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# Characterization of Bacteriocin-Producing Lactic Acid Bacteria Strain N1-33 Isolated from Edible Fermented Bamboo Shoot

**Melaku Alemu[1,5], Panthipa Laemkom[2,5], Katsumi Doi[3], Sadahiro Ohmomo[2,4], Mizuo Yajima[4] and Sunee Nitisinprasert[5,*]**


## ABSTRACT

Lactic acid bacteria (LAB) strain N1-33, isolated from edible fermented bamboo shoot, displayed broad spectrum of inhibitory activity against LAB and other Gram-positive food spoilage and pathogenic bacteria. On the basis of its physiological and biochemical properties together with 16S rRNA sequence analysis, N1-33 was identified as *Enterococcus faecalis*. It was cultivated in MRS broth at 30°C to early stationary growth phase and the resulting cell-free culture supernatant (CS) showed a maximum inhibitory activity of 12800 AU/ml against *Lactobacillus sakei* subsp. *sakei* JCM 1157$^T$, with bactericidal mode of action and concomitant cell lysis. The antibacterial activity of the CS was completely destroyed with proteinase K and partially inactivated with pepsin treatments. Moreover, the study on its physicochemical stability indicated that it was heat tolerant up to 121°C and remained bioactive over a pH range of 2-11. Thus, the spectrum of inhibitory activity, sensitivity to proteolytic enzymes and thermostability of the CS clearly suggested that the bioactive substances had proteinaceous structures and hence classified as bacteriocins. The fact that *Enterococcus faecalis* N1-33 was isolated from edible fermented bamboo shoot together with these desirable physicochemical properties of its bacteriocin-activity and its ability of metabolizing low cost carbohydrates strongly suggested its great potential for use in food biopreservation. This was known to be the first report of the production of bacteriocins from LAB of fermented bamboo shoot.


**Key words:** bacteriocin-activity, bamboo shoot, *Enterococcus faecalis*, lactic acid bacteria

## INTRODUCTION

Lactic acid bacteria (LAB) have been extensively investigated for their potential uses as food biopreservatives and probiotics because of their abilities to produce several functional metabolites and health promoting effects. The preservative effect of LAB is attributed to the production of bacteriocins and other antimicrobial compounds (Soomro *et al.*, 2002).

Bacteriocins of LAB constitute a heterogeneous group of ribosomally synthesized


1 Ethiopian Agricultural Research Organization, Ambo PPRC, P.O. Box 2003, Addis Ababa, Ethiopia.

2 Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agriculture, Forestry and Fisheries (MAFF), Division of Soil Science, Department of Agriculture, Bangkok 10900, Thailand.

3 Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Graduate School, Kyushu University, Japan.

4 Asama Chemical Co., Ltd., 20-3 Nihonbashi Kodenmacho, Chou-ku, Tokyo 103, Japan.

5 Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: fagisnn@ku.ac.th


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and extracellularly released proteins or peptides that display antibacterial activity against not only closely related LAB species but also other Gram-positive bacteria, including food spoilage and pathogenic bacteria (O'Sullivan *et al.,* 2002). Because of this beneficial trait, either bacteriocin-producing LAB starter cultures or their bacteriocins have gained increasing interest as a new food preservation method to control food spoilage and pathogenic bacteria (Chen and Hoover, 2003). As a matter of fact, the role of LAB and their bacteriocins as food biopreservatives may also increase in the future as a result of consumer awareness of the potential risks derived not only from foodborne pathogens but also from the artificial chemical preservatives currently used to control them (Hugas *et al.,* 2003). Consequently, this new trend has encouraged a great deal of research to discover new LAB strains endowed with broader or novel technological applications. As a result, numerous bacterioicin-producing LAB have been isolated and characterized from various sources including fermented food products. Among these sources, fermented vegetables have been reported to contain bacteriocin-producing LAB such as *Enterococcus mundtii* (Bennik *et al.,* 1998), *Lactococcus lactis* (Franz *et al.,* 1997) and *Leuconostoc* sp. J2 (Choi *et al*., 1999) and *Pediococcus parvulus* (Bennik *et al*., 1997).

Bamboo shoot is a kind of edible greens that serves as vegetable which can be consumed fresh or cooked, dried, pickled and fermented (http://www.basicallybamboo.com, 01 June 2005). Taking into account the major role of LAB in Thai fermented foods, Dhavises (1972) examined fermented bamboo shoot and showed that *Lactobacillus brevis, Lactobacillus plantarum* and *Pediococcus pentosaceus* were the major species. Interestingly, these same LAB species were also reported to be the predominant species in fermented bamboo shoot of India (Tamang and Sarkar, 1996). It was evident that these two similar microbiology studies merely revealed the succession of LAB species in fermented bamboo shoot but did not investigate the presence of bacteriocin-producing LAB. On the other hand, Rattanachaikunsopon and Phumkhachorn (2000) evaluated various Thai fermented foods, including fermented bamboo shoot, for the presence of bacteriocin-producing LAB species. However, they reported that LAB strain isolated from fermented bamboo shoot did not show bacteriocin-activity against *Leuconostoc mesenteroides* TISTR 473 and hence was not identified. In contrast, these studies on LAB strain N1-33, isolated from the same source, revealed that this strain displayed broad spectrum of bacteriocin-activity against LAB, including *Leuconostoc mesenteroides* TISTR 473, and other Gram-positive food spoilage and pathogenic bacteria. This study describe the characterization of this bacteriocin-producing LAB strain N1-33 and preliminary observation on its potential technological application in the food industry as a natural food preservative.

