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Isolation and Characterization of an Antimicrobial Compound Produced by *Lactobacillus casei* C7-2

Tie-Dan Zhang[1#], Yun-Zhi Shi[3#], Zhi-Wei Ye[1,2], Li-Qiong Guo[1,2] and Jun-Fang Lin[1,2*]

[1]Department of Bioengineering, College of Food Science, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou - 510 640, China.
[2]Institute of Biomass Research, South China Agricultural University, Guangzhou - 510 640, China.
[3]Beijing tiantan Biological Products Corporation Limited, Beijing - 100 024, China.

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In this study, a *Lactobacillus casei* strain (C7-2) that produced broad spectrum antifungal compounds was isolated from fermented pickle samples. This strain was identified by the analyses of morphologic characteristics, physiological-biochemical tests, and molecular-biological test. The highest yield of the antimicrobial substances produced by this strain was recorded at 36 h. The antimicrobial compound remained stable at pH values between 2.0 to 6.0, and also for 30 min at 121 °C. The antimicrobial compound produced by the strain C7-2 was found to have a wide antimicrobial spectrum, which effectively inhibit *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus, Salmonella*, *Saccharomyces cerevisiae*, and *Botryodiplodia theobromae* Pat. This antimicrobial compound was purified using silica gel column chromatography, VLC, and HPLC. The structure of this compound was elucidated using NMR (^{1}HNMR, ^{13}CNMR). The active compound was identified as 2,5-diketopiperazines (DKPS) [cyclo (L-Pro-D-Leu)]. The diketopiperazine derivative identified in the study may be a promising alternative to chemical preservatives as a potential bio-preservative which prevent fungal growth and mycotoxin formation in food and feed. This is the first report on the isolation of this DKPS from *L. casei.*

Key words: *Lactobacillus casei*; Fermented pickle; Antimicrobial compound; Chromatography; Diketopiperazines.

At present the increasing consumer awareness of food risks derived not only from food-borne pathogens, but also from the artificial chemical preservatives used to control them (Shin *et al.*, 2012), which led to renewed interest in so-called "green technologies" including novel approaches for a minimal processing for biopreservation (Wouters *et al.*, 2013; Birri *et al.*, 2013).

The use of lactic acid bacteria (LAB) is a natural method of extending the shelf life of food (Birri *et al.*, 2013; O' Shea *et al.*, 2013). The LAB bacterial strains produce various antimicrobial compounds such as organic acids, hydrogen peroxide, diacetyl, bacteriocin, and so on (Ryan *et al.*, 2011; Ugi *et al.*, 2000; Mehanna *et al.*, 2013). Ryan *et al.* have been reported that *Lactobacillus amylovorus* DSM19280 can produce a wide spectrum of antimicrobial compounds active against common bread spoilage fungi (Ryan *et al.*, 2011).

Molds and yeasts are important spoilage organisms in food where the potential mycotoxin production from molds is of particular concern (Askari *et al.*, 2012; Dömling and Ugi, 2000). There are many reports on the production of antibacterial

* To whom all correspondence should be addressed.
# These authors contributed equally
Tel.: +86-20-85285382; Fax: +86-20-85280270;
E-mail: linjf@scau.edu.cn

compounds by LAB (Haney *et al.*, 2009; Miri *et al.*, 2012; Yang *et al.*, 2010), but reports on inhibition of yeasts and molds are comparatively few. Various kinds of antibiotics against bacteria and fungi are synthesized and secreted by Xenorhabdus and Photorhabdus

The aim of this study was to screened a strain and identify the antimicrobial compound produced by *Lactobacillus casei*, which active over a wide pH range, and is tested against diverse Gram-positive, Gram-negative bacteria, and against medicinally and agriculturally important fungi. To our knowledge, this is the first report about this kind of antimicrobial compound produced by *L. casei.*

MATERIAL AND METHODS

The antimicrobial compound producer strain was isolated from fermented pickles. The strain was selected from among 224 strains of *Lactobacillus* on the basis of its antibacterial activity. The sensitive indicator strains used for the inhibitory text were: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus, Salmonella*, *Saccharomyces cerevisiae*, and *Botryodiplodia theobromae* Pat. The LAB bacterial strains were maintained frozen at -20 °C in MRS broth media with 20% glycerol. The S. cerevisiae and B. theobromae Pat. were stored at -20 °C in PDA broth containing 10% glycerol. Other indicator strains were stored at Luria-Bertani (LB) broth and maintained as frozen stocks at -20°C in LB. Before experimental use, frozen cultures were plated onto MRS agar, PDA agar, or LB agar respectively.

