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Utilization of Xylose as an Alternative Carbon Source for Nisin Z Production by *Lactococcus lactis* IO-1

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Lactococcus lactis IO-1 was able to use xylose as a carbon source for nisin Z production, whose yield was superior to that made with glucose under the same fermentation conditions. Nisin Z production was affected by several environmental factors, such as pH, temperature, initial xylose concentration and the addition of a cation. In view of possible future use, these factors have to be considered for designing the production process. From this study, the optimal condition for nisin Z production was obtained with 4% xylose at pH 6.0 and 37°C. Addition of 0.1 M $CaCl_2$ increased nisin Z production specifically, but not cell growth and acid production, which resulted in the maximum nisin Z activity of about 1.5 times that without $CaCl_2$.

INTRODUCTION

As the world population continuously increases and new technologies are being developed, many natural resources are daily decreased. To prevent the problem of resource shortage, the effective utilization of natural resources should be studied. Reuse of waste has been introduced to human life. The main portion of waste generally comes from the agricultural field, and many researchers are trying to utilize it. Agricultural waste is composed of many kinds of polysaccharides, such as cellulose, hemicellulose and lignin. Xylose is one kind of sugar which is obtained after the hydrolysis of these waste products. Ishizaki *et al.* (1992b, 1993, 1995b) reported the utilization of xylose for L-lactate production by lactic acid bacteria. *Lactococcus lactis* IO-1, a lactic acid bacterium that was isolated in our laboratory, produces mainly L-lactic acid from many kinds of sugar, including xylose (Ishizaki and Ohta, 1989). Furthermore, *L. lactis* IO-1 was found to produce a peptide antibiotic which was identified as nisin Z, a natural nisin variant bacteriocin (Ishizaki *et al.*, 1990; Ishizaki *et al.*, 1992a; Matsusaki *et al.*, 1996b). Nisin was approved as a food preservative (Delves-Brounghton, 1990) because of its high effectiveness against several strains of Gram-positive food pathogen such as *Bacillus* (de Vuyst and Vandamme, 1994), *Clostridium* (de Vuyst and Vandamme, 1994), and *Listeria* (Winkowski *et al.*, 1994; Benkerroum and Sandine, 1988).

Recently the influence of several parameters on nisin Z production from glucose fermentation was reported focussing on the enhancement of nisin Z productivity, as a result, a maximal nisin Z production was obtained from 4% glucose fermentation supplemented with 0.1 M $CaCl_2$ (Matsusaki *et al.*, 1996a). The major effect of calcium ion

on nisin-producing cells was reported on nisin maturation and immunity. Calcium ion was believed to play a role in the integrity of the lipid membrane of the producing strain (de Vuyst and Vandamme, 1993). Uptake of calcium ion was linked to nisin inactivation and thus suggested that nisin was bound to nisin-producing cells in the form of a calcium complex (de Vuyst and Vandamme, 1994). Calcium ion binding sites are probably present in Nis P peptidase, which cleaves the precursor nisin, followed by the liberation of mature nisin (Matsusaki *et al.*, 1996a).

Natural rubber serum powder (NRSP) is the spray-dried product of the serum obtained during the process of separating natural rubber latex. Because the serum containing many kinds of amino acids, peptides, inorganic salts, etc. causes environmental pollution with its putrid and foul smell, the treatment of the serum has become a serious problem in natural rubber-producing countries. In our laboratory, it has been found that NRSP can be used as a nutritional source for many kinds of microorganisms. Tripetchkul *et al.* (1992) reported the use of NRSP as a nutritional source in ethanol production by *Zymomonas mobilis*. They stated that the yeast extract in the medium can be replaced by NRSP and mieki (soybean protein hydrolysate). Ishizaki (1995a) and Oiki *et al.* (1996) reported the growth-promoting effect of NRSP on bifidobacterium, consequently with enhancing metabolite production and yields. Recently lactic acid fermentation with xylose by *L. lactis* IO-1 was stimulated by NRSP (unpublished data).

