แบคเทอริโอซินจากแบคทีเรียกรดแล็กติกที่แยกได้จากผลิตภัณฑ์ปลาหมัก

นางสาวนุจรี สอนสะอาด

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BACTERIOCINS FROM LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FISH PRODUCTS

Nootjaree Sonsa-ard

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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BACTERIOCINS FROM LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FISH PRODUCTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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นุจรี สอนสะอาด : แบคเทอริโอซินจากแบคทีเรียกรดแล็กติกที่แยกได้จากผลิตภัณฑ์ปลาหมัก (BACTERIOCINS FROM LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FISH PRODUCTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 164 หน้า.

แบคเทอริโอซินเป็นสารเพปไทด์ที่สังเคราะห์จากไรโบโซม มีฤทธิ์ในการยับยั้งจุลินทรีย์ซึ่งผลิตได้ทั้งแบคทีเรียแกรมลบและแกรมบวก วัตถุประสงค์ของการศึกษานี้คือ เพื่อคัดแยกแบคทีเรียกรดแล็กติกที่ผลิตแบคเทอริโอซินจากอาหารปลาหมักพื้นบ้านไทยได้แก่ ปลาส้ม ส้มไข่ปลา ปลาร้า และกุ้งจ่อม เพื่อศึกษาสภาวะที่เหมาะสมสำหรับผลิตแบคเทอริโอซิน ทำบริสุทธิ์และศึกษาคุณลักษณะของแบคเทอริโอซินที่ได้ ศึกษากลไกการออกฤทธิ์ จากจำนวนทั้งหมด 285 ไอโซเลท ที่คัดเลือกได้ เมื่อคัดกรองจากการสร้างแบคเทอริโอซินพบว่า 4 ไอโซเลทแสดงการยับยั้ง *Listeria monocytogenes* เป็นอย่างมาก เมื่อวิเคราะห์ด้วยเทคนิคการแพร่ของสารในเนื้อวุ้น(ซึมผ่านบนอาหารแข็ง) ได้แก่ ไอโซเลท CN-25, GY-20, MSKC-13 และ MSK-3-18 โดยทั้ง 4 ไอโซเลทนี้ถูกระบุชนิดโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ของ 16S rRNA gene เป็น *Enterococcus faecium*, *Lactococcus lactis* subsp. *lactis* และ *Pediococcus pentosaceus* ซึ่งเป็นครั้งแรกที่พบ *Enterococcus faecium* ที่แยกจากส้มไข่ปลา และผลิตแบคเทอริโอซินที่สามารถยับยั้ง *L. monocytogenes* นอกจากนี้ตรวจพบยีนที่การควบคุมการสร้างสารเอนเทอโรซินเอและเอนเทอโรซินบีใน *E. faecium* CN-25 ซึ่งมีความเหมือนร้อยละ 100 กับลำดับนิวคลีโอไทด์ของยีนที่ควบคุมการสร้างสารเอนเทอโรซินเอ ของ *E. faecium* CRL1385 และสารเอนเทอโรซินบีของ *E. faecium* T136 การผลิตแบคเทอริโอซินเกิดขึ้นสูงสุดในอาหารเลี้ยงเชื้อที่มีรำข้าวร้อยละ 0.5 น้ำตาลกลูโคลสร้อยละ 0.2 สารสกัดของยีสต์ ร้อยละ 0.5 ไตรแอมโมเนียมซิเตรทร้อยละ 0.2 โซเดียมอะซิเตทร้อยละ 2.0 ไดโพแทสเซียมไฮโดรเจนฟอสเฟตร้อยละ 0.2 แมกนีเซียมซัลเฟตร้อยละ 0.02 แมงกานีสซัลเฟตร้อยละ 0.05 และ โพลีซอเบท (Tween 80) 0.1 มิลลิลิตร การผลิตแบคเทอริโอซินมีผลผลิตสูงสุดที่ 1828.15 AU ต่อมิลลิลิตร ที่กล้าเชื้อเริ่มต้นร้อยละ 0.5 และอุณหภูมิ 25 องศาเซลเซียส ก่อนการเข้าสู่ระยะที่เซลล์มีการเจริญคงที่ (stationary phase) โดยมีจำนวนเซลล์ 9.4 log CFU ต่อมิลลิลิตร แบคเทอริโอซินที่ผลิตได้มีความคงตัวที่ช่วงพีเอช 2-11 และมีความเสถียรต่อความร้อนช่วงกว้าง แต่กิจกรรมลดลงเมื่อบ่มที่ 121 องศาเซลเซียส เป็นเวลา 15 นาที เกิดการสูญเสียกิจกรรมด้วยโปรติเนส เมื่อทำบริสุทธิ์เพปไทด์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟตและการแยกโดยการแลกเปลี่ยนแบบประจุลบ พบว่าเพปไทด์ที่ได้มีค่ากิจกรรมการยับยั้งเฉพาะเพิ่มขึ้น มีความบริสุทธิ์เพิ่มขึ้น 8.1 เท่า โดยมีค่าความเข้มข้นต่ำสุดที่ยับยั้งการเจริญได้ของ *L. monocytogenes* ได้สมบูรณ์ (MIC) เท่ากับ 2.34 ไมโครกรัมต่อมิลลิลิตร ศึกษากลไกการยับยั้งของสารเพปไทด์ CN-25 ต่อการทำลาย

เซลล์ของ *L. monocytogenes* โดยการตรวจวัดการเหนี่ยวนำให้เกิดการไหลออกของสารอะดีโนซินไตรฟอสเฟต (ATP) ออกมานอกเซลล์และการเปลี่ยนแปลงของแรงขับเคลื่อนโปรตรอน (proton motive force) ในเซลล์ พบว่าเพปไทด์ CN-25 สามารถลดค่าพลังงานทั้งหมดของเซลล์ *L. monocytogenes* แต่ไม่พบการไหลออกของสารให้พลังงานออกนอกเซลล์ และพบการลดลงของแรงที่เกิดจากความต่างศักย์เยื่อหุ้มชั้นใน ($\Delta\Psi$) แต่ไม่มีผลต่อการลดลงของแรงที่เกิดจากความแตกต่างของความเข้มข้นของโปรตอนด้านในและด้านนอกของเยื่อหุ้มเซลล์ (ΔpH) เมื่อเติมเพปไทด์ CN-25 ปริมาณ 914.2 AU ต่อมิลลิลิตรลงในน้ำนม ที่ผ่านการพาสเจอไรซ์ พบว่า *L. monocytogenes* ในนมพลาสเจอไรซ์ลดจำนวนจาก 4.1 log CFU ต่อมิลลิลิตร เหลือ 3.7 log CFU ต่อมิลลิลิตร ($p<0.05$) ในระยะเวลา 5 วัน ของการเก็บรักษา ดังนั้นเพปไทด์ที่ผลิตโดย *E. faecium* CN-25 สามารถใช้เป็นสารต่อต้านจุลชีพในนม เพื่อควบคุมการเจริญของ *L. monocytogenes*

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2556

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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NOOTJAREE SONSA-ARD : BACTERIOCINS FROM LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FISH PRODUCTS. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 164 PP.

BACTERIOCIN/LACTIC ACID BACTERIA/FERMENTED FOOD

Bacteriocins are ribosomally-synthesized peptides with antimicrobial activity, produced by both Gram-negative and Gram-positive bacteria. The objectives of this study were to isolate bacteriocin-produced by lactic acid bacteria from traditional Thai fermented fish products, namely pla-som, somkai-pla, pla-ra and kong-jom, and to optimize the bacteriocin production of selected isolates. In addition, the objectives were to purify and elucidate their stability and modes of action of bacteriocins produced by the selected isolate. A total of 285 isolates were obtained and screened for bacteriocin production. Four isolates which produced remarkably wide zones of inhibition based on the agar well diffusion technique against *Listeria monocytogenes* were CN-25, GY-20, MSKC-13 and MSK-3-18. These isolates were identified on the basis of 16S rRNA gene sequence as *Enterococcus faecium*, *Lactococcus lactis* subsp. *lactis* and *Pediococcus pentosaceus*. This is the first report of *E. faecium* isolated from somkai-pla, which produced bacteriocins and showed *L. monocytogenes* inhibition as compared to other isolates. Therefore, this strain was selected for the production and characterization of the antibacterial compounds. *E. faecium* CN-25 was found to harbour genes encoding for enterocin A and enterocin B with a similar sequence (100%) homology to gene encoding enterocin A of *E. faecium* CRL1385 and enterocin B of *E. faecium* T136. *E. faecium* CN-25 showed the maximum bacteriocin production in

a modified broth containing 0.5% rice bran, 0.2% glucose, 0.5% yeast extract, 0.2% tri-ammonium citrate, 2% sodium acetate, 0.2% di-potassium hydrogen phosphate, 0.02% magnesium sulfate, 0.05% manganese sulfate and 0.1 ml polysorbate (Tween 80). The optimal bacteriocin production for *E. faecium* CN-25 was at 0.5% inoculum and 25 °C. Maximum production of bacteriocin of 1828.15 AU/ml was reached at the beginning of the stationary phase and the cell growth was determined to be 9.4 log CFU/ml. The bacteriocin CN-25 had stable activity with a wide pH range of 2-11.The activity was largely stable to heating, however, it decreased when treated at 121^{o}C for 15 min. It was found that the antibacterial activity was sensitive to various proteinases. Purification of CN-25 peptide by ammonium sulfate precipitation and anion exchange chromatography increased specific activity by 8.1 folds. The lowest concentration at which bacteriocin completely inhibited *L. monocytogenes* (MIC) was 2.38 µg/ml. The mode of action of CN-25 peptide on *L. monocytogenes* was investigated based on the efflux of intracellular ATP and the change of the proton motive force (PMF) in cell membranes. The CN-25 peptide decreased the total ATP of *L. monocytogenes*, but had no significant effect on the efflux of intracellular ATP. In addition, it depleted the cellular $\Delta\Psi$ (transmembrane electrical potential) but had no effect on the ΔpH (pH gradient). The CN-25 peptide at 914.2 AU/ml reduced *L.monocytogenes* in inoculated pasteurized milk from 4.1 to 3.7 log CFU/ml ($p<0.05$) during 5 day storage. The peptide produced by *E. faecium* CN-25 could be used in milk as an antimicrobial agent for controlling *L. monocytogenes*.

School of Food Technology — Student's Signature _____

Academic Year 2013 — Advisor's Signature _____

Co-advisor's Signature _____

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Nootjaree Sonsa-ard

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มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS

ΔpH	=	pH gradient
ΔΨ	=	Transmembrane electrical potential
α	=	Alfa
ATP	=	Adenosine 5'-triphosphate
BLAST	=	Basic local alignment search tool
bp	=	Base pair
BSA	=	Bovine serum albumin
BCECF-AM	=	(2',7'-bis-(2-carboxyethyl)- 5-(and-6) carboxyfluorescein
CFU	=	Colony forming unit
cm	=	Centrimeter
°C	=	Degree Celsius
dNTPs	=	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DNA	=	Deoxyribonucleic acid
DEAE	=	Diethylaminoethyl
DiSC3(5)	=	3,3'-dipropylthiadicarbocyanine iodine
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylenediaminetetraacetic acid
h	=	Hour
kDa	=	kilodalton
LAB	=	Lactic acid bacteria

LIST OF ABBREVIATION (Continued)

MES	=	Morpholinoethanesulfonic acid
M	=	mol l^{-1}
Mw	=	Molecular weight
mg	=	Milligram (10^{-3}gram)
min	=	Minute
ml	=	Milliliter (10^{-3})
mM	=	Millimolar (10^{-3}mol l^{-1})
NCBI	=	National Center for Biotechnological Information
OD	=	Optical density
RP-HPLC	=	Reversed-phase high performance liquid chromatography
SDS	=	Sodium dodecyl sulfate
s	=	Second
sp.	=	Species
Tris	=	Tris (hydroxymethyl)aminomethane
TISTR	=	Thailand Institute of Scientific and Technological Research
TSB	=	Tryptic soy broth
µg	=	Microgram (10^{-6} gram)
µl	=	Microliter (10^{-6}l)
µM	=	Micromolar (10^{-6}mol l^{-1})
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Bacteriocins are ribosomally synthesized peptides that exert their antimicrobial activity against either strains of the same or related species (broad range) (Belguesmia et al,. 2011, Galvez et al., 2008). According to Klaenhammer (1993), bacteriocins can be divided into four classes. Class I is lantibiotics, very low molecular weight (<5 kDa). Class II is composed of small thermostable peptides (<10 kDa) divided into three subclasses: IIa, IIb and IIc. Class III is represented by high molecular weight (>30 kDa). And class IV is large peptides complexed with carbohydrates or lipids.

Lactic acid bacteria (LAB) of different species and archaea may produce bacteriocins (Belguesmia et al., 2011). Moreover, the bacteriocins from LAB are considered to be Generally Recognized as Safe microorganisms (GRS) (Anastasiadou et al., 2008). At the time of nisin discovery, its producer strains were identified as *Lactococcus lactis* (Schleifer et al., 1985). Only nisin is approved for use as food preservative by the US Food and Drug Administration (Federal Register, 1988). The commercially available nisin is Nisaplin™ (Danisco, Copenhagen, Denmark). However, the use of nisin is limited since it is only effective against some Gram-negative spoilage, pathogenic bacteria, yeasts and molds. Thus, its application in the food preservation is limited. The effective bacteriocin against all spoilage and pathogenic bacteria ought to be sought. LAB-producing bacteriocin have been

isolated from Thai fermented foods. Nisin-producing *Lactococcus lactis* WNC 20 strain isolated from traditional Thai fermented sausage (Nham) which not only inhibited closely related LAB, but also some food-borne pathogens including *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus* (Noonpakdee et al., 2003). *P. pentosaceus* BCC 3772 isolated from Nham was found to produce pediocin PA-1/AcH which effectively inhibited growth of *L. monocytogenes*. Moreover, traditional fermented fish products in South East Asia are unique with organoleptic properties and widely prepared by spontaneous microbial fermentation. They offer a new source of bacterial strains and/or with special properties for potential biotechnological use such as starter culture (Noopakdee et al., 2009). The commonly LAB in Thai fermented fish products are *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Weisella confusa*, *Carnobacterium piscicola* (Ostergaard et al., 1998). Some of these strains have the anti-listerial activity but their identities of bacteriocins have not yet been elucidated.

Many studies have been optimized for bacteriocin production by manipulating growth media composition, temperature or pH. Investigations of alternative carbon, nitrogen and mineral sources have successfully led to increased bacteriocin yields or more cost effective production (Furuta et al., 2008, Gonzalez-Toled et al., 2010). Todorov and Dicks (2009) reported that the highest antimicrobial activity (102,400 AU/ml) of *Enterococcus mundtii* was recorded in MRS broth supplemented with fructose , yeast extract , tryptone or a combination of yeast extract and tryptone, K_2HPO_4. Bacteriocin production is often regulated by medium pH and growth temperature, as shown in pediocin AcH (Biswas et al., 1991) and pediocin PD-1 (Nel

et al., 2001) produced by *Pediococcus* spp., enterocin 1146 (Parente and Ricciardi, 1994), enterocin AS-48 (Abriouel et al., 2005), a bacteriocin produced by *E. faecium* RZS C5 (Leroy and de Vuyst 2003), and enterocin P produced by *Enterococcus* spp. (Herranz et al., 2001). Moreover, the study of medium components on the production of biomass and bacteriocin is a step required for the efficient bacteriocin production. Therefore, the parameters effecting bacteriocin production should be studied to obtain maximum yield and low cost bacteriocin production.

Purification is necessary for identification and characterization of bacteriocins. Bacteriocin characterized at the biochemical level proves to be strong inhibitors of foodborne pathogens such as *Listeria monocytogenes*, Moreover, purification and characterization of bacteriocin can potentially be important for studying mechanism of bioactive peptides.

The potential application of LAB bacteriocins as food preservatives requires in-depth knowledge of how they exert their bactericidal effects. In general, most bacteriocins from LAB appear to share a common mechanism of action which is depleting proton motive force (PMF) in target cells through the formation of pores in the cell membrane releasing intracellular ions (Abee, 1995; Montville and Bruno, 1994; Moll et al., 1999; Nes and Holo, 2000). However, the mechanisms through which they achieve this appear to differ among different bacteriocins. Structural studies of treated sensitive cells indicate different mechanisms of membrane destabilization and cell death (Jack et al., 1995). In addition, the potential application of bacteriocins as food preservatives requires knowledge of their characteristics. In this study, research strategies for newly discovered bacteriocins, are constructed and executed. The strategies include: 1) screening and determining LAB isolated from the

traditionally fermented Thai food including pla-som, somkai-pla, pla-ra, and kung-chom, 2) studying optimization conditions and production of bacteriocins, 3) purifying and investigating their characteristics, and 4) studying mode of actions of antimicrobial spectrum.

1.2 Research objectives

The objectives of this study were:

1) To screen and identify bacteriocin-producing LAB isolated from traditionally fermented Thai food.
2) To study the optimal conditions of the selected isolate for bacteriocin production.
3) To purify, characterize and evaluate mode of action of bacteriocins.

1.3 Research hypotheses

Distinctive bacteriocins are produced by selected lactic acid bacteria isolated from traditionally fermented fish products. Under the optimal condition, the high production bacteriocins be obtained. Purified bacteriocins show antimicrobial activity properties against food spoilage and pathogenic bacteria. Bacteriocin could be applied to control the grow of food pathogen bacteria in food.

1.4 Scope of the study

In this study, isolated strains of LAB are from four sources: traditionally fermented food (pla-som, som-kai-pla, pla-ra and kung-chom). The isolated LAB

were screened for their bacteriocin producing activity, focusing on the activity against food spoilage bacteria and food-borne pathogens. The bacteriocins production bacteria were selected and identified. The effect of culture medium composition, temperature and inoculum size were investigated.

The bacteriocins was evaluated for its antimicrobial spectrum, sensitivity of enzymes, temperature and pH. The partially-purified bacteriocin was purified using two steps including ammonium sulfate precipitation, anion exchange chromatography. Modes of action of the purified bacteriocin were studied.

1.5 Expected results

Bacteriocins with broad spectrum produced from traditionally fermented Thai food were obtained. In addition, bacteriocins are likely to be used as food bio-preservatives which may lead to the replacement the use of synthetic chemical preservatives for the application in food industry.

CHAPTER II

LITERATURE REVIEWS

2.1 Bacteriocins

Since the discovery of the first bacteriocin by Gratia in 1925, bacteriocins have been found in numerous species (Garneau et al., 2002). Bacteriocins are ribosomally synthesized, extracellularly released low molecular mass peptides or proteins (usually 30 – 60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria (Klaenhammer, 1988; Tagg et al., 1976) either in the same species (narrow spectrum) or across genera (Cotter et al., 2005; Galvez et al., 2008; Belguesmia et al., 2011). Bacteriocins have been divided into four classes based on structures and modes of action as shown on Table 2.1.

a) Class I: Lantibiotics

Class I generally consists of small compound of one or two peptides of approximately 3 kDa. Bacteriocins' unusual feature of this group is that they are post-translationally modified to contain lanthionine, β-methyllanthionine and dehydrated amino acids, while at least two members of the group also contain D-alanine (Skaugen et al., 1994; Ryan et al., 1996). This latter amino acid residue is derived from dehydroalanine resulting from the dehydration of a serine residue. The lantibiotics are originally subdivided into two subclasses, a and b based on their chemical structures and antimicrobial activities (Moll et al., 1999 and Guder et al., 2000). Subclass Ia are elongated peptide molecules that have positive charges. They exert their activity

through the formation of pore in bacteria membrane, such as nisin (Hurts, 1981), lactocin S (Mortvedt et al., 1991), epidermin (Allgaier et al., 1986), gallidermin (Kellner et al., 1988), lacticin 481 (Xie et al., 2004), Pep5 and epilancin K7 (Sahl et al., 1995; Sahl and Bierbaum, 1998).

Table 2.1 Classes of bacteriocins produced by lactic acid bacteria

Class	Subclass	Description
I		Lantibiotics - small, heat stable, containing unusual amino acids
	Ia	Cationic and hydrophobic peptide,flexible structure
	Ib	No net charge or net negative charge, globular peptide
II		Small (30–100 amino acids), heat stable, non-lantibiotic.
	IIa	Pediocin-like bacteriocins, with anti-listerial effects.
	IIb	Two -peptide bacteriocins
	IIc	Other peptide bacteriocins
III		Large (30 kDa) heat-labile proteins
IV		Complex bacteriocins with glyco- and/or lipid moieties

Source: Caplice and Fitzgerald. (1999).

Nisin is a pentacyclic peptide consisting of 34 amino acid residues of which 13 have been post-translationally modified (Figure 1). Two naturally occurring nisin variants, nisin A and nisin Z, are produced by lactococci (Buchman et al., 1988; Mulders et al., 1991). Nisin A differs from nisin Z in a single amino acid residue at position 27, being histidine in nisin A and asparagine in nisin Z. The structure of the nisin molecule determined by NMR suggests that the peptide contains two well defined amphipathic domains (Van den Hooven et al., 1996b). The N-terminal

domain, amino acids 1 to 19, forms the first three lanthionine rings (A, B, and C). A flexible hinge region connects this domain with the second domain formed by residues 23–28. The second domain consists of intertwined rings D and E and is followed by a flexible region of six C-terminal amino acids.

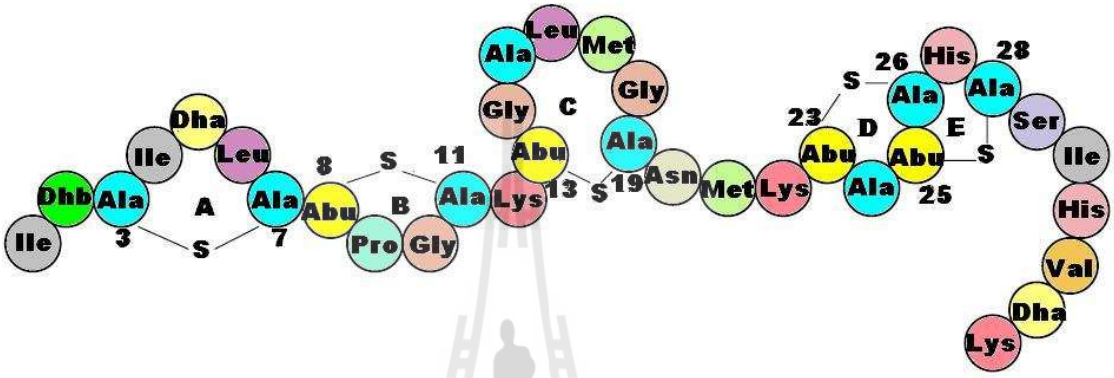


Figure 2.1 Structure of nisin: Abu: aminobutyric; Dha: dehydroalanine; Dhb: dehydrobutyrine (β-methyldehydroalanine); Ala-S-Ala: lanthionine; Abu-S-Ala: β-methyllanthionine

Source: Jack et al. (1998)

Subclass b lantibiotic are characteristically globular, more rigid in structure, and either negatively charged or have no net charge (Klaenhammer, 1993). Examples include mersacidin, actagardin, cinnamycin, duramycin and ancovenin (Fredenhagen et al., 1991). In addition, some members of the lantibiotic family require two separate peptides for activity. The continual discovery of extra members of this complex group of peptides implies that their classification ought to be periodically updated (Sahl and Bierbaum, 1998; Guder et al., 2000). However, nisin is the only commercially exploited lantibiotic to date.

b) Class II: Small heat-stable bacteriocins

Class II bacteriocins are non-lantibiotic, cationic and hydrophobic peptides of 20 – 60 amino acids in length. They contain small (<10 kDa) thermostable peptide with an amphiphilic helical structure that allows for their insertion in the cytoplasmic membrane of target cell, thereby promoting membrane depolarization and cell death (Nes and Holo, 2000; Drider et al., 2006). They are divided into three subclasses, IIa, IIb, and IIc, on the basis of their primary structures.

The class IIa includes pediocin-like peptide having an N-terminal consensus sequence, Tyr-Gly-Asn-Gly-Val-Xaa-Cys. This subgroup has attracted much attention due to their anti-listeria activity (Ennahar et al., 2000b). Recent reports have suggested that class IIa bacteriocins are more interesting anti-listerial agents than class I bacteriocins because they do not have as broad an inhibitory spectrum and thus do not kill many starter cultures while they are more effective in killing *Listeria* strains. Up to now, several bacteriocins in Class IIa are known to be produced by *L. sakei* strains including sakacin A (Schillinger and Lucke, 1989), sakacin M (Sobrino et al., 1992), sakacin P (Tichaczek et al., 1994), sakacin 674 (Holck et al., 1994), sakacin B (Samelis et al., 1994), sakacin K (Hugas et al., 1995), sakacin T (Aymerich et al., 2000), sakacin G (Simon et al., 2002), sakacin X (Vaughan et al., 2003), sakacin Q (Mathiesen et al., 2005) and sakacin C2 (Gao, et al., 2009).

The first enterocin was identified by Kjems (1955) and subsequently classified as a member of the pediocin family. Since then, several enterocins have been described, which have represented in more than one class of bacteriocins. Enterocins cluster in two subgroups according to sequence similarities, corresponding to the subgroups IIa and IIc described by Fimland et al. (2005). The first subgroup

includes enterocin A, the mundticins and enterocin CRL35. The second subgroup is represented by bacteriocin 31, bacteriocin T8, bacteriocin RC714, enterocin SEK4 and enterocin P. Bacteriocins from subgroup IIa contain seven unique conserved amino acid residues that differentiate them from subgroup IIb bacteriocins (which in turn contain six unique conserved amino acid residues, including Ala and Thr as first and second N-terminal positions) (Franz et al., 2008). Enterocin A (EntA) can be unequivocally grouped as a class IIa bacteriocin. It contains the characteristic YGNGVXC-motif at the N-terminus and four cysteine residues. These characteristics make it equivalent to pediocin PA-1/AcH (Marugg et al., 1992). EntA is produced by several *E. faecium* strains CTC492: T136 and P21, which were isolated from Spanish fermented sausages (Aymerich et al., 1996; Casaus et al., 1997; Herranz et al., 2001); by strain BFE 900 from black olives; by strains DPC 1146, WHE 81 and EFM01 from dairy sources (Franz et al., 1999b; O'Keeffe et al., 1999; Ennahar and Deschamps, 2000; Ennahar et al., 2001); and by N5 from nuka (a Japanese rice-bran paste) (Losteinkit et al., 2001). Class IIa are thermostable (121 ^{o}C/15 min) and resistant to lyophilization and storage at -20 ^{o}C for long periods. These compounds have selective antimicrobial activity; do not show antagonism with *Leuconostoc* and *Lactococcus*, but attack *C. perfringens*, *Clostridium botulinum*, *S. aureus* and especially species of the genus *Listeria* (Cintas et al., 1997).

Class IIb bacteriocins are bacteriocins whose activity depend on the complementary activities of two peptides (Nissen-Meyer et al. 1992). Each peptide displays very low activity, if any, when tested individually. Most of these bacteriocins require a 1:1 peptide ratio for optimal bactericidal effect (Garneau, Martin and Vederas, 2002). Lactococcin G is a bacteriocin whose activity depends on

the complementary action of two peptides, termed α and β, that consist of 39 and 35 amino acids, respectively (Nissen-Meyer et al., 1992).

Lactococcin MMT24 is a novel bacteriocin produced by *Lactococcus lactis* MMT24, a strain isolated from a Tunisian traditional cheese. The lactococcin MMT24 consists of two distinct peptides, named pep α and pep β (Ghrairi et al., 2005), whose complementary action is necessary for full antibacterial activity. Optimal antibacterial activity is obtained when the complementary peptides, pep α and pep β, is present in equal amount. The combined action of two peptides has been demonstrated for other bacteriocins including lacticin F (Muriana and Klaenhammer, 1991), lactococcin G (Nissen-Meyer et al., 1992), thermophilin 13 (Marciset et al., 1997) and lactacin 3147 (Ryan et al., 1996). For these bacteriocins, it has been observed that equivalent amount of both peptides are required for an interaction with target cells.

Class IIc consists of all non-lantibiotic bacteriocins that do not belong to class IIa and IIb. Class IIc represents a rather diverse set of bacteriocins derived from a variety of LAB despite the fact that some bacteriocins in this class display significant sequence similarities (Franz et al. 1999). So far, no sub-groups have been established. Some examples are enterocin AS-48, reutericin 6, circularin A (Balciunas et al., 2013), acidiocin B (Lee et al., 1995), canobacteriocin A (Worobo et al., 1994), divergicin A (Worobo et al., 1995), enterocins P (Cintas et al., 1997) and enterocin B (Nes and Holo, 2000).

c) Class III: Large heat-labile bacteriocins

Class III bacteriocins are not as well-characterized group and consisted of heat-labile proteins which are generally > 30 kDa. The group includes Helvetin J produced by *Lactobacillus helveticus* (Joerger and Klaenhammer, 1986) and

enterolysin produced by *Enterococcus faecium* (Nilson, 1999). Some examples of class III bacteriocins are helveticin J, helveticin V-1829, acidophilucin A, lactacins A and B. Although narrow spectrum and heat-instability of Helveticin J limits its usage as a food preservative, these bacteriocins may contribute to the host's ability to compete in complex dairy ecosystems.

d) Class IV: Complex bacteriocins

Class IV consists of bacteriocins that form large complexes with other macromolecules. Complex bacteriocins, plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1, are composed of protein and one or more chemical moieties (lipids, carbohydrates) required for activity. However, no such bacteriocins have been presently purified. This type of bacteriocins is an artifact due to the cationic and hydrophobic properties of bacteriocins which result in complexing with other macromolecules in the crude extract. This phenomenon has been shown in the case of plantaricin S. It is originally claimed to be a large complex molecule, but it later is purified as a small peptide, and the complex disintegrated while the activity is maintained (Jimenez-Diaz et al., 1995). Bacteriocins in this class have not been characterized adequately at the biochemical level (Jimenez-Diaz et al., 1995; McAuliffe, 2001).

2.2 Source of bacteriocins

Lactic acid bacteria (LAB) are Gram-positive; cocci or rods; anaerobic, microaerophilic, or aerotolerant; and catalase negative. Lactic acid is usually produced as the major end product during fermentation of carbohydrate. Some LAB can grow in the temperature below 5°C and some as high as 45°C. With respect to pH, some LAB can grow in the pH as low as 3.2, some as high as 9.6, but most LAB can grow in the

pH ranging between 4.0 and 4.5. The classification of LAB is largely based on their cell morphology, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations and acid or alkaline tolerance. Genera of LAB are *Aerococcus*, *Alloiococcus*, *Bifidobacterium*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 1998).

