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ARTICLE

In vitro Characterization of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Nem Chua, a Traditional Vietnamese Fermented Pork

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Abstract

The aim of this study was to screen and *In vitro* characterize the properties of bacteriocin produced by lactic acid bacteria isolated from Vietnamese fermented pork (Nem chua). One hundred and fifty LAB were isolated from ten samples of Nem chua and screened for bacteriocin-producing lactic acid bacteria. Antimicrobial activity of bacteriocin was carried out by spot on lawn method against both gram positive and gram negative bacteria. One isolate, assigned as KL-1, produced bacteriocin and showed inhibitory activity against *Lactobacillus sakei*, *Leuconostoc mesenteroides* and *Enterococcus faecalis*. To characterize the bacteriocin-producing strain, optimum temperature, incubation period for maximum bacteriocin production and identification of bacteriocin-producing strain were determined. It was found that the optimum cultivation temperature of the strain to produce the maximum bacteriocin activity (12,800 AU/mL) was obtained at 30°C. Meanwhile, bacteriocin production at 6,400 AU/mL was found when culturing the strain at 37°C and 42°C. The isolate KL-1 was identified as *L. plantarum*. Antimicrobial activity of cell-free supernatant was completely inhibited by proteolytic enzyme of trypsin, alpha-chymotrypsin and proteinase K. Bacteriocin activity was stable at high temperature up to 100°C for 10 min and at 4°C storage for 2 d. However, the longer heating at 100°C and 4°C storage, its activity was reduced.

Keywords: lactic acid bacteria, bacteriocin, Nem Chua

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Introduction

"Nem Chua" is the name of sour fermented meat, which is popular traditional fermented meat product in Vietnam. The ingredient of Nem Chua consists of lean ground pork, boiled pig skin cut into strings, garlic and spices. After mixing well all ingredients, meat is shaped into cubes and wrapped with *Pridium guajava leaves.* Then covered again with banana leave to provide the anaerobic environment for the growth of lactic acid bacteria (LAB) and also inhibit the growth of pathogenic bacteria. The fermentation process takes 3-4 d at ambient temperature. This product is ready-to-eat without cooking (Nguyen *et al*., 2010; Nguyen *et al*., 2013a; Nguyen *et al*., 2013b). A dominance of Lactobacilli in Nem chua were *L. plantarum, L. farciminis* and *L. pentosus* (Nguyen *et al.,* 2013a).

LAB are important agents for fermented meat during fermentation as they are natural microflora of many fermented food (Huot *et al*., 1996). They are able to inhibit the growth of other microorganisms including pathogenic and food spoilage bacteria because they produce various antibacterial compounds, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Fadda *et al*., 2010). Among the anti-microbial substances, bacteriocins have demonstrated great potential as food preservatives. Bacteriocins are ribosomally synthesized antibacterial polypeptides (Nes and Johnsborg 2004). Many bacteriocins of LAB are safe and effective natural inhibitors against pathogenic and food spoilage bacteria in various foods (Leroy *et al.,* 2006). Therefore, the objectives of this research were to screen, study of *in vitro* properties of LAB and bacteriocin produced by LAB isolated from Vietnamese fermen-

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ted pork (Nem Chua) in order to benefit further use in meat industry.

Materials and Methods

Screening of LAB from Nem Chua

Ten samples of Vietnamese fermented pork products, call Nem Chua, were screened for LAB. Twenty-five gram of each sample was homogenized and diluted to 10-fold serial dilution with 0.1% (w/v) of peptone solution, before spreading plate using MRS agar (de Man-Rogosa-Sharpe; Merck, Germany) containing 0.5% (w/v) calcium carbonate (Scharlau Chemie S.A., Spain) and 1% (w/v) NaCl (Prolabo, Belgium) (Modified from Itoi *et al*., 2008). The MRS plates were then incubated under anaerobic condition (Candle jar) at 37°C for 48 h. The LAB isolate which showed antimicrobial activity was collected. Antimicrobial activity was observed as clear zone of bacteriocin.

