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Identification and Fermentation Optimization of an Antibacterial *Aspergillus tubingensis* Associated with the Crab *Portunus triuberbuculatus*

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To identify marine fungus SZX-6 isolated from the inner of *Portunus triuberbuculatus* with antibacterial activity and to optimize the fermentation conditions of producing antibacterial active substances. Based on its morphological characters and internal transcribed spacer (ITS) sequence analysis results, strain SZX-6 was identified as *Aspergillus tubingensis*. The optimized fermentation conditions were determined as sucrose 3%, beef extract 1.5%, KCl 0.1%, $MgSO_4$ 0.02%, old seawater, inoculum size 1%, 28°C, 160 r/min for 8 d by the the one-factor-at-a-time method, the orthogonal matrix method and the time-bioactivity cources assay. The initial identification data demonstrated that the antibacterial active substances were mainly composed of moderate polar components with plentiful quantity and alkaloid-colored. The strain of *A. tubingensis* being isolated from the seawater-cultured animal was firstly reported here, and its antimicrobial activity was initially invenstigated in this paper.

Key words: *Aspergillus tubingensis*, Antibacterial activity, Identification, Fermentation optimization.

Since antibacterial cephalosporin C was founded from marine fungi *Cephalosporium acremonium* in the 1950 s, the researches on antimicrobial substances of marine microbe increasingly become the hot spot of the drug research and development. Among them, marine fungi have the unique living environments different from terrestrial fungi (such as oligotrophic, high salt, weak alkali etc.), making the marine fungi form special metabolism and defense systems, which can produce the metabolites with unique structure and specific biological activity that can not produced by the terrestrial fungi[1-3]. So far, more than one thousand new secondary metabolites from marine fungi have been founded and structure types involve alkaloids, polyketides, terpenes, macrolides, etc., these compounds possess good bioactivities such as antibacterial, anti-tumor, antivirus etc.[4,5].

During the past two decades, the aquaculture industry of China acquired the rapid development, the output of aquatic products ranks the first in the world, but also some animal diseases continuously appeared. *Vibrio anguillarum* and *Aeromonas hydrophila* are the common pathogenic bacteria causing the cultured fish diseases[6,7]. The long-term use or abuse of antimicrobial drugs resulted in the drug resistance of pathogenic bacteria, high residues and increasingly serious environmental pollutions[8]. Therefore, the research and development of new high efficiency, broad spectrum and low pollution

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of aquatic antibiotics attracts great attention worldwide.

The strain SZX-6 associated with the crab *Portunus triuberbuculatus* was obtained by bioactive-chemical integrated screening methods in our laboratory, which presents the favorable antibacterial activities and produces alkaloids compounds. This study will identify SZX-6 strain, optimize the culture medium components for the production of antibacterial active substances, in order to provide the clues for further separate and identify the active compounds.

MATERIALS AND METHODS

Microorganism

SZX-6 strain was separated from the inner of crab *Portunus triuberbuculatus* and conserved at the marine microbial active substances laboratory of Huaihai Institute of Technology, Jiangsu Province, P. R. China. Indicator strains *Vibrio anguillarum* and *Aeromonas hydrophila* kept by our laboratory.

Culture medium

Potato Dextrose Agar medium (PDA) contained (per liter): potato extract (200 g, counted by fresh potato weight), glucose (20 g), agar (15 g). Sabouraud's Dextrose Agar medium (SDA) contained (per liter): peptone (10 g), glucose (40 g), agar (15 g). Liquid PD and SD were prepared similar to PDA and SDA but without the agar. Czapek's Yeast extract medium (CY) contained (per liter): sucrose (30 g), yeast extract (1 g), $NaNO_3$ (3 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), K_2HPO_4 (1 g). Beef extract Peptone medium contained (per liter): beef extract (3 g), peptone (10 g), NaCl (5 g) and agar (15 g). The pH was adjusted to 7.0 with NaOH. All above medium were dissolved in natural seawater.

The observation of conventional morphology and scanning electron microscope

The taxonomic systems of *Aspergillus* were followed for observation and identification. The strain SZX-6 was cultured on SDA at 28°C. Macroscopical characters were assessed at 4 and 7 d of training time and microscopical characters were assessed after staining with lactophenol cotton blue. For scanning electron microscopic observations, the strain SZX-6 was cultured on SDA with the inclined inserting cover slips at 28°C for 7 d. The coverslip were picked up and dried for viewing in the scanning electron microscope (HITACHI JSM-6390LA, Japan)[9].

