred that the active substance was a basic antibiotics.

3.6. Separation and purification of antibacterial active substances

In the thin layer chromatography experiment with dichloromethane: methanol at a ratio of 1:2, three spots with Rf values of 0.3, 0.6, and 0.7 appeared on the thin layer plate, all of which had antibacterial activity. However, when other ratios of solvents were used in the mobile phase, no spots appeared. Therefore, the best developing agent was dichloromethane: methanol at a ratio of 1:2.

The subsequent silica gel column chromatography results are shown in Table 2. According to the size of the inhibition zone, the eluent

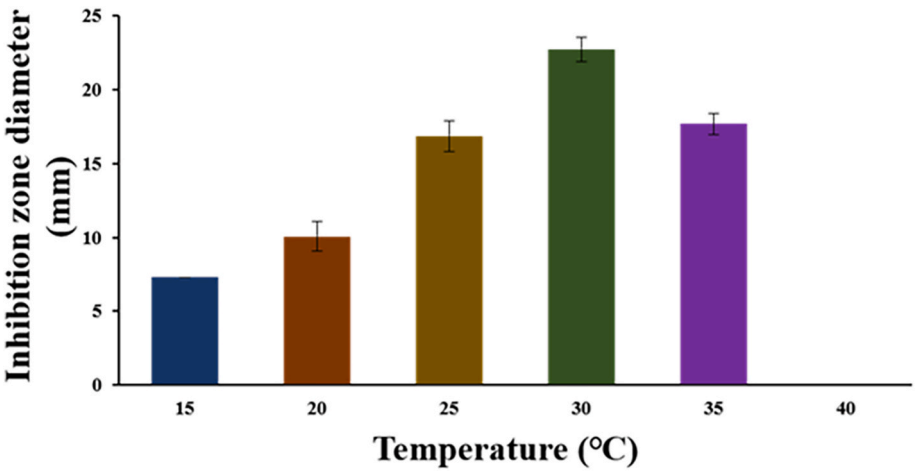

Fig. 5. The effect of culture temperature on the antibacterial activity of strain fermentation broth.

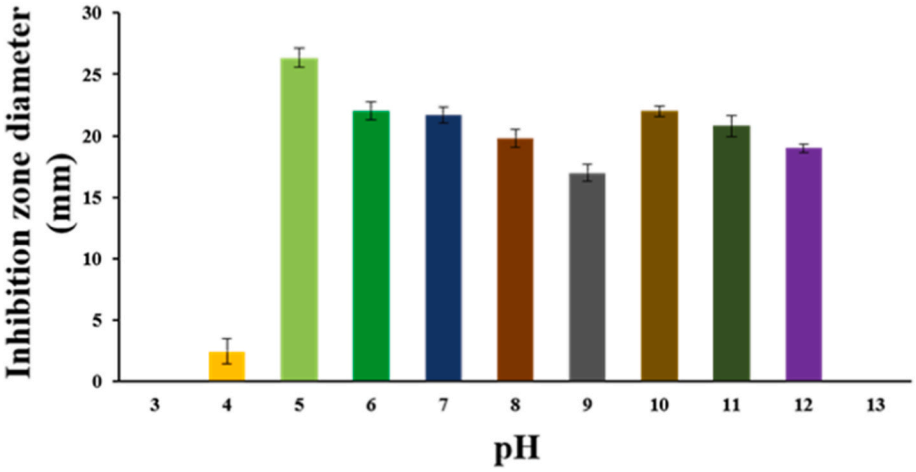

Fig. 6. pH control.

