Biocontrol Science, 2011, Vol. 16, No. 1, 33−40

Original

Thermotolerant Bacteriocin-Producing Lactic Acid Bacteria Isolated from Thai Local Fermented Foods and Their Bacteriocin Productivity

VICHAI LEELAVATCHARAMAS[1,2,3], NIDA ARBSUWAN[1], JIRAWAN APIRAKSAKORN[1], PATTANA LAOPAIBOON[1,2], AND MASAO KISHIDA[3]*

[1]*Department of Biotechnology, Faculty of Technology, Khon Kaen University, 123 Mittrapap Rd. Amphu Muang, Khon Kaen, 40002, Thailand*
[2]*Fermentation Research Center for Value Added Agricultural Products. Faculty of Technology, Khon Kaen University, 132 Mittrapap Rd. Amphur Muang, Khon Kaen 40002, Thailand*
[3]*Department of Applied Biochemistry, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*

Received 17 November, 2010/Accepted 22 January, 2011

Twenty-one samples of Thai local fermented foods were screened for thermotolerant bacteriocin-producing lactic acid bacteria. From 529 isolates of lactic acid bacteria, 121 isolates were able to inhibit the growth of certain bacterial strains. Of these 121 isolates, only 11 produced antibacterial agents that were capable of inhibiting the growth of multiple bacterial strains in a liquid medium. One strain (KKU 170) of these 11 isolates produced an antibacterial agent that could strongly inhibit the growth of selected strains of gram-positive bacteria including *Listeria* sp. The antibacterial agent produced by the strain KKU 170 was identified as a bacteriocin since it was inactivated by proteinase K treatment. The strain KKU 170 was identified as *Pediococcus acidilactici* by both biochemical tests and molecular biological techniques. Optimal production of bacteriocin by the strain KKU 170 was found in culture medium containing 0.2% glucose, at an initial culture pH of 6.5, and temperature of 45 ℃. The maximum bacteriocin activity (1600 AU ml^{-1}) was reached at the late exponential phase of growth and displayed primary metabolite production. The partially purified bacteriocin of the strain KKU 170 was tolerant to heat treatment at 121 ℃ for 30 min.

Key words : Bacteriocins/Lactic acid bacteria/Thermotolerant/Thai local fermented foods.

INTRODUCTION

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) bacteria and play an important role in food and feed fermentation and preservation, either as the natural microflora or as starter cultures added under controlled conditions (Hastings and Stiles, 1991; Cintas et al., 2001). GRAS status bacteria that play important roles in food fermentation and preservation include cocci or rods that are nonsporing, micro-aerophilic, or facultatively anaerobic bacteria that lack cytochromes and the catalase *sensu stricto*. These bacteria produce lactic acid as the major end-product (at least 50%) during the fermentation of carbohydrates and produce antimicrobial compounds, including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acid, reuterin, and bacteriocins (Klaenhammer, 1988, 1993; Hastings and Stiles, 1991; Piard and Desmazeaud, 1991, 1992). Bacteriocin-producing lactic acid bacteria strains protect themselves against the toxicity of their own bacteriocins by the expression of a specific immune protein, which is generally

*Corresponding author. *Tel* : +81-72-254-9455, *Fax*: +81-72-254-9921, *E-mail*: masyksd(a)biochem.osakafu-u.ac.jp

encoded by the bacteriocin operon (Cintas et al., 2001). Bacteriocin has been defined as an extracellularly released primary or modified product of bacterial ribosomal synthesis that can have a relatively narrow spectrum of bacteriocidal activity. These are antibacterial proteins produced by bacteria, which kill or inhibit the growth of other bacteria (Cleveland et al., 2001). The classification of bacteriocins is currently being revised to reflect similarities and differences observed commensurate with the discovery of new molecules.

Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyllanthionine, dehydrobutyrine, and dehydroalanine. Class II bacteriocins contain small heat-stable, nonmodified peptides and are subdivided into IIa and IIb. Class IIa includes bacteriocins that are pediocin-like anti-*Listeria* peptides with a conserved N-terminal, and Class IIb bacteriocins are composed of 2 different peptides required to form an active poration complex. Class III bacteriocins are large proteins about which there is much less information available. An additional class (Class IV) of bacteriocins, which forms large complexes with other macromolecules, has been proposed (Klaenhammer, 1993; Cleveland et al., 2001). Bacteriocin production from LAB is affected by the medium composition and culture conditions, such as pH, temperature, and agitation (Kim et al., 2006). For example, Pediocin AcH can be produced from glucose, sucrose, and xylose, and the best carbon sources for the production of this bacteriocin are galactose and glucose (Biswas et al., 1991; Parante and Ricciardi, 1999). The optimal pH for bacteriocin production is usually 5.5-6.0, which is often lower than the optimal pH for growth. Agitation and aeration affect bacteriocin production. During fermentation at pH 5.5 using glucose media with *Lactobacillus* lactis IO-1, maximum production of its bacteriocin (nisin Z) and $Y_{B/X}$ was obtained at 320 rpm (Parante and Ricciardi, 1999), with only a small decrease in nisin Z production and yield at 1000 rpm.