## MATERIALS AND METHODS

### Bacterial strains, media and growth conditions

LAB strain N1-33, isolated from edible fermented bamboo shoot, was used in the study. This strain and sixteen indicator strains were obtained from the culture collection of the Department of Biotechnology, Kasetsart University, Thailand. The additional three indicating strains namely *Lactobacillus sakei* subsp. *sakei* JCM 1157$^T$, *Listeria innocua* ATCC 33090$^T$ and *Listeria innocua* LTH 3096 were obtained from Prof. Dr. Kenji Sonomoto, Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Japan. LAB strains were grown in MRS broth (Merck, Darmstadt, Germany) at 30-37°C, under anaerobic condition and routinely maintained at 4°C on MRS agar slant cultures. Other indicator strains were cultivated in nutrient broth (Pronadisa, Madrid, Spain) at 37°C, except *Campylobacter* which was

grown in Columbia blood agar base and Brucella broth base (Himidia, India) at 42°C.

**Antibacterial spectrum of the cell-free culture supernatant of N1-33**

LAB strain N1-33 was cultivated in MRS broth at 30°C until early stationary growth phase under anaerobic condition (to exclude the effect of hydrogen peroxide) and then the cells were removed by centrifugation (5369 x g for 15 min at 4°C). The resulting cell-free culture supernatant (CS) was neutralized to pH 6.0 with NaOH solution (to exclude the effect of organic acids) and evaluated for its antibacterial activity using the spot-on-lawn method described in the previous study (Alemu *et al.,* 2003).

**Determination of morphology and other physical characteristics**

LAB strain N1-33 was subject to Gram-staining reaction and examined by phase contrast microscope to determine the cell morphology. The tests of motility, catalase and spore-forming and ability to produce $CO_2$ gas were investigated using the methods described by Brown (2005). Hemolysin activity was tested on NA and MRS agar plates containing 7% sheep blood, similar to the method used by De Vuyst *et al.* (2003).

**Determination of the growth on various temperatures, pH and NaCl concentrations**

The growth of LAB strain N1-33 at temperatures 10, 30, 37, 45 and 50°C; at pH 4.5 and 9.6; and in MRS broth supplemented with 6.5 and 18 % NaCl was determined using the methods described in the previous study (Alemu *et al.*, 2003).

**Carbohydrate fermentation pattern**

Carbohydrate fermentation patterns were determined, simultaneously at 30 and 37°C, with API 50CH Rapid fermentation strips (API, BioMérieux, France) in CHL medium as specified by the manufacturer.

**16S rDNA sequence analysis**

The entire 16S rDNA of LAB strain N1-33 was amplified by PCR and directly sequenced using the method of Mori *et al.* (1997). The 16S rDNA sequences of related microorganisms were obtained from the database of GenBank and sequences were aligned using the DNASIS program (Hitachi Software, Japan).

**Physicochemical stability of the inhibitory activity of CS of N1-33**

The effects of enzymes, heat treatment and pH on the inhibitory activity of the CS of N1-33 were evaluated. Proteolytic, lipolytic and amylolytic enzymes (all from Sigma, St. Louis, Montana, USA) were added to CS of N1-33 at a final concentration of 1 mg/ml. All preparations were incubated at appropriate temperature for 24 h and then enzymes were heat-inactivated at 100° C for 3 min. Thermostability of the inhibitory activity of CS was determined by heating aliquots of CS at 100, 110 and 121°C for a duration of 15-30 min. The effect of pH on bacteriocin activity was evaluated by adjusting the CS to pH 2-12 with NaOH/HCl solutions and incubation at 37°C for 24 h. The CS was also stored at 4 and -20°C for 1 month to determine its stability under food storage conditions. To test the effect of pH on the thermostability, the CS adjusted to pH 2-12 was heated at 100°C for 15 min. After all these treatments, samples were re-adjusted back to pH 6.0 and the residual bacteriocin activity was determined against *Lb. sakei* subsp. *sakei* JCM $1157^T$, where untreated CS pH 6.0 was used as a control, similar to the method described by Yamamoto *et al.* (2003).

**Effect of temperature on the growth of N1-33 and production of bacteriocin**

The effect of temperature on the growth of N1-33 and production of bacteriocin was studied. Thus, MRS broths were inoculated at 1% (v/v) level with an overnight culture of LAB strain N1-33 to $1.07\times10^7$ cfu/ml and then incubated at

various temperatures: in the refrigerator (4-8°C), 10, 25, 30, 37 and 45°C. Aliquots of samples were collected from the refrigerator and 10°C after 24 h, whereas samples from cultures incubated at 25-45°C at every 2 h intervals for a period of 24 h. All samples were examined for viable cell count (cfu/ml), cell density (OD at 600 nm), pH and bacteriocin activity of the culture supernatant (CS), as previously described by Sabia *et al.* (2002).

