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Influence of Physical Factors and Various Complex Media on Growth and Bacteriocin Production of Two-synergistic Peptide with Heat Stable Bacteriocin Producer, *Enterococcus faecium* NKR-5-3, Isolated from Thai Fermented Fish

Pongtep Wilaipun[1] , Takeshi Zendo[2] , Mathana Sangjindavong[3] , Sunee Nitisinprasert[1], Vichien Leelawatcharamas[1], Jiro Nakayama[2] and Kenji Sonomoto[2]

ABSTRACT

Enterococcus faecium NKR-5-3, isolated from Thai fermented fish (Pla-ra), produced an interested two-synergistic peptide bacteriocin with heat stable and broad spectrum activity. The influence of complex media and culture condition on growth as well as bacteriocin production of *E. faecium* NKR-5-3 has been studied. From 8 indicator strains used, *Enterococcus faecalis* ATCC 19433 was found to be the most sensitive strain. The bacteriocin to be diluted with distilled water containing 0.1% (v/v) Tween 80 increased 2-fold to 4-fold of its activity against indicator strains when compared with distilled water. In M-MRS broth, bacteriocin production reached maximum level at 30°C and decreased with the increasing of culture temperature. Among different 6 complex media for lactic acid bacteria cultured at 30°C, M 17 broth with the initial pH of 7.5 yielded the maximum growth and bacteriocin production of *E. faecium* NKR-5-3. Growth and bacteriocin production decreased when it was cultured in M 17 broth containing more than 1% NaCl and they were completely inhibited in M 17 broth containing more than 9% NaCl. Bacteriocin activity reached a detectable level at the early exponential phase and increased due to the cell growth to the maximum level at the end of exponential phase in 22 hr of incubation, which displayed a primary metabolite production.

Key words: two-synergistic peptide bacteriocin, growth and bacteriocin production, *Enterococcus faecium*, Thai fermented fish, Pla-ra

INTRODUCTION

Bacteriocins are proteinaceous antimicrobial compounds with a bactericidal mode of action against bacteria closely related to the producer strain (Tagg *et al*., 1976). Some bacteriocins produced by lactic acid bacteria (LAB), such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, Staphylococcus aureus* and many other gram-positive spoilage bacteria (Cleveland *et al*., 2001; O'Sullivan *et al*., 2002). Recently, the use of either bacteriocin-producing LAB starter cultures or their bacteriocins for food preservation has received much interest (Ennahar *et al*., 1999; Franz *et al*., 1999).

Among LAB, *Enterococcus* is widely

1 Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan.
3 Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.

distributed and associated with food substrate. Enterococci are also used in food fermentation and some strains are routinely employed as starter culture in the manufacture of fermented food (Ennahar *et al*., 1999; Franz *et al*., 1999). Several strains of enterococci are known to produce bacteriocin, enterocin, and most of them belong to class II bacteriocin with heat stability and anti-*Listeria* activity (Franz *et al*., 1999).

Bacteriocin production in LAB usually occurs throughout the growth phase and ceases at the end of the exponential phase (Parente *et al*., 1994; De Vuyst *et al*., 1996). However, many factors affect on bacteriocin production in LAB such as medium component and culture condition (Parente and Hill, 1992). All of these factors are the most important factors for large-scale production of bacteriocin in food industry.

In recent years, there have been numerous reports on bacteriocin production by *E. faecium* isolated from various sources (Franz *et al*., 1996; Ennahar *et al*., 1998). However, only one report on bacteriocin producing *E. faecium* isolated from Thai fermented fishes (Pla-ra) of Thailand was described (Wilaipun *et al*., 2002). In summary, 11 bacteriocin producing LAB were isolated from 80 Pla-ra samples and one potent strain with high bacteriocin activity was selected. According to the result of API 20 Strep system and 16S rDNA sequences it was identified to be *E. faecium* and named *E. faecium* NKR-5-3. Beside its high bacteriocin activity production, *E. faecium* NKR-5-3 produced two-synergistic peptide bacteriocin with heat stable and a broad spectrum activity against some food pathogenic bacteria and spoilage LAB. The purpose of this study was to characterize the growth and bacteriocin production of *E. faecium* NKR-5-3.

