SUPPLEMENTARY MATERIAL

Separation, determination and antifungal activity test of the products from a new *Bacillus amyloliquefaciens*

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Abstract One new *Bacillus amyloliquefaciens* named ZJU-2011 was discovered and the culture supernatant showing strong inhibitory effect against *Candida albicans*. In this study, a novel method was developed to purify the antifungal compounds in high purity. The obtained products were analyzed by HPLC proven to be of high purity. MS chromatograms showed that the molecular weight of the bioactive components was 270 and 288, respectively, and their structures were determined to be bacilysin and chlorotetaine by using ¹H NMR and ¹³C NMR. To our best knowledge, this is the first time that *Bacillus amyloliquefaciens* has been reported to produce bacilysin and chlorotetaine simultaneously. The MIC values of chlorotetaine against six common fungal pathogens were determined firstly to be 7.8 µg/mL, 3.9 µg/mL, 3.9 µg/mL, 3.9 µg/mL, 1.8 µg/mL, and 1.8 µg/mL, respectively.

1 Experimental details

1.1 Microorganisms, cultivation and conditions

1.1.1 Determination of the antibiotic-producing bacterium: ZJU-2011

DNA extraction and 16S rRNA gene PCR were carried out in standard molecular manipulations. 16S rRNA gene sequence analysis and whole-genome sequencing were completed by Sangon Biotech (Shang Hai) Co., Ltd. The identification of ZJU-2011 was carried out by the BLAST (Zhang et al., 2000) program against the database containing the published type strains with default settings.

1.1.2 The antibiotic-producing strain, test strain and fermentation process

The fermentation process and agar-well diffusion assay for the determination of antibiotic activity were carried out according to our previous work (Tao et al. 2014).

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1.2 Separation and purification of the antifungal compounds

1.2.1 Pretreatment of the fermentation broth

The fermentation broth was centrifuged at 5000 rpm/min and then concentrated in a rotary evaporator. The concentrated supernatant (less than 200 mL) was applied to an XAD1600 resin column (400 mm x Φ 55 mm) eluted with distilled water as the mobile phase; the flow rate was set at 5 mL/min. The 3BV (bed volume) eluent was collected and preserved for use. The antimicrobial activity of the water-soluble phase was made to a volume equal to that of the fermentation broth and tested as stated above. Samples were freeze-dried and weighed.

The rate of killing was determined by the following formula:

$$\text{Killing (\%)} = \frac{\text{diameter of inhibitory zone(sample)}}{\text{the diameter of inhibitory zone(the maximal zone)}}$$

1.2.2 Separation and purification of the active component in lab-scale experiments

The separation and purification process is shown in Fig. S1.

(1) Purification of the active component by reversed-phase chromatography with acid-resistant MCI resin

The crude culture broth was applied to a reversed phase MCI resin column (400 mm x Φ 100 mm). The sample (250 mL) was added to this column and eluted with 0.1 % trifluoroacetic acid as the mobile phase with a flow rate of 22 mL/min and an elution volume of 2.5 BV. The eluent was collected and assessed for antifungal activity. Then the mobile phase was replaced for 10% methanol (v/v) + 0.1 % trifluoroacetic acid with a flow rate of 22 mL/min and an elution volume of 2.5 BV. The eluent was collected and assessed for antifungal activity.

(2) Purification of the active component by reversed-phase chromatography with C18 resin

The active sample collected from the above procedure was then applied to a reversed-phase chromatography column (400 mm x Φ 40 mm). The concentrated sample (3 mL) was added to the column and eluted with purified water, and the flow rate was set at 12 mL/min. The eluent was collected by using an automatic sampling instrument (every 50 s). Samples in tubes were applied on a silica G plate and developed at room temperature with n-butyl alcohol:glacial acetic acid:water (2:2:1, v/v). Samples of bioactivities were collected, concentrated and then applied to another

reversed-phase chromatography column (400 mm x Φ 40 mm) (method was the same as that mentioned above). The breakthrough curve for the reversed-phase chromatography was drawn at the same time.

1.2.3 HPLC analysis of samples collected in every step with antibacterial activity

Samples of the isolated products of antifungal activity were evaluated by HPLC-CAD (Corona® ultra™ Detector) to test the purity. The column used was XBridge™ Amide 3.5 μ m 4.6*250 mm column, and the mobile phase was 80% acetonitrile (v/v), with the flow rate set at 0.8 mL/min.