The aim of this study was to describe a method for the screening of bacteriocin-producing thermotolerant lactic acid bacteria from local Thai fermented foods, to characterize the bacteriocins produced by the isolated strains, and to determine the optimum conditions for bacteriocin production in laboratory-scale cultures.

MATERIALS AND METHODS

Media and indicator strains

The media used for bacterial culture were MRS (peptone, 5 g l^{-1}; beef extract, 8 g l^{-1}; yeast extract, 4 g l^{-1}; K_2HPO_4, 1 g l^{-1}; tween 80, 1 g l^{-1}; di-ammonium hydrogen citrate, 2 g l^{-1}; sodium acetate, 5 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.04 g l^{-1}; pH 6.2), M17 (peptone, 14 g l^{-1}; beef extract, 7 g l^{-1}; yeast extract, 3.5 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.35 g l^{-1}; pH 7.0) and Nutrient Broth (Kanto Chemicals, Tokyo, Japan). Each plate of media contained 1.5% agar.

Indicator strains used in this study were provided from the Thailand Institute of Scientific and Technological Research (TISTR) and American Type Culture Collection (ATCC) as follows: *Streptococcus cremoris* TISTR 058, *S. lactis* TISTR 457, *S. thermophilus* TISTR 458, *E. coli* TISTR 073, *Staphylococcus aureus* TISTR 746, *Bacillus subtilis* TISTR 001, *Listeria innocua* ATCC 33090, and *Salmonella* Typhimurium ATCC 14028, cultured at 37 ℃ in Nutrient Broth, and *Pediococcus acidilactici* TISTR 783 and *P. pentosaceus* TISTR 423, cultured at 37 ℃ in MRS.

Screening of the antimicrobial producing lactic acid bacteria (LAB)

LAB were screened as follows. Acid-producing bacteria on MRS plates with 2.0% glucose were isolated from local Thai fermented foods, and colonies growing at 45 ℃ after treatment with 3% hydrogen peroxide were selected as LAB strains. Antibacterial activity of LAB against the other bacterial strains was tested by using a soft agar-based procedure. Briefly, LAB were spotted onto MRS agar plates containing 0.2% glucose and incubated at 45 ℃ overnight. The indicator strains (approximately 10^7 CFU ml^{-1}) were mixed with MRS containing 0.75% soft agar (Difco Agar Noble, Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 45 ℃ and then overlayed on LAB-spotted plates. Plates were incubated overnight at 37 ℃ and inhibition zones around the LAB were identified.

Antibacterial activity against LAB cultures was performed by using the paper disc method. Sterile filter paper discs (diameter, 6.0 mm) were placed on the surface of the agar plates; approximately 10^7 CFU ml^{-1} of the indicator strain was overlayed on MRS. For testing the antibacterial activity, 10 μl of cell-free supernatants of LAB cultures were spotted on the paper discs, incubated at 37 ℃, and inhibition zones around the paper discs were identified. The cell-free supernatants were prepared by centrifugation (13,000 × *g* for 10 min at 4 ℃) of LAB cultures in MRS with 0.2% glucose at 45 ℃. Supernatants were collected, neutralized to pH 7.0 by adding 0.1 M NaOH), and maintained at 4 ℃ for later experiments.

Proteinase treatment of bacteriocins

Cell-free supernatants having antibacterial activity were tested for susceptibility to proteinase K. Supernatants were incubated at 37 ℃ for 12 h with proteinase K at a final concentration of 1 mg ml^{-1}, and subsequently heated to 100 ℃ and 121 ℃ for 10, 15, and 30 min, respectively. Residual activity was determined by the paper disc method using *P. pentosaceus* TISTR 423 as an indicator.

Identification of the bacteriocin producing LAB

Bacterial isolates were identified using the following biochemical tests: Gram staining, catalase activity, growth sensitivity at 10 ℃ and 45 ℃, O-F glucose test, CO_2 production from glucose, growth at pH 4.4 and 9.6, and salt tolerance at 6.5% and 18% sodium chloride.

