ELSEVIER

Process Biochemistry 40 (2005) 13–24

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Continuous nisin production in laboratory media and whey permeate by immobilized *Lactococcus lactis*

Xia Liu, Yoon-Kyung Chung, Shang-Tian Yang, Ahmed E. Yousef*

Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, 110 Parker Food Science and Technology Building, Columbus, OH 43210, USA

Received 27 June 2003; received in revised form 18 October 2003; accepted 19 November 2003

Abstract

Continuous production of nisin in laboratory media and whey permeate was investigated using a packed-bed bioreactor. *Lactococcus lactis* subsp. *lactis* ATCC 11454 was immobilized by natural attachment to fibre surfaces and entrapment in the void volume within spiral wound fibrous matrix. The inoculated bioreactor was fed M17 medium and effect of pH, temperature, dilution rate, and substrate concentrations on nisin production was examined. Bioreactor performance was monitored by checking cell density, lactic acid production, lactose consumption, and nisin activity. Optimal nisin activity was observed at pH 5.5, 31 °C, 0.2–0.3/h dilution rate, and 30 g/l lactose in M17. The maximum nisin titre was 2.6×10^4 AU/ml against a nisin sensitive strain, *Lactobacillus leichmannii* ATCC 4797. The bioreactor was fed whey permeate, supplemented with casein hydrolysate, and growth of *L. lactis* and associated nisin production were monitored. Optimal conditions for continuous nisin production in whey permeate were pH 5.5, 31 °C, 10–20 g/l casein hydrolysate, and 0.2/h dilution rate. Under these conditions, a maximum nisin titre of 5.1×10^4 AU/ml was observed. The bioreactor was operated continuously for 6 months without encountering any clogging, degeneration, or contamination problems. The cell density in the bioreactor was 52.0 g/l with 96.4% of the cells immobilized. This study illustrates the possibility of continuous production of high concentration of bacteriocins by lactic acid bacteria for use as food biopreservatives.
© 2003 Published by Elsevier Ltd.

Keywords: *Lactococcus lactis*; Nisin; Bacteriocins; Bioreactor; Cell immobilization; Whey permeate

1. Introduction

Bacteriocins are antagonistic compounds of proteinaceous nature that show a bactericidal activity against organisms most closely related to the producer [1–6]. One of the most industrially relevant bacteriocins is nisin, which is produced by some strains of *Lactococcus lactis* [7–10]. Nisin is active against some Gram-positive bacteria, including pathogenic and food spoilage microorganisms such as clostridia, bacilli, *Staphylococcus aureus*, micrococci, lactobacilli, and *Listeria monocytogenes* [9,11,12]. Nisin is a safe and effective food preservative, and it has been used for decades in many countries [13,14]. Use of nisin was approved in 1988 by the US Food and Drug Administration (FDA) for pasteurized cheese spreads [15]. Subsequently, FDA approved nisin use in other foods such as soups that are heat-treated and stored at chill temperatures [16].

Commercial nisin is produced by large-scale fermentation of media containing food-grade ingredients. Whey permeate is a by-product of the cheese industry and may serve as an inexpensive medium for bacteriocin production. Approximately, 9 kg of whey result for every kilogram of cheese produced and the cost associated with disposing this large volume of whey is substantial. Furthermore, the high chemical oxygen demand (COD) (50 kg O_2/ton permeate) of whey or whey permeate makes its disposal a pollution problem [17]. Therefore, use of whey or whey permeate as fermentation feedstock has long been of industrial interest. Products resulting from fermentation of whey or whey permeate include lactic acid [18–21], acetic acid [22], propionic

Abbreviations: CDW, cell dry weight (g); CH, casein hydrolysate; P, nisin productivity (AU l^{-1} h^{-1}); Q_p, specific rate of product (nisin) formation (AU g-CDW^{-1} h^{-1}); Q_s, specific rate of substrate (lactose) utilization (g-lactose consumed g-CDW^{-1} h^{-1}); WP, whey permeate; $Y_{p/s}$, nisin yield (AU/g-lactose consumed); $Y_{p/x}$, nisin yield (AU/g-CDW); $Y_{x/s}$, cell yield (g-CDW/g-lactose consumed); $Y_{l/s}$, lactic acid yield (g-lactic acid/g-lactose consumed); YE, yeast extract

* Corresponding author. Tel.: +1-614-292-7814; fax: +1-614-292-0218.

E-mail address: yousef.1@osu.edu (A.E. Yousef).

0032-9592/$ – see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/j.procbio.2003.11.032

acid [23–26], ethanol [27], and single cell protein [28]. Currently, there is an increasing interest in producing bacteriocin from whey permeate since bacteriocin-producing lactic acid bacteria (LAB) can grow in this low-cost medium. Nisin and pediocin production in whey medium was investigated in batch fermentations [29]. These researchers indicated that whey supported the growth and bacteriocin production, but biomass and bacteriocin levels were lower than those obtained in MRS broth. Liao et al. [30] also studied the production of pediocin from whey permeate. According to these researchers, whey permeate supports pediocin production when the medium is supplemented with yeast extract.

Nisin biosynthesis was mostly studied in batch cultures using synthetic media [31–36]. The biosynthesis of nisin in batch cultures is associated with the growth of the producing bacteria [29,34]. However, there are limited reports on continuous nisin production [37,38]. Continuous culture techniques provide several advantages over conventional batch processes, such as high productivity, reduced product inhibition, and no batch-to-batch variation; this leads to low fermentation costs [21]. Free-cell continuous fermentation, however, is limited by cell wash-out. In order to increase the productivity, it is necessary to use a high cell concentration by cell immobilization [39]. A packed-bed, immobilized-cell bioreactor has been developed and successfully applied in fermentation of whey permeate to produce organic acids [25,26,40,41], and pediocin PO2, a bacteriocin produced by *Pediococcus acidilactici* PO2 [42]. The goals of the current study are to (a) develop a method for continuous nisin production using immobilized cells in a packed-bed bioreactor, (b) optimize conditions for continuous nisin production in laboratory media, and (c) explore the feasibility of continuous nisin production from whey permeate, and optimize this fermentation process.

2. Materials and methods

2.1. Bacterial strains and media

A bacteriocin-producing strain, *L. lactis* subsp. *lactis* ATCC 11454, and a bacteriocin-sensitive strain, *Lactobacillus leichmannii* ATCC 4797, were used in this study. *L. lactis* was propagated in M17 broth (Difco Laboratories, Sparks, MD) by incubation at 30 °C for 24 h, while *L. leichmannii* was grown in *Lactobacillus* MRS broth (Difco) at 37 °C for 24 h. The cultures were transferred at least twice before use.

Unmodified M17 broth contains 5 g/l lactose. This medium was modified by increasing lactose concentration up to 50 g/l depending on the experiment. Sterile lactose solution was added to medium after sterilization. MRS soft overlay agar was prepared by adding 7.5 g of agar in a liter of MRS broth. Modified Elliker broth [43] contained (per liter) tryptone, 20 g; glucose, 20 g; yeast extract, 5 g; sodium chloride, 4 g; gelatin, 2.5 g; sodium acetate, 1.5 g; ascorbic acid, 0.5 g. The medium pH was 6.8 before autoclaving.

Whey permeate was prepared from fresh Cheddar cheese whey. Stirred-curd cheddar cheese was made in the pilot plant of the Department of Food Science and Technology, The Ohio State University (Columbus, OH) using the procedure of Kosikowski and Mistry [44]. The whey was collected, defatted by a cream separator (The DE Laval Separator Co., Chicago, IL), pasteurized at 71.7 °C for 15 s (The APV Co., Buffalo, NY), and ultrafiltered through a hollow fibre cartridge (Romicon, Inc., Woburn, MA). The whey permeate was stored at −20 °C until use. Yeast extract (Difco) and casein hydrolysate (Mead Johnson International, Evansville, IN) were used as supplements to whey permeate at 5–40 g/l. Sterilized whey permeate with or without supplement was allowed to set for at least 24 h, and only the clear layer was used as medium.

2.2. Analytical methods

2.2.1. Cell density

Cell density was measured as $OD_{600\,nm}$ units using a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY). For cell dry weight (CDW) determination, microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) were weight-stabilized by heating at 70 °C. Portions (18 ml) of culture were dispensed into 12 weight-stabilized tubes, with 1.5 ml in each, and centrifuged (Heraeus Equipment, Germany) at 12,000 rpm for 15 min. The supernatant was removed and the cells were then washed twice with demineralized water, and dried in an oven at 70 °C for 24 h with vacuum.

