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NOTE

Evaluation of Optimum Conditions for Bacteriocin Production from *Lactobacillus* sp. JB-42 Isolated from Kimchi

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Bacteriocin-producing microorganism was isolated from Kimchi. The microorganism was identified as a *Lactobacillus* sp. and named *Lactobacillus* sp. JB-42. The optimum conditions for the bacteriocin production from the isolated microorganism were evaluated. For the maximum yield of bacteriocin from *Lactobacillus* sp. JB-42, the cell should be harvested at the early stationary phase and the temperature, pH and NaCl concentration should be 30°C, pH 7 and without the addition of NaCl, respectively. Sucrose, glucose or fructose should be used as a carbon source and organic nitrogen such as tryptone should be used as a nitrogen source for the best yield. The production of bacteriocin was related to the cell growth of *Lactobacillus* sp. JB-42 indicating the role of *Lactobacillus* in the Kimchi fermentation process.

Bacteriocins are proteinaceous compounds with bactericidal activity principally against strains that are the same or closely related to the bacteriocin producer bacterium. These substances are of particular interest as they are proteinaceous and may thus be degraded during digestion by humans and other animals. Many of the lactic acid bacteria (LAB) produce bacteriocins (8). Bacteriocins have a great potential as food preservatives due to the occurrence of antibiotic resistant pathogenic mutants when traditional antibiotics are used for the food preservatives. Bacteriocin is produced by microorganisms found in the processing of foods such as cheese, sausage, and Kimchi, therefore, they might be usable as a food preservatives (4) and a new cloning vehicle for genetic engineering (6).

Many bacteriocins are produced by gram-positive bacteria, especially from lactic acid bacteria (2) and bacteriocins from various food sources have been isolated and their structures identified (12).

Kimchi is a traditional fermented Korean vegetable food. Kimchi fermentation is initiated by various microorganisms originally present in the raw materials, but the fermentation is gradually dominated by lactic acid bacteria (10). Complex biochemical changes occur depending on the environmental conditions before, during and after fermentation. The most important characteristics of Kimchi fermentation are the compositional changes in sugar and vitamins, formation and accumulation of organic acids, and texture degradation. Quality of Kimchi is highly dependent on the storage conditions and can be deleteriously affected through undesirable biochemical and biological actions after the fermentation. The main microorganism responsible for ripening is *L. mesenteroides* but *L. plantarum* is responsible for excessive acidification in the later stages (9). In food and feed fermentations such as vegetables (16), sausage products (14), and silages (5), *Lactobacillus* species play a major role in the preservation of the fermented products.

This report describes a study on the isolation of bacteriocin-producing lactic acid bacteria from the most representative of traditional Korean foods, Kimchi, the evaluation of optimum conditions for bacteriocin production through the investigation of the physiological and biochemical characteristics of the isolated strain, and the factors affecting bacteriocin production in the *Lactobacillus* strain isolated of Kimchi.

MATERIALS AND METHODS

Isolation of *Lactobacillus* from Kimchi

Household Kimchis were collected from various sources. After whole components were ground, the sample was filtered with sterilized gauze. The filtrate was diluted to $1/10^6$ to $1/10^8$. The strains of *Lactobacillus* sp. were isolated from the diluted samples using modified *Lactobacillus* selection (LBS) medium containing acetic

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acid and sodium acetate to prevent the growth of *Pediococcus* sp. and 2% $CaCO_3$ added deMan-Rogosa-Sharpe (MRS) medium. The plates were incubated at 37°C for 2-3 days and the colonies were isolated. The strains were stored at -70°C in MRS broth (Difco laboratories) containing 50% glycerol before use.

Screening of the Strain Producing Bacteriocin from the Isolated *Lactobacillus*

Screening for inhibitory compound production and sensitivity analysis was performed by the modified Staskawicz's method (15). The agar drop diffusion test was performed as follows: A strain of *Lactobacillus* sp. was grown overnight in MRS broth at 37°C. 10 μl of cultured broth was spotted onto the MRS agar plate and cultured at 37°C for 6 h. The lawn of the indicator strain (*Lactobacillus helveticus* CNRZ 1096) was prepared by pouring the mixture of 100 μl precultured indicator strain in 4 ml of soft-overlay MRS (0.7% agar) onto the surface of precultured MRS agar plates (1.5% agar). The plate was incubated at 37°C for 12 h. The size of inhibitory zone was measured and compared for each plate. The strain that produced the largest inhibitory zone was selected for further experimentation.