Isolates were also identified by the following molecular biological techniques. Extraction of the genomic DNA from LAB was done according to the slightly modified method described by Aly (2004). The 16S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) with a forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-TGACGGGCGGTGTGTACAAG-3′. PCR conditions were as follows: pre-heating at 94 ℃ for 5 min, denaturing at 94 ℃ for 30 s, annealing at 50 ℃ for 45 s, extension at 72 ℃ for 90 s, and a final extension at 72 ℃ for 10 min. The nucleotide sequences of the PCR products were determined using a DY Enamic ET Terminator Sequencing kit, Mega BACE (GE Healthcare Life Sciences, Buckinghamshire, UK). The nucleotide sequences were compared using the BLAST program of National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

Laboratory scale Bacteriocin production

The maximum laboratory scale bacteriocin production of LAB was assayed using *P. pentosaceus* TISTR 423 as the indicator for bacteriocin activity. The bacteriocin-producing LAB and the indicator strain were pre-cultured in MRS broth at 37 ℃. Two types of media were used for the optimization experiments—MRS and M17—both containing 0.2% glucose.

For all fermentation experiments, we used 1% (v/v) inoculum of LAB, which was grown in MRS broth (2.0% glucose) for 6-12 h (OD_{660} 1.0). All experiments, except where indicated, were conducted at 45 ℃. LAB were cultured in 500-ml Erlenmeyer flasks containing 300 ml MRS. The effects of glucose concentration (0.2, 2.0, 5.0, and 10.0%), initial pH (3.2, 4.2, 5.2, and 6.5), temperature (30, 37, 45, and 50 ℃), and agitation rate (0 and 100 rpm) were examined. The sample was analyzed for reduced sugar content, cell growth (OD_{660}), total acidity, pH, and bacteriocin activity. Bacteriocin activity was measured by the two-fold-dilution method (Atrik et al., 2001). Sugar content was measured by the method described by Somogii (1952). Total acidity was measured by the international methods of the Association of Official Analytical Chemists described by Fablo et al. (2006).

Partial purification of bacteriocins produced from LAB

The bacteriocins were partially purified from the laboratory scale cultures of LAB after 6-12 h, harvested by centrifugation (22,000 × *g* for 10 min at 4 ℃), and precipitated using ammonium sulfate saturations (20-40%, 40-60%, 60-80%, and 0-80%) slowly added to the cell-free supernatant and gently stirred overnight. The precipitated material was then harvested by centrifugation at 22,000 × *g* for 20 min at 4 ℃, and the precipitate was solubilized in 50 mM sodium acetate buffer (pH 5.5) and dialyzed for 24 h against the same buffer. The dialyzed material was sterilized by the filter membrane (pore size: 0.2 μm) (Millex-FG; Millipore, Billerica, MA, USA), and antibacterial activity was determined by the two-fold dilution method (Radttanachaikunsopon and Phumkhachorn., 2000; de Carvalho et al., 2006). The partially purified substance from LAB was serially diluted into 50 mM sodium acetate buffer (pH 5.5) and tested against *P. pentosaceus* TISTR 423. The bacteriocin activity (AU ml^{-1}) was calculated by the following formula: the highest dilution which still showed the positive result × 1,000 / sample volume. Protein concentration was measured by the Lowry method (Waterborg and Matthews, 1994).

Thermal and proteinase stability of bacteriocins from LAB

Heat sensitivity was examined by incubating the LAB culture supernatant at 100 ℃ and 121 ℃ for 10, 15, and 30 min. To test the sensitivity of the bacteriocin to proteinase K, the culture supernatant was adjusted pH to 5.0-6.0 using 6 M HCl or NaOH and then treated with proteinase K to a final concentration of 1.0 mg ml^{-1}. The remaining activity was assayed by the paper disc method using *P. pentosaceus* TISTR 423 as an indicator strain.

RESULTS

Screening of lactic acid bacteria (LAB) for antimicrobial production

A total of 529 strains of lactic acid bacteria were isolated in the primary screening of fermented foods.

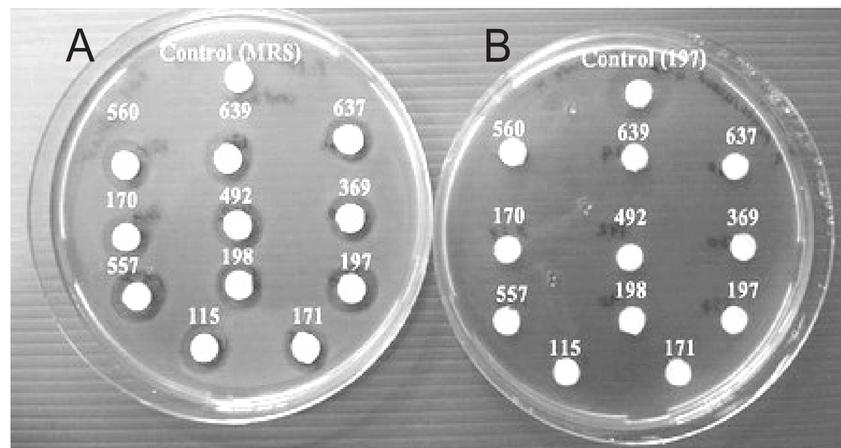

FIG. 1 The antibacterial agent of 11 isolates of lactic acid bacteria (LAB). Antibacterial activity was assayed by the paper disc method. Neutralized culture filtrates of LAB were treated without (A) and with (B) 1 mg ml^{-1} proteinase K.