2.2.2. Nisin activity

Samples of the fermentation mixture were withdrawn from the batch fermentation vessel, or collected from the continuous bioreactor, into a container and placed on ice. The pH of the fermentate was adjusted to 4.0 with 5N HCl. Fermentate samples were dispensed into microcentrifuge tubes, centrifuged at 12,000 rpm for 15 min, and the supernatant was assayed for nisin activity using an agar diffusion method [45]. Briefly, two-fold serial dilutions of cell-free culture supernatant were made with sterile water, and 5 μl of each dilution were spotted onto MRS soft overlay agar (6 ml) that was seeded earlier with 10 μl of overnight culture of the sensitive indicator, *L. leichmannii* ATCC 4797. Assay plates were incubated at 37 °C for 24 h. Nisin activity was defined as the reciprocal of the highest dilution exhibiting complete inhibition of the indicator lawn, expressed in activity units (AU) per milliliter.

2.2.3. Lactose and lactic acid

Lactose consumption and lactic acid production during fermentation were determined by high performance liquid chromatography (HPLC) [42]. Samples were centrifuged at 12,000 rpm in microcentrifuge tubes for 15 min, filtered

through a 0.45 μm-pore size filter (Gelman Sciences, Ann Arbor, MI), and analyzed by HPLC. The HPLC system (Waters Associates, Inc., Milford, MA) consisted of a pump (Model 6000A, Waters), an autosampler (WISP 712, Waters), an HPX-87H ion exclusion column (Bio-Rad, Richmond, CA), a programmable multi-wave length detector (Model 490, Waters), a differential refractometer (Model 410, Waters), and a microcomputer loaded with a data acquisition software (Maxima 820 workstation, Waters). Culture filtrate of 10 μl was injected into the HPLC system. The mobile phase, 0.005 M H_2SO_4, was pumped at 0.6 ml/min.

2.3. Nisin production by batch fermentation

2.3.1. Selection of a laboratory medium

Modified Elliker, MRS and M17 broths were compared for nisin production. Flasks containing 250 ml of media were inoculated with *L. lactis* culture at 0.1% level, and incubated at 30 °C. Samples (10 ml) were withdrawn from the flasks during incubation period at selected intervals. The samples were placed immediately on ice, and tested for growth ($OD_{600\,nm}$), pH, activity of nisin, and concentration of lactose and lactic acid.

2.3.2. Supplemented whey permeate

Yeast extract or casein hydrolysate was used to supplement the whey permeate. The effect of supplement on the growth and nisin production by *L. lactis* was tested. Yeast extract or casein hydrolysate was added to 500 ml of whey permeate at 0–40 g/l. The mixture was autoclaved, and kept undisturbed for at least 24 h. The clear layer from each flask (ca. 250 ml) was transferred into a sterile flask for use as a testing medium. M17 was used as a control medium. The media were inoculated with *L. lactis* culture at 0.1% and incubated at 30 °C. Samples (10 ml) were withdrawn at selected intervals and tested for growth ($OD_{600\,nm}$), pH, and nisin activity.

2.4. Nisin production by continuous fermentation

2.4.1. Bioreactor construction

Continuous nisin production was investigated using an immobilized-cell, packed-bed bioreactor. The basic construction of the bioreactor can be found elsewhere [26,40,42]. The bioreactor was made of a glass column (5 cm i.d. × 15 cm) fitted with a water jacket. The fibrous matrix used for cell immobilization was made of a piece of cotton towel (9.8 cm × 44 cm), mounted on a stainless steel wire screen. The matrix was spirally wound around the vertical axis and packed in the glass column on top of 2 cm-thick ceramic saddles and a filter as support. The temperature of the bioreactor was controlled by continuously circulating water from a thermostatically controlled waterbath through the water jacked. An Erlenmeyer flask was connected to the reactor column for media circulation to monitor and control pH. The pH in the bioreactor was controlled using an automatic pH controller (Cole Palmer, Chicago, IL) and 15% ammonium hydroxide. The fermentation medium in the reactor was recycled at ca. 3.7 l/h to ensure sufficient mixing and adequate pH control. The medium was pumped into the reactor using a low speed, peristaltic pump with No. 13 pump head (Masterflex, Cole Palmer, Chicago, IL). The product was collected automatically into an Erlenmeyer flask. The total reactor working volume was 360 ml.

2.4.2. Bioreactor startup and operation

The bioreactor was autoclaved twice at 121 °C for 45 min, with 1 day waiting period between autoclaving to allow any surviving bacterial spore to germinate. The bioreactor was then filled with sterile laboratory medium, selected on the basis of batch fermentation studies, and inoculated with 30 ml of *L. lactis* culture. The cells in the bioreactor were allowed to grow for 1 day without feeding medium or pH control. Then, the medium was fed at about 90 ml/day (i.e., dilution rate of 0.01/h) and the pH loop was circulated slowly at about 2 l/day for 3 days with pH controlled at 5.5. The medium feeding rate was gradually increased as the cell density in the bioreactor was allowed to build up. It took about 3 weeks for the new bioreactor packing to be saturated with cells and to reach a steady state that gave high cell density (at a dilution rate of 0.15/h). At each testing condition, the reactor was operated until a steady state was reached. It took about 6–8 retention times for the bioreactor to reach a steady state at each conditions. Once the reactor had reached a steady state, fermentate samples were collected for analysis. The bioreactor performance was monitored by checking the effluent for optical density, cell dry weight, nisin activity, lactose consumption, and lactic acid formation.

2.4.3. Continuous fermentation study

The bioreactor was fed with a laboratory medium, selected during the batch fermentation, to evaluate the fermentation kinetics and bioreactor performance under various conditions (i.e., different dilution rates, pH values, temperatures, and substrate concentrations). At a selected temperature of 31 °C and pH of 5.5, the bioreactor performance was studied at a wide range of dilution rates (0.06–0.38/h) to determine the optimal dilution rate for nisin activity. After an optimal dilution rate was selected, the pH (5.0–7.0) and temperature (25–38 °C) were step changed after reaching each new steady state to determine the optimal pH and temperature for nisin activity. Similarly, an optimal substrate concentration was also determined. After the fermentation conditions were optimized in laboratory medium, the feed was switched to the supplemented whey permeate. As in case of experiments on the laboratory medium, the fermentation conditions, including dilution rate (0.03–0.30/h), pH (5.0–6.5), supplement level (5–40 g/l), and Tween 80 level (0 or 5 g/l), were optimized in whey permeate for nisin production. The bioreactor performance was monitored by checking the fermentate for optical density, cell dry weight, nisin activity, lactose consumption, and lactic acid formation.

2.4.4. Efficiency of immobilization

After continuous operation for 6 months, the immobilized-cell bioreactor was disassembled and its cell density and efficiency of immobilization were estimated [42]. Cell mass suspended in the bioreactor (M_s) was calculated from the reactor working volume and cell dry weight of the fermentate. All liquid in the bioreactor was drained, and its volume and the cell dry weight were measured and used to estimate the total cell mass suspended or sedimented in the bioreactor (M_c). The drained fibrous matrix was then removed from the reactor chamber and washed several times until almost all cells had been removed, as indicated by the washing water being almost clear. The total volume of the washing water and its cell dry mass were measured and used to estimate the total cell mass immobilized in the fibrous matrix (M_i). The efficiency of cell immobilization was calculated as $1 - [M_s/(M_c + M_i)]$.

2.4.5. Scanning electron micrograph

Before the drained matrix was washed, six small pieces (ca. 1 cm × 1 cm) of this fibrous material were prepared for scanning electron microscopy (SEM). Matrix samples were immersed overnight in 3% glutaraldehyde in phosphate buffer (pH 6.5) at 5 °C, rinsed thoroughly with the phosphate buffer, and post-fixed with 1% osmium tetraoxide in phosphate buffer for 1 h at 22 °C. After post-fixation, the samples were rinsed thoroughly with buffer and gradually dehydrated with 50, 70, 80, 95, and 100% ethanol by holding the samples at each concentration for 10 min. The samples were then cryogenically dried with liquid CO_2. The completely dried samples were coated with gold/palladium, and SEM micrograph was taken using a scanning electron microscope (JOEL model 820, Japan).