LAB are important in food industry particularly, traditionally fermented foods. LAB are considered as 'food grade' organisms that are safe to consume (Adams and Marteau, 1995). LAB play a defining role in the preservation and microbial safety of fermented foods (Caplice and Fitzgerald, 1999), thus promoting the microbial stability of the finished products of fermentation (Mensah et al., 1991). Protective of LAB in foods is due to the production of organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl (Atrih et al., 2001), antifungal compounds such as fatty acids (Corsetti et al., 1998) or phenyllactic acid (Lavermicocca et al., 2000), bacteriocins (De Vuyst and Vandamme, 1994) and antibiotics such as reutericyclin (Höltzel et al., 2000). Most of LAB are related to various fermented foods, such as fermented sausages, yogurts and fermented vegetables. In recent years, many bacteriocins-producing LAB have been isolated from various food as summarized in Table 2.2.

LAB-producing bacteriocin have been isolated from Thai fermented foods. Nisin-producing *Lactococcus lactis* WNC 20 isolated from Thai fermented sausage (Nham) which not only inhibited closely related LAB, but also some food-borne pathogens including *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus* (Noonpakdee et al., 2003).

Table 2.2 Bacteriocin producing LAB isolated from food and fermented foods

Source of bacteriocin	Bacteriocin	Source of bacteria	Reference
Lactococcus. lactis ssp. *lactis* LJH80 and NCK 400	Nisin	Sauerkraut	Harris et al. (1992)
Lactococcus. lactis ssp. *lactis* M30	Lacticin 3147	Barley	Settanni et al. (2005)
Lactococcus lactis MMT24	Lactococcin MMT24	Traditional cheese	Ghrairi et al. (2005)
Lactococcus. lactis KAC 2386	Lactococcin K	White kimchi	Ko and Ahn. (2000)
Leuconostoc spp. *J2*	Leuconocin J	Kimchi	Choi et al. (1999)
Leuconostoc mesenteroides 6	Leuconocin A and leucocin	Malted barley	Vaughan et al. (2001)
Leuconostoc mesenteroides E131	Mesenterocin E131	Fermented sausage	Xiraphi et al. (2008)
Leuconostoc mesenteroides 7 and 10	Cleucocin C	Malted barley	Vaughan et al. (2001)
Leuconostoc mesenteroides ssp. *Dextranicum* ST99	Mesentericin ST99	Fermented sausage	Todorov and Dick. (2004)
Pediococcus acidilactici	Pediocin PA-1	Fermented sausage	Albano et al. (2007)
Pediococcus pentosaceus ST18	Pediocin ST 18	Fermented milk	Todorov and Dick (2005)
Pediococcus pentosaceus ST44AM	Pediocin PA-1	Fermented fruit (*Scerocarya birrea*)	Todorov and Dick (2009)
Pediococcus pentosaceus 05-10	Pediocin 05-10	Pickle	Huang et al. (2009)
Pediococcus pentosaceus Iz3.13	Pediocin AcH	Fermented seafood	Bagenda et al. (2008)
Pediococcus damnosus NCFB 1832	Pediocin PD-1	Wine	Bauer et al. (2007)
Pediococcus. pentosaceus Mees 1934	Pediocin SM-1	Meat	Anastasiadou et al. (2008)
Pediococcus acidilactici HA-6111-2	PA-1 bacteriocin	Fermented meat sausage	Albano et al. (2009)
Lactobacillus sake C2	Sakacin C2	Fermented cabbage	Gao et.al. (2009)
Lactobacillus sakei	Bacteriocins ST22Ch, ST153Ch and ST154Ch	fermented meat product	Todorov et al. (2013)
Lactococcus lactis 69	Nisin-like	Charqui (traditional fermented food)	Biscola et al. (2013)

P. pentosaceus BCC 3772 isolated from Nham was found to produce pediocin PA-1/AcH which effectively inhibited growth of *L. monocytogenes* in spiked Nham

samples when applied as a starter culture (Kingcha et al., 2012). Morover, Wilaipun et al., (2004) reported that bacteriocin produced by *Enterococcus faecium* NKR-5-3 isolated from Thai fermented fish (pla-ra) was enterocin NKR-5-3A and enterocin NKR-5-3B. Two antibacterial peptides were against some of Gram-positive such as *B. cereus* and *Listeria inoccua* but no activity against any Gram-negative bacteria. Noonpakdee et al., (2009) reported that *Lactobacillus plantarum* PMU33 strain isolated from Som-fak (fermented fish) produced bacteriocin that inhibited Gram-positive bacteria including food borne pathogens, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. However, variation of LAB strains found in Thai fermented fish products were *Lb. plantalum*, *Lb. pentosus*, *Lc. lactis*, *P. petosaceus*, *Weisella confuse*, *Carnobacterim piscicola* (Ostergaard et al., 1998). Some LAB strains had the anti-listerial activity but their identities of bacteriocins have not yet been elucidated.

2.3 Production of bacteriocins

Bacteriocin production can be influenced by culture conditions among them including medium composition, pH, temperature, and microorganism growth phase (Ganzle et al., 1999). Supplementation of the medium with growth limiting factors such as carbohydrates, nitrogen sources, vitamins and potassium phosphate or adjustment of the medium pH, can increase the levels of bacteriocin production (Todorov and Dicks, 2005). Stimulation of bacteriocin production by yeast extract and meat extract has been reported for pediocin AcH (Bhunia et al., 1988) and helveticin J (Joerger and Klaenhammer, 1986). Kim et al. (2006) found that maximum bacteriocin activity was obtained in the modified MRS medium containing 0.5% tryptone and

1.0% yeast extract as nitrogen sources instead of other nitrogen sources present in MRS medium. Todorova et al. (2012) reported that yeast extract yielded two time increased activity (3200 AU/ml) compared with control (tryptone, meat extract and yeast extract) (1600 AU/ml) for bacteriocin ST22Ch produced by *Lactobacillus sakei* ST22Ch. A combination of tryptone and meat extract (12.5:7.5 g/L), meat extract and yeast extract (10.0:10.0 g/L) or tryptone as single organic nitrogen source yielded 1600 AU/ml. However, the result suggests that combination of yeast extract required for optimal bacteriocin ST22Ch production. Moreover, the concentration of yeast extract affected cell mass and bacteriocin production. The maximum bacteriocin was obtained at 3.0% of yeast extract where cell mass was 2.43 g/L (Cheigh et al., 2002). Todorov and Dicks (2006) reported that the presence of meat extract as the only nitrogen source, or a combination of meat extract and yeast extract (1:1), resulted in approximately 50% reduction of bacteriocin ST23LD production. Pei et al. (2013) reported that yeast extract played an important role on paracin C production of *L. paracasei* CICC 20421. This could be due to the availability of larger quantity of free amino acids, short peptides and more growth factors from yeast extract that induce bacteriocin production (Castro et al., 2011). In addition bacteriocin producer strains are nutritionally fastidious microorganisms, cell growth and bacteriocin production have been shown to be influenced by organic nitrogen source. Therefore, nitrogen source is the most impotant factor for affecting on bacteriocin production.

Bacteriocins can be produced from media containing different carbon sources. Glucose, the rather cheap carbohydrate, was shown to be a favorable carbohydrate for production of paracin C. The same results were reported on plantaricin UG1 (Enan et al., 1996) and bacteriocins ST202Ch and ST216Ch (Todorov et al., 2010). Nisin Z can

be produced from glucose, sucrose and xylose by *Lactococcus lactis* IO-1 (Matsusaki et al., 1996; Chinachoti et al., 1997b) but better results are obtained with glucose (4000 IU/ml) compared to xylose (3000 IU/ml). Per Drosinos et al. (2005) reported that level, while type of sugar used as a carbon source affected both growth and bacteriocin production with glucose being better source for biomass production, while fructose more suitable for bacteriocin production produced by *Leuconostoc mesenteroides* E131. Nevertheless, sucrose is found to be a better carbon source than glucose for enterocin 1146 production. Fructose or lactose results in as comparable levels of biomass but low bacteriocin (Parente and Ricciardi, 1994b). Per Todorov et al. (2005) reported that an increase in bacteriocin ST16Pa (*L. plantarum* ST16Pa) production was record in the presence of 30 g/L glucose (102 400 AU/ml), 50 g/L of glucose (51200 AU/ml) or 20 g/L of mannose (51200 AU/ml). Lactose does not stimulate bacteriocin ST11BR, produced by *Lactobacillus paracasei* subsp. *paracasei* ST11BR, whereas growth in the presence of 20.0 g/l maltose, sucrose or mannose yield bacteriocin of 25 600 AU/ml. Production of bacteriocin ST16Pa is stimulated by the presence of glucose and mannose (Todorov et al., 2011). Thus, carbon source on bacteriocin production might play important role increasing bacteriocin yield.

The pH control improves the growth of LAB, it also results in improved bacteriocin production. The optimal pH for bacteriocin production is usually 5.5-6.0 (Meghrous et al. 1992; Kaiser and Montville 1993; Parente et al. 1994; Parente and Ricciardi 1994a; Matsusaki et al. 1996; Chinachoti et al. 1997b) which is often lower than the optimal pH for growth. Todorov and Dicks, (2009) reported that in MRS broth (Biolab) adjusted to pH 6.0 or 6.5, peptide ST4SA high levels of 51,200 AU/ml were recorded. At pH 5.0 and 4.5 low levels of peptide ST4SA (3,200 AU/ml and

1,600 AU/ml) were recorded. The optimal pH for production of enterocin P ranged between pH 5.7 and 6.0. Maximum growth of producer strain was recorded at pH 6.2–7.0 (Herranz et al., 2001). The optimal pH for production of bacteriocin ST311LD, produced by *E. faecium* ST311LD was 5.0–6.0 (Todorov and Dicks, 2005). Optimal levels of bacteriocin production by *E. faecium* RSC5 was obtained at pH 6.5. At 35^{o}C enterocin RS C5 activity was recorded only between pH 5.5 and 8.0 (Leroy and de Vuyst, 2003). The results concluded that the optimal production of *E. faecium* bacteriocins occur during early logarithmic growth, usually at a pH above 5.5 (Todorov et al., 2000; Jimenez-Diaz et al., 1993). Therefore, the pH control is the effective factor on bacteriocin production.

Moreover, growth at the optimal temperature usually results in optimal bacteriocin production (Meghrous et al. 1992; Daba et al. 1993; Matsusaki et al. 1996). Bacteriocin production is found at 30^{o}C and not at 37^{o}C. Indeed, growth temperature and bacteriocin production are often correlated as observed for lactocin A (Parente et al., 1994), enterocin 1146 (Parente and Ricciardi, 1994), lactocin S (Mortvedt-Abildgaard, 1995), amylovorin 147 (Nel et al., 2001), nisin Z (Matsusaki et al., 1996) and mesenterocin (Kim et al., 1997). Per Drosinos et al. (2005) reported that the effect of the environmental factors on growth and bacteriocin production of *Leuc. mesenteroides* E131. Bacteriocin production is favored when the microorganism is grown at temperatures closed to the optimum for growth (25^{o}C). The specific rate of bacteriocin production increases with higher temperature and is optimal at the optimum growth temperature (25^{o}C). Production of pediocin SA-1 from the strain *P. acidilactici* NRRL B5627 is also at 30°C (Anastasiadou et al., 2008). A higher temperature found to be as the optimum for pediocin production for *P. acidilactici* F

isolated from a sausage isolates (Osmanagaoglou et al., 1998). Production of pediocins from *P. pentosaceus* strains is at 37°C for *P. pentosaceus* ACCEL (Wu et al., 2004), and *P. pentosaceus* L and S (Yin et al., 2003), 35°C for *P. pentosaceus* Pep1 (Osmanagaoglou et al., 2001), 30°C for *P. pentosaceus* ST18 (Todorov and Dicks. 2005) and pediocin PD-1 from *Pediococcus damnosus* NCFB1832 (Bauer et al., 2005).

2.4 Determination of bacteriocin activity

The detection of bacteriocins and other antimicrobial substances from LAB has involved the use of a variety of methodologies with both agar (Kekessy and Piguet, 1970; Tagg and McGiven, 1971) and broth cultures (Toba et al., 1991). Many screening techniques have been used for the detection of bacteriocin. They typically fall into one of two main assays: the direct and deferred antagonism assays (Ray, 1992). In the former, the putative bacteriocin producing and indicator strains are grown simultaneously in appropriate conditions using liquid (associative growth) or semisolid media (e.g., spot-on-lawn, cross streak, well-diffusion and disc assays, etc.). Production of the antimicrobial substance is evidenced by the inhibition of the growth of the indicator strain after incubation as indicated by the decline in the colony-forming units or in OD (liquid media) or by the development of clearing zones around the spot, streak or well of the active strain (agar media).

In the deferred antagonism assay, the putative bacteriocin-producing strain is pre-incubated in a suitable medium to allow bacteriocin production. The cells are removed or inactivated before inoculating the medium with the indicator strain and allowing for further incubation. In some instances, the putative producer is not eliminated after the

first incubation but only physically separated from the indicator organism (e.g., by flipping the agar medium and inoculating the reversed surface). Although the direct and deferred antagonism techniques are widely used, they have many limitations in relation to their sensitivity and ability to exclude the inhibitory effect of undesired substances. Furthermore, their accuracy depends largely on the medium (i.e., composition, buffering capacity, suitability for the diffusion of antimicrobial substance) as well as on the strains used and their respective inoculum sizes (Geis et al. 1983; Davidson and Parish 1989; Spelhaug and Harlander 1989). Therefore, many variants of either direct or deferred antagonism assays have been developed to address such limitations (Tagg and Mac Given 1971; Davidson and Parish 1989; Spelhaug and Harlander 1989; Tagg and Bannister 1989; Kang and Fung 1998).

However, the development of faster and less laborious techniques was useful for the detection of strains producing bacteriocin. The polymerase chain reaction (PCR) has provided a method to detect DNA sequences with high speed and sensitivity (Hofstra et al. 1994). This technique is emerging as a powerful tool to identify and select bacterial isolates with specific functions. Because the DNA sequences that encode the genetic determinants for the biosynthesis of some bacteriocins are known (Klaenhammer, 1993, Jack et al., 1995), primers and DNA probes may easily be designed for their specific detection. Gene detection is necessary for synthesis of lacticin 481 and nisin using PCR techniques with specific probes in an isolate of *Lc. lactis* subsp. *lactis*. Methods to detect inducing activities of nisin via nisin-specific two-component regulatory system have been developed and achieved high sensitivity and specificity (Garde et al., (2001); Wahlstrom and Saris 1999; Reunanen and Saris 2003; Hakovirta et al., 2006). Out of 339 screened isolates, 26 strains show activity

towards various wine related and non-wine-related indicator strains. A PCR-based screening reveals the presence of the plantaricin encoding genes plnA, plnEF, plnJ and plnK in five selected *Lactobasillus plantarum* strains. Furthermore, a co-culture experiment with *Lactobasillus plantarum* and *Enterococcus faecalis* is performed. A complete inhibition of cell growth of *Enteroccus faecalis* was observed within 72 h. Four putative bacteriocin-encoding genes in the genome of *O. oeni* are identified and sequenced (Knoll et al., 2008).

Recent studies suggest that some enterococcal strains may produce more than one bacteriocin. For example, the production of two synergistic bacteriocins has been reported for several Enterococcus species: enterocins 1071A and 1071B (Balla, Dicks, du Toit, van der Merwe, and Holzapfel, 2000), enterocins L50A and L50B (Cintas et al., 2000), enterocins A and B (Ennahar, Asou, Zendo, Sonomoto, and Ishizaki, 2001; Ghrairi, Frere, Berjeaud, and Manai, 2008) and enterocin A and P (Makanera, Arlet, Gautier, and Manai, 2003). Moreover, Franco et al., (2012) reported that the structural of enterocin A, B, P and LB50B genes were found in *E. faecium* ETW20 and ETW22 strains while *E. faecium* ETW15 exhibited the presence of enterocin A and P genes. Franz et al., (2007) reported that the gene expression depends on environmental conditions or genetics mechanisms like induction or transcription control. The efficient gene transfer mechanisms and the fact that bacteriocin genes are often located on transmissible genetic entities may explain the production of multiple bacteriocins by single strains. However, 16S rRNA gene sequence and gene detection are useful molecular techniques for LAB bacteriocinogenic organisms identification.

2.5 Bacteriocin purification and characterization

Bacteriocin-producers are LAB that needs complex nutritional exigencies to grow, and this not only increases the production cost, but also makes the purification of bacteriocins more difficult (Li, Bai, Cai, and Ouyang, 2002). Since bacteriocins form an extremely heterogeneous group of substances, specific purification protocols generally need to be designed for each of them, which may explain why only few bacteriocins have been purified to homogeneity like nisin. Three major methods for the purification of LAB bacteriocins can be distinguished according to their biochemical structure. First, purification can be done by a conventional method that is based on a rather laborious series of subsequent steps of ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography (Parente and Ricciardi, 1999). Second, a simple three-step protocol has been developed, including ammonium sulfate precipitation, chloroform/methanol extraction/precipitation, and reversed phase high-pressure liquid chromatography, as the sole chromatographic step involved (Callewaert et al., 1999). Third, bacteriocins can be isolated through a unique unit operation, i.e. expanded bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titer through pH adjustment of the crude fermentation medium (Foulquié-Moreno, Callewaert and de Vuyst, 2001). Following the last two methods, which are more rapid and successful than the first conventional one, several bacteriocins with interesting industrial potential have been purified such as amylovorin L (produced by *Lactobacillus amylovorus* DCE 471 and belonging to the class II), several enterocins (produced by the *E. faecium* RZS C5, RZS C13 and FAIR-E 406 strains) and the lantibiotic macedocin (produced by *Streptococcus macedonicus* ACA-DC 198)

(Callewaert et al., 1999). Nisin, for example, has been purified using immunoaffinity chromatography (Prioult et al., 2000), expanded bed ion exchange (Cheigh, Kook, Kim, Hong, and Pyun, 2004) and reversed phase high-performance liquid chromatography (López et al., 2007). However, the several approaches for the analytical purification and some characteristic of bacteriocin of LAB bacteriocins have been published shown in Table 2.3.

Table 2.3 Purification strategies and characterization of bacteriocins produced by Lactic acid bacteria

Bacteriocin	Producer	Purification strategy	Characteristic of bacteriocin			Reference
			MW (KDa)	pH	Temp (°C)/ Time (min)	
Enterocin 1071A and B	*E. faecalis* BFE 1071	Ammonium sulfate precipitation, cation-exchange SP-Sepharose	3.8,4.2	3-12	121 °C for 15 min	Balla et al. (2000)
Enterocin AS-48RJ	*E. faecium* RJ16	Cation exchange chromatography, reversed phase high-performance chromatography	7.1	3.0-90	NA	Abriouel et al. (2005)
Enterocin (enterocin A and enterocin B)	*E. faecium*	Cation exchange chromatography, C18 Sep-pack chromatography and C18 RP-FPLC	4.8, 5.4	2-10	100 °C for 15 min	Ghrairi et al. (2008)
Enterocin LR/6	*E. faecium*	Ammonium sulfate precipitation, cation-exchange chromatography, gel-filtration, and reverse phase high-performance liquid chromatography.	6.1	2.0–8.0	121 °C for 15 min	Kumar et al. (2010)
Bacteriocins LM-2	*E. faecium*	Ammonium sulfate precipitation and cation exchange chromatography.	3.5 ,6.4	2.0-12.0	121 °C for 15 min	Liu et al. (2011)
Sakacin LSJ618	*Lb. sakei* LSJ618	Ammonium sulfate precipitation and Sephadex G-25 chromatography	5.2	2-8	121 °C for 30 min	Jiang et al. (2012)
Pediocin PA-1/AcH	*P. pentosaceus* BCC 3772	Hydrophobic interaction chromatography, cation-exchange chromatography , RP-HPLC (Resource RPC column)	4.6	2-10	121 °C for 15 min	Kingcha et al. (2012)
Paracin C,	*Lb. paracasei* CICC 20241	Cation exchange column, reverse-phase HPLC on a C18 reverse-phase column	5.0	2-10	100 °C for 30 min	Pei et al. (2013)
Bacteriocin ST71KS	*Lb. plantarum* ST71KS	Ammonium sulfate, SepPakC18 cartridge	5.0	2.0-12.0	121 °C for 20 min	Martinez et al. (2013)

NA, not available

2.6 Mode of action of bacteriocin

Bacteriocin producing cells are not affected by action of bacteriocin due to the development of specific immunity mediated by a protein (Hancock and Chapple, 1999). Bacteriocins have generally a cationic character and easily interact with Gram-positive bacteria that have a high content of anionic lipids in the membrane determining the formation of pores (Cleveland et al., 2001; Chen and Hoover, 2003). Pores in the cytoplasmic membrane clearly affect the energetic status of the cell i.e., dissipation of proton motive force (PMF) causes an arrest of ΔpH and Δψ (transmembrane electrical potential) dependent (e.g. transport) processes while certain bacteriocins cause ATP efflux (Moll et al., 1999). The mechanisms of bacteriocins have been reported act on target cells including permeabilization of the cytoplasmic membrane followed by leakage of low molecular weight cellular compounds, dissipation of the proton motive force (PMF), cell lysis, degradation of vital macromolecules (such as DNA, RNA), inhibition of biological processes such as synthesis of protein DNA, RNA and peptidoglycan (Schillinger et al., 1996). However, mode of action of bacteriocins is not yet fully comprehended. Class I and Class II bacteriocins can work via different mechanisms to exert an antimicrobial effect. The cell envelope is generally the target.

a) Mode of action class I

Nisin and many other lantibiotics have been well studied in terms of their modes of action. The pores formed by lantibiotics may have lifetime of a few to several hundred milliseconds with diameters of up to 2 nm (Sahl et al., 1987). The initial electrostatic attraction between the target cell membrane and the bacteriocin peptide is thought to be the driving force for subsequent events. Various

models for pore formation have been proposed during the years. For the lantibiotic nisin, pore formation is thought to occur through a series of distinct steps. In equilibrium, nisin seems to orient parallel to the surface of the membrane (Breukink et al. 1998). The PMF consists of two components: a transmembrane electrical potential ($\Delta\Psi$) and the pH gradient (ΔpH) (Montville et al. 1995). In principle, if a compound interacts with and damages the membrane, changes will occur in either the $\Delta\Psi$, the ΔpH or both. The $\Delta\Psi$ and the ΔpH are able to induce pore formation possibly by driving the membrane insertion of nisin domains. The C-terminus of nisin inserts deeply into the membrane (Martin et al. 1996; Winkowsky et al. 1996), and it has been suggested that the entire nisin translocates across the membrane (van Kraaij et al., 1998). Nisin induces the transmembrane movement of fluorescent phospholipid suggesting that the membrane insertion of the C-terminus of nisin causes inter monolayer contact of phospholipids (Moll et al., 1998c). A wedge-like model for nisin-induced pore formation may involve a proton motive force driven co-insertion of lipids and nisin domains. The hinge(s) in the nisin molecule might allow bending of the C-terminal part and thus its insertion into the membrane. Multiple inserted nisin molecules may give rise to a large local disturbance of the lipid bilayer organization causing formation of transient lipid-protein pores. Such structures are intrinsically unstable due to the hydrophobic forces that will drive the re-arrangements of the lipids into their original bi-layer organization (Driessen et al., 1995).

Nisin forms pores that disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death (Benz, Jung and Sahl, 1991; Sahl, Kordel and Benz, 1987). Other lantibiotics that also form pores include lacticin 3147, Pep5, subtilin and epidermin (Brotz et al., 1998, Kordel, Benz

and Sahl, 1988, McAuliffe et al., 1998; Schuller, Benz and Sahl, 1989). However, it has long been recognized that nisin also interferes with cell wall biosynthesis (Linnett and Strominger, 1973; Reisinger, Seidel, Tschesche and Hammes, 1980). It has now been established that this phenomenon is mediated by the ability of nisin to bind lipid II, a peptidoglycan precursor, thus inhibiting cell wall biosynthesis. Such binding is also intrinsic to the ability of nisin to form pores. The possession of dual mechanisms of action renders nisin active at nM concentrations (Breukink et al., 1999).

b) Mode of action class II

Class II bacteriocins predominantly act by forming pores, causing dissipation of the cell membrane, depletion of intracellular ATP and leakage of amino acids and ions. The activity of class II (in the nanomolar range) mainly induces membrane permeabilisation and leakage of molecules from sensitive bacteria (Table 2.4). The inhibition spectrum is rather narrow limited to species or strains related to the producers. Class IIa bacteriocins are bactericidal peptides which act primarily by permeabilizing the membranes of susceptible microorganisms, probably through the formation of poration complexes, causing an ionic imbalance and leakage of inorganic phosphate (Klaenhammer, 1993; Jack, Tagg and Ray, 1995). A consequence of such disruptions is the dissipation of proton motive force (PMF), which involves the partial or total dissipation of either or both the transmembrane potential ($\Delta\Psi$) and the pH gradient (ΔpH). Unlike lantibiotics, which totally dissipate both $\Delta\Psi$ and ΔpH (Montville and Chen, 1998). The lethal activity of class IIa bacteriocins is thus mainly ascribed to the dissipation of the PMF (Abee, 1995; Venema, Venema and Kok, 1995). Particularly, the intracellular ATP is depleted by rates of up to 98.9% (Bennik et al., 1998; Chen and Montville, 1995).

Table 2.4 Mode of action of Class II bacteriocins

Subclass	Mode of action	ΔpH	Δψ	ATP	Efflux
Subclass IIa					
Bavaricin MN	Pore formation	Dissipation	Dissipation	ND	CF
Enterocin P	K^+ pores	No effect	Dissipation	Depletion of intracellular ATP	K^+, CF
Mesentericin Y105	Pore formation PTS permease-dependent	ND	Dissipation	ND	Amino acids
Pediocin PA-1	Pore formation	ND	Dissipation	Depletion of intracellular ATP	Amino acids K^+
Subclass IIb					
Lactacin F	Pore formation	ND	Dissipation	Depletion of intracellular ATP	K^+ and Pi efflux
Lactococcin G	Cation pores	No effect	Dissipation	Depletion of intracellular ATP	Amino acids
Plantaricin EF	Cation pores	Dissipation	Dissipation	ND	Cations
Plantaricin JK	Anion pores	Dissipation	Dissipation	ND	Anions
Subclass IIc					
Lactococcin A	Pore formation	ND	Dissipation	Dissipation	Amino acids
Lactococcin 972	Inhibition of septum formation	ND	ND	ND	No efflux
Plantaricin A	Pore formation	Dissipation	Dissipation	ND	ND

ND, not determined; CF, carboxyfluorescein; ΔpH, pH gradient; (Δψ), membrane potential; ATP, intracellular ATP; Efflux, leakage of amino acids and ions

Source: George et al. (2008)

Per Bhunia et al. (1991) first reported on the mode of action of subclass IIa bacteriocins. They described that treatment with pediocin AcH resulted in the leakage of K^+ and some UV absorbing materials. Besides, pediocin PA-1 is shown to dissipate

the membrane potential ($\Delta\psi$) of *Pediococcus pentosaceus* and to cause release of amino acids accumulated either in a proton motive force (PMF) dependent or independent manner (Chikindas et al., 1993). It is finally proposed that pediocin PA-1 might modify the permeability of sensitive cells by forming pores in the cytoplasmic membrane, and that it needs a specific target molecule at the surface of the sensitive cells. Furthermore, more purified pediocins, has been examined for their mode of action (Green et al., 1997; Bauer et al., 2005, and Anastasiadou et al., 2008). Pediocin PD-1 acts on cytoplasmic membrane, and the antimicrobial activity is due to the generation of pores in the membrane. The ability of pediocin PD-1 to form pores in sensitive cells of *O. oeni* as observed by K^+ loss is found to be pH dependent and increases when the extracellular pH is reduced (Bauer et al., (2005). However, class II bacteriocins are believed to bind to a chiral receptor in cell membranes and create a pore that depolarizes the target cell. The exact nature of the bacteriocin receptor interaction is not yet understood, but it appears to be mediated by the membrane bound proteins (Derksen et al., 2008).

2.7 Applications in food industry

Nowadays, bacteriocins have been widely for food preservation. The use of bacteriocins in food industry especially on dairy, egg, vegetable and meat products has been extensively investigated. Since the majority of bacteriocin-producing LAB are natural food isolates, their antimicrobial peptides could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner, which is likely to be more acceptable to consumers (Cleveland et al., 2001; Deegan et al., 2006). Nisin, a bacteriocin produced by *Lactococcus lactis subsp. lactis* was the

first bacteriocin approved for food use and is the only bacteriocin widely employed as a food preservative (Arauz, et al., 2009). Many studies have also focused on the selection and development of bacteriocin for food applications (Leroy et al., 2006; Ross et al., 2002) such as inhibition of spoilage and pathogenic bacteria during the shelf life period of non-fermented foods.

Over the past decade the recurrence of listeriosis outbreaks, combined with the natural resistance of the causative agent, *L. monocytogenes*, to traditional food preservation methods such as its ability to grow at near freezing temperatures has focused the attention of bacteriocin researchers on this organism. This attention has resulted in the isolation of a large number of bacteriocins, all of which are highly active against *L. monocytogenes*. Several studies demonstrate the effectiveness of these compounds in food biopreservation shown in Table 2.5. Moreover, the members of the genus Enterococcus are found in many food products. As already mentioned, enterococci have been an important component of many artisanal starter cultures, used for the preparation of dairy products (Foulquié Moreno et al., 2006).

Table 2.5 Application of bacteriocins in food

Bacteriocin	Producer	Test microorganism	Product	Reduction (log CFU/g)	Reference
Nisin	*Lac. lactis*	*L. innocua*	Milk	3.7-3.8	Zapico et al. (1998)
Nisin	*Lac. lactis*	*L. monocytogenes*	Fermented milk	6.0	Benkerroum et al. (2002)
AcH Pediocin	*Lb. plantarum*	*L. monocytogenes*	Cheese	1.0-2.0	Loessner et al. (2003)
Bacteriocin	*Lac. lactis* sp. *lactis*	*L. monocytogenes*	Sausages	1.5	Benkerroum et al. (2003)
Enterocin	*E. faecium*	*L. monocytogenes*	Milk	2.0	Elotmani et al. (2002)
Enterocin AS-48	*E. faecalis*	*L. monocytogenes*	Sausage	1.0	Ananou et al. (2005)
Amysin	*B. amyloliquefaciens*	*L. monocytogenes*	Sliced bologna sausage	2.3	Kaewklom et al. (2013)

Discovery of bacteriocins, the use of enterococci as starter cultures or co-cultures has been studied by various researchers not only for their organoleptic properties, but also for their negative effect on food pathogens by production of enterocins as shown in Table 2.6. Bacteriocin producing enterococcal strains is suitable for use as starter culture due to its high acid and flavor production. They also offer a double advantage of being used as sole culture for both fermentation and food preservation. However, if the enterocin producing strain is being used along with another starter culture, then it should not interfere with the acid and flavor producing properties of the starter strain, otherwise the preservative effect of the enterocin will be nullified by a low quality product.

