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# Isolation, Identification, and Characterization of a Bacteriocin-Producing *Enterococcus* sp. from Kimchi and Its Application to Kimchi Fermentation

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**Abstract** A bacteriocin-producing lactic acid bacterium, which strongly inhibited the *Lactobacillus plantarum* recognized as an important acid spoilage microorganism in kimchi fermentation, was isolated from kimchi. From morphological, physiological, sugar fermentation, biochemical tests, and 16S rDNA sequencing results, the isolate was identified as an *Enterococcus* sp. and designated as *Enterococcus* sp. K25. The bacteriocin produced by *Enterococcus* sp. K25 inhibited several Gram-positive bacteria, including *Lb. plantarum*, whereas it did not inhibit Gram-negative bacteria and yeasts. Optimal temperature and pH for the bacteriocin production were 25°C and 5.5, respectively. *Enterococcus* sp. K25 was applied to kimchi manufacturing alone and together with other preservatives (i.e., chitosan and fumaric acid). In addition, growth of lactic acid bacteria, pH, and titratable acidity (TA) were measured during aging at 5°C and 10°C. Inoculation of *Enterococcus* sp. K25 together with fumaric acid showed the most synergistic effect on extension of kimchi shelf-life. Compared to control (no addition), the treatment prolonged the kimchi shelf-life up to 6 days, whereupon the eight-point TA value recognized as the edible limit was reached.

**Key words:** Bacteriocin, *Enterococcus* sp., kimchi, shelf-life

Lactic acid bacteria (LAB) have been used as starter cultures for dairy, meat, and vegetable fermentations in many countries. They contribute to flavor development as well as preservation of foods. They also produce a variety of antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins [6, 17, 32]. In particular, extensive efforts to find bacteriocins with a broad antimicrobial spectrum and superior stabilities against heat treatment and pH variation have been made during the last decade [17, 22, 27, 33]. Since many foods fermented by lactic acid bacteria have been consumed for thousands of years without any known harmful effects, bacteriocins are generally regarded as safe for human consumption. Therefore, bacteriocins from lactic acid bacteria are the prime candidates for developing safe food biopreservatives that can replace chemical preservatives [5, 8, 11, 17, 30].

Kimchi is one of the most globally famous traditional Korean food that is produced by the fermentation of various lactic acid bacteria. It is generally accepted that kimchi fermentation is initiated by *Leuconostoc mesenteroides* and terminated by *Lactobacillus plantarum* [7, 29]. It is still controversial whether or not *Lb. plantarum* plays a beneficiary role, i.e., flavor production, in kimchi fermentations. However, as far as acid production is concerned, *Lb. plantarum* plays a significant role in acidification, but eventually the shelf-life or edible period of kimchi expires. This has attracted many food scientists to focus on the extension of kimchi shelf-life. To date, many studies have been conducted on extending shelf-life of kimchi by addition of a starter with increased acid resistance [15, 19, 20, 21], chitosan [16, 25, 35], medicinal herb extracts [26], preservatives and heat treatment [12], and application of adipic acid and storage temperature [2].

In this study, we have isolated a bacteriocinogenic lactic acid bacterium, which strongly inhibited *Lb. plantarum*, and identified and characterized it. The lactic acid bacterium was applied to kimchi manufacturing as a biopreservative alone and together with other preservatives (i.e., chitosan and fumaric acid). Population of lactic acid bacteria, pH, and titratable acidity (TA) were measured during fermentation at 5°C and 10°C to determine the effect of the bacteriocinogenic lactic acid bacterium on extending the shelf-life of kimchi.

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## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

All lactic acid bacteria were cultured in MRS medium (Merck, Darmstadt, Germany) at 37°C for 18 h. *Listeria monocytogenes* was cultured in brain heart infusion broth (BHI broth, Merck, Darmstadt, Germany) at 37°C for 18 h. *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, and *Streptococcus mutans* were cultivated in tryptic soy broth (TSB, Merck, Darmstadt, Germany) at 37°C for 18 h. *Clostridium perfringens* was cultivated in reinforced clostridial medium (RCM, Merck, Darmstadt, Germany) at 37°C for 18 h. *Escherichia coli* and other Gram-negative bacteria were cultured in nutrient broth (NB, Merck, Darmstadt, Germany) at 37°C for 18 h. Yeasts were cultured in yeast malt extract broth (YM broth, Merck, Darmstadt, Germany) at 30°C for 24 h.