**Mode of action of the CS of N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$**

The mode of action of N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was determined basically as described by Balla *et al.* (2000). Briefly, *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was inoculated to 100 ml of MRS broth, at 1% (v/v) level corresponding to $3.65\times10^6$ cfu/ml, and grown at 30°C until mid-exponential growth phase, from which a total of 10 ml sample was collected at 0 and 2 h to monitor the growth. Then, at 4 h growth, 10 ml of neutralized and filter-sterilized CS of N1-33 (crude bacteriocin) was added to it, which would correspond to a final bacteriocin concentration of 1280 AU/ml. Another culture of *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was simultaneously grown to serve as a control, into which 10 ml MRS broth was added instead of crude bacteriocin. Aliquots of samples, from both treated and untreated cultures, were collected at every 2 h intervals and examined for viable cell count (cfu/ml), cell density (OD at 600 nm) and pH of the culture broth.

## RESULTS

**Identification of LAB strain N1-33**

LAB strain N1-33 was Gram-positive, coccus, catalase negative, non-motile and non-sporulating. It grew at both 10°C and 45°C, in 6.5% NaCl broth and at pH 4.5 to 9.6 and fermented glucose in a homofermentative manner. These properties strongly suggested that it belongeds to the genus *Enterococcus* (Domig *et al.*, 2003). According to latest taxonomic reports, the genus *Enterococcus* comprise more than 26 species (Klein, 2003), with *Enterococcus faecalis* and *Enterococcus faecium* being the two species mostly found especially in food related habitat (Giraffa, 2003). It has been well-established that *Enterococcus faecalis* and *Enterococcus faecium* can be easily discriminated with regard to carbohydrate fermentation pattern particularly on the basis of their abilities to ferment L-arabinose and sorbitol (Manero and Blanch, 1999). The carbohydrate fermentation pattern of N1-33 (Table 1) revealed that this strain metabolized sorbitol but not L-arabinose, which unambiguously identified it as the strain of *Enterococcus faecalis*. Moreover, its inability to grow at 50°C also supported this biochemical identification (Klein, 2003).

Accordingly, all of the phenotypic characteristics of N1-33 were found to be in good agreement with those reported for *Enterococcus faecalis* strains. Their difference was related to the ability of metabolizing 3 out of 49 substrates namely glycogen, D-Turanose and 5-keto-gluconate, while the profile with respect to the other 46 substrates were identical for N1-33 and other *E. faecalis* strains. Comparison of their carbohydrate fermentation pattern indicated that (i). N1-33 metabolized glycogen (Table 1), whereas *E. faecalis* SE-K4 and other strains did not ferment this substrate, and (ii). *E. faecalis* SE-K4 metabolized D-Turnose and 5-keto-gluconate (Eguichi *et al.*, 2001), whereas N1-33 and other *E. faecalis* strains (Manero and Blanch, 1999) did not ferment these two substrates. Furthermore, sequencing of the entire 16S rDNA of N1-33 revealed close similarity to that of *Enterococcus faecalis* V583 (99.93% identity) (Meziane-Cherif *et al.*, 1994). Therefore, on the basis of its morphology, physiology and 16S rDNA sequence analysis, strain N1-33 was identified as *Enterococcus faecalis* and hence designated *Enterococcus faecalis* N1-33. This strain grew on NA and MRS agar containing 7% sheep blood,

**Table 1** Carbohydrate fermentation pattern of LAB strain N1-33 at 37°C.

| Test | Characteristic | Test | Characteristic |
|---|---|---|---|
| 1. Glycerol | + | 26. Salicin | + |
| 2. Erythritol | - | 27. Cellubiose | + |
| 3. D-Arabinose | - | 28. Maltose | + |
| 4. L-Arabinose | - | 29. Lactose | + |
| 5. Ribose | + | 30. Melibiose | - |
| 6. D-Xylose | - | 31. Sucrose | + |
| 7. L-Xylose | - | 32. Trehalose | + |
| 8. Adonitol | - | 33. Inulin | - |
| 9. β-Methyl-D-xylose | - | 34. Melezitose | + |
| 10. Galactose | + | 35. D-Raffinose | - |
| 11. D-Glucose | + | 36. Starch | + |
| 12. D-Fructose | + | 37. Glycogen | + |
| 13. D-Mannose | + | 38. Xylitol | - |
| 14. L-Sorbose | - | 39. β-Gentiobiose | + |
| 15. Rhamnose | + | 40. D-Turnaose | - |
| 16. Dulcitol | - | 41. D-Lyxose | - |
| 17. Inositol | + | 42. D-Tagatose | + |
| 18. Mannitol | + | 43. D-Fucose | - |
| 19. Sorbitol | + | 44. L-Fucose | - |
| 20. α-Methyl-D-mannoside | - | 45. D-Arabitol | - |
| 21. α-Methyl-D-glucoside | - | 46. D-Arabitol | - |
| 22. N-Acetyl-glucosamine | + | 47. Gluconate | + |
| 23. Amygdalin | + | 48. 2-Keto-gluconate | - |
| 24. Arbutin | + | 49. 5-Keto-gluconate | - |
| 25. Esculin | + | | |

+, acid production (positive); -, no acid produced

without forming any clear zone of hydrolysis around the colonies, indicating the absence of hemolysin activity and hence no potential pathogenicity (De Vuyst *et al.*, 2003).