1.3 Structural determination of the isolated antifungal compounds

The structure of the antifungal metabolite was determined by MS, ^1H NMR and ^{13}C NMR.

1.4 Antifungal activity of chlorotetaine

The MICs for antifungal activity were determined by the 2-fold broth dilution method by using a 96-well microplate. Test strains used in this study included two strains of *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Candida parapolymorpha*, and *Aspergillus niger*. Fluconazole (purchased from National Institutes for Food and Drug Control, China) was used as a positive control, and sterile water was used as a negative control. One milliliter of cell suspension ($\sim 2.03 \times 10^5$ cells/mL) cultured in SDA medium containing 1% bacteriological peptone and 4% glucose was incubated overnight at 37°C. Firstly, 100 μ l cell suspension was added to A1 of the 96-well microplate containing 100 μ l SDA medium solution in each well and mixed well. Then 100 μ l culture from A1 was transferred into A2 and mixed well in turn. Carried out as shown in Fig.S5, making the final solution volume in the each well be 100 μ l and the final antibiotic concentration ranging from 100 μ g/mL to 0.2 μ g/mL (antibiotic: 125 μ g/mL-0.0611 μ g/mL). Then the 96-well microplate was incubated at 37°C for 18-24h. Each test was repeated three times and the MIC was defined as the lowest antibiotic concentration that prevented visible growth.

2 SUPPLEMENTARY TABLES AND FIGURES

Fig. S1 Flow chart of the process of separation and purification of bacilysin and chlorotetaine (1 indicates that the chromatography column was eluted with 0.1% trifluoroacetic acid, and 2 indicates that the chromatography column eluted with 10% methanol + 0.1% trifluoroacetic acid).

1 gcagtcgagc ggacagatgg gagcttgctc cctgatgtta gcggcggacg ggtgagtaac
 61 acgtgggtaa cctgcctgta agactgggat aactccggga aaccggggct aataccggt
 121 ggttggttga accgcatggt tcagacataa aagtggtgctt cggctaccac ttacagatgg
 181 accgcggcgc cattagctag ttggtgaggt aacggctcac caaggcgacg atcgtagcc
 241 gacctgagag ggtgatcggc cacactggga ctgagacacg gccagactc ctacgggagg
 301 cagcagtagg gaattctccg caatggacga aagtctgacg gagcaacgcc gcgtgagtga
 361 tgaaggtttt cggatcgtaa agctctgttg ttagggaaga acaagtgccg ttcaaatagg
 421 gcggcacctt gacgtacct aaccagaaag ccacgggtaa ctacgtgcca gcagccgagg
 481 taatacgtag gtggcaagcg ttgtccggaa ttattgggcg taaagggtc gcagggggtt
 541 tcttaagtct gatgtgaaag ccccggtc aaccggggag ggatcattgga aactggggaa
 601 cttgagtga gaagaggaga gtggaattcc acgtgtagcg gtgaaatgcg tagagatgtg
 661 gaggaacacc agtggcgaag gcgactctt ggtctgtaac tgacgtgag gagcgaaagc
 721 gtggggagcg aacaggatta gataccctgg tagtcacgc cgtaaacgat gagtgcctaag
 781 tgttaggggg ttccgcccc ttagtgtgc agtaacgca ttaagcactc cgctggggga
 841 gtacggtgc aagactgaaa ctcaaaggaa ttacggggg cccgcacaag cgggtggagca
 901 tgtggtttaa ttcgaagca cgcaagaac cttaccaggt cttgacatcc tctgacaatc
 961 ctgagatag gacgtcccct tcgggggcag agtgacaggt ggtgcatggt tgcgtcagc
 1021 tctgtctgtg agatgttggg ttaagtccg caacgagcg aacccttgat cttagttgcc
 1081 agcattcagt tgggcactct aaggtgactg ccggtgacaa accggaggaa ggtgggggatg

1141 acgtcaaate atcatgcccc ttatgacctg ggctacacac gtgctacaat ggacagaaca
1201 aagggcagcg aaaccgcgag gtaagccaa tcccacaaat ctgttctcag ttcggatcgc
1261 agtctgcaac tcgactgcgt gaagctggaa tcgctagtaa tcgcggatca gcatgccgcg
1321 gtgaatacgt tccggggcct tgtacacacc gcccgtcaca ccacgagagt ttgtaacacc
1381 cgaagtcggt gaggtaacc

Fig.S2 The 16S rRNA gene sequence of ZJU-2011

Fig. S3 Phylogenetic tree of ZJU-2011 based on the 16S rRNA gene sequences. The tree was constructed with BLAST pairwise alignments.