### Isolation of Bacteriocin-Producing Lactic Acid Bacteria

To isolate bacteriocin-producing lactic acid bacteria from kimchi, a deferred antagonism assay, namely the sandwich method, was applied [4, 9, 18, 23, 24, 32]. Various kimchi samples were collected and the juices were serially diluted and aliquots of the dilutents were pour-plated in 5 ml melted and tempered MRS agar, solidified, and 5 ml MRS soft agar (0.7%) was overlaid on it. The plates were incubated at 30°C anaerobically.

### Identification of Bacteriocin-Producing Lactic Acid Bacterium

Conventional morphological and physiological tests and G+C content measurement were done to identify the bacteriocin-producing lactic acid bacterium. Carbohydrate fermentation patterns were determined by API kit (API 50 CHL, BioMerieux, SA, France). 16S rDNA sequence analysis was performed using Big Dye Terminator Cycle Sequencing Kit with an automatic DNA kit (Applied Biosystems, Model 310, Perkin-Elmer, Foster City, CA, U.S.A.). The 16S rDNA sequence of the strain was aligned with those of lactic acid bacteria and several other related taxa to construct the phylogenetic tree and compare the level of similarity.

### Preparation of Crude Bacteriocin

Overnight culture of *Enterococcus* sp. K25 in 100 ml MRS broth was centrifuged (30 min, 10,000 rpm, and 4°C) and the supernatant was filtered through a 0.20 μm-pore size membrane filter (Sartorius, Göttingen, Germany). While stirred at 4°C, ammonium sulfate was added to the filtrate to achieve 50% saturation. The saturated solution was centrifuged (30 min, 10,000 rpm, and 4°C) and the precipitate was recovered. The precipitate was reconstituted in distilled and deionized water ($ddH_2O$) and dialyzed with Spectra/Por membrane (MWCO. 1,000, Spectrum Medical Industries, Houston, U.S.A.) against $ddH_2O$ for 24 h at 4°C. After dialysis, the crude bacteriocin was freeze-dried and stored at -20°C until used [30, 31].

### Antimicrobial Spectrum of Bacteriocin

Using the prepared crude bacteriocin of *Enterococcus* sp. K25, antimicrobial activities against various bacteria were determined by agar well diffusion assay [32]. After forming a well in the MRS plate by steal punch, 50 μl of the redissolved crude bacteriocin was loaded into the well and target bacteria ($10^7$ cfu/ml) mixed in 5 ml top agar (0.7% agar) were poured on the plate. The plates were incubated at 30 or 37°C depending on the tested bacteria and the plates were examined for inhibition zone after 24 h.

### Culture Condition for Bacteriocin Production by *Enterococcus* sp. K25

The effect of growth temperature and pH on the bacteriocin production was investigated. Viable cell counts and bacteriocin titer of *Enterococcus* sp. K25 were determined at 20, 25, 30, and 37°C in GM17 broth (0.5% glucose/M17 broth, Merck, Darmstadt, Germany). Initial pH of GM17 broth was adjusted to 5.0, 5.5, 6.0, and 7.0, using 6 N HCl and 1 N NaOH, and desired pHs were maintained by adding 1 N NaOH. All cultivations were performed with a commercial fermenter (New Brunswick Scientific, Edison, NJ, U.S.A.). For the measurements of cell counts and bacteriocin titer, subsamplings were performed at every 4 h during 24 h fermentation. The bacteriocin titer was expressed as AU (arbitrary unit) per ml. To determine the bacteriocin titer of the original preparation, the reciprocal of the highest dilution that gave a definite zone of inhibition of at least 1 to 2 mm was multiplied by a conversion factor (e.g., 200 if 5 μl is used) [32].

### Application of *Enterococcus* sp. K25 to Kimchi Fermentation

To investigate the effect of *Enterococcus* sp. K25 on the extension of kimchi shelf-life, $1-2\times10^7$ CFU/g whole cell of *Enterococcus* sp. K25 was applied to kimchi manufacturing alone and together with other preservatives (i.e., 0.2% chitosan and 0.05 and 0.1% fumaric acid). At every 3 days, subsamplings were performed and the population of lactic acid bacteria, pH, and titratable acidity (TA) were measured during fermentation at 5°C and 10°C.