**Antibacterial spectrum of CS of N1-33**

Table 2 shows the antibacterial activity of neutralized and filter-sterilized cell free culture supernatant (CS) of *Enterococcus faecalis* N1-33 against selected indicator strains. The CS of *Enterococcus faecalis* N1-33 displayed broad spectrum of inhibition activity against LAB and other Gram-positive food spoilage and pathogenic bacteria. However, it did not inhibit the Gram-negative bacteria. Since maximum inhibition activity was displayed against *Lb. sakei* subsp. *sakei* JCM 1157$^T$, this indicator strain was chosen for the study on other properties of the bacteriocin activity of N1-33.

**Physicochemical stability of the bacteriocin activity of the culture supernatant**

The effects of enzymes and heat treatments on the antibacterial activity of CS are summarized in Table 3. The antibacterial activity of the CS was completely abolished with proteinase K and reduced by 75% with pepsin treatments, whereas, the CS was not affected by

lipolytic and amylolytic enzymes such as lipase and α-amylase, respectively. This result together with the spectrum of inhibitory activity clearly suggested that the bioactive substances had proteinaceous structures and hence classified as bacteriocins.

The antibacterial activity of CS remained unaffected when heated at 100°C for 15 min, but

**Table 2** Inhibition spectrum of the culture supernatant of *E. faecalis* N1-33 CS pH 6.0.

| Indicating strain | AU/ml |
|---|---|
| *Enterococcus faecalis* TISTR 927 | 400 |
| *Lactobacillus plantarum* TISTR 541 | 400 |
| *Lactobacillus sakei* TISTR 890 | 100 |
| *Lactobacillus sakei* TISTR 911 | 800 |
| *Lactobacillus sakei* TISTR 912 | 800 |
| *Lactobacillus sakei* subsp. *sakei* JCM 1157$^T$ | 12800 |
| *Leuconostoc mesenteroides* TISTR 473 | 100 |
| *Pediococcus acidilactici* TISTR 953 | 100 |
| *Bacillus cereus* (isolated from silage) | 100 |
| *Bacillus subtilis* TISTR 025 | 400 |
| *Listeria innocua* ATCC 33090$^T$ | 400 |
| *Listeria innocua* LTH 3096 | 400 |
| *Staphylococcus aureus* TISTR 029 | 100 |
| *Staphylococcus aureus* TISTR 118 | 200 |
| *Escherichia coli* TISTR 780 | 0 |
| *Escherichia coli* TISTR 727 | 0 |
| *Salmonella* Typhimurium TISTR 292 | 0 |
| *Campylobacter jejuni* ATCC 33291 | 0 |
| *Shigella* sp. | 0 |

**Table 3** Effect of enzymatic and heat treatments on the inhibitory activity of the CS.

| Treatment | Residual inhibitory activity (AU/ml)[a] |
|---|---|
| Enzymatic treatment | |
| Control, untreated CS | 12800 |
| Proteinase K (pH 7.5, 37°C)[b] | 0 |
| Pepsin (pH 2, 37°C) | 3200 |
| Lipase (pH 7.2, 37°C) | 12800 |
| α-Amylase (pH 6.9, 20°C) | 12800 |
| | |
| Heat treatment | |
| Control, unheated CS | 12800 |
| 100°C for 15 min | 12800 |
| 100°C for 30 min | 6400 |
| 110°C for 15 min | 6400 |
| 121°C for 15 min | 6400 |

[a] Inhibitory activity was determined against *Lb. sakei* subsp. *sakei* JCM 1157$^T$

[b] The parentheses indicate the pH of CS and incubation temperature for that enzyme

reduced by 50% after extended heating at 100°C for 30 min and 121°C for 15 min. On the other hand, incubation of the CS adjusted to pH 2-8, at 37°C for 24 h did not affect the inhibitory activity of the CS, while it reduced by more than 75% for CS of pH 9-12. On the other hand, when CS adjusted to pH 2-8 was heated at 100°C for 15 min, the inhibitory activity remained unaffected, but reduced by 75-95% for CS at pH 9-11 and completely lost at pH 12 (Figure 1). The bacteriocin activity of the CS was unaffected (12800 AU/ml) after storage at 4 and -20°C for 1 month.

**Effect of temperature on growth of N1-33 and production of bacteriocin**

The study on the effect of temperature on growth of N1-33 and production of bacteriocin indicated that no growth and bacteriocin production was found in the culture kept in the refrigerator (4-8°C) during the 24 h study period. But, when this culture was stored in the refrigerator for 1 month the viable cells count increased from $1.07\times10^7$ to $1.56\times10^7$ cfu/ml and a maximum bacteriocin activity of only 800 AU/ml was observed, in which the OD raised from 0.016 to 0.12 and pH dropped from 5.7 to 5.48, concurrently. This clearly indicated that the cells were still viable and slowly grew during 1 month storage time in the refrigerator. Whereas, bacteriocin production was observed at 10, 25, 30, 37, 45°C over the 24 h study period at varying degrees. In the case of 10°C the viable cells count increased from $1.07\times10^7$ to $1.68\times10^7$ cfu/ml, the OD raised from 0.017 to 0.59, the pH dropped from 5.7 to 5.5, and a maximum bacteriocin activity of only 800 AU/ml was observed after 24 h growth.