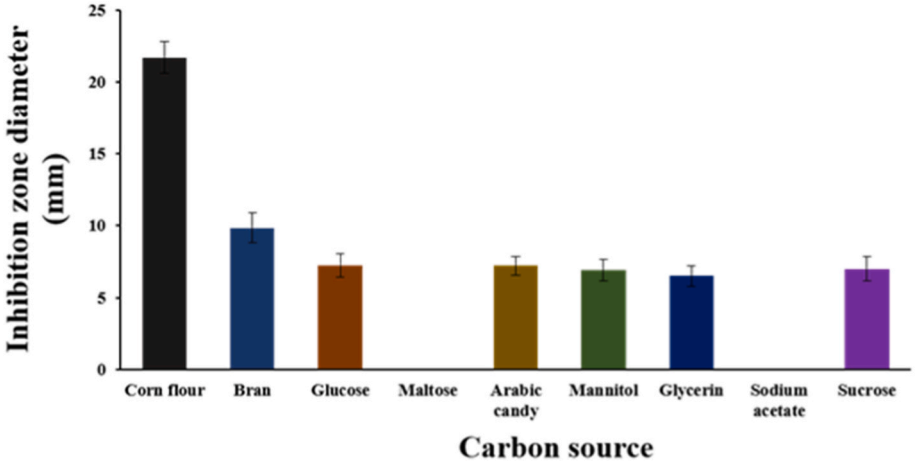

Fig. 7. Effect of carbon source change on antibacterial active of fermentation broth of the strain.

fractions ① ② ③ obtained with dichloromethane: methanol had the strongest antibacterial activity. After these three eluates with good antibacterial effects were combined and diluted, a multifunctional microplate reader was used to record their absorption spectra. The antibacterial active substances mainly absorbed at 280 nm, indicating the presence of phenyl groups or aromatic amino acids.

The combined eluent was analyzed by semi-preparative high performance liquid chromatography, and the gradient elution result is shown that when the mobile phase system was set to methanol: water at a ratio of 1:1, a large characteristic peak appeared at a retention time of