Internal transcribed spacer (ITS) sequence analysis

The purified SZX-6 was inoculated on SDA culture medium and cultivated at 28°C for 7 d, then colony of SZX-6 were collected. Extraction of DNA from SZX-6 was performed according to the reference[10]. The ITS rDNA of the strain was amplified by PCR with the forward primer (5' -TCCGTAGGTGAACCTGCGG-32) and reverse primer (5' -TCCTCCGCTTATTGATATGC-3'). Genomic DNA of strain SZX-6 was added to PCR reactions containing forward and reverse primers. After 5 min incubation at 94°C, thirty-five PCR cycles (94°C, 1 min; 57°C 45 s, 72°C 45 s) were performed, followed by one cycle of 10 min at 72°C. The PCR product was separated by electrophoresis and then isolated and sequenced (Beijing Sunbiotech Co., Ltd, China). A homology search was performed with GenBank database. The ITS sequence was deposited into GeneBank Data Library with the accession number JX287371.1. The ITS sequence of strain SZX-6 was aligned manually with available nucleotide sequences retrieved from the GenBank and the multiple sequence alignment using CLUSTAL X (Ver.1.83). Phylogenetic tree was constructed using the Neighbor-joining method from MEGA (Ver.4.0). The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replications.

Screening of basal culture medium

The seed culture was prepared by adding mycelia on a SDA slant into a 250-ml Erlenmeyer flask with 100 ml of SD medium. Cultures were incubated for 24 h in a rotary shaker (160 r/min) at 28°C. The harvested seed culture was added into 250-ml Erlenmeyer flasks containing 100 ml of liquid PD, SD and CY fermentation medium respectively to be optimized in the following procedures (described below). The inoculum size was 1% (v/v). The culture was incubated at 28°C on a rotary speed 160 r/min for 7 d. Culture supernatant was obtained by filtered through a 0.22 μm Millipore Filter. The supernatant samples were tested for antibacterial activity by Oxford cup method, antibacterial activity was expressed as the inhibition zone (mm).

Optimization of fermentation medium using the one-factor-at-a-time method

In order to study the major nutrients requirement for enhancing antibacterial activity of fermentation broth against *V. anguillarum* and *A. hydrophila*, various carbon sources (glucose, sucrose, galactose, lactose, fructose and mannose at 3% concentration), nitrogen sources (peptone, yeast extract, beef extract, KNO_3, $NaNO_3$ and NH_4Cl at 1% concentration) or inorganic salts (KH_2PO_4, $MgSO_4$, $CaCl_2$, KCl, Na_2HPO_4 and K_2HPO_4 at 0.05% concentration) were respectively supplemented to the basal medium (SD medium) using the one-factor-at-a-time method. After 7 d of fermentation, the antibacterial activities of fermentation broth were investigated.

Optimization of concentrations of selected medium components using the orthogonal matrix method

To raise the antibacterial activity of fermentation broth, the orthogonal design L_9 (3^4) was carried out to optimize the concentrations of four selected nutrients (sucrose, beef extract, KCl and $MgSO_4$) in flask experiments. The factors and levels of orthogonal experiment were shown in Table 2. Based on the L_9 orthogonal array design, 9 experiments in triplicate were carried out. After 7 d of fermentation, the antibacterial activity of fermentation broth was evaluated.

Determination of fermentation time using the time-bioactivity cources assay

The seed culture was added into the optimized medium and the inoculum size was 1% (v/v). The culture was incubated at 28°C on a rotary speed 160 r/min for 1-10 d. Culture supernatant and mycelia were obtained by filtered through a 0.22 µm Millipore Filter per day respectively. The supernatant samples were tested for antibacterial activity by Oxford cup method, the wet mycelia were dried in hot air oven at 90°C for 12 h and the dried cell weight (DCW) was determined. The antibacterial activity was expressed as inhibition zone (mm).