For the efficient utilization of natural resources and as an alternative trial for agricultural waste treatment, xylose and NRSP are expected to be potentially an alternative carbon source and growth promoter for nisin Z production, respectively. Until now, no information on bacteriocin production from agricultural waste or its hydrolysate has been reported. The objective of this study is to optimize conditions for nisin Z production using xylose as a carbon source and to determine the effect of additives such as NRSP and calcium chloride.

MATERIALS AND METHODS

Microorganism and media

Lactococcus lactis IO-1 (JCM 7638) was maintained in 30% glycerol at -80 °C. The culture was refreshed in 10 ml TGC medium without glucose (Difco Laboratories, Detroit, MI, USA) at 37 °C for 18 h and was propagated in CM medium which consisted of 0.5% polypeptone (Nihon Seiyaku Co., Ltd., Tokyo, Japan), 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA) and 0.5% NaCl. Xylose was added in preculture medium in the amount of 1% w/v. Preculture was incubated at 37 °C for 3 h as an inoculum of the main culture.

Fermentation in reciprocated-flask

Experiments were performed in 300-ml Erlenmeyer flasks with 100-ml working volume. Calcium carbonate was added to the CM medium containing the indicated sugar about half the sugar concentration to prevent rapid decrease in pH. The inoculated volume was 5%. The main culture was incubated at a speed of 100 strokes/min.

pH-controlled fermentation

A 1-l fermenter containing 300 ml CM medium was added with 4% xylose. The fermenter was operated at 37 °C with an agitation speed of 400 rpm. The pH of the fermentation broth was maintained with 3 N NaOH.

Analytical methods

The absorption of light at 562 nm was measured and was converted to dry cell weight (DCW). Xylose, lactate and acetate were analyzed by Shimadzu high-performance liquid chromatography (HPLC) with Aminex ion-exclusion column HPX-87H (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Nisin Z was determined by reverse-phase HPLC with Shodex Asahipak ODP 50-6E column (Showa Denko Co., Ltd., Tokyo, Japan) (Matsusaki *et al.*, 1996b). One unit of nisin Z activity is defined as an arbitary unit (AU) of activity that is equivalent to the activity of 1 μg of commercial nisin (ICN Biomedicals, Inc., Costa Mesa, CA, USA; activity, 1000 IU/mg-solid; nisin content, 2.5%). Thus one AU is equivalent to one IU.

RESULTS

The possibility of producing nisin Z from xylose

L. lactis IO-1 grew well at 37 °C (Ishizaki *et al.*, 1990), and the highest nisin Z was produced at 30 °C when glucose was used as the carbon source (Matsusaki *et al.*, 1996b). To study the possibility of nisin Z production from xylose, *L. lactis* IO-1 was grown in 300-ml Erlenmeyer flask containing 100 ml CM medium, 1% xylose and 0.5% $CaCO_3$. The cultures were incubated at 30 °C and 37 °C at a speed of 100 strokes/min. Nisin Z was first detected early during the exponential growth phase and showed the maximum level during the mid-exponential phase, which indicates that bacteriocin displays primary metabolite kinetics (de Vuyst and Vandamme, 1994). Maximal nisin Z activity and yields of nisin Z and lactate production in each xylose fermentation were compared with those in glucose fermentation which was incubated at 30 °C (Table 1). Using xylose as a carbon

Table 1. Comparison of maximum nisin Z activity and yields of nisin Z and lactate production under different conditions

Condition	Maximum nisin Z (AU/ml)	Nisin Z yield ($\times 10^5$AU/g-sugar)	($\times 10^6$AU/g-DCW)	Lactate yield (g/g-sugar)	(g/g-DCW)
Glucose 1% at 30 °C	737	0.750	0.860	0.960	13.4
Xylose 1% at 30 °C	810	0.930	1.11	0.140	1.67
Xylose 1% at 37 °C	744	1.13	1.13	0.120	1.63

DCW, dry cell weight

source, nisin Z was produced at both incubating temperatures. In 1% glucose fermentation, *L. lactis* IO-1 grew faster than in 1% xylose fermentation. The specific growth rate of IO-1 cells in glucose fermentation was about 0.67 h^{-1}, whereas that in xylose fermentation was about 0.10 h^{-1} at both temperatures. The maximal amounts of nisin Z were obtained at 12 h in glucose fermentation at 30 °C, and at 15 h and 18 h in xylose fermentation at 37 °C and 30 °C, respectively. It is interesting that lactate yields from xylose fermentations were much lower than those in glucose fermentation. This should be an advantage for nisin Z production from xylose fermentation, as lactate inhibits the growth of the producer strain (Ishizaki *et al.*, 1993). The rate of xylose consumption was lower than that of glucose consumption, which resulted in higher nisin Z yield based on carbon source consumption.