## RESULTS AND DISCUSSION

### Isolation of Bacteriocin-Producing Lactic Acid Bacteria

Bacteriocin-producing lactic acid bacteria were isolated from various kimchi samples (Fig. 1). Bacteriocin production by isolates was confirmed via a well diffusion assay [32] using a pH-neutralized, catalase-treated supernatant to eliminate the possibility of either hydrogen peroxide or

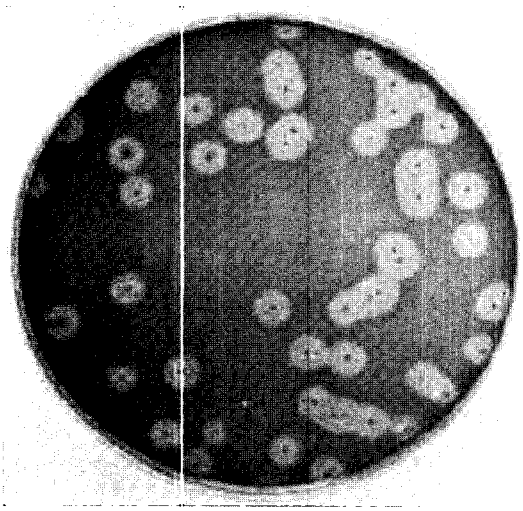

**Fig. 1.** Bacteriocin-producing lactic acid bacterial colonies formed the growth inhibition zone on a lawn of *Lactobacillus plantarum* NCDO 955.

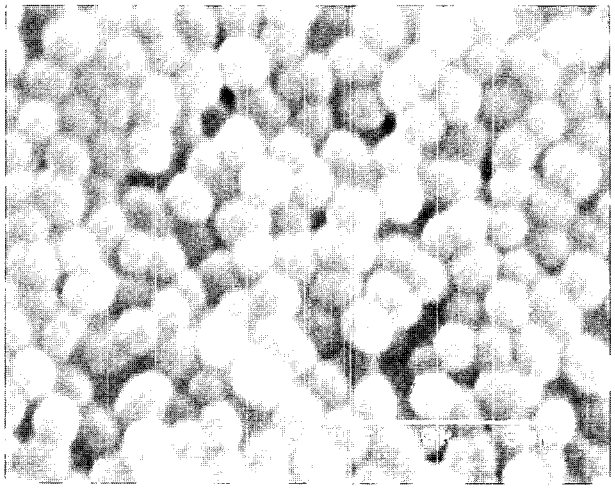

**Fig. 2.** Scanning electron microphotograph (SEM) of K25 strain isolated from kimchi.

lactic acid inhibition (data not shown). Among the isolates, K25 strain showed the largest zone of inhibition against *Lb. plantarum* NCDO 955 and it was selected for the test of whether or not it affected extension of kimchi shelf-life as a starter.

### Identification of Bacteriocin-Producing K25 strain

Morphological and bicchemical properties of K25 strain were examined according to *Bergey's Manual of Determinative Bacteriology* [13] and the results are summarized in Table 1. This strain was a Gram-positive, catalase-negative, facultatively anaerobic coccus (Fig. 2). The strain grew at 45°C and 10°C. It was able to grow at pH 9.6 and in the presence of 6.5% NaCl. These properties agreed with the characteristics of enterococci. Also, the result of the API test showed the characteristics of enterococci (data not shown). Furthermore, K25 strain did not show hemolysis activity and the GC content of the K25 genome was 37 mol%. In order to confirm the identification, 16S rDNA sequencing was performed. The 16S rDNA sequence of K25 strain showed 99.4, 99.3, 99.3, and 98.8% similarity with *Enterococcus durans* NCDO 596$^T$, *Enterococcus hirae* NCDO 1258$^T$, *Enterococcus faecium* DSM 20477$^T$, and *Enterococcus*