Table 2.6 Utilization of enterococcal strains for in-situ bacteriocin production

Enterocin produced	**Producer strain**	**Product test**	**Typical target**	**Reference**
Undefined bacteriocin	*E. faecium* 7C5	Italain cheese	*L. monocytogenes*	Giraffa and Carminati, (1997)
Undefined bacteriocin	*E. faecium* 7C5	Milk	*L. innocula*	Giraffa et al. (1995)
Enterocin	*E. faecium* FAIR-E 198	Greek Feta cheese	*L. innocua* CTC 1014	Sarantinopoulos et al. (2002)
Enterocin 416 K1	*E. casseliflavus* IM 416K1	Italain cheese	*L. monocytogenes*	Sabai et al. (2002)
Undefined bacteriocin	*E. faecium* RZS C5 and *E. faecium* DPC 1146	Cheddar cheese	*L. inocua* LMG 13568	Foulquie´ Moreno et al. (2003)
Enterocin A-48-32	*E. faecalis* A-48-32	Non-fat hard cheese	*B. cereus*	Munoz et al. (2004)
Enterocins L50A and B	*E. faecium* F58	Goat's milk and Jben (goat milk's cheese)	*L. monocytogenes*	Achemchem et al. (2006)
Enterocin A-48-32	*E. faecalis* A-48-32	Skimmed milk and Non-fat unripened soft cheese	*S. aureus*	Munoz et al. (2007)

Table 2.6 (Continued)

Enterocin produced	Producer strain	Product test	Typical target	Reference
Enterocin A and B	*E. faecium* WHE 81	Munster cheese	*L. monocytogenes*	Izquierdo et al. (2009)
Undefined bacteriocin	*Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch	Fresh Minas cheese	*L. monocytogenes*	Pingitore et al. (2012)

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains and growth media

Reference strains used were *Lb. fermentum* TISTR 876, *Lb. plantarum* TISTR 050, *Lb. plantarum* TISTR 877, *Lb. acidophilus* TISTR 1034, *B. cereus* TISTR 687, *L. monocytogenes* TISTR 1327, *S. aureus* TISTR 517, *E. coli* TISTR 887, *S. enteritidis* JCM 1652, *S. typhimurium*, *M. morganii* and *S. xylosus*. Lactic acid bacteria were grown on MRS medium, while other cultures were grown on tryptic soy broth (TSB) (Difco, Becton, Dickinson and Company sparks, MD, USA). The media and optimum temperature used for culturing test organisms were shown in Table 3.1. The reference strains were obtained from stock cultures of Thailand Institute of Scientific and Technological Research (TISTR) and Suranaree University of Technology, the School of Microbiology (SUT).

3.2 Isolation of lactic acid bacteria from traditional fermented foods

Lactic acid bacteria (LAB) strains were isolated from pla-som (acid fermented fish), pla-ra (salted fermented fish), somkai-pla (fermented fish roe) and kong-jom (fermented shrimp). A total of 11 samples were collected from six local markets in Nakhon Ratchasima, Yasoton, Kalasin, Burirum, Surin and Chaiyapom province LAB were isolated by the spread plate technique using four selective media, including carnobacterium medium (CN), MRS, nd GYP, MRS containing 0.5% NaCl. The

plates were all supplemented with 0.5% $CaCO_3$ and incubated at 35°C for 48 h under anaerobic condition. After incubation, colonies with different morphological characteristics were randomly selected for further investigation.

3.3 Screening of LAB producing bacteriocin

To determine bacteriocin-producing LAB isolates ability were tested using the agar well diffusion technique. One loopful of 48 h culture was inoculated into 7 ml of MRS broth and incubated at 30°C under anaerobic condition for 18 h. Bacterial growth was monitored spectrophotometically at 600 nm, and all-free extract was obtained by centrifugation at 12,000 rpm at 4°C for 10 min. The supernatants were collected, determined for pH before adjusted to pH 6.5 using 3 N NaOH.

The agar well diffusion assay was tested according to Schillinger and Lucke (1989). Twenty ml of TSA soft agar (0.75%) was contained with 10^6 CFU/ml of test strains. Soft agar were poured into petri-dishes and allowed to solidify for 30 min at room temperature. Wells were prepared using a 6-mm sterilized cork borer, and 70 µl of supernatant was placed into each well. Diameters of the inhibition zones were measured after 18 h incubation under optimal temperatures for each test organism (Table 3.1). Activity was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain (Todorov et al., 2005).

For primary screening, 285 isolates of LAB were tested for bacteriocin production using agar well diffusion assay. Test organisms in the primary screening were *Lb. fermentum* TISTR 876, *Lb. plantarum* TISTR 050, *Lb. plantarum* TISTR 877, *Lb. acidophilus* TISTR 1034, *B. cereus* TISTR 687 and *S. aureus* TISTR 517. Inhibition

zones were measured for each test organism and isolates showing inhibition zone were chosen for secondary screening.

For secondary screening, test organisms were *L. monocytogenes* TISTR 1327, *E. coli* TISTR 887, *S. enteritidis* JCM 1652, *S. typhimurium*, *M. morganii* and *S. xylosus*.

Table 3.1 Test organisms used for bacteriocin activity detection

Test microorganism	Cultivation medium	Cultivation Temperature (°C)
B. cereus TISTR 687	TSB	37
E. coli TISTR 887	TSB	37
Lb. fermentum TISTR 876	MRS	37
Lb. plantarum TISTR 050	MRS	37
Lb. plantarum TISTR 877	MRS	37
Lb. acidophilus TISTR 1034	MRS	37
L. monocytogenes TISTR 1327	TSB	37
M. morganii	TSB	37
S. enteritidis JCM 1652	TSB	37
S. typhimurium TISTR 022	TSB	37
S. xylosus	TSB	37
S. aureus TISTR 517	TSB	37

3.4 Identification of the selected bacteriocin-producing LAB isolates

3.4.1 Morphological characteristics

Four isolates were selected based on their antibacterial activity against *L. monocytogenes* TISTR 1327. The selected isolates were cultured on MRS agar and incubated at 30°C for 48 h under anaerobic condition. Cell morphology and cell

arrangement were observed by Gram staining. Cell size was determined using a light microscope (Model BX51TRF, Olympus Optical Co., Ltd., Tokyo, Japan) in conjunction with Image-Pro Plus Version 6.0.0.260 (Media Cybernetics, Inc. Tokyo, Japan).

3.4.2 Physiological and biochemical characteristics

3.4.2.1 Catalase test

Cells were transferred to the surface of a glass slide and 3% hydrogen peroxide was dropped onto glass slide. Positive result showed rapid gas formation.

3.4.2.2 Oxidase test

Oxidase test was determined by dropping 1% of tetramethyl-p phenylenediamine dihydrochloride on filter paper (Whatman no.4) and the bacterial cells were streaked on the filter paper. Appearance of dark blue indicated a positive result.

3.4.2.3 Nitrate reduction

Cultures were inoculated into nitrate reduction broth (Appendix A 2.5) and incubated at 35^{o}C for 7 days. After incubation, 1 drop of solution A (Appendix 1.5) was added. Development of pink color in 5 min indicated the positive result. Samples with negative result was added zinc powder, development of red color indicated the presence of nitrate in the medium.

3.4.2.4 Extracellular enzymes

The production of proteinase, amylase, and lipase was tested using MRS agar containing 1% skim milk, 1% starch, 1% Tween80, respectively. Each LAB isolate was point inoculated onto these media and incubated at 30°C for 48 h under anaerobic condition. A positive reaction of the proteolytic test was indicated by clear

zone around colony. Starch hydrolysis was visualized by adding iodine solution to the plate and a clear zone indicated amylase production.

3.4.2.5 Carbohydrate fermentation

Carbohydrate fermentation tests were performed using API 50 CH/CHL Kit (BIO-Merieux, Marcy-I, E toile, France) according to the instruction manual.

3.4.2.6 Pyruvate utilization

Utilization of pyruvate was tested in 1% pyruvate broth. The medium used are described in the Appendix A 2.6.

3.4.2.7 Survival at 60^{o}C for 30 min

The overnight cell culture in 5 ml of broth MRS of four selected isolates (approximate 10^6 CFU/ml) were heat at 60°C for 30 min. Survival bacteria were enumerated on MRS agar.

3.4.2.8 Growth at various conditions

The effect of NaCl concentrations, temperature and initial pH on growth were determined in 5 ml of MRS broth and the inoculum size of 2% (approximate 10^6 CFU/ml).

The isolates were tested for the effect of growth on different NaCl concentrations, of 0, 2, 4, 6, 6.5,7,8 and 10% NaCl in MRS. The inoculated cultures were incubated at 35°C for 48 h under anaerobic condition. Optical density at the wavelength of 600 nm was used to evaluate bacterial all growth evaluation. Samples with positive result were transferred to 5 ml of fresh MRS and incubated at the same condition for 2 more cycles.

For the effect of temperature: cultures were added into 5 ml of MRS broth incubated at various temperatures, 10, 15, 20, 25, 30, 35, 40 45 and 55°C under

anaerobic condition for 48 h. Optical density at the wavelength of 600 nm was used to evaluate bacterial all growth evaluation. Samples with positive result were transferred to 5 ml of fresh MRS and incubated at the same condition for 2 more cycles.

For optimum pH, cultures were added into 5 ml of MRS broth and adjusted to different pHs of 2, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 The culture was incubated at 35°C for 48 h under anaerobic condition. Optical density at the wavelength of 600 nm was used to evaluate bacterial all growth evaluation. Samples with positive result were transferred to 5 ml of fresh MRS and incubated at the same condition for 2 more cycles.

3.4.3 Ribosomal RNA gene analysis

3.4.3.1 Bacterial genomic DNA extraction

Four LAB isolates were cultured in 5 ml MRS broth at 35^{o}C for 24 h. One ml of overnight cultured cells was added to a 1.5 ml microcentrifuge tube and cells were collected by centrifugation at 10,000×g for 5 min at 4°C. Total cellular DNA was extracted using the Wizard genomic DNA purification Kit (Promega, Madison, WI). DNA quality was tested using 1% agarose gel electrophoresis. The purified DNA was kept at -20°C until use.

3.4.3.2 Amplification of 16S rDNA

DNA from the −20°C stock was diluted to 10-150 ng using nuclease-free double distilled water (Invitrogen, NY, USA). The 16S rDNA fragment was amplified by PCR with the universal primer set of FD1(5′-AGAGTTTGATCCTGGCTCAG-3′) and RP2 (5′-CGGCTACCTTGTTACGACTT-3′) using an Cycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, USA). The amplification was done in 50-µl reaction. The reaction contained 4 µl of 25 mM

$MgCl_2$, 5 µl of 2 µM of each nucleoside triphosphate (dATP, dCTP, dGTP, dTTP; Invitrogen™ Life Technologies, Foster, CA., U.S.A.), 5 µl of 10X PCR buffer (200 mM Tris-HCl, pH 8.0, 500 mM KCl; Invitrogen™ Life Technologies, Foster, CA., U.S.A.), 2 µl of 10 pmole of each primer, 0.5 µl of 5 U Taq polymerase (Invitrogen™ life technologies, Foster, CA., U.S.A.) and 10-150 ng of 4 µl of Genomic DNA. The thermal cycling included an initial denaturation step at 95°C for 2 min; 35 cycles of a denaturation step at 95°C for 45 s, an annealing step at 55°C for 45 s, an extension step at 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR product size was 1,500 bp. This product was checked using 1% (w/v) agarose gel electrophoresis and the gel was visualized using ethidium bromide.

3.4.3.3 Cloning of 16S rRNA gene

The bacterial 16S rRNA gene from PCR amplification were purified with Wizard Gel/PCR product Kit (Promega corporation, Madison, WI., U.S.A.) and ligated into pGEM-T easy vector (Promega corporation, Madison, WI., U.S.A.) following the company's instruction. The recombinant vector was transformed into *E. coli* DH5α by an electroporator (Electroporator 2510, Eppendorf AG, Hamburg, Germany) set at 1800 V. The transformed *E. coli* DH5α was selected by blue-white selection method (Sambrook and Russell, 2001). The plasmid vector was purified using Wizard DNA purified Kit (Promega corporation, Madison, WI., U.S.A.) and the vector was cut using EcoRI (Invitrogen™ life technologies, Foster, CA., U.S.A.) and incubated at 37°C for 6 h to verify DNA insertion. DNA fragments were detected on 2% agarose gel electrophoresis.

3.4.3.4 DNA sequence analysis

The pGEM plasmid was used for sequence analysis of cloned 16S rRNA

fragments. T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAAT -3′) primer was used for sequencing. Nucleotide sequence data were obtained from DNA sequencing software of ABI 3730xl DNA analyzer (Model 373, Forster, CA., U.S.A.). The sequences were compared to local alignment search of GenBank database using the BLAST version 2.2.9 program of the National Center for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment was performed using CLUSTAL_X (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins, 1997). Phylogenetic tree was constructed by the Maximum Pasimony method with software MEGA version 4.0 (Kumar, Tamura, Jakobsen, and Nei, 2004). The robustness of relationships was evaluated by a bootstrap analysis through 1,000 bootstrap replications.

3.5 PCR detection of enterocin structural genes

PCR amplification of structural genes of enterocin A (entA), enterocin B (entB), enterocin P (entP), enterocin L50 (entL50A entL50B), was performed with specific bacteriocin PCR primers listed in Table 3.2. PCR was performed on a DNA thermal cycler in a final volume of 50 µl containing 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 mM each of the four dNTPs, 0.5 mM of each primer and 1.25 units of Taq DNA polymerase . The cycles used were 95 ^{o}C for 5 min for the 55^{o}C (for the primers of enterocin A) or 56^{o}C for the primers of other enterocins B, P, L50A and L50B) for 30 s, and 72^{o}C for 30 s for the next 35 cycles; 72^{o}C for 5 min were used for the last cycle. PCR products were resolved by electrophoresis in 1.5% agarose gels and digitized by the GelDoc 1000 documentation

system. DNA from strain *E. faecium* T136 and *E. faecium* L50 were used as positive control.

Table 3.2 Specific primers for the PCR detection of enterocin structural genes

Enterocin	Forward primer	Reverse primer	Reference
A	5'-GGT ACC ACT CAT AGT GGA AA-3'	5'-CCC TGG AAT TGC TCC ACC TAA-3'	de Vuyst et al.(2003)
B	5'-CAA AAT GTA AAA GAA TTA AGT ACG-3'	5'-AGA GTA TAC ATT TGC TAA CCC-3'	de Vuyst et al.(2003)
P	5'-GCT ACG CGT TCA TAT GGT AAT-3'	5'-TCC TGC AAT ATT CTC TTT AGC-3'	Cintas et al. (2000)
L50A	5'-ATG GGA GCA ATC GCA AAA TTA-3'	5'-TTT GTT AAT TGC CCA TCC TTC-3'	de Vuyst et al.(2003)
L50B	5'-ATG GGA GCA ATC GCA AAA TTA-3'	5'-TAG CCA TTT TTC AAT TTG ATC-3'	de Vuyst et al.(2003)

3.6 Optimization of bacteriocin production conditions

E. faecium CN-25 isolate was selected. It showed the highest bacteriocin production based on inhibition zone against *L. monocytogenes* TISTR 1327. Optimal conditions of its bacteriocin production were determined. Bacteriocin activity was detected using agar well diffusion assay and *L. monocytogenes* TISTR 1327 was used indicator strain.

3.6.1 Types and concentration of nitrogen source

To obtain the optimal nitrogen source and concentration, inexpensive agricultural byproducts were investigated, including defatted rice bran (RB); fish sauce sludge (FS) from fish sauce industry, yeast sludge (YS) from brewing industry and soybean pomace (SP) from soy sauce plant. Medium for bacteriocin production was the modified MRS medium containing % (w/v); glucose, 2; yeast extract, 0.5; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.05; tri-ammonium citrate, 0.2; sodium acetate 0.2 and Tween 80, 0.1 ml. The modified medium (300 ml) containing SP, RB, MP, YS, at 2 % (w/v) added with glucose, 2, yeast extract 0.5, K_2HPO_4 0.2, $MgSO_4.7H_2O$ 0.02,$MnSO_4.4H_2O$ 0.5, tri-ammonium citrate 0.2, sodium acetate and Tween 80, 0.1 ml and then the pH was adjusted to 7.0. The medium was satirized at 121^oC and used for bacteriocin production. Inoculum culture was prepared by inoculating a loopfull of *E. faecium* CN-25 into 10 mL MRS and incubating at 35 ^oC for 48 h. The inoculum size of 2% (approximate 10^6 CFU/ml) was transferred to the modified medium (300 ml) and incubated at 25^oC for 24 h with a shaking speed of 100 rpm. The bacteriocin activity of supernatant was determined using agar well diffusion assay with *L. monocytogenes* TISTR 1327 as an indicator strain.

3.6.1.1 Concentration of nitrogen source

The suitable byproduct was determined for bacteriocin production. Inoculum of (approximate 10^6 CFU/ml) 2% was transferred to the modified medium (300 ml) containing the suitable byproduct at various concentrations (0.5, 1, 1.5, 2 and 2.5%) and incubated at 25^oC for 24 h with a shaking speed of 100 rpm. Bacterial growth was determined by total viable counts using spread plate technique on MRS

agar and incubated at 35°C for 48 h. The bacteriocin activity of supernatant was determined using agar well diffusion assay.

3.6.2 Concentration of yeast extract

Yeast extract was applied as the growth factors. *E. faecium* CN-25 was cultivated by transferring a loopful of colony into MRS medium and incubating at 25°C for 24 h. Cells (approximate 10^6 CFU/ml) with inoculum size of 2% were transferred to the modified medium (300 ml) containing 0.2, 0.4, 0.5, 0.6 and 0.8% of yeast extract with the suitable concentration of nitrogen sources. The culture was incubated at 25°C for 24 h with shaking speed of 100 rpm. Bacterial growth was determined by total viable counts using spread plate technique on MRS agar and incubated at 35 °C 48 h. Bacteriocin activity was detected using agar well diffusion assay and *L. monocytogenes* TISTR 1327 was used indicator strain.

3.6.3 Concentration of carbon source

The optimum concentration of carbon source contained in the modified medium was investigated. Four concentrations of glucose as a carbon source (0, 0.5, 1.0 and 1.5%) were applied. The inoculum size of 2% (approximate 10^6 CFU/ml) was transferred to modified medium containing suitable concentration of nitrogen source, yeast extract in Erlenmeyer flask (300 ml) at different amounts of glucose. The culture was incubated at 25°C for 24 h with shaking speed of 100 rpm. The bacteriocin activity were investigated as described previously**.**

3.6.4 Effect of salts

Effect of salts was investigated using sodium acetate, di-potassium hydrogen phosphate and tri-ammonium citrate. The concentration of sodium acetate at 0.1, 0.2, 1.5, 2% (w/v) and di-potassium hydrogen phosphate at 0.1, 0.2, 0.5, 1% (w/v) of tri-

ammonium citrate at 0.1, 0.2, 0.3, 0.5% (w/v) were investigated using medium with optimum concentration of carbon and nitrogen sources. The cultures were incubated at 25^{o}C for 24 h with a shaking speed of 100 rpm. The bacteriocin activity was investigated.

3.6.5 Effect of inoculum size

The effect of different inoculum volumes on the production of bacteriocin was evaluated by inoculating 0.5, 1, 2, 3 and 4% inoculum in the modified medium broth. The cultures were incubated at 25^{o}C for 24 h. Changes in bacterial growth and activity of bacteriocin were determined.

3.6.6 Effect of temperature

The bacteriocin production at difference growth temperatures was determined in the modified medium at 25, 30 and 35^{o}C for 24 h. The culture was inoculated at 0.5% (v/v) and incubated with 25, 30 and 35^{o}C for 24 h under anaerobic condition. The bacteriocins activity was monitored.

3.7 Bacteriocin production

The optimum condition was used for bacteriocin production. Starter culture was prepared by inoculating a loopfull of *E. faecium* CN-25 into a 20 mL of the modified medium listed in Table 3.3 and incubating at 35^{o}C for 48 h. The culture of *E. faecium* CN-25 was inoculated at 0.5% (v/v). The bacteriocin production of *E. faecium* CN-25 was carried out in a fermenter using 3000 ml of modified broth. Fermentation was controlled at 25^{o}C, pH 7.0 with agitation of 100 rpm without aeration. At predetermined intervals, culture broth was taken from the fermentor and analyzed for cell growth. After centrifugation (6000×g for 20 min at 4^{o}C), the antibacterial activity

of the supernatant was measured by agar well diffusion assay as described above using *L. monocytogenes* TISTR 1327 as an indicator microorganism.

Table 3.3 Composition of the modified medium for bacteriocin production.

Component	**Content (g/l)**
Rice bran	5
Yeast extract	5
Glucose	5
Sodium acetate	20
K_2HPO_4	2
Tri-ammonium citrate	2
Tween 80	1
$MgSO_4$-$7H_2O$	0.1
$MnSO_4$ $4H_2O$	0.05

3.8 Effects of enzymes, pH and temperature on bacteriocin stability

Sensitivity of antimicrobial substance produced by *E. faecium* CN-25 towards different enzymes was tested. Crude bacteriocin was treated with proteinase K, trypsin, chymotrypsin, lipase and catalase. The enzymes were filtered through 0.22 µm filters and added to crude bacteriocin at a final concentration of 1 mg/ml. The mixture was incubated at 37^{o}C for 2 h. After incubation, the enzyme activity was terminated by heating at 100^{o}C for 5 min. Untreated samples were used as control. The residual bacteriocin activity was assayed against indicator strain *L. monocytogenes* TISTR 1327.

The effect of pH on bacteriocin activity of *E. faecium* CN-25 was tested by adjusting pH of cell-free supernatants to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 6 M NaOH or 5 N HCl, and incubating at 37^{o}C for 2 h. Residual antimicrobial activity was monitored using the agar well diffusion method. The modified medium at various pH 2–12 was used as the control.

The effect of temperature on bacteriocin activity was tested by incubating cell-free supernatant adjusted to pH 6.0 at 121^{o}C for 15 min, 100, 80, and 60^{o}C for 30 min. The residual antilisterial activity was tested.

3.9 Determination of antimicrobial spectrum

Cell-free supernatant of *E. faecium* CN-25 was used to determine antimicrobial spectra. Cells were grown in the modified medium broth for 24 h at 25^{o}C and cultures were centrifuged at 6000×g for 20 min at 4^{o}C. Activity of cell-free supernatant was tested against variation of indicator strains.

3.10 Purification of the bacteriocin

Cells were removed by centrifugation, and the pH of the cell-free supernatant was adjusted to 6.5. The crude bacteriocin was precipitated with 65% ammonium and overnight at 4^{o}C. After overnight stirring at 4^{o}C, pellets were collected by centrifugation at 10,000×g, 4^{o}C for 20 min and dissolved in 50 mM Tris-HCl buffer (pH 8.0). Subsequently, it was passed through a 0.22-μm filter (0.22 μm, Millipore, Belford, MA, USA) and filtrates were kept at −20°C until further use. The bacteriocin was loaded into DEAE–Sephacel ion exchange column (2.6 x 6.5 cm, GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Purification

was achieved using AKTA^{TM} FPLC with UNICRON^{TM} software version 3.2 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The elution was performed at a linear gradient of NaCl (0–1.0 M) at a flow rate of 1 ml/min. Peptides were monitored spectrophotometrically at 215 nm and collected in 5 ml-volume fractions. The bacteriocin activity of pooled fractions was determined using agar well diffusion assay with *L. monocytogenes* as an indicator strain. The pooled fraction with the highest bacteriocin activity was used for further study.

3.11 Minimal inhibitory concentration (MIC) determination

The MIC of bacteriocin produced by the *E. faecium* CN-25 was conducted as described by van Kuijk *et al.* (2011) with modifications. *L. monocytogenes* Scott A was grown in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 0.6% yeast extract (TGY, Difco) at 30°C for 24 h. Cells approximately 10^8 CFU/ml were diluted 100 folds in fresh TGY broth to a final of cells 10^6 CFU/ml. Stock solution of partially-purified bacteriocin in steriled water were prepared to contain final concentration of 2.38, 2.04, 1.70, 1.36, 1.02, 0.68, 0.34, µg/ml. The positive control nisin (Sigma, St Louis, MO, USA) was prepared at a 10 mg/m1 using 0.02 M hydrochloric acid (pH 1.7) of which 50 µl was added to wells containing 150µl of the diluted *L. monocytogenes* Scott A culture. Uninoculated TGY broth was used as a negative control. The positive control was prepared with wells containing 200 µl of the *L. monocytogenes* Scott A dilution without bacteriocin. Each plate was covered with a clear thermal adhesive sealing film (lot no. 204786; Fisher, Pittsburgh, PA, USA) to prevent evaporation of the contents. The plates were observed using a

Molecular Devices THERMO max Microplate Reader (Sunnyvale, CA, USA). The optical density of each well was measured at 595 nm every 30 min, with 5 s shaking prior to each reading, for 24 h at 30^{o}C. Each experiment was performed at least twice in duplicate.

3.12 ATP measurements

The effect of partially-purified bacteriocin on intracellular ATP levels was determined using an ATP Bioluminiscent Assay Kit (Sigma-Aldrich, Saint Louis, USA) as described by Guihard et al. (1993) with some modifications. All measurements were taken using a Luminoskan^{TM} single-tube luminometer (Luminoskan, Helsinki, Finland). An ATP standard curve was generated prior to each assay. Dilutions of ATP from 10^{-8} to 10^{-12} mol/ml were prepared using the ATP stock solution (3.79×10^{-7} moles/ml). One hundred µl of these dilutions were mixed with 100 µl of the ATP assay mix (luciferin, luciferase, $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine salt buffer salts), and the luminescence was measured. The concentrations were plotted against luminescence expressed in relative luminescence units. *L. monocytogenes* Scott A was grown in TSB broth to a final OD_{600} of 0.6. Cells were washed with 50 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.5) and kept on ice. The cells were then equilibrated at room temperature for 5 min. Subsequently, cells were energized by suspending in half of the original volume of 50 mM MES buffer (pH 6.5) with 0.2% glucose and holding at room temperature for 20 min. Two different measurements were performed: external ATP and total ATP (internal + external ATP). Prior to both measurements, 100 µl of the cell suspension

was mixed with 100 µl of ATP assay mix. For total ATP levels including 20 µl of the cell mixture and partial bacteriocin (final concentration 2.38 µg/ml) or water (2 µl) (control) was mixed with 80 µl of DMSO (FisherBiotech, Fair Lawn, NJ) and 4.9 ml of cold water. For the external ATP measurements, 100 µl of the cell mixture including partial bacteriocin (final concentration 2.38 µg/ml) or control was mixed with 4.9 ml of 50 mM MES buffer (pH 6.5). Nisin (final concentration 1.5 µg/ml) and DMSO were used as positive controls for total ATP. For the external ATP determination, water, nisin (final concentration 1.5 µg/ml) and MES buffer were used as controls. The results were compared with ATP standard curve.

3.13 Effect of bacteriocin on proton motive force (PMF)

3.13.1 Transmembrane electrical potential (ΔΨ) measurement

The effect of bacteriocin treatment on transmembrane electrical potential (ΔΨ) was assessed as described by van Kuijk et al. (2011) with modifications. *L. monocytogenes* ScottA was grown in TGY broth at 30°C until an approximate OD_{600} of 0.6 was reached. Cells were harvested by centrifugation for 15 min at 4500 × g at 4°C (Hermle Z400K; LabNet, Woodbridge, NJ, USA), washed twice with 50 mM K-HEPES buffer at pH 7.0 (Sigma-Aldrich, St Louis, MO, USA) and concentrated in 1⁄100 of their original volume in the same buffer. During the experiment, cells were stored on ice. The ΔΨ of the cells was observed in relation to the fluorescence of the probe $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodine; Molecular Probes, Eugene, OR, USA). The experiment was performed using a PerkinElmer LS50B spectrofluorometer (PerkinElmer Life and Analytical Science, Inc., Boston, MA,

USA) at excitation and emission wavelengths of 643 and 666 nm, respectively, and a slit width of 10 nm. Two ml of 50 mM K-HEPES buffer (pH 7.0) was used to equilibrate the fluorometer and acted as the starting point for the measurement of fluorescence. The reagents were added in the following order as soon as the fluorescent signal had stabilized from the previous step: 5 µl of 2 mM $DiSC_3$ (5) (final concentration of 5 µM), 10 µl of concentrated *L. monocytogenes* Scott A, 20 µl of 20% glucose, 2 µl of 5 µM nigericin dissolved in ethanol (Molecular Probes), an appropriate amount of bacteriocin or control to reach the desired end concentration, and 2 µl of 2 µM valinomycin in ethanol (Molecular Probes). The final concentration of bacteriocin used was 2.38 µg/ml. A similar amount of sterile ddH_2O was used as a negative control while nisin (final concentration 32.61 µg/ml) was used as a positive control. Each assay was performed at least twice in duplicate.

3.13.2 ΔpH measurement

ΔpH measurements were conducted as described by van Kuijk et al. (2011) with the following modifications. *L. monocytogenes* Scott A was grown overnight in 30 ml of TGY broth until an OD_{600} of 0.6 was reached. The cells were harvested by centrifugation for 15 min at 4500×g at 4°C (Hermle Z400K; LabNet, Woodbridge, NJ, USA), washed twice with 50mM KPi buffer at pH 6.0 (Sigma-Aldrich) and concentrated 100-fold by resuspending them in 300 µl of the same buffer. Ten µl of BCECF-AM (2',7'-bis-(2-carboxyethyl)- 5-(and-6) carboxyfluorescein; Molecular Probes) was added to 200 µl of the concentrated cells, which were then incubated for 5 min at room temperature to allow the probe to be internalized. After this incubation, 1 ml of 50 mM KPi buffer (pH 6.0) was added and the cells were harvested by

centrifugation as previously described. The cells were washed twice with 1 ml of 50 mM KPi buffer (pH 6.0) and resuspended in 200 µl of the same buffer. ΔpH measurements were performed using a PerkinElmer LS50B spectrofluorometer at excitation and emission wavelengths of 502 and 252 nm, respectively, and slit widths of 5 nm for excitation and 15 nm for emission. The experiment was started by measuring the fluorescence of 2 ml of 50 mM KPi buffer (pH 6.0) in quartz cuvettes to provide a baseline fluorescence signal prior to adding the BCECF-loaded cells. As soon as the fluorescent signal stabilized, the compounds were added in the following step: 3 µl of BCECF-loaded cell suspension, 20 µl of 20% glucose (in water), 2 µl of 5 µM valinomycin in ethanol, an appropriate amount of bacteriocin or control to reach the desired end concentration and 2 µl of 2 µM nigericin in ethanol. Bacteriocin was used at a concentration of 0.68 µg/ml (20 µl) and 2.38 µg/ml(70 µl). A amount 20 µl and 70 µl of water was used as a negative control, and nisin (32.6 µg/ml) was used as positive control. Each experiment was performed at least twice in duplicate.