Determination of Bacteriocin Activity

Due to the inaccuracy of the agar drop diffusion test, a new unit of bacteriocin activity was defined using the turbidity of the indicator strain at 660 nm. The selected *Lactobacillus* strain was cultured in 5 ml tubes for 12 h. After centrifugation, 4 ml of the supernatant was collected. After the addition of 1 ml 5×MRS broth and 0.5 ml of precultured indicator strain (*Lactobacillus helveticus* CNRZ 1096) to the supernatant, the mixture was cultured for 18 h and the turbidity was measured at 660 nm.

Inhibitory activity was determined by dividing the absorbance of the reference culture (indicator strain culture at 5 ml MRS broth) by that of the tested *Lactobacillus* culture broth. The unit of bacteriocin activity was defined by the log value of inhibitory activity.

Evaluation of Optimum Conditions for Bacteriocin Production

The optimum conditions for bacteriocin production from the selected *Lactobacillus* strain was determined by: 1) the growth phases of *Lactobacillus*, 2) the temperature of cultures, 3) pH of cultures, 4) carbon source, 5) nitrogen source, and 6) NaCl concentration.

RESULTS AND DISCUSSION

Selection of *Lactobacillus* Producing Bacteriocin from Kimchi

Various Kimchis were collected from restaurants and private homes. The ground Kimchi filtrates were diluted and plated on the modified LBS and $CaCO_3$-MRS medium. The plates were cultured at 37°C for 24 h. From these plates, 768 colonies of *Lactobacillus* were isolated and stored at -70°C until use.

The *Lactobacillus* producing bacteriocin were differentiated from each other by agar drop diffusion test using *L. helveticus* CNRZ 1096 as an indicator strain. Among 768 strains, 96 strains showed bacteriocin activity. Among the 96 strains, a strain showing the largest inhibitory zone was selected and named *Lactobacillus* sp. JB-42 as shown in Fig. 1. The biological and biochemical analysis of *Lactobacillus* sp. JB-42 showed indicative matching characteristics to *Lactobacillus* according to the 8th edition of Bergey's manual of determinative bacteriology (3) and Bergey's manual of systematic bacteriology vol. 2 (7).

The confirmation of bacteriocin production from *Lactobacillus* sp. JB-42 was carried out by heating, pH adjustment and a pronase test on the supernatant of the fermentation broth. The results indicated that the bactericidal agent from *Lactobacillus* sp. JB-42 was bacteriocin. The inhibitory spectrum of bacteriocin produced from *Lactobacillus* sp. JB-42 was investigated using various indicator strains as shown in Table 1.

The bacteriocin from *Lactobacillus* sp. JB-42 was active for gram positive microorganisms such as *Lactobacillus* sp., *Leuconostoc* sp., *Lactococcus* sp., *Staphylococcus aureus*, and *Clostridium acetobutyricum* and also active for gram negative microorganism such as *Escherichia coli*. However, it did not show activity for spore forming *Bacillus subtilis* and *Acetobacter aceti*.

Evaluation of Optimum Condition for the Production of Bacteriocin

Lactobacillus sp. JB-42 was inoculated to 200 ml MRS broth, and the growth and bacteriocin activity was

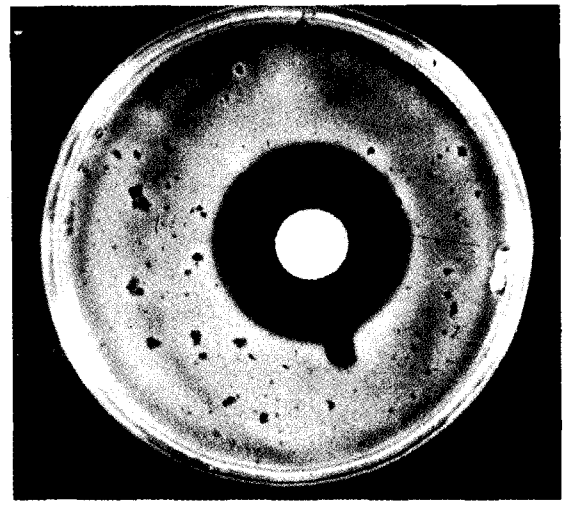

Fig. 1. Antagonistic activity of *Lactobacillus* sp. JB-42 overlaid with *L. helveticus* CNRZ1096.
The inhibitory zone was shown as clear halo in the center of petri dish.