MATERIALS AND METHODS

Bacterial strains and media

The two-synergistic peptide bacteriocin producer, *E. faecium* NKR-5-3, has been previously isolated from Thai fermented fish (Pla-ra) (Wilaipun *et al*., 2002). The culture was maintained as frozen stocks and held at -80°C in TSBYE (tryptic soy broth, Difco Laboratory, Detroit, MI, USA, supplemented with 6 g l^{-1} yeast extract, Difco Laboratory, Detroit, MI, USA) containing 15% (v/v) of glycerol. Throughout the experiments, this strain was subcultured every 2 weeks on TSAYE (TSBYE plus 12 g l^{-1} agar) slant and kept at 4°C. The cultures were propagated twice in TSBYE (pH 6.5) at 30°C for 18-24 hr before used. The following selected indicator strains were grown in the indicated media and temperature: *Bacillus cereus* JCM 2152 and *Bacillus coagulans* JCM 2257 in brain heart infusion broth (Difco Laboratory, Detroit, MI, USA) at 30°C and 37°C, respectively, *Lactobacillus sakei* subsp. *sakei* JCM 1157, *Lactobacillus plantarum* ATCC 14917, *Lactococcus lactis* subsp. *cremoris* TUA 1344L, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124 in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) at 30°C, *Enterococcus faecalis* ATCC 19433 and *Listeria innocua* ATCC 33090 in TSBYE at 37°C for determining bacteriocin activity.

For bacteriocin production, *E. faecium* NKR-5-3 was cultured in a production medium and incubated at each indicated temperature with no aeration and pH control. Unless otherwise noted *E. faecalis* ATCC 19433 was used as an indicator strain for bacetriocin activity determination.

Bacteriocin activity assay

The cell-free neutralized supernatant (CFNS) of *E. faecium* NKR-5-3 grown in each medium at 30°C for 18-24 hr was obtained by centrifugation at 10,000x*g* for 15 min at 4°C, neutralization with 1 M NaOH to pH 6.5 and subsequent sterilization by heating at 100°C in water bath for 5 min. The bacteriocin activity of CFNS was determined against indicator strains using critical dilution method (Mayr-Harting *et al*., 1972). The CFNS were twofold serially diluted with sterile diluent in microtiter

plate and aliquots (10 µl) of each dilution were spotted onto TSAYE plate overlaid with 5 ml of TSAYE soft agar media (1% agar, w/v) seeded with 10^7 CFU ml^{-1} of overnight (18 hr) cultured indicator strain. The arbitrary activity unit was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn and expressed in terms of arbitrary units per milliliter (AU ml^{-1}).

Selection of the most sensitive strain and influence of diluents on bacteriocin activity

Modified MRS broth described by Tichaczek *et al*. (1992) with 2% glucose (M-MRS+2% Glu broth) at pH 6.5 was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at 30°C for 24 hr. The bacteriocin activity of CFNS was determined comparing with 8 different indicator strains (Table 1) using two different diluents of sterile distilled water and sterile distilled water containing 0.1% Tween 80.

Production of bacteriocin at different temperatures

M-MRS+2% Glu broth was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at different temperatures (25, 30, 35, 40 and 45°C). After 24 hr of incubation, the pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined.

Production of bacteriocin in various media conditions

For complex media study, APT broth (Difco; pH 7.7), Elliker broth (Difco; pH 6.8), M 17 broth (Merck; pH 7.2), MRS broth (Oxoid; pH 6.2), M-MRS broth (pH 6.5) and M-MRS+2%Glu broth (pH 6.5) were inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at 30°C. At the selected time intervals, the pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined.

For the influence of pH and sodium chloride, M 17 broth (Merck) adjusted to pH 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 9.5 were inoculated with 1% (v/v) of bacteriocin producing culture grown for 18 hr at 30°C. To determine the effect of sodium chloride concentration in the medium, sodium chloride was added to M 17 broth (pH 7.5) to a final concentration of 1, 3, 5, 7, 9, 11, 13 and 15% (w/v). The pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined after incubation at 30°C for 24 hr.

Kinetics of bacteriocin production

M 17 broth (1,000 ml, pH 7.5) was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium*

Table 1 Bacteriocin activity of *E. faecium* NKR-5-3 CFNS against various indicator strains and the enhancement of Tween-80 on bacteriocin activity.