3. Results

3.1. Batch fermentation in laboratory media

3.1.1. Selection of a laboratory medium

Preliminary experiments were carried out to compare MRS, M17, and modified Elliker broths for their ability to support growth of *L. lactis* and nisin production in batch cultures (Table 1). MRS is a commonly used medium for growth of lactic acid bacteria and M17 is particularly suitable for growth of lactococci. According to earlier research, Elliker broth is suitable for growth of bacteriocin-producing lactic acid bacteria [43,46]. Current investigation shows that M17 and MRS broths are suitable for nisin production. M17, however, was selected for further investigation because it contains lactose as the carbon source which is the same as that in whey permeate, whereas MRS contains glucose.

Table 1
Parameters for batch fermentation of microbiological media by *L. lactis* subsp. *lactis* ATCC 11454

Media	Specific growth rate (1/h)	Maximum growth ($OD_{600\,nm}$)	Final pH	Maximum nisin (AU/ml)
MRS	0.65	1.6–1.7	4.6–4.7	1.3×10^4
M17	0.60	1.9–2.0	5.0–5.1	1.3×10^4
Modified Elliker	0.39	1.3–1.4	4.3–4.4	6.4×10^3

3.1.2. Batch fermentation in M17 broth

Varying lactose concentration from 5 to 40 g/l in M17 broth produced different fermentation parameters (Table 2). Specific growth rate of *L. lactis* was highest during fermentation of M17 broth containing 20 g/l lactose. Compared to other levels of supplementation, 10 g/l lactose in M17 broth gave a relatively high maximum growth and nisin activity (1.3×10^4 AU/ml). Nisin production increased with growth and reached to a maximum value at the early stationary phase (Fig. 1). This is consistent with earlier findings for nisin production in batch cultures [34,36]. Nisin activity in cultures was at the highest when pH was lowest and most of lactose was consumed (Fig. 1).

3.2. Continuous fermentation in M17 broth

3.2.1. Dilution rate

L. lactis was immobilized in a packed-bed bioreactor and continuous fermentation of M17 broth was monitored at dilution rates of 0.06–0.38/h (Fig. 2a). Nisin activity increased with increases in dilution rate and reached a maximum value of 1.3×10^4 AU/ml at 0.20–0.31/h. Further increase in dilution rate decreased nisin activity. Cell dry weight increased with increase in dilution rate from 0.06 to 0.16; then decreased at higher dilution rates. Lactic acid production and lactose concentration increased slightly at ≥0.20/h. The specific nisin formation rate, Q_p, reached a maximum of 5.3×10^6 AU g-CDW^{-1} h^{-1} when the dilution rate was 0.25/h (Table 3(a)). The nisin yield $Y_{p/s}$ increased from 6.4×10^5 to 5.5×10^6 AU/g-lactose consumed when the dilution rate increased from 0.06 to 0.25/h. The highest nisin yield was obtained at dilution rate of 0.20–0.25/h.

3.2.2. pH

pH of M17 broth was varied from 5.0 to 7.0 and nisin production by immobilized *L. lactis* was monitored (Fig. 2b) and productivity parameters were calculated (Table 3(b)).

Table 2
Parameters for batch fermentation of lactose-supplemented M17 broth by *L. lactis* subsp. *lactis* ATCC 11454

Lactose (g/l)	Specific growth rate (1/h)	Maximum growth ($OD_{600\,nm}$)	Final pH	Maximum nisin (AU/ml)
5	0.54	2.07	5.3	6.4×10^3
10	0.58	2.01	4.8	1.3×10^4
20	1.05	1.97	4.6	1.3×10^4
30	0.68	1.96	4.6	6.4×10^3
40	0.55	1.85	4.6	6.4×10^3

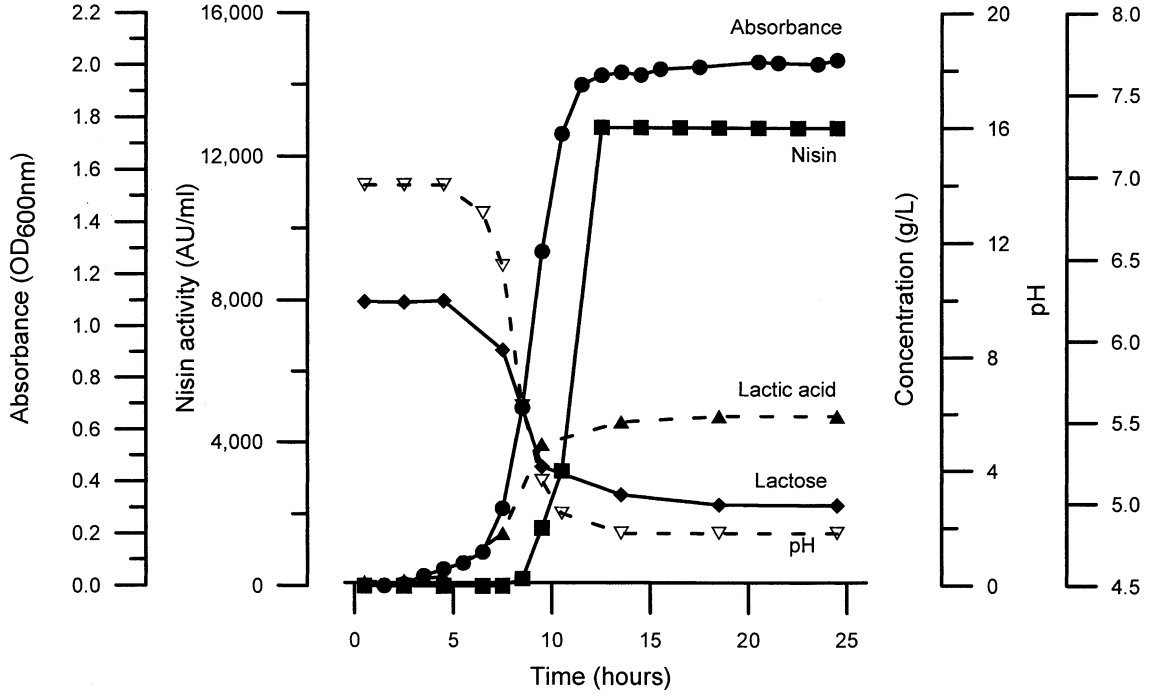

Fig. 1. Nisin production during batch fermentation of M17 broth, containing 10 g/l lactose, by *L. lactis* subsp. *lactis* ATCC 11454.

Nisin activity was highest at pH 5.5, whereas cell dry weight was highest at pH 6.5. With increase of pH from 5.0 to 6.0, lactose consumption and lactic acid production increased, but further increases in pH decreased these values (Fig. 2b). Biomass and lactic acid yields increased when the pH was increased up to 6.5 (Table 3(b)). Nisin yield and specific nisin formation rate reached a maximum of 5.4×10^6 AU/g lactose consumed and 4.2×10^6 AU g-CDW^{-1} h^{-1}, respectively, at pH 5.5. Therefore, optimal pH for continuous nisin production is 5.5, while pH 6.5 is best for biomass accumulation.

3.2.3. Temperature

Continuous fermentation of M17 broth was tested at 25–38 °C and changes in growth and nisin production parameters were monitored (Fig. 2c and Table 3(c)). The optimal fermentation temperature was 31 °C; at this temperature, maxima for nisin activity (1.3×10^4 AU/ml) and lactic acid production (3.5 g/l) were attained.

