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Production of Bacteriocin by *Staphylococcus* sp. NPSI 38 in Koji Extract Medium with Rice Protein Hydrolyzate and Its Growth-inhibitory Activity against Hiochi-bacteria

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Enhanced production of bacteriocin by *Staphylococcus* sp. NPSI 38 (NPSI 38) using koji extract medium was investigated. The bacteriocin produced by NPSI 38 in MRS medium was found to be effective for inhibiting the growth of *Lactobacillus hilgardii* NBRC 15886^T, one of the representative hiochi-bacteria. In cultivations using test tubes without pH control, meat extract and Polypepton were effective for bacteriocin production by NPSI 38 with 10%(v/v) koji extract medium. When the koji extract medium supplemented with hydrolyzate of a rice protein preparation was used instead of meat extract and Polypepton, NPSI 38 produced 160 U/ml of bacteriocin in the cultivation with pH control, which was almost as high as that (156 U/ml) observed in cultivation using MRS medium with pH control. When the cells of *L. hilgardii* NBRC 15886^T collected at logarithmic growth and stationary phases were inoculated into fresh modified MRS medium with 11 U/ml of the bacteriocin, negligible cell growth was observed, irrespective of different growth phases of cells inoculated. Little or no increase in cell concentrations in the media containing bacteriocin showed that the action of the bacteriocin produced by NPSI 38 was bacteriostatic against the hiochi-bacterium.

Keywords: bacteriocin, hiochi-bacteria, *Lactobacillus hilgardii*, koji extract medium, rice protein

Introduction

Hiochi-bacteria are recognized as major potential spoilage microorganisms of sake. Hiochi-bacteria have been identified as several *Lactobacillus* species (*Lactobacillus fructivorans*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum* and *Lactobacillus hilgardii*) by molecular biology and biochemistry methods (Goto-Yamamoto *et al.*, 1994, Niidome *et al.*, 1995). Hiochi caused by outbreaks of the bacteria results in lowering of the pH, occurrence of turbidity, and formation of off-flavors such as diacetyl. The spoilaging phenomena seriously deteriorate the quality of sake. To prevent hiochi from emerging in the sake brewing industry, pasteurization (hiire) at around 65°C for a short duration of 2-3 min or microfiltration for removing hiochi-bacteria has been carried out in the sake brewing process. However, the spoilage of sake caused by hiochi-bacteria is not completely repressed at present.

A large number of bacteriocins, antimicrobial peptides, are produced by many lactic acid bacteria (LAB) (Onda *et al.*, 2002, Soriano *et al.*, 2004, Sparo *et al.*, 2006). Since bacteriocins are isolated from foods, such as meat and dairy products, which normally contain LAB, they have unknowingly been consumed for centuries by humans (Goktepe, 2006). Thus, bacteriocins from LAB have been shown to be safe, and have the potential to be effective as natural food preservatives (Ennahar *et al.*, 2000, Cleveland *et al.*, 2001). Many bacteriocins are small proteins, stable at high temperature, digested by protease (Ray, 1992, Heng *et al.*, 2007), and only effective against a limited narrow spectrum of bacteria, typically those related to the producer strain. For example, nisin A, produced by *Lactococcus lactis*, is approved for use in over 40 countries and has been used as a food additive for over 50 years to preserve the quality of processed cheese,

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dairy products and canned foods (Delves-Broughton *et al.*, 1996, Cleveland *et al.*, 2001).

The inhibitory effect of commercially pure nisin A on growth of hiochi-bacteria was confirmed by Kanatani *et al.* (1992), who showed the sensitivity of hiochi-bacteria to nisin A using the agar diffusion method. They also showed that no obvious growth of hiochi-bacteria was observed in either the heat-treated or ultrafiltrated sake containing nisin A. Kaneoke *et al.* (2002) isolated a novel bacteriocin-producing bacterium, *Staphylococcus* sp. NPSI 38 (NPSI 38: referred as *Pediococcus* sp. NPIB 38 in the ref.) from rice koji. They found that the bacteriocin showed antimicrobial activity against selected strains of hiochi-bacteria (*L. fructivorans*, *L. hilgardii*, etc.). However, apart from utilization of cultures with bacteriocin formed during fermentation, the addition of purified bacteriocins to foods is currently illegal in Japan.

In this study, to produce bacteriocin active against hiochi-bacteria using rice koji extract as a medium, we investigated the positive effects of components in MRS medium on production of bacteriocin by NPSI 38. On the basis of the results obtained, we also examined the improvement of bacteriocin production by the addition of hydrolyzates of ori (precipitate from koji extract), pressed sake cake, and rice protein as a stimulating factor to koji extract medium. Further, we evaluated the inhibitory effects of the bacteriocin solution obtained on the growth of *L. hilgardii*.