3.14 Application of bacteriocin in food model

Preparation of bateriocin and nisin

Crude of bacteriocin was prepared from the cell-free supernatant of *Enterococcus faecium* CN-25. The culture was inoculated into 300 ml of modified broth and incubated at 25^{o}C under anaerobic condition for 24 h. Cell-free supernatant was obtained by centrifugation at 12,000 rpm at 4°C for 10 min. The supernatants were collected, determined pH and adjusted to pH 6.5 with 6 M NaOH and then filter by filtration (0.22 µm, Millipore, Belford, MA, USA) before use. Nisin (Nisaplin, 1×10^{6}

IU nisin /g) was obtained from Aplin and Barrett Ltd. (Dorset, England). A stock solution (1×10^4 IU/ml) was prepared in HCl 0.02 N, pH=2, and stored at -40^{o}C.

Inoculum preparation

To prepare inoculate, *Listeria monocytogenes* was grown overnight at 37^{o}C in trypticase soya broth. The cells were diluted to appropriate concentration (approximately 10^6 CFU/ml) before adding directly to samples.

3.14.1 Milk model

Pasteurized milk (3.2% milk fat), were purchased from a local market and produced by SUT farm Nakhon ratchasima (Thailand). The samples (20 ml) in duplicate were inoculated with a cocktail of *L. monocytogenes* cells (10^6 CFU/ml) and then supplemented with bacteriocin 914.2 AU/ml. Nisin 10^4 AU/ml was treated in samples. Untreated controls ,bacteriocin-treated and nisin-treated samples were stored in refrigerator at 4^{o}C for 10 day. Milk was sampled at selected intervals to perform microbial counts and listerial count by aseptically removing 10 ml. Samples were mixed with saline solution as 0.85% NaCl. The homogenization was done in a Stomacher Lab-blender (model 400, Cooke Laboratory products, Alexandria, Virginia) for 3 min. Samples were serially diluted with sterile saline solution and then plated in duplicate on PCA agar and listeria agar plates. The PCA agar plates were incubated at 37^{o}C for 24 h. For listeria agar plates were incubated at 30^{o}C for 72 h. The average number of colonies was used to calculate the viable cell concentrations. These were expressed in log colony-forming units (CFU) per ml of sample.

3.15 Statistical analyses

The effect of each condition on bacteriocin production and bacterial growth was

analyzed using completely randomized design (CRD). All experiments were done in duplicate and mean values were presented. Analysis of variance (ANOVA) was carried out using SPSS program (version 13; SPSS Inc., Chicago, IL, USA). Differences among mean values were established using Duncan's multiple range test (DMRT) at $p<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) counts of fermented fishery products were in the range of 5.0-8.0 log CFU/ml (Table 4.1). The fermented fish with salt are found in different part of Thailand. LAB are typically found these products. Tanaspawat, Okada, and Komagata, (1998) reported that LAB counts in high salted (11.5-23.9 NaCl %) fermented fish (pla-ra) was approximately 1.02×10^{7}-2.84×10^{10} CFU/g, while the LAB counts in low salted products (3.2-9.4 NaCl %) including pla-som and kong-jom was 4.2×10^{7}-2.72×10^{10} and 7.30×10^{7}-9.56×10^{11} CFU/g. Some strains of LAB were found only in low salt products. The concentration of NaCl in fermented fish controls the growth of these bacteria (Tanasupawat and Daengsubha, 1983; Tanasupawat et al., 1993). Vissanguan et al. (2004) reported that the traditional fermentation relies on natural fortuitous microorganisms, LAB found in the ingredients, on the processing utensils as natural starters. There were 285 isolates exhibiting a clear zone on MRS agar supplemented with $CaCO_3$. Calcium carbonate is used as an indicator for acid-producing strains since it becomes solubilized in the presence of acid, appearing as a clear zone (Onda et al., 1999). All 285 isolates were tentatively classified as LAB based on their Gram positive stain and negative catalase activity. LAB were randomly selected based on different colonies appearance on four selective media. Of these isolates, 68 isolates were obtained from somkai-pla, 97 isolates were obtained from

pla-som, 44 isolates were obtained from pla-ra and 74 isolates were obtained from kong-jom. All LAB isolates were subsequently screened for their ability of bacteriocin production.

Table 4.1 Bacterial counts of various fishery fermented food samples collected from different location.

Sample	Place of purchase	Total bacteria counts (log CFU/g)				No. of isolate collected			
		MRS[a]	MRS[b]	CN[c]	GYP[d]	MRS[a]	MRS+ NaCl[b]	CN[c]	GYP[d]
somkai-pla (fermented fish roe)	Nakhon Ratchasima	7.2	6.8	7.0	7.9	13	13	24	18
	Chiyaphom	8.0	6.7	7.1	6.8				
pla-som	Yasoton	7.9	7.8	7.3	7.9	23	21	27	26
	Nakhon Ratchasima	7.6	7.2	6.5	6.8				
pla-ra	Kalasin	6.7	6.8	6.7	7.5	12	12	10	10
	Nakhon Ratchasima	7.7	6.7	6.5	7.7				
kong-jom (fermented shrimp)	Surin	6.8	6.7	5.3	6.7	18	18	12	28
	Burirum	6.6	6.7	5.0	6.7				

[a]MRS; [b]MRS+ NaCl, MRS containing 5% NaCl; [c]CN (Canobacterium medium); [d]GYP containing 5% NaCl.

4.2 Screening of LAB-producing bacteriocin

Among 285 isolates, only 11 isolates showed bacteriocin activity against test organisms of as *Lb. fermentum* TISTR876 and against *Lb. plantarum* TISTR 050 but MSKC-13 isolates showed antimicrobial activity against *Lb. fermentum* (Table 4.2). All of 11 isolate showed strong bacteriocin activity against *L. monocytogenes* TISTR 1327 in the secondary screening (Table 4.3). Cell density of all 11 strains measured by OD_{600} ranged 2.20-2.84 and the final pH in MRS broth after cultivation were 7.0 to 5.1. After adjust in pH to 6.5 of cell-free supernatant, it was found that the highest bacteriocin activity was observed in CN-25 isolate. Only MSK-3-18 showed

inhibitory against *L. monocytogenes* TISTR 1327 and only *M. morganii* strain.

Table 4.2 Zone of inhibition (mm) for bacteriocin-producing isolates against indicator strains for the primary screening.

Samples	Number of isolate	Number of positive strains	Isolate code	Diameter of inhibition zone (mm) Test organism strains[a]					
				1	2	3	4	5	6
somkai-pla (fermented fish roe)	68	3	CN-25	9.0±00	7.0±0.00	0	0	0	0
			CN-22	8.25±0.35	7.4±0.28	0	0	0	0
			MSK-3-18	21.50±0.19	21.5±0.71	0	0	0	0
pla-som	97	0	0	0	0	0	0	0	0
pla-ra	44	7	GY-7	7.2±0.35	6.2±0.28	0	0	0	0
			GY-20	6.0±.00	6.2±0.28	0	0	0	0
			GY-30	8.5±0.71	6.2±0.28	0	0	0	0
			MF-3	8.5±0.71	7.4±0.57	0	0	0	0
			MF-22	7.2±0.35	6.4±0.28	0	0	0	0
			MF-16	8.2±0.35	7.4±0.64	0	0	0	0
			MC-20	8.1±0.14	7.0±0.00	0	0	0	0
kong-jom (fermented shrimp)	76	1	MSKC-13	7.4±0.19	0	0	0	0	0

[a]1, *Lb. fermentum* TISTR 876; 2, *Lb. plantarum* TISTR 050; 3, *Lb. plantarum* TISTR 877; 4, *Lb. acidophillus* TISTR 1034; 5, *B. cereus* TISTR 687; 6, *S. aureus* TISTR 517; Standard streptomycin was used as the positive control (15 mm inhibition zone diameter).

No inhibition zone was observed against *E. coli* TISTR 887, *S. enteritidis* JCM 1652 and *S. typhimurium*. Criteria for selection was based on strong inhibition against of *L. monocytogenes* TISTR 1327 and the different source of LAB isolates. MSKC-13 was selected as it showed no inhibition against *Lb. plantarum* TISTR 050 because *Lb. plantarum* widely used as functional starter culture or co-culture in food industry (Ruiz-Barba et al., 1994), fermented of vegetables, sauerkraut, pickles, soy sauce, and

fermented cereals (Leroy, Foulquie Moreno, and De Vuys, 2003). MSK-3-18 inhibited *L. monocytogenes* and *M. morganii*, which can be found in fermented fish. *M. morganii* has been reported to be produce for histamine formation fish (Lehane and Olley, 2000). Therefore, MKC-3-18 might be useful for improving product safety of fermented fish products. Therefore, 4 isolates, namely CN-25, GY-20, MSKC-13 and MSK-3-18 were selected for bacterial identification and characterization.

Table 4.3 Zone of inhibition (mm) for bacteriocin-producing isolates against indicator strains of secondary screening.

Bacterial isolate code	Isolation source	Isolation medium[a]	pH	Growth (OD)	Diameters of inhibition zone (mm)					
					Test strain[b]					
					1	2	3	4	5	6
GY-30	pla-ra	GYP	5.13	2.40	13.0±0.00	0	0	0	0	0
GY-20	pla-ra	GYP	5.13	2.73	14.0±0.00	0	0	0	0	0
CN-25	somkai-pla	CN	5.16	2.76	15.0±0.00	0	0	0	0	0
CN-22	somkai-pla	CN	5.15	2.44	12.5±0.70	0	0	0	0	0
MF-22	pla-ra	CN	5.14	2.84	13.0±0.00	0	0	0	0	0
MC-20	pla-ra	MRS	5.14	2.60	11.75±0.35	0	0	0	0	0
MF-3	pla-ra	MRS	5.14	2.75	12.75±0.35	0	0	0	0	0
MF-16	pla-ra	MRS	5.19	2.72	13.0±0.00	0	0	0	0	0
GY-7	pla-ra	GYP	5.13	2.65	12.0±0.00	0	0	0	0	0
MSKC-13	kong-jom	MRS	5.18	2.20	12.0±0.00	0	0	0	0	0
MSK-3-18	somkai-pla	MRS	5.15	2.70	10.0±0.00	0	0	0	16.0±0.00	0

[a]GYP+5% NaCl; CN; MRS. [b]1, *L. monocytogenes* TISTR 1327; 2, *E. coli* TISTR 887; 3, *S. enteritidis* JCM 1652; 4, *S. typhimurium*; 5, *M. morganii* ; 6, *S. xylosus*. Standard streptomycin was used as the positive control (15 mm inhibition zone diameter).

4.3 Identification bacteriocin-producing strains

4.3.1 Phenotypic and biochemical characteristics

The selected bacteriocin-producing strains were Gram positive cocci with cell arrangement as pairs (Figure 4.1). Colonies on MRS agar were 1-2 mm, white, circular, low convex, and smooth. Only MSKC-13 showed ability to hydrolyze starch (Table 4.4). The bacteriocin-producing isolates were initially identified using API

system. Isolates of GY-20 and CN-25 were identified to be *L. lactis* subsp. *lactis* with 82.4% identity, while MSK-3-18 isolate was *L. lactis* subsp. *lactis* with 99.4%. MSKC-13 was identified to be *P. pentosaceus* with 92.4%.

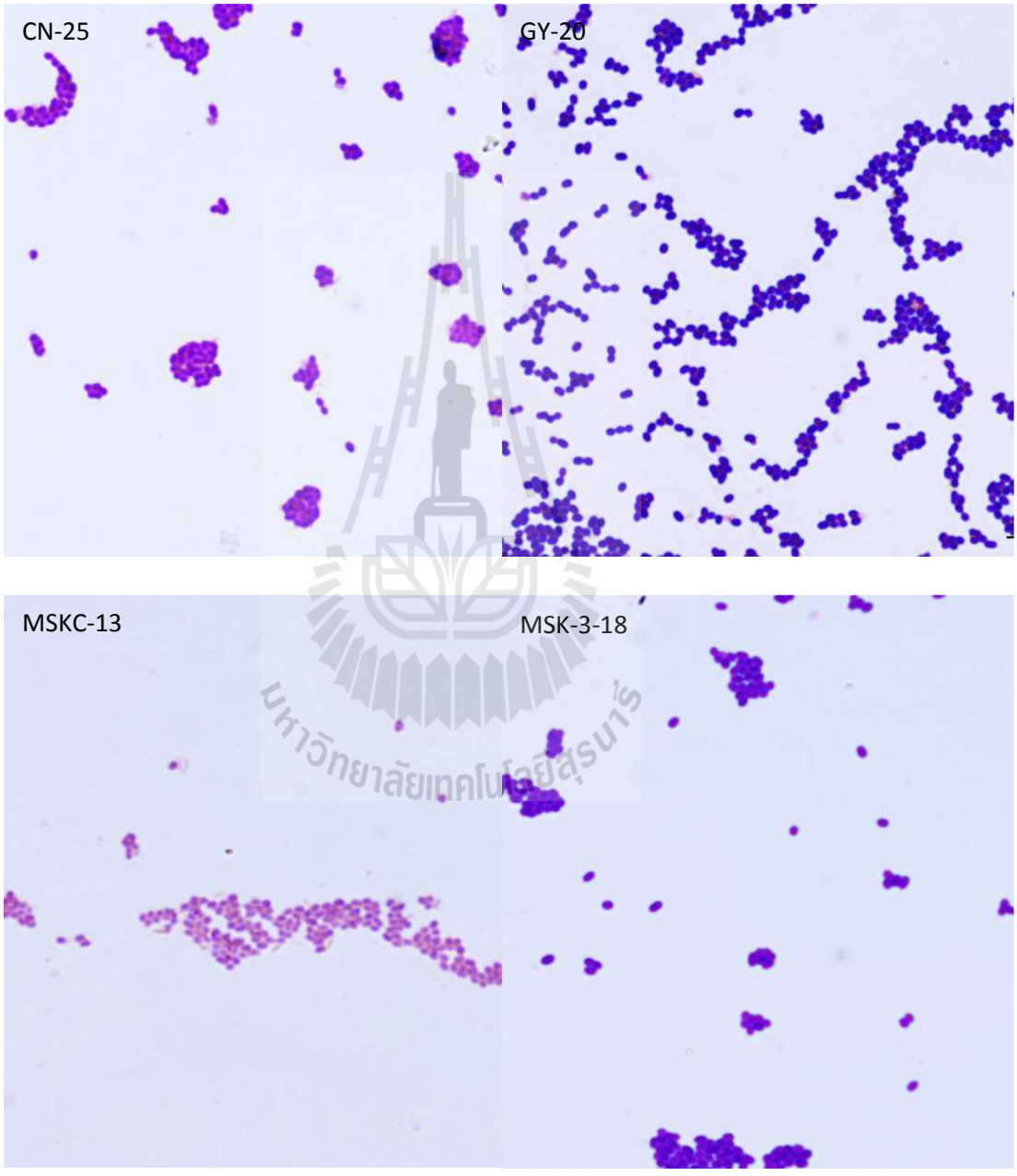


Figure 4.1 Gram stain and cell arrangement of selected LAB isolated from somkai-pla (CN-25), pla-ra (GY-20), kong-jom (MSKC-13), somkai-pla (MSK-3-18)

Table 4.4 Comparison of physiological characteristics of 4 selected isolates and identification results according to biochemical characteristics.

Source	Bacterial Isolate code	Gram strain	Cell shape	Oxidase	Catalase	Proteinase[a]	Lipase[b]	Amylase[c]	Fermentative metabolism		Identification result based on API 50 CHL	
									Homo	Hetero	Closest relative	Identity (%)
somkai-pla	CN-25	+	Cocci	-	+	-	-	-	+	-	*Lactococcus lactis* subsp. *lactis*	82.4
plara	GY-20	+	Cocci	-	+	-	-	-	+	-	*Lactococcus lactis* subsp. *lactis*	82.4
kong-jom	MSKC-13	+	Cocci	-	+	-	-	+	+	-	*Pediococcus pentosaceus*	92.4
somkai-pla	MSK-3-18	+	Cocci	-	+	-	-	-	+	-	*Lactococcus lactis* subsp. *lactis*	99.4

[a] Casein hydrolysis; [b] Tween80 hydrolysis; [c] Starch hydrolysis; [d] API system (API 50CH/L for lactic acid bacteria; bioMérieux)

4.3.2 Genotypic characterization

The genomic DNA was extracted from 4 isolates and used for 16S rDNA amplification. The size of amplified DNA fragments was 1,500 bp (Figure 4.2). Similarity of CN-25 and GY-20 when compared to strain *E. faecium* DSM 20477^T were 99.2% and 98.9% (Table 4.5). When phylogenetic tree analysis was performed, isolates CN-25 and GY-20 fell in the same cluster of *E. faecium* DSM 20477^T (Figure 4.3). These results demonstrated that CN-25 and GY-20 isolates appeared to be closely related to the genus *Enterococcus*.

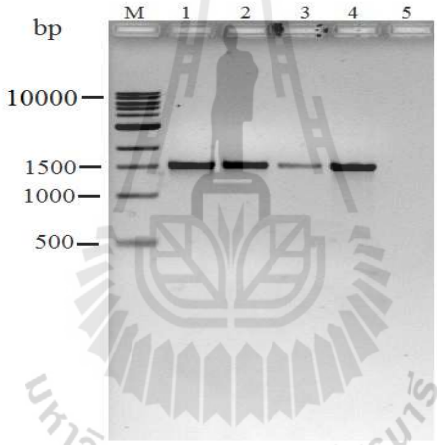


Figure 4.2 Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rDNA using primer fD1/rP2. Lanes M, Molecular weight marke (1-kb ladder, Fermentas Life Sciences); 1, CN-25; 2, GY-20; 3, MSKC-13; 4, MSK-3-18 and 5, negative control.

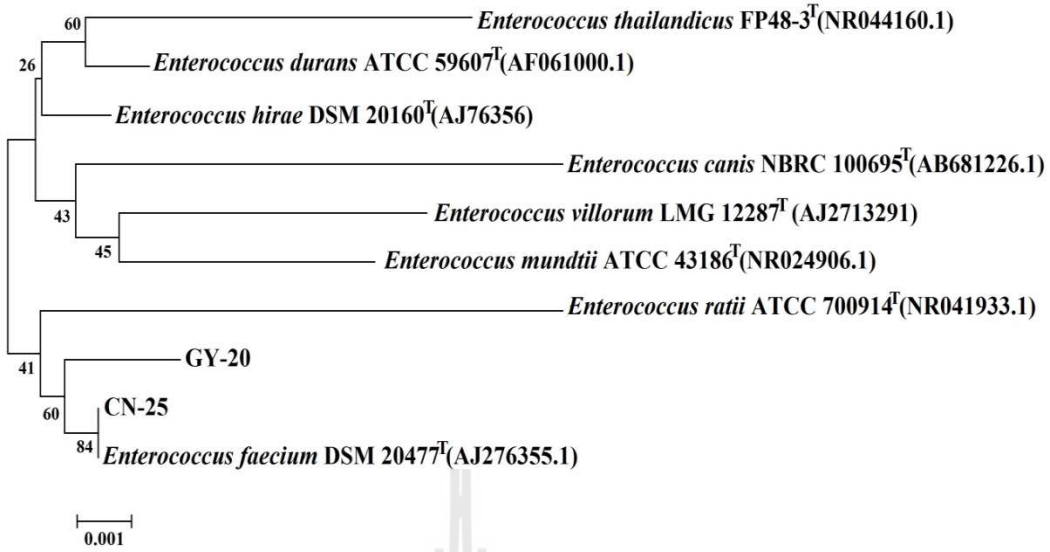


Figure 4.3 Phylogenetic tree of CN-25, GY-20 isolated from fermented fish based on 16S rRNA gene sequence data. Bar indicates 0.001 substitutions per nucleotide position.

Table 4.5 Similarity of 16S rRNA gene sequence of CN-25 and GY-20 isolates andrelated species.

Bacterial code	**Bacterial isolate**		**Type culture strains[a]**							
	CN-25	**GY-20**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
CN-25	100									
GY-20	99.70	100								
1	99.20	98.90	100							
2	98.10	98.00	98.80	100						
3	98.90	98.80	99.40	98.70	100					
4	97.70	97.50	98.40	97.80	98.40	100				
5	97.70	97.60	98.50	97.50	98.50	98.80	100			
6	96.10	95.90	96.80	95.80	96.80	96.80	97.10	100		
7	97.50	97.30	98.20	97.20	98.10	98.00	98.40	97.90	100	
8	96.69	96.67	97.70	96.66	97.60	96.80	97.20	95.40	97.00	100

[a]1, *E. faecium* DSM 20477^T (AJ276355.1); 2, *E. ratti* ATCC 70091^T (NR_041933.1); 3, *E. hirae* DMS 20160^T (AJ76356); 4, *E. villorum* LMG 12287^T (AJ2713291); 5, *E. mundtii* ATCC 43186^T (NR_041933.1); 6, *E. thailandicus* FP48-3 (NR_044160.1); 7, *E. durans* ATCC 59607^T (AF061000.1); 8, *E. canis* NBRC 100695^T (NR_044160.1)

Currently, 37 species of Enterococcus are validly described (Devriese et al., 2002; Franz and Holzapfel, 2004), which fall into seven species groups on the basis of 16S rDNA gene similarity. The group of *E. faecium* that currently contains *E. faecium*, *E. hirae*, *E. durans*, *E. mundtii*, *E. villorum*, *E. ratti* and *E. canis* (Collins et al., 1984, Farrow and Collins, 1985; Vancanneyt et al., 2001; Teixeira et al., 2001; De Graef et al., 2003).

Enterococci can be found in many different habitats such as in soil, surface waters, ocean water, sewage, on plants and in the gastrointestinal tract of animals and humans. Based on their association with the gastrointestinal tract, enterococci often occur in foods of animal origin, such as meats, fermented and cooked meats, as well as cheese (Franz et al., 1999). Tanasupawat, Sukontasin and Lee, (2008) reported that a novel species of *E. thailandicus* isolated from fermented sausage (‘mum’) in Thailand. The 16S rRNA gene sequence (1326 bp) of *E. thailandicus* sp. nov. (strain FP48-3^T) indicated that the strain belonged to the genus Enterococcus and was closely related to *E. hirae* LMG 6399^T (99.6 %), *E. durans* LMG 10746^T (99.6 %) and *E. faecium* LMG 11423^T (99.3 %). Strain FP48-3^T could be differentiated from closely related Enterococcus species by low DNA–DNA relatedness and phenotypic characteristics. Therefore, this strain represents a novel species of the genus Enterococcus.

Although 16S rDNA sequence confirmed the taxonomic identity of CN-25, GY-20, these isolates showed a wide variation in phenotypic characteristics among strain. Therefore, physiological and biochemical characteristics of the selected LAB were compared with type strains. The physiological and biochemical characteristics of CN-25 and GY-20 were compared with *E. faecium* ATCC 19434^T (Table 4.6).

Isolates of CN-25 and GY-20 showed similar physiological characteristics. They grew at the temperature 10-45 ^{o}C, 2-6.5% NaCl, pH 5.0-10, and produced acid from N-acetyl-glucosamine, L-arabinose, ribose, arbutin, galactose, β-gentiobiose D-glucose, lactose, maltose, mannitol, sucrose, treharose. Only D-tagatose was utilized by GY-20 but not by CN-25 for acid production. The physiological and biochemical characteristics of *E. thailandicus* FP48-3^{T} was shown in Table 4.6. It grows at pH 5.0–9.6, 15–45 ^{o}C and in 2–6.5% NaCl. Acid is not produced from L-arabinose sucrose, treharose, which is different from *E. faecium*. Albano et al. (2009) reported that *E. faecium* isolated from fermented sausage. It grows at 4% NaCl, at 10-45 ^{o}C, pH 4.4-9.6 and produce acid from fructose, galactose, D-glucose, lactose, maltose, mannitol, sucrose, treharose, which is similar with *E. faecium* ATCC 19434^{T}. Phenotypic characterization of Enterococcus species has been discussed (Leclerc, Devriese and Mossel, 1996). It is generally agreed that the genus Enterococcus comprises of gram-positive cocci that are catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts, and 0.1% methylene blue milk and at pH 9.6. They grow at 10 and 45°C and can resist at 60°C for 30 min (Devriese, Collins, and Wirth, 1991; Schleifer and Kilpper-Balz, 1984; Schleifer and Kilpper-Balz, 1987). There is clear evidence of the genotypic identity of Enterococcus, based on molecular studies (Farrow, Jones, Phillips, and Collins, 1983; Ludwig et al., 1985; Schleifer and Kilpper-Balz, 1987). Base on phylogenic tree (Figer 4.3), biochemical and physiological characteristics (Table 4.6) of CN-25 and GY-20 appeared to be closely related to *E. faecium* ATCC 19434^{T}. Therefore, they were identified as *E. faecium*.

Genotypic similarity of MSKC-13 when compared to *P. pentosaceus* ATCC 25745^{T} was 99.9% (Table 4.7). When phylogenetic tree analysis was performed, MSKC-13 fell in the same cluster of *P. pentosaceus* ATCC 25745^{T} (Figure 4.4).

Table 4.6 Characteristics of *E. faecium* strains isolated from fishery products.

Characteristics	**Selected LAB isolates**		**Type culture strain**			
	CN-25	**GY-20**	***Enterococcus faecium* ATCC 19434^T**	***Enterococcus ratii* ATCC 700914^T**	***Enterococcus hirae* ATCC 8043^T**	***Enterococcus thailandicus* FP48-3^T**
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Spherical/ovoid
Cell size (µm)	0.5-2 µm	0.5-2 µm				0.5-1µm
Cell arrangement	Pairs/short chains	Pairs/short chains	Pairs/short chains	Pairs/short chains	Pairs/short chains	Pairs/chains
Gram stain	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Proteinase	-	-	NA	NA	NA	NA
Lipase	-	-	NA	NA	NA	NA
Amylase	-	-	NA	NA	NA	-
Hydrolysis of esculine	+	+	+	+	+	+
Arginine dehydrolase	+	+	+	+	+	+
Reduction of nitate	-	-	-	-	-	-
Growth						
at 10 ^oC	+	+	+	NA	NA	-
at 15 ^oC	+	+	+	+	+	+
at 25 ^oC	+	+	+	+	+	+
at 30 ^oC	+	+	NA	NA	NA	NA
at 35 ^oC	+	+	NA	NA	NA	NA
at 40 ^oC	+	+	NA	NA	NA	NA
at 45 ^oC	+	+	NA	NA	NA	NA
at 50 ^oC	-	-	NA	NA	NA	NA
Growth at 2% NaCl	+	+	+	+	+	+
Growth at 4% NaCl	+	+	+	+	+	+
Growth at 6% NaCl	+	+	+	+	+	+

Table 4.6 (Continued)

Characteristics	Selected LAB isolates		Type culture strain			
	CN-25	GY-20	*Enterococcus faecium* ATCC 19434^T	*Enterococcus ratii* ATCC 700914^T	*Enterococcus hirae* ATCC 8043	*Enterococcus thailandicus* FP48-3^T
Growth at 6.5% NaCl	+	+	+	+	+	+
Growth at pH 4	-	-	NA	NA	NA	-
Growth at pH 7	+	+	NA	NA	NA	+
Growth at pH 8	+	+	NA	NA	NA	+
Growth at pH 9	+	+	NA	+	+	+
Growth at pH 9.6	+	+	+	+	+	+
Growth at pH 10	+	+	NA	NA	-	-
Survival at 60 ^oC for 30 min	+	+	+	+	NA	NA
Acid from:						
N-Acetyl-glucosamine	+	+	+	+	+	+
Amygdalin	-	-	+	+	+	+
D-Arabinose	-	-	NA	NA	-	-
L-Arabinose	+	+	+	-	-	-
L-Arabitol	-	-	-	-	-	-
Adonital	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	+
Erythritol	-	-	-	-	-	-
D-Fucose	-	-	-	-	+	-
L-Fucose	-	-	-	-	-	-
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	-	+
D-Glucose	+	+	+	+	+	+
Glycerol	-	-	d	-	d	+
Glycogen	-	-	-	-	-	-

Table 4.6 (Continued)

Characteristics	Selected LAB isolates		Type culture strain			
	CN-25	GY-20	*Enterococcus faecium* ATCC 19434^T	*Enterococcus ratii* ATCC 700914^T	*Enterococcus hirae* ATCC 8043	*Enterococcus thailandicus* FP48-3^T
Lactose	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Mannitol	+	+	+	d	-	+
D-Mannose	+	+	+	+	+	+
L-sorbose	-	-	-	-	+	-
Melibiose	-	-	(+)	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	-
α-Methyl-D-manoside	-	-	-	-	-	+
D-Raffinose	-	-	d	-	d	-
Rhamnose	-	-	-	-	-	-
Ribose	+	+	+	+	+	+
Sorbital	-	-	-	-	-	-
Starch (Amidon)	-	-	d	(+)	(+)	-
Sucrose	+	+	+	-	+	-
D-Tagatose	-	+	-	(-)	(-)	-
Trehalose	+	+	+	d	+	-
D-Turanose	-	-	-	(-)	(-)	-
Xylitol	-	-	-	-	-	-
D-Xylose	-	-	d	-	-	-
L-Xylose	-	-	-	-	-	-

+,90% or more of the strains of isolate are positive (+), 75 to 89% are positive; V, 26 are negative; (-), 11.25% are negative; -, d, discrepancies among reference studies. NA, Not aviliable, Phenotypic characteristics were tested of this study[a] *E.faecium* ATTC 19434^T; *E.ratii* ACTT 70914^T (data from Manero and Blanch 1999);*E. thailandicus* FP48-3^T (data from Tanasupawat et al., 2008)

Table 4.7 Similarity of 16S rRNA gene sequence of MSKC-13 isolate and related species.