3.2.4. Substrate concentration

Continuous production of nisin in M17 broth was examined at different levels of lactose supplementation (5–50 g/l),

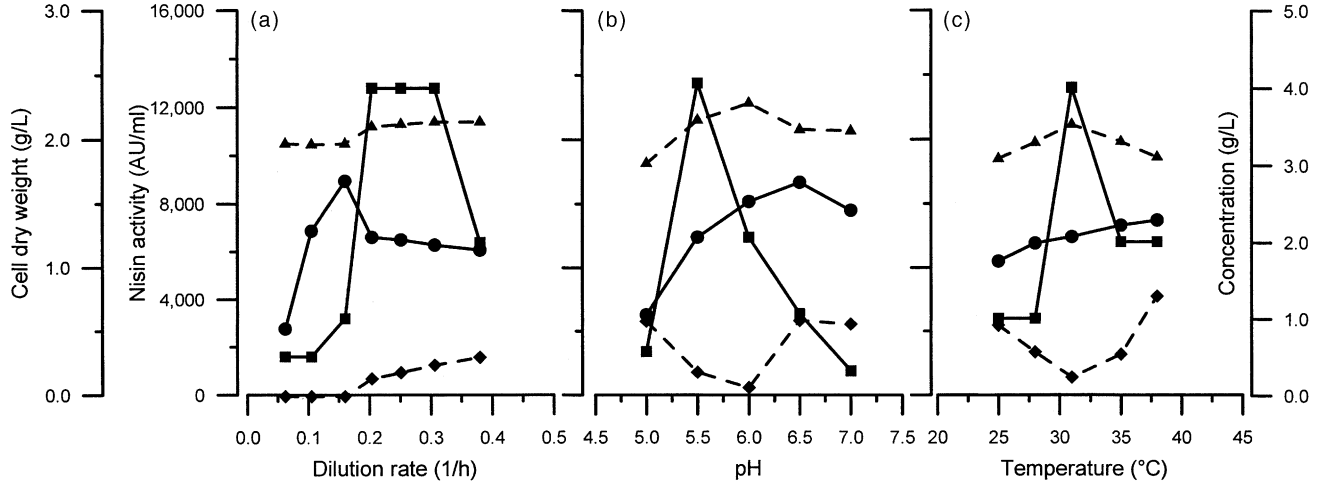

Fig. 2. Kinetics for the continuous fermentation of M17 broth by immobilized *L. lactis* subsp. *lactis* ATCC 11454 in a packed-bed bioreactor. Fermentation was conducted at: (a) different dilution rates at 31 °C and pH 5.5, (b) different pHs at 31 °C and a dilution rate of 0.2/h, (c) different temperatures at pH 5.5 and a dilution rate of 0.2/h. (■) nisin activity; (▲) lactic acid; (●) cell dry weight and (◆) lactose.

Table 3
Yield and productivity parameters for fermentation of M17 broth by *L. lactis* subsp. *lactis* ATCC 11454 when the bioreactor was operated under different conditions

Fermentation condition	Biomass, $Y_{x/s}$	Lactic acid, $Y_{l/s}$	Nisin			
			$Y_{p/x}$	$Y_{p/s}$	P	Q_p
(a) pH 5.5, 31 °C						
Dilution rate (1/h)						
0.06	0.10	0.66	6.2×10^6	6.4×10^5	2.0×10^5	3.8×10^5
0.11	0.26	0.66	2.8×10^6	6.4×10^5	3.4×10^5	3.0×10^5
0.16	0.34	0.66	3.8×10^6	1.3×10^6	1.0×10^6	6.1×10^5
0.20	0.26	0.74	2.1×10^7	5.4×10^6	5.2×10^6	4.2×10^6
0.25	0.26	0.76	2.1×10^7	5.5×10^6	6.4×10^6	5.3×10^6
0.31	0.26	0.78	1.1×10^7	2.8×10^6	3.9×10^6	3.3×10^6
0.38	0.25	0.80	1.1×10^7	2.9×10^6	4.9×10^6	4.3×10^6
(b) 31 °C, dilution rate 0.2/h						
pH						
5.0	0.16	0.72	5.1×10^6	7.8×10^5	6.5×10^5	1.0×10^6
5.5	0.26	0.74	2.1×10^7	5.4×10^6	5.2×10^6	4.2×10^6
6.0	0.38	0.75	8.4×10^6	2.6×10^6	2.6×10^6	1.7×10^6
6.5	0.41	0.83	3.8×10^6	1.6×10^6	1.3×10^6	7.8×10^5
7.0	0.35	0.82	2.2×10^6	7.7×10^5	6.5×10^5	4.5×10^5
(c) pH 5.5, dilution rate 0.2/h						
Temperature (°C)						
25	0.26	0.75	6.1×10^6	1.6×10^6	1.3×10^6	1.2×10^6
28	0.27	0.74	5.4×10^6	1.4×10^6	1.3×10^6	1.1×10^6
31	0.26	0.74	2.1×10^7	5.4×10^7	5.2×10^7	4.2×10^6
35	0.30	0.74	9.6×10^6	2.9×10^7	2.6×10^7	2.0×10^6
38	0.37	0.84	9.3×10^6	3.5×10^7	2.6×10^7	1.9×10^6

using the optimal dilution rate (0.2/h), pH (5.5), and temperature (31 °C), as concluded earlier. Nisin activity, lactose consumption, and lactic acid production varied markedly with the change in lactose concentration in feeding medium, while the cell dry weight was not affected appreciably (Fig. 3). With increasing lactose concentrations up to 40 g/l, lactose consumption and lactic acid production increased progressively, while nisin activity reached a maximum of 2.6×10^4 AU/ml at 30–40 g/l of lactose (Fig. 3a). Rate of lactose utilization (Q_s) increased with increasing initial lactose concentration up to 50 g/l, whereas nisin formation rate (Q_p) reached a maximum at the initial lactose concentration of 30 g/l (Fig. 3b). Therefore, nisin production was optimal when the medium contained 30 g/l lactose.

3.3. Batch fermentation in whey permeate

3.3.1. Selection of a supplement

Batch cultures of *L. lactis* were grown in whey permeate, with or without supplementation, and fermentation parameters were measured (Table 4). Whey permeate alone was a poor medium for the growth and nisin production. When different levels of supplementation (5–40 g/l) of yeast extract or casein hydrolysate in whey permeate were compared, yeast extract gave higher maximum growth, however, casein hydrolysate gave a greater nisin activity and a higher specific growth rate. Therefore, casein hydrolysate was selected as a supplement to whey permeate in continuous fermentation studies. When casein hydrolysate concentration increased from 5 to 40 g/l, maximum growth of *L. lacits* increased, but nisin activity did not increase.

3.4. Continuous fermentation of whey permeate

3.4.1. Dilution rate

Fig. 4a and Table 5(a) summarize the influence of dilution rate on nisin production and other fermentation parameters

Table 4
Effects of supplements on the growth parameters and bacteriocin production in whey permeate by *L. lactis* subsp. *lactis* ATCC 11454 in batch cultures

Media	Specific growth rate (1/h)	Maximum growth ($OD_{600\,nm}$)	Final pH	Maximum nisin (AU/ml)
Whey permeate	0.52	1.1	4.2	3.2×10^3
WP + casein hydrolysate (g/l)				
5	0.77	1.4	4.2	1.3×10^4
10	1.24	1.4	4.3	1.3×10^4
20	1.00	1.6	4.4	1.3×10^4
30	0.76	1.6	4.5	1.3×10^4
40	0.68	1.8	4.5	6.4×10^3
WP + yeast extract (g/l)				
5	0.97	1.4	4.2	6.4×10^3
10	0.79	1.5	4.3	6.4×10^3
20	0.81	1.8	4.4	6.4×10^3
30	0.72	2.0	5.5	1.3×10^4
40	0.67	2.0	4.5	6.4×10^3
M17	0.60	2.0	5.0	1.3×10^4