Materials and Methods

Microorganisms and media *Staphylococcus* sp. NPSI 38 (NPSI 38) isolated from rice koji was used as a bacteriocin-producing strain in this study. *Lactobacillus hilgardii* NBRC 15886^T was used as an indicator microorganism. NPIS 38 was grown in normal MRS medium (De Man *et al.*, 1960), containing 10 g/l glucose at 30 °C for 24 h. MRS medium contained 10 g of Polypepton (Nihon Pharmaceuticals, Tokyo), 10 g of fish meat extract (#01230, Kyokuto Pharmaceutical Industrial, Tokyo), 5 g of yeast extract (Oriental Yeast, Tokyo), 2 g of K_2HPO_4, 2 g of diammonium hydrogen citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot 4H_2O$, and 1 g of Tween 80 per liter (pH 6.8). *L. hilgardii* NBRC 15886^T was grown in the modified MRS medium (pH 6.8) from which Tween 80 and diammonium hydrogen citrate were omitted. The bacterial strains were maintained at -80 °C in 25% glycerol.

Determination of antimicrobial activity Antimicrobial activity was determined by the agar diffusion method using a stainless cup according to the procedure described previously (Taniguchi *et al.* 1994). The supernatant obtained by centrifugation (at 15,500 rpm for 10 min) of culture broth was analyzed for antimicrobial activity. Commercial nisin A (Sigma Chemical Co., St. Louis, USA) was used as a standard. Nisin A was dissolved in 0.02 N HCl solution and diluted with 0.02 N HCl solution to produce the required concentrations. After autoclaving, MRS medium with 1% agar was poured in petri dish and allowed to solidify. Soft agar (1%) containing the test organism at an appropriate concentration was overlaid on MRS agar medium. Then, a sterile stainless cup (6 mm in inner diameter) was placed on the surface of the agar medium, and a sample solution was added to the cup. The resulting plates were anaerobically incubated in a jar with a deoxidizing reagent (AnaeroPak A-03, Mitsubishi Gas Chemicals, Tokyo) at 30 °C for 2 days. The diameter of growth-inhibitory zone around the cup was measured directly after incubation. One unit of antimicrobial activity was defined as the amount of bacteriocin which showed a diameter of growth-inhibitory zone equal to that obtained by 1 ng of pure nisin A standard solution. The antimicrobial activity of culture supernatant was expressed as a corresponding nisin activity.

Preparation of koji extract medium and hydrolysis of protein-containing materials Three liters of distilled water was added to 1 kg of rice koji and the suspension was incubated at 55 °C for 8 h. The solubilized fraction was separated from insoluble portions by pressing using a hemp cloth bag and allowing it to stand at 4 °C overnight. After removing the precipitate (this is referred to as ori) formed during the incubation at 4 °C by filtration, the resultant filtrate was used as koji extract (100% (v/v)). Total soluble sugar, glucose, and protein concentrations of the koji extract were 114, 77 and 5.2 g/l, respectively. Three types of additives (ori, pressed sake cake, and rice protein) were used as a nitrogen source for supplying 10% (v/v) koji extract medium. Pressed sake cake was produced as a by-product at the Niigata Prefectural Sake Research Institute. Commercial rice protein (I) (code MA-41, Meelunie B.V., Amsterdam, Netherlands) and rice protein (II) prepared preliminarily, which is a gift from Shimada Chemical Industries, Nagaoka, Japan, were used. Rice protein (II) was recovered as a precipitate by extraction with 1% NaOH solution from polished rice, followed by neutralization. Rice protein (II) recovered was stored after drying. The additives were suspended in McIlvaine buffer (pH 4.0) and hydrolyzed by protease (protease M amino: Amano Enzyme, Nagoya) at 50 °C for 6 h. The supernatants obtained by centrifuging at 8,000 rpm for 20 min at 4 °C were added to the koji extract medium as a promotive factor of bacteriocin production.

Bacteriocin production Bacteriocin production by NPSI 38 was carried out using MRS medium and 10% (v/v) koji extract medium at the initial pH of 6.8. The components of MRS medium and the hydrolyzates described above were

added to the koji extract medium when needed. NPSI 38 was precultured statically in test tubes containing MRS medium at 30 °C for 24 h, and the precultured cells were inoculated at an initial turbidity of 0.1 at 660 nm (T_{660} = 0.1). To estimate the effects of the addition of components of MRS medium and the hydrolyzates on bacteriocin production, NPSI 38 was cultivated in a test tube without pH control. In experiments on the comparison of bacteriocin production between MRS medium and the koji extract medium with rice protein hydrolyzate, NPSI 38 was cultivated in medium-sized bottles or a fermentor (TBR-1; Chiyoda Seisakusho, Nagano) with a working volume of 700 ml. pH was maintained at 6.8 by additions of 4 N NaOH using a peristaltic pump coupled to a pH controller. Cultures were sparged with nitrogen gas at 0.3 vvm throughout the cultivation to maintain anaerobic conditions.