Indicator strains	Bacteriocin activity (AU ml^{-1})	
	Distilled water	0.1% Tween 80
Bacillus cereus JCM 2152	800	1,600
Bacillus coagulans JCM 2257	1,600	6,400
Enterococcus faecalis ATCC 19433	6,400	12,800
Lactobacillus plantarum ATCC 14917	1,600	6,400
Lactobacillus sakei subsp. *sakei* JCM 1157	1,600	6,400
Lactococccus lactis subsp. *cremoris* TUA 1344L	800	3,200
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124	400	1,600
Listeria innocua ATCC 33090	800	3,200

NKR-5-3 and incubated at 30°C. At the selected time intervals, the pH and OD_{600} of culture broth and bacteriocin activity in CFNS were determined.

RESULTS

Selecting for the most sensitive strain and influence of diluent on bacteriocin activity

Bacteriocin activity in CFNS of *E. faecium* NKR-5-3 was determined comparing to eight indicator strains using two different diluents. According to the highest antibacterial activity of bacteriocin produced by *E. faecium* NKR-5-3 to *E. faecalis* ATCC 19433 (Table 1), it suggested that *E. faecalis* ATCC 19433 was the most sensitive strain among eight indicator strains used. Comparing of the diluted solution between sterile distilled water containing 0.1% Tween 80 and distilled water (Table 1), the higher bacteriocin activity of 2-4 folds was obtained when Tween 80 was added. According to these results, *E. faecalis* ATCC 19433 was selected as an indicator strain and sterile distilled water containing 0.1% Tween 80 was chosen as a diluent for bacteriocin activity determination in further experiments.

Production of bacteriocin at different temperatures

E. faecium NKR-5-3 exhibited the maximum cell density when grew in M-MRS+2%Glu at 25°C for 24 hr. However, the maximum bacteriocin activity (12,800 AU ml^{-1}) was obtained when it was grown at 30°C and yielded lower cell density (Table 2). On the other hand, at the high growing temperatures of 40 and 45°C bacteriocin activity in CFNS were found to be as low as 200 and 0 AU ml^{-1}, respectively.

Production of bacteriocin in different complex media

E. faecium NKR-5-3 was cultured in six different complex media at 30°C. After 12 hr of incubation, bacteriocin activity could be detected from most of the six complex media and after 24 to 36 hr *E. faecium* NKR-5-3 exhibited both of the maximum cell density (OD_{600} = 3.10) and highest bacteriocin activity (51,200 AU ml^{-1}) when grown in M 17 broth (Table 3). Therefore, M 17 broth was selected for further experiment. Meanwhile, in M-MRS+0.2%Glu it gave the lowest growth and lowest bacteriocin activity.

Influence of initial pH medium on bacteriocin production

E. faecium NKR-5-3 grew to the maximum cell density (OD_{600} = 3.3) in M 17 broth with the initial pH = 7.5 and 8.0, meanwhile, bacteriocin production was maximum (51,200 AU ml^{-1}) at initial pH = 7.5. In M 17 broth adjusted initial pH below 7.5 and above pH 8.0 the growth was reduced

Table 2 Growth and bacteriocin production of *E. faecium* NKR-5-3 in M-MRS+2% glucose at different temperatures.

Temperature (°C)	pH	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
25	4.65	3.47	6,400
30	4.55	3.18	12,800
35	4.45	2.96	6,400
40	4.35	2.63	200
45	5.55	1.50	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

Table 3 Growth and bacteriocin activity on different complex medium of *E. faecium* NKR-5-3 at 30°C.