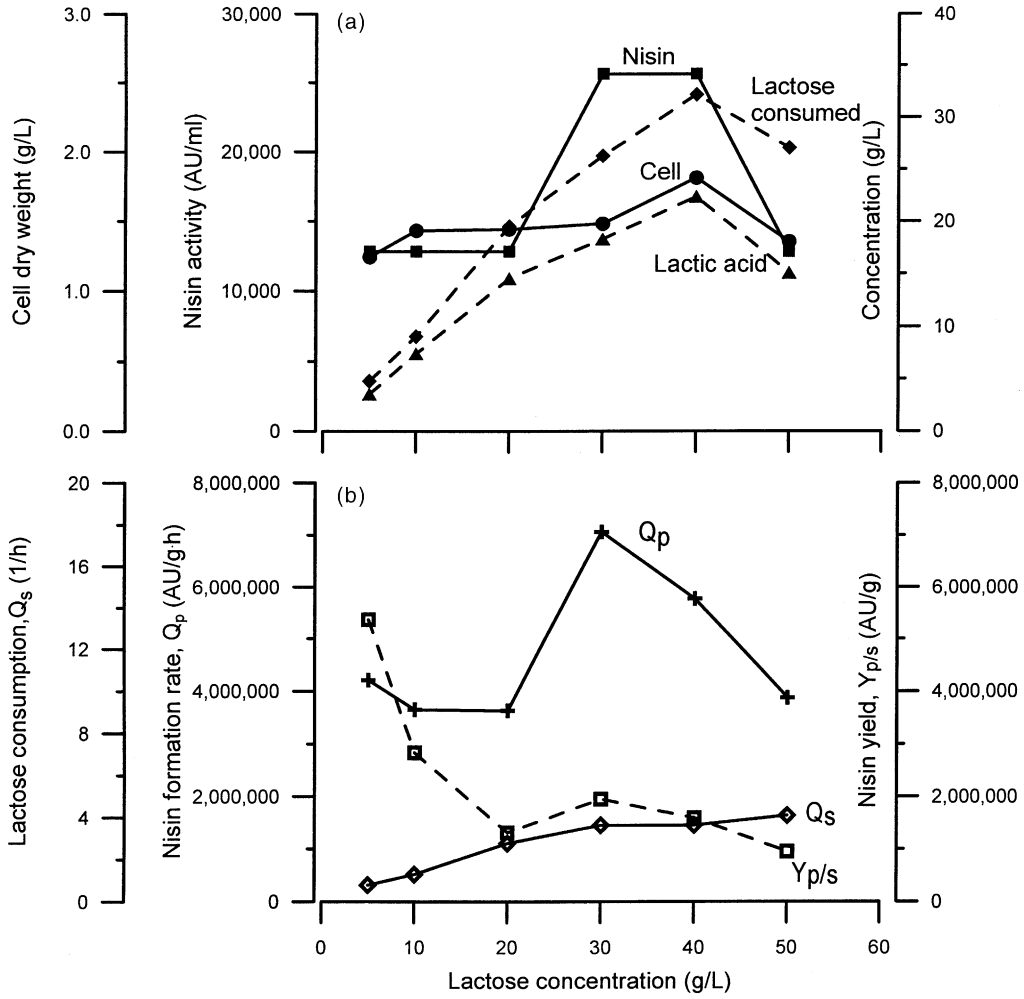

Fig. 3. Effect of lactose concentration on the continuous fermentation of M17 broth by immobilized *L. lactis* subsp. *lactis* ATCC 11454 in a packed-bed bioreactor at 31 °C, pH 5.5, and a dilution rate of 0.2/h.

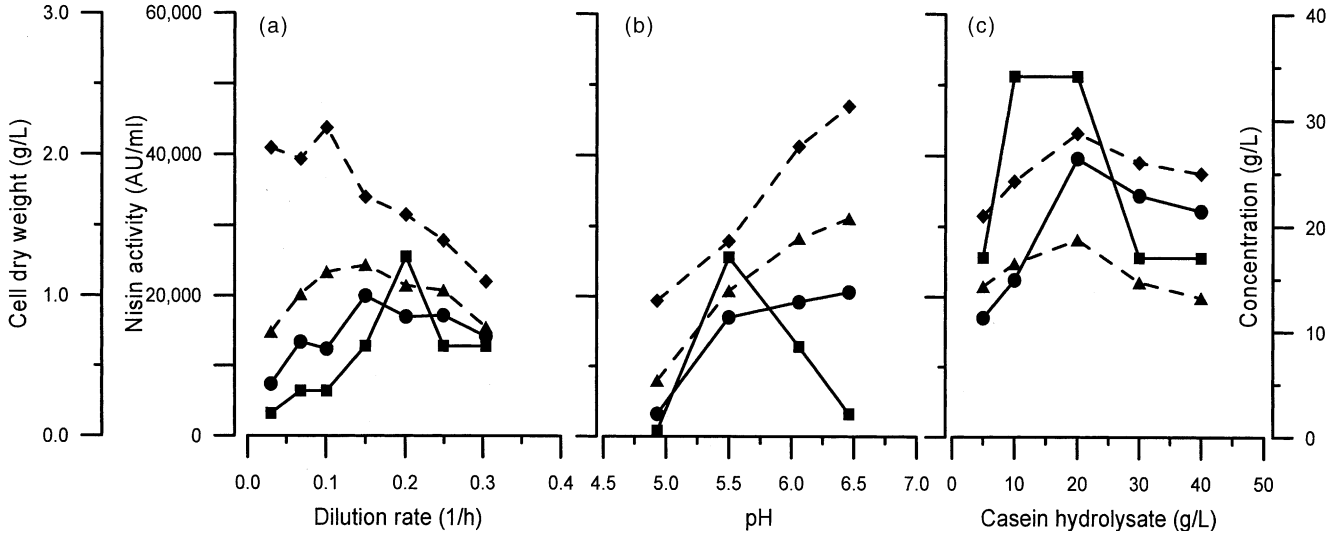

Fig. 4. Kinetics for the continuous fermentation of whey permeate, supplemented with 5 g/l of casein hydrolysate, by immobilized *L. lactis* subsp. *lactis* ATCC 11454 in a packed-bed bioreactor. Fermentation was conducted at: (a) different dilution rates at 31 °C and pH 5.5, (b) different pHs at 31 °C and a dilution rate of 0.2/h, (c) 31 °C, pH 5.5, and a dilution rate of 0.2/h with the supplementation of different concentration of casein hydrolysate. (■) nisin activity; (▲) lactic acid; (●) cell dry weight and (◆) lactose consumed.

Table 5
Yield and productivity parameters for fermentation of whey permeate, supplemented with casein hydrolysate, by *L. lactis* subsp. *lactis* ATCC 11454 when the bioreactor was operated at various conditions

Condition	Biomass, $Y_{x/s}$	Lactic acid, $Y_{l/s}$	Nisin			
			$Y_{p/x}$	$Y_{p/s}$	P	Q_p
(a) pH 5.5, 31 °C, casein hydrolysate 5 g/l						
Dilution rate (1/h)						
0.03	0.01	0.36	8.7×10^6	1.2×10^5	9.6×10^4	2.6×10^5
0.07	0.03	0.51	9.6×10^6	2.4×10^5	4.4×10^5	6.5×10^5
0.10	0.02	0.53	1.0×10^7	2.2×10^5	6.5×10^5	1.0×10^6
0.15	0.04	0.72	1.3×10^7	5.6×10^5	1.9×10^6	1.9×10^6
0.20	0.04	0.68	3.0×10^7	1.2×10^6	5.2×10^6	6.1×10^6
0.25	0.05	0.75	1.5×10^7	1.4×10^6	6.4×10^6	7.4×10^6
0.30	0.05	0.70	1.8×10^7	8.7×10^5	3.9×10^6	5.5×10^6
(b) 31 °C, dilution rate 0.2/h, casein hydrolysate 5 g/l						
pH						
5.0	0.01	0.41	1.0×10^7	1.2×10^5	3.2×10^5	2.0×10^6
5.5	0.05	0.75	3.0×10^7	1.4×10^6	5.1×10^6	6.0×10^6
6.0	0.04	0.69	1.3×10^7	4.7×10^5	2.6×10^6	2.7×10^6
6.5	0.03	0.66	6.2×10^6	2.1×10^5	1.3×10^6	1.2×10^6
(c) pH 5.5, 31 °C, dilution rate 0.2/h						
Casein hydrolysate (g/l)						
5	0.04	0.68	3.0×10^7	1.2×10^6	5.1×10^6	6.0×10^6
10	0.05	0.68	4.6×10^7	2.1×10^6	1.0×10^7	9.1×10^6
20	0.07	0.65	2.6×10^7	1.8×10^6	1.0×10^7	9.1×10^6
30	0.07	0.57	1.5×10^7	9.8×10^5	5.1×10^6	3.0×10^6
40	0.06	0.53	1.6×10^7	1.0×10^6	5.1×10^6	3.2×10^6

in the continuous culture of whey permeate supplemented with 5 g/l casein hydrolysate. Similar as that in M17 broth, nisin activity increased with initial increase in dilution rate. Nisin activity reached a maximum value at a dilution rate of 0.2/h (Fig. 4a). Maximal nisin activity was 2.6×10^4 AU/ml, which is double the amount obtained using M17 as a medium (Fig. 2a). The maximum specific rate of nisin formation (Q_p) in supplemented whey permeate was 7.4×10^6 AU g-CDW^{-1} h^{-1} (Table 5(a)), which is also higher than that in M17 (5.3×10^6 AU g-CDW^{-1} h^{-1}) (Table 3(a)). Supplemented whey permeate, however, gave a nisin yield ($Y_{p/s}$) of 1.2×10^6 AU/g-lactose consumed (Table 5(a)), which was lower than that in M17 (5.4×10^6 AU/g-lactose consumed; Table 3(a)) at 0.2/h of dilution rate.

3.4.2. pH

The effect of pH on continuous production of nisin from whey permeate is shown in Fig. 4b and Table 5(b). Similar to the results obtained during fermentation of M17 broth, pH 5.5 was optimum for continuous nisin production in whey permeate.