Effect of initial xylose concentration on nisin Z production

In order to prolong the exponential phase which might result in the increase in cell density and concomitantly nisin production, flask-based cultivations were carried out at 37 °C with increasing initial xylose concentration of 10 to 70 g/*l*. Calcium carbonate was added to each flask at half the xylose concentration. Cell growth, lactate and nisin Z production after 24 h of incubation are shown in Fig. 1. Lactate produced increased with an increase in xylose concentration. The cell formation gradually decreased when xylose concentration was increased higher than 20 g/*l*. On the other hand, nisin Z activity increased with increasing xylose concentration over the range of 10 to 40 g/*l*. The highest value was achieved with 40 g/*l* xylose in the amount of 942 AU/ml. Above 40 g/*l* of xylose, growth and nisin Z production were limited by low pH, although xylose remained sufficiently available.

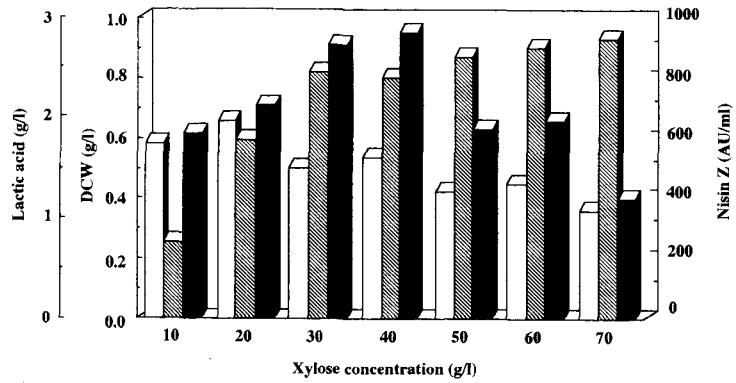

Fig. 1. Cell formation, lactate formation and nisin Z production of *Lactococcus lactis* IO-1 with different xylose concentrations. The cultivation was carried out at 37 °C and 100 strokes/min for 24 h.

Symbols: Cell growth (□), lactate (▨), nisin Z (■)

Effect of incubating temperature

Bacteriocin production was influenced by several environmental parameters of the fermentation, including pH and temperature (de Vuyst and Vandamme, 1994; Biswas *et al.*, 1991; Parente *et al.*, 1994). To study the effect of the temperature, *L. lactis* IO-1 was cultivated in 300-ml Erlenmeyer flasks containing 40 g/l xylose at different temperatures. Cell growth, lactate and nisin Z production after 24 h of incubation are shown in Fig. 2. The growth was not good at low temperature (20 and 25 °C) and at 40 °C. The trends of lactate and nisin Z production corresponded with cell growth. No nisin Z was produced at 20 °C. The highest nisin Z activity was detected at 37 °C at 988 AU/ml. The optimal temperature for nisin and biomass production was 37 °C. From these results, nisin Z was proportionally related to biomass, as de Vuyst and Vandamme (1992) stated that achieving a high biomass may be a prerequisite for high nisin production.