	APT broth	Elliker broth	M 17 broth	MRS broth	M-MRS broth (0.2% glucose)	M-MRS broth (2% glucose)
12 hours						
pH	4.85	5.15	6.38	4.96	6.05	5.15
OD_{600}	2.44	1.63	2.34	2.48	1.64	2.52
Activity[a] (AU ml^{-1})	3,200	1,600	12,800	3,200	1,600	3,200
18 hours						
pH	4.68	5.00	6.02	4.52	6.05	4.64
OD_{600}	2.63	1.67	2.98	2.67	1.58	2.70
Activity[a] (AU ml^{-1})	6,400	3,200	25,600	6,400	1,600	6,400
24 hours						
pH	4.61	5.00	5.85	4.52	6.05	4.53
OD_{600}	2.68	1.68	3.10	2.65	1.56	2.72
Activity[a] (AU ml^{-1})	6,400	3,200	51,200	6,400	1,600	12,800
36 hours						
pH	4.56	4.95	5.80	4.48	5.98	4.49
OD_{600}	2.46	1.70	2.96	2.61	1.48	2.64
Activity[a] (AU ml^{-1})	6,400	3,200	51,200	6,400	1,600	12,800

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

and bacteriocin production was lower than at pH 7.5. *E. faecium* NKR-5-3 was not capable of growth and bacteriocin production at pH 4.0. Although the growth of *E. faecium* NKR-5-3 was detected at pH 5.0 and 9.5, but it gave very low bacteriocin activity of 100 AU ml^{-1} and no activity were obtained at all in these two pH (Table 4).

Influence of sodium chloride on bacteriocin production

E. faecium NKR-5-3 exhibited the maximum growth (OD_{600} = 3.3) and bacteriocin production (51,200 AU ml^{-1}) when it was grown in M 17 broth containing 0 to 1% NaCl. However, having NaCl concentration of 3 to 7%, drastically decreased both of growth and bacteriocin production. No growth or bacteriocin could be detected when higher NaCl concentration of 9% was used (Table 5).

The kinetics of bacteriocin production

Optical density (OD_{600}), pH of culture medium and bacteriocin production during the growth of *E. faecium* NKR-5-3 in M 17 broth at 30°C are shown in Fig. 1. OD_{600} increased from an initial ca. 0.03 to 3.0 during the first 24 hr of incubation. The OD_{600} was then stabilized at ca. 3.0 and remained at this level up to 36 hr of incubation. OD_{600} was found to decrease and reached the level of 2.5 at the end of the 72 hr incubation period. The initial pH of culture medium at 7.4 was decreased to 5.7 at the end of incubation period. Bacteriocin production was initially detected at 4 hr after inoculation and increased to a maximum of 51,200 AU ml^{-1} after 22–24 hr. Furthermore, bacteriocin activity remained stable at this level till the end of incubation period.

Table 4 Influence of initial pH of M 17 broth on growth and bacteriocin production of *E. faecium* NKR-5-3.

Initial pH	pH of culture broth after 24 hr	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
4.0	3.98	0.03	0
5.0	4.83	0.67	100
6.0	4.99	2.18	6,400
7.0	5.65	2.70	12,800
7.5	5.84	3.28	51,200
8.0	6.07	3.30	25,600
9.0	6.52	3.00	6,400
9.5	8.06	1.44	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

Table 5 Influence of sodium chloride concentration in M 17 broth on growth and bacteriocin production of *E. faecium* NKR-5-3.

NaCl concentration (%)	pH of culture broth after 24 hr	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
0	5.90	3.33	51,200
1	5.84	3.26	51,200
3	6.45	1.56	1,600
5	6.59	0.95	400
7	6.89	0.37	200
9	7.27	0.03	0
11	7.26	0.03	0
13	7.23	0.03	0
15	7.20	0.03	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

DISCUSSION

According to the highest antibacterial activity (6,400 AU ml^{-1}) of bacteriocin produced by *E. faecium* NKR-5-3 to *E. faecalis* ATCC 19433. It suggested that *E. faecalis* ATCC 19433, which is the closely related strain to *E. faecium*, was the most sensitive strain among 8 indicator strains used. This result comply with bacteriocins produced by another LAB, those are usually exhibit the highest antibacterial activity against the target strains which are closely related to the producing strain (De Vuyst and Vandamme, 1994; Tagg *et al.*, 1976). On the comparison of two diluents on bacteriocin activity, sterile distilled water containing 0.1% Tween 80 increased 2-4 folds of bacteriocin activity against indicator strains when compared to distilled water. The enhancement of Tween 80 on bacteriocin