3.4.3. Casein hydrolysate concentration

Casein hydrolysate supplementation in whey permeate (5–40 g/l) was tested for its effect on continuous nisin production. As shown in Fig. 4c and Table 5(c), nisin activity (AU/ml) and productivity parameters ($Y_{p/x}$, $Y_{p/s}$, P, and Q_p) increased when supplementation of casein hydrolysate to whey permeate increased from 5 to 10 g/l. Optimum nisin production was observed in whey permeate containing 10–20 g/l casein hydrolysate; these fermentation conditions produced the highest nisin activity observed in this investigation (5.1×10^4 AU/ml). Further increase in casein hydrolysate concentration decreased nisin activity (Fig. 4c).

3.4.4. Supplement combinations

Supplementation of whey permeate with casein hydrolysate (5 g/l), yeast extract (5 g/l), or their combinations (total of 5 g/l) were tested in the continuous culture for nisin production. Although the cell dry weight increased with the use of a combination of yeast extract and casein hydrolysate, nisin activities were similar among these different supplementations (Fig. 5). When Tween 80 (5 g/l) was added to the medium, nisin activity was doubled at all medium compositions tested, although the presence of Tween 80 did not have an effect on the biomass (Fig. 5).

3.5. Cell density and efficiency of immobilization

After continuously operated for 6 months, the bioreactor was disassembled and cell dry weight in the bioreactor was 52.0 g/l with about 96.4% of the cells being immobilized. Scanning electronic micrographs (Fig. 6) show that cell immobilization was achieved by natural attachment on the fiber surfaces and entrapment in the void volume within the fibrous matrix. Cell immobilization was effective as shown by the high cell density in the bioreactor, the SEM graphs, and

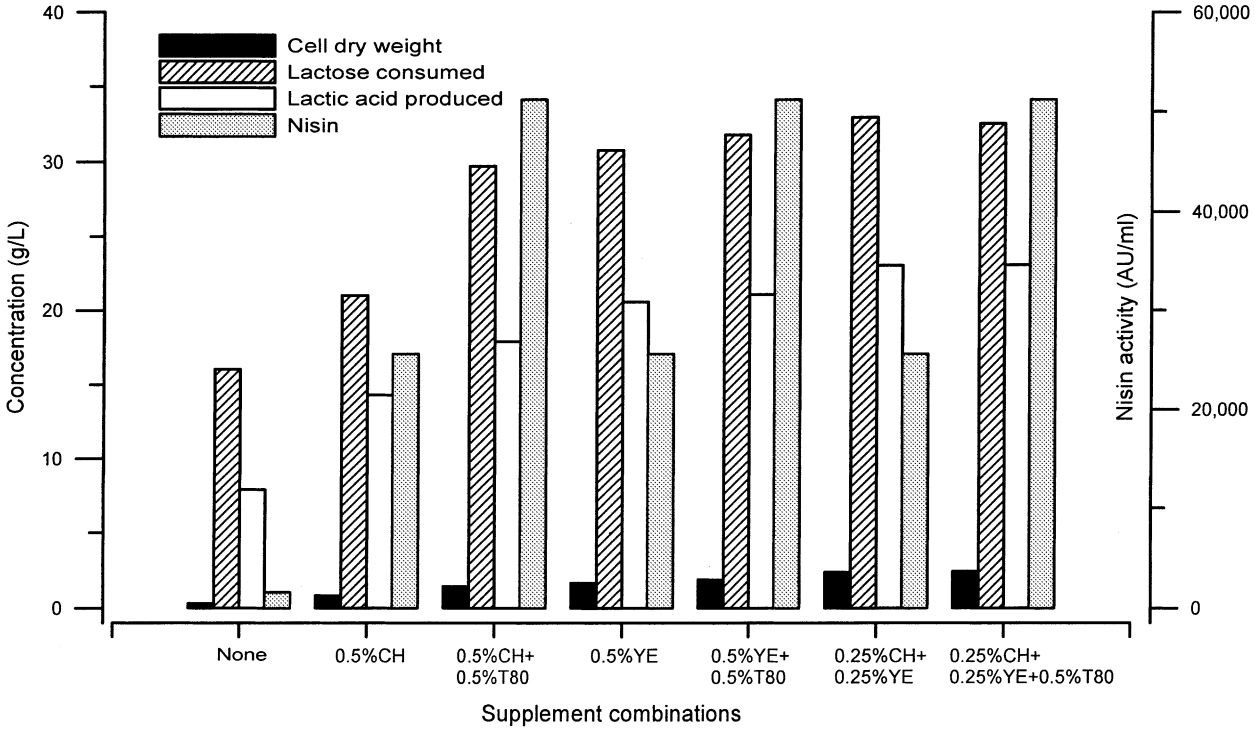

Fig. 5. Nisin production and growth parameters during fermentation of whey permeate, supplemented with different nitrogen sources and Tween 80, by immobilized *L. lactis* subsp. *lactis* ATCC 11454 in a packed-bed bioreactor at 31 °C, pH 5.5, and a dilution rate of 0.2/h. CH, casein hydrolysate; YE, yeast extract; T80, Tween 80.

sustaining the high speed medium circulation (ca. 3.7 l/h) in the bioreactor.

4. Discussion

Bacteriocins are generally considered primary metabolites, that is, their production is associated with the growth of producing strains. Therefore, condition that promote higher cell density, usually result in greater bacteriocin production. This growth-bacteriocin association was observed in cultures producing nisin [29,34,35], pediocin PO2 [30], pediocin AcH [47], leuconosin S [48], sakacin A [47], and bavaricin [49]. Maximal bacteriocin production corresponds to the late exponential or early stationary growth phase [30,48] and maximum cell concentration [50,51]. Results of batch fermentations in the current study (Fig. 1) are consistent with these earlier observations. Production of nisin in the bioreactor is also associated with cell mass, but depends to a greater extent on the metabolic activity of the immobilized cells. Production of nisin during the continuous fermentation of M17, for example, varied with reactors operating conditions such as dilution rate, temperature and pH of fed medium (Fig. 2). These operating variables altered biomass and nisin production independently.

Information about nisin production were mostly obtained from batch cultures using synthetic media with glucose or sucrose as a carbon source [7,31–36,52]. De Vuyst and Vandamme [34] described the drawbacks of batch culture for nisin production due to the lack of control over growth rate, and suggested that a continuous process could be suitable for nisin production. Continuous nisin production was addressed in a limited number of studies. Meghrous et al. [37] produced nisin by a free-cell continuous culture of *L. lactis* using Elliker broth which contains lactose as a carbon source. Our study, however, showed that Elliker broth was less suitable than M17 or MRS broth in supporting the growth and nisin formation by *L. lactis* in batch culture (Table 1). Using M17 broth, which also contains lactose as a carbon source, in the continuous fermentation by immobilized *L. lactis*, we found that nisin formation was greatly influenced by medium dilution rate. Nisin production was optimum at a dilution rate of 0.2–0.3/h, which is similar to that reported by Meghrous et al. [37] in free-cell continuous culture. The maximum nisin activity in these two studies could not be compared directly because of differences in methodologies and indicator microorganisms used for nisin bioassay.

Wan et al. [38] used calcium alginate-immobilized bacteria to continuously produce bacteriocins, including nisin. The authors cautioned that stability of calcium alginate beads was low or moderate and, therefore, the continuous fermentation lasted for 92 h only. Calcium alginate and similar natural matrices are not suitable for long-term fermentation because of instability of the gels [53]. In addition, Zezza et al. [54] showed that calcium alginate-immobilized *L. lactis* produced only 0.1% of the amount of nisin obtained