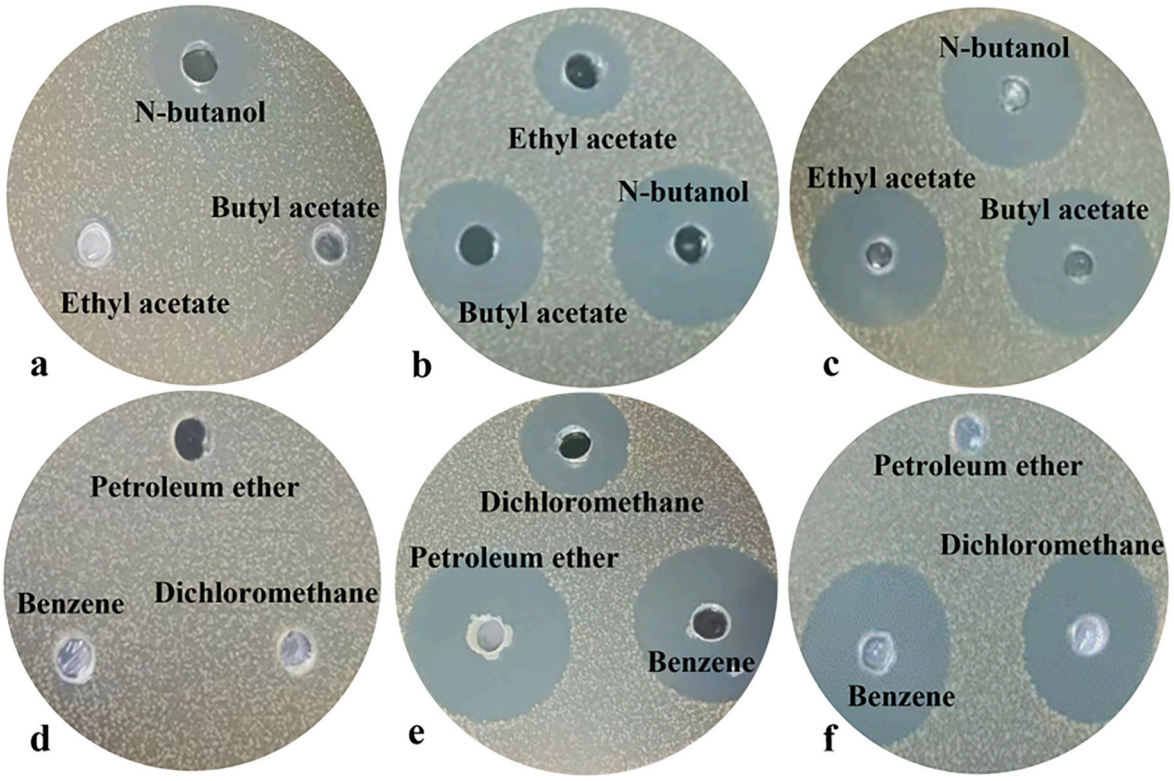

Fig. 8. The effect of different extractants (a. Control: Ethyl acetate, N-butanol, Butyl acetate; b. Water phase: Ethyl acetate, N-butanol, Butyl acetate; c. Organic phase: Ethyl acetate, N-butanol, Butyl acetate; d. Control: Benzene, Petroleum ether, Dichloromethane; e. Water phase: Benzene, Petroleum ether, Dichloromethane; f. Organic phase: Benzene, Petroleum ether, Dichloromethane).

Table 2
Silica gel column chromatography.

Eluent ratio	TLC same component number	Diameter of inhibition zone of each same component (mm)
Dichloromethane: Methanol 1:0	①	29
	②	29
	③	21
	④	13
Dichloromethane: methanol 2:1	①	25
	②	19
	③	15
Dichloromethane: methanol 1:1	①	12
	②	12
Dichloromethane: methanol 1:2	①	12
Dichloromethane: methanol 1:3	②	12
Dichloromethane: methanol 0:1	③	0

28.192 min. The eluted fractions corresponding to the large characteristic peaks were collected, and their antibacterial activity was determined by the bacteriostatic zone diffusion method. As shown in Fig. 9a, the eluate fractions collected at 25–30.34 min and 30.34–34.73 min all had antibacterial activity, while there was no antibacterial ability in the other fractions.

Subsequently, the fractions with an antibacterial effect were eluted with a mobile phase consisting of methanol: water at a ratio of 80:20 for 30 min, and then with a mobile phase of methanol: water at a ratio of 1:1 for 20 min. As shown in Fig. 9b, there was no peak after 30 min, demonstrating that the antibacterial active substance had been completely eluted before 30 min.

When the mobile phase of methanol: water at a ratio of 80:20 was used to elute the column for 20 min, two characteristic peaks appeared at retention times of 8.244 min and 14.684 min, respectively (Fig. 10b). Then, the eluate fractions corresponding to these two characteristic peaks were collected and their antibacterial activity was determined. As shown in Fig. 10b, the characteristic peak with a retention time of 14.684 min had antibacterial activity, and the corresponding eluent was prepared in larger quantities.

After the preparation of the corresponding fractions, its purity was checked by high performance liquid chromatography. As shown in Fig. 10c, the characteristic peak only appeared at 13.261 min, this eluate had antibacterial ability, which demonstrated that the antibacterial active substance had been successfully separated and purified.

As expected, the concentration after collection was lower than that before loading, and the antibacterial effect decreased. Notably, the pH paper chromatography also showed that the strain XJ-16 could produce a variety of antibacterial active substances, and if the antibacterial active substances had a combined structure, the antibacterial effect would also be weakened as the number of purification steps increased. However, the separation of substances is the most critical step in the study of microbial secondary metabolites. The bacteriostatic substances could only be separated from the multi-component mixture of fermentation broth by targeted separation methods, and the purification of crude extracts of antibacterial substances to obtain the pure substance is a prerequisite for chemical molecular composition and molecular structure analysis in future studies.

4. Conclusions

In this study, it was determined that the alkali-resistant strain XJ-16 belongs to cyaneus group of filamentous *Actinomycetes*, which can produce a striking blue pigment. In addition, this strain had high enzyme production capacity, and good inhibitory effects against Gram-positive and Gram-negative bacteria, as well as the yeast *Candida albicans*. The antibacterial active substance exhibited low polarity and had a strong