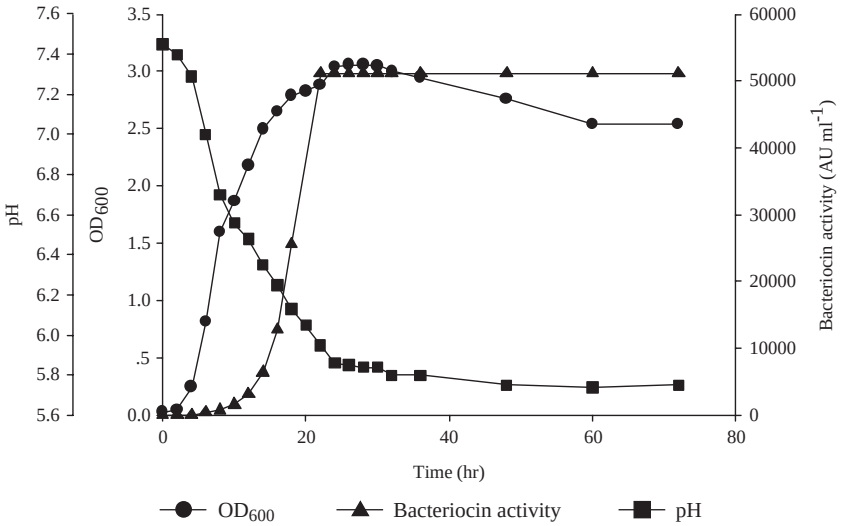

Figure 1 The growth (OD_{600}), bacteriocin production and pH of culture broth of *E. faecium* NKR-5-3 during incubation in M 17 broth at 30°C.

activity was due to a reduction of the binding of bacteriocin to the surface of plastic microtiter wells since most bacteriocins are proteinaceous with hydrophobic compound which tend to aggregate to form large complexes and to adhere to hydrophobic surfaces (Nissen-Meyer *et al*., 1992). Nisin and enterocin 4 were previously reported to be rapidly adsorbed to polypropylene and glassware. The addition of 0.1% Tween 80 to the diluent buffer could reduce these adsorption from 45-75% to less than 5% (Joosten and Nunez, 1995). In addition, the adsorption of nisin to hydrophilic surfaces by electrostatic interactions and desorption by Tween has also been studied by Daeschel *et al*. (1992). Furthermore, Nissen-Meyer *et al*. (1992) suggested that Tween 80 may involve in the stabilization of a favorable configuration of the bacteriocin molecules and/or the sensitization of the target cell, perhaps through destabilization of its membrane.

In general, bacteriocin production is growth-associated (Parente *et al*., 1994; De Vuyst *et al*., 1996). However, in some bacteriocin producing strains such as *Lactobacillus acidophilus* and *Lactococcus lactis* the maximum bacteriocin levels or production rates did not correlate directly with cell mass or growth rate (Kim *et al*., 1997; Bogovic-Matijasic and Rogelj, 1998). As it also was found in *E. faecium* NKR-5-3 grown at 25 and 30°C. Moreover, the lower growth rate or some unfavorable growth conditions was also reported to enhance bacteriocin production in some bacteriocin producing strains (De Vuyst *et al*., 1996). At 40 and 45°C, *E. faecium* NKR-5-3 showed the low level and lost of bacteriocin production, respectively. The similar result was also found in *Enterococcus faecium* DPC 1146 showing the low level of enterocin production at 42-45°C (Parente and Hill, 1992). However, enterocin P and enterocin Q production of *E. faecium* L50 were reported to reach the maximum level at 47°C and 37-47°C of incubation temperature, respectively (Cintas *et al*., 2000).

The growth and bacteriocin production of *E. faecium* NKR-5-3 was studied in six different complex media. After 24 hr of incubation, growth of *E. faecium* NKR-5-3 in M 17 broth gave the maximum cell density and maximum bacteriocin production. This result resembles enterocin 1146 production in which *E. faecium* DPC 1146 grow best with the highest antimicrobial compound in M 17 broth supplemented with 0.5% glucose (Parente and Hill, 1992).