RESULTS

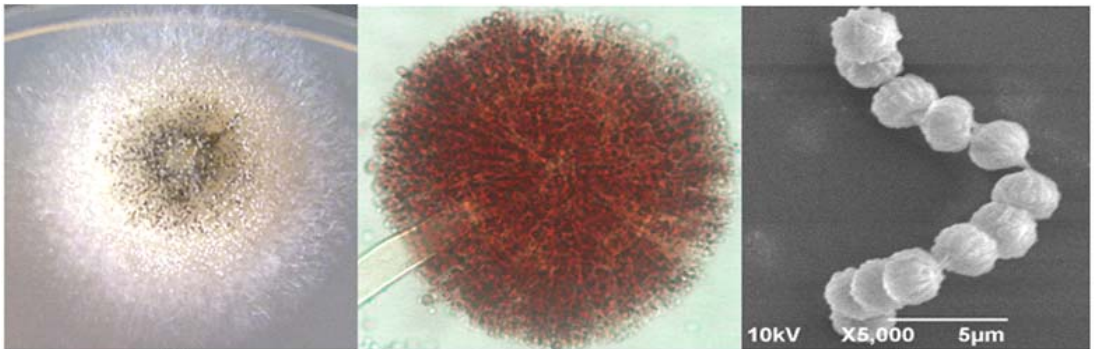
Identification of strain SZX-6

The purified colonies growed rapidly in Sabouraud's agar plate and had radial villus and white mycelia at the edge of each colony in early culture stages (3-5 d) (Fig. 1A). After 7 d, the colony diameter could reach 65-70 mm at 28°C and the colony became pitchy at the head of mycelium and yellowish-brown in the reverse side, respectively. The vesicle of SZX-6 was round and radial. Diaphragms in hypha were visible with microscope (Fig. 1B). Electron microscope scanning image showed that sporangia of SZX-6 consisted of many spores and the value of conidial diameter was about 3-4 µm. The conidium was elliptical with introcession in head (Fig. 1C).

The ITS gene of strain SZX-6 was generated and the size of amplified fragment was 557 bp. The results of the ITS gene sequence analysis were submitted to GenBank (access number JX287371). Phylogenetic analysis of ITS gene sequence of the strain and related taxa showed 99% similarity to the *A. tubingensis* A1KA (Genbank accession no. AM745112) (Fig. 2). As the result, strain SZX-6 was identified as *A. tubingensis*.

Screening of basal medium

Antibacterial metabolites production was investigated in the different medium compositions dissolved in natural seawater. The fermentation



(a) (b) (c)

Fig. 1. Morphological characters of strain SZX-6. A. Colony; B. Conidiphore; C. Conidium (Bar=5µm)

broth in Sabouraud's Dextrose medium (SD) presented the inhibition zones of 17.72 mm and 14.96 mm against *V. anguillarum* and *A. hydrophila*, meanwhile the fermentation broth in Potato Dextrose medium (PD) just possessed the inhibition zone of 16.97 mm against *A. hydrophila*, whereas the fermentation broth in Czapek's Yeast extract medium showed no antibacterial activity for both two indicator bacteria. According to the reference[11], the inhibition zone >12 mm against *A. hydrophila* were considered as susceptibility to the isolates. Thus, SD medium was selected as the basal medium for further experiments.

Optimization by the one-factor-at-a-time method

With the one-factor-at-a-time method, the effects of carbon sources, nitrogen sources and inorganic salts on antibacterial activities against *V. anguillarum* and *A. hydrophila* were tested. Sucrose (16.28±0.68 mm, 20.84±0.87 mm) and beef extract (16.07±1.03 mm, 19.74±0.81 mm) were the most suitable carbon source and nitrogen source respectively, while both KCl (18.06±0.56 mm, 16.83±0.81 mm) and $MgSO_4$ (16.25±0.47 mm, 14.05±0.57 mm) had a benefical effect on antibacterial activity of fermentation broth (Table 1). As a result, the concentrations of the four nutrients were optimized by the L_9 (3^4) orthogonal matrix method in the following experiment.

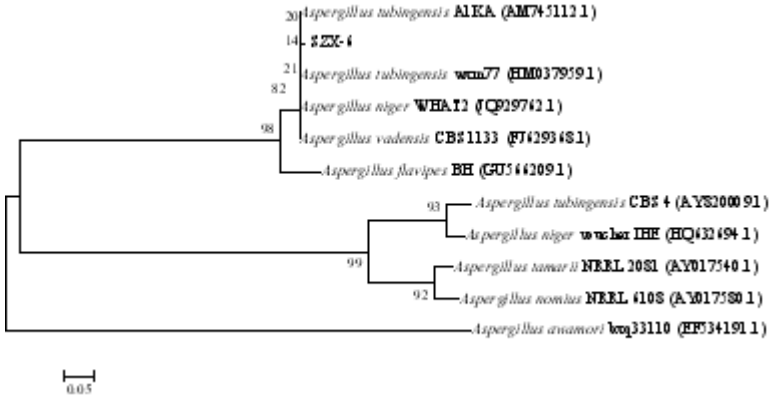
Optimization by the L_9 (3^4) orthogonal matrix method

The L_9 (3^4) orthogonal matrix method was often used to optimize the medium components in the fermentation experiments[9]. In this study, four factors (sucrose, beef extract, KCl and $MgSO_4$) with three levels of each factor were investigated. The results of antibacterial activities against *V. anguillarum* and *A. hydrophila* by the orthogonal matrix method were given in Table 2.