Inhibitory activity of bacteriocin on the growth of a hiochi-bacterium *L. hilgardii* NBRC 15886^T, a representative hiochi-bacterium, was cultivated in medium-sized bottles in order to evaluate the inhibitory effect of bacteriocin on the cell growth of the hiochi-bacterium. The bacteriocin solution was prepared by cultivating NPSI 38 in the koji extract medium supplemented with the hydrolyzate of rice protein (II) at a protein concentration of 40 g/l. The culture supernatant was obtained by centrifugation (15,500 rpm, 10 min) of the culture broth. The resultant supernatant was sterilized by membrane filtration, and used as a bacteriocin solution. *L. hilgardii* NBRC 15886^T was inoculated in modified MRS medium containing 11 U/ml of a bacteriocin solution or 100 ng/ml of nisin and incubated at 30 °C for 24-48 h. To examine the sensitivity of cells to bacteriocin, the cells at different growth phases were prepared as follows: the cells of *L. hilgardii* NBRC 15886^T were recovered at cultivation times of 24 h (logarithmic growth phase) and 48 h (stationary phase) by centrifugation (15,500 rpm, 10 min). The cells of each growth phase were resuspended in fresh modified MRS medium containing 11 U/ml of bacteriocin solution. The inhibitory effect of bacteriocin on the growth was evaluated by measuring T_{660} and the concentration of viable cells.

Other analytical methods Cell concentration was determined by measuring T_{660}. The number of viable cells was counted by the plate culture method using MRS agar medium containing 10 g/l glucose. Viable cell concentration was expressed as colony forming units per milliliter (CFU/ml). Protein concentration was determined by the method of Lowry *et al.* (1951) using a bovine serum albumin as a standard. The supernatant obtained by centrifugation (15,500 rpm, 10 min) of culture broth was analyzed for total soluble sugar, glucose and lactic acid concentrations. The concentrations of total soluble sugar, glucose and lactic acid were measured as described previously (Taniguchi *et al.*, 1998, 2005).

Results

Bacteriocin production using koji extract medium To evaluate the effect on bacteriocin production by NPSI 38, the components of MRS medium were added to 10% (v/v) koji extract medium. Figure 1 shows the effect of addition of natural ingredients (Polypepton (Pp), fish meat extract (ME), and yeast extract (YE)) and other components in MRS medium on cell growth and bacteriocin production. In addition, we investigated the improvement of bacteriocin production by combining two and three components as additives. The concentration of each additive coincided with that in MRS medium. The supplementation of each natural ingredient to the koji extract medium resulted in an increase in cell growth, as shown in Fig. 1. Bacteriocin production was observed in the koji extract medium with Pp and ME, although no bacteriocin was produced when YE was added. ME and Pp were the most preferential for bacteriocin production by NPSI 38 and the bacteriocin activity obtained in the koji extract medium with ME and Pp was approximately 2.2 and 2.0 U/ml, respectively. When Pp was added to the koji extract medium together with ME, the cell growth was further stimulated and the bacteriocin activity was 15.5 U/ml. When ME

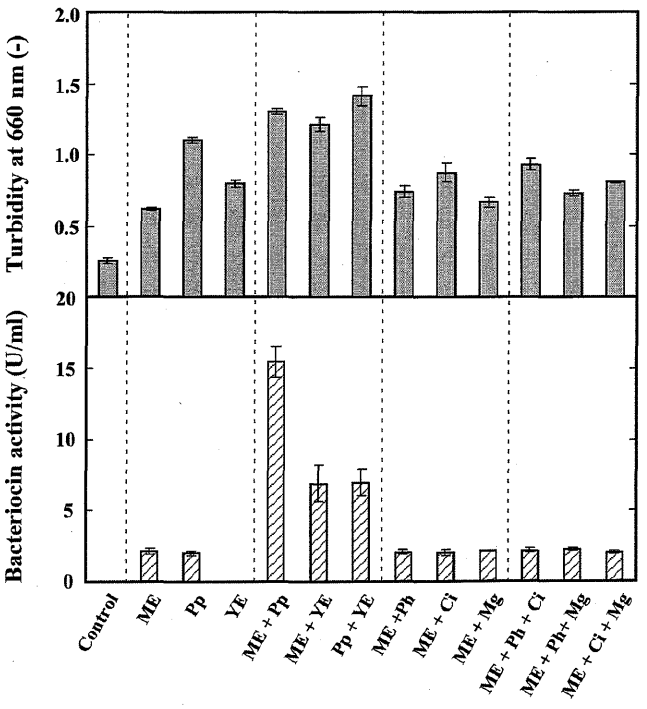

Fig. 1. Effect of addition of components of MRS medium to 10%(v/v) koji extract medium on the bacteriocin production by NPSI 38. Medium components: Pp; Polypepton, ME; Fish meat extract, YE; Yeast extract, Ph; K_2HPO_4, Ci; diammonium hydrogen citrate, and Mg; $MgSO_4$. The data represent mean values (n = 3). The error bars indicate standard deviations.

or Pp was further added to the koji extract media with YE, the cell concentration increased to more than T_{660} = 1.2, but the bacteriocin activity (7 U/ml) was less than that obtained in the koji extract medium with ME and Pp. When K_2HPO_4 (Ph), $MgSO_4$ (Mg) or diammonium hydrogen citrate (Ci) was added to the medium with ME, bacteriocin activity hardly increased although the stimulation of cell growth in each cultivation was observed. The addition of two components of Ph and Ci, Ph and Mg, or Ci and Mg to the koji extract medium with ME resulted in negligible increases in bacteriocin activity. In cultivation using the koji extract medium with Pp, no significant effect of addition of Ph, Mg, and Ci on bacteriocin production was observed in a similar manner as the koji medium with ME (data not shown). These results suggested that ME and Pp were effective additives for bacteriocin production by NPSI 38 using the koji extract medium. However, ME and Pp are legally prohibited for use in the sake brewing process. Therefore, we attempted to exploit an alternative to ME and Pp, which can be lawfully used in the sake production process and facilitates bacteriocin production by NPSI 38 in the koji extract medium.