In general, LAB are fastidious microorganisms with respect to nutrient requirement so that a rich medium with yeast extract and protein hydrolysates is required for good growth and bacteriocin production (Parente and Hill, 1992; De Vuyst *et al*., 1996). The growth and bacteriocin production of LAB is often limited by organic nitrogen sources rather than by the carbon sources (Parente and Ricciardi, 1999). Furthermore, M 17 broth contains more various organic nitrogen sources, as in digested form of beef, casein and soybean, than those of another 5 media used. Moreover, M 17 broth contains large amount of sodium beta-glycerophosphate, which increases the buffering capacity of medium and also promotes the growth of lactic streptococci (Merck, 2000). Consequently, M 17 broth is suitable for the growth and bacteriocin production of *E. faecium* NKR-5-3. Beside the previously optimum properties, M 17 medium does not contain Tween 80 as seen in most complex media for bacteriocin production, which will be the advantage in a bacteriocin purification step since Tween 80 was reported to interfere with the bacteriocin purification procedure (Muriana and Klaenhammer, 1991).

The growth and bacteriocin production of *E. faecium* NKR-5-3 occurred in the neutral or slightly alkaline medium condition. This finding is different from what is known for the optimum pH for bacteriocin production in previous reports which were usually at pH 5.5-6.0 and were often lower than the optimum pH for growth (Parente *et al*., 1994; Matsusaki *et al*., 1996). On the other hand, it complies with the optimum pH range of enterocin 900 production by *E. faecium* BFE 900 (Franz *et al*., 1996). However, the optimum pH for bacteriocin production may also be affected by the culture medium (Parente and Ricciardi, 1999). According to this study, the large scale production of bacteriocin by *E. faecium* NKR-5-3 in batch culture should be done in M 17 broth with initial pH value of 7.5. Moreover, the application of this strain as a protective culture in situ, should therefore be used in food systems with pH more than 6.0.

Although *E. faecium* NKR-5-3 was isolated from Thai fermented fish (Pla-ra) containing large amount of NaCl it exhibited a poor growth and bacteriocin production in the medium containing more than 1% NaCl. In addition, the growth and bacteriocin production were completely inhibited in the medium containing more than 9% NaCl. Hence, *E. faecium* NKR-5-3 could not be classified as a halophilic bacteria that played an important role in fermented fish process but rather a halotolerant bacteria that could survive during fermented process. In a previous report, the nukacin ISK-1 production by *Staphylococcus warneri* ISK-1 was shown to increase with increasing NaCl and the highest level of production was reached in the medium having 1.4 M NaCl. Meanwhile, cell growth, glucose consumption and lactate production were inhibited by the increase in NaCl concentration (Sashihara *et al*., 2001). In our previous study, bacteriocin from *E. faecium* NKR-5-3 cultured in M 17 broth without sodium chloride could stabilize under different sodium chloride concentration (0-24%) environment at 4°C for more than 30 days (data not shown). By this finding, the application of *E. faecium* NKR-5-3 in high salt containing food system should be done by using its bacteriocin as a biopreservative compound rather than using it as a starter or biopreservative culture.

The growth and bacteriocin production of *E. faecium* NKR-5-3 was studied at selected time intervals throughout the incubation period. From our finding, bacteriocin activity could be detected at early exponential phase and increased with the increasing of cell growth until reached the maximum production in early stationary growth phase. It suggested that bacteriocin production of *E. faecium* NKR-5-3 was the primary metabolite production. This result complied with almost of bacteriocin production by LAB those were reported to be the primary metabolite production (De Vuyst and Vandamme, 1994). The maximum production of bacteriocin occurred at high cell density in early

stationary phase, as was reported also for bacteriocin produced by *Enterococcus faecium* BFE 900 (Franz *et al*., 1996), *Enterococcus faecium* RZS C5 (Leroy and De Vuyst, 2002). However, some types of bacteriocin were reported to be a secondary metabolite production such as bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* (Rattanachaikunsopon and Phumkhachorn, 2000), pediocin AcH and Mesenteroicin 5 which were produced by *Pediococcus acidilactici* and *Leuconostoc mesenteroides* strain UL5 (Biswas *et al*., 1991; Lewus *et al*., 1991).

CONCLUSION

It was shown that *E. faecalis* ATCC 19433 was the most sensitive strain to the bacteriocin of *E. faecium* NKR-5-3 and Tween 80 could increase bacteriocin activity against the selected indicator strains. Bacteriocin production of *E. faecium* NKR-5-3 is a growth-associated process and it was influenced by some controllable environmental factors such as temperature, nutrient component and initial pH of culture media.

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