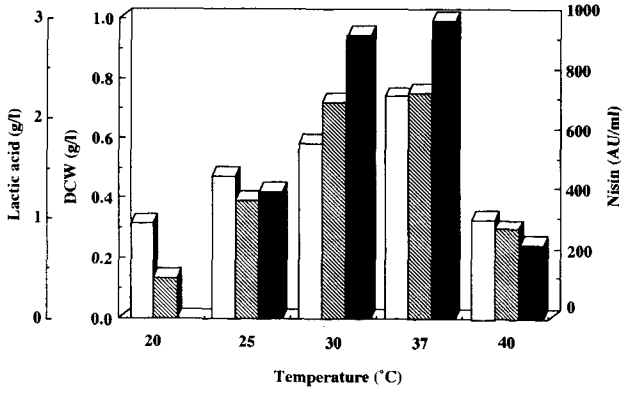

Fig. 2. Effect of temperature on growth, lactate production and nisin Z production of *Lactococcus lactis* IO-1. The cultivation was performed at 100 strokes/min with 4% xylose and 2% $CaCO_3$. Samples were withdrawn after 24 h of incubation.

Symbols: Cell growth (□), lactate (▨), nisin Z (■)

Effect of natural rubber serum powder

There were some reports on the enhancing effect of natural rubber serum powder (NRSP) on the production of ethanol and the growth of bifidobacterium (Tripetchkul *et al.*, 1992; Ishizaki, 1995a; Oiki *et al.*, 1996). NRSP enhanced not only the growth of bifidobacterium, but also the metabolite production and its yield. NRSP was examined to learn whether this powder promotes nisin Z production. As shown in Fig. 3a, the growth of IO-1 cells with 1.5% NRSP was greater than that without NRSP. Nisin Z production is shown in Fig. 3b. When 1.5% NRSP was added, nisin Z production was not affected, whereas lactate production was increased about 3 times that of the control (Fig. 3c). The lactate formation rate increased from 0.12 $g \cdot \ell^{-1} \cdot h^{-1}$ without NRSP to 0.31 $g \cdot \ell^{-1} \cdot h^{-1}$ with

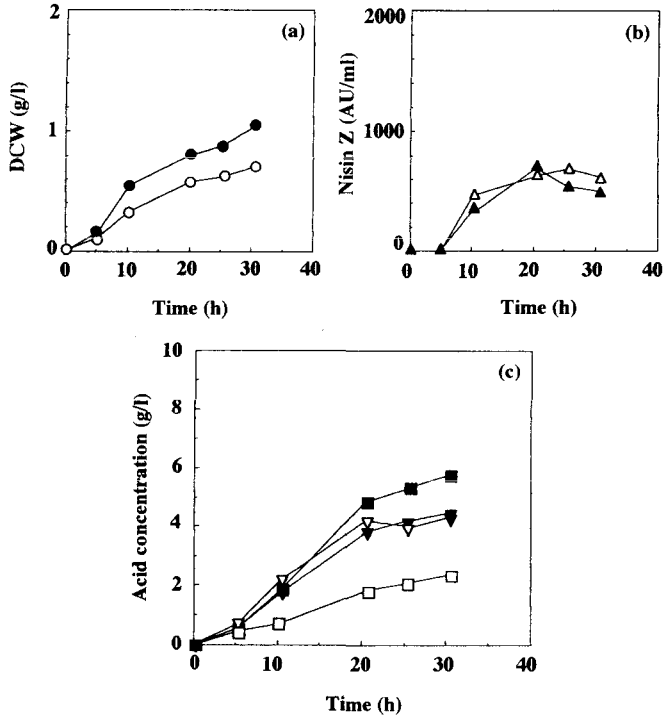

Fig. 3. Effect of NRSP on cell mass (a), nisin Z production (b), and lactate and acetate production (c). The cultivation was performed at 37 °C and 100 strokes/min with 4% xylose and 2% $CaCO_3$.

Symbols: Control (open symbols), with 1.5% NRSP (closed symbols)
Cell growth (●,○), nisin Z (▲,△), lactate (■,□), acetate (▼,▽)

1.5% NRSP. To confirm the effect of NRSP on nisin Z production, pH-controlled fermentation was carried out in the presence of 1.5% NRSP. NRSP affected only the stimulation of lactate production, whereas cell growth, acetic acid and nisin Z production were not affected (Fig. 4).