Screening for bacteriocin-producing LAB

Screening of bacteriocin was carried out by spot-on-lawn method. Stock cultured was transferred to MRS broth with 1% (w/v) NaCl over night at 37°C to refresh culture. Then, transferred 2% (v/v) culture in MRS broth again and incubated overnight at 37°C. Later, culture broth was centrifuged at 1,500 *g* at 4°C (Jouan CR 3i, France). Cell-free supernatant (CFS) was adjusted at pH 7 by NaOH (Ajax Finechem, Australia) and sterilized by boiling for 5 min. The two layers of agar plate were prepared and 5 mL of soft agar (0.8% agar) was added to make top layer which seeded with 10 μL of freshly grown of tested bacterial strain (about 10^7 CFU/mL). Bacteriocin activity was tested against 17 indicator microorganisms by spotting 10 μL of CFS onto the top surface of agar plate. Inhibition zone was observed (Ennahar *et al*., 2001) after overnight incubation at proper temperature for each indicator microorganism as shown in Table 1. The spectrum of CFS was expressed in an arbitrary unit (AU/mL). The AU was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strains, it was calculated by described method (Parente *et al*., 1995).

Optimum temperature and incubation time for maximum bacteriocin production

The optimum condition for maximum bacteriocin production was demonstrated by culturing the bacteria among various temperature at 30, 37 and 42°C for 24 h, after refreshing stock culture in MRS broth overnight at 37°C. Each of 2% (v/v) stock culture was transferred to 100 mL MRS broth and incubated at 30, 37 and 42°C. Bacteriocin was determined every 2 h for 24 h and expressed as Log CFU/mL. The optical density (OD) at 600 nm was measured to evaluate the growth of bacteriocin-producing LAB. Antibacterial activity of CFS (pH 7) against *L. sakei* subsp. *sakei* JCM1157^T was examined and expressed as arbitrary unit (AU/mL).

Morphological and biochemical identification

Cell morphology, gram strain, catalase activity, gas production of bacteriocin - producing LAB and the growth was determined according to the method of and Kandler and Weiss (1986) and Forbes *et al*. (1998). Biochemical identification was attributed to carbohydrate fermentation using API 50 CH Rapid fermentation strips (API, Bio Merieux, France).

Determination of 16S rDNA identification

To confirm the species of bacteriocin produced bacteria, 16S rDNA identification was carried out by PCR amplification and sequencing. Total DNA was extracted following Carolissen-Mackay *et al*. (1997). Bacterial universal primer of BSF8/20 (5'-AGAGTTTGATCCTGGCTC AG-3') and REVB (5'-GGTTACCTTGTTACGACTT-3') (Kanokratana *et al*., 2004) were applied for PCR amplification. PCR reaction was comprised 1X buffer with ammonium sulfate, 2 mM magnesium chloride, 400 μM deoxynucleoside triphosphate, 0.05 μM of Taq polymerase (Fermentas, USA), 0.4 μM of each primer and genomic DNA about 100 ng were used as a template for PCR amplification. PCR amplification was done on thermocycler, (Biometra, Germany) for 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s and extension at 72°C for 1.45 min. PCR products were performed using QIAquick Gel Extraction Kit (Qiagen, USA). DNA fragments were ligated into pTZ57R/T cloning vector (Fermentas, USA). Ligation mixes were transformed into competent *E. coli* DH5α cells using heat-shock method (Sambrook *et al*., 2001). Positive clones were picked by Blue/White colonies selection and checked for size of the right insert by PCR.

Effect of proteolytic enzymes on antibacterial activity of CFS

The effect of enzymes on bacteriocin activity tested following from Toit *et al*. (2000). The CFS was treated with trypsin (Sigma, USA), alpha-chymotrypsin (Sigma, USA) pepsin and proteinase K (Sigma, USA) at final concentra-

tion of 1 mg/mL. All enzyme solutions were adjusted to pH 7 except pepsin which was adjusted to pH 3. Therefore, this study included both bacteriocin adjustd to pH 7 and 3 as control (without enzyme treatment). The samples were sterilized by filtering through filter membrane (0.2 μm, Pall, USA) and incubated at 37°C for 3 h. Subsequently, enzyme activity was terminated by heating at 100°C for 5 min. The residual bacteriocin activity was determined by applying spot-on-lawn method against *L. sakei* subsp. *sakei* JCM1157^T which was used as indicator microorganism.