**Table 1.** The general characteristics of K25 strain isolated from kimchi.

| Characteristics | Result | Characteristics | Result |
|---|---|---|---|
| Cell form | Cocci | Cell size (μm) | 0.7-1.2 |
| Cell arrangement | Pairs, chains | Gram reaction | + |
| Motility | - | Spore formation | - |
| Gas from glucose | - | Catalase | - |
| Reaction in litmus milk | | DAP in peptidoglycan | - |
| reduction | + | Ammonia from arginine | - |
| peptonization | + | Hydrolysis of gelatin | - |
| acid curd | + | Hydrolysis of esculin | + |
| Hydrolysis of hippurate | - | Hydrolysis of arginine | + |
| Nitrate reduction | - | Reduction of tellurite | - |
| Growth at pH 3.6 | - | Reduction of tetrazolium | - |
| Growth at pH 3.9 | - | Dextran formation | - |
| Growth at pH 4.2 | - | Growth at 10°C | + |
| Growth at pH 4.8 | - | Growth at 15°C | + |
| Growth at pH 8.2 | + | Growth at 40°C | + |
| Growth at pH 9.6 | + | Growth at 45°C | + |
| Growth at 6.5% NaCl | + | Growth at 50°C | - |
| Isomer of lactic acid | L | Sheep blood hemolysis | - |

Symbols: +, positive; -, negative.

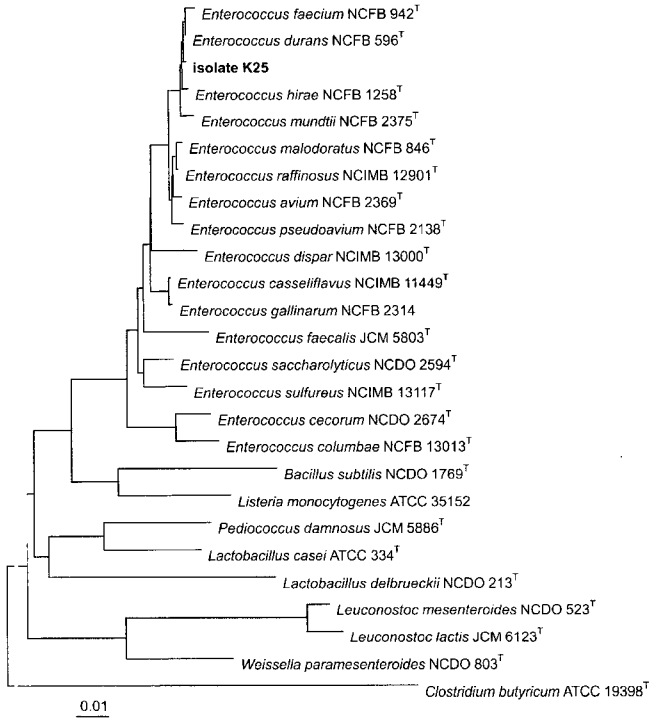


**Fig. 3.** Dendrogram showing the similarity among K25 strain, *Enterococcus* species, and representatives of some related taxa based on 16S rDNA sequences.
The scale bar represents 0.01 substitution per nucleotide position.

*mundtii* ATCC 43186$^T$, respectively. However, due to the highly close similarity among the four *Enterococcus* spp., allocation of K25 into the species level was unsuccessful at the moment. Therefore, K25 strain was tentatively designated as *Enterococcus* sp. K25. The dendrogram showing the similarity among K25 strain, *Enterococcus* spp., and representatives of some related taxa based on 16S rDNA sequences is shown in Fig. 3. Currently, we are working on more detailed analysis methods such as Random Amplified Polymorphic DNA (RAPD) [14] and Denaturing Gradient Gel Electrophoresis (DGGE) [1] to identify K25 at the species level.

## Antimicrobial Spectrum of Bacteriocin Produced by *Enterococcus* sp. K25

Various Gram-positive and Gram-negative bacteria, and yeasts were tested for their susceptibilities to the bacteriocin and the results are shown in Table 2. The bacteriocin inhibited some strains of *Bacillus*, *Lactobacillus*, *Listeria*, *Pediococcus*, and *Streptococcus*. In particular, the bacteriocin showed strong inhibition against the *Lb. plantarum* recognized as a major causative organism for acid deterioration of kimchi. However, it did not inhibit any other Gram-negative bacteria and yeasts used in this study. These results strongly indicate that the bacteriocin produced by *Enterococcus* sp. K25 belonged to class II bacteriocin. To date, many