We selected three types of protein-containing additives (ori, sake cake and rice protein) as an alternative to ME or Pp for supplementing the koji extract medium. NPSI 38 was cultivated in the koji extract medium with the hydrolyzate of each protein-containing additive. In the case of adding the hydrolyzate of ori or sake cake ranging from 3 to 6 g/l in protein concentration, no bacteriocin was produced by NPSI 38, although the cell concentration was enhanced up to several times as high as that in the koji extract medium without the hydrolyzate (data not shown). When the hydrolyzate of rice protein (I) or (II) was supplemented at concentrations of less than 5 g/l, NPSI 38 produced no bacteriocin although the cell growth was stimulated, as was observed in cultivation using the koji extract medium with the hydrolyzate of ori or sake cake (data not shown). Since the protein contents of rice protein (I) and (II) are about 72 and 82% (supplier's specifications), respectively, it was relatively easy to prepare the hydrolyzate at high protein concentrations from rice proteins compared to preparations with ori and sake cake. Figure 2 shows the effect of adding rice protein hydrolyzates to the koji extract medium on cell growth and bacteriocin production by NPSI 38. The hydrolyzates of rice proteins (I) and (II) were added, ranging from 10 to 40 g/l in protein concentration. The cell concentrations (T_{660}) increased gradually, as the concentrations of rice protein hydrolyzates added were elevated, although the turbidities were slightly overestimated due to cloudiness of hydrolyzates of rice protein. When the hydrolyzate of rice protein (I) or (II) was added at a protein concentration of 40 g/l, the bacteriocin activity was approximately 11 or 68 U/ml, respectively. The latter value corresponds to about 70% as high as the amount (98 U/ml) of bacteriocin produced in MRS medium.

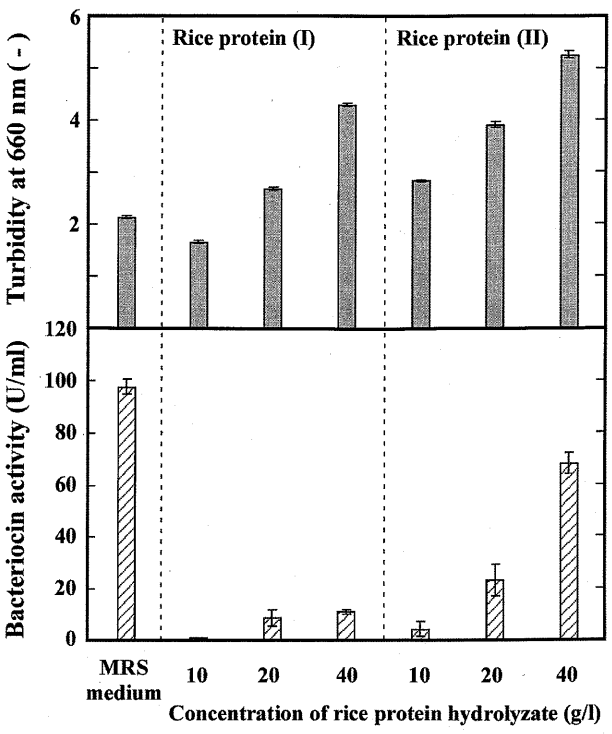

Fig. 2. Effect of addition of the hydrolyzate of rice protein to 10%(v/v) koji extract medium on the bacteriocin production by NPSI 38. Rice protein (I) (MA-41) and rice protein (II) (a sample preparation of Shimada Chemical Industries, Nagaoka, Japan) were used. The data represent mean values (n = 3). The error bars indicate standard deviations.

Bacteriocin production by cultivation with pH control

Figure 3 shows the results of bacteriocin production by NPSI 38 using MRS medium with 20 g/l glucose and the koji extract medium supplemented with the hydrolyzate of rice protein (II) at a protein concentration of 40 g/l. Cultivation with and without pH control was carried out in a fermentor and medium-sized bottles, respectively, as described in Materials and Methods. The results of turbidities and viable cell concentrations indicated that the cells of NPSI 38 grew vigorously at the initial stage of all the cultivations, followed by a gradual decrease in growth rate. This decrease in growth rate seems to not be caused by the depletion of nutrients in the media but by the accumulation of lactic acid, similarly to other organic acid-producing bacteria described previously (Taniguchi *et. al.*, 1998, 2001, 2005; Kouya *et al.*, 2007). As NPSI 38 consumed glucose gradually in the media, only lactic acid was produced corresponding to the consumption of glucose, and no other organic acids were detected throughout the cultivations, indicating that NPSI 38 is a typical homo-fermentative LAB. When the pH of the media