Effect of cation

From the previous research (Matsusaki *et al.*, 1996a) the production of nisin Z from glucose was increased when $CaCl_2$ was added. The effect of calcium ion and other cations on nisin Z production with xylose was studied. The cultures were cultivated with 0.1 M of $CaCl_2$, $MgSO_4$, and $MnCl_2$. To maintain pH instead of $CaCO_3$, 0.2 M 3-(*N*-morpholino) propanesulfonic acid (MOPS) was used as a buffer. The results are shown in Fig. 5. No nisin was produced from the fermentation with 0.1 M $MnCl_2$. Nisin Z production was increased 11% and 40% with 0.1 M $MgSO_4$ and 0.1 M $CaCl_2$, respectively. In flask-based fermentation with MOPS, the cell growth and nisin Z production were lower than those with $CaCO_3$. Application of 50 mM potassium phosphate buffer instead of MOPS also resulted in low growth rate and led to low nisin Z production.

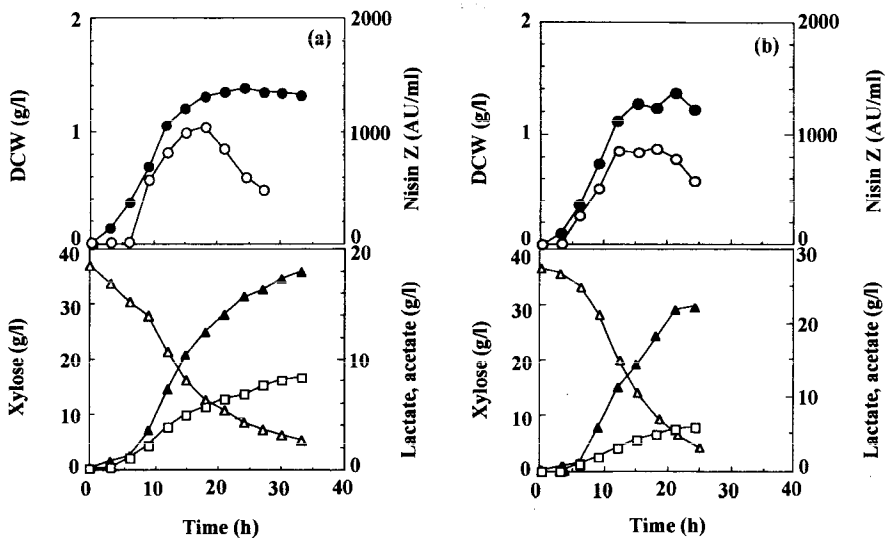

Fig. 4. Effect of NRSP on nisin Z production in pH-controlled fermentation. The cultivation was carried out at 37°C and pH 6.0 with 4% xylose and without NRSP (a) and with 1.5% NRSP (b)

Symbols: Cell growth (●), nisin Z (○), lactate (▲), acetate (□), xylose (△)

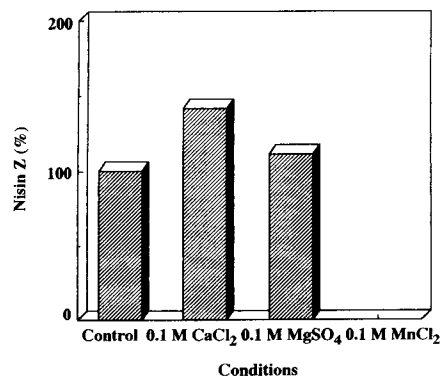

Fig. 5. Effect of cation on nisin Z production by *Lactococcus lactis* IO-1. The cultivation was performed at 37°C and 100 strokes/min for 24 h with 4% xylose and 0.2 M MOPS. Nisin Z production in control, that is without any cation, was expreesd as 100%.