**Table 2.** Antimicrobial spectrum of the bacteriocin produced by *Enterococcus* sp. K25 against bacteria and yeasts.

| Indicator strain | Bacteriocin activity |
|---|---|
| **GRAM-POSITIVE BACTERIA** | |
| *Bacillus cereus* ATCC[a] 11778 | +[b] |
| *Bacillus coagulans* ATCC 7050 | + |
| *Clostridium perfringens* ATCC 13124 | - |
| *Enterococcus faecalis* IFO 3971 | - |
| *Enterococcus faecalis* var. *liquefaciens* KFRI 675 | - |
| *Lactobacillus acidophillus* KCTC 3179 | + |
| *Lactobacillus brevis* ATCC 8287 | - |
| *Lactobacillus curvatus* NRRL B-4562 | + |
| *Lactobacillus delbrueckii* ATCC 9640 | + |
| *Lactobacillus gasseri* NCFB 2233 | - |
| *Lactobacillus helveticus* ATCC 15009 | + |
| *Lactobacillus pentosaceus* ATCC 8041 | - |
| *Lactobacillus plantarum* ATCC 14917 | + |
| *Lactobacillus plantarum* NCDO 955[c] | + |
| *Lactobacillus reuteri* NCFB 2589 | - |
| *Lactobacillus sake* KFRI 816 | - |
| *Lactococcus diacetylactis* NRRL B-2356 | - |
| *Leuconostoc mesenteroides* IAM 1046 | - |
| *Listeria monocytogenes* ATCC 19111 | + |
| *Pediococcus acidilactici* ATCC 8081 | + |
| *Pediococcus cerevisiae* KCTC 1628 | + |
| *Pediococcus pentosaceus* NRRL B-14009 | + |
| *Propionibacterium freudenreichii* subsp. *freudenreichii* NCFB 546 | - |
| *Propionibacterium acnes* ATCC 6919 | - |
| *Staphylococcus aureus* ATCC 14458 | - |
| *Streptococcus agalactiae* ATCC 14364 | - |
| *Streptococcus mutans* KCTC 3298 | + |
| **GRAM-NEGATIVE BACTERIA** | |
| *Aeromonas hydrophila* ATCC 7966 | - |
| *Escherichia coli* ATCC 25922 | - |
| *Pseudomonas aeruginosa* ATCC 27853 | - |
| *Shigella flexneri* ATCC 9199 | - |
| **YEASTS** | |
| *Candida boidinii* ATCC 26175 | - |
| *Candida famata* ATCC 10539 | - |
| *Candida albicans* ATCC 10231 | - |
| *Debaryomyces hansenii* ATCC 10620 | - |
| *Hansenula capsulata* ATCC 24202 | - |
| *Saccharomyces cerevisiae* ATCC 9763 | - |

[a]ATCC, American Type Culture Collection (Rockville, MD, U.S.A.); IFO, Institute for Fermentation Osaka (Osaka, Japan); IAM, Institute of Applied Microbiology (Tokyo, Japan); KCCM, Korean Culture Center of Microorganism (Yonsei University, Seoul, Korea); KCTC, Korea Collection for Type Cultures (Daeduk, Korea); KFRI, Korea Food Research Institute (Songnam, Korea); NCFB, National Collection of Food Bacteria (Reading, U.K.); NRRL, Northern Regional Research Laboratory (Peoria, U.S.A.); NCDO, National Collection of Dairy Organisms, see NCFB.
[b]+, sensitive; -, not sensitive.
Sensitivity was estimated for each strain by the agar well diffusion assay described in Materials and Methods.
[c]Indicates the indicator strain used for isolation of bacteriocin-producing lactic acid bacteria and measurement of bacteriocin activity.