measured at 2 h intervals as shown in Fig. 2. The logarithmic phase was observed after 6 h of growth and the stationary phase was observed after 16 h. According to the literature (11, 13), bacteriocin has been produced at the late-logarithmic or stationary phase as a typical secondary metabolite. However, the bacteriocin from *Lactobacillus* sp. JB-42 could be detected at the early logarithmic phase and showed a growth-related trend. The productivity of bacteriocin started to decrease when the growth of cell was in the middle of the stationary phase and became zero during the early stationary phase, according to cumulative bacteriocin activity measurement. The cumulative bacteriocin activity decreased at the stationary phase probably due to released protease from cell autolysis. The reason that pattern of bacteriocin production was closely related to cell growth is that *Lactobacillus* species are a dominating colony in early Kimchi fermentation process and provide acids and flavors to Kimchi. Therefore, *Lactobacillus* should produce bactericidal agents to prevent the growth of other microorganisms in the open, mixed fermentation system of Kimchi.

Table 1. Inhibitory spectrum of bacteriocin from *Lactobacillus* sp. JB-42 using agar drop diffusion test.

Strains	Inhibitor activity
Lactobacillus acidophillus	(+)
Lactobacillus bulgaricus IFO 13953	+
Lactobacillus delbrueckii subsp. *delbrueckii*	(+)
Lactobacillus helveticus IMA 12090	+
Lactobacillus helveticus CNRZ 1096	+
Lactobacillus helveticus CNRZ 1094	+
Lactobacillus plantarum	(+)
Lactobacillus brevis	-
Lactobacillus casei KCTC 1121	+
Lactococcus lactis subsp. *lactis*	+
Streptococcus lactis	+
Streptococcus mutans	(+)
Leuconostoc mesenteroides	-
Acetobacter aceti	-
Clostridium acetobutyricum	+
Escherichia coli	+
Staphylococcus aureus IAM 1011	+
Pediococcus acidilactis KCTC 3101	+
Bacillus subtilis	-

The relative halo size was observed and size of halo was expressed as the degree of inhibition. +, good inhibition; (+), weak inhibition; -, no inhibition.

The effect of cultural temperature on the growth and bacteriocin productivity was determined as shown in Fig. 3. *Lactobacillus* sp. JB-42 showed the maximum growth and bacteriocin production at 37°C which is the optimum temperature for common *Lactobacillus*. The growth and bacteriocin production of the cells decreased at temperatures higher than 40°C. However, temperatures lower than 37°C did not drastically decrease the growth and bacteriocin production of the cells. This was most likely due to the storage condition of Kimchi in the refrigerator. The temperature effect again showed a growth related pattern for the production of bacteriocin from *Lactobacillus* sp. JB-42.

Fig. 4. shows the effect of pH on the growth and bacteriocin production of the cells. *Lactobacillus* sp. JB-42 showed rapid growth at pH range of 5.0 to 8.0. Maximum cell growth could be observed at pH 8.0. A drastic decrease in cell growth could be detected at pH higher

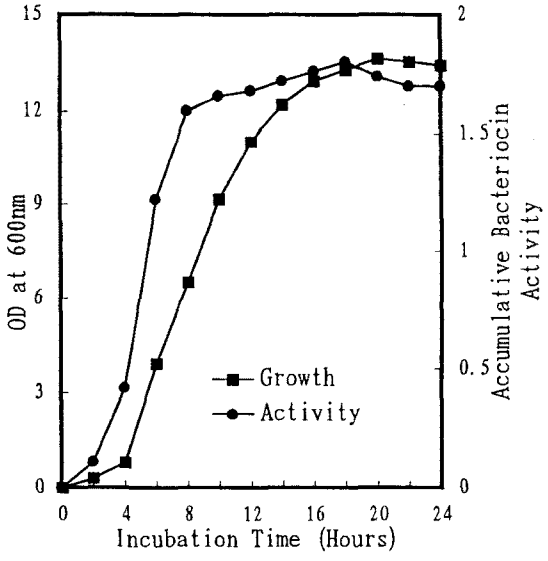

Fig. 2. Effect of incubation time on the production of bacteriocin and the cell growth of *Lactobacillus* sp. JB-42.