Effect of pH in pH-controlled fermentation

To determine the optimal conditions for bacteriocin production, pH is one of the important factors (Biswas *et al.*, 1991; Parente *et al.*, 1994). The cultures were grown in

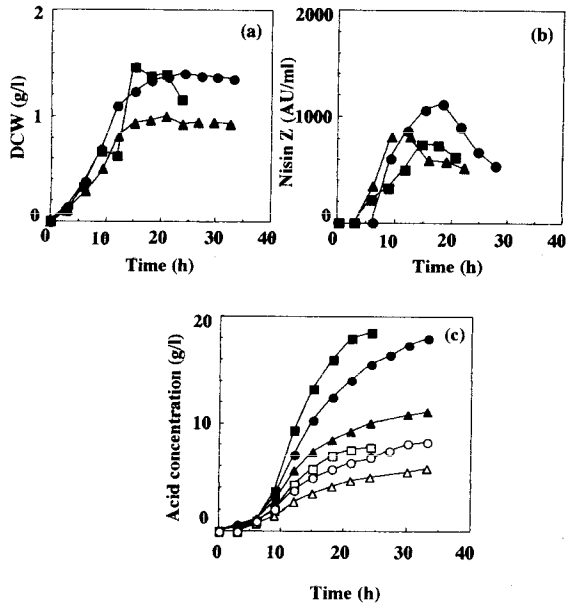

Fig. 6. Effect of pH on cell growth (a), nisin Z production (b), and lactate and acetate production (c) of *Lactococcus lactis* IO-1. The cultivation was carried out in 1-L jar fermenter with 4% xylose at 37 °C. The indicated pH was maintained with 3 N NaOH.

Symbols: pH 5.5 (▲), pH 6.0 (●), pH 6.5 (■)
In Fig. 6c, acetate (open symbols), lactate (closed symbols)

a pH-controlled fermenter at pH 5.5, 6.0, and 6.5 (Fig. 6). Cell formation at pH 5.5 was lower than that at pH 6.0 and 6.5. Consequently, lactate production and nisin biosynthesis were poor at pH 5.5. Lactate was rapidly produced with pH controlled at 6.5, while the highest nisin production was achieved from the fermentation with pH controlled at 6.0. The optimal pH of nisin production with xylose (pH 6.0) differred from that with glucose which was reported at pH 5.5 by Matsusaki *et al.* (1996a). Also, pH 6.5 was the optimal pH for lactate production, which differred from the value (pH 6.0) with glucose reported by Ishizaki and Ohta (1989). Biomass and lactate were increased when the fermentation was changed from flask culture to pH-controlled fermentation. The rate of nisin biosynthesis was faster in the pH-controlled fermentation than in the reciprocated-flask fermentation, but the highest activity obtained was not altered.

Effect of $CaCl_2$ concentration

Because nisin Z production was increased when adding 0.1 M $CaCl_2$ to the flask fermentation as previously described, the optimal concentration of $CaCl_2$ studied was varied from 0.1 M to 0.3 M. As a result, a high concentration of $CaCl_2$ decreased the growth of *L. lactis* IO-1, thus, low amount of nisin Z was detected. Because the buffer capacity of MOPS was less than that of $CaCO_3$, the experimental system was then carried

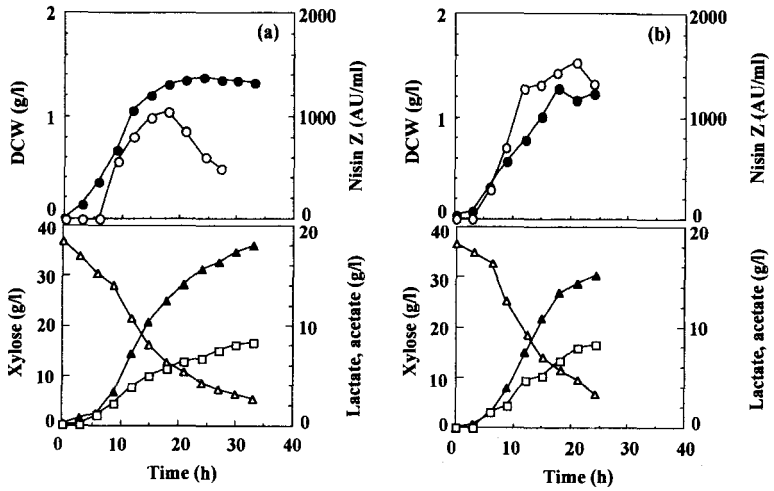

Fig. 7. Effect of $CaCl_2$ on nisin Z production in pH-controlled fermentation. The cultivation was carried out at 37°C and pH 6.0 with 4% xylose without $CaCl_2$ (a) and with 0.1 M $CaCl_2$ (b).