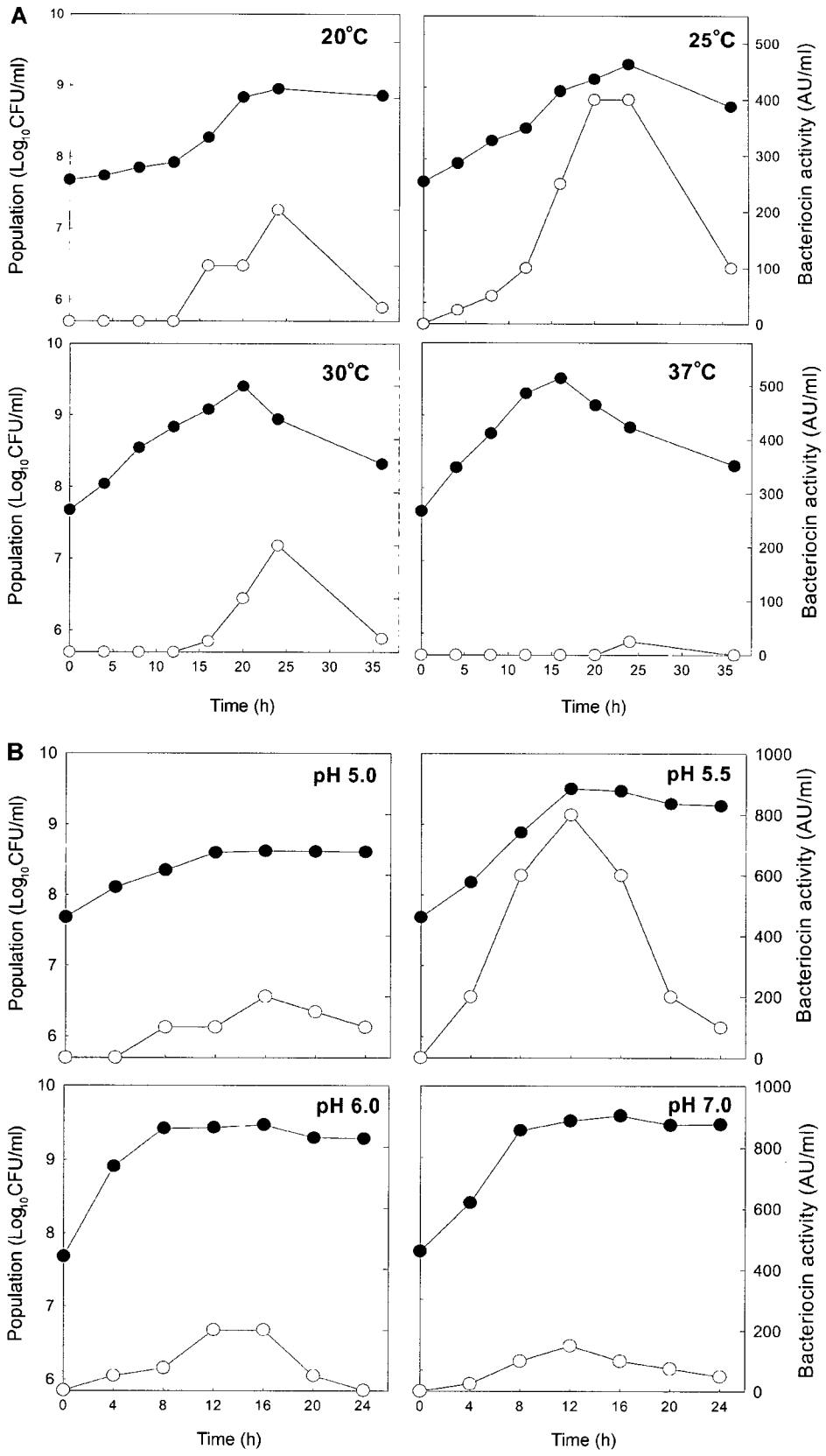


**Fig. 4.** Growth and bacteriocin production of *Enterococcus* sp. K25 at different temperatures (A) and pHs (B).
Symbols: ●, cell growth; ○, bacteriocin activity.

bacteriocins produced by lactic acid bacteria, including enterococci, have been characterized and many of them are classified as class II [3, 17, 34]. In particular, pediocin PA-1, a representative class IIa bacteriocin having antilisterial motif (-YGNGV-), has a broad-range antimicrobial spectrum and is very well characterized on the gene level [10, 23, 28].

### Optimal Culture Condition for Bacteriocin Production by *Enterococcus* sp. K25

The effect of temperature and pH of the growth medium on the bacteriocin production of *Enterococcus* sp. K25 were investigated (Figs. 4A and 4B). The bacteriocin production started from the early exponential growth phase and reached its maximum level at the later part of the exponential growth phase in all the temperatures tested, except for 37°C. Interestingly, the level of bacteriocin titer (400 AU/ml) was the highest when the strain was grown at 25°C, but cells grown at 37°C showed very low activity (below 100 AU/ml). Therefore, *Enterococcus* sp. K25 could be suitable for energy-saving storage (e.g., w/o refrigeration) of kimchi at room temperature. Moreover, the level of bacteriocin titer (800 AU/ml) was the highest for cells grown at pH 5.5; whereas cells grown at other pH conditions (i.e., pH 5.0, 6.0, 7.0) were low (maximal 200 AU/ml) (Fig. 4B). This is another advantage of *Enterococcus* sp. K25 in kimchi fermentation, considering that the pH of fresh kimchi is around pH 5.5.