Bacterial code	Bacterial isolate	Type culture strains								
	MSKC-13	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
MSKC-13	100									
1	99.90	100								
2	97.90	97.90	100							
3	96.90	96.90	95.80	100						
4	94.00	94.10	93.80	94.00	100					
5	94.40	94.40	94.20	94.10	99.00	100				
6	94.40	94.40	93.90	95.40	97.90	97.80	100			
7	95.40	95.30	95.10	93.70	97.40	97.80	96.80	100		
8	44.10	44.00	43.50	43.50	42.80	42.70	43.10	43.3	100	
9	43.30	43.30	42.70	42.60	42.10	42.00	42.20	42.50	90.40	100

[a]1, *P. pentosaceus* ATCC 25745^T (NR_0750521.1); 2, *P. acidilactici* DSM 20284^T (NR_042057.1); 3, *P. stilesii* LMG 23082^T (NR_042401.1); 4, *P. damnosus* DSM 20332^T (NR_04087.1); 5 *P. inopinatus* DSM 20285T (NR_042087.1); 6, *P. parvulus* NBRC 100673^T (AB681216.1); 7, *P. cellicola* Z-8^T (NR_043290.1); 8, *P. claussenii* ATCC BAA-344^T (NR_0750291); 9, *P. dextrinicus* DSM 20335^T (EF116579.1)

The genus Pediococcus consists of eight species, *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. dextrinicus*, *P. damnosus*, *P. inopinatus*, *P. halophilus*, and *P. urinaeequi*, although the taxonomic status of the latter two species remains uncertain (Collins, Williams, and Wallbanks, 1990; Dellaglio, Trovatelli, and Sarra, 1981; Garvie, 1986). Two Pediococcus species, *P. acidilactici* and *P. pentosaceus*, have been widely used in the fermentation of vegetables (Knorr, 1998), meats (Luchansky et al., 1992; Mattila-Sandholm, Haikara, and Skyttae, 1993), fruit juices (Knorr, 1998), dairy products (Litopoulou-Tzanetaki, Graham, and Beyatli, 1989; Bhowmik and Marth, 1990), and silage (Cai, Kumai, Ogawa, Benno, and Nakase, 1999). *P. pentosaceus* has been isolated from Thai traditional fermented foods such as pork sausage (Nham) (Kingcha et al., 2012) and fermented fish (pla-som) (Kopermsub and Yunchalard, 2010).

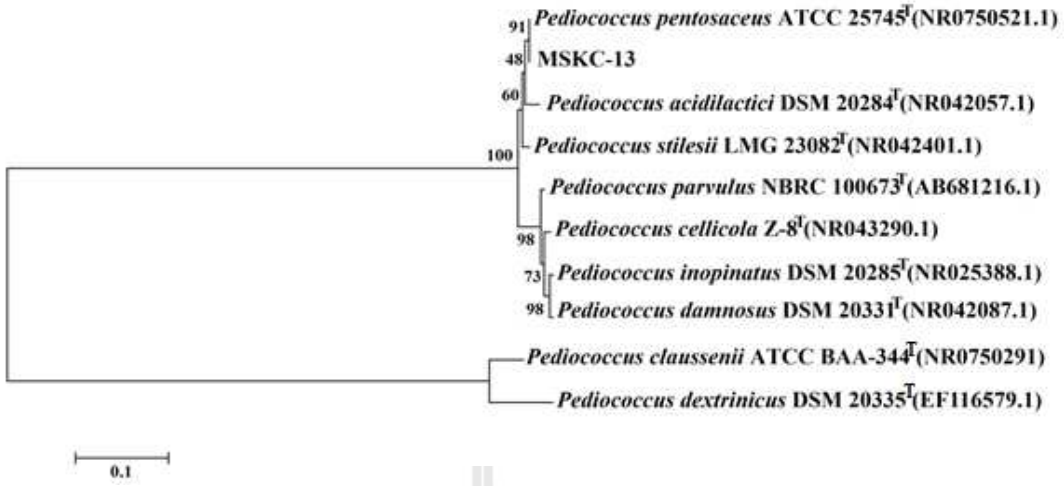


Figure 4.4 Phylogenetic tree of MSKC-13 isolated from kong-jom, based on 16S rRNA gene sequence data. Bar indicates 0.1 substitutions per nucleotide position.

Physiological characteristics of MSKC-13 appeared to be closely related to *P. pentosaceous* ATCC 25745^{T}. It grew at 15-45^{o}C, pH 5.0-9.0 and 2-6.5% NaCl (Table 4.8). It hydrolyzed esculin and produced acid from N-acetyl-glucosamine, arbutin, cellobiose, β-gentiobiose, D-glucose, galactose, maltose, D-mannose, ribose, salicin, D-tagatose, D-xylose. Variation in phenotypic characteristics such as sugar fermentation, pH, growth and temperature is reported. Todorov and Dicks (2005) reported that *P. pentosaceus* ST18 isolated from boza (fermented cereal). Grows is optimal at 30^{o}C but no growth was observed at 45^{o}C. *P. pentosaceus* ST18 produced acid from rhamnose, lactose, melibiose, inulin, raffinose, while *P. pentosaceus* ATCC 33316^{T} did not. Tamang et al. (2005) isolated *P. pentosaceus* from vegetable products, which grew at 45^{o}C and produced acid from xylose but not lactose. These characteristic were different from those of *P. pentosaceus* ATCC 33316^{T}. Based on

the phylogenic tree (Finger 4.4), and the physiological and biochemical characteristics that MSKC-13, it was appeared closely related to *P. pentosaceus* ATCC 25745^T (Table 4.8). Therefore, MSKC-13 was identified as *P. pentosaceus*.

Table 4.8 Characteristics of *P. pentosaceus* strains isolated from kong-jom product.

Characteristics	**Selected LAB isolate**	**Type culture strain**	
	MSKC-13	***P. pentosaceus* ATCC 33316^T**	***P. acidilactici* DSM 20284^T**
Cell shape	Cocci	Cocci	Cocci
Cell arrangement	Pairs	Pairs/tetrads	Pairs/tetrads
Gram stain	+	+	+
Catalase	-	-	-
Motility	-	-	-
Pyruvate utilization	-	NA	NA
Hydrolysis of esculine	+	+	+
Arginine hydrolase	-	-	+
Reduction of nitate	-	-	-
Proteinase	-	NA	NA
Lipase	-	NA	NA
Amylase	+	NA	NA
Growth			
at 10 ^oC	-	NA	NA
at 15 ^oC	+	NA	NA
at 25 ^oC	+	NA	NA
at 30 ^oC	+	NA	NA
at 35 ^oC	+	NA	NA
at 45 ^oC	+	-	+
at 50 ^oC	-	-	+
Growth at 2% NaCl	+	+	+
Growth at 4% NaCl	+	+	+
Growth at 6% NaCl	+	+	+
Growth at 6.5% NaCl	+	NA	NA
Growth at 7% NaCl	-	-	-
Growth at pH 2	-	NA	NA
Growth at pH 3	-	NA	NA
Growth at pH 4	-	+	+
Growth at pH 5	+	+	+
Growth at pH 6	+	+	+
Growth at pH 7	+	+	+
Growth at pH 8	+	+	+
Growth at pH 9	+	-	-
Growth at pH 9.6	-	-	-
Growth at pH 10	-	-	-
Acid from:			
N-Acetyl-glucosamine	+	NA	NA

Table 4.8 (Continued)

Characteristics	Selected LAB isolate MSKC-13	Type culture strain	
		P. pentosaceus **ATCC 33316^T**	***P. acidilactici*** **DSM 20284^T**
D-Arabinose	-	NA	NA
L-Arabinose	+	+	+
L-Arabitol	-	NA	NA
D-Arabitol	-	NA	NA
Arbutin	+	NA	NA
Adonital	-	NA	NA
Cellobiose	+	+	+
Dulcitol	-	NA	NA
Esculin	+	NA	NA
D-Fucose	-	-	-
L-Fucose	-	NA	NA
Fructose	+	+	+
β-Gentiobiose	+	NA	NA
Gluconate	-	-	-
D-Glucose	+	+	+
Galactose	+	+	+
Glycerol	-	-	-
Glycogen	-	NA	NA
Inositol	-	NA	NA
2-Keto-gluconate	-	NA	NA
5-Keto-gluconate	-	NA	NA
Lactose	-	-	-
Maltose	+	NA	NA
Mannitol	-	-	-
D-Mannose	+	NA	NA
Melibiose	-	+	-
Melezitose	-	-	-
α-Methyl-D-glucoside	-	-	-
α-Methyl-D-manoside	-	NA	NA
B-Methyl-D-xylose	-	NA	NA
D-Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	+	+	+
Salicin	+	+	+
Sorbitol	-	-	-
L-Sorbose	-	NA	NA
Sucrose	-	+	-
D-Tagatose	+	NA	NA
Trehalose	+	+	+
D-Turanose	-	NA	NA
Xylitol	-	NA	NA
D-Xylose	-	W	+
L-Xylose	-	NA	NA

[a]1, *P. pentosaceus* ATCC 33316^T (data from Phalakornkule and Tanasupawat 2006-2007); 2, *P. acidilactici* DSM 20284^T (data from from Phalakornkule and Tanasupawat 2006-2007).

Based on 16S rDNA sequence, MSK-3-18 represented 99.7% similarity to *L. lactis* subsp. *lactis* ATCC 1943^T (Table 4.9). MSK-3-18 fell in the same cluster of *L. lactis* subsp. *lactis* ATCC 1943^T and *L. lactis* subsp. *hordniae* NBRC 100931^T (Figure 4.5). The genus Lactococcus currently classified into five species of *L. lactis*, *L. garvieae*, *L. piscium*, *L. plantarum* and *L. raffinolactis* (Schleifer et al. 1985). *L. lactis* strains used for dairy production have been genetically subdivided into *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Cogan and Hill 1993). Citrate-utilizing *L. lactis* subsp. *lactis* is now recognized as *L. lactis* subsp. *lactis* biovar diacetylactis (Mundt, 1986). The phenotypic characteristic of MSK-3-18 appeared to be closely related to *L. lactis* subsp. *lactis* ATCC 13675^T. It grew at 15-40^{o}C, 2-4% NaCl and pH 5.0-9.0 (Table 4.10). It produced acids from *N*-acetyl-glucosamine, amygdalin, arbutin, cellobiose, β-gentiobiose, D-glucose, galactose, maltose, D-mannose, manitol, lactose, ribose, salicin, and treharose. *L. lactis* subsp. *lactis* can be found in various environments, including animal sources, dairy products and silages (Salama et al. 1995; Ulrich and Muller 1999; Ennahar et al. 2003). *L. lactis* subsp. *lactis* have been isolated from bean-sprouts (Cai, Ng, and Farber, 1997). It grew at 4% NaCl and 30^{o}C but not at 45^{o}C. The fermentation of sugar profiles were similar *L. lactis* subsp. *lactis* ATCC 1943^T except for fermentation of several sugars including fructose, esculin, β-gentiobiose, D-glucose, galactose, maltose, D-mannose, mannitol, lactose, ribose, salicin, and treharose.

Similarly, Noonpakdee et al. (2003) reported *L. lactis* WNC 20 strain isolated from a traditional Thai fermented sausage (nham). These strain hydrolyzed arginine and grew at 10- 40 ^{o}C at pH 4-9 and 4% NaCl but not 6.5%. NaCl. Itoi et al. (2008) reported that *L. lactis* subsp. *lactis* isolated from intestinal tract of coastal fish. Grew

at pH 4.0 and produced acids from L-arabinose, ribose, d-xylose, galactose, glucose, fructose, mannose, mannitol, *N*-acetyl-D-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, starch, gentiobiose and gluconate. These profiles were different from the freshwater fish-derived *L. lactis* subsp. *lactis* in the fermentation of trehalose and inulin (Sugita et al. 2007), and from the cheese starter culture-derived *L. lactis* subsp. *lactis* in the fermentation of L-arabinose, mannitol, amygdalin, saccharose and gluconate (Itoi et al., 2008). Moreover, *L. lactis* subsp. *hordniae* have been described by Latorre-Guzman, Kado and Kunkee in 1997. It corresponds to the description of *L. lactis* subsp. *lactis* (Finger 4.5). They differ in the following characteristic, grows at 2% but not 4% NaCl (w/v). No growth at 40^{o}C and acid not produced from galactose, lactose, maltose or ribose.

Table 4.9 Similarity of 16S rRNA gene sequences of MSK-3-18 isolate and related species.

Bacterial code	**Bacterial isolate**	**Type culture strains[a]**						
	MSK-3-18	**1**	**2**	**3**	**4**	**5**	**6**	**7**
MSK-3-18	100							
1	99.70	100						
2	98.40	98.30	100					
3	97.90	97.70	99.20	100				
4	93.10	92.80	91.60	91.80	100			
5	88.00	87.80	89.10	89.20	87.60	100		
6	88.70	88.40	89.80	90.20	87.80	95.80	100	
7	90.10	89.90	91.30	91.20	88.90	96.00	94.80	100

[a]1, *L. lactis* subsp. *lactis* ATCC 1943^{T} (AB008215.1); 2, *L. lactis* subsp. *hordniae* NBRC 100931^{T} (AB681293.1); 3, *L. lactis* subsp. *cremoris* NCD 607^{T} (NR_040954.1); 4, *L. garvieae* JCM 10343^{T}(AB598994.1); 5, *L. piscium* CCUG 32732^{T} (NR_043739.1); 6, *L. raffinolactic* DSM 2043^{T} (NR_044359.1); 7, *L. plantarum* DSM 20686^{T} (NR_044358.1)

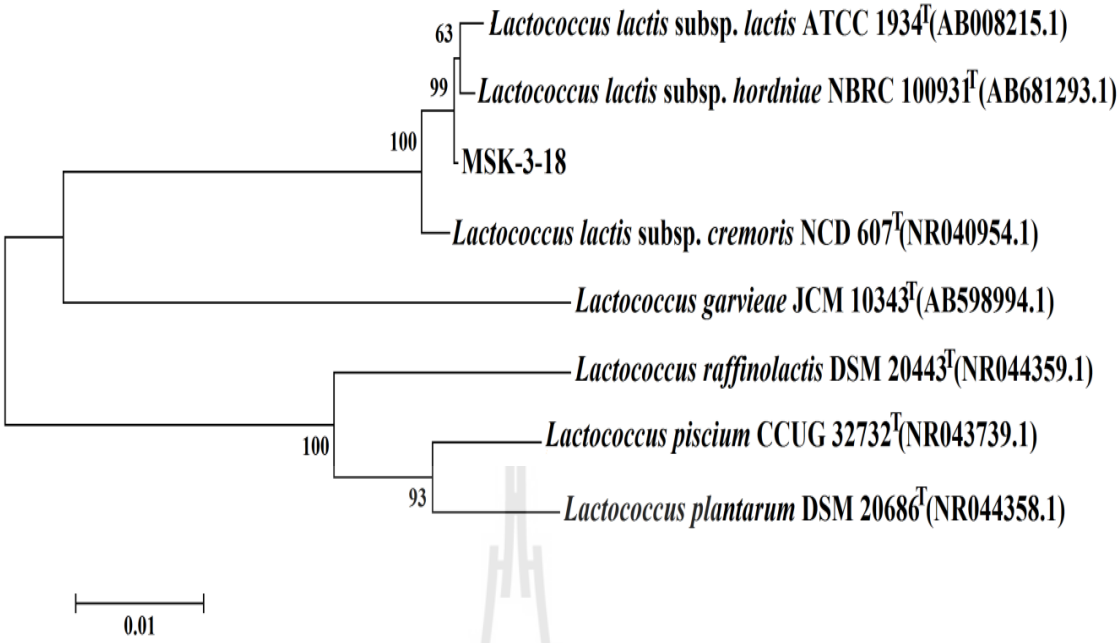


Figure 4.5 Phylogenetic tree of MSK-3-18 isolated from somkai-pla, based on 16S rRNA gene sequence data. Bar indicates 0.01 substitutions per nucleotide position.

Based on phenotypic and genotypic characteristics, MSK-3-18 appeared to be closely related to *L. lactis* subsp. *lactis*. Therefore, the MSK-3-18 strain was identified as *L. lactis* subsp. *lactis*. LAB have been isolated from various traditionally fermented fish products. LAB isolated from Thai fermented fish product have shown to produce antimicrobial substances against pathogen and spoilage bacteria in foods, such as *Lb. spp* (Østergaard et al, 1998), *W. cibaria* 110 (Srionnuala et al, 2007), *S. salivarius* LD219, *E. faecalis* LPS04, *E. faecalis* LPS17 and *E. faecalis* LPS18 (Hwanhlem at el., 2011), *Lb. sp.*, *Lb. farciminis*, *Leuconostoc* sp., *Lb. pentosus*, *Lb. sp.*, *Lb. pentosus*, *Lb. farciminis*, *Lb. sp.* (Tanasupawat et al., 1998).

Table 4.10 Characteristics of *Lactococcus lactis* species isolated from somkai-pla product.

Characteristic	**Selected LAB isolate**	**Type culture strain**	
	MSK-3-18	***Lactococcus lactis* subsp. *lactis* ATCC 13675^{T}**	***Lactococcus lactic* subsp.*hordniae* DSM 20450^{T}**
Cell shape	Cocci	Ovoid	Cocci
Cell arrangement	Pairs/short chains	Pairs/short chains	Pairs/short chains
Gram stain	+	+	+
Catalase	-	-	-
Motility	-	-	-
Pyruvate utilization	-	NA	NA
Hydrolysis of esculin	+	+	+
Arginine hydrolase	+	+	+
Reduction of nitate	-	NA	NA
Proteinase	-	NA	NA
Lipase	-	NA	NA
Amylase	-	NA	NA
Growth	-		
at 10 ^{o}C	-	+	+
at 15 ^{o}C	-	+	+
at 25 ^{o}C	+	+	+
at 35 ^{o}C	+	+	+
at 40 ^{o}C	+	+	+
at 45 ^{o}C	-	-	-
at 50 ^{o}C	-	-	-
Growth at 2% NaCl	+	+	+
Growth at 4% NaCl	+	+	-
Growth at 6% NaCl	-	-	-
Growth at 6.5% NaCl	-	-	-
Growth at 7% NaCl	-	-	-
Growth at pH 2	-	NA	NA
Growth at pH 3	-	NA	NA
Growth at pH 4	-	NA	NA
Growth at pH 5	+	NA	NA
Growth at pH 6	+	NA	NA
Growth at pH 7	+	NA	NA
Growth at pH 8	+	NA	NA
Growth at pH 9	+	NA	NA
Growth at pH 9.6	-	NA	NA
Growth at pH 10	-	NA	NA
Acid from:			
N-Acetyl-glucosamine	+	+	+
Amygdalin	+	+	+
D-Arabinose	-	-	-
L-Arabinose	-	-	+
L-Arabitol	-	-	-
D-Arabitol	-	-	-

Table 4.10 (Continued)

Characteristic	Selected LAB isolate	Type culture strain	
	MSK-3-18	*L. lactis* subsp. *lactis* ATCC 13675^T	*L. lactic* subsp.*hordniae* DSM 20450^T
Cellobiose	+	+	+
Dulcitol	-	-	-
Arbutin	+	+	+
Adonital	-	-	-
Esculin	+	+	+
D-Fucose	-	-	-
L-Fucose	-	-	-
Fructose	+	+	+
β-Gentiobiose	+	+	-
Gluconate	-	-	-
D-Glucose	+	+	+
Galactose	+	+	+
Glycerol	-	-	-
Glycogen	-	-	-
Inositol	-	-	-
Inulin	-	-	-
2-Keto-gluconate	-	-	-
5-Keto-gluconate	-	-	-
Lactose	+	+	-
D-Lyxose	-	-	-
Maltose	+	+	-
Mannitol	+	+	-
D-Mannose	+	+	+
Melibiose	-	-	-
Melezitose	-	-	-
α-Methyl-D-glucoside	-	-	-
α-Methyl-D-manoside	-	-	-
B-Methyl-D-xylose	-	-	-
D-Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	+	+	-
Salicin	+	+	+
Sorbitol	-	-	-
L-Sorbose	-	-	-
Starch (Amidon)	-	-	-
Sucrose	+	+	+
D-Tagatose	+	-	-
Trehalose	+	+	+
D-Turanose	-	-	-
Xylitol	-	-	-
D-Xylose	+	+	-
L-Xylose	-	-	-

1, *L. lactis* subsp. *lactis* ATCC 13675^T (data from Nomura et al., 1999); 2, *L. hordniae* DSM 20450^T (data from from Schleifer et al., 1985); +, Positive; -, negative; W, weakly positive; NA, not available

Table 4.11 Similarity of 16S rRNA gene sequences of four LAB isolates compared with other bacteria from NCBI nucleotide sequence database.

Bacterial isolate code	Length of sequence (bp)	Identification result	Nucleotide sequence comparison			
			Closest relative	Length of sequence (bp)	Sequence homology	NCBI accession no.
CN-25	1533	*E. faecium*	*E. faecium* DSM 20477^{T}	1533	99%	AJ276355.1
			E. faecium LMG 11423^{T}	1551	99%	NR_042054.1
GY-20	1532		*E. hirae* DSM 20160^{T}	1433	99%	AJ276356.1
MSKC-13	1456	*P. pentosaceus*	*P. pentosaceus* ATCC 25745^{T}	1580	99%	NR_ 075052.1
			P. pentosaceus DSM 20336^{T}	1569	99%	NR_ 042058.1
MSK-3-18	1443	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *lactis* 3B7^{T}	1528	99%	EU337111.1
			L. lactis subsp. *lactis* ATCC 19435^{T}	1540	99%	AB008215.1

This study showed that four strains isolates from pla-ra, somkai-pla, kong-jom were *E. faecium* GY-20, *E. faecium* CN-25, *L. lactis* subsp. *lactis* MSK-3-18 and *P. pentosaceous* MSKC-13 respectively. These four isolates produced antimicrobial substance against pathogen and spoilage bacteria. This is the first report of *E. faecium* isolated from somkai-pla producing bacteriocins and showing strong inhibition against *L. monocytogenes*. Therefore, only *E. faecium* CN-25 strain was selected for bacteriocin production and characterization of antibacterial compounds.

4.4 PCR detection of enterocin structural genes

DNA of *E. faecium* CN-25 was screened by PCR for the presence of known enterocin genes, namely enterocin A , enterocin B, enterocin P, enterocin L50A and enterocin L50B structural gene, which would result in PCR fragments of 135 bp, 205 bp, 132 bp, 135 bp and 135 bp, respectively, Presence of enterocins A and B structural genes in *E. faecium* T136 has been studied, while *E. faecium* L50 normally exhibited enterocin P and enterocin L50A or entercin L50B (Casaus et al., 1997). Therefore, the strains of *E. faecium* T136 and *E. faecium* L50 were used as controls. The PCR results revealed the presence of structural enterocin A and enterocin B gene in *E. faecium* CN-25 strain, while *E. faecium* T136 and *E. faecium* L50 exhibited the presence of enteriocin A , enteriocin B, enterocin P and enterocin L50A gene (Figure 4.6). This finding indicated that *E. faecium* CN-25 carried enterocin A and enterocin B gene on structural gene. It, therefore, is likely to produce enterocins.

Similarity of enterocin gene sequence of *E. faecium* CN-25 compared with other bacteria from NCBI nucleotide sequence database was illustrated in Table 4.12. The 87-bp nucleotide sequence of fragment was found to have 100% homology with

enterocin A of *E. faecium* strain CRL 1385 accession number GQ369790.1 (Audisio et al., 2001) and have 100% homology with enterocin A of *E. faecium* strain airy1 accession number HM060244.1 (Mirhosseini et al., 2010).

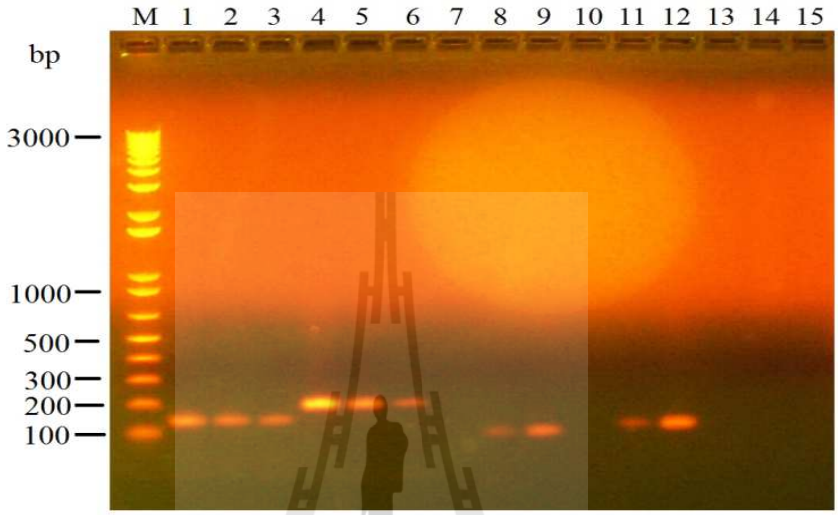


Figure 4.6 Enterocin gene screening. Electrophoresis in 1.5% agarose of PCR products with different enterocin primers of the strain *E. facecium* CN-25, *E. facecium* T136, *E. facecium* L50. M = marker 100bp, Lane, 1, CN-25 (Ent A); 2, T136 (Ent A); 3, L50 (Ent A); 4,CN-25 (Ent B); 5,T136 (Ent B); 6,L50 (Ent B); 7,CN-25 (Ent P); 8,T136 (Ent P); 9,L50 (Ent P); 10, CN-25 (L50A); 11,T136 (L50A); 12, L50 (L50A); 13, CN-25 (L50B); 14, T136 (L50B); 15, L50 (L50B)

The fragment size of 118- bp was found to be 100% similary to enterocin B of *E. faecium* T136 accession number U87997.1(Casaus et al., 1997), *E. faecium* CRL1385 accession number GQ36979.1 (Diop et al., 2007) and *E. faecium* CWBI141116S accession number EF3710041.1 (Diop et al., 2007) (Table 4.12).

Table 4.12 Similarity of enterocin gene sequence of *E. faecium* CN-25 compared with other bacteria from NCBI nucleotide sequence database.

Gene	Length of sequence (bp)	Identification result	Nucleotide sequence comparison			
			Closest relative	Length of sequence (bp)	Sequence homology	NCBI accession no.
Enterocin A	87	Enterocin A	*E. faecium* strain CRL1385 class IIa bacteriocin enterocin A	87	100%	GQ369790.1
			E. faecium strain airy1 enterocin A gene	98	100%	HM060244.1
Enterocin B	118	Enterocin B	*E. faecium* enterocin strain T136 B (entB) gene	353	100%	U87997.1
			E. faecium strain CRL1385 class IIa bacteriocin enterocin B precursor (entB)	159	100%	GQ36979.1
			E. faecium strain CWBI14111 16S ribosomal RNA gene, partial sequence; and enterocin B gene	204	100%	EF371004.1

The presence of enterocins is typically observed among enterococci, isolated from a variety of food products, animal and vegetable origin. Viola Saıd Ennahar et al. (2001) reported that *E. faecium* WHE 81, isolated from cheese produced enterocin. The sequencing DNA revealed two genes with sequences identical to entA and entB. Consequently, amino acid sequencing combined with DNA analysis clearly showed that *E. faecium* WHE 81 produces bacteriocins identical to enterocins A and B. *E. faecium* FAIR-E 406, was isolated from the gut of a chicken, and harbors the structural genes for the enterocins A and B (den Berghe et al., 2006). Strompfova et al. (2008) reported that the gene of enterocin A was exclusively detected among *E. faecium* strains, while the gene of enterocin P, B, L50B were detected in strains of both *E. faecium* and *E. faecalis.* The structural gene of enterocin A is widely distributed among *E. faecium* strains, whereas that of enterocin B always occurs in the presence of enterocin A. This is most probably due to the fact that no transport genes have been found for enterocin B production (Franz et al., 1999b). Enterocin A and B are bacteriocin belonging to class IIa (pediocin family) and class IIc (non-pediocin-type) (Gamze et al, 2011). Literatures indicate that pediocin-like bacteriocins from class IIa exhibit strong antagonistic activity towards *L. monocytogenes* (Drider et al., 2006; Ennahar et al., 2000; Ennahar et al., 1999; Fimland et al., 2005; Sip, Wieckowicz and Krasowska, 2009). Presence of enterocins A and B structural genes in *E. faecium* T136 has been studied by Casaus et al (1997). Two enterocins not only act synergistically, but also have an independent pattern of activity against various bacteria. They have been found to be active against a wide range of Gram-positive bacteria, including species of *Clostridium*, *Propioni* bacterium, *Listeria*, *Staphylococcus* and most lactic acid bacteria. However, the enterocin A and enterocin

B from *E. faecium* CN-25 showed strong antagonistic activity towards *L. monocytogenes*, which suggests that both bacteriocins may possess different mechanisms of action on sensitive cells.

4.5 Optimization of bacteriocin production

To obtain the maximum yield of bacteriocin, the effect of nitrogen source, carbon source, salt (tri-ammonium citrate, K_2HPO_4 and sodium acetate) on bacteriocin production of *E. faecium* CN-25 was investigated. MRS was chosen as the control medium. The MRS medium composes of (%, w/v); glucose, 2; peptone, 1; beef extract, 1; yeast extract, 0.5; K_2HPO_4, 0.2; $MgSO_4$, 0.02; $MnSO_4$, 0.05; Tri-ammonium citrate, 0.2; sodium acetate 0.2 and polysorbate (Tween 80), 0.1 ml.

4.5.1 Effect of byproducts on bacteriocin production

Various byproducts including rice bran (RB), mung bean protein (MP), yeast sludge (YS), fish sauce sludge (FS) were studied as replacement of peptone and beef extract in the MRS. Among 4 byproducts studied, RB resulted in the highest yield bacteriocin production ($p<0.05$) (Figure 4.7), while YS showed the lowest yield ($p<0.05$). This result suggests that the bacteriocin production does not fully depend on a rich of amino acids, peptides, vitamins, and minerals obtained from yeast sludge (YS) and mungbean protein (MP). They contained total nitrogen of 7.79% (w/w) and 12.29% respectively, while rice bran contained 2.16% (w/w) of total nitrogen content. Rice bran resulted in lower yield bacteriocin when compared with control (MRS broth) ($p>0.05$). This result suggests that peptone and beef extract in MRS medium, which are principally derived from casein, soya, gelatin and meat, could generate peptides suitable for bacteriocin production of *E. faecium* CN-25. Li, et al. (2002)

reported that total protein content of rice bran was 4.36%, and this low soluble amino nitrogen generally high peptide length. Most of lactic acid bacteria were limited of photolytic system, and only amino acids and some small molecular peptide could be used.