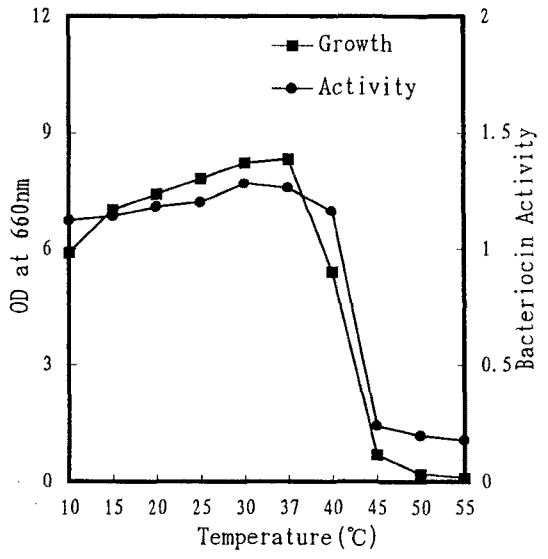

Fig. 3. Effect of temperature on the production of bacteriocin and the cell growth of *Lactobacillus* sp. JB-42.

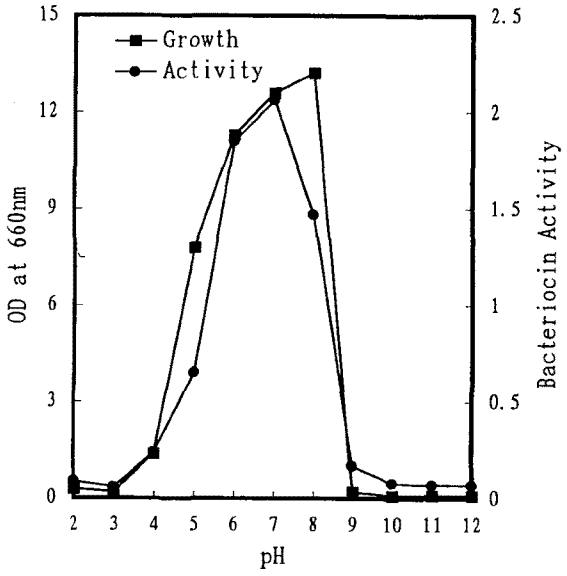

Fig. 4. Effect of pH on the production of bacteriocin and the cell growth of *Lactobacillus* sp. JB-42.

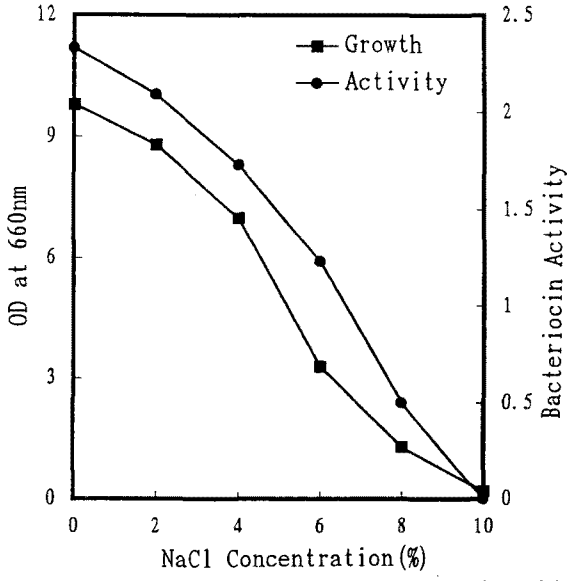

Fig. 5. Effect of sodium chloride on the production of bacteriocin and the cell growth of *Lactobacillus* sp. JB-42.

than 9.0. However, considerable cell growth could be detected at lower pH indicating the acidophilic property of *Lactobacillus*. The optimum pH for the production of bacteriocin was a little different from that for the growth of cells. The maximum bacteriocin production could be observed at pH 7.0. Even though there was a slight difference in the optimum pH for bacteriocin production and cell growth, an overall growth related pattern of bacteriocin production could be observed.

The effect of NaCl concentrations on cell growth and bacteriocin production from *Lactobacillus* sp. JB-42 was assessed because Kimchi was fermented in 2 to 10% NaCl solution. Contrary to the result of Amechi and Montville mentioning increases in bacteriocin production in tandem with the increase in NaCl concentration, the growth and bacteriocin production of *Lactobacillus* sp. JB-42 decreased as the NaCl concentration increased as shown in Fig. 5. Almost no growth and bacteriocin production were observed at 10% NaCl concentration.

The type of carbon source also affected cell growth and bacteriocin production as shown in Fig. 6. Glucose in MRS medium was replaced with 2% tested carbon sources. *Lactobacillus* sp. JB-42 grew well on glucose, fructose, sucrose, maltose, cellobiose or mannose as a carbon source. Maximum cell growth could be observed when maltose was used as a carbon source. However, the highest productivity of bacteriocin was observed when glucose, fructose or sucrose was used as a carbon source. The maximum production of bacteriocin was obtained when sucrose was used as a carbon source.