### Application of *Enterococcus* sp. K25, Chitosan, and Fumaric acid to Kimchi Fermentation

To investigate the effect of *Enterococcus* sp. K25 on the extension of kimchi shelf-life, the whole cell of *Enterococcus* sp. K25 was applied to kimchi alone or together with other preservatives as described in Materials and Methods. As shown in Fig. 5, treatment of *Enterococcus* sp. K25 ($10^7$ cfu/g) together with fumaric acid (0.1%) showed a synergistic effect on the extension of kimchi shelf-life at 5°C. Compared to control (no addition), the treatment delayed kimchi to reach the eight-point TA value, recognized as the edible limit, up to 5 days. However, other treatments, such as treatment of whole cell alone, chitosan (0.2%) alone, fumaric acid (0.1%) alone, whole cell with chitosan, chitosan with fumaric acid, and whole cell with chitosan and fumaric acid, were not effective for extending shelf-life. For further analysis, several combinations of whole cell and fumaric acid at different concentrations were tested at 10°C. As shown in Fig. 6, all treatments were effective for extending the shelf-life of kimchi. Compared to control (no addition), kimchi shelf-life was prolonged by a minimal of 2 days in all treatments. In particular, the treatment of whole cell ($2\times10^7$ cfu/g) and 0.1% fumaric acid showed a favorable easy slope and prolonged the kimchi shelf-life up to 6 days. In the future, consumption of commercially prepared

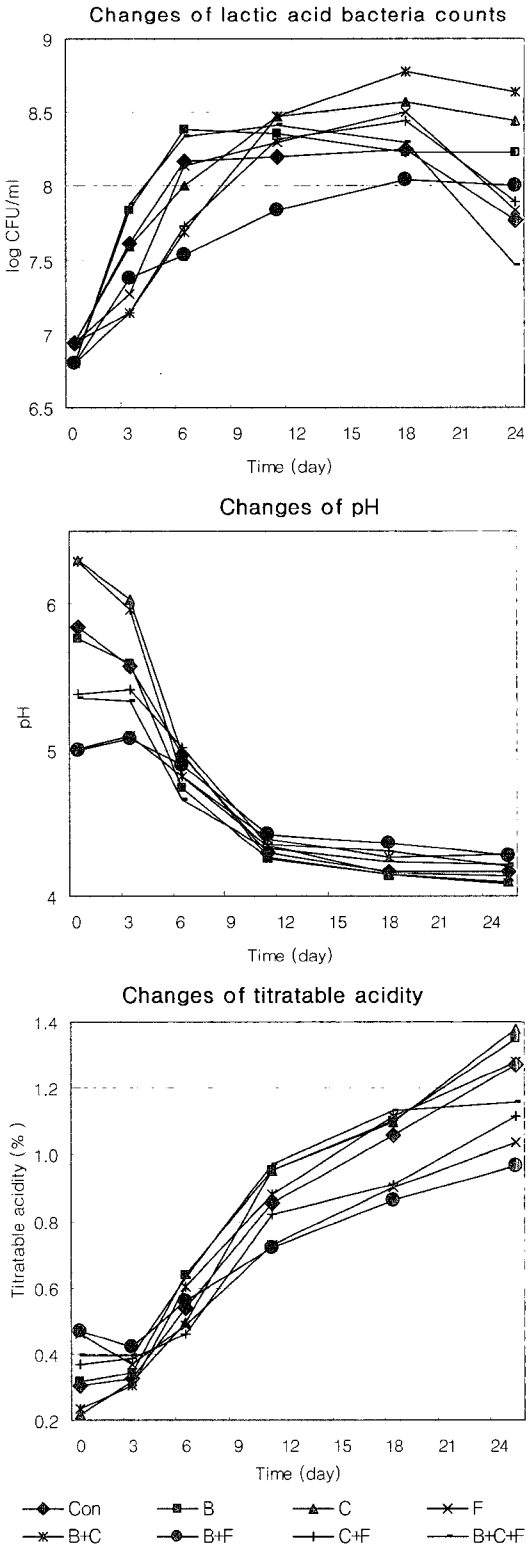


**Fig. 5.** Changes of cell counts, pH, and titratable acidity of kimchi during fermentation at 5°C.
*Enterococcus* sp. K25 cells were inoculated to kimchi alone or together with other preservatives (i.e., chitosan and fumaric acid). Con, control (no addition); B, *Enterococcus* sp. K25 ($10^7$ CFU/g); C, chitosan (0.2%); F, fumaric acid (0.1%).