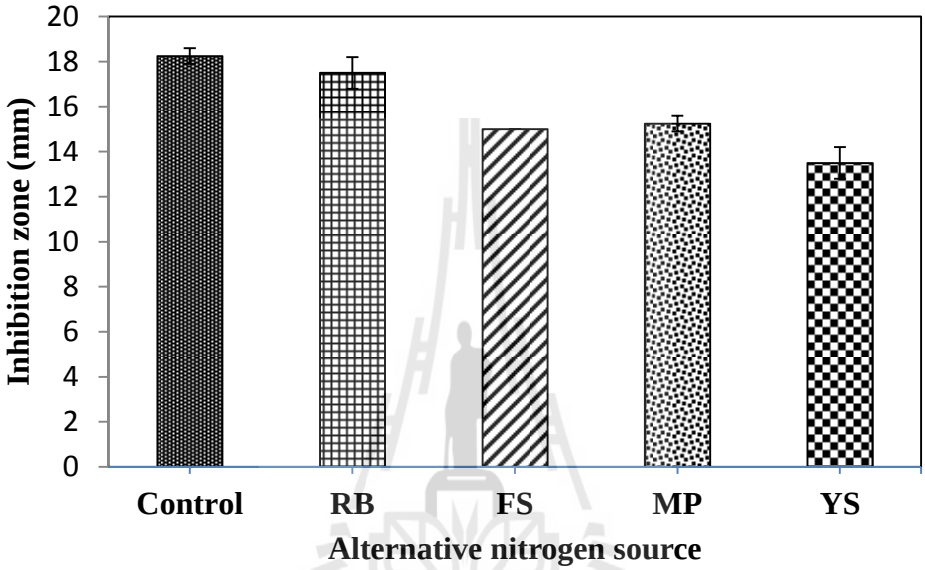


Figure 4.7 Effect of byproducts on bacteriocin production, Control (MRS broth), medium containing rice bran (RB), mungbean protein (MP), yeast sludge (YS) and fish sauce sludge (FS) at 2% (w/v). Basal formula: 2.0% glucose, 0.5% yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.05% $MnSO_4$, 0.2% tri-ammonium citrate, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0 and incubated at 25^{o}C for 24 h.

Nicolas et al. (2004) reported that the muricin produced by *S. mutans* can be produced in medium supplemented cheese whey permeate. The good yield of muricin was obtained the medium in consisting of cheese whey permeate (6% w/v), yeast extract (2% w/v). It was found to be an inexpensive medium for the efficient

production of mutacin. In this case, RB contained the lowest protein content which may induce bacteriocin production. Therefore, it was chosen for further optimization.

4.5.2 Effect of rice bran content

The maximal yield of bacteriocin production of *E. faecium* CN-25 was obtained in the medium containing 0.5% rice bran with maximum cell counts of 9.4 log CFU/ml ($p<0.05$, Figure 4.8). The lowest yield was obtained in 0.2% rice bran ($p<0.05$) with cell counts of 7.5 log CFU/ml ($p<0.05$). The higher content of rice bran in the medium had no affect on an increase in cell growth and bacteriocin production.

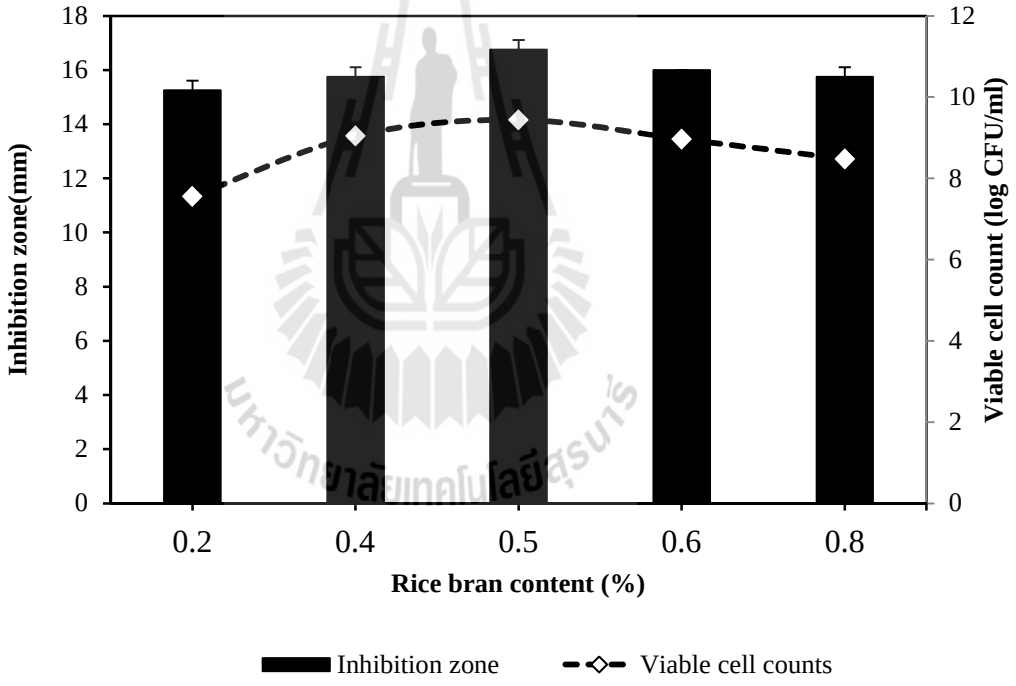


Figure 4.8 Effect of rice bran (RB) content on bacteriocin production and viable cell counts of *E. faecium* CN-25. The medium contained 2.0% glucose, 0.5% yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.05% $MnSO_4$, 0.2% tri-ammonium citrate, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0 and incubated at 25^{o}C for 24 h.

These results indicated an increase in content of rice bran content does not necessarily correspond with an increase in cell count and bacteriocin production of *E. faecium* CN-25.This could be because the limited nitrogen source in rice bran. Therefore, 0.5% rice bran was chosen for further optimization.

4.5.3 Effect of yeast extract content in the medium

Concentration of yeast extract significantly affected bacteriocin production of *E. faecium* CN-25. The highest bacteriocin production was achieved in the medium containing 0.8% yeast extract ($p<0.05$) with 0.5% RB. The highest viable cell count was achieved in the medium containing 0.6% yeast extract (10.3 log CFU/ml), while the yield of bacteriocin was lower than the medium containing 0.8 % yeast extract. The lowest bacteriocin production was at 0.1% yeast extract ($p<0.05$, Figure 4.9). These results suggest that bacteriocin production of *E. faecium* CN-25 is stimulated by yeast extract. Yeast extract provides not only a relatively larger proportion of free amino acids and short peptides (two to three amino acids), but also more growth factors than other protein hydrolysates (Cheigh et al., 2002; Aasen et al., 2000; De Vuyst, 1995; De Vuyst and Vandamme, 1993). The effect of yeast extract on bacteriocin production have been reported. Todorova et al. (2013) reported that highest bacteriocin ST22Ch production (3200 AU/ml) produced by *Lb. sakei* ST22Ch was records in presence of yeast extract as single organic nitrogen source (20.0 g/l).The optimum bacteriocin was obtained at 3.0% yeast extract where cell mass was 2.43 g/l (Cheigh et al., 2002), while Todorov and Dicks (2006) reported that the presence of meat extract as the only nitrogen source, or a combination of meat extract and yeast extract (1:1), resulted in approximately 50% reduction of bacteriocin ST23LD production. Pei et al. (2013) reported that yeast extract played an important

role on paracin C production of *L. paracasei* CICC 20421. This could be due to the availability of larger quantity of free amino acids, short peptides and more growth factors from yeast extract that induce bacteriocin production (Castro et al., 2011).

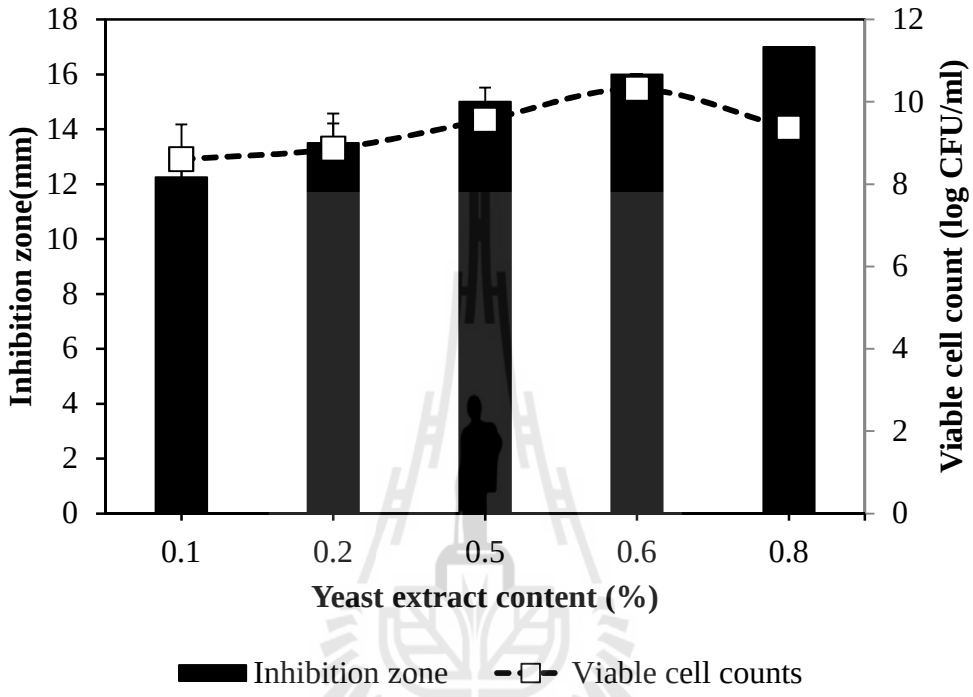


Figure 4.9 Effect of yeast extract on bacteriocin production and viable cell count of *E. facecium* CN-25. The medium contained 2.0% glucose, 0.5% yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.05% $MnSO_4$, 0.2% tri-ammonium citrate, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0 and incubated at 25^{o}C for 24 h.

In addition, bacteriocin have been shown to be influenced by organic nitrogen source. Therefore, nitrogen source is the most important factor affecting on bacteriocin production. Induction by nisin have been shown to be influence induction by related peptides (Diep et al. 1995; Eijsink et al. 1996). Yeast extract could contain

peptides that are essential for the synthesis of bacteriocin or may act as inducers of bacteriocin production. From these results, bacteriocin production of *E. faecium* CN-25 is stimulated by yeast extract.

4.5.4 Effect of glucose content

The maximum bacteriocin production was found at 0.5% glucose ($p<0.05$) (Figure 4.10) and *E. faecium* CN-25 grew and produced bacteriocin in the medium without glucose. This results suggest that *E. faecium* CN-25 may be derived from another nutrient such as yeast extract or rice bran presenting in the modified medium in sufficient quantities to allow *E. faecium* CN-25 growth but insufficient for normal synthesis of bacteriocin. Bacteriocin production appeared to decrease at 1%-1.5% glucose (w/v, $p<0.05$).

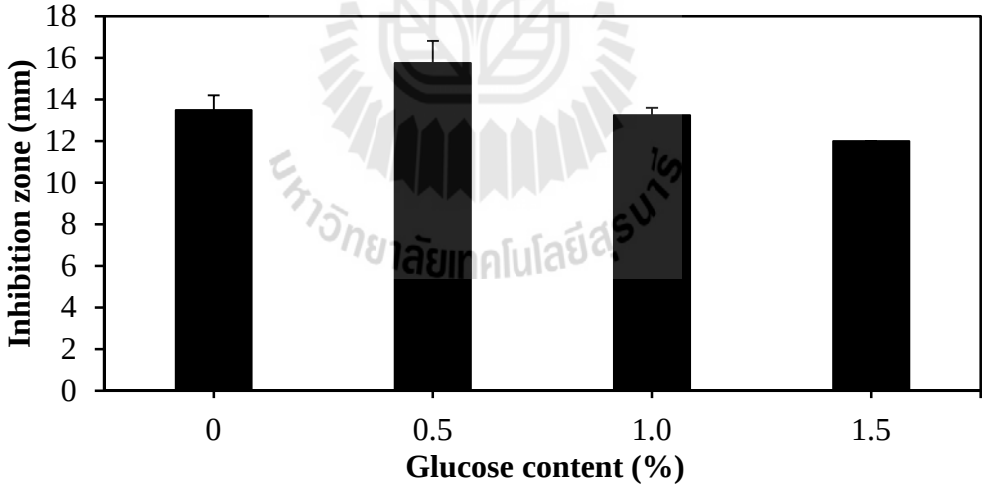


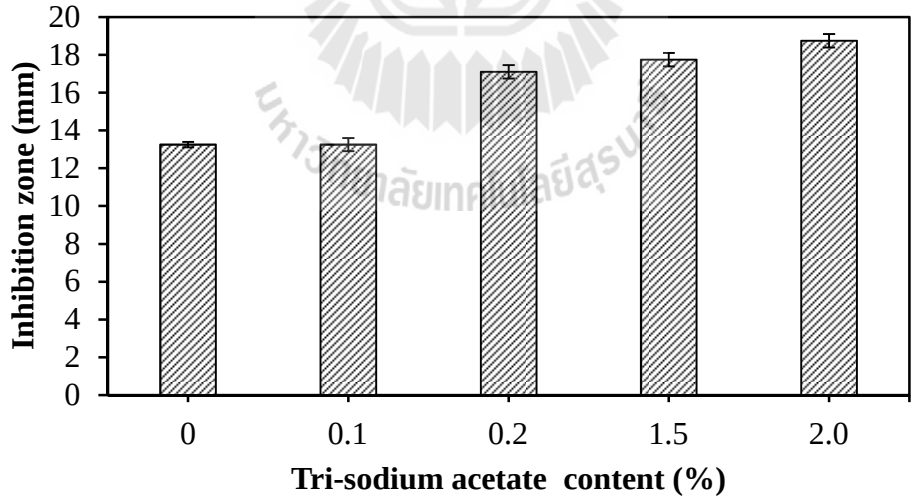
Figure 4.10 Effect of varied concentration of glucose on bacteriocin production and viable cell count of *E. facecium* CN-25. The medium contained 0.5% rice bran, 0.5% yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.05%, $MnSO_4$, 0.2% tri-ammonium citrate, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0 and incubated at 25^{o}C for 24 h.

Similar results were reported for bacteriocins produced by *Lb. plantarum* ST341LD (Todorov and Dicks, 2006). The production of bacteriocin ST341LD was found to be optimal at 20 g/l glucose with bacteriocin of 2835 AU/DO (Antimicrobial activity was express as arbitrary unit (AU) per optical density (OD) at 600 nm). Glucose concentrations of 10 and 40 g/l yielded 1420 AU/DO, whereas glucose at 1.0 and 5.0 g/l yielded less than 90 AU/DO. It was concluded bacteriocin ST341LD may be regulated by the glucose concentration in the medium, with levels below 20 g/l and above 30 g/l leading to repression. An increase of the glucose levels in culture medium could trigger regulatory mechanisms, which reduce glucose intake towards the cell, and therefore, reduce the growth and the metabolic activities (Axelsson, 1998). In addition, the effect of substrate inhibition was proposed to explain the reduction of plantaricin C production by *Lb. plantarum* LL441 at high glucose concentrations (Bruno et al., 1998). The optimum glucose content for bacteriocin production of *E. faecium* CN-25 was 0.5 % (w/v). Glucose is considered to be the main carbon source by all microorganisms due to its size, rapid uptake utilization and cellular energy conversion. The effect of carbon source on bacteriocin production has been investigated and responses have been varied strain. Han et al. (2011) reported that the optimal concentration of 0.36% glucose and 0.14% NaCl stimulated the production of bacteriocin produced by *Lb. plantarum* YJG, which these of optimum conditions produced bacteriocin at 1.4 fold higher than common MRS broth. Concentration of glucose over 0.36% inhibited the bacteriocin production of *Lb. plantarum* YJG. Nisin Z can be produced from glucose, sucrose and xylose by *L. lactis* IO-1 (Matsusaki et al., 1996; Chinachoti et al., 1997b) but better results were obtained with glucose. Drosinos et al. (2005) reported that Glucose is considered as a

better source for biomass production, while fructose was more suitable for bacteriocin production by *L. mesenteroides* E131. The highest bacteriocin production of *L. mesenteroides* E131strain rate was achieved with fructose 20 g/l fructose. Moreover, Todorov et al. (2005) reported that an increase in bacteriocin ST16Pa (*Lb. plantarum* ST16Pa) production was found in the presence of 30 g/l glucose. Production of bacteriocin ST16Pa is stimulated by the presence of glucose and mannose (Todorov et al., 2011). Thus, carbon source might play an important role in increasing bacteriocin yield.

4.5.5 Effect of inorganic salts

The rate of bacteriocin production of *E. faecium* CN-25 significantly varied with sodium acetate content in the medium. The maximum bacteriocin production was found at 2.0% sodium acetate ($p<0.05$, Figure 4.11), while low bacteriocin production

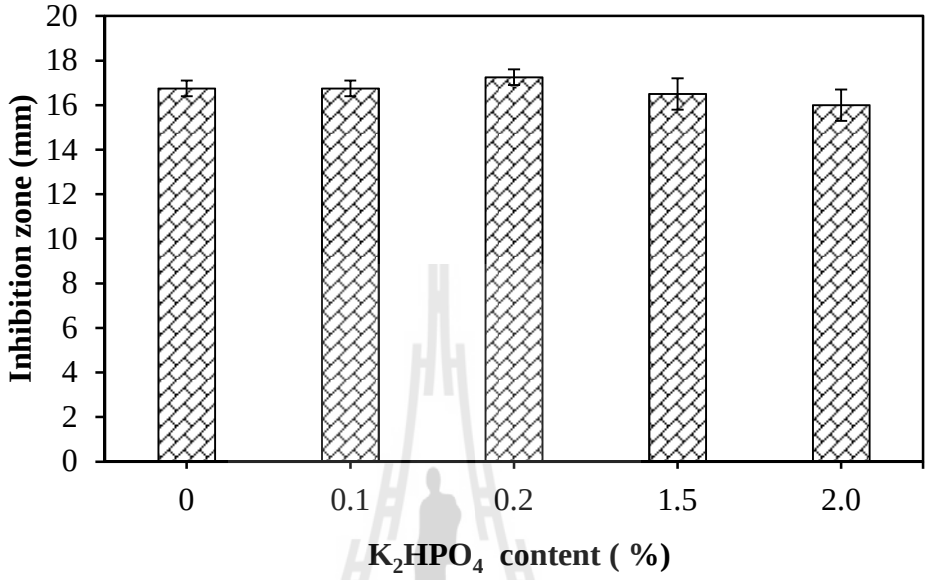


Finger 4.11 Effect of varied concentration of sodium acetate on bacteriocin production of *E. facecium* CN-25 and incubated at 25^{o}C for 24 h.

was observed in the medium without sodium acetate. Similarly, the lack of sodium acetate in the MRS medium markedly reduced bacteriocin production of *E. faecium* GM-1, whereas other inorganic salts had only a minor effect (Kang and Lee, 2005). Optimal production of peptide ST4SA was recorded in the presence of 0.5% sodium acetate. Concentration of 1% sodium acetate resulted in reduction of peptide ST4SA activity by 50% and concentrations of 0.2% sodium acetate or lower reduced activity by 75% (Todorov and Dick, 2009). The optimum concentration of sodium acetate on bacteriocin production of *E. faecium* CN-25 was 2.0%.

The bacteriocin production was not affected by K_2HPO_4 contents from 0.2% ($p>0.05$, Figure 4.12). These results suggests that inorganic salt could regulate pH which it had effect on cell growth, which these concentration (0.2%) may be sufficient quantities to allow *E. faecium* CN-25 growth but no effect on bacteriocin production. Therefore, the optimum of K_2HPO_4 on bacteriocin production by *E. faecium* CN-25 was 0.2%. Similarity, maximal bacteriocin activity (12 800 AU/ml) was recorded when strain ST194BZ was grown in the presence of 0.2% K_2HPO_4 (Todorov and Dicks, 2005). Little is known about the influence of potassium ion on the production of bacteriocins. In the case of a bacteriocin produced by *Lb. plantarum* UG1, 0.7% K_2HPO_4 was needed to increase bacteriocin production (Enan et al., 1996). Lower peptide ST4SA activity was recorded in 0.2% K_2HPO_4 in MRS broth and increasing of in K_2HPO_4 1% and 2% resulted in higher peptide ST4SA activity (Todorov and Dick, 2009). In the case of plantaricin UG1, 0.7% K_2HPO_4 resulted in increased activity (Enan et al., 2009). The optimal level of K_2HPO_4 for plantaricin ST31 was between 0.2%-0.5% (Audisto et al., 2001). Mahrous et al. (2013) reported that larger

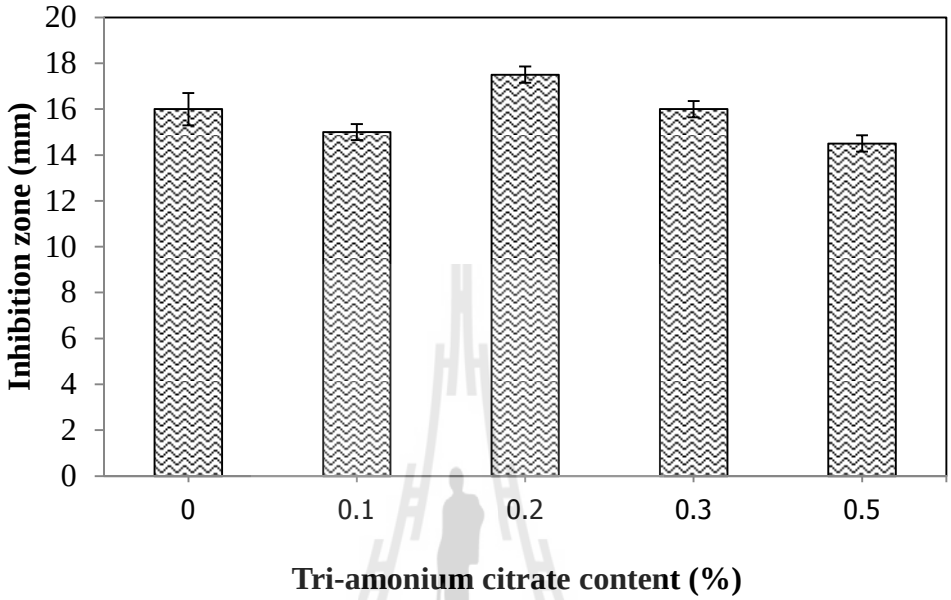
amount of bacteriocin was synthesized in the MRS medium supplemented with 1% K_2HPO_4.



Finger 4.12 Effect of varied concentration of K_2HPO_4 on bacteriocin production of *E. facecium* CN-25. The medium contained 0.5% rice bran, 0.5% yeast extract, 0.02% $MgSO_4$, 0.05%, $MnSO_4$, 0.2% tri-ammonium citrate, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0 and incubated at 25^{o}C for 24 h.

High bacteriocin production was observed in the medium containing 0.2 % tri-ammonium citrate (p<0.05, Figure 4.13). When concentration increased up to 0.5%, bacteriocin activity appeared to decrease (p<0.05). Bacteriocin production of *E. faecium* CN-25 may be regulated by tri-ammonium citrate concentration, levels below or above 0.2 % tri-ammonium citrate might lead to repression. Similarly, Todorov and Dicks, (2009) reported that the maximum bacteriocin activity produced by *E. mundtii*

ST4SA was obtained in MRS added 0.5 % tri-ammonium citrate, whereas tri-ammonium citrate at 0.1-0.2% or above 0.5% yielded lower bacteriocin production.



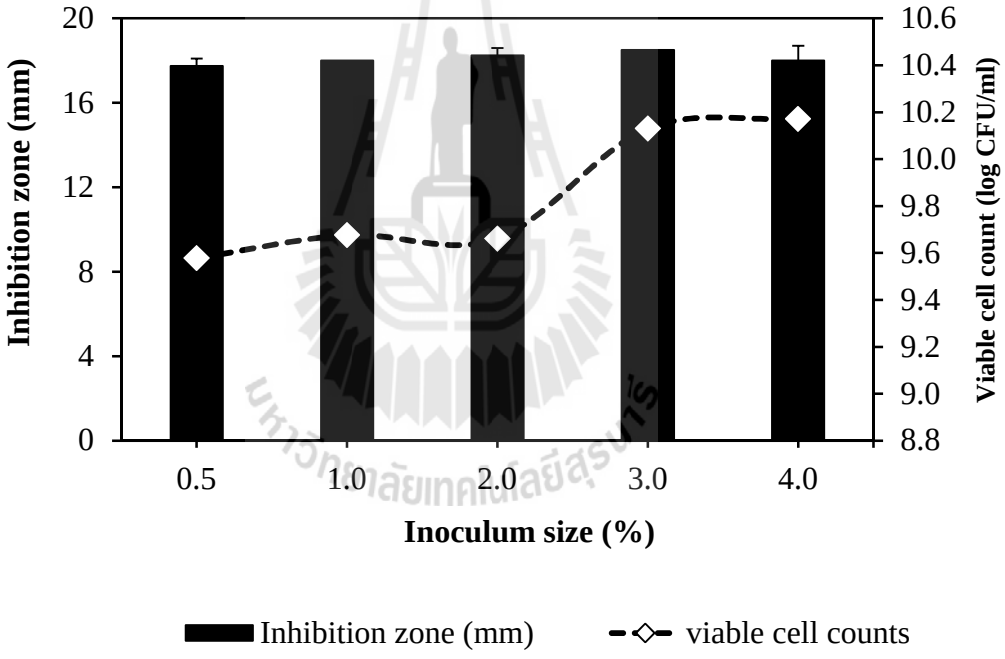
Finger 4.13 Effect of varied concentration of tri-ammonium citrate on bacteriocin production of *E. facecium* CN-25. The medium contained 0.5% rice bran, 0.5% yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$, 0.05%, $MnSO_4$, 0.2% sodium acetate, 0.1 ml of Tween 80, pH 7.0, and incubated at 25°C for 24 h.

Similar effect of tri-ammonium citrate was found for bacteriocin ST8KF production (Powell et al., 2007). In summary, the optimized medium composed of 0.5%, rice bran; 0.2%, glucose; 0.5%, yeast extract; 0.2%, tri-ammonium citrate; 2%, sodium acetate; 0.2%, K_2HPO_4; 0.02%, $MgSO_4$; 0.05%, $MnSO_4$ and 0.1 ml, Tween 80.

4.5.6 Effect of inoculum size

The effect of inoculum size on bacteriocin production was determined.

Inoculum at 0.5-4% had no effect on bacteriocin production but greatly affected cells growth (p>0.05, Figure 4.14). The maximum cell count of 10.1 log CFU/ml (p>0.05) was observed at 4% inoculum. Didem et al. (2011) reported that the highest bacteriocin yield was obtained by adding *L. lactis* subsp. *lactis* BZ at the level of 0.1% (v/v). This result suggests that the biosynthesis of bacteriocin is often an inducible trait that depends on the cell density of the cell culture and concentration of the inducer. Therefore, an inoculum of 0.5% was chosen for bacteriocin production of *E. faecium* CN-25.

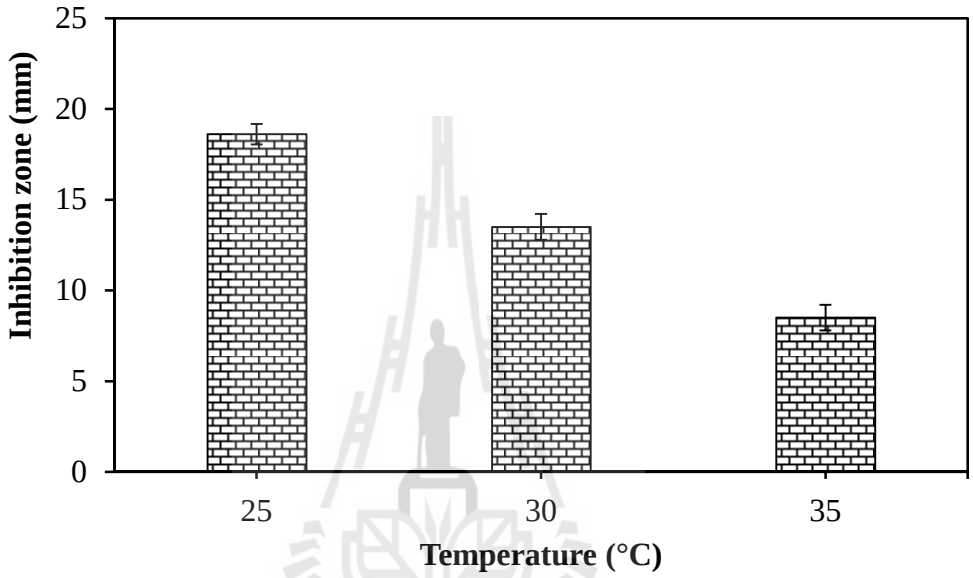


Finger 4.14 Effect of inoculum size on bacteriocin production and viable cell count in the optimized medium at pH 7.0, and incubated at 25^{o}C for 24 h.

4.5.7 Effect of temperature

Temperature strongly affected bacteriocin production of *E. faecium* CN-25. The maximum bacteriocin production was observed at 25^{o}C (p<0.05, Figure 4.15).

Lower bacteriocin production was observed at 30-35^{o}C. An optimum growth temperature of *E. faecium* CN-25 was 30-35^{o}C, while the optimum temperature for bacteriocin production was at 25^{o}C. Temperatures lower than the optimal growth may increase better utilization of energy and essential metabolites (Aasen et al., 2000).



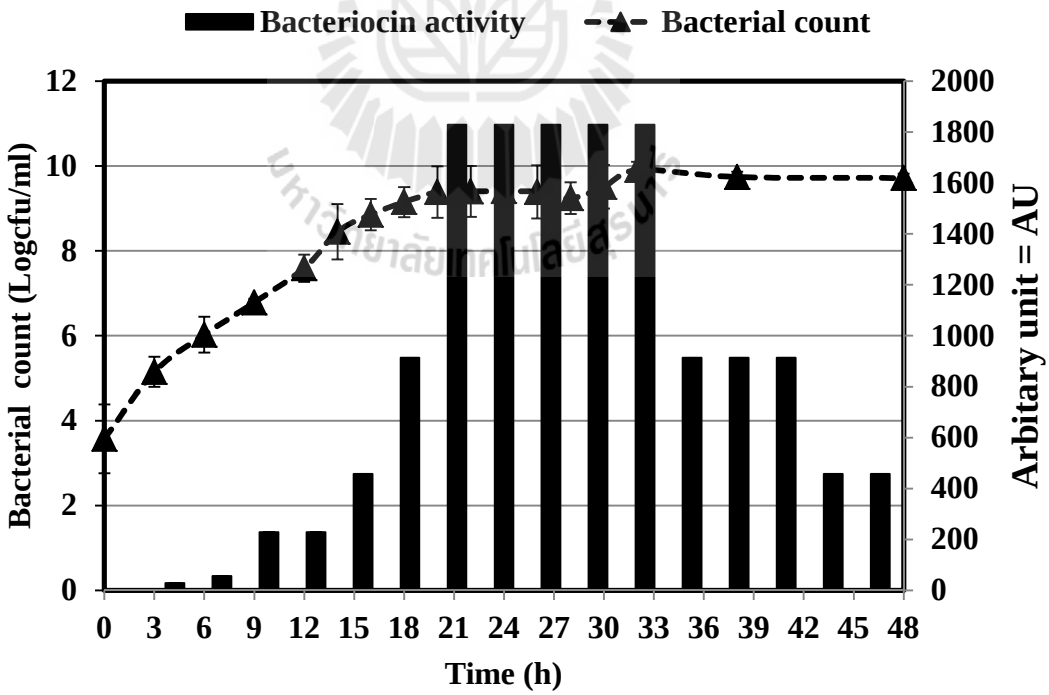
Finger 4.15 Effect of temperature on bacteriocin production of *E. facecium* CN-25

Generally, bacteriocin production by LAB is reported as a temperature-sensitive process, whereby the optimal temperature for bacteriocin production does not necessarily coincide with the optimal growth temperature (Leroy and de Vuyst, 1999b). It has been suggested that bacteriocin production by LAB is enhanced by suboptimal temperatures (de Vuyst et al., 1996; Delgado et al., 2007). A similar result was observed in a multiple bacteriocin producing strain *E. faecium* L50, where each bacteriocin had different optimal temperatures for its production. Maximum enterocin L50A and L50B production was detected at 25^{o}C, while enterocins P and Q were maximally produced at 37^{o}C and 47^{o}C, respectively (Criado et al, 2006). Similarly,

the highest bacterioin production by *E. faecium* NKR-5-3 appeared to be at 25°C and production significantly reduced at temperature greater than 30°C (Ishibashi et al., 2012).