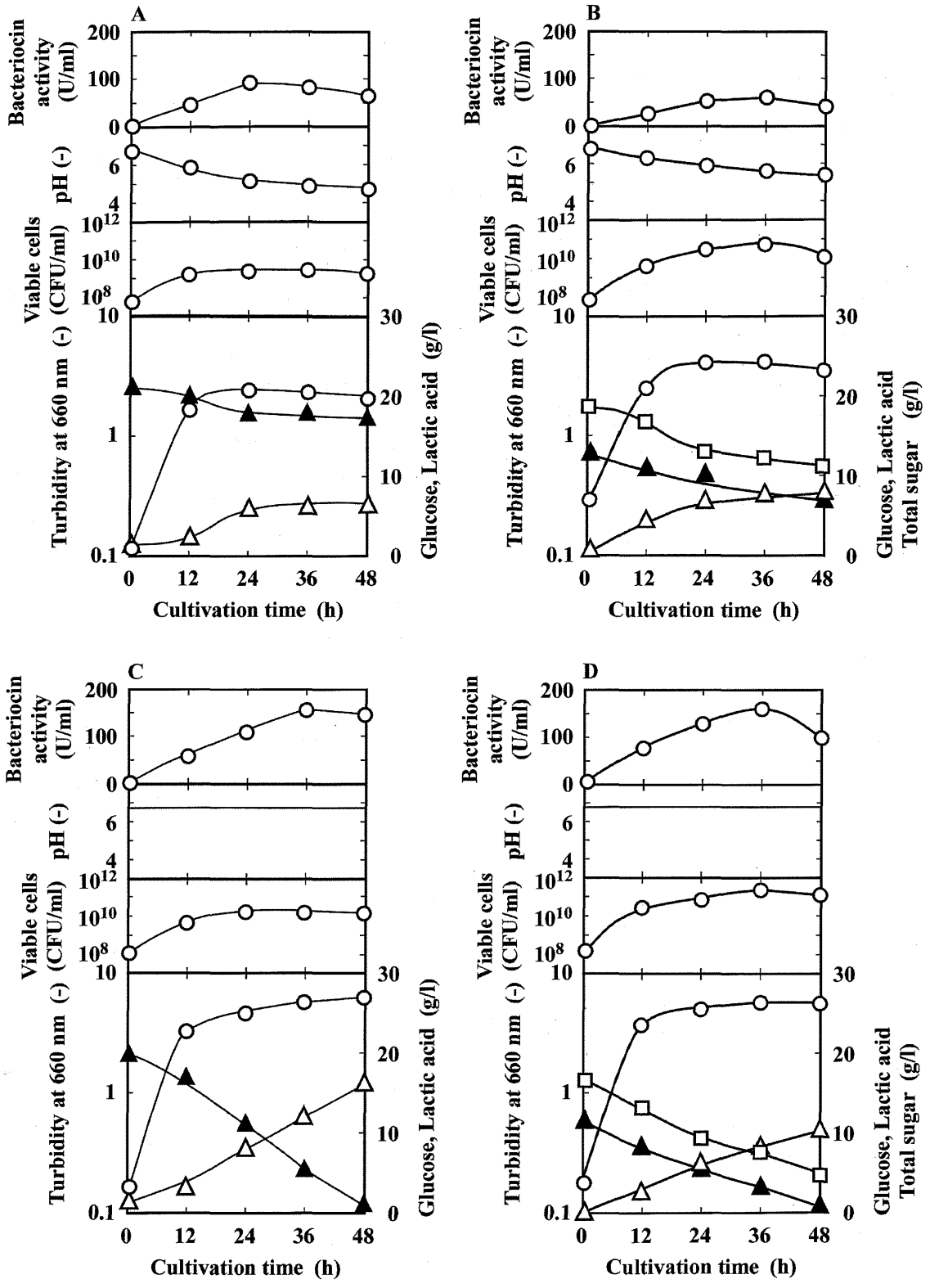

Fig. 3. Bacteriocin production by NPSI 38 using MRS medium and the koji extract medium supplemented with the hydrolyzate of rice protein (II). NPSI 38 was cultivated in MRS medium (A and C) and the koji extract medium with the hydrolyzate of rice protein (II) at a concentration of 40 g/l (B and D). Cultivations (A) and (B) were carried out without pH control. Cultivations (C) and (D) were carried out with pH control. Symbols: open circles, bacteriocin activity, pH, viable cells, and turbidity; closed triangles, glucose; open triangles, lactic acid; open squares, total sugar.

was controlled at a constant level of 6.8 (Figs. 3C and 3D), glucose was consumed completely in the cultivations using both media and the final concentrations of lactic acid were higher than those in the corresponding cultivations without pH control (Figs. 3A and 3B). In the koji extract medium supplemented with the hydrolyzate of rice protein (II) (Fig. 3D), the maximum bacteriocin activity was obtained at 36 h and the bacteriocin concentration was 160 U/ml. This value is almost as high as the concentration (156 U/ml) in the cultivation using MRS medium with pH control (Fig. 3C).