32 fermented pickles were purchased from local markets in Guangzhou, China. All simples were exposed to the air for 5 days. Then, seven pickles maintained best qualities were selected for strain isolation. These pickles were diluted 10^4 fold with a sterile 0.85% NaCl solution and mixed thoroughly. Then, 0.2 ml of each dilution was spread onto MRS agar with 1% (v/v). Litmus and incubated for 48h at 30 °C. Red circle surrounded colonies were selected and re-streaked on MRS agar plates to obtain pure cultures. The cell morphology and gram-staining reaction were examined by light microscopy. The strain was tested for catalase reaction. Esculin hydrolysis was tested on bile-esculin agar (40% bile, v/v). Starch hydrolysis was tested on starch-MRS agar (0.5% starch, w/v). Voges-Proskauer was tested on MRS agar with 40% NaOH and a little creatine. Lead acetate test strips was tested for hydrogen sulfide yielding. Indole test was operated by addition of indole into fermented Peptone medium. Gelatin liquefaction was tested on Gelatin medium. Arginine hydrolysis was operated on puncture inoculation in the arginine hydrolysis medium and phenol red as indicator. Growth at alkaline pH and in the presence of 6.5% NaCl was tested on MRS broth modified accordingly. The 16S ribosomal DNA (16S rDNA) was amplified using standard PCR protocol, and a similarity of 99% to existing 16S rDNA sequences of type strains in the NCBI GenBank database was used as a criterion for identification (Verdenelli *et al.*, 2009).

The *L. casei* C7-2 was grown in 1000 ml MRS broth at 30 °C for 64 h, and production of antimicrobial compound was monitored during this growth. For analyses, 5 ml samples were aseptically taken from the culture at 4-48 h (time interval of 4 h. The growth of the strain was described by measuring its optical density at 600 nm at 30 °C at the aforementioned time intervals during the 48 period. Antimicrobial compound activity was detected by agar well diffusion assay and expressed as AU/ml.

The inhibition spectrum of strain *L. casei* C7-2 was determined by using the overlay method as previously described (Magnusson and Schnurer, 2001). The pH of each supernatant sample was adjusted to 5.0 with 1 N NaOH. Activity was measured by the agar well diffusion method. 15 ml of medium agar (0.75%, w/v) was inoculated with 0.1 ml of a fresh overnight culture of the indicator strains (10^7 cfu/ml) and poured into a Petri dish. The wells of 8 mm in diameter were filled with 120 ìl of samples. The plates were placed for 2 h at 4°C and incubated overnight at 25 °C or 37 °C. Then, they were examined for inhibition of the bacterial lawn; inhibition zone diameters were measured by subtracting the well diameter. One arbitrary unit (AU) is defined as the lowest concentration able to produce a visible halo of inhibition (8mm diameter) on the seeded lawn. The titer was then expressed as AU/ml.

The effect of pH on the antimicrobial compound was tested by adjusting cell-free supernatants to pH 2.0-12.0 (at increments of one

pH unit) with sterile 1 N NaOH or 1 N HCl. After 30 min and 2 h of incubation at room temperature 30 °C, the samples were re-adjusted to pH 5.0with 1 N NaOH or 1 N HCl and tested for antimicrobial activity using the agar well diffusion test method.

The effect of temperature on the activity of the antimicrobial compound was tested by heating the cell-free supernatant to 40, 60, 80, 100 and 121 °C, respectively. Antimicrobial compound activity was tested after 5, 10, 15, 20 and 30 min at each of these temperatures. The agar well diffusion test method was used.

L. casei C7-2 was inoculated to an initial concentration of 10^5cfu/mL in two litres of MRS broth and was grown as still culture at 30 °C for 48 h. Afterwards, broth culture was centrifuged at 3000 ×*g* for 15 min and sterile filtrated (0.45 ìm pore size filter Millipore).

The fermented material was extracted repeatedly with EtOAc, and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (7.0 g), which was fractionated by silica gel VLC using CH_2C_{l2}-MeOH gradient elution. The fraction (300 mg) eluted with CH_2C_{l2} was separated again by Sephadex LH-20 column chromatography using MeOH as eluents, and the resulting subfractions were further purified by semipreparative RP HPLC (Agilent Zorbax SB-C_{18} column; 5 ìm; 9.4×250 mm; 25-35 % MeOH in H_2O over 30 min; 2 mL/min) to acquire the compound (65.0 mg, tR 22.01 min).

The structures of the antimicrobial compounds were determined using nuclear magnetic resonance NMR (^{1}HNMR, ^{13}CNMR) spectroscopy. (Bruker DRX 400 NMR instrument, Bruker, Rheinstetten, Germany) equipped with a 2.5-mm microprobe. $CDCl_3$ was used as solvent to measure ^{1}HNMR, ^{13}CNMR experiments and all spectra were recorded at 25 °C.