As shown in Figure 2, the growth trend in terms of viable cell count (cfu/ml) at these 4 temperatures were 25, 30, 45 and 37°C, with increasing order, whereas in terms of OD values, the trend were, 25, 30, 37 and 45°C with increasing order. In both ways, 37°C could be considered the optimum temperature for the best growth of *E. faecalis* N1-33.

It can be seen from Figure 3 that the

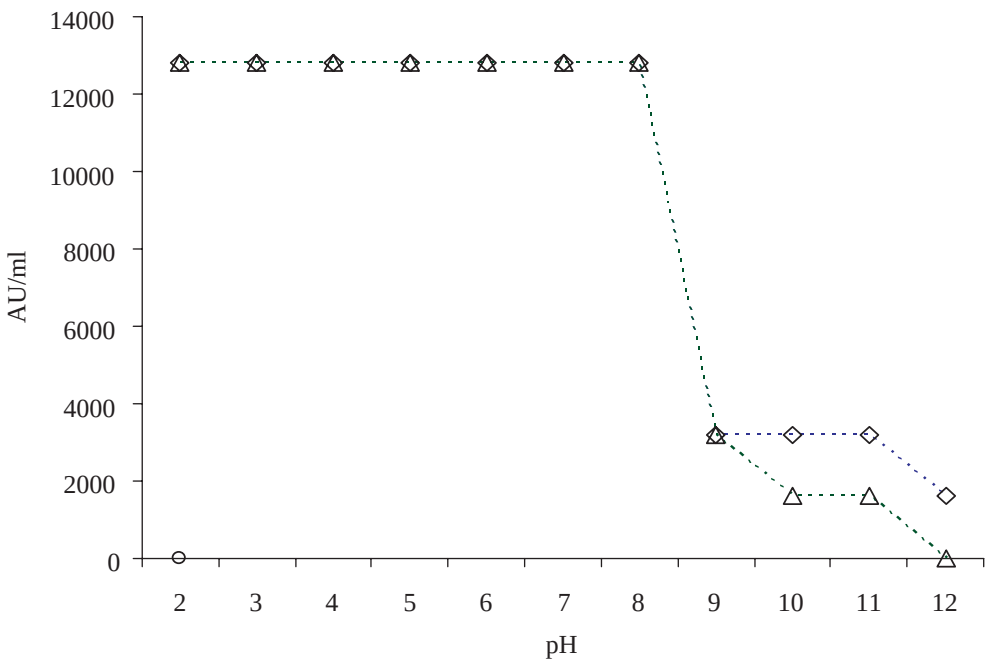


**Figure 1** Stability of CS (with 12800 AU/ml) at pH 2-12.
◊, after incubation at 37°C for 24 h ; Δ, after heating at 100°C for 15 min

production of bacteriocin at 25°C reaches a maximum of 6400 AU/ml after 18 h of growth and remain unchanged until the 24 h. At 30°C, bacteriocin activity of 6400 AU/ml was obtained after 10 h growth and reached the maximum of 12800 AU/ml in the early stationary growth phase (14 h) at 30°C, which was constantly maintained until the 24 h. The maximum bacteriocin produced at 37 and 45°C correspond to 6400 AU/ml, which remain unaffected at 37°C but declined at the end of stationary growth phase at 45°C. The production trends of acids at these temperatures were 25, 30, 37, 45°C with increasing order, meaning decreasing order of pH values. The highest growth at 37 and 45°C corresponded to the lowest pH values, meaning increased acids production at these 2 temperatures.

The results presented in Figures 2 and 3, clearly indicated that temperature critically affect the growth of *E. faecalis* N1-33 and production of bacteriocins and acids. Moreover, these results magnificently demonstrated the potentials of *E. faecalis* N1-33 for applications in both mesophilic and thermophilic conditions.

**Mode of action of CS of N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$**

The mode of action of CS of N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was investigated by comparing the viable cell count and turbidity of the treated and untreated cultures. It was found that within 10 min of addition of the CS of N1-33, *Lb. sakei* subsp. *sakei* JCM 1157$^T$ culture did not show viable cell, while the control contained 8.5 × $10^7$ cfu/ml (Figure 4). The treated culture was incubated for 12 h and examined for the viable cell count but no colony was found. This indicated that cells of *Lb. sakei* subsp. *sakei* JCM 1157$^T$ were all killed by the bacteriocin of N1-33. Accordingly, a concomitant and dramatic reduction of the OD was observed during the 24 h of growth, while the pH of the culture broth remained unchanged in the treated culture (Figure 5). These results clearly confirmed that re-growth of cells of *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was not observed during the rest of the study period.