Symbols: Cell growth (●), nisin Z (○), xylose (△), lactate (▲), acetate (□)

out in pH-controlled batch fermentation. The results of the fermentation with and without 0.1 M $CaCl_2$ are shown in Fig. 7. The profiles of cell growth and lactate production of the fermentation with 0.1 M $CaCl_2$ were nearly the same as those without $CaCl_2$. Specific nisin Z production rate was changed from 0.16 AU·ℓ^{-1}·h^{-1} without $CaCl_2$ to 0.28 AU·ℓ^{-1}·h^{-1} with 0.1 M $CaCl_2$. The maximum nisin Z activity with $CaCl_2$ was about 1.5 times greater than without $CaCl_2$, which reached 1520 AU/ml after 20 h of incubation.

DISCUSSION

Although the factors affecting the production of nisin have been studied (de Vuyst and Vandamme, 1992; Matsusaki *et al.*, 1996a), there were no reports about xylose utilization as a carbon source. In order to make more profit on natural resources, the production of nisin Z from xylose was studied. From the results, nisin Z could be produced in xylose fermentation, as in the case of glucose fermentation (Table 1). Nisin Z is like other bacteriocin from lactic acid bacteria, which was proved to be a primary metabolite judging from the profiles of cell growth and nisin Z poduction. It is clear that nisin Z was produced during the exponential cell growth phase and reached a maximum at the end of this phase or at the beginning of the stationary phase. Although maximal nisin Z did not correspond with maximal biomass, a critical amount of biomass seems to be a very important parameter (de Vuyst and Vandamme, 1994). Thus low specific growth rate in xylose fermentation led to low nisin Z production.

In order to prolong the exponential growth phase, the initial xylose concentration was increased. The produced lactate increased with an increase in initial xylose

concentration, whereas nisin Z production decreased at a xylose concentration higher than 40 g/l (Fig. 1). This might be responsible for the difference in the biosynthesis of these two metabolic products. Thus the biosynthesis of nisin Z is complicated much more than that of lactate, which is a well-known primary metabolite.

Nisin possesses an amphiphilic character with hydrophobic residues at the N-terminus and hydrophilic ones at the C-terminus (de Vuyst and Vandamme, 1992). Yang *et al.* (1992) stated that, in general, bacteriocins were adsorbed on the producing cell at a pH near 6.0 and the desorption took place at pH 1.5 to 2.0. The maximum and minimum adsorption with respect to pH slightly varied among the bacteriocins. Maximum adsorption of nisin to the producer occured at pH 6.5, and complete loss of adsorption was found at pH 3.0 and below. In the case of xylose fermentation, pH controlled at 6.0 showed the best nisin Z production (Fig. 6). Because nisin Z is in the class of lantibiotics which are posttranslationally modified peptides, there are many enzymes involved in transcription, translation, maturation, and transportation out of the producing cells. These enzyme activities may be affected by the pH of the cultivation broth.

Furthermore, addition of cation may also affect the nisin Z formation process. As a result, the Ca^{2+} ion (0.1 M) promoted nisin Z production specifically, but not cell growth and acid production (Fig. 7). A higher than 0.1 M concentration of calcium ion repressed the cell growth and led to low nisin Z production. Calcium ion may be involved in the nisin maturation process and inactivation of nisin by the producing cells (Delves-Broughton, 1990; de Vuyst and Vandamme, 1993; Matsusaki *et al.*, 1996a). The growth of *L. lactis* IO-1 was repressed by 50 mM of potassium phosphate, whereas de Vuyst and Vandamme (1993) reported that inorganic phosphate stimulated both cell growth and nisin production.

Some reports stated the stimulating effect of NRSP on the growth and metabolite production of several microorganisms (Tripetchkul *et al.*, 1992; Ishizaki, 1995a; Oiki *et al.*, 1996). In this study, however, NRSP stimulated lactate production, but not production of nisin Z (Figs. 3 and 4). This might be also responsible for the complex expression system for nisin Z compared to lactate as a well-known primary metabolite.

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