Among the 529 strains, 121 isolates could inhibit the growth of *S. thermophilus* TISTR 458, *P. acidilactici* TISTR 783, *P. pentosaceus* TISTR 423, and *S. aureus* TISTR 746 based on the soft agar testing method. When these 121 isolates were examined for antibacterial production in liquid medium by the paper disc method, only 11 isolates—KKU 115, KKU 170, KKU 197, KKU 198, KKU 369, KKU 492, KKU 557, KKU 560, KKU 637, and KKU 639—exhibited positive results (Fig. 1, A). These 11 isolates were further identified by biochemical testing. The results showed that the 11 isolates were all Gram-positive cocci, catalase negative, able to grow at 45 ℃ but not at 10 ℃, O-F glucose positive, CO_2 production negative, able to grow at pH 4.4, 6.5% salt tolerant, and 18% salt intolerant. The results of biochemical tests indicate that 11 isolates were identified as belonging to the genus *Pediococcus*. All 11 isolates were also identified as *P. acidilactici* by comparing the 16S rDNA sequences using the BLAST program in NCBI.

Characterization of the bacteriocins

Antibacterial agents of the 11 LAB isolates were tested for susceptibility to proteinase K and for heat sensitivity at 100 ℃ and 121 ℃ for 10, 15, and 30 min. When antibacterial agents were treated with proteinase K, inhibition zones around paper discs were not observed (Fig. 1, B). These results suggest that the antibacterial agent is a protein-like compound. When the antibacterial agent was exposed to heat, the activity of the antibacterial agent of all 11 isolates was maintained at 100 ℃ for 30 min with the use of *P. pentosaceus* TISTR 423 as a tester strain, but the activity of the antibacterial agent of KKU 115, KKU 170, KKU 197, KKU 198, and KKU 560 was maintained at a temperature up to 121 ℃ for 15 min. Since the antibacterial agent of KKU 170 showed the largest inhibitory zone, KKU 170 was used in laboratory scale experiments for bacteriocin production.

The effect of MRS and M17 medium on laboratory scale bacteriocin production

The growth of *P. acidilactici* KKU 170 cultured in MRS and M17 containing 0.2% glucose (pH 6.2) at 45 ℃ was similar. The maximum bacteriocin activity (1600 $AUml^{-1}$) for KKU 170 occurred after 10-12 h culture in MRS and M17; however, the antibacterial activity was 4-fold higher in MRS than in M17 (data not shown). These results indicate that the bacteriocin production of KKU 170 reached a maximum in the late exponential phase and was more efficient in MRS than in M17. Therefore, laboratory scale experiments were performed using the MRS medium.

The effect of glucose concentration on laboratory scale bacteriocin production

The effect of glucose concentration on cell growth, culture conditions, and bacteriocin production is shown in Fig. 2. The maximum cell growth of KKU 170 was found in MRS broth containing 2.0% glucose (Fig. 2-A). Glucose concentrations in cultures containing 0.2, 2.0, 5.0, and 10.0% were decreased to 0.27, 6.08, 34.33, and 80.85 g l^{-1} (Fig. 2-B), respectively. The culture broth containing 2.0, 5.0, and 10.0% glucose resulted in higher acid production than the culture broth containing 0.2% glucose (Fig. 2-C). In medium containing 2.0, 5.0, and 10% glucose, the total acidity of the culture was increased, and the pH of the culture was decreased, but there were only subtle changes in the medium containing