Table 1. Characteristics of the bacteriocin-producing strain

Characteristics	+/-
Catalase test	-
Sugar alcohol fermentation test	+
D-fructose	+
Glucose	+
Lactose	+
Mannitol	+
Sorbitol	+
Sucrose	+
Maltose	+
Use glucose to produce acid and gas	+
Hydrolysis of starch	+
V-P test	+
H_2S test	-
Indole test	+
Gelatin liquefaction test	+
Litmus milk experiments	+
Arginine hydrolysis	-
Growth at 16 °C	+
Growth at 70 °C	-
Growth at pH 4.5	+
Growth at pH 9.0	+
Growth at 4% NaCl	+
Growth at 6.5% NaCl	+
Growth at 10% NaCl	-
Growth at 15% NaCl	-

Note: +, positive; -, negative

RESULTS AND DISCUSSION

A total of 224 lactic bacteria from fermented pickle samples were isolated, with blue lawn on the MRS agar supplemented with Litmus. Among them, 17 were able to inhibit growth of the indicator strain *E. coli*. Strain C7-2 was selected for the large and clear zones of inhibition it produced.

The strain isolated was a Gram-positive bacterium; short rod-shaped, no spore and no flagellum. Some of the strain characteristics were shown in Table 1. C7-2 was identified as with 99% similarity with *L. casei*. Therefore strain C7-2 was determined to belong to *L. case*i.

The growth curve and antimicrobial compound production time of *L. casei* C7-2 was shown on Fig. 1. The antimicrobial compound produced by C7-2 maximum activity yield against *E. coli* was obtained at 36h of growth (440AU/ml) and remained stable as shown in Fig. 1. The analyses of stability of antimicrobial compound to pH and temperature are shown in Fig. 2 and Fig. 3. The graph in Fig. 1 illustrates that antimicrobial compound production occurs mostly during the exponential phase of the growth curve and antimicrobial compound amount parallels the growth rate. Maximum antimicrobial compound activity of *L. casei* C7-2 was achieved at the end of the exponential phase, which is characteristic of primary metabolites.

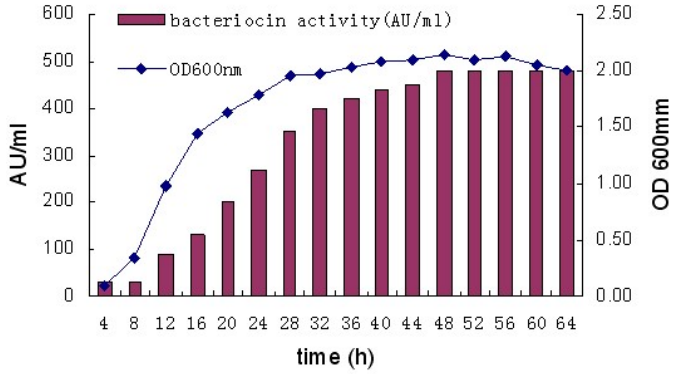

Fig. 1. Growth and antimicrobial compound production of *Lactobacillus casei* C7-2