The intuitive analysis (Table 2) and the variance analysis results (data not shown) showed that antibacterial activity of fermentation broth could be enhanced using a combination of those factors at different levels. Among the four nutrients, KCl was the most significant effect factor. The best combination of antibacterial activity against *V. anguillarum* was A2B2C1D1, meanwhile the best combination of antibacterial activity against *A. hydrophila* was A2B3C3D1. The checking experiments by using these two combinations were taken and the results showed that the combination of A2B3C3D1 was better than the combination of A2B2C1D1. So the combination of A2B3C3D1 was used as the final optimal combination, namely (w/v): 3% sucrose, 1.5% beef extract, 0.1% KCl, 0.02% $MgSO_4$.

Determination of fermentation time

After carrying out the fermentation of strain SZX-6 for 1-10 d under the optimum medium, the DCW of strain SZX-6 was gradually increased, achieved the maximal level at 7 d and kept stable during 7-10 d. For antibacterial activity of fermantation broth, the inhibition zones arrived the highest level at 8-9 d. Therefore, the fermentation days were determined as 8 d.



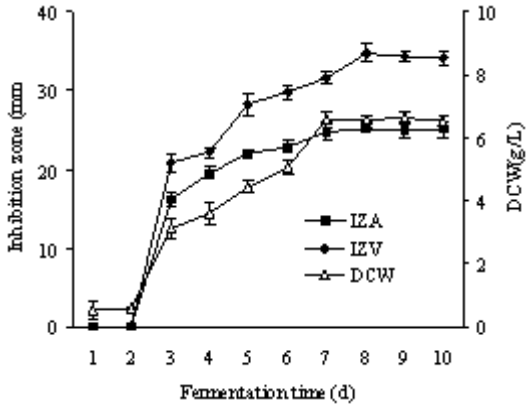
Note: The sequence number in the bracket means the GenBank accession number of the strain. The number at the node means the percentage of occurrence in 1,000 boot-straped trees. The scale bar means 5% sequence difference

Fig. 2. Polymeric analysis of strain SZX-6 and other *Aspergillus* strains based on the sequence of ITS

Table 1. Effects of nutrients on inhibition zone of the fermentation broth of strain SZX-6 against *V. anguillarum* and *A. hydrophila* (mean±SD, n=3, mm)

Carbon sources	Inhibition zone		Nitrogen sources	Inhibition zone		Inorganic salts	Inhibition zone	
	V. anguillarum	*A. hydrophila*		*V. anguillarum*	*A. hydrophila*		*V. anguillarum*	*A. hydrophila*
glucose	11.80±0.61	17.46±0.49	peptone	16.89±0.46	16.27±0.93	KH_2PO_4	14.48±0.65	13.09±0.65
sucrose	16.28±0.68	20.84±0.87	yeast extract	9.01±0.78	12.92±0.57	$MgSO_4$	16.25±0.47	14.05±0.57
galactose	9.56±0.19	20.84±1.01	beef extract	16.07±1.03	19.74±0.81	$CaCl_2$	18.06±0.93	-
lactose	10.10±0.35	19.08±0.71	KNO_3	15.78±0.58	12.08±0.67	KCl	18.06±0.56	16.83±0.81
fructose	-	20.76±0.54	$NaNO_3$	12.22±0.88	10.03±0.52	Na_2HPO_4	15.59±0.49	12.68±0.96
mannose	10.47±1.08	22.42±0.29	NH_4Cl	8.91±0.39	9.97±0.58	K_2HPO_4	15.02±0.54	11.78±0.41

Note: "-" shows no antibacterial activity



Note: IZV and IZA show the antibacterial activity against *V. anguillarum* and *A. hydrophila* of SZX-6 broth, respectively. DCW shows dry cell weight

Fig. 3. The variation of antibacterial activity and dry cell weight of strain SZX-6 during the fermentation time course

DISCUSSION

It is estimated that marine fungi have at least 1,500 species. But until 2,000 year, only 444 kinds of marine fungi were described and less than 1% of approximately fifty thousand terrestrial fungi[12]. Therefore, marine fungi are still in the stage of exploration and research. Researches showed that the secondary metabolites of marine fungi are mainly made up of alkaloid, polyketide and terpene[13]. Nowadays 7 kinds of marine drugs in market are alkaloid compounds, which shows that alkaloids possess the highest potent to develop the drugs[14].