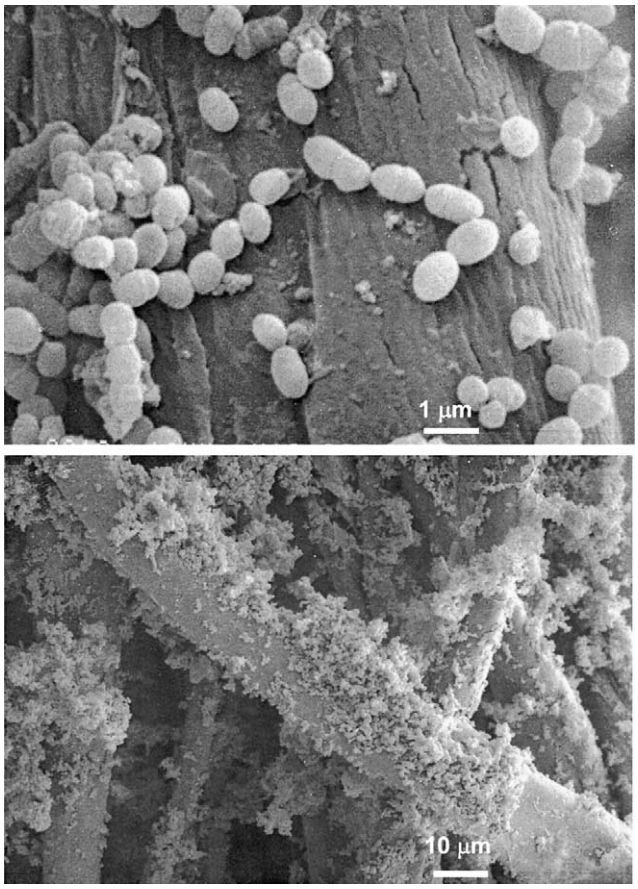

Fig. 6. Micrographs from the scanning electron microscope, illustrating *L. lactis* subsp. *lactis* ATCC 11454 cell attachment onto the fibrous matrix.

from free-cell fermentation. In the current investigation, a fibrous, packed-bed, immobilized-cell bioreactor was used for continuous nisin production. High cell density of ca. 52.0 g/l has been achieved in the bioreactor and cells were immobilized through natural attachment to fiber surfaces (Fig. 6) or entrapment in the void volume within the fibrous matrix. Cell immobilization was effective as indicated by cells sustaining high speed medium circulation in the bioreactor, and shown by the high cell density and the SEM micrographs. The bioreactor was continuously operated for 6 months without encountering any clogging, degeneration, or contamination problems. A similar bioreactor setup has been successfully used by Cho et al. [42] to continuously produce pediocin, a bacteriocin from *P. acidilactici*. The long term stability of similar bioreactor setups was proven in several studies [25,26,40,41].

Factors influencing nisin production have been reported previously [33–36,47,55]. These include nutritional factors such as carbon, nitrogen, and phosphorous sources, initial and final pH, temperature, and aeration. pH is an important factor for the production of bacteriocins [36,47,49,50,56–58]. The optimal pH is lower for bacteriocin production than for bacterial growth [56,58]. pH may affect bacteriocin production by different mechanisms. Environmental pH during fermentation may alter microbial growth which subsequently modifies bacteriocin production, adsorption and desorption of bacteriocins to cell membrane [22,58], enzymic activities required for post-translational modification or secretion of bacteriocins from the cells [59], and gene regulation for the biosynthesis of bacteriocins. The optimal pH for nisin production has been reported to be 5.5–6.0 in both batch [60] and continuous [37] cultures. In this study, similar optimum pH values for nisin production were observed. According to Kolot [61], changes in cell metabolism may be seen after immobilization; these include shift of pH optimum by 0.5–1.0 and temperature optimum by 10 °C, and decrease in cell generation time. Attachment of cells to a matrix may enhance cell-to-cell interaction and communication, a phenomenon that may alter cell metabolism. In the current study, immobilization of *L. lactis* in the fibrous packed-bed bioreactor did not seem to cause noticeable metabolic shift such as those reported earlier.

Typically, whey permeate from sweet whey contains 4.9% lactose, 0.03% protein, 0.1% non-protein nitrogen, 0.5% ash, 0.15% lactic acid, and less than 0.01% fat [62]. The lactose in whey permeate is a suitable carbon source for many microorganisms. In addition, whey permeate is rich in minerals and contains vitamins which may provide valuable nutrients to stimulate bacterial growth and bacteriocin production. Our study showed that whey permeate without supplementation supported the growth and nisin production by *L. lactis* in batch culture, although the amounts of growth and nisin activity were less than those obtained when the permeate was supplemented (Table 4). Guerra et al. [29] also demonstrated the feasibility of the production of nisin and pediocin in whey, although the biomass and bacteriocin activity were lower than those obtained in MRS medium. With supplementation of certain nitrogen source, particularly casein hydrolysate, growth of *L. lactis* and nisin production were greatly increased (Table 4). Guerra et al. [29] also showed enhanced production of nisin in whey supplemented with yeast extract and casitone. Whey permeate was also used as a medium for pediocin production from *P. acidilactici* when it was supplemented with yeast extract [29,30].

In the current investigation, maximal nisin activities in M17 broth and in whey permeate supplemented with 0.5% casein hydrolysate were comparable at a dilution rate of 0.2/h (Figs. 2 and 4; Tables 3 and 5). When the supplementation of casein hydrolysate increased to 10–20 g/l, the maximal nisin activity was twice as much as that from M17 broth. Nisin activity doubled when Tween 80 (5 g/l) was added to whey permeate supplemented with casein hydrolysate, yeast extract, or their combinations (Fig. 5). Several studies showed that Tween 80 stimulated bacteriocin production [56,63,64]. Daba et al. [56] observed a four-fold amplification of mesenterocin-5 activity when Tween 80 was added to whey permeate (1 g/l), with no difference observed when this surfactant was used in the range of 1–20 g/l. Vignolo et al. [63] reported similar results for the production of lactocin with no difference found in the use of Tween 80 at

5–20 g/l. It appeared that Tween 80 affects cell permeability to promote both uptake and release of compounds from the cell through modification of cytoplasmic membrane permeability [65]. Amiali et al. [51] proposed that Tween 80 may increase soluble bacteriocin due to the release of cell-bound product.

Supplementation of whey permeate with casein hydrolysate increased maximum specific growth rate of the nisin producing strain (Table 4); this permits operation of the bioreactor at a high dilution rate which maximizes bioreactor productivity without cell wash-out. About 70% of lactose in whey permeate was utilized when pH was maintained at 6.5 in the continuous culture. In conclusion, high concentrations of nisin were successfully produced during continuous fermentation of supplemented whey permeate by a lactic acid bacterium that was immobilized in a packed-bed bioreactor. Optimal conditions for continuous nisin production from whey permeate were defined. With the use of whey permeate and the immobilized-cell bioreactor, nisin could be produced economically and effectively. The bioreactor showed long term stability and should be scalable for industrial applications.