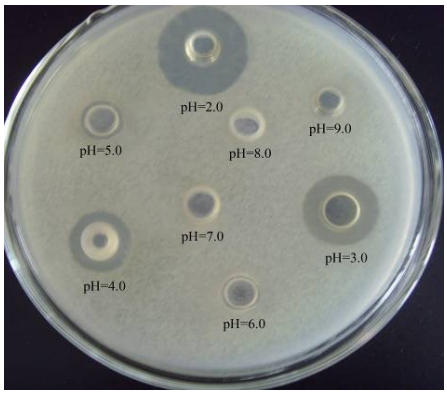

Fig. 2. The antimicrobial compound stability to pH

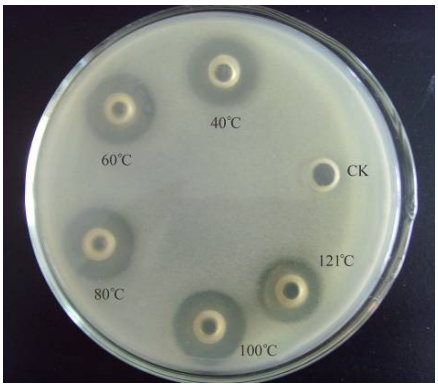

Fig. 3. The antimicrobial compound to temperature

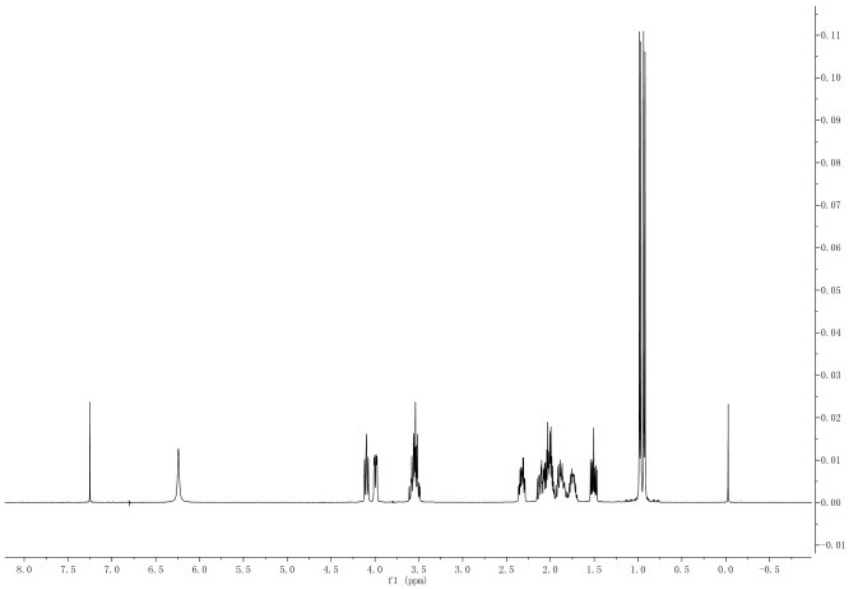

Fig. 4. The ^{1}HNMR spectrum of antimicrobial compound

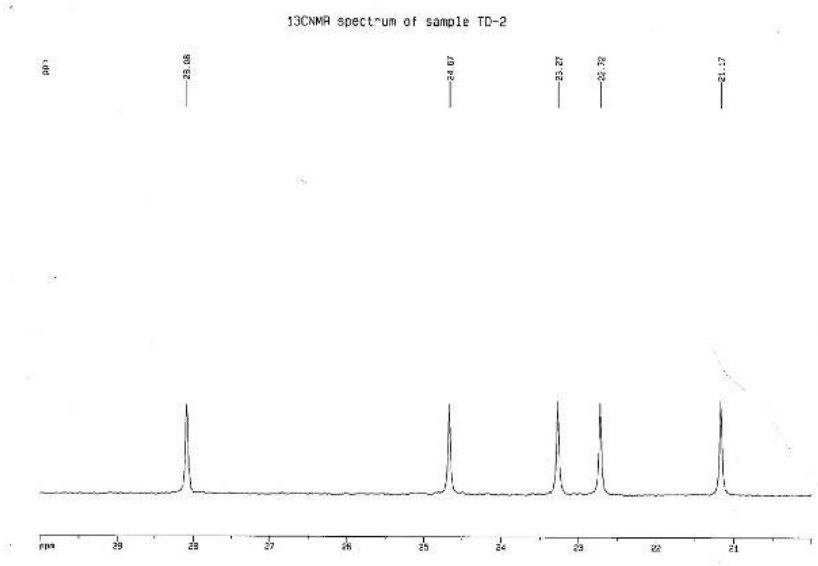

Fig. 5. The ^{13}CNMR spectrum of antimicrobial compound

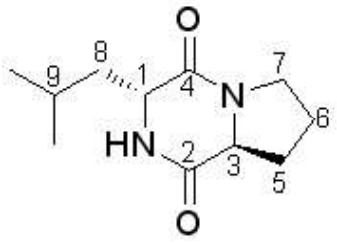

Fig. 6. The structure of antimicrobial compound

As a result, the antimicrobial compound produced by C7-2 has a wide antibacterial spectrum, being able to strongly inhibit the *E. coli*, *B. subtilis*, *S. aureus*, *Salmonella*, *S. cerevisiae*, and *B. theobromae* Pat.

Cyclo (L-Pro-D-Leu): White amorphous powder; [α] D-101 (c 0.02, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.93 (3H, d); 0.97 (3H, d); 1.51 (1H, m); 1.76 (1H, m); 1.87 (1H, m); 1.99 (1H, m); 2.01 (1H, m); 2.09 (1H, m); 2.30 (1H, m); 3.48-3.61 (2H, m); 4.00 (1H, dd); 4.10 (1H, t); 6.24 (1H, s). ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 21.2 (C-11), 22.7 (C-10), 23.3 (C-9), 24.7 (C-6), 28.1 (C-5), 38.6 (C-8), 45.5 (C-7), 53.4 (C-1), 59.0 (C-3), 166.2 (C-2), 170.2 (C-4). The molecular formula was determined to be $C_{11}H_{18}N_2O_2$ by HRMS (Fig. 4 and Fig. 5). According to the reference (Kumar *et al.*, 2013), the structure was established as shown in Fig. 6.

L. casei C7-2 isolated from fermented pickle was found to produce an Antimicrobial compound was found to have a wide inhibitive spectrum against diverse Gram-positive, Gram-negative bacteria, and agriculturally fungi. The results of the present study show that 2,5-diketopiperazines (DKPS) [cyclo (L-Pro-D-Leu)] exhibit against agriculturally important fungi in economically low concentrations. This is the first report on the isolation of this compound from *L. casei.*

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