## DISCUSSION

In this study *Enterococcus faecalis* N1-33 was characterized that displayed wide spectrum of inhibitory activity against both LAB and other Gram-positive food spoilage and pathogenic bacteria belonging to the genera *Bacillus*, *Listeria* and *Staphylococcus.* The inhibitory activity was resistant to heat treatment but sensitive to proteolytic enzymes. On the basis of its spectrum of inhibitory activity, proteinaceous nature and heat resistance, the bioactive substances of N1-33 can be classified as small heat-stable antilisteria peptides, presumably belong to class II bacteriocins (Chen and Hoover, 2003). This bacteriocin-activity remained unaffected upon exposure to pH-range of 2-8 but half of the activity was lost at pH 9-12. The reduction of bacteriocin-activity at higher pHs has been attributed to the low solubility and aggregation of the hydrophobic patches and conformational changes of bacteriocins molecules, which reduce their affinities for the cell surfaces of indicator strains (Jack *et al.*, 1995). The combined effect of pH and heating at elevated temperature has also exhibited similar trend. It has been reported that as pH increases, the heat stability of bacteriocins decreases (Chen and Hoover, 2003).

The profile of bacteriocin production at various temperatures showed a typical growth associated pattern because the onset of production occurred during the active growth phase. At 30-45°C, the inhibitory activity reached maximal at the beginning of stationary growth phases, as observed for other bacteriocins. The culture grown at 30°C produced maximum bacteriocin-activity, without declining up to the end of study period. But, the cell density, viable cell count, at 30°C were less than at 37°C and 45°C. In contrast, at 37°C and 45°C the bacteriocin-activities were almost half of that produced at 30°C. Furthermore,

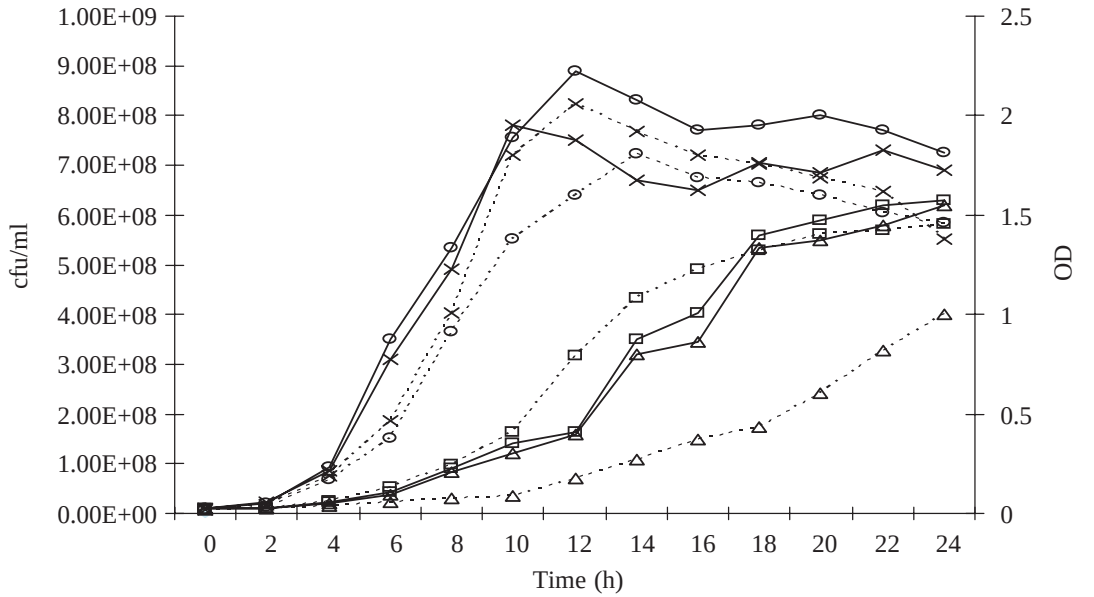


**Figure 2** Effect of temperature on growth of *E. faecalis* N1-33.
Δ, 25°C; ❑, 30°C; ❍, 37°C; ×, 45°C
solid lines indicate viable cell count (cfu/ml); broken lines indicate OD.