The effect of heat and chill temperature on bacteriocin activity

To test for heat sensitivity, CFS (pH 7) was heated to 100 and 121°C by autoclave (High-Pressure Steam Sterilizer ES-315, Japan). The boiling time intervals were 5, 30 and 60 min at 100°C and were 15 min. at 121°C. Antibacterial activity of CFS (pH 7) against *L. sakei* subsp. *sakei* JCM1157^T was examined and expressed as arbitrary unit (AU/mL) by using spot-on-lawn method after heating. Whereas, The effect of chilled temperature on bacteriocin activity was performed at 4°C for 0, 1, 3, 5, 7 and 10 d. (adapted from Campos *et al*., 2006).

Results and Discussion

Antibacterial activity of bacteriocin producing LAB isolated from Nem Chua, Vietnamese fermented pork

The isolation of LAB from 10 Nem Chua samples were collected. A total of 150 LAB isolates exhibited a inhibition zone and grew on MRS agar with 0.5% (w/v) calcium carbonate. All isolates were determined antimicrobial activity of bacteriocin against 17 indicator bacteria as shown in Table 1. Only one isolate assigned as KL-1 was found to produce antimicrobial activity of bacteriocin against tested bacteria. The highest antibacterial activity of 12,800 AU/mL inhibited growth of *L. sakei* subsp. *sakei* JCM1157^T and *L. sakei* TISTR890. However, antibacterial activity against *L. plantarum* ATCC14917^T, *E. faecalis* JCM5803^T, *E. faecalis* TISTR888, *Leuc. mesenteroides* subsp. *mesenteroides* JCM6124^T and *Leuc. mesenteroides* subsp. *mesenteroides* TISTR942 was lower (Table 1).

LAB are able to inhibit the growth of other microorganisms by excretion of metabolite products such as organic acids, hydrogen peroxide, diacetyl and bacteriocin (Huot *et al*., 1996). However, these results were not from acidic effect as CFS was adjusted to neutralize to get rid of the acidic effect. Some bacteriocins produced by LAB inhibit not only closely related species but also effective against food-borne pathogens and other Gram-positive spoilage microorganisms including *Bacillus* sp. and *E. faecalis* (Delves-Broughton 1990). This result is useful in meat industry in the future. Rao *et al*. (1998) and Corry (2006) reported that in anarobically packed meat, vacuum or modified atmospheres packaging (MAP), was deteriorated due to Lactobacili including *L. sake*, *L. curvatus* and *Leuc. mesenteroides* at the time of spoilage. Nguyen *et al*. (2010) isolated LAB from Nem chua and screened for bacteriocin-producing LAB. However, there was no lactic acid bacteria which produced bacteriocin detected by this study.

Table 1. Antimicrobial activity of isolate KL-1 against indicator microorganisms

Indicator microorganism	Bacteriocin activity (AU/mL)
Lactobacillus plantarum ATCC14917^T	6,400
Lactobacillus sakei subsp. *sakei* JCM1157^T	12,800
Lactobacillus sakei TISTR 890	12,800
Lactococcus lactis subsp. *cremoris* TISTR1344	0
Leuconostoc mesenteroides subsp. *mesenteroides* JCM6124^T	1,600
Leuconostoc mesenteroides subsp. *mesenteroides* TISTR942	400
Enterococcus faecalis JCM5803^T	6,400
Enterococcus faecalis TISTR888	6,400
Streptococcus sp. TISTR1030	0
Bacillus coagulans JCM2257^T	0
Bacillus coagulans TISTR1447	0
Listeria innocua ATCC33090^T	0
Brochotrix campeatris NBRC11547^T	0
Staphylococcus aureus TISTR118	0
Pseudomonas fluorescens JCM 5963^T	0
Pseudomonas fluorescens TISTR 358	0
Aeromonas hydrophila TISTR 1321	0