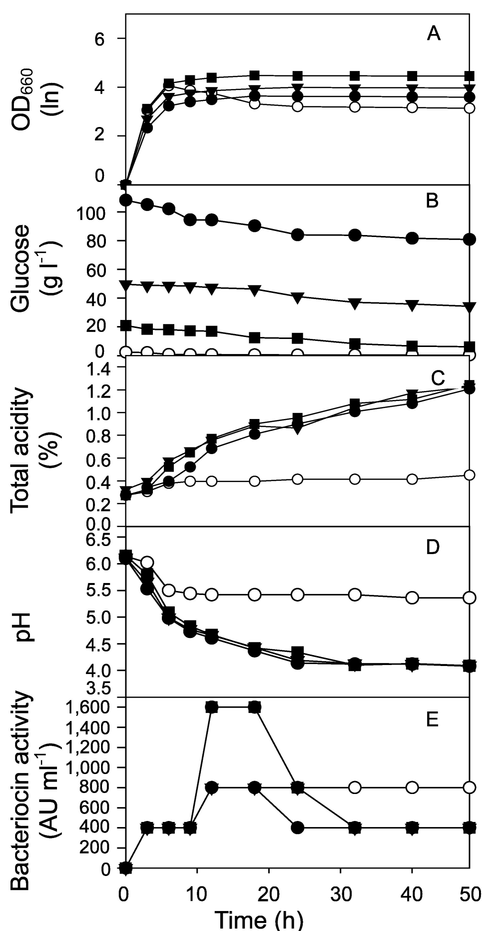

FIG. 2. The effect of glucose concentration on the bacteriocin production of *P. acidilactici* KKU 170. LAB were cultured in MRS containing 0.2% (open circles), 2.0% (filled squares), 5.0% (filled triangles), and 10.0% (filled circles) glucose. A; Natural logs of optical density at 660 nm, B; Glucose contents of cultures, C; Total acidity of cultures, D; pH, E; Bacteriocin activity.

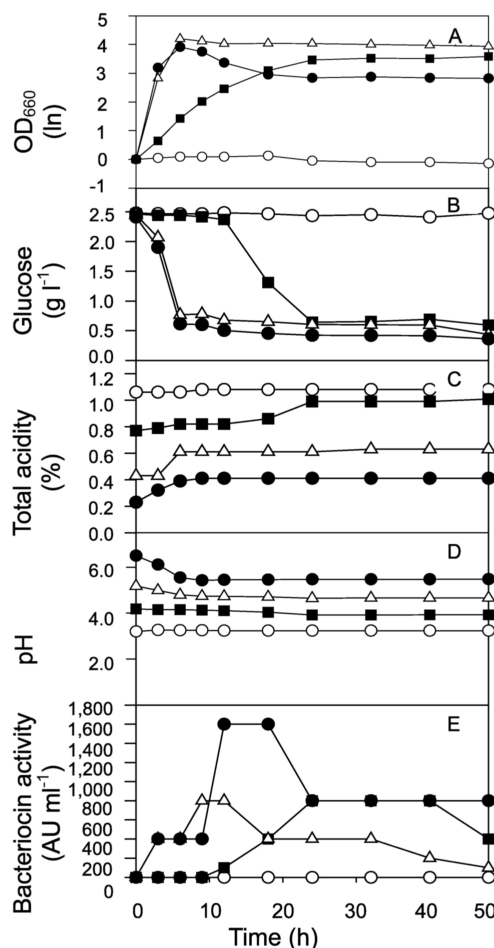

FIG. 3. The effect of initial pH on the bacteriocin production of *P. acidilactici* KKU 170. Initial pH of the culture was 3.2 (open circles), 4.2 (filled squares), 5.2 (open triangles), and 6.5 (filled circles). A; Natural logs of optical density at 660 nm, B; Glucose contents of cultures, C; Total acidity of cultures, D; pH, E; Bacteriocin activity.

0.2% glucose (Fig. 2-C and -D, respectively). The maximum bacteriocin activity of 1600 AU ml^{-1} was obtained in 0.2 and 2.0% glucose, whereas only half the total activity was obtained in 5.0 and 10.0% glucose. These results suggest that higher concentrations of glucose inhibit KKU 170 bacteriocin production. Thus, MRS containing 0.2% glucose was used for all the remaining experiments.

The effect of the initial culture pH on laboratory scale bacteriocin production

The effect of initial culture pH on bacteriocin production is shown in Fig. 3. The highest cell growth of KKU 170 was obtained at the initial pH 5.2; conversely, there was no cell growth of KKU 170 at pH 3.2 (Fig. 3-A). The level of glucose consumption by KKU 170 correlated with cell growth (Fig. 3-B). Changes in total acidity and pH were related to the cell growth at the initial pH of 6.5, 5.2, and 4.2 (Fig. 3-C and D). The maximum bacteriocin activity of KKU 170 was 1600 AU ml^{-1} at pH 6.5. These results suggest that the initial acidity of the culture affects KKU 170 bacteriocin production and higher acidity inhibits production.