Nitrogen sources affected cell growth and the production of bacteriocin as shown in Fig. 7. Various nitrogen sources in MRS medium such as peptone, beef extract, yeast extract and ammonium sulfate were replaced with tested nitrogen sources. Organic nitrogen sources

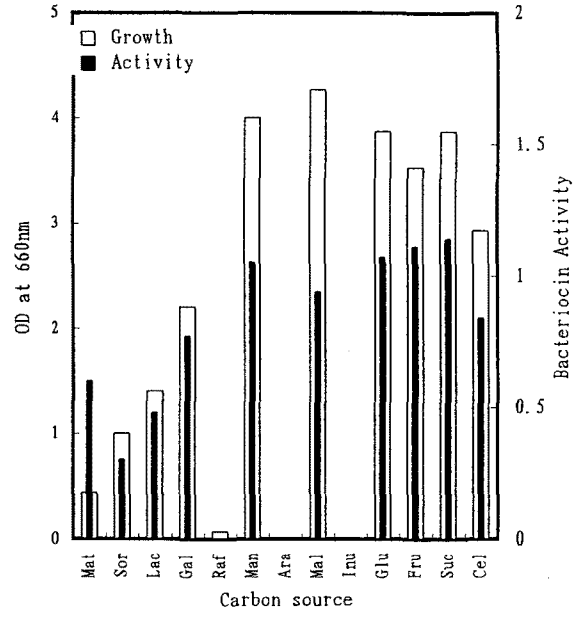

Fig. 6. Effect of carbon source on the production of bacteriocin and the cell growth.
Glucose in MRS medium was replaced with 2% tested carbon sources. The tested carbon sources and abbreviations are Mat, manitol; Sor, sorbitol; Lac, lactose; Gal, galactose; Raf, raffinose; Man, mannose; Ara, arabinose; Mal, maltose; Inu, inulin; Glu, glucose; Fru, fructose; Suc, sucrose and Cel, cellobiose.

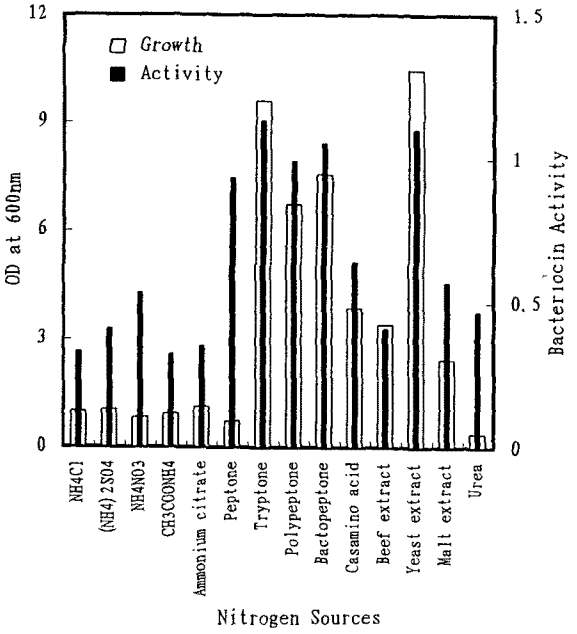

Fig. 7. Effect of nitrogen source on the production of bacteriocin and the cell growth of *Lactobacillus* sp. JB-42
Various nitrogen sources in MRS medium such as peptone, beef extract, yeast extract and ammonium sulfate were replaced with tested nitrogen sources.

showed superior cell growth and bacteriocin production as compared to inorganic nitrogen sources.

Evaluating the factors for the cell growth and production of bacteriocin from *Lactobacillus* sp. JB-42 which was isolated from Kimchi, the optimum conditions for the maximum yield of bacteriocin could be determined. The cell should be harvested at the early stationary phase and the temperature, pH and NaCl concentration should be 30°C, pH 7 and zero M, respectively, for the maximum yield of bacteriocin from *Lactobacillus* sp. JB-42. Sucrose, glucose or fructose should be used as a carbon source and organic nitrogen such as tryptone should be used as a nitrogen source for the best yield of bacteriocin from *Lactobacillus* sp. JB-42.

Based on the optimum conditions defined in this study, a large scale production of bacteriocin is being carried out along with the studies on the purification of bacteriocin from *Lactobacillus* sp. JB-42.

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