4.6 Bacteriocin production

E. faecium CN-25 started to produce bacteriocin after 3 h of cultivation, corresponding to early logarithmic growth phase with cell growth of 5.4 log CFU/ml. Maximum production of bacteriocin (1828.15 AU/ml) was reached at the beginning of stationary phase (18 h) and the cell growth was determined to be 9.4 log CFU/ml (Figure 4.16). The activity remained stable during the stationary phase of 21- 33 h of cultivation and slightly decreased thereafter. This result indicated that bacteriocin



Finger 4.16 Growth curve and bacteriocin production of *E. facecium* CN-25 in the modified medium incubated at 25°C and shaking speed at 100 rpm.

produced by *E. faecium* CN-25 is a primary metabolite. Various antimicrobial agents were reported to be a primary metabolite, including *E. faecium* AQ71 (Ahmadova et al., 2013), peptides from *E. faecium* TW15, TW22 TW20 (Rivas et al., 2012), pediocin-like bacteriocin from *E. faecium* ST5Ha (Todorov et al., 2013), and enterocins from *E. faecium* PC4.1 (El-Ghaish et al., 2011), *E. faecium* ALP7 (Pinto et al., 2009) and *E. mundtii* ST15 (Kwaadsteniet et al., 2005). Bacteriocin production began to decrease after 36 h, which may be due to degradation by endogenous proteases induced during the growth phase and/or the adsorption of bacteriocin on the surface of producer (Deraz et al,. 2005). In this study, the maximum bacteriocin production by *E. faecium* CN-25 was achieved at the end of exponential phase.

4.7 Stability of bacteriocin

The effect of enzymes, pH and temperature on bacteriocin activity in cell-free supernatant was present in Table 4.13. The bacteriocin was very sensitive to all tested proteases. Bacteriocin activity totally disappeared after 1 h in the presence of proteases, indicating the proteinaceous nature of the antimicrobial compound. Catalase and lipase had no effect on the bacteriocin. Heating the bacteriocin at 60, 80, 100^{o}C for up to 30 min did not affect activity.

However, activity was decreased when treated at 121^{o}C for 15 min, while nisin showed high thermostability after treatment at 121^{o}C for 15 min. These results indicated that bacteriocin produced by *E. faecium* CN-25 was heat stable peptides. Generally enterococcal bacteriocins are heat-stable peptides (Ahmadova et al., 2013; Hadji-Sfaxi et al., 2011; Rehaiem et al., 2009; Renye et al., 2009). The bacteriocin produced by *E. faecium* CN-25 was active over a wide pH range of 2-12. The high

bacteriocin activity was observed at pH 2 and the activity was lower at pH 11 and 12. The antimicrobial activity has been reported to be active at wide pH range. The antimicrobial activity of *E. faecium* from varied strains was also stable over a wide pH range of 2-12, *E. faecium* AQ7 (Ahmadova et al., 2013), *E. faecium* PC4.1 (Hadji-Sfaxi et al., 2011), *E. faecium* 3D (Bayoub et al., 2011) and, *E. faecium* LM-2 (Guorong et al., 2011). High stability of bacteriocins at low pH was observed. This implied that CN-25 bacteriocin can be applied in a wide range of thermally-processed food and at a wide pH range.

Table 4.13 Effect of various enzymes, pH and heat treatment on the antibacterial activity produced by *E. facecium* CN-25 against *L. monocytogenes*.

Treatment	**Antibacterial activity (AU/ml)**
Enzyme	
Proteinase K	0
Trypsin	0
Chymotrypsin	0
Lipase	57.14
Catalase	57.14
Control	57.14
Heat	
Heating at 60 ^{o}C	
Control	57.14
5 min	57.14
10 min	28.57
15 min	28.57
20 min	28.57
30 min	28.57
Heating at 80 ^{o}C	28.57
5 min	
10 min	28.57
15 min	28.57
20 min	28.57
30 min	28.57
Heating at 100 ^{o}C	28.57
5 min	
10 min	28.57
15 min	28.57
20 min	28.57
30 min	28.57

Table 4.13 (Continuted)

Treatment	Antibacterial activity (AU/ml)
Heating at 121 ^{o}C	
15 min	14.28
Nisin	114.28
pH	
Control	57.14
pH 2	114.28
pH 3	57.14
pH 4	57.14
pH 5	57.14
pH 6	57.14
pH 7	57.14
pH 8	57.14
pH 9	57.14
pH 10	28.57
pH 11	14.28
pH 12	14.28

- No inhibition zone

4.8 Spectrum of inhibitory activity

The enterococcal CN-25 bacteriocin showed activity against most tested strains including *L. monocytogenes*, *E. faecalis* TISTR 379, *E. faecium* TW22, *E. faecium* TW20, *E. faecium* L50, *Lb. fermentum* TISTR 876 and *Lb. plantarum* TISTR 050. All tested strains of *L. monocytogenes* appeared to be highly sensitive to the enterococcal CN-25 bacteriocin (Table 4.14). Tested strains of Gram-negative and some strains of LAB were resistant. The lack of inhibitory activity against Gram-negative bacteria was a typical characteristic of enterococcal bacteriocins. Ahmadova et al., (2013) reported that bacteriocin from *E. faecium* AQ71 showed activity against closely related species of *Enterococcus*, *Lactobacillus* and strong anti-listeria activity. No activity was observed against *Salmonella* and *Escherichia coli*. Most of the studied bacteriocinogenic *E. faecium* strains showed activity against *L. monocytogenes* (Ben Belgacem et al., 2010; Leroy et al., 2003, Rodríguez, 2009) and *B. cereus* (Chen, Yanagida, Srionnual, 2007; Ghrairi, Frère, Berjeaud, Manai, 2008).

However, enterococcal CN-25 peptide showed strong activity against *L. monocytogenes*, corresponding to the distinctive characteristic of class IIa. It should be noticed that class IIa bacteriocins are more powerful anti-listerial agents than nisin, which is considered as class I (Ennahar et al., 2000).

Table 4.14 Inhibitory spectrum of enterococal CN-25 bacteriocin isolated from somkai-pla against lactic acid bacteria and pathogenic bacteria

Test microorganism	Growth medium	Growth temperature(°C)	Diameter of inhibition zone (mm)	
			CN-25 peptide	Nisin
Bacillus cereus TISTR 687	TSB	37	-	-
Enterococcus faecalis TISTR 379	MRS	37	8	9
Enterococcus faecium T 22	MRS	30	12	15
Enterococcus faecium Tw20	MRS	30	14	15
Enterococcus faecium T136	MRS	30	-	-
Enterococcus faecium L50	MRS	30	14	14
Escherichia coli O157:H7 ATCC 43895	TSB	37	-	-
Escherichia coli 15-12	TSB	37	-	-
Escherichia coli TISTR 887	TSB	37	-	-
Lactobacillus fermentum TISTR 876	MRS	37	9	-
Lactobacillus plantalum TISTR 050	MRS	37	7	-
Lactobacillus plantalum TISTR 877	MRS	37	-	-
Lactobacillus acidophillus TISTR 1034	MRS	37	-	-
Lactobacillus plantalum ATCC 14917	MRS	37	-	-
Lactobacillus gasseri ATCC 3323	MRS	37	-	-
Lactobacillus gasseri ATCC 44540	MRS	37	-	-
Listeria monocytogenes DSM 1327	TSB	37	12	13
Listeria monocytogenes TISTR 23136	TSB	37	15	15
Listeria monocytogenes ScottA	TGY	30	13	14
Morganella morganii	TSB	37	-	-
Micrococcus luteus	TSB	37	-	-
Pseudomonas aeruginosa ATCC 15442	TSB	37	-	-
Staphylococcus aureus TISTR 517	TSB	37	-	-

Table 4.14 (Continued)

Test microorganism	Growth medium	Growth temperature(°C)	Diameter of inhibition zone (mm)	
			CN-25 peptide	Nisin
Staphylococcu. canosus TISTR 833	TSB	37	-	11
Staphylococcus gallinarum TISTR 830	TSB	37	-	-
Staphylococcus xylosus	TSB	37	-	-
Salmonella enteritidis JCM 1652	TSB	37	-	-
Salmonella typhimurium	TSB	37	-	-
Salmonella Stanley ATCC 7308	TSB	37	-	-

-No inhibition zone

4.9 Bacteriocin purification

Purification of antimicrobial peptides from *E. faecium* CN-25 was carried out using ammonium sulfate precipitation and anion exchange chromatography. Ammonium sulfate precipitation resulted in 6.8% recovery and an approximately 1.7-folds increase in the specific activity (Table 4.15). The peptide recovery by DEAE-Sephacel anion exchange chromatography was relatively low. Similarly, the bacteriocin from *L. lactis* was successfully purified using ammonium sulfate precipitation, DEAE-cellulose chromatography. Purity of bacteriocin increased about 11.11 folds (Rajaram et al., 2010). *E. faecium* LM-2 bacteriocin purified using two step procedures of ammonium sulfate precipitation and cation exchange chromatography. The final cation exchange step revealed the activity of 10,240 AU/ml and the specific activity of 4.58×10^4 AU/mg (Liu et al., 2011). Enterocin LR/6 a bacteriocin obtained from *E. faecium* strain LR/6, has been purified to homogeneity using ammonium sulfate precipitation, cation-exchange chromatography, gel-filtration. Purity of enterocin LR/6 increased about 6.37 folds with a yield of 0.98% (Kumar, Tiwari and Srivastava, 2010). Purification of

bacteriocin derived from *E. faecium* P2 include ammonium sulfate precipitation, gel filtration, and cation-exchange, hydrophobic interaction and reverse-phase chromatography. Ammonium sulfate precipitation resulted in seven-fold increase in specific antimicrobial activity and 44% yield of the initial bacteriocin activity. The last step of bacteriocin purification reverse-phase chromatography, revealed two activities (fractions A and B). The purified bacteriocins represented 1.25 and 1.81% yield respectively, of the bacteriocin activity (Herranz et al., 2001).

Table 4.15 Purification table of the enterococal peptides produced by *E. facecium* CN-25.

Sample	**Total protein (mg)**	**Total bacteriocin activity (AU)**	**Specific activity (AU/mg)**	**Fold**	**Yield (%)**
Crude	1807	457100	252.96	1	100
65% $(NH_4)_2SO_4$ precipitation	123.0	54857.1	445.66	1.7	6.81
DEAE-Sephacel	0.555	1142.8	2059.09	8.1	0.03

4.10 Minimal inhibitory concentration (MIC)

The MIC is considered as the lowest concentration at which bacteriocin completely inhibites *L. monocytogenes* Scott A. Partialy purified CN-25 peptide was found to completely inhibit the growth of the pathogen at a concentration of 2.38 µg/ml (Figure 4.17). van Kuijk, Noll and Chikindas (2011) reported that the MIC of subtilosin from *B. amyloliquefaciens* fully inhibited *L. monocytogenes* Scott A at a concentration of 19 µg/ml. Minahk et al. (2004) reported that MIC values of enterocin CRL35 was 8 ng/ml, whose concentration inhibited growth of *L. inocua* 7

more than 50% at 37 ^{o}C. Galvez et al. (1989) reported that the MICs of enterocin AS-48 for *Escherichia coli* K-12, *E. durans* CECT 411, and *Corynebacterium laevaniformans* CECT 445 were calculated to be 1.2, 0.087, and 0.13 mg/ml, respectively. The MICs of enterocin 4 for the *C. laevaniformans* and *E. durans* were in the same order of magnitude of 0.096 and 0.048 mg/ml but even concentrations as high as 200 mg/ml did not inhibit *E. coli* K-12 or any of the other gram-negative bacteria tested (Joosten et al., 1996).

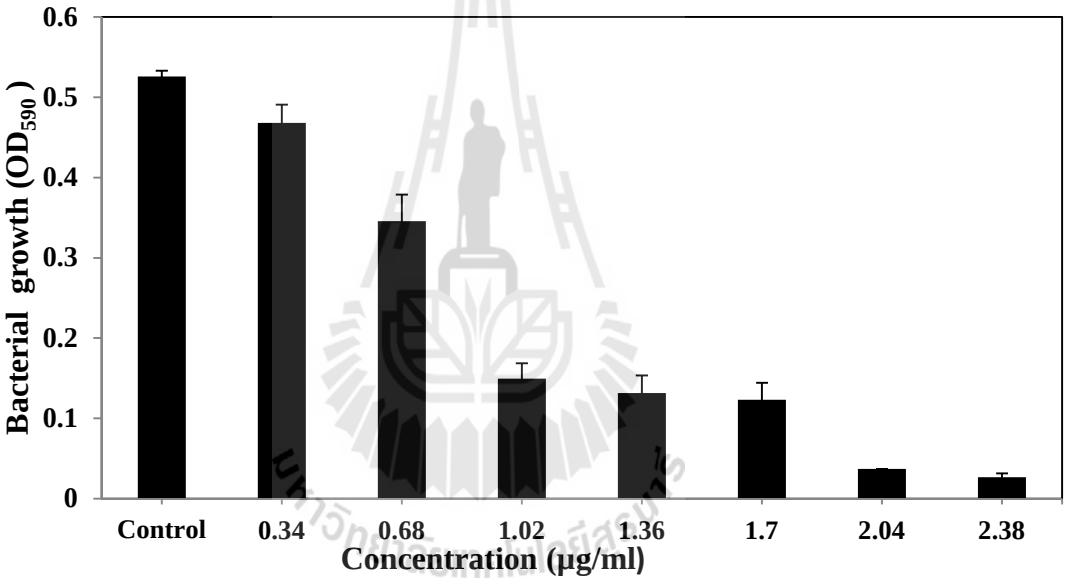


Figure 4.17 Minimal inhibitory concentrations (MIC) of CN-25 peptide on *L. monocytogenes* Scott A.

4.11 Mode of action

4.11.1 ATP measurements

The effect of anti-listeria peptide produced by *E. faecium* CN-25 on intracellular ATP levels in *L. monocytogenes* Scott A cells was assessed. ATP levels were determined using an ATP bioluminescence assay. The activity of anti-listeria

peptide CN-25 had no effect on efflux of ATP, but instead triggered intracellular hydrolysis of ATP as evidenced by a decrease in the luminescence of total ATP (Figure 4.18a). The negative controls (water) were determined. It showed in an extremely low of luminescence, signifying that the controls did not cause ATP efflux and that the *L. monocytogenes* Scott A were not leaking ATP prior to bacteriocin treatment. Nisin caused an efflux of ATP equivalent to 12% of the total ATP released (Figure 4.18b). Anti-listeria peptide CN-25 caused an efflux equivalent to 6.01% of the total ATP. The negative control of nisin diluent (0.02 M hydrochloric acid) had most of their ATP content, indicating *L. monocytogenes* Scott A poorly tolerates the conditions of the assay. van Kuijk, Noll and Chikindas (2011) reported that antimicrobial peptide subtilosin did not cause an efflux of intracellular ATP from *L. monocytogenes* Scott A cells This result suggests that nisin diluent has no effect on intracellular ATP but nisin increase efflux of ATP of *L. monocytogenes* Scott A cells, which probably unable to form pores in the membrane of *L. monocytogenes* Scott A cells. Herranz et al. (2001) reported that the addition of enterocin P to energize *E. faecium* T136 cells induced intracellular ATP depletion without ATP efflux. This effect has been reported several class II bacteriocins, such as lactacin F (Abee et al., 1994), pediocin PA-1 (Chen et al., 1995), lactococcin G (Moll et al., 1996), and mundticin (Bennik et al., 1998). Sutyak Noll et al. (2011) showed that subtilosin inhibits the vaginal pathogen *Gardnerella vaginalis*, which subtilosin caused an efflux of intracellular ATP in *G. vaginalis* but it did not cause an efflux of intracellular ATP from *L. monocytogenes* cells. The subtilosin was able to induce transient pores in the *G. vaginalis* cellular membrane. This concurred with previous research showing

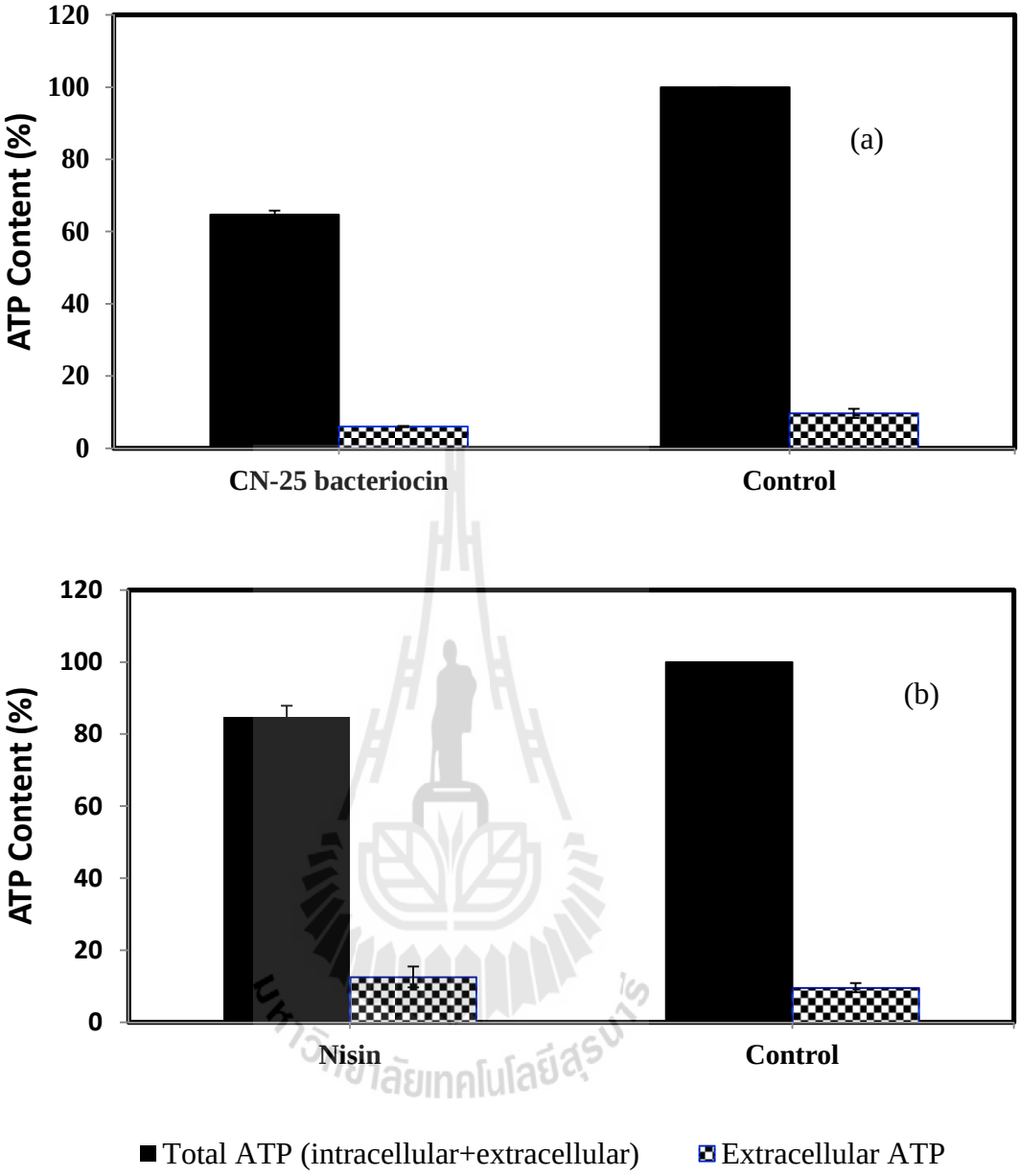


Figure 4.18 The effect of CN-25 peptide (a) and nisin (b) on ATP content of *L. monocytogenes* Scott A cells at 2.38 µg/ml

that in an in *vitro*, cell-free environment, subtilosin can cause damage by binding and inserting itself into the lipid bilayer (Thennarasu et al. 2005; Yamamoto et al. 2010).

4.11.2 Effect of CN-25 peptide on transmembrane electrical potential (ΔΨ)

The proton motive force (PMF) consists of two components: a transmembrane

electrical potential (ΔΨ) and the pH gradient (ΔpH). In principle, if a compound interacts with and damages the membrane, changes will occur in either the transmembrane electrical potential (ΔΨ), the pH gradient (ΔpH) or both. Furthermore, as the energy status of a cell is generally considered to be an indication of the cell's well-being. The effect of CN-25 peptide produced on the ΔΨ was observed using fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Figure 4.19). The fluorescence was increased after addition glucose. The ionophore nigericin was added to initially convert all ΔpH into ΔΨ, which does not influence fluorescence. After this step, CN-25 peptide was added. The CN-25 peptide increased fluorescence signal at 0.68 µg/ml and 2.38 µg/ml. The ionophore valinomycin was then added to completely dissipate the ΔΨ. Valinomycin increased in fluorescence as result of complete depolarization of the cells. The negative control (water) did not show fluorescence. Nisin was used as the positive control as it is known to induce pores in the target cell membranes (Moll et al. 1999). Nisin caused an increase in the fluorescent signal of the probe as result of the cellular membrane being depolarized bacteriocin. Further addition of valinomycin caused no increase in fluorescence, indicating that nisin completely dissipates ΔΨ. However, it is generally accepted that bacteriocins produced by LAB act by altering the permeability barrier of cell membranes (Gonzalez et al., 1996, Moll et al., 1996, van Belkum et al., 1991, Venema et al., 1993, Winkowski et al., 1996, Herranz et al., 2001) and one of the common mechanisms of inhibition of their target cells is the dissipation of PMF, the proton motive force (Abee et al., 1994, Bruno et al., 1992, Montville et al., 1995). Enterocin P efficiently dissipated the ΔΨ in *E. faecium* T136 cells (Herranz et al., 2001). Since enterocin P is an amphipathic, cationic peptide, it may interact with the

negatively charged bacterial membranes of the sensitive cells and alter their properties (Cintas et al., 1997). The CN-25 peptide produced by *E. faecium* CN-25 had effect on increasing cell membrane permeability, leading to depletion of ΔΨ.

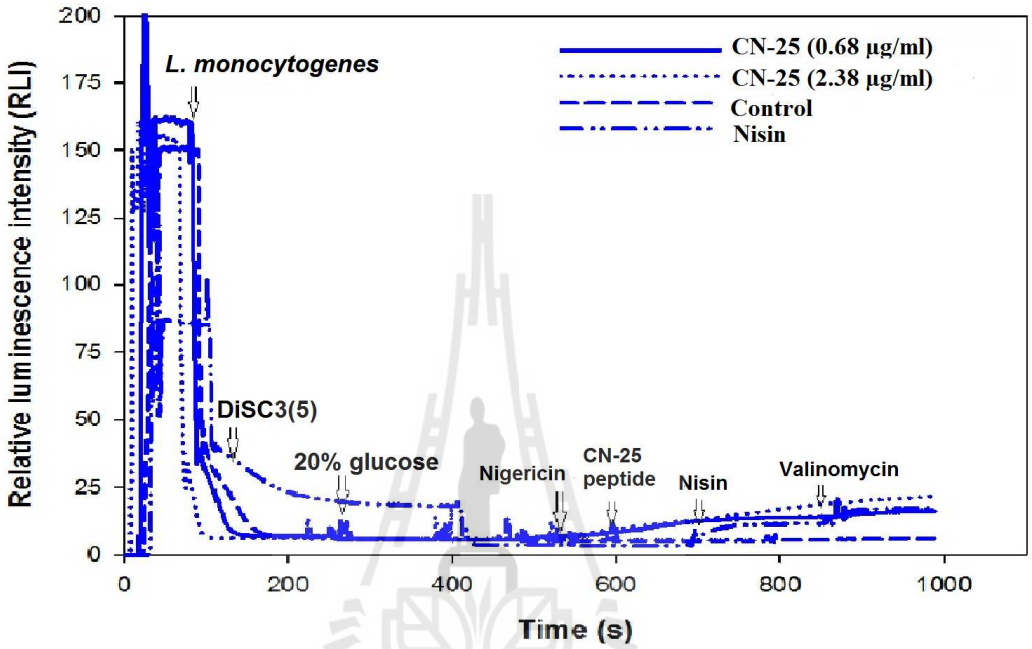


Figure 4.19 Effect of CN-25 peptide on a partial dissipation of the ΔΨ of *L. monocytogenes* Scott A cells

4.11.3 Effect of CN-25 peptide on transmembrane pH gradient (ΔpH)

The effect of CN-25 peptide on changes of intracellular pH in cellular suspension was recorded by measuring the fluorescence of the probe (2',7'-bis-(2-carboxyethyl)- 5-(and-6) carboxyfluorescein (BCECF-AM) (Figure 4.20). No effect of ΔpH dissipation was observed after addition 0.68 and 2.38 µg/ml^{-1} CN-25 peptide. The control (buffer) did not affect fluorescent signal. Addition nigericin to deplete any remaining ΔpH did not cause a further drop in fluorescence, indicating that all samples caused depletion of the ΔpH. These results showed that CN-25 peptide

depleted the transmembrane electic potential (ΔΨ) but has no effect on transmembrane pH gradient (ΔpH). The CN-25 peptide does not affect both portions of the proton motive force (PMF), which is similar to other bacteriocins that are known to selectively dissipate only one PMF component. Enterocin P, loctococcin G were reported to dissipate ΔΨ but no effect on ΔpH and depletion of intracellular ATP level (Herranz et al., 2001, Herranz and Chen et al., 2001). van Kuijk, Noll and Chikindas, (2011) reported that the subtilosin has only a minor effect on the ΔΨ but no had effect on the ΔpH of *L. monocytogenes* Scott A cells. The changes in PMF observed in this study suggest that instead of forming pores, subtilosin is likely to bind the cellular membrane, perhaps via a membrane-bound receptor molecule, and inserting itself into the lipid bilayer. Such that perturbation of the membrane could disruptt cellular processes, leading to cell death. It should be mentioned that the

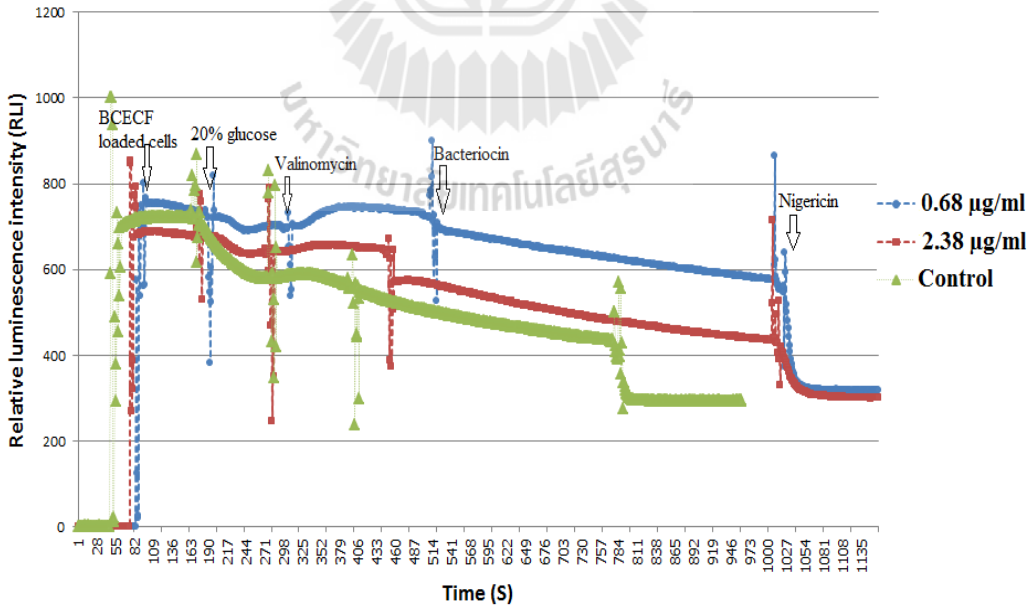


Figure 4.20 Effect of CN-25 peptide on the transmembrane pH gradient (ΔpH) of *L. monocytogenes* Scott A cells.

peptide produced by *E. faecium* CN-25 only dissipated $\Delta\psi$ and dissipation, while the ΔpH is not the main mode of action against *L. monocytogenes* Scott A. It can be hypothesized that this could be the main mode of anti-listeria to kill *L. monocytogenes* Scott A of CN-25 peptide.

4.12 Effect of CN-25 peptide on control of *L. monocytogenes* in milk

To investigate the efficacy of CN-25 peptide on food application, it was tested on the pasteurized milk. The evolution of total counts and listeria counts of pasteurized milk samples with addition of CN-25 peptide and nisin during storage at 4 ^{o}C for 10 days. Viable cell counts in all samples were increased up to 6.4 log CFU/ml was observed after storage for 5 days (Figure 4.21a). No viable cell counts were detected in uninoculate samples in the first day, but rapid increase of viable cell counts up to 6.4 log CFU/ml was observed after 10 days. In contrast, listerial counts of samples containing CN-25 peptide deceased from 4.1 to 3.7 log CFU/ml ($p<0.05$) within 5 days (Figure 4.21b). No growth of *L. monocytogenes* was observed in uninoculated samples during the entire period of the study. This result suggested that CN-25 had antilisterial effect in milk. Therefore, peptide produced by *E. faecium* CN-25 cloud be used as a preservative in milk or milk products for controlling *L. monocytogenes*. Bacteriocin have been used application in foods against *L. monocytogenes*, including the use of bactreiocin-producing strains as starter cultures for fermented foods or addition bacteriocins to foods. Zapico et al. (1999) reported that nisin addition of 100 IU/ml achieved a reduction in *L. innocua* counts of 3.7–3.8 log CFU/ml in whole milk compared to untreated samples.