References

[1] Tagg JR, Dajani AS, Wannamaker LW. Bacteriocins of Gram-positive bacteria. Bacteriol Rev 1976;40:722–56.
[2] Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 1993;12:39–86.
[3] Abee T. Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Microbiol Lett 1995;129:1–10.
[4] Jack RW, Tagg JR, Ray B. Bacteriocins of Gram-positive bacteria. Microbiol Rev 1995;59:171–200.
[5] Daw MA, Falkiner FR. Bacteriocins: nature, function and structure. Micron 1996;27:467–79.
[6] Hugenholtz J. Bacteriocins as preservatives in food. Bull Int Dairy Fed 1998;329:6–8.
[7] Hurst A. Nisin Adv Appl Microbiol 1981;27:85–123.
[8] Hurst A. Nisin and other inhibitory substances from lactic acid bacteria. In: Braun AL, Davidson PM, editors. Antimicrobials in foods. London, UK: Marcel Dekker, 1983.
[9] Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. Applications of the bacteriocin, nisin. Anton van Leeuw 1996;69:193–202.
[10] Delves-Broughton J. Nisin Bull Int Dairy Fed 1998;329:9–12.
[11] Buchman GW, Banerjee S, Hansen JN. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J Biol Chem 1988;263:16260–6.
[12] Nettles GG, Barefoot SF. Biochemical and characteristics of bacteriocins of food-associated lactic acid bacteria. J Food Prot 1993;56:338–56.
[13] Delves-Broughton J. Nisin and its uses as a food preservative. Food Technol 1990;44:100–17.
[14] Turtell A, Delves-Broughton J. International acceptance of nisin as a food preservative. Bull Int Dairy Fed 1998;329:20–3.
[15] FDA. Nisin preparation; affirmation of GRAS status as a direct human food ingredient. Federal Register 1988;53:11247–51.
[16] Thomas LV, Clarkson MR, Delves-Broughton J. Nisin. In: Naidu N, editor. Natural food antimicrobial systems. Florida: CRC Press. 2000. pp. 463–523.
[17] Jelen P. Industrial whey processing technology: an overview. J Agric Food Chem 1979;27:658.
[18] Roy D, Goulet J. Continuous production of lactic acid from whey permeate by free and calcium alginate entrapped *Lactobacillus helveticus*. J Dairy Sci 1987;70:722–56.
[19] Aeschlimann A, Distasi L, Vonstockar U. Continuous production of lactic acid from whey permeate by *Lactobacillus helveticus* in two chemostats in series. Environ Microbiol Technol 1990;12:926–32.
[20] Norton S, Lacroix C, Vuillemard JC. Reduction of yeast extract supplementation in lactic acid fermentation of whey permeate by immobilized cell technology. J Dairy Sci 1993;77:2494–508.
[21] Tejayadi S, Cheryan M. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. Appl Microbiol Biotechnol 1995;43:242–8.
[22] Yang ST, Tang IC, Zhu H. A novel fermentation process for calcium magnesium acetate (CMA) production from cheese whey. Appl Biochem Biotechnol 1992;34–35:569–83.
[23] Colomban A, Roger L, Boyaval P. Production of propionic acid from whey permeate by sequential fermentation ultrafiltration, and cell recycling. Biotechnol Bioeng 1993;42:1091–8.
[24] Paik HD, Glatz BA. Propionic acid production by immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl Microbiol Biotechnol 1994;42:22–7.
[25] Yang ST, Huang Y, Hong G. A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol Bioeng 1995;45:379–86.
[26] Yang ST, Zhu H, Li Y, Hong G. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotechnol Bioeng 1994;43:1124–30.
[27] Giec A, Kosikowski FV. Activity of lactose-fermenting yeast in producing biomass from concentrated whey permeate. J Food Sci 1982;47:1992–3.
[28] Ghaly AE, Ben-Hassan RM, Ben-Abdallah N. Utilization of cheese whey lactose by *Kluyveromyces fragilis* for energy and growth under continuous fermentation. Appl Biochem Biotechnol 1992;36:13–34.
[29] Guerra NP, Rua ML, Pastrana L. Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. Int J Food Microbiol 2001;70:267–81.
[30] Liao CC, Yousef AE, Richter ER, Chism GW. *Pediococcus acidilactici* PO2 bacteriocin production in whey permeate and inhibition of *Listeria monocytogenes* in foods. J Food Sci 1993;58:430–4.
[31] Hirsh A. Growth and nisin production of a strain of *Streptococcus lactis*. J Gen Microbiol 1951;5:208–21.
[32] Egorov NS, Baranova IPK, Yu I. Optimization of nutrient medium composition for the production of the antibiotic nisin by *Streptococcus lactis*. Microbiologia 1971;40:993–8.
[33] De Vuyst L. Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. *lactis* NIZO 22186 in a synthetic medium. J Appl Bacteriol 1995;78:28–33.
[34] De Vuyst L, Vandamme EJ. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentation. J Gen Microbiol 1992;138:571–8.
[35] De Vuyst L, Vandamme EJ. Influence of the phosphorus and nitrogen source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations using a complex medium. Appl Microbiol Biotechnol 1993;40:17–22.
[36] Cabo ML, Murado MA, González MP, Pastoriza L. Effects of aeration and pH gradient on nisin production. A mathematical model. Enz Microbiol Technol 2001;29:264–73.
[37] Meghrous J, Huot E, Quittelier M, Petitdemange H. Regulation of nisin biosynthesis by continuous cultures and by resting cells of *Lactococcus lactis* subsp. *lactis*. Res Microbiol 1992;143:879–90.
[38] Wan J, Hickey MW, Coventry MJ. Continuous production of bacteriocins, brevicin, nisin and pediocin, using calcium alginate-immobilized bacteria. J Appl Bacteriol 1995;79:671–6.

[39] Dervakos GA, Webb C. On the merits of viable-cell immobilization. Biotech Adv 1991;9:559–612.
[40] Lewis VP, Yang ST. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. Biotechnol Bioeng 1992;40:465–71.
[41] Silva EM, Yang ST. Kinetics and stability of a fibrous-bed bioreactor for continuous production of lactic acid from unsupplemented acid whey. J Biotechnol 1995;41:59–70.
[42] Cho HY, Yousef AE, Yang ST. Continuous production of pediocin by immobilized *Pediococcus acidilactici* PO2 in a packed-bed bioreactor. Appl Microbiol Biotechnol 1996;45:589–94.
[43] Elliker PR, Anderson AW, Hammesson G. An agar culture medium for lactic acid streptococci and lactobacilli. J Dairy Sci 1956;39: 1611–2.
[44] Kosikowski FV, Mistry VV. Cheese and fermented milk foods, vol. I, 3rd ed. Connecticut: FV Kosikowski L.L.C., 1997.
[45] Barefoot SF, Klaenhammer TR. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol 1983;45:1808–15.
[46] Geis A, Singh J, Teuber M. Potential of lactic streptococci to produce bacteriocin. Appl Environ Microbiol 1983;45:205–11.
[47] Yang R, Ray B. Factor influencing production of bacteriocins by lactic acid bacteria. Food Microbiol 1994;11:281–91.
[48] Baker RC, Winkowski K, Montville TJ. pH-controlled fermentors to increase production of leuconocin S by *Leuconostoc paramesenteroides*. Proc Biochem 1996;31:225–8.
[49] Kaiser AL, Montville TJ. The influence of pH and growth rate on production of the bacteriocin, bavaricin MN, in batch and continuous fermentations. J Appl Bacteriol 1993;75:536–40.
[50] Parente E, Ricciardi A, Addario G. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* ssp. *lactis* 14NWC during batch fermentation. Appl Microbiol Biotechnol 1994;41:388–94.
[51] Amiali MN, Lacroix C, Simard RE. High nisin Z production by *Lactococcus lactis* UL719 in whey permeate with aeration. World J Microbiol Biotechnol 1998;14:887–94.
[52] Chandrapati S, O'Sullivan DJ. Procedure for quantifiable assessment of nutritional parameters influencing nisin production by *Lactococcus lactis* subsp. *lactis*. J Biotechnol 1998;63:229–33.
[53] Sonomoto K, Chinachoti N, Endo N, Ishizaki A. Biosynthetic production of nisin Z by immobilized *Lactococcus lactis* IO-1. J Mol Catal B: Enzymatic 2000;10:325–34.
[54] Zezza N, Pasini G, Lombardi A, Mercenier A, Spettoli P, Zamorani A, Nuti MP. Production of a bacteriocin active on lactate-fermenting clostridia by *Lactococcus lactis* subsp. *lactis* immobilized in coated alginate beads. J Dairy Res 1993;60:581–91.
[55] Desjardins P, Meghrous J, Lacroix C. Effect of aeration and dilution rate on nisin Z production during continuous fermentation with free and immobilized *Lactococcus lactis* UL719 in supplemented whey permeate. Int Dairy J 2001;11:943–51.
[56] Daba H, Lacroix C, Huang J, Simard RE. Influence of growth conditions on production and activity of mesenterocin 5 by a strain of *Leuconostoc mesenteroides*. Appl Microbiol Biotechnol 1993;39:166–73.
[57] Mortvedt-Abildgaard CI, Nissen-Meyer J, Jelle B, Grenov B, Skaugen M, Nes IF. Production of pH-dependent bactericidal activity of lactocin S, a lantibiotic from *Lactobacillus sake* L45. Appl Environ Microbiol 1995;61:175–9.
[58] Parente E, Ricciardi A. Influence of pH on the production of enteriocin 1146 during batch fermentation. Lett Appl Microbiol 1994;19:12–5.
[59] Ray B. Pediocin(s) of *Pediococcus acidilactici* as a food biopreservatives. In: Ray B, Daeschel MA, editors. Food biopreservatives of microbial origin. Florida: CRC Press, 1992. pp. 265–332.
[60] Benkerroum N, Sandine WE. Inhibitory action of nisin against *Listeria monocytogenes*. J Dairy Sci 1988;71:3237–45.
[61] Kolot FB. Immobilized microbial system—principles, techniques and industrial applications. Florida: RE Krieger Publishing Co., 1988.
[62] Coton G. The utilization of permeates from the ultrafiltration of whey and skim milk. Int Dairy Fed Bull Doc 1980;126:23–33.
[63] Vignolo GM, De Kairuz MN, De Ruiz Holgado AAP, Oliver G. Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. J Appl Bacteriol 1995;78:5–10.
[64] Huot E, Barrena-Gonzalez C, Petitdemange H. Tween 80 effect on bacteriocin synthesis by *Lactococcus lactis* subsp. *cremoris* J46. Lett Appl Microbiol 1996;22:307–10.
[65] Reese ET, Maguire A. Surfactants as stimulants of enzyme production by microorganisms. Appl Microbiol 1969;17:242–5.