Fig. S4 The breakthrough curve for the first C18 reversed-phase chromatography.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100μL antibiotic medium + 100μL(a) cell suspension	100μL	100μL	100μL	100μL	100μL	100μL	100μL	100μL	100μL	100μL	100μL
B	Replicate of A											
C	Replicate of A											
D	100μL antibiotic medium + 100μL(b) cell suspension											
E	Replicate of B											
F	Replicate of B											
G	Negative control											
H	Positive control											
FAC (μg/mL)	100(positive control)	50	25	12.5	6.2	3.1	1.6	0.8	0.4	0.2	0.1	0.05
	125(antibiotic)	62.5	31.25	15.625	7.8125	3.9063	1.9531	0.9766	0.4883	0.2441	0.1221	0.0611

(FAC: the final antibiotic concentration)

Fig. S5 Representation of the work flow for the determination of MICs

Fig.S6 Mass spectrum of the molecular from target 1

Fig.S7 Mass spectrum of the molecular from target 2

Fig.S8 HPLC-CAD analysis of the purity of bacilysin

Fig.S9 HPLC-CAD analysis of the purity of chlorotetaine

Table S1

	Fermentation broth	Ethanol supernatant	Eluted from XAD 1600
Dry weight (mg/mL)	32.4	12.3	5
Antifungal activity (mm)	42	41	41

(Activity (mm): the mean diameters of the inhibition zone, and antifungal activity in each part was determined at the same concentration as fermentation broth.)

Table S2 Results of the antifungal activity of chlorotetaine

Test strains	MICs (μg/mL)	MICs (μg/mL)
	chlorotetaine	Fluconazole
<i>Candida albicans</i> ATCC 10731	7.8	6.2
<i>Candida albicans</i> ATCC 10231	3.9	1.6
<i>Candida parapsilosis</i> JCM1785	3.9	0.8
<i>Candida krusei</i> CBS573	1.8	1.6
<i>Candida parapolymorpha</i> ATCC 26012	1.8	0.4
<i>Aspergillus niger</i>	3.9	1.6

Table S3

¹³C (600 MHz) and ¹H (600 MHz) NMR data for bacilysin in D₂O.

¹³ C			¹ H			
Position	δ _C	mult.	Position	δ _H	Position	δ _H
1	16.2	CH3	1	1.27	13	3.38
2	20.08	CH2	2	1.29	14	3.79
3	30.06	CH	3	1.45	15	3.80
4	34.59	CH2	4	1.77	16	3.81
5	35.21	CH2	5	1.95	17	4.00
6	48.72	CH	6	2.20	18	4.70
7	53.18	CH	7	2.23		
8	55.11	CH	8	2.96		
9	59.16	CH	9	2.97		
10	170.08	C(quaternary)	10	3.07		
11	178.44	C(quaternary)	11	3.08		
12	209.52	C(quaternary)	12	3.16		

Table S4

^{13}C (600 MHz) and ^1H (600 MHz) NMR data for chlorotetaine in D_2O . P.: position; **qua.** : Quaternary.

^{13}C			^1H	
Position	δ_{C}	mult.	Position	δ_{H}
1	16.4	CH3	1	1.50
2	26.83	CH2	2	1.76,2.14
3	34.75	CH	3	2.68
4	35.56	CH2	4	1.90,1.90
5	36.23	CH2	5	2.53,1.65
6	49.04	CH	6	4.03
7	53.15	CH	7	4.21
8	130.37	C(qua)	8	
9	153.72	CH	9	7.20
10	170.29	C(qua)	10	
11	178.17	C(qua)	11	
12	196.40	C=O	12	

References

- Tao, W., Shuyan, Z., Mianbin, W., Zhengjie, C., Jianping, L., Lirong, Y. (2014). Studies on batch fermentation kinetics of a novel water soluble antifungal antibiotic producing strain bacillus amyloliquefaciens ZJU-2011. *Journal of Chemical Engineering of Chinese Universities*, 6, 1294-1301.