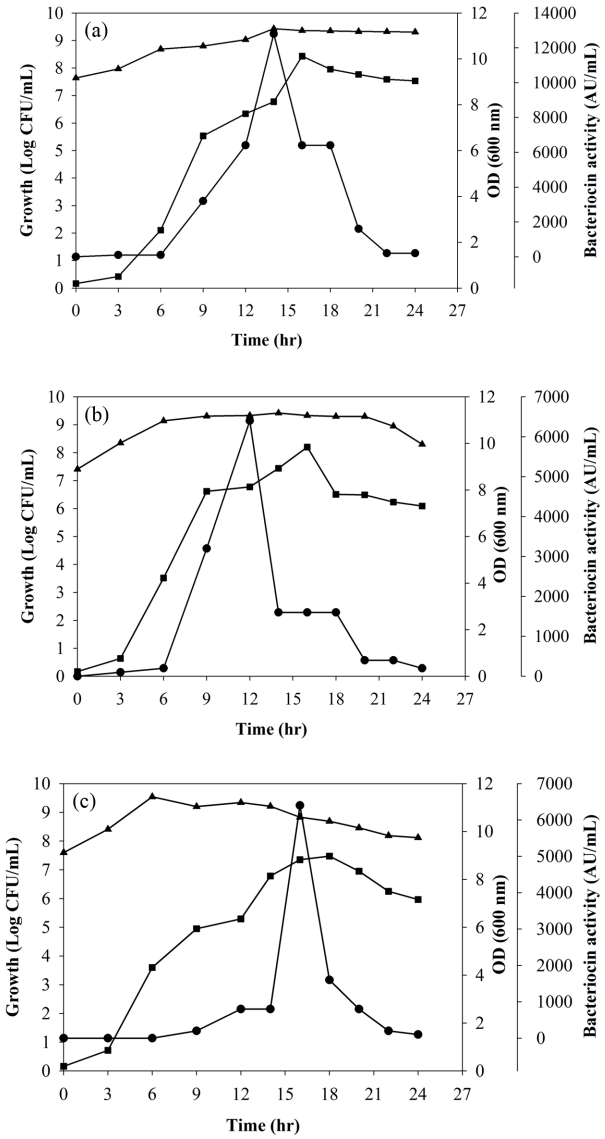

Fig. 1. The growth temperature of isolate KL-1 to produce bacteriocin: (a) 30°C, (b) 37°C and (c) 42°C, ▲ bacterial growth (Log CFU/mL), ■ OD at 600 nm, ● bacteriocin activity (AU/mL).

Profile of growth and bacteriocin production of isolate KL-1

The growth of isolate KL-1 and production of bacteriocins were studied at various temperatures: 30, 37 and 42°C as shown in Fig. 1(a), 1(b) and 1(c). The profile of growth was measured by cell number and its OD at 600 nm at every 3 h. At temperature 37 and 42°C, the bacteria grew to stationary phase in less time than the growth at 30°C. The highest bacteriocin activity was observed in late exponential phase at all temperatures. The highest bacteriocin activity (12,800 AU/mL) was observed at 30°C after incubated at 14 h with cell number of 9.43 Log CFU/mL. However, the bacteriocin activity decreased when cell entered stationary phase. In addition, it was found that maximal bacteriocin production showed paralleled with the growth rate of the strain.

Our results were supported by Cheigh *et al.* (2002) who reported that the production of bacteriocin is growth-associated because production occurred during mid-exponential phase and increased until reach a maximal level at the end of exponential phase or the beginning of the early-stationary phase where the maximal biomass was observed. Our result revealed that bacteriocin production decreased when cell entered the stationary phase that displayed similar result to Suma *et al.* (1998). They reported that the maximum antimicrobial activity of *L. plantarum* NCIM2084 against *B. cereus* was observed during late exponential phase and early stationary phase (The strain was grown for 48 h at incubation temperature of 37 and 40°C). Messens *et al*. (2003) reported that the loss of bacteriocin activity may be due to degradation by endogenous protease induced during the growth phase.

Identification of isolate KL-1

Morphological identification of isolate KL-1 was short rod, Gram-positive, catalase negative and did not produce gas from glucose. It grew at temperature ranging from 30°C to 42°C and at pH 4.5 and 9.6. Based on comparison of their characteristics with Bergey's manual (Kandler and Weiss 1986), this isolate could be identified as genus *Lactobacillus*. For species determination, biochemical characterization was used. Carbohydrate fermentation patterns indicated that isolate KL-1 was identified as *L. plantarum* with 99% identities (data not shown).