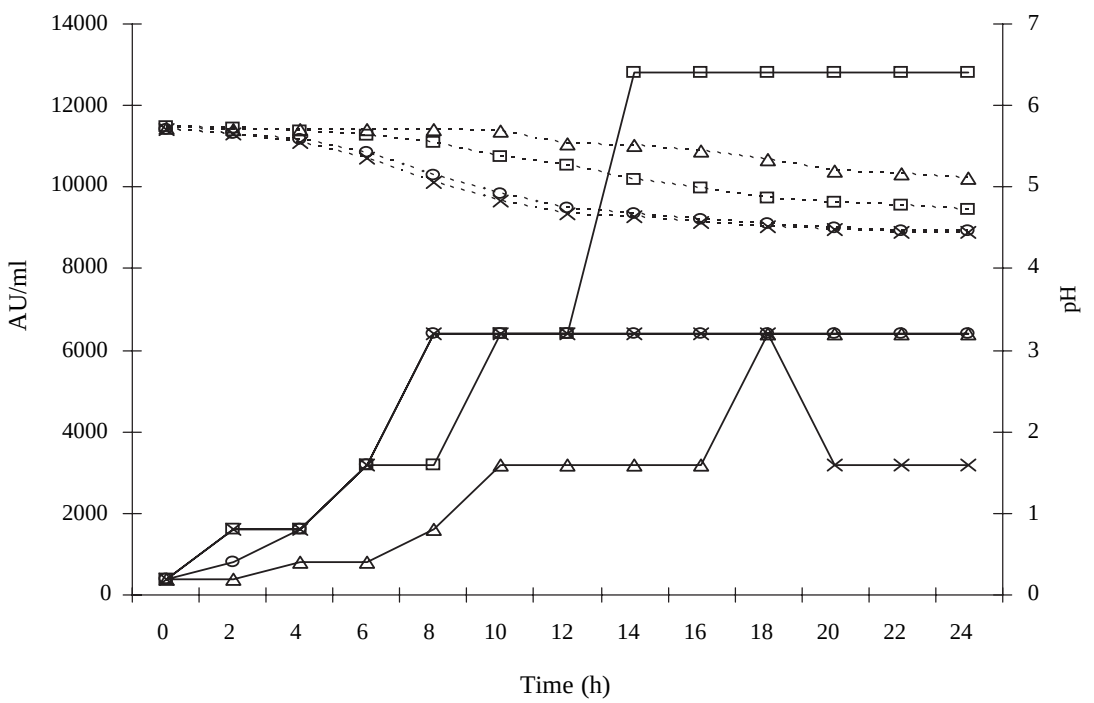


**Figure 3** Effect of temperature on production of bacteriocins and pH of *E. faecalis* N1-33.
Δ, 25°C; ❑, 30°C; ❍, 37°C; ×, 45°C
solid lines indicate bacteriocin-activity of CS pH 6 (AU/ml); broken lines indicate pH of the culture broth.

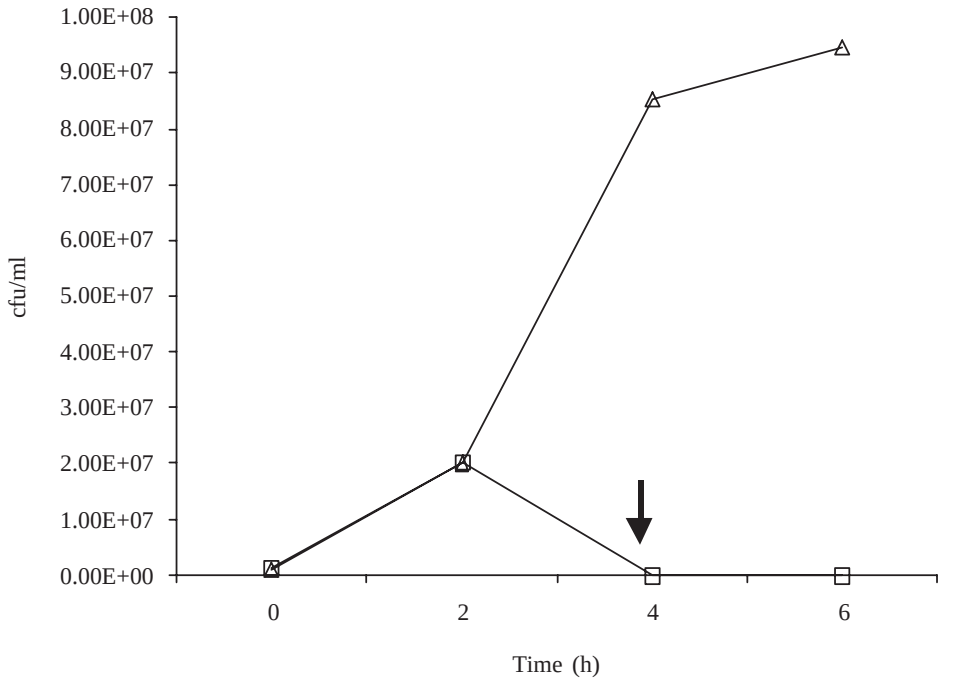


**Figure 4** Mode of action of *E. faecalis* N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$.
Δ, cfu/ml of *Lb. sakei* control; ❑, cfu/ml of *Lb. sakei* with crude bacteriocin (arrow indicate time of addition).