Inhibitory effect of bacteriocin on growth of a hiochi-bacterium *L. hilgardii*, one of the well-known hiochi-bacteria, has frequently been detected in spoiled sake. The inhibitory effect on the growth of *L. hilgardii* NBRC 15886^T was examined using a bacteriocin solution which was prepared by cultivating NPSI 38 in the koji extract medium with the hydrolyzate of rice protein (II) as described above. Figure 4 shows the inhibitory effects on the growth of cells at different growth phases. To avoid the influences of metabolites produced by *L. hilgardii* NBRC 15886^T on the bacteriocin activity as well as to examine the sensitivity of cells at different growth phases, the cells at logarithmic growth and stationary phases were once collected by centrifugation and then inoculated into a fresh modified MRS medium. The initial cell concentration was 8.0 ± 0.2×10^7 CFU/ml for cultivations with 11 U/ml of bacteriocin from NPSI 38 and 5.6 ± 0.8×10^8 CFU/ml for cultivations with 100 ng/ml of nisin A. In media without the bacteriocin, the cells of *L. hilgardii* NBRC 15886^T at different growth phases grew gradually after the inoculation, as shown in Figs. 4A and 4B. In the bacteriocin-free cultivation, the final turbidity was more than T_{660} = 3 and the maximum number of viable cells reached a level of 10^{10} CFU/ml for the cultivation with inoculation of cells at logarithmic growth phase (Fig. 4A) and 10^9 CFU/ml for the cultivation with inoculation of cells at stationary growth phase (Fig. 4B). In contrast, judging from the viable cell concentrations, negligible cell growth was observed in media containing bacteriocin from NPSI 38, irrespective of different growth phases of cells inoculated, although turbidity slightly increased in cultivation with inoculation of cells

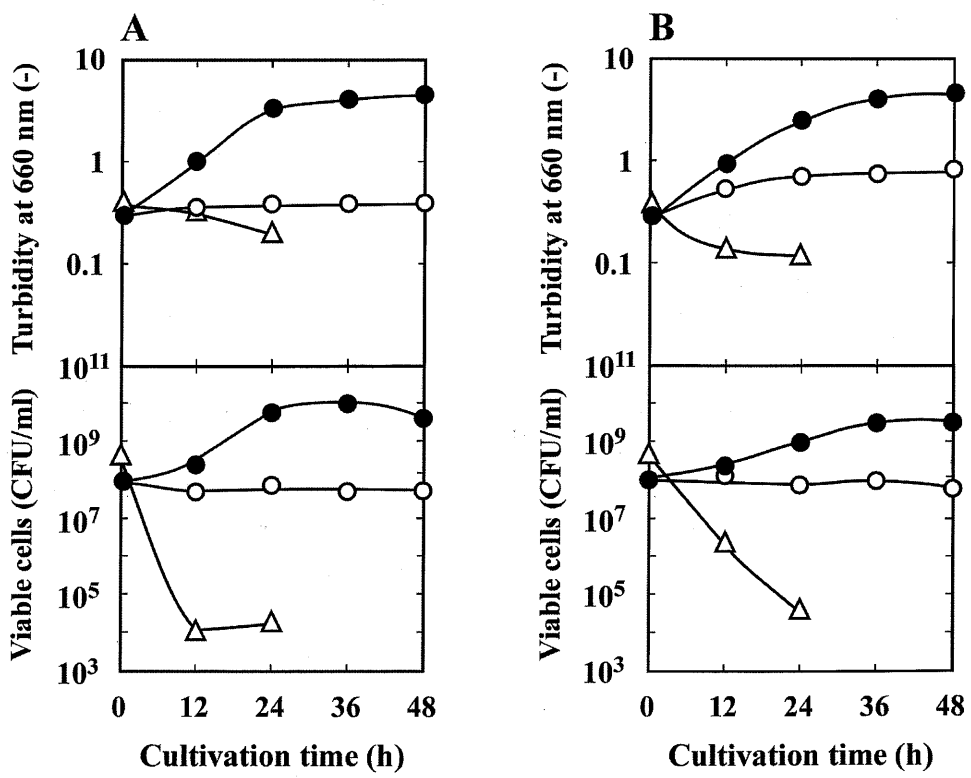

Fig. 4. Inhibitory effect of the bacteriocin from NPSI 38 and nisin A on the cell growth of *L. hilgardii* NBRC 15886^T at different growth phases. Precultured cells were inoculated into the fresh modified MRS medium with 11 U/ml of the bacteriocin from NPSI 38 (open circles) or 100 ng/ml of nisin (open triangles). Closed circles show the results for medium without the bacteriocin. The cell concentrations were determined on the basis of turbidity and CFU/ml. The cells at logarithmic growth phase (A) and stationary phase (B) were collected by centrifugation and then inoculated into the medium. The initial cell concentration was 8.0 ± 0.2×10^7 CFU/ml for cultivations with the bacteriocin from NPSI 38 and 5.6 ± 0.8×10^8 CFU/ml for cultivations with nisin A.

at stationary growth phase. The difference between viable cell concentrations and the turbidity is perhaps attributable to dead cells. On the other hand, when nisin A was added to the medium, the number of viable cells decreased more than four orders of magnitude within 24 h as compared with the initial value and a gradual decrease in turbidity was observed, irrespective of differences in growth phase of inoculated cells.