To confirm the conventional identification results, 16S rDNA gene investigation was used in this study. Comparison of the sequence (about 1500 bp) with the database in GenBank (http://www.ncbi.nlm.nih.gov) by BLAST program, the alignment of 16S rDNA gene sequence of isolate KL-1 indicated the identical to *L. plantarum* JDM1 (sbjct) 99% identities (Accession No. CP001617.1). Therefore, this isolate was named as *L. plantarum* KL-1. Detection of *L. plantarum* showed similar result to Nguyen *et al*. (2013*a*) studied on the diversity of the native LAB population in Nem chua. A total of two hundred seventy-three LAB isolates were identified. They found that the highest prevalence of LAB was *L. plantarum* (29.7%), followed by *L. farciminis* (23.0%) and *L. pentosus* (21.0%).

Effect of proteolytic enzyme on bacteriocin activity

It was found that bacteriocin activity was completely

Table 2. Effect of proteolytic enzyme on bacteriocin activity against *L. sakei* subsp. *sakei* JCM1157^T

Proteolytic enzyme	Bacteriocin activity (AU/mL)
Control pH 7	12,800
Control pH 3 (without adjusted pH)	12,800
Trypsin	0
α-chymotrypsin	0
Proteinase K	0
Pepsin	100

*ATCC, American Type Culture Collection, Rockville; JCM, Japan Collection of Micro-organisms, Wako, Japan; NBRC, NITE Biological Resource Center, Chiba, Japan; TISTR, Thailand Institute of Scientific and Technological Research.

destroyed by trypsin, alpha-chymotrypsin and proteinase K, while control at pH 7 and 3 (bacteriocin without treated with enzyme) revealed bacteriocin activity (12,800 AU/mL). However, some antimicrobial activity (100 AU/mL) remained after digesting by pepsin compared to bacteriocin without digested by enzyme (12,800 AU/mL as shown in Table 2). The antibacterial activities of CFS produced by *L. plantarum* KL-1 were inactivated by proteolytic enzymes, indicating that it has proteinaceous structure as a bacteriocin (Alvarez-Cisneros *et al*., 2010). The results supported by Lash *et al*. (2005). They digested bacteriocin from *L. plantarum* ATCC 8014 by pepsin, trypsin and alpha-chymotrypsin and found that antimicrobial activity was lost after treated by those proteolytic enzymes.

Effects of heat and chill temperature on bacteriocin activity of CFS

Bacteriocin was heat stabilized at 100°C for 10 min. However, after heating at 100°C for 30 and 60 min, the bacteriocin activity was reduced by 50% (from 12,800 to 6,400 AU/mL). In addition, bacteriocin activity remained only 400 AU/mL compared to initial activity 12,800 AU/ml at 121°C for 15 min as shown in Table 3. Abo-Amer (2007) reported that antimicrobial activity of BLIS of *L. plantarum* AA135 was resistant to heat at 121°C for 30 min, our bacteriocin activity decreased after heat at 121°C for 30 min. The bacteriocin activity was stable in chill temperature (4ºC) up to 2 d that displayed 12,800 AU/mL. Its activity gradually decreased to 50% after storing at 4°C for 4 d (the activity was reduced from 12,800 to 6,400 AU/mL) and decreased to 25% at day 6 (3,200 AU/mL). The activity was remained 1,600 AU/mL when stored for 7 d. Therefore, the stability in heat and chill condition of this bacteriocin is very useful for food production industry.

Table 3. Effects of heat and chill temperature on bacteriocin activity against *L. sakei* subsp. *sakei* JCM1157^T

Conditions	Bacteriocin activity (AU/mL)
Heat stability	
Control (100°C 5 min)	12,800
100°C 10 min	12,800
100°C 30 min	6,400
100°C 60 min	6,400
121°C 15 min	400
Chill stability (4°C)	
Day 0	12,800
1	12,800
2	12,800
3	6,400
4	6,400
5	3,200
6	3,200
7	1,600
8	1,600
9	1,600
10	1,600

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