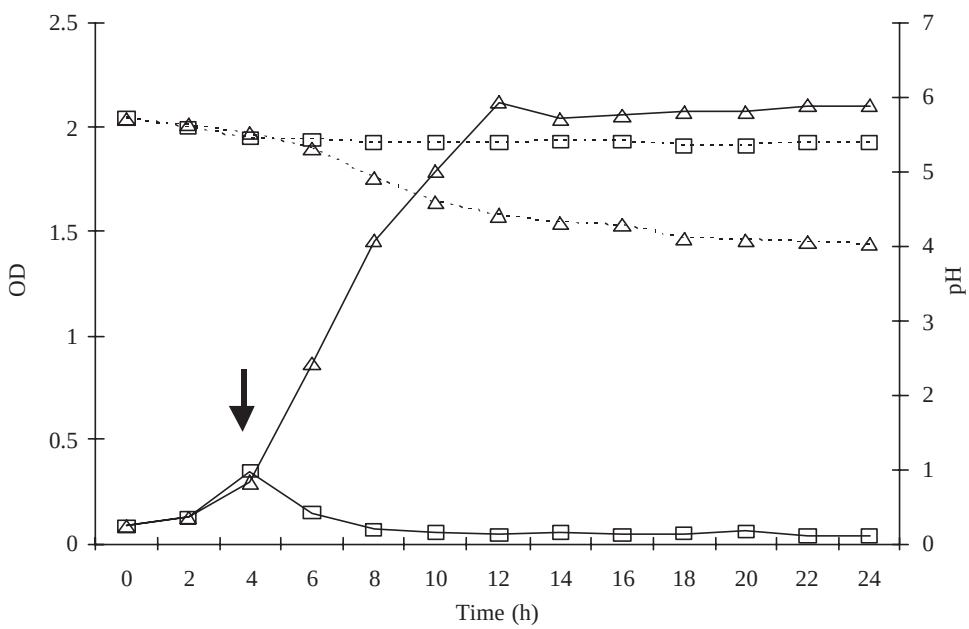


**Figure 5** Effect of crude bacteriocin of *E. faecalis* N1-33 to the growth of *Lb. sakei* subsp. *sakei* JCM 1157$^T$.
Δ, *Lb. sakei* control; ❑, *Lb. sakei* with crude bacteriocin at 4 h
solid lines indicate OD, broken lines indicate pH of the culture broth.

at 45°C the bacteriocin activity declined at the end of the stationary growth phase, which appeared to be in good agreement with a previous finding which reported that enterocin inactivation increased linearly with increasing temperature (Leroy and De Vuyst, 2002). Several mechanisms have been suggested for the decrease of bacteriocin activity such as protein aggregation, proteolytic degradation by specific or non-specific native enzymes and bacteriocin adsorption on the producer cells. It has been reported that conditions favoring bacterial growth and high cell densities may be beneficial to high bacteriocin production as well. However, elevated growth rates and high cell mass concentrations do not necessarily result in a high bacteriocin activity. The environmental conditions, such as pH, temperature and water activity, may thoroughly affect the specific bacteriocin production, i.e. the bacteriocin production per gram of cell, and lead to low bacteriocin production even if the producer cells grow well. Hence, there exists a rather complex relationship between environmental conditions and bacteriocin activity levels, which is partly associated to the quorum-sensing production mechanism (Leroy and De Vuyst, 2002).

The mode of action of *Enterococcus feacalis* N1-33 on *Lb. sakei* subsp. *sakei* JCM 1157$^T$ was determined to be bactericidal because the viable cells count immediately reduced to zero just after addition of the CS of N1-33. A dramatic reduction of the cells density, OD, also suggested the complete inhibition of *Lb. sakei* subsp. *sakei* JCM 1157$^T$. Consequently, it could not revive again and thus the pH of culture broth remained unchanged, while it dropped in the control (untreated) culture. This finding clearly indicated bactericidal mode of action with concomitant and fast cell lysis, which was obviously the most desirable feature for effective food preservation. Such type of inhibitory activity demonstrated by only few bacteriocins of enterococcal species, while most of them did not exert concomitant cell lytic effects (Sabia *et al*., 2002).

*Enterococcus faecalis* N1-33 exhibited several desirable properties of interest that appeared to be compatible with a wide range of food processing and storage conditions. These properties apparently demonstrated the great potential for use in food preservation. First, its broad spectrum of inhibitory activity against both LAB and Gram-positive food spoilage and pathogenic bacteria suggested the wider extent of its applications. Second, its ability of metabolizing low cost carbohydrates such as maltose, lactose, sucrose, starch and glycogen (Table 1) suggested its capacity to proliferate and produce bacteriocins in cheaper substrates naturally available in the foods to be preserved. Third, its thermostability indicated the possibility of its use in pasteurized food products. Forth, its ability for exerting inhibitory activity under a wide pH range suggested that it might be useful in acidic as well as low-acidic foods. The fifth equally important feature was the production and stability of its bacteriocin activity at low temperatures for a long period of time, indicating that it could be employed in refrigerated foods against psychrotrophic food spoilage and pathogenic bacteria such as *Listeria*.

Bacteriocin-producing enterococci with strong anti-*Listeria* activity are widespread in nature and have been isolated from various sources such as dairy products, fermented sausages, fish, vegetables and silage (Sabia *et al*., 2002). To date about ten bacteriocins with molecular mass in the range of 3-7 KDa have been characterized from *Enterococcus faecalis* strains which are isolated mainly from food-associated sources (Balla *et al.*, 2000; Eguichi *et al.*, 2001; Yamamoto *et al.*, 2003). However, as far as it has been known, this is the first report on the production of bacteriocins by LAB of fermented bamboo shoot of Thailand or elsewhere.

## CONCLUSION

The results of these studies demonstrated that *Enterococcus faecalis* N1-33 was endowed

with several desirable characteristics that would make it a good candidate for application as a natural food preservative under a wide range of conditions. The most attractive properties of this strain were the adaptability to different substrates (metabolizing low cost carbohydrates) and growth conditions, production of bacteriocins over a wide range of temperature 4-45°C that were bioactive over a wide range of pH 2-11 and thermostable up to 121°C, and broad spectrum of bacteriocin-activity against LAB and other Gram-positive food spoilage and pathogenic bacteria. All of these remarkable features and the long history of various technological uses of other *Enterococcus feacalis* strains ultimately urged further investigation to be conducted on N1-33. Interestingly, preliminary purification of its bacteriocin-activity revealed the presence of multiple bacteriocins, with were enhanced bioactivities than CS, and hence suggested its additional advantages. In order to utilize all these enormous potentials, applied studies with regard to its technological applications in food biopreservations and detailed characterization of the bacteriocins are currently in good progresses, which will be communicated elsewhere when completed.

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