Figure 5 shows the effect of the initial cell concentrations on the growth-inhibitory activity of the bacteriocin from NPSI 38. To clarify the influence of cell concentration in the media containing the bacteriocin, the concentrations of cells at logarithmic growth phase used for inoculation were adjusted to 3.6×10^6 and 7.8×10^8 CFU/ml. When the initial cell concentration was 3.6×10^6 CFU/ml, the viable cell concentration decreased slightly with time although the turbidity was maintained at a constant level throughout the cultivation, as shown in Fig. 5A. The results of cultivation with the initial cell concentration of 7.8×10^7 CFU/ml were already shown in Fig. 4A. Negligible cell growth was observed in the medium containing the bacteriocin, as described above. In cultivation at the initial cell concentration of 7.8×10^8 CFU/ml, the cell growth in the early stage of the cultivation was retarded by the addition of bacteriocin, as compared with that in the cultivation without the bacteriocin, as shown in Fig. 5B. However, thereafter, the cell concentration increased gradually and then reached at the same level as that in the control cultivation without the bacteriocin. Almost the same result as the response described above was also obtained with respect to cells at stationary phase (data not shown).

Discussion

The bacteriocin solution for inhibiting growth of hiochibacterium could be prepared from culture broth produced by NPSI 38 cultivated in MRS medium (Fig. 2). However, MRS medium contains natural nitrogen sources such as Pp, ME, and YE and chemical reagents. Since several natural and chemical components in MRS medium are lawfully prohibited in the sake brewing process, we tried to use diluted koji extract as a basal medium for bacteriocin production by NPSI 38. When 10%(v/v) koji extract medium was used,

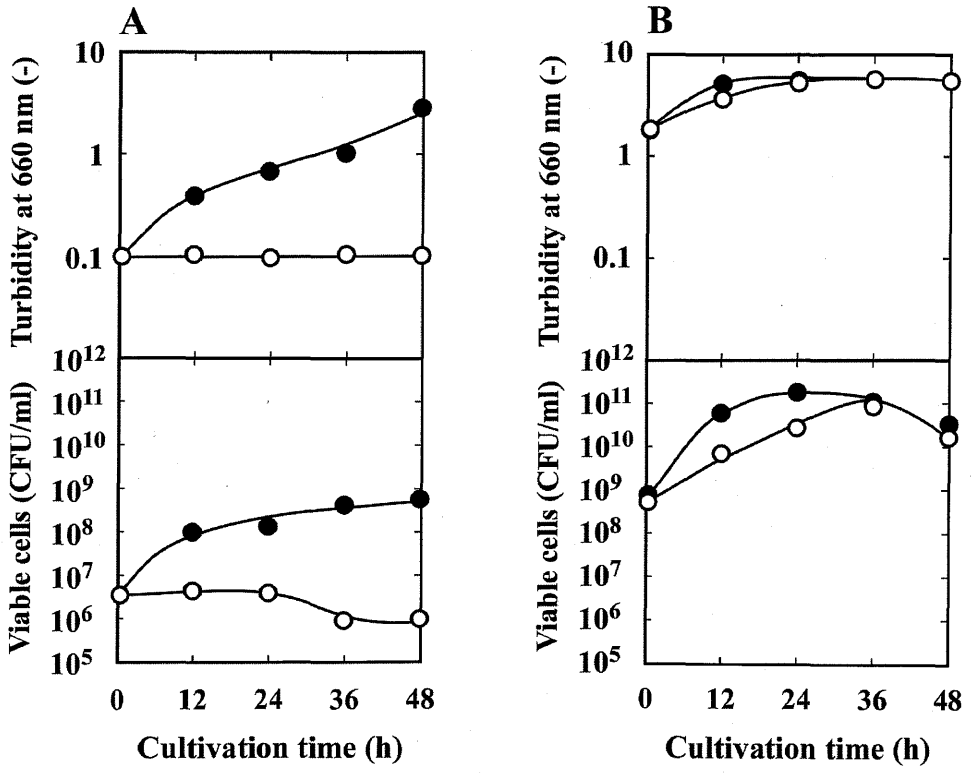

Fig. 5. Inhibitory effect of the bacteriocin from NPSI 38 on the growth of *L. hilgardii* NBRC 15886^T at different initial concentrations of cells at logarithmic growth phase. Turbidities and viable cell concentrations in culture broths without (closed symbols) and with (open symbols) 11 U/ml of the bacteriocin were determined. The initial viable cell concentrations were adjusted to 3.6×10^6 CFU/ml (A) and 7.8×10^8 CFU/ml (B).