Talented strains refer to the microbial strains which can produce abundant secondary metabolites with high output and good bioactivity. Talented strains are rarely although microbial species are various. Then talented strains can be found from various marine fungi just through the bioactive-chemical integrated screening methods[2].

In the present study, the strain SZX-6 associated with the crab *Portunus triuberbuculatus* was obtained by bioactive-chemical integrated screening methods in our laboratory, which was found to possess the favorable antibacterial activities and produces series alkaloids compounds. The strain was further identified as *A. tubingensis* according to the traditional classification and the ITS sequence analysis. *A. tubingensis* is a black *Aspergillus*

Table 2. L_9 (3^4) orthogonal matrix experimental design and results

Exp. No.	Factors				Inhibition zone (mean±SD, n=3, mm)	
	Sucrose/%	Beef extract/%	KCl/%	$MgSO_4$/%	*V. anguillarum*	*A. hydrophila*
1	1 (2) *	1 (0.5)	1 (0.02)	1 (0.02)	17.88±0.61	15.67±0.31
2	1 (2)	2 (1.0)	2 (0.06)	2 (0.06)	13.38±0.19	12.08±0.18
3	1 (2)	3 (1.5)	3 (0.10)	3 (0.10)	15.86±0.67	14.40±0.60
4	2 (3)	1 (0.5)	2 (0.06)	3 (0.10)	13.04±0.56	12.24±0.05
5	2 (3)	2 (1.0)	3 (0.10)	1 (0.02)	19.88±0.43	17.63±0.56
6	2 (3)	3 (1.5)	1 (0.02)	2 (0.06)	18.77±1.68	16.47±0.78
7	3 (4)	1 (0.5)	3 (0.10)	2 (0.06)	14.84±0.21	16.67±1.39
8	3 (4)	2 (1.0)	1 (0.02)	3 (0.10)	16.35±0.01	14.71±0.50
9	3 (4)	3 (1.5)	2 (0.06)	1 (0.02)	13.75±0.68	13.96±0.62
K_1-*V.* (*A.*)	47.12	45.76	53.00	51.51		
	(42.15) #	(44.58)	(46.85)	(47.26)		
K_2-*V.* (*A.*)	51.69	49.61	40.17	46.99		
	(46.34)	(44.42)	(38.28)	(45.22)		
K_3-*V.* (*A.*)	44.94	48.38	50.58	45.25		
	(45.34)	(44.83)	(48.70)	(41.35)		
Sj-*V.* (*A.*)	7.91	2.58	30.98	6.96		
	(3.19)	(0.03)	(20.60)	(6.01)		
Order-*V.* (*A.*)	C>A>D>B	(C>D>A>B)				
Optimal combination-*V.* (*A.*)	A2B2C1D1	(A2B3C3D1)				
Final optimal combination	A2B3C3D1					

Note: *The inside of bracket shows the actual level of factors; # the outside of bracket shows the response of *V. anguillarum* and the inside shows the response of *A. hydrophila*

frequently isolated from different agricultural products. Nowadays the researches on *A. tubingensis* were mainly focused on the ochratoxin A production and molecular identification[15,16], there have few reports published in recent years about its secondary metabolites. Huang *et al*. reported that three new dimeric naphtho-γ-pyrones together with five known compounds were isolated from the mangrove endophytic fungus *A. tubingensis* GX1-5E strain[17]. In this study, we firstly reported an antibacterial *A. tubingensis* strain associated with the crab *P. triuberbuculatus* being isolated and identified. Since *A. tubingensis* SZX-6 lived in extreme marine environment different from the land, the ecological differentiation could lead *A. tubingensis* strain SZX-6 to produce novel metabolites, which was consistent with the previous reports that each metabolites may have a distinct ecological function[18,19].

Generally, medium components need to be optimized to achieve more objective products. The optimized medium was obtained in this study using the one-factor-at-a-time method, the L_9 (3^4) orthogonal matrix method and the time-bioactivity cources assay. The further study indicated that the antibacterial active substances were mainly composed of moderate polar components with plentiful quantity and alkaloid-colored, which provides the clues for further separation and purification of the active metabolites.

In conclusion, *A. tubingensis* SZX-6 strain demonstrated its higher antibacterial properties and a great potential source of antibacterial compounds. To the best of our knowledge, this is the first report of antibacterial activity detected in *A. tubingensis*. Further study on the metabolites from strain SZX-6 will help to find novel active compounds, the relevant work is in progress.

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