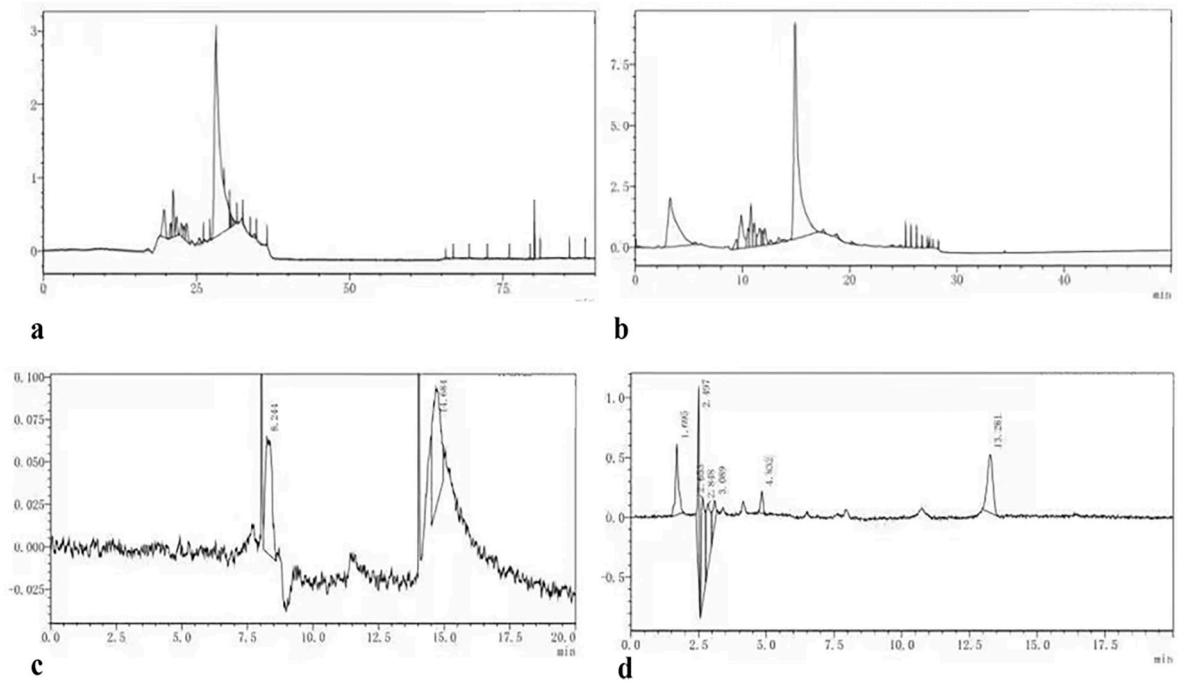

Fig. 9. HPLC chromatogram.

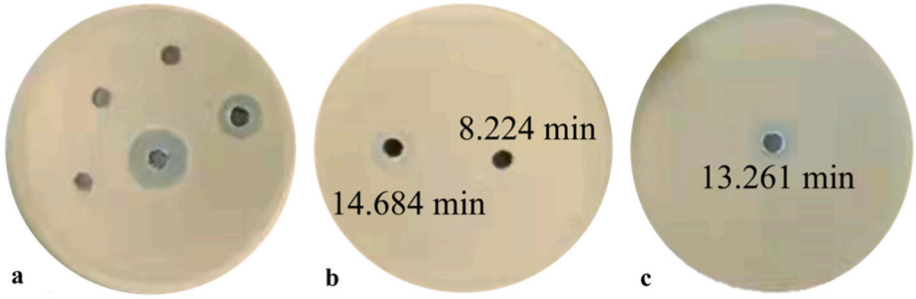

Fig. 10. Antibacterial activity graph.

tolerance to alkali. Overall, XJ-16 is a strain with great potential for development, and this study lays the foundation for further analysis of the chemical molecular composition and molecular structure of its active substances.

Authors' contributions

Ke Jiang and Ruoxuan Bai contributed to the microbiological laboratory and data analyses, helped in the study design, and integrated the first draft. Xianglian Luo and Ting Gao did the molecular analysis of the strains. Ke Jiang, Fangxu Xu, and Hongxin Zhao prepared tables and contributed to design the work that led to the submission. Dianpeng Zhang helped in the drug-resistance tests, interpreted results, and contributed to revise the manuscript and approved the final version. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2023.03.001.

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