The effect of temperature on laboratory scale bacteriocin production

The effect of the culture temperature is shown in Fig. 4. KKU 170 could be grown at 30, 37, 45, and 50 ℃ (Fig. 4-A). However, at 45 and 50 ℃, cell growth could be detected slightly earlier than at other temperatures. The glucose consumption, the increase of the total acidity, and the decrease of culture pH were related to KKU 170 growth at each temperature (Fig.

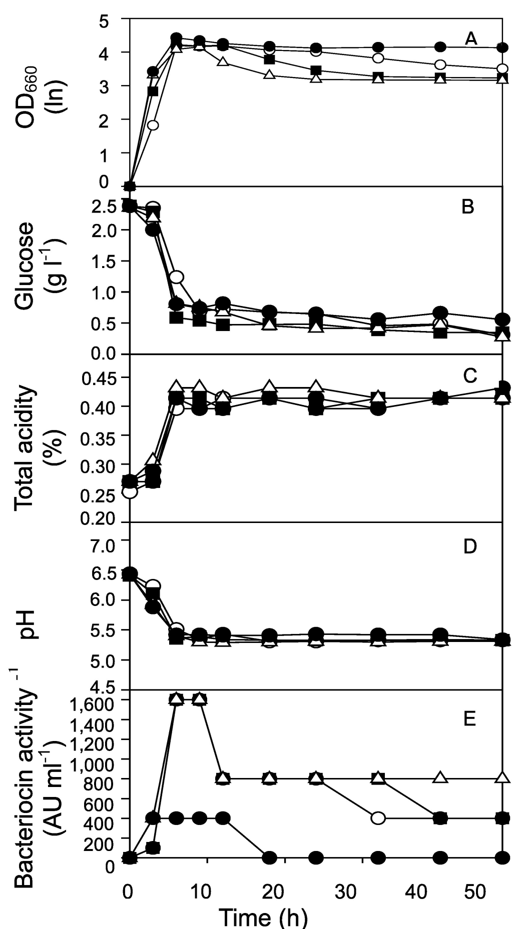

FIG. 4. The effect of temperature on the bacteriocin production of *P. acidilactici* KKU 170. LAB were cultured at 30 ℃ (open circles), 37 ℃ (filled squares), 45 ℃ (open triangles), and 50 ℃ (filled circles). A; Natural logs of optical density at 660 nm, B; Glucose contents of cultures, C; Total acidity of cultures, D; pH, E; Bacteriocin activity.

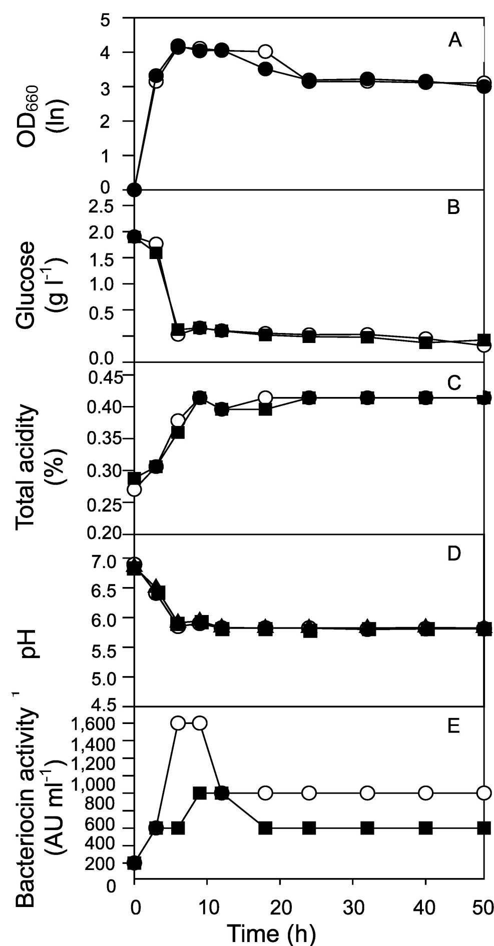

FIG. 5. The effect of agitation on the bacteriocin production of *P. acidilactici* KKU 170. LAB were cultured without (open circles) and with (filled squares) 100 rpm of agitation. A; Natural logs of optical density at 660 nm, B; Glucose contents of cultures, C; Total acidity of cultures, D; pH, E; Bacteriocin activity.

4-B, -C, and -D, respectively). The maximum bacteriocin activity (1600 AU ml^{-1}) of KKU 170 was obtained at 30, 37, and 45 ℃ (Fig. 4-E). However, at 45 ℃, the bacteriocin reached maximal activity level with faster kinetics than at other temperatures. These results suggest that the optimum temperature for KKU 170 bacteriocin production is at 45 ℃.