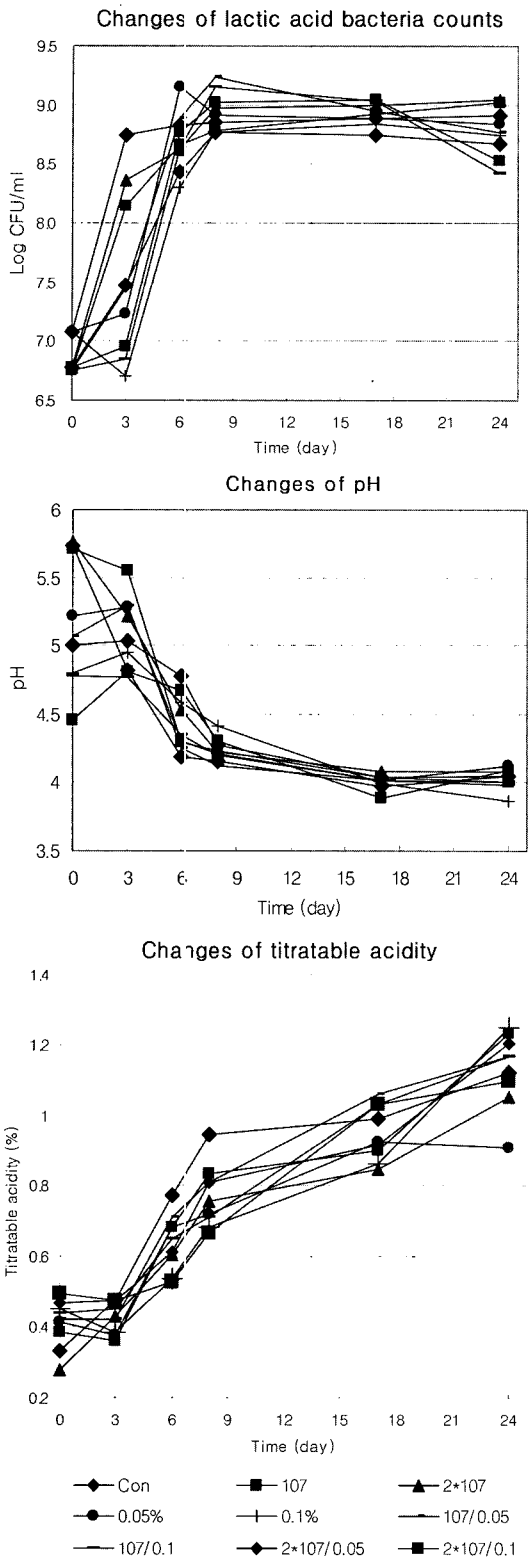


**Fig. 6.** Changes of cell counts, pH, and titratable acidity of kimchi during fermentation at 10°C.
*Enterococcus* sp. K25 cells were inoculated to kimchi alone or together with fumaric acid. Con, control (no addition); 107, *Enterococcus* sp. K25 ($10^7$ CFU/g); 2*107, *Enterococcus* sp. K25 ($2\times10^7$ CFU/g); 0.05%, fumaric acid (0.05%); 0.1%. fumaric acid (0.1%).

kimchi products will be expected to remarkably increase and, therefore, the extension of kimchi shelf-life will be an important industrial concern. In this respect, the use of bacteriocin-producing LAB inhibiting kimchi spoilage microorganisms with appropriate preservatives as synergistic hurdles could be a novel method for the extension of kimchi shelf-life. However, not all bacteriocinogenic LAB can be used since they produce lactic acid as well. In this regard, and as a conclusion, K25 strain fulfils several requirements as a starter for a shelf-life extender of kimchi: 1) It strongly inhibits the growth of *Lb. plantarum*, the major acidifier of kimchi; 2) it is a heterofermentative lactic acid bacterium that produces less lactic acid, but instead produces flavor compounds that contribute to enhance the taste of kimchi; 3) it produces bacteriocin in the early period; 4) its bacteriocin production is maximum at low temperature (ca. 25°C) at pH 5.5; and finally, 5) it has a synergistic effect with fumaric acid.

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