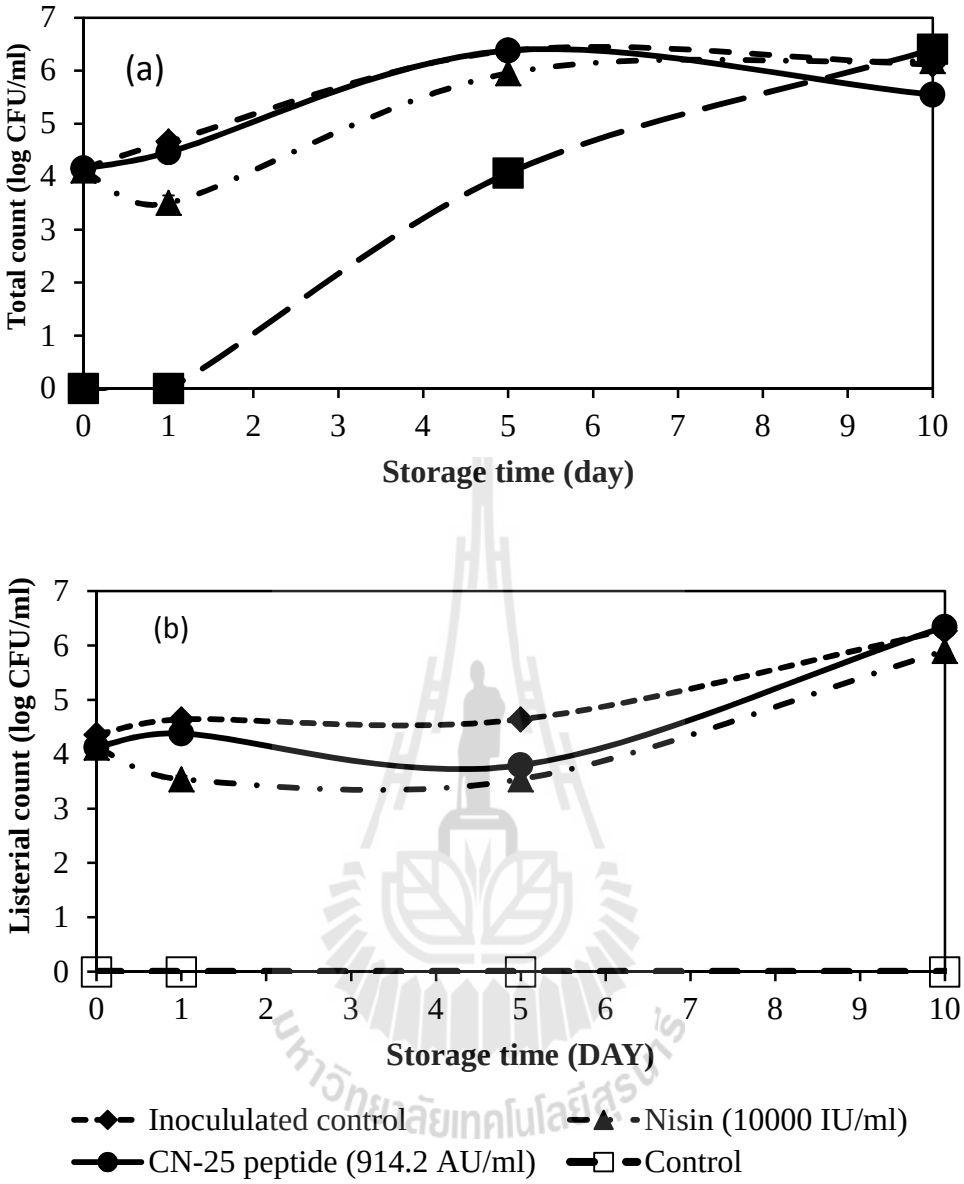


Figure 4.21 Effect of CN-25 peptide on total viable cell count (a) and listerial count (b) in pasteurized milk samples inoculated with *L. monocytogenes* and keep at 4 ^{o}C for 10 days.

CHAPTER V
CONCLUSIONS

Total of 285 LAB isolates were isolated and screen for bacteriocin production. Four strains producing good inhibition zones were CN-25, GY-20, MSKC-13, and MSKC-3-18, which showed the greatest activity against *L. monocytogenes*. These isolates were identified as *E. faecium*, *L. lactis* subsp. *lactis*, and *P. pentosaceus*. *E. faecium* isolated from Somkai-pla producing bacteriocins showed the highest inhibition against *L. monocytogenes*. *E. faecium* CN-25 contained DNA fragments corresponding in size to genes coding for enterocin A and enterocin B, which showed high activity against *L. monocytogenes*. *E. faecium* CN-25 showed maximum bacteriocin production in the modified broth containing 0.5% rice bran; 0.2% glucose; 0.5% yeast extract; 0.2% tri-ammonium citrate; 2% sodium acetate; 0.2% di-potassium hydrogen phosphate; 0.02% magnesium sulfate; 0.05% manganese sulfate and 0.1 ml polysorbate (Tween 80). Optimal bacteriocin production for *E. faecium* CN-25 was at the level of 0.5% inoculum and 25 °C. Maximum production of bacteriocin, 1828.15 AU/ml, was reached at the beginning of stationary phase and the cell growth was determined to be 9.4 log CFU/ml. Purity of bacteriocin was increased about 8.1 folds. In addition, the bacteriocin of *E. faecium* CN-25 was heat stable peptides and active over a wide pH range of 2-12. The minimum concentration (MIC) of bacterocin from *E. faecium* CN-25 which fully inhibited *L. monocytogenes* Scott A was 2.38 µg/ml. Although, the activity of peptide CN-25 had no effect on efflux of ATP, it triggered intracellular hydrolysis of ATP and dissipated $\Delta\psi$, while the ΔpH is

not effect on *L. monocytogenes* Scott A cells. The addition of 914.2 AU/ml of CN-25 peptide reduced *L. monocytogenes* in the inoculated pasteurized milk from 4.1 to 3.7 log CFU/ml ($p<0.05$) within 5 days. This peptide could be used as antimicrobial in milk or milk products for controlling *L. monocytogenes*.

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APPENDICES

Appendix A

Reagent preparations and culture media

1. Reagents

1.1 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol (95%)	20.00	ml
Mixed thoroughly		
Ammonium oxalate	80.00	ml
(1% Aqueous solution)		

1.2 Hydrogen peroxide (3% solution)

Hydrogen peroxide	3.00	g
Distilled water	1,000.00	ml

1.3 Iodine solution (Gram stain)

Iodine	1.00	g
Potassium iodide	2.00	g
Add distilled water and bring volume up to	300.00	ml

1.4 Safranin (Gram stain

Safranin O (0.25% solution in 95% ethanol)	10.00	ml
Distilled water	90.00	ml

1.5 Nitrate reduction test reagent

Sulfanilic acid solution		
5 N Acetic acid	100	ml
Dissolve by gentle heating in a fume hood.		
N,*N*-dimethyl-1-naphthylamine solution		
N,*N*-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	ml

1.6 TE buffer (10mM Tris-HCl, 1mM EDTA)

Tris-HCl	0.79	g
EDTA (di-soduim salt)	0.37	g
Boric acid	5.54	g
Add distilled water and bring volume up to	1,000.0	ml

1.7 Tris-HCl buffer (0.1M, pH 9.0)

Tris	1.21	g
Distilled water	100.00	ml
Adjust to pH 9.0 using 1N HCl.		

1.8 Tris-Borate buffer

Tris-Bas	10.77	g
EDTA (di-Sodium salt)	0.93	g
Boric acid	5.54	g
Add distilled water and bring volume up to	1,000.0	ml

2. Culture media

2.1 Carnobacterium medium

Beef extract	10	g
Peptone	10	g
NaCl	5	g
Glucose	5	g
Yeast extract	3	g
Agar	15	g
pH 6.8±0.2 at 25 ^{o}C		

2.2 GYP sodium acetate mineral salt broth

Peptone	10	g
Glucose	10	g
Yeast extract	10	g
Sodium acetate	10	g
$MgSO4.7H_2O$	0.2	g
$FeSO_4.7H_2O$	10	g
$MnSO_4.7H_2O$	10	g
NaCl	10	mg

pH 6.8±0.2 at 25 oC

The medium was autoclaved at 121°C for 15 min.

2.3 O-F test medium (Atlas and Park, 1997)

Sodium chloride	5.0	g
Pancreatic digest of casein	2.0	g
di-Potassium hydrogen phosphate	0.30	g
Bromthymol blue	0.03	g
Agar	2.5	g
Glucose solution	100.0	ml
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

2.4 De Man Rogosa and Sharpe medium (MRS)

Peptone	10.00	g
Meat extract	10.00	g
Yeast extract	5.00	g
Glucose	20.00	g
Tween 80	1.0	ml
K_2HPO_4	2.00	g
Sodium acetate	2.00	g
di-Ammonium citrate	0.20	g
$MgSO_4.7H2O$	0.20	g
$MnSO_4.4H_2O$	0.05	g
Add distilled water to bring volume up to	1,000.0	ml

pH 7.0

The medium was autoclaved at 115 °C for 10 min.

2.5 Nitrate broth

Beef extract	3	g
Peptone	5	g
Potassium Nitrate (KNO_3)	1	g
Add distilled water to bring volume up to	1,000.0	ml

The medium was autoclaved at 115 °C for 10 min.

2.6 Pyruvate fermentation medium

Tryptone	10	g
Yeast extract	5	g
Sodium pyruvate	10	g
Bromthymol blue	10	ml
K_2HPO_4	5	g

pH 7.2-7.4 The medium was autoclaved at 115 °C for 20 min.

2.7 Starch agar (Atlas and Park, 1997)

Soluble starch	2.0	g
Beef extract	3.0	g
Peptone	5.0	g
Sodium chloride	50.0	g
Agar	20.0	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2 The medium was autoclaved at 121°C for 15 min.

2.8 Trypticase (tryptic) soy broth (TSB)

(Atlas and Parks,1997)

Tryptone	17.0	g
Phytone (Papaic digest soya meal)	3.0	g
Sodium chloride	5.0	g
di-Potassium hydrogen phosphate	2.5	g
Glucose	2.5	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2 The medium was autoclaved at 121°C for 15 min.

2.9 Tween-80 agar (Atlas and Park, 1997)

Peptone	10.0	g
Sodium chloride	50.0	g
Calcium chloride	0.1	g
Yeast extract	2.5	g
Tween-80	10.0	ml
Agar	20.0	g
Add distilled water and bring volume up to	1,000.0	ml

pH 7.0±0.2

The medium was autoclaved at 121°C for 15 min.

Appendix B

Colony morphology on MRS agar at 35^{o}C for 24 h

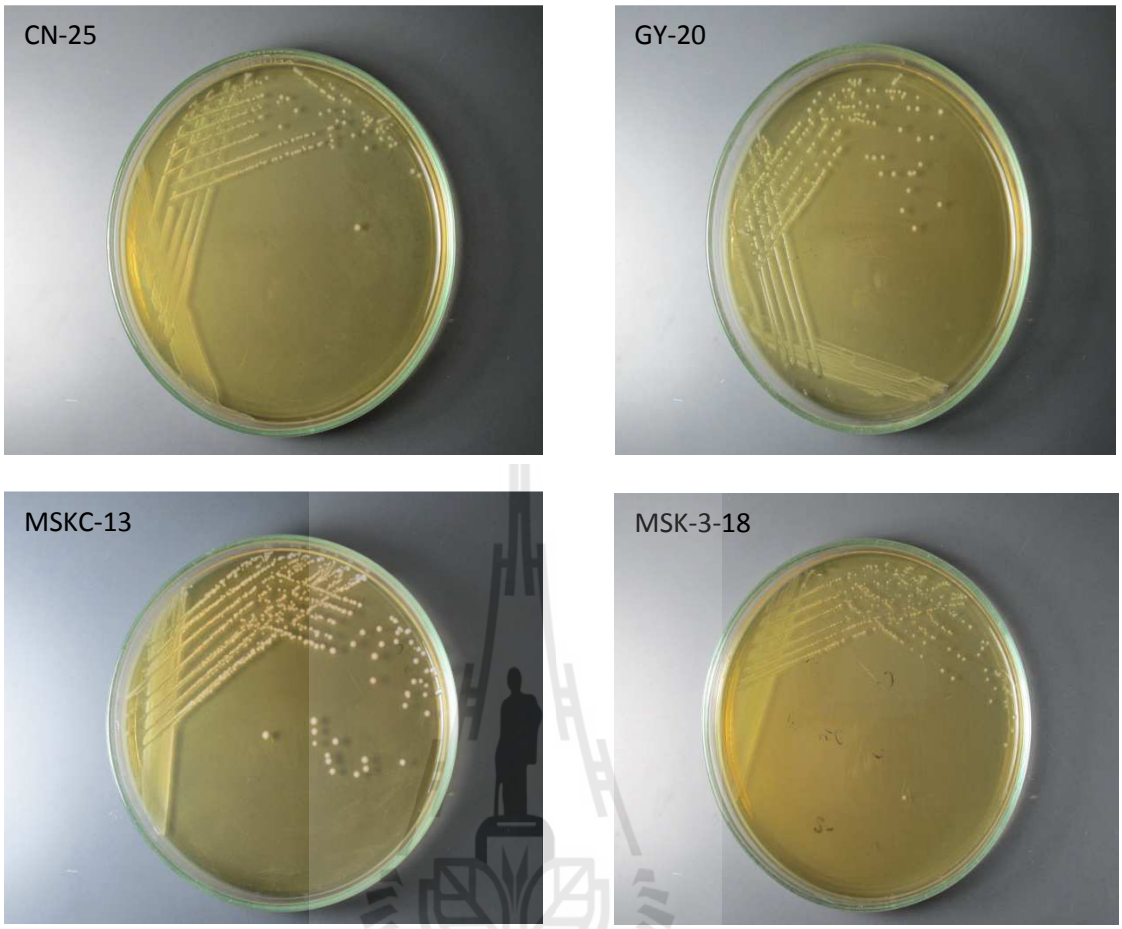

Figure 1The colony of 4 isolate selected from fishery products.

Appendix C

Nucleotide sequence of 16S rRNA gene

1. Nucleotide sequence of 16S rRNA gene (partial sequence) of CN-25 isolated from fermented fishery product.

```
1      AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG      50
51     CAAGTCGAAC GCTTCTTTTT CCACCGGAGC TTGCTCCACC GGAAAAAGAG      100
101    GAGTGGCGAA CGGGTGAGTA ACACGTGGGT AACCTGCCCA TCAGAAGGGG      150
151    ATAACACTTG GAAACAGGTG CTAATACCGT ATAACAATCG AAACCGCATG      200
201    GTTTTGATTT GAAAGGCGCT TTCGGGTGTC GCTGATGGAT GGACCCGCGG      250
251    TGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCCA CGATGCATAG      300
301    CCGACCTGAG AGGGTGATCG GCCACATTGG GACTGAGACA CGGCCCAAAC      350
351    TCCTACGGGA GGCAGCAGTA GGGAATCTTC GGCAATGGAC GAAAGTCTGA      400
401    CCGAGCAACG CCGCGTGAGT GAAGAAGGTT TTCGGATCGT AAAACTCTGT      450
451    TGTTAGAGAA GAACAAGGAT GAGAGTAACT GTTCATCCCT TGACGGTATC      500
451    TAACCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA      550
551    GGTGGCAAGC GTTGTCCGGA TTTATTGGGC GTAAAGCGAG CGCAGGCGGT      600
601    TTCTTAAGTC TGATGTGAAA GCCCCCGGCT CAACCGGGGA GGGTCATTGG      650
651    AAACTGGGAG ACTTGAGTGC AGAAGAGGAG AGTGGAATTC CATGTGTAGC      700
701    GGTGAAATGC GTAGATATAT GGAGGAACAC CAGTGGCGAA GGCGGCTCTC      750
751    TGGTCTGTAA CTGACGCTGA GGCTCGAAAG CGTGGGGAGC AAACAGGATT      800
801    AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA GTGTTTGGAG      850
851    GGTTTCCGCC CTTCAGTGCT GCAGCTAACG CATTAAGCAC TCCGCCTGGG      900
901    GAGTACGACC GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA      950
951    AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG      1000
1001   GTCTTGACAT CCTTTGACCA CTCTAGAGAT AGAGCTTCCC CTTCGGGGGC      1050
1051   AAAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG TGAGATGTTG      1100
1101   GGTTAAGTCC CGCAACGAGC GCAACCCTTA TTGTTAGTTG CCATCATTCA      1150
1151   GTTGGGCACT CTAGCAAGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA      1200
1201   TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA      1250
1251   ATGGGAAGTA CAACGAGTTG CGAAGTCGCG AGGCTAAGCT AATCTCTTAA      1300
1301   AGCTTCTCTC AGTTCGGATT GCAGGCTGCA ACTCGCCTGC ATGAAGCCGG      1350
1351   AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC GTTCCCGGGC      1400
1401   CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG      1450
1451   GTGAGGTAAC CTTTTGGAGC CAGCCGCCTA AGGTGGGATA GATGATTGGG      1500
1501   GTGAAGTCGT AACAAGGTAG CCGT                                  1524
```

2. Nucleotide sequence of 16S rRNA gene (partial sequence) of GY-20 isolated from fermented fishery product.

```
1      AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG      50
51     CAAGTCGAAC GCTTCTTTTT CCACCGGAGC TTGCTCCACC GGAAAAAGAG      100
101    GAGTGGCGAA CGGGTGAGTA ACACGTGGGT AACCTGCCCA TCAGAAGGGG      150
151    ATAACACTTG GAAACAGGTG CTAATACCGT ATAACAATCA AACCGCATGG      200
201    TTTTGATTTG AAAGGCGCTT TCGGGTGTCG CTGATGGATG GACCCGCGGT      250
251    GCATTAGCTA GTTGGTGAGG TAACGGCTCA CCAAGGCCAC GATGCATAGC      300
301    CGACCTGAGA GGGTGATCGG CCACATTGGG ACTGAGACAC GGCCCAAACT      350
351    CCTACGGGAG GCAGCAGTAG GGAATCTTCG GCAATGGACG AAAGTCTGAC      400
401    CGAGCAACGC CGCGTGAGTG AAGAAGGTTT TCGGATCGTA AAACTCTGTT      450
451    GTTAGAGAAG AACAAGGATG AGAGTAACTG TTCATCCCTT GACGGTATCT      500
501    AACCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG      550
551    GTGGCAAGCG TTGTCCGGAT TTATTGGGCG TAAAGCGAGC GCAGGCGGTT      600
601    TCTTAAGTCT GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA      650
651    AACTGGGAGA CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ATGTGTAGCG      700
701    GTGAAATGCG TAGATATATG GAGGAACACC AGTGGCGAAG GCGGCTCTCT      750
751    GGTCTGTAAC TGACGCTGAG GCTCGAAAGC GTGGGGAGCA AACAGGATTA      800
801    GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG TGTTGGAGGG      850
851    TTTCCGCCCT TCAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGAGGA      900
901    GTACGACCGC AAGGTTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG      950
951    CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGAAGAAC CTTACCAGGT      1000
1001   CTTGACATCC TTTGACCACT CTAGAGATAG AGCTTCCCCT TCGGGGGCAA      1050
1051   AGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTGGG      1100
1101   TTAAGTCCCG CAACGAGCGC AACCCTTATT GTTAGTTGCC ATCATTCAGT      1150
1051   TGGGCACTCT AGCAAGACTG CGGTGACAAA CCGGAGGAAG GTGGGGATGA      1200
1201   CGTCAAATCA TCATGCCCCT TATGACCTGG GCTACACACG TGCTACAATG      1250
1251   GGAAGTACAA CGAGTCGCGA AGTCGCGAGG CTAAGCTAAT CTCTTAAAGC      1300
1301   TTCTCTCAGT TCGGATTGCA GGCTGCAACT CGCCTGCATG AAGCCGGAAT      1350
1351   CGCTAGTAAT CGCGGATCAG CACGCCGCGG TGAATACGTT CCCGGGCCTT      1400
1401   GTACACACCG CCCGTCACAC CACGGGAGTT TGTAACACCC GAAGTCGGTG      1450
1451   AGGTAACCTT TTGGAGCCAG CCGCCTAAGG TGGGATAGAT GATTGGGGTG      1500
1501   AAGTCGTAAC AAGGTAGCCG T                                    1521
```

3. Nucleotide sequence of 16S rRNA gene (partial sequence) of MSK-3-18 isolated from fermented fishery product.

```
1      AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG      50
51     CAAGTTGAGC GCTGAAGGTT GGTACTTGTA CCGACTGGAT GAGCAGCGAA      100
101    CGGGTGAGTA ACGCGTGGGG AATCTGCCTT TGAGCGGGGG ACAACATTTG      150
151    GAAACGAATG CTAATACCGC ATAACAACTT TAAACACAAG TTTTAAGTTT      200
201    GAAAGATGCA ATTGCATCAC TCAAAGATGA TCCCGCGTTG TATTAGCTAG      250
251    TTGGTGAGGT AAAGGCTCAC CAAGGCGATG ATACATAGCC GACCTGAGAG      300
301    GGTGATCGGC CACATTGGGA CTGAGACACG GCCCAAACTC CTACGGGAGG      350
351    CAGCAGTAGG GAATCTTCGG CAATGGACGA AAGTCTGACC GAGCAACGCC      400
401    GCGTGAGTGA AGAAGGTTTT CGGATCGTAA AACTCTGTTG GTAGAGAAGA      450
451    ACGTTGGTGA GAGTGGAAAG CTCATCAAGT GACGGTAACT ACCCAGAAAG      500
501    GGACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG GTCCCGAGCG      550
551    TTGTCCGGAT TTATTGGGCG TAAAGCGAGC GCAGGTGGTT TATTAAGTCT      600
601    GGTGTAAAAG GCAGTGGCTC AACCATTGTA TGCATTGGAA ACTGGTAGAC      650
651    TGAGTGCAGG AGAGGAGAGT GGAATTCCAT GTGTAGCGGT GAAATGCGTA      700
701    GATATATGGA GGAACACCGG TGGCGAAAGC GGCTCTCTGG CCTGTAACTG      750
751    ACACTGAGGC TCGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA      800
801    GTCCACGCCG TAAACGATGA GTGCTAGATG TAGGGAGCTA TAAGTTCTCT      850
851    GTATCGCAGC TAACGCAATA AGCACTCCGC CTGGGGAGTA CGACCGCAAG      900
90     GTTGAAACTC AAAGGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT      950
951    GGTTTAATTC GAAGCAACGC GAAGAACCTT ACCAGGTCTT GACATACTCG      1000
1001   TGCTATTCCT AGAGATAGGA AGTTCCTTCG GGACACGGGA TACAGGTGGT      1050
1051   GCATGGTTGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA AGTCCCGCAA      1100
1101   CGAGCGCAAC CCCTATTGTT AGTTGCCATC ATTAAGTTGG GCACTCTAAC      1150
1151   GAGACTGCCG GTGATAAACC GGAGGAAGGT GGGGATGACG TCAAATCATC      1200
1201   ATGCCCCTTA TGACCTGGGC TACACACGTG CTACAATGGA TGGTACAACG      1250
1251   AGTCGCGAGA CAGTGATGTT TAGCTAATCT CTTAAAACCA TTCTCAGTTC      1300
1301   GGATTGTAGG CTGCAACTCG CCTACATGAA GTCGGAATCG CTAGTAATCG      1350
1351   CGGATCAGCA CGCCGCGGTG AATACGTTCC CGGGCCTTGT ACACACCGCC      1400
1401   CGTCACACCA CGGGAGTTGG GAGTACCCGAA GTAGGTTGCC T             1441
```

4. Nucleotide sequence of 16S rRNA gene (partial sequence) of MSKC-18 isolated from fermented fishery product.

```
1      AGAGTTTGAT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG      50
51     CAAGTCGAAC GAACTTCCGT TAATTGATTA TGACGTACTT GTACTGATTG      100
101    AGATTTTAAC ACGAAGTGAG TGGCGAACGG GTGAGTAACA CGTGGGTAAC      150
151    CTGCCCAGAA GTAGGGGATA ACACCTGGAA ACAGATGCTA ATACCGTATA      200
201    ACAGAGAAAA CCGCATGGTT TTCTTTTAAA AGATGGCTCT GCTATCACTT      250
251    CTGGATGGAC CCGCGGCGTA TTAGCTAGTT GGTGAGGTAA AGGCTCACCA      300
301    AGGCAGTGAT ACGTAGCCGA CCTGAGAGGG TAATCGGCCA CATTGGGACT      350
351    GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTAGGGA ATCTTCCACA      400
401    ATGGACGCAA GTCTGATGGA GCAACGCCGC GTGAGTGAAG AAGGGTTTCG      450
451    GCTCGTAAAG CTCTGTTGTT AAAGAAGAAC GTGGGTAAGA GTAACTGTTT      500
501    ACCCAGTGAC GGTATTTAAC CAGAAAGCCA CGGCTAACTA CGTGCCAGCA      550
551    GCCGCGGTAA TACGTAGGTG GCAAGCGTTA TCCGGATTTA TTGGGCGTAA      600
601    AGCGAGCGCA GGCGGTCTTT TAAGTCTAAT GTGAAAGCCT TCGGCTCAAC      650
651    CGAAGAAGTG CATTGGAAAC TGGGAGACTT GAGTGCAGAA GAGGACAGTG      700
701    GAACTCCATG TGTAGCGGTG AAATGCGTAG TATATGGAAG AACACCAGTG      750
751    GCGAAGGCGG CTGTCTGGTC TGCAACTGAC GCTGAGGCTC GAAAGCATGG      800
801    GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA AACGATGATT      850
851    ACTAAGTGTT GGGAGGGTTT CCGCCCTTCA GTGCTGCAGC TAACGCATTA      900
901    AGTAATCCGC CTGGGGAGTA CGACCGCAAG GTTGAAACTC AAAAGAATTG      1000
1001   ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC GAAGCTACGC      1050
1051   AAGAACCTTA CCAGGTCTTG ACATCTTCTG ACAGTCTAAG AGATTAGAGG      1100
1101   TTCCCTTCGG GGACAGAATG ACAGGTGGTG CATGGTTGTC GTCAGCTCGT      1150
1151   GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTATTACTA      1200
1201   GTTGCCAGCA TTAAGTTGGG CACTCTAGTG AGACTGCCGG TGACAAACCG      1250
1251   GAGGAAGGTG GGGACGACGT CAAATCATCA TGCCCCTTAT GACCTGGGCT      1300
1301   ACACACGTGC TACAATGGAT GGTACAACGA GTCGCGAGAC CGCGAGGTTA      1350
1351   AGCTAATCTC TTAAAACCAT TCTCAGTTCG GACTGTAGGC TGCAACTCGC      1400
1401   CTACACGAAG TCGGAATCGC TAGTAATCGC GGATCAGCAT GCCGCGGTGA      1450
1451   ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GAGAGTTTGT      1500
1501   AACA                                                       1504
```

```
airy1           ---------------------------------TTGCACTAAAAATAAATGTACGGTCG 26
CRL1385         ----------------------AATGGAGTGTATTGCACTAAAAATAAATGTACGGTCG 37
CN-25           TCATAGTGGAAAATATTATGGAAAATGGAGTGTATTGCACTAAAAATAAATGTACGGTCG 60
                                      *************************

airy1           ATTGGGCCAAGGCAACTACTTGTATTGCAGGAATGTCTATAGGT---------------- 70
CRL1385         ATTGGGCCAAGGCAACTACTTGTATTGCAGGAATGTCTATAGGTGGTTTTGGTTTTTTAG 97
CN-25           ATTGGGCCAAGGCAACTACTTGTAT-GC------------------------------ 87
                ************************* **

airy1           ------------------
CRL1385         GTGGAGCAATTCCAGGGA 115
CN-25           ------------------
```

Figure 1C Sequence comparison between enterocin A gene of *Enterococcus faecium* strains CRL1385, *Enterococcus faecium* strains CWBI1435 and *Enterococcus faecium* CN-25 for enterocin B. Identical nucleotides are indicated by asterisks

```
T136            TTCAAATGTAAAAGAATTAAGTACGAAAGAGATGAAACAAATTATCGGTGGAGAAAATGA 60
CWBI1411        TACAAATGTAAAAGAATTAAGTACGAAAGAGATGAAACAAATTATCGGTGGAGAAAATGA 60
CN-25B          ----------AAAGAATTAAGTACGAAAGAGATGAAACAAATTATCGGTGGAGAAAATGA 50
                          **************************************************

T136            TCACAGAATGCCTAATGAGTTAAATAGACCTAACAACTTATCTAAAGGTGGAGCAAAATG 12
CWBI1411        TCACAGAATGCCTAATGAGTTAAATAGACCTAACAACTTATCTAAAGGTGGAGCAAAATG 12
CN-25           TCACAGAATGCCTAATGAGTTAAATAGACCTAACAACTTATCTAAAGGTGGAGCAAAATG 11
                ************************************************************

T136            TGGTGCTGCAATTGCTGGGGGATTATTTGGAATCCCAAAAGGACCACTAGCATGGGCTGC 18
CWBI1411        TGGTGCTGCAATTGCTGGGGGATTATTTGGAATCCCAAAAGGACCACTAGCATGGGCTGC 18
CN-25           TGGTGCTG-------------------------------------------------- 11
                ********

T136            TGGGTTAGCAAATGTATACTCTAAA 205
CWBI1411        TGGGTTAGCAAATGTATACTCTAA- 204
CN-25B          -------------------------
```

Figure 2C Sequence comparison between enterocin B gene of *Enterococcus faecium* strains T136 *Enterococcus faecium* strains CWBI1411 and *Enterococcus faecium* CN-25 for enterocin B. Identical nucleotides are indicated by asterisks

มหาวิทยาลัยเทคโนโลยีสุรนารี

BIOGRAPHY

Nootjaree Sonsa-ard was born in February 22, 1976, in Roi-et, Thailand. She received B.S. (Food Science and Technology) from Maejo University, Chiang Mai, Thailand. Master Degree in M.S. (Food Science and Technology) from Naresuan University, Phitsanulok Thailand. She received scholarship from Ragamangala University of Technology Isan (RMUTI) for the Ph. D at Suranaree University of Technology. Part of her thesis work was presented in the oral presentation at The 3rd conference on taxonomy and systematics in Thailand, May 11-13, 2013, Faculty of science, Chulalongkorn University, Thailand and poster presentation at The 4th International on Fermentation Technology for Value Added Agricultural Products with Joint Sessions from Asia Core Program, August 29-31, 2011, Kosa Hotel, Khon Kaen, Thailand.