NPSI 38 showed slight growth, but no production of bacteriocin was observed (Fig. 1). The effect of medium components on bacteriocin production by *Lactobacillus* strains was confirmed by Todorov and Dicks (2004, 2006) who showed that bacteriocin production was stimulated in the presence of tryptone or tryptone plus ME but that the addition of 5 or 10 g/l K_2HPO_4 resulted in a 50% reduction of bacteriocin production. Aasen *et al.* (2000) reported that increasing the concentrations of YE and/or tryptone had a positive influence on sakacin P production. In this study, we found that the addition of ME and Pp to the koji extract medium resulted in the enhanced production of the bacteriocin by NPSI 38, together with increasing the cell growth of NPSI 38. Based on the results on the stimulating effects of the nitrogen sources, we investigated an alternative additive to ME and Pp for supplementing koji extract medium. Of the protein-containing additives tested, two types of rice protein hydrolyzate were effective for bacteriocin production by NPSI 38, as well as the cell growth (Fig. 2). However, the reason for the difference in positive effects on bacteriocin production between both rice protein hydrolyzates is unclear. The identification of effective components (proteins and/or peptides) in the hydrolyzates for the bacteriocin production seems to be an interesting subject of future research. We are now investigating the stimulating effect of components fractionated from the hydrolyzates on bacteriocin production by NPSI 38. We also found that pH control was favorable for bacteriocin production by NPSI 38 in cultivations using both the koji extract medium supplemented with the hydrolyzate of rice protein (II). In cultivation with pH control, an amount of bacteriocin comparable to that produced in the cultivation using MRS medium could be obtained (Fig. 3).

In the experiments on the evaluation of the growth-inhibitory effect, the addition of pure nisin A caused a significant reduction of the number of viable cells (Fig. 4), suggesting the bacteriocidal effect of nisin A against *L. hilgardii* NBRC 15886^T. The bacteriocidal effect of nisin A against sensitive strains, such as *Lactobacillus*, *Micrococcus*, *Listeria*, and *Bacillus*, was previously reported (Ray, 1992, Delves-Broughton *et al.*, 1996, Cleveland *et al.*, 2001, Heng *et al.*, 2007). In contrast, when the bacteriocin produced by NPSI 38 was added to the medium with inoculation of *L. hilgardii* NBRC 15886^T, the initial viable cell concentrations were maintained at a constant value in the media containing bacteriocin (Fig. 4), indicating the probability that the action of bacteriocin produced by NPSI 38 is bacteriostatic. Since the bacteriostatic action caused no complete leakage of cell contents of hiochi-bacteria, the property of bacteriocin is considered to be favorable for maintaining the quality of sake. However, when the initial cell concentration of *L. hilgardii* NBRC 15886^T was high, recovery of the number of viable cells was observed, probably due to survival of a fraction of the hiochi-bacterial cells (Fig. 5B). The survival was assumed to be attributed to the lack of the molecular number of the bacteriocin required for the complete growth inhibition as well as decrease in the total bacteriocin activity by enzymatic digestion with proteases from *L. hilgardii* NBRC 15886^T.

Several bacteriocins have been reported to have a bacteriostatic effect on spoilage bacteria containing food-borne pathogens. Todorov and Dicks (2005) reported that a slight or no growth of *Lactobacillus casei* and *Pseudomonas aeruginosa* was observed after addition of the two bacteriocins produced by *Lactobacillus plantarum*. Sakacin P from *Lactobacillus sakei* (Katla *et al.*, 2002) and a bacteriocin from *Streptococous thermphilus* 81 (Ivanova *et al.*, 1998) were also shown to bacteriostatically inhibit the growth of *Listeria monocytogenes* and *Escherichia coli*, respectively. In the near future, considering the previous reports described above and after the bacteriocin produced by NPSI 38 is purified, it will be necessary to clarify the minimum bacteriocin concentration required for exhibiting complete bacteriostatic activity against several hiochi-bacteria containing *L. hilgardii* NBRC 15886^T. To evaluate the mode of action of the bacteriocin from NPSI 38, further studies on not only its interactions with the membrane of sensitive cells but also the leakage of intracellular ions, proteins and UV-absorbing materials from sensitive cells are necessary.

Conclusions

In the agar diffusion method using a stainless cup, the bacteriocin produced by NPSI 38 in MRS medium was found to be effective for inhibiting the growth of *L. hilgardii* NBRC 15886^T. In the cultivations using test tubes without pH control, ME and Pp were effective for the bacteriocin production by NPSI 38 using 10% (v/v) koji extract medium. When the hydrolyzate of rice protein (II) was supplemented with the koji medium, the concentration (68 U/ml) of bacteriocin produced was about 70% as high as that (98 U/ml) with MRS medium. By using the koji extract medium with the hydrolyzate of rice protein (B) in a fermentor with pH control, the amount (160 U/ml) of bacteriocin produced by NPSI 38 was 2.7-fold than that (60 U/ml) in the cultivation without pH control. When the cells of *L. hilgardii* NBRC 15886^T obtained at logarithmic growth and stationary phases were inoculated into fresh modified MRS medium with 11 U/ml of bacteriocin, the initial cell concentrations were maintained at a constant level, irrespective of different growth phases of the cells inoculated. The negligible cell growth in the media containing bacteriocin showed that the action of the bacteriocin produced by NPSI 38 was bacteriostatic

against the hiochi-bacterium. Since the bacteriostatic action caused no complete leakage of cell contents of *L. hilgardii* NBRC 15886^T, the property of bacteriocin is advantageous for maintaining quality of sake.

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