The effect of agitation on laboratory scale bacteriocin production

The effect of agitation is shown in Fig. 5. The increase in KKU 170 cell numbers in the static culture agitated at 0 rpm was slightly higher than that in the culture agitated at 100 rpm (Fig. 5-A). The glucose consumption, acid production, and pH were not different between static and agitated cultures (Fig. 5-B, -C, and -D, respectively). The maximum KKU 170 bacteriocin activity (1600 AU ml^{-1}) was observed in the static culture (Fig. 5-E). These results suggest that agitation inhibits KKU 170 bacteriocin production.

Partial purification and heat stability of bacteriocins produced by LAB

The bacteriocin was partially purified from KKU 170 by precipitation with different percentages of ammonium sulfate saturations (Table 1). The whole precipitate derived by 80% ammonium sulfate saturation showed 6,400 AU of bacteriocin activity and 1,280 AU mg^{-1} protein of specific activity (Table 1, 0-80%). Bacteriocin could be precipitated at all levels of ammonium sulfate saturation (20%-40%, 40%-60%, and 60%-80%), and the 40%-60% ammonium sulfate saturation fraction showed the maximum bacteriocin activity (800 AU ml^{-1}). Protein concentration and specific activity was 0.7 mg ml^{-1} and 1,140 AU mg^{-1}

TABLE 1. Summary of bacteriocin precipitation from *P. acidilactici* KKU 170 by different saturations of ammonium sulfate.

Saturation of ammonium sulfate (%)	Bacteriocin activity (AU ml^{-1})	Protein concentration (mg ml^{-1})	Specific activity (AU mg^{-1})
20−40	200	0.7	286
40−60	800	0.7	1140
60−80	400	2.7	148
0−80	6400	5.0	1280

protein in the 40%-60% fraction, respectively (Table 1). The bacteriocin partially purified by 80% ammonium sulfate saturation could inhibit different indicator strains—*P. acidilactici* TISTR 783, *P. pentosaceus* TISTR 423, *S. cremoris* TISTR 058, *S. lactis* TISTR 457, *S. thermophilus* TISTR 458, *S. aureus* TISTR 746, and *L. innocua* ATCC 33090. The inhibition spectrums of the 20%-40%, 40%-60%, and 60%-80% saturated fractions were similar to that of the 0%-80% fraction.

The partially purified bacteriocin produced by KKU 170 was tested for heat stability (thermotolerance). When the bacteriocin of the 80% ammonium sulfate precipitated fraction was incubated at 121 ℃ for 60 min, the bacteriocin activity was maintained. The bacteriocin present in the 40%-60% saturated fraction was tolerant under the same conditions (121 ℃ for 60 min). These results suggest that the major bacteriocin of 80% ammonium sulfate precipitate is fractionated by the 40%-60% saturation of ammonium sulfate.

DISCUSSION

Eleven bacteriocin-producing LABs showing thermotolerance were isolated by screening 21 samples of Thai local fermented foods. Based on biochemical and molecular assessments, 11 of the LABs were identified as *P. acidilactici*. These isolates were able to inhibit the growth of different indicator strains, especially the growth of *Listeria innocua*, a closely related species to *L. monocytogenes*, with maximum bacteriocin activity (1600 AU ml^{-1}). *L. mononcytogenes* is a widespread food-borne pathogen, but listeriosis outbreaks in humans have commonly been associated with contaminated meat and meat products (de Carvalho et al., 2006). Thus, to understand optimum conditions for bacteriocin production, we studied bacteriocin production using the strong bacteriocin-producing strain, *P. acidilactici* KKU 170.

The bacteriocin production of KKU 170 in MRS medium was higher than that in M17 medium although cell growth was similar. This result suggests that the supplements in MRS medium are more suitable for bacteriocin production than those in M17 medium. In general, culture pH is known to be important to cell growth as well as to bacteriocin production because of aggregation, adsorption of bacteriocin to the cells, and/or proteolytic degradation dependent on pH. The pH of the culture is also affected by the bacteriocin activity in culture supernatants (de Vuyst et al., 1996; Parante et al., 1994; Kim et al., 2006). The bacteriocin production in the medium containing 0.2% or 2.0% glucose was higher than that in the medium containing 5.0% and 10.0% glucose. These results suggest the possibility that the lower growth rate at 5.0% and 10.0% glucose is due to the end-product inhibition of lactic acid (Matsusaki et al., 1996). This was demonstrated by the arrest of cell growth, even with the substrate still available, when total acidity was higher than 1.0%. The optimal temperature for growth and bacteriocin production was the same (45 ℃) in the case of KKU 170. We have not found reports that the optimal temperature for the growth and bacteriocin production is more than 40 ℃ in LAB, although there are a few reports that the optimal temperature for the growth and bacteriocin production is 37 ℃ (Biswas et al., 1991; Juarez Tomas et al., 2002). Thus, in terms of usefulness it is an advantage for KKU 170 that its bacteriocin production is at a higher temperature than other bacteriocin producers. The inhibition of bacteriocin production by the agitation of the bacterial culture suggests that the oxygen content in the culture is important to the overall bacteriocin production by KKU 170.

We found that bacteriocin was sensitive to proteinase K and stable to heat (100 ℃ and 121 ℃). These properties are similar to Class II bacteriocins, defined as small, heat stable, non-modified peptides and the subclass IIa, which includes pediocin-like *Listeria* active peptides (Cleveland et al., 2001).

ACKNOWLEDGMENTS

This work was financially supported by the Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand.

REFERENCES

Aly, S., Cheik, A. T., Ouattara, P. W. S., Nicolas, B., Aboubacar, S. O., and Alfred, S. T. (2004) Identification of exopolysaccharides-producing lactic acid bacteria from Burkina Faso fermented milk sample. *African J. Biotechnol*. **3**, 189-194.

Atrik, A. N., Rekhif, A., Moir, J. G., and Lebrihi, G. L. (2001) Mode of action purification and amino acid sequence of plantaricin C 19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C 19. *Int. J. Food Microbiol*. **68**, 93-104.

Biswas, S. R., Ray, P., Johnson, M. C., and Ray, B. (1991) Influence of growth conditions on the production of a Bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. *Appl. Environ. Microbiol*. **57**, 1265-1267.

Cintas, L. M., Casaus, M. P., Herranz, C., Nes, I. F., and Hernánde, P. E. (2001) Bacteriocins of Lactic Acid Bacteria. *Food Sci. Tech. Int*. **7**, 281-305.

Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol*. **71**, 1-20.

de Carvalho, A. A. T., de Paula, R. A., Mantovani, H. C., and de Moraes, C. A. (2006) Inhibition of *Listeria monocytogenes* by a lactic acid bacterium isolated from Italian salami. *Food Microbiol*. **23**, 213-219.

Fabro, M. A., Milanesio, H. V., Robert, L. M., Speranza, J. L., Murphy, M., Rodriguez G., and Castaneda, R. (2006) Technical Note: Determination of acidity in whole raw milk: comparison of results obtained by two different analytical methods. *J. Dairy Sci*. **89**, 859-861.

Hastings, J. W., and Stiles, M. E. (1991) Antibiosis of *Leuconostoc gelidum* isolated from meat. *J. Appl. Bacteriol*. **70**, 127-134.

Juarez Tomas, M. S., Bru, E., Wiese, B., de Ruiz Holgado A. A. P., and Nader-Mcias, M. E. (2002) Influence of pH, temperature and culture media on the growth and bacteriocin production by virginal *Lactobacillus salivarius* CRL 1328. *J. Appl. Microbiol*. **93**, 714-724.

Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie*. **70**, 337-349.

Klaenhammer, T. R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*. **12**, 39-86.

Kim, M. H., Kong, Y. J., Baek, H., and Hyun, H. H. (2006) Optimization of culture conditions and medium composition for the production of micrococcin GO5 by *Micrococcus sp*. GO5. *J. Biotechnol*. **121**, 54-61.

Matsusaki, H., Endo, N., Sonomoto, K., and Ishizaki, A. (1996) Lantibiotic nisin Z fermentative production by *Lactococcus lactis* IO-1: relationship between production of the lantibiotic and lactate and cell growth. *Appl. Microbiol. Biotechnol*. **45**, 36-40.

Parante, E., Ricciardi, A., and Addario, G. (1994) Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140 NWC during batch fermentation. *Appl. Microbiol. Biotechnol.* **41**, 388-394.

Parante, E., and Ricciardi, A. (1999) Production recovery and purification of bacteriocins from lactic acid bacteria. *Appl. Microbiol. Biotechnol*. **52**, 628-638.

Piard, J. C., and Desmazaud, M. (1991) Inhibition factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Lait*. **71**, 525-541.

Piard, J. C., and Desmazaud, M. (1992) Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. *Lait*. **72**, 113-142.

Rattanachaikunsopon, P., and Phumkhachorn, P. (2000) A bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* isolated from Thai fermented foods. *Sci. Asia* **26**, 195-200.

Somogii, M. (1952) Notes on sugar determination. *J. Biol. Chem*. **159**, 19-23.

de Vuyst, L., Callewaert, R., and Crabbe, K. (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavorable growth conditions. *Microbiol*. **142**, 817-827.

Waterborg, J. H. and Matthews, H. R. (1994) The Lowry method for protein quantitation. *Methods Mol. Biol*. **32**, 1-4.