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Biosynthetic production of nisin Z by immobilized *Lactococcus lactis* IO-1

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Abstract

Nisin Z, a natural nisin variant, is a lantibiotic that contains unusual amino acids. The immobilization technique was applied to improve the productivity of nisin Z, the peptide antibiotic produced by *Lactococcus lactis* IO-1. Both entrapment and adsorption methods of immobilization were studied with several kinds of materials in repeated fermentation. The long-term application of natural materials was limited because of the instability of the gel. Low growth rate and low productivity were obtained from the entrapment method due to the improper diffusion of nutrients through the materials. Moreover, the method to entrap the cells with photo-crosslinkable resin prepolymers caused damage to the cells. The adsorption method showed better results compared with the entrapment method. The adsorption of the cells on porous chitosan beads, Chitopearl SH-2510, gave nisin Z productivity about 1.7 times greater than the free cells. However, during the repeated cultivation, a decrease in nisin Z activity was observed. The cells adsorbed on photo-crosslinked resin gel beads, ENTG-3800, produced a greater amount of nisin Z and a lower level of lactic acid than the free cells with a good operational stability. The nisin Z production tended to increase with repeated cultivation. Continuous fermentation was introduced to improve the nisin Z productivity. Free cells showed a good productivity at the dilution rate of 0.1 h^{-1}. However, nisin Z production was affected by cell washout at the dilution rate of 0.2 h^{-1}. The cells adsorbed on ENTG-3800 gel beads displayed an improvement in productivity at higher dilution rates.

Keywords: Lantibiotic; Nisin Z; *Lactococcus lactis*; Immobilization; Continuous fermentation; Photo-crosslinked resin gel beads

1. Introduction

Lantibiotics are class I bacteriocins, which contain unusual amino acids such as dehydroalanine (Da), dehydrobutyrine (Db), lanthionine, and 3-methyllanthionine [1]. Nisin, or more precisely nisin A, is a lantibiotic that is produced by *Lactococcus lactis* [2,3]. In view of its strong activity against food pathogens, nisin A has been approved by the World Health Organization as a food preservative for use in the food industry [4]. Nisin is synthesized on ribosomes as a precursor peptide of 57 amino acids. It is posttranslationally converted to the biologically active peptide (34 amino acids) through enzymatic modifications [5,6]. Firstly, some of serine and threonine residues in the prepeptide are dehydrated to Da and Db, respectively. The

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double bonds of some of the Da and Db residues react with the thiol group of a neighboring cysteine residue to form the respective thioether rings of lanthionine and 3-methyllanthionine. Finally, the active nisin is released and secreted by proteolytic cleavage of the leader peptide (23 amino acids). However, the mechanism of the biosynthesis has not been satisfactorily characterized, especially formation of the unusual amino acids and the regio- and stereo-specificities of the modification enzymes. According to the biosynthetic peculiarity, fermentation method is very preferable for practical production of nisin to enzymatic method with integration of multi-enzyme steps.

L. lactis IO-1 (JCM 7638) [7] isolated in our laboratory produces a peptide antibiotic, which was purified and identified to be nisin Z, a natural nisin variant [8]. Nisin Z differs from nisin A by a single amino acid substitution (His27Asn). Because nisin A is hardly soluble at neutral pH, its use as a preservative is limited at present. Nisin Z is more soluble than nisin A above pH 6 because the asparagine side chain is more polar than the histidine side chain [9]. We have reported the characterization of pH and heat stability, sensitivity to proteolytic enzymes and antimicrobial spectrum of nisin Z [10]. The results indicated nearly the same characteristics as those of nisin A. Therefore, nisin Z should be used rather than nisin A as a food preservative. We also investigated the relationship between the primary metabolite production of nisin Z and lactic acid and cell growth, which had so far not been clarified in detail. This study resulted in a threefold yield of nisin Z under optimal conditions [11]. In our previous paper [12], xylose as a carbon source was found to be utilized by *L. lactis* IO-1 for the efficient production of lactic acid as well as nisin Z.

Recently, immobilization of lactic acid bacteria for the production of nisin was studied [13–15], which is expected to stabilize the above-mentioned complex system for de novo synthesis of nisin and to improve the productivity as a result of the confinement of high cell density in the reactor. However, their results are unsatisfactory for high yield and productivity of nisin and long-term operational stability of the bioreactors from the standpoint of the potent commercial application. This might be partly due to the use of alginate as a natural and conventional cell-immobilizing material. Continuous attempts have been made to find new materials and techniques for the cell immobilization that avoid unfavorable effects such as support instability, limitation of nutrient transfer, and sloughing of the excess cells from the supports.

In the present paper, the production of nisin Z was studied with *L. lactis* IO-1 immobilized with various kinds of materials in both the entrapment and adsorption methods in order to select the most suitable material and procedure. Continuous production of nisin Z was also carried out by the cells immobilized with the selected support.

2. Experimental

2.1. Natural materials for cell immobilization

Agar and sodium alginate were obtained from Nacalai Tesque (Kyoto, Japan), agarose type VI was purchased from Sigma (St. Louis, MS, USA) and κ-carrageenan was from Tanabe Seiyaku (Osaka, Japan).

2.2. Synthetic and chemically modified materials for cell immobilization

Urethane prepolymers (PU-3 and PU-6) were products of Toyo Tire and Rubber (Osaka, Japan). These prepolymers are synthesized from toluene diisocyanate and polyether diol moiety (chain length, ca. 25–26 nm) that are composed of polyethylene glycol and polypropylene glycol [16]. PU-3 is hydrophobic because of high polypropylene glycol content (43%), and PU-6 is hydrophilic (polypropylene glycol, 9%).

Photo-crosslinkable resin prepolymers and photo-crosslinked resin beads were synthesized

Table 1
Properties of photo-crosslinkable resin prepolymer

Prepolymer	Main chain	Chain length (nm)	Property
ENT-3400	Polyethylene glycol	34	Hydrophilic
ENTG-3800	Polyethylene glycol mixed with polypropylene glycol (4:1 w/w)	38	Hydrophilic
ENTP-4000	Polypropylene glycol	40	Hydrophobic

by Kansai Paint (Tokyo, Japan). The photo-crosslinkable resin prepolymers [16,17] used in this experiment are shown in Table 1.

Photo-crosslinked resin beads (ENT-2000, VGE-112 and ENTG-3800) were prepared as follows [18]. For bead forming, 500 parts by weight of 3% sodium alginate solution was mixed with 100 parts by weight of each photo-crosslinkable resin prepolymer solution (40% w/v), 3 parts by weight of a photo-initiator, and 100 parts by weight of deionized water. Gel beads were then formed by dropping the mixture into 2% $CaCl_2$ solution. VGE-112 beads were prepared using the prepolymer (chain length, ca. 25 nm) whose main chains were polyethylene glycol, polyvinyl alcohol and polypropylene glycol (39:4:7 w/w). The beads thus formed were irradiated for 5 min by near-ultraviolet light at 300–400 nm. After the irradiation, the beads were washed twice with tap water. The beads with a diameter of ca. 4 mm showed a compressive strength of 15–25 kg/cm^2. Their hydrophilicity decreases in order of ENT-2000, VGE-112, and ENTG-3800.

Porous chitosan beads (Chitopearl) [19] were gifts from Fuji Spinning (Tokyo, Japan) (Table 2).

2.3. Preparation of cell suspension

L. lactis IO-1 stock culture was subcultured into 10 ml of thioglycolate medium without glucose (Difco Laboratories, Detroit, MI, USA). Static incubation was done at 37°C for 18 h before the culture was added into 100 ml of CM medium supplemented with 1% glucose for preculture. The CM medium contained 0.5% yeast extract (Difco Laboratories), 0.5% polypeptone (Nihon Seiyaku, Tokyo, Japan) and 0.5% NaCl in distilled water at pH 7.0. The culture was incubated at 30°C and 320 rpm for 3 h. The seed culture then obtained was centrifuged at $17{,}800 \times g$ for 15 min. The cell pellet was washed twice and resuspended with 0.85% NaCl solution to obtain 6.72 g-dry cell weight (DCW)/l of cell suspension. The resulting cell suspension was used for cell-entrapment as described below. Cell immobilization was aseptically performed in all the methods.

2.4. Cell-entrapment method

2.4.1. Calcium alginate

Six milliliters of sodium alginate solution (0.03 g/ml) was autoclaved at 110°C for 10 min and was kept at 45°C until used. The cell suspension (1.25 ml) previously described was added to the sterile sodium alginate solution.

Table 2
Properties of porous chitosan beads

Chitopearl	Functional group	Binding mode	Particle size (mm)	Pore size (μm)
SH-2503	Primary, secondary and quaternary amines	Ionic	0.3	5
SH-2510			1.0	5
HP-2520			2.0	50
HP-3020	Primary amine and linear alkyl	Hydrophobic and ionic	2.0	50
SH-3503	Primary amine and aromatic alkyl	Hydrophobic	0.3	5
SH-3510			1.0	5
HP-3520			2.0	50
HP-5020	Chitin-like structure	Hydrophobic and ionic	2.0	50

The mixture was gently mixed and quickly dropped into 50 ml of 0.1 M $CaCl_2$ solution with a No.18 syringe (Terumo, Tokyo, Japan). The spherical beads (diameter, ca. 3.2–3.5 mm) were obtained and kept in $CaCl_2$ solution for 3 h at room temperature.

2.4.2. κ-Carrageenan

κ-Carrageenan solution (0.07 g/ml) of 2.5 ml was autoclaved at 110°C for 10 min. The solution was quickly mixed with 1.25 ml of cell suspension in a 50-ml beaker at 45°C. The mixture was left until complete gel formation at room temperature, and then the gel was soaked with sterile 0.3 M KCl solution at room temperature for 30 min. The gel was aseptically cut into small pieces (each ca. $5 \times 5 \times 2$ mm).

2.4.3. Agar and agarose

Five milliliters of agar or agarose solution at a concentration of 0.03 g/ml was autoclaved at 110°C for 10 min. The cell suspension (1.25 ml) was added into the solution kept in a 50-ml beaker at 45°C. The mixtures were gently mixed and were left at room temperature until the gel formation was completed. The gels were aseptically cut into pieces, which have the same size as the κ-carrageenan-immobilized cells.

2.4.4. Urethane prepolymer

Two kinds of urethane prepolymers (PU-3 and PU-6), 0.625 g, each sterilized in an oven at 160°C for 10 min were quickly mixed with 1.25 ml of cell suspension. The mixtures stood at 4°C for 30 min. The gels formed were aseptically cut into small pieces of ca. $5 \times 5 \times 5$ mm.

2.4.5. Photo-crosslinkable resin prepolymer

Each of the photo-crosslinkable resin prepolymers in the amount of 2.5 g was autoclaved at 110°C for 10 min. The prepolymers were mixed with 0.02 g of benzoin isobutyl ether as a photosensitizer, 1.25 ml of cell suspension and 0.24 ml of 0.85% NaCl solution. The mixtures were spread on the transparent glass plate and illuminated with near-ultraviolet light over the wavelength range 300–400 nm, maximum intensity at 360 nm, for 3 min, as previously described [16,18]. Immobilization with ENTP-4000 was performed in the same manner, except benzoin isobutyl ether and 0.85% NaCl solution were added in the amount of 0.05 g and 0.05 ml, respectively. The resin film formed (thickness, ca. 1 mm) was aseptically cut into small pieces (each ca. 5×5 mm) and washed with sterile 0.85% NaCl solution.

2.5. Cell-adsorption method

Five milliliters of porous chitosan beads or photo-crosslinked resin gel beads was autoclaved in distilled water at 110°C for 10 min before addition to 100 ml of CM medium supplemented with 4% glucose and 2% $CaCO_3$. Five percent of the seed culture was then added and the culture was incubated at 30°C and 120 strokes/min for 16 h. The cell-adsorbed gel beads obtained were washed with sterile distilled water before use as immobilized cells.

2.6. Repeated batch cultivation

2.6.1. Free cells

Five milliliters of the seed culture was inoculated to 100 ml of CM medium supplemented with 40 g/l of glucose and 20 g/l of $CaCO_3$. The culture was incubated at 30°C with shaking at 100 strokes/min. Five milliliters of the culture was withdrawn and transferred to a fresh medium every 24 h.

2.6.2. Entrapped cells

Each type of immobilized cell was inoculated into 100 ml of CM medium supplemented with 50 g/l of glucose and 25 g/l of $CaCO_3$. The culture was incubated at 30°C and 100 strokes/min. After incubation for 24 h, the immobilized cells were washed with sterile 0.85% NaCl solution and were employed in the 10 successive batches of cultivation.

2.6.3. Adsorbed cells

Each type of immobilized bead was inoculated into 100 ml of CM medium supplemented

with 40 g/l glucose and 20 g/l $CaCO_3$. The cultures were incubated at 30°C and 100 strokes/min. Repeated batch cultivation was performed as in the case of the entrapped cells.

2.7. Continuous fermentation of the cells adsorbed on ENTG-3800

Five milliliters of the seed culture and 100 ml of ENTG-3800 gel beads were added to 100 ml of CM medium containing 4% glucose and 2% $CaCO_3$ for cell adsorption. The culture was incubated at 30°C and 100 strokes/min for 24 h. The immobilized cells obtained were further cultivated in a fresh medium for 24 h and then washed with sterile 0.85% NaCl solution. The cell-adsorbed beads were packed into a sterile glass column (40 mm id × 200 mm, working volume 80 ml). CM medium containing 3% glucose and 0.1 M $CaCl_2$ was circulated through the column at a speed of 16 ml/min. The effluent was returned back to a 500-ml Erlenmeyer flask for controlling the pH at 5.5 with 5 N NaOH (working volume, 220 ml). The temperature was controlled at 30°C. Continuous fermentation was started at a dilution rate of 0.1 h^{-1} after the cultivation for 12 h. The schematic diagram of this system is shown in Fig. 1.

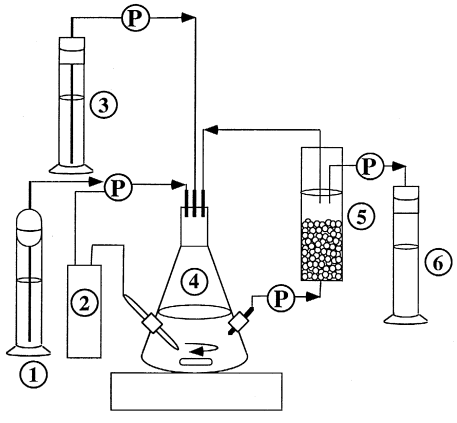

Fig. 1. Schematic diagram of continuous fermentation with *L. lactis* IO-1 cells immobilized on photo-crosslinked resin gel beads (ENTG-3800). (1) 5 N NaOH reservoir, (2) pH controller, (3) fresh medium reservoir, (4) mixing reservoir for pH control, (5) packed-bed reactor, (6) product reservoir, and (P) pump.

2.8. Analytical methods

All samples were analysed for the residual glucose and the produced lactic acid by a glucose-lactic acid analyzer (model 23A-23L, Yellow Spring International, OH, USA) and for DCW by conversion from optical absorbance at 562 nm with a spectrophotometer (Uvidec 320, Japan Spectroscopic, Tokyo, Japan). Nisin Z produced was determined by reversed-phase high-performance liquid chromatography as described in our previous reports [8,11]. One unit of nisin Z activity is defined as an arbitrary unit (AU) of activity that is equivalent to the activity of 1 mg of commercial nisin (ICN Biomedicals, Costa Mesa, CA, USA; activity, 1000 IU/ mg-solid; nisin content, 2.5%).

3. Results

Many bacteriocins of lactic acid bacteria are produced during the active growth phase. Maximum bacteriocin production generally corresponds to maximum cell mass concentration. Thus, the bacteriocin production shows primary metabolite kinetics like lactic acid production [20]. Although nisin Z activity was increased with cell growth and lactic acid production, the optimal condition was different from that of cell growth and lactic acid production [11]. For potent commercial application, the higher yield and productivity are often required.

We tried to develop a continuous bioreactor system for nisin Z production by *L. lactis* IO-1. Immobilization technique was applied to improve the productivity of nisin Z. Both entrapment and adsorption methods of immobilization were studied with several kinds of support materials in repeated fermentations. The results are summarized in Table 3 and the detail is described in the following parts. From the results of the repeated batch cultivation described in Section 2, free cells produced about 30.3 g/l lactic acid and about 1790 AU/ml nisin Z on average.

Table 3
Nisin Z production by immobilized cells of *L. lactis* IO-1 in repeated fermentation

Support materials	Average nisin Z yield[a] ($\times 10^4$ AU/g-glucose consumed)	Average nisin Z productivity[a]	
		(AU · ml-broth^{-1} · day^{-1})	(AU · ml-broth^{-1} · ml-gel^{-1} · day^{-1})
(Free cells)[b]	4.70	1790	–
Entrapment[c]			
Ca-alginate	3.70	1700	405
Agar	4.30	1820	479
Agarose	3.95	1660	346
κ-Carrageenan	4.20	1820	536
PU-3	4.11	1800	600
PU-6	3.89	1660	333
ENT-3400	4.22	1270	190
ENTG-3800	4.35	1440	178
ENTP-4000	5.11	1250	298
Adsorption[c]			
ENT-2000	6.90	2340	468
VGE-112	5.60	1990	398
ENTG-3800	6.55	2310	463
Chitopearl 2503	8.30	3070	307
Chitopearl 2510	9.10	3180	318
Chitopearl 2520	8.50	2600	260
Chitopearl 3503	2.50	1000	100
Chitopearl 3510	5.80	1540	154
Chitopearl 3520	5.40	2080	207

[a]Average nisin Z yield and productivity were estimated with the results obtained by the repeated fermentation (24-h interval).
[b]Eight times repeated use.
[c]Ten times repeated use.

3.1. Nisin Z production by the entrapped cells

Most of the cells entrapped in natural materials showed lower average nisin Z production than the free cells. Among natural materials tested, agar and κ-carrageenan provided almost the same nisin Z productivity as the free cells. All of the immobilized cells had low activity during the first incubation, but their activities increased in the subsequent incubations. However, much cell-mass has been leaked out of all of the natural materials throughout the repeated cultivation. This resulted in almost the same cell density of the culture as free cells. Long-term application of natural materials was limited by the instability of the gel, which was especially obvious in calcium alginate and κ-carrageenan. No natural materials showed higher nisin yield than free cells.

The cells entrapped with photo-crosslinkable resin prepolymers gave low lactic acid and nisin Z production, which might result from the low growth of the cells in the gels. Actually, cell leakage from the gels was less than that from natural materials as previously described. Furthermore, there was about one-third to half of the amount of initial glucose left after each cultivation. Lactic acid and nisin Z produced by urethane polymer-immobilized cells were nearly equal to those by free cells. However, the quantity of cells leaked from the urethane gels was as much as in free cells. Nisin yield based on glucose consumption was slightly higher with ENTP-4000 than the others. The cells entrapped in ENTP-4000 produced small amount of nisin Z, whereas, the nisin yield was high because the low growth rate of the cells contributed to low glucose consumption. Among the synthetic ma-

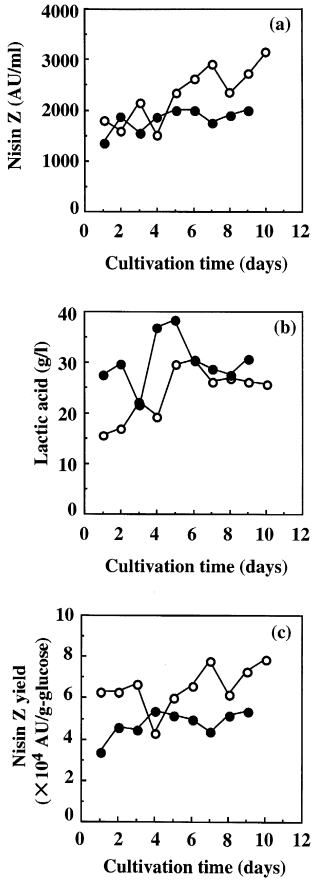

Fig. 2. Comparison between the free cells (●) and ENTG-3800-adsorbed cells (○). (a) Nisin Z produced, (b) lactic acid produced, (c) nisin Z yield based on glucose consumption.

terials tested, PU-3 exhibited the best nisin Z production, and the fermentation could run longer than 10 days without gel instability.

3.2. Nisin Z production by the adsorbed cells

The adsorption technique was applied to improve the nutrients diffusion through the cell-support materials. The synthetic and chemically modified materials were chosen because of their operational stability. Chitopearl HP-2520 and HP-3520 were found to be suitable support materials among the porous chitosan beads shown in Table 2 in terms of the cell adsorption ability (data not shown). Thus, Chitopearl type 2500 and type 3500 were selected in the subsequent experiment. Nisin Z production was associated with the number of cells leaked from Chitopearl type 2500 over the range of 2000–4100 AU/ml. Lactic acid was stably produced in the range of 27–35 g/l. On the other hand, nisin Z production was very low in the first cultivation of the cells adsorbed on Chitopearl type 3500. However, they exhibited lactic acid production and cell leakage similar to those in the Chitopearl type 2500. Nisin Z produced was increased in the subsequent cultivation of the cells adsorbed on Chitopearl type 3500. From the results where no residual glucose was left over from the repeated fermentation, nisin Z yield was in the same pattern as nisin Z production. Chitopearl type 3500 showed lower average nisin Z yield than Chitopearl type 2500. However, the nisin Z yield from Chitopearl type 2500 decreased with an increase in cultivation time. Among the photo-crosslinked resin gel beads, VGE-112 showed the highest lactic acid production, while ENTG-3800 showed the lowest. Each nisin Z production was gradually increased with an increase in cultivation time. Nisin Z yield also increased with increasing cultivation time, similarly to nisin Z production.

Among all the materials tested, thus, ENTG-3800 could be the best cell-immobilizing support in continuous fermentation because of the

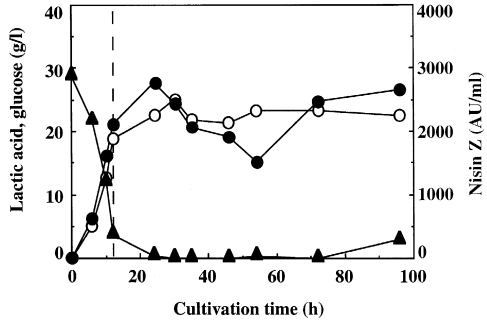

Fig. 3. Continuous fermentation of Nisin Z by *L. lactis* IO-1 cells immobilized on photo-crosslinked resin gel beads (ENTG-3800). Continuous fermentation was started at 12 h as indicated by the dotted line. The dilution rate was maintained at 0.1 h^{-1}. The same volume of fresh CM medium with 3% glucose and 0.1 M $CaCl_2$ as the filtrate was supplied. pH was maintained at 5.5. Nisin Z (●), lactic acid (○), and glucose (▲).

Table 4
Comparison of nisin Z productivities between ENTG-3800-adsorbed cells and free cells in continuous fermentation

Dilution rate, D (h^{-1})	Average glucose consumed, S (g/l)	Average nisin Z, P ($\times 10^6$ AU/l)	Average L-lactic acid, L (g/l)	Nisin Z productivity, PD ($\times 10^5$ AU $\cdot$ $l^{-1} \cdot h^{-1}$)	Nisin Z yield, P/S ($\times 10^4$ AU/g)
Free cells					
0.1	27.9	2.81	23.3	2.81	10.0
ENTG-3800-adsorbed cells					
0.1	27.0	2.16	23.3	2.16	8.00
0.2	25.1	1.86	18.6	3.71	7.40
0.3	24.7	1.30	13.3	3.89	5.26

high nisin Z productivity, which tended to increase with an increase in cultivation time. The comparison of nisin Z production between the free cells and ENTG-3800-adsorbed cells is shown in Fig. 2. The adsorbed cell system showed higher nisin Z production than the free cell system. Nisin Z produced by the adsorbed cell system was increased with an increase in repeated use (Fig. 2a). Nisin Z activity from the free cells was almost constant during the operation. The immobilized cells produced a moderately low concentration of lactic acid (Fig. 2b). Nisin Z yield based on glucose consumption was higher in the immobilized system than in the free cells (Fig. 2c). From the results in Fig. 2, ENTG-3800 can, therefore, be presumed to adsorb much more active cells during the repeated use in comparison with that at the initial cultivation. This could be responsible for the increase in nisin Z production in the repeated cultivation. Actually, viable cells of *L. lactis* IO-1 are tightly adsorbed on ENTG-3800. The cell-loading capacity of ENTG-3800 was calculated to be greater than 48 g-DCW/m^2. Cells were hardly desorbed from the gel without any detergent (data not shown).

3.3. Continuous fermentation of nisin Z by the cells adsorbed on ENTG-3800

Continuous fermentation of the free cells displayed a good cell growth followed by a high nisin Z productivity at a dilution rate of 0.1 h^{-1}. However, cell growth, lactic acid and nisin Z production were observed to rapidly decrease when the dilution rate was increased to 0.2 h^{-1} (data not shown). In the immobilized cell system, produced nisin Z displayed a high level in the range of 1500–2900 AU/ml (Fig. 3). Almost all the glucose was utilized during the continuous fermentation. At the dilution rates of 0.2 and 0.3 h^{-1}, nisin Z and lactic acid were moderately produced with some fluctuations (data not shown). Nisin Z productivities at the dilution rates of 0.2 and 0.3 h^{-1} were higher than that of the free cells (Table 4).

4. Discussion

The limitation of nutrient diffusion through the gel and the permeability of the products out of the gel caused the immobilized cells to grow slower than free cells. This led to the lower nisin Z productivity of the immobilized cells during the first incubation. These problems affected entrapped types more than adsorbed types. Moreover, the long-term application of natural materials such as calcium alginate was limited because of the instability of the gels.

In the immobilization of cells with photocrosslinkable resin prepolymers, it is necessary to illuminate the cell suspension with near-ultraviolet light for 3 min as described in Section 2. *L. lactis* IO-1 cells were affected by the near-ul-

traviolet light, which was dependent on the irradiation time. Irradiation for 3 and 5 min decreased viable cell concentration about 16% and 25%, respectively. These kinds of immobilized cells showed low glucose consumption and, consequently, low nisin Z productivity. Sensitivity to near-ultraviolet light during cell entrapment with the prepolymers is dependent on the strains. Wakao et al. [21] reported that the iron oxidation activity of *Thiobacillus ferrooxidans* was completely inhibited during immobilization with the prepolymers. The negative effects of this prepolymer method on immobilized bacterial cells were also reported by Takashima et al. [22] and Uchiyama et al. [23]. On the other hand, various kinds of microbial cells such as bacteria, yeasts and fungi could be applied to bioconversion and fermentation in the states of living and growing using the prepolymer methods [16,17,24].

The cells adsorbed on Chitopearl 2500 type materials showed a high level of nisin Z production compared to those on various kinds of porous chitosan beads. However, nisin Z production was gradually decreased with repeated cultivation. The decrease in nisin Z activity during the repeated use might be caused by sloughing of the microbial layer from the surface of chitosan beads as stated by Mori et al. [19]. The binding mode of Chitopearl type 3500 is very hydrophobic; hence, nisin Z should be easily adsorbed along the repeated batch fermentation. Nisin was allowed to adsorb on silanized silica surfaces for use as a food grade antimicrobial agent on food contact surfaces [25]. Chitopearl SH-3503 with 0.3-mm particle sizes showed lower nisin Z production than Chitopearl SH-3510 and HP-3520 whose particle sizes are 1.0 and 2.0 mm, respectively. Thus, Chitopearl SH-3503 has a greater surface area for nisin adsorption than Chitopearl SH-3510 and HP-3520. Nisin Z activity in the broth of Chitopearl type 3500-adsorbed cells increased with an increase in cultivation time because the materials were gradually saturated with the nisin Z produced.

Photo-crosslinked resin gel beads as new cell-adsorbing support materials have several advantages as follows. During the course of immobilization the cells are not exposed to near-ultraviolet light, which is occasionally responsible for cell damage. The gel beads are given the desired properties through the gel formation process prior to cell adsorption. The pore size of the gel can be controlled by the chain length of the composed prepolymer. The hydrophilicity and hydrophobicity of gel can be obtained by choosing and mixing polyethylene glycol and polypropylene glycol as the main skeleton of the prepolymers. Selecting proper functional groups resulted in the desired ionic property of the beads.

Among the cell-immobilizing supports tested, ENTG-3800 was the best material for nisin Z production. ENTG-3800 has a higher gel strength than natural support materials, which could be used for long-term application. During the repeated use of ENTG-3800-adsorbed cells, low lactic acid and high level of nisin Z were produced (Fig. 2). Although high nisin Z production was observed in the cultivation with porous chitosan beads, the nisin Z activity was on the same level as that by ENTG-3800 at the sixth cultivation. Nisin Z production by the cells adsorbed on ENTG-3800 gradually increased after the sixth cultivation (Fig. 2a). The capability to release the adsorbed cells from ENTG-3800 in the presence of a surfactant suggested the possibility of bead regeneration, which could not be performed with any type of Chitopearl. Tween 80 was more suitable at a concentration of 0.5% than Triton X-100 for cell desorption from ENTG-3800 gel beads.

Current investigation with immobilized cells for nisin production showed improvement of the stability of alginate gels by using double-coating method [13] and barium cation as the gel-hardening agent [14] and by adding $CaCl_2$ to the medium [15]. However, the nisin productivity was extremely low (0.1–1.0%) compared to that of free-cell fermentation [13,14]. Wan et al. [15] succeeded in 2-day continuous fermenta-

tion, but the activity was only 125 U/ml. These low productivities might be partly due to limitation of nutrient diffusion through the gel and permeability of the products out of the gel entrapping cells. On the other hand, ENTG-3800-adsorbed cells produced a high concentration of the product and showed a good operational stability in the continuous fermentation (Fig. 3). This cell-immobilization method for nisin production could avoid unfavorable effects such as support instability, limitation of nutrient transfer, and sloughing